Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 21 No 4, 514 doi: 10.2166/wh.2023.314

Frequency and degradation of SARS-CoV-2 markers N1, N2, and E in sewage

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ABSTRACT

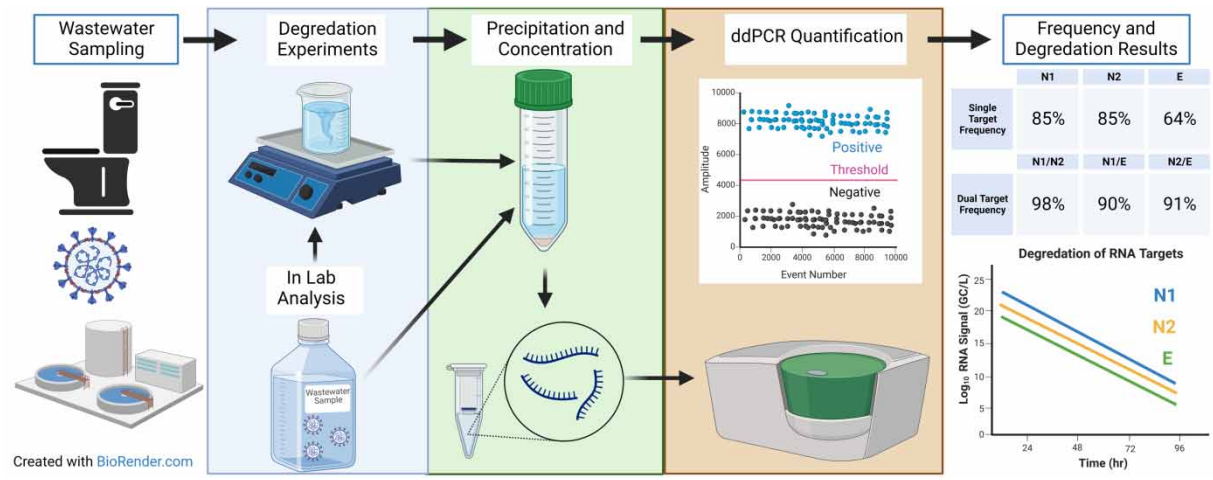
Coronavirus disease 2019 (COVID-19) is an infectious disease that is mainly spread through aerosolized droplets containing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is excreted in feces by infected individuals. Sewage surveillance has been applied widely to obtain data on the prevalence of COVID-19 in whole communities. We used SARS-CoV-2 gene targets N1, N2, and E to determine the prevalence of COVID-19 at both municipal and building levels. Frequency analysis of wastewater testing indicated that single markers detected only 85% or less of samples that were detected as positive for SARS-CoV-2 with the three markers combined, indicating the necessity of pairing markers to lower the false-negative rate. The best pair of markers in both municipal and building level monitoring was N1 and N2, which correctly identified 98% of positive samples detected with the three markers combined. The degradation rates of all three targets were assessed at two different temperatures (25 and 35 °C) as a possible explanation for observed differences between markers in frequency. Results indicated that all three RNA targets degrade at nearly the same rate, indicating that differences in degradation rate are not responsible for the observed differences in marker frequency.

Key words: degradation kinetics, droplet digital PCR (ddPCR), SARS-CoV-2, temperature, wastewater-based epidemiology (WBE)

HIGHLIGHTS

- There is a knowledge gap regarding the expression frequency and degradation of SARS-CoV-2 in wastewater.
- This study looks at the frequency and degradation of three RNA molecular markers commonly used in wastewater-based epidemiology (WBE).
- Results indicate the best combination of RNA markers and their average decay kinetics.
- This study will aid in future WBE monitoring and modeling efforts for SARS-CoV-2.

GRAPHICAL ABSTRACT



INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a member of the *Coronaviridae* family, a group of enveloped viruses with a single-stranded RNA genome (Pal *et al.* 2020). The high transmissibility of this virus has caused it to quickly spread across the globe, inflicting many individuals with respiratory and cardiovascular ailments. The first confirmed case in the United States was identified on January 18, 2020 in Washington state, and a global pandemic was officially declared by the World Health Organization on March 11, 2020 (Carvalho *et al.* 2021). SARS-CoV-2 is mainly spread through the transport of aerosolized droplets in the air (Morawska & Cao 2020). The pandemic has also resulted in major supply chain issues, making public health monitoring challenging due to the lack of available testing equipment, available personnel, and long analysis times for testing results (Khan *et al.* 2021). This has provided a challenge to public health departments to properly track and control the spread of this virus in their communities.

Although the major transmission route of SARS-CoV-2 is via airborne transport, the virus has also been found in the feces of both symptomatic and asymptomatic infected individuals (Fei Xiao *et al.* 2020; Park *et al.* 2021). This has led to the successful implementation of wastewater-based epidemiology (WBE) to monitor the spread of coronavirus disease 2019 (COVID-19) in communities (Schmitz *et al.* 2021; Wu *et al.* 2021). WBE has previously been used to improve public health policies to reduce the spread of infectious diseases. WBE played an important role in the detection, and near eradication, of polioviruses globally in 2019 (Asghar *et al.* 2014). This technique has been used to monitor COVID-19 in many different populations, such as universities, assisted living facilities, and correctional facilities, providing improved insights for health officials to make more informed decisions to protect their communities (Bivins *et al.* 2020b; Daughton 2020; Betancourt *et al.* 2021; Harris-Lovett *et al.* 2021; West *et al.* 2022). This type of WBE has also been suggested to provide an early warning by identifying spikes in detection before increases in clinical cases occur (Sodré *et al.* 2020; Bibby *et al.* 2021; Kumar *et al.* 2021).

Samples for analysis of wastewater from these populations typically are acquired by sampling sanitary sewers or wastewater treatment plants (WWTPs). Current techniques for COVID-19 detection use viral RNA targets to identify the presence of SARS-CoV-2 in wastewater. Most current studies have focused on detecting the presence of SARS-CoV-2 and its variants of concern, extrapolating the WBE results to estimate the number of infected individuals through modeling, optimizing WBE analysis to improve detection limits and reduce inhibition, and normalizing WBE data (Ahmed *et al.* 2020a; Medema *et al.* 2020).

Current applications of WBE use either the quantitative polymerase chain reaction (qPCR) or droplet digital polymerase chain reaction (ddPCR) analytical method to amplify targeted RNA sequences to quantify the amount of viral signal present in wastewater. The selection of target RNA sequences is integral to providing accurate estimations of viral impacts on communities. Picking non-conserved sequences (i.e., sequences that are highly susceptible to mutation or are selectively neutral or nearly neutral) could result in an underestimation of viral presence in communities, limiting the effectiveness of diagnostic results (Thakur *et al.* 2022). The Centers for Disease Control (CDC) has identified several nucleocapsid sequences that are suitable for WBE (CDC 2019). These proteins are responsible for maintaining the genomic structure and play a role in a variety of viral processes (Gao *et al.* 2021). Based on genomic studies, these targets appear to be highly conserved, with very low mutation rates compared to the S protein (Dutta *et al.* 2020; Oliveira *et al.* 2020; Rahman *et al.* 2020). In addition to these sequences, independent research has identified other structural proteins such as the viral envelope protein as potential WBE RNA targets (Corman *et al.* 2020). The E-protein was selected as a target because it is conserved, has a low mutation rate (<2%) and generally exhibits fewer mutations than the S protein (Jakhmola *et al.* 2021; Rahman *et al.* 2021).

Early studies have shown the relative stability and low degradation rates of SARS-CoV-2 when spiked into municipal wastewater in a series of laboratory microcosm experiments (Ahmed *et al.* 2020b). Other research has also been performed on the handling and storage of wastewater samples in the laboratory, as well as preliminary research on the effect of treatments such as pasteurization of samples for safer handling (Hokajärvi *et al.* 2021; Markt *et al.* 2021; Simpson *et al.* 2021; Islam *et al.* 2022). There are limited studies regarding the persistence of SARS-CoV-2 RNA targets in raw wastewater (Yang *et al.* 2022). These limited studies support the conclusion of Ahmed *et al.* (2020b) that persistence is high for a variety of RNA primer targets (Bivins *et al.* 2020a; McCall *et al.* 2022; Babler *et al.* 2023). Various biological pathways and external environmental conditions (e.g., temperature, pH, and UV radiation) could potentially degrade SARS-CoV-2 in the environment (Parsa *et al.* 2021; Amoah *et al.* 2022). Additionally, while all selected targets for SARS-CoV-2 are validated for specificity and exhibit low cross-reactivity, their frequency of occurrence in wastewater is still unknown. Determining RNA target frequency and rate of decay is important in order to identify the most effective combinations of RNA markers to minimize both

in-lab analysis and detection error rates, thereby maximizing the effectiveness of WBE by providing rapid (<48 h), accurate results.

Initial monitoring efforts in Michigan used three markers: two that were recommended by the CDC, N1, and N2, and one identified by Corman *et al.* (2020), E, to identify SARS-CoV-2 prevalence in wastewater using qPCR or ddPCR technology (CDC 2019). Three different markers were used to reduce the SARS-CoV-2 false-negative rate. Unfortunately, a current limitation of these monitoring methods is that for practical purposes most PCR technologies can only quantitatively analyze two targets at a time. Triplex assays are possible, but spectral overlap and efficiency issues effectively limit their use for quantitation. Consequently, a duplex assay for N1 and N2 was run in conjunction with a single-plex assay for E. This lengthened analysis time and increased the costs of WBE due to the additional costly enzymes and time required to run all markers. If only two RNA targets are used, then analytical labs can provide a more rapid and cost-effective assessment to officials helping control the spread of SARS-CoV-2 in communities.

This raises the question as to which two markers yield the highest detection rate and provide stakeholders with the most accurate information. An additional question that has been raised is the apparent degradation rates of the selected SARS-CoV-2 RNA targets in wastewater. Anecdotal evidence from multiple Michigan Sewage Surveillance groups indicated that E was appearing in smaller frequencies less frequently over time, which was corroborated by other published studies (MDHHS 2020; Chavarria-Miró *et al.* 2021). These groups in Michigan thought this could be due to degradation factors *in situ* or a mutation from a single nucleotide polymorphism (SNP) (Artesi *et al.* 2020). Markers may degrade at meaningfully different rates, which can affect WBE analysis, making it vital to determine the approximate degradation rates of N1, N2, and E.

The purpose of the present study was to address the occurrence and degradation rates of SARS-CoV-2 RNA signal as identified by the markers N1, N2, and E in wastewater. Specifically, the study objectives were to (1) determine the frequency of detection of SARS-CoV-2 by well-established primer sets, N1, N2, and E in wastewater, (2) determine the best single and dual combination of primers to maximize detection and minimize cost/false-negative rate, and (3) determine the degradation rates of individual RNA targets and their sensitivity to temperature.

METHODS

Sample sites and sample collection

Wastewater samples were collected twice a week (October 2020–May 2021) from 36 sample locations across four counties in southeast Michigan (Supplementary material, Figure S1). These locations can be separated into municipal and institutional facilities. There were 14 samples that came from municipal facilities and 22 samples that came from institutional facilities (universities, assisted living facilities, and correctional facilities). Sample sites were a combination of building-specific sanitary sewer lines, community wastewater sewer mains, and municipal WWTPs. Composite samples were collected using a high-frequency autosampler (ISCO 3700C). Approximately 5 L were collected over a period of time ranging from 8 to 24 h, depending on the sample site. The composite sample was collected and mixed thoroughly before pouring 500 mL of sewage into a bottle to be analyzed. Samples were transported on ice to the lab for analysis and were processed within 72 h of collection.

Sample concentration and RNA extraction

Samples were prepared and quantified following methods described by Flood *et al.* (2021) with modifications. In brief, each sample was inverted 30 times to ensure a homogeneous mixture. A 45-mL portion of the sample was poured into a 50-mL falcon tube and concentrated by adding ~4.0 g of polyethylene glycol and ~0.585 g of sodium chloride. Samples were agitated for 2 h at 4 °C before being centrifuged for 1 h at 3,650 rpm at 4 °C; the negative processing control was also prepared following this method, and type II deionized water was used (Borchardt *et al.* 2017). The supernatant was removed from each sample using a serological pipet, leaving the concentrated viral pellet. The final volume of the pellet was recorded for concentration calculations using Equation (1). Samples were processed using QIAamp® Viral RNA mini kit (Qiagen) as described by Flood *et al.* (2021). 200 μL of the concentrated pellet was used in the viral concentration. A final eluted volume of 80 μL was collected for quantification of viral RNA targets.

ddPCR analysis

Molecular quantification of the SARS-CoV-2 virus was conducted using the CDC's qPCR detection method and adapted for the BIO-RAD QX200™ ddPCR as outlined by Flood *et al.* (2021). Reactions that were 22 μL were completed using Bio-Rad's

One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories, Richmond, CA). ddPCR reactions contained 16.5 μL of an assay mix containing 3.3 μL of primer/probe mix (900 nM forward primer, 900 nM reverse primer, 250 nM of the probe), 5.5 μL Supermix, 2.2 μL of Reverse Transcriptase, 1.1 μL of DTT, and 4.4 μL of PCR-certified nuclease-free water. If the reaction was run in a duplex, two different primer/probe mixes were added (both in 3.3 μL quantities), and the amount of water used per reaction was reduced to 1.1 μL. A 5.5 μL portion of extracted RNA was then added to the assay mix. N1, N2, and E were the SARS-CoV-2 targets analyzed in this study. N1 and N2 were analyzed as a duplex reaction and E as a single-plex reaction. Primer and probe sequences are listed in Supplementary material, Table S1. All ddPCR reactions were completed in triplicate. Each ddPCR plate contained three positive controls, three negative controls, and three no-template controls (NTCs) for each assay mix. Droplets were generated using Bio-Rad's Automated Droplet Generator (ADG), and amplification was done using a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories) at the following conditions: 25 °C for 3 min, 50 °C for 60 min, 95 °C for 10 min, 40 cycles of alternating temperature (95 °C for 30 s, 55 °C for 1 min), 98 °C for 10 min, 4 °C for 30 min, and hold 4 °C until ready for the QX200 Droplet Reader. After thermocycling was completed, the 96-well plate was put onto the droplet reader. The droplet reader provides information on the number of copies of the gene target per 20 μL, the number of positive droplets, and the number of accepted droplets. The data produced by the droplet reader and other values throughout the process were used to calculate the gene copies per liter (GC/L) for each sample using Equation (1):

$$\mathrm{GC/L} = \frac{((\mathrm{Copies}/20\ \mu\mathrm{L})/5) \times \mathrm{Final\ RNA\ Volume}\ (\mu\mathrm{L}) \times ((\mathrm{Concentrated\ Volume}\ (\mathrm{mL})/\mathrm{Extracted\ Volume}\ (\mathrm{mL})))}{\mathrm{Initial\ Volume}\ (\mathrm{mL})} \times 1,000 \quad (1)$$

Sample selection and preparation thermal degradation

Samples were selected for thermal degradation microcosm experiments that had an initial concentration of at least 80,000 GC/L for all three gene targets. A stir bar was added to the 500-mL bottle of the selected sample. The sample was placed on a stir plate and rotated at 60 rpm to agitate the sample at a speed comparable to environmental conditions. It was then placed in an incubator at the target temperature. A sample was taken every 24 h and processed using the same sampling, extraction, and amplification protocols described above. In total 12 samples were run: six at 25 °C and six at 35 °C.

Quality assurance and quality control

Quality assurance and quality control measures (QA/QC) were performed for all samples. Samples with less than 10,000 accepted droplets were excluded from the data analysis. In order for a sample to be considered positive, it must have greater than or equal to three positive droplets in at least one of the three wells. Non-detection does not necessarily indicate that the virus is not present in the sample, since it can also occur when the virus is present if the sample is inhibited or the concentration of the gene target is below the minimum detection limit (MDL). The MDL varies for each sample based on the initial pellet volume.

Frequency analysis, decay rate, and T_{90} calculations

All data were initially log-transformed, plotted, and fitted with a linear regression model to assess general trends in the RNA expression of the three targets measured. Frequency was calculated for all the samples that were positive for SARS-CoV-2 for one or more of the markers. These samples were tabulated by site and location. Samples were then analyzed for the presence–absence of each marker using a combination of computational logic gates (1,0) and Boolean statements (IF, AND, OR). The binary results were then compiled to calculate the occurrence of each marker and all possible combinations of markers. The Agresti–Coull interval was used to estimate 95% confidence intervals (CIs) calculated with R software (R Core Team 2021) using the Binom package (Brown *et al.* 2001).

A first-order decay model was fitted to log-transformed data from each degradation experiment using ordinary least-squares linear regression. The specific decay rate, k, for each run was the slope of the fitted regression line given by Equation (2) and was used in turn to calculate the half-life T_{50} (Equation (3), $\theta = 50$) and time, T90 to 90% decay (Equation (3), $\theta = 90$). Due to the high amount of variation in initial gene target concentration and the likely presence of unknown inhibitors in the wastewater samples, statistical analysis was conducted separately on each sample to estimate k, T_{50}, and T_{90}. Visual assessment was

then used to determine whether pooling samples at a given temperature was warranted:

$$log_e(C(t)) = log_e(C(0)) - kt \tag{2}$$

$$T_\theta = \frac{-log_e(1 - \theta/100)}{k}, 0 < \theta < 100 \tag{3}$$

RESULTS

Frequency analysis

Linear regression analysis revealed that N1, N2, and E were all highly correlated, with N1 and N2 showing similar concentrations that were somewhat higher than concentrations of E (Figure 1). In total, of 1,451 samples were analyzed, of which 431 (30%) samples were removed from analysis because none of the three markers tested were positive for SARS-CoV-2. Of the remaining 1,020 samples, N1 and N2 had comparable ability to detect SARS-CoV-2, which was nearly always better than the ability of E (Table 1). Individually, N1 or N2 was able to account for 85% of the SARS-CoV-2 detections without the use of another marker (Table 1). When duplexed together, these markers (N1 and N2) had the highest ability to detect SARS-CoV-2 compared to the other possible duplex combinations (Table 1), accounting for 98% of the SARS-CoV-2-positive samples.

When comparing institutional to municipal sites, there was a distinct difference seen in single-marker frequency (Table 2). In institutional sites, the best single target was N2, which was present in 84% of samples (Table 2). In municipal sites, the best single target was N1, which was present in 93% of samples (Table 2). When looking at dual marker frequency in institutions versus municipalities, N1/N2 was the best combination for both groups (97 vs. 98% respectively, Table 2). It is also interesting to note that in municipal sites, N1/E was a close second showing up in 97% of samples (Table 2). Finally, when looking at

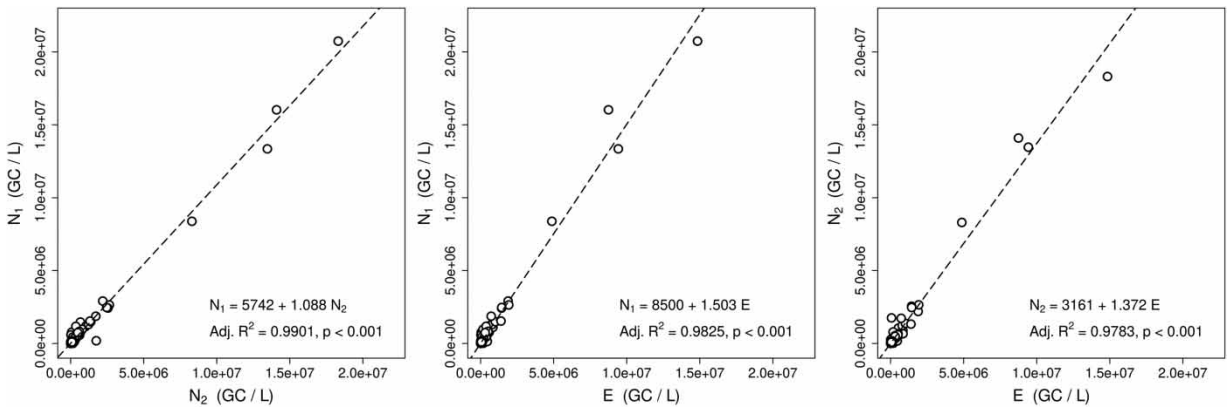


Figure 1 | General trends of virus expression for all combinations of markers tested, fitted with a linear regression model. The plots represent all three pair-wise relationships, with the left plot involving N2 and N1, the center plot E and N1, and the right plot E and N2.

Table 1 | Frequency of positive samples as well as the percent of samples under the minimum detection limit (MDL) at each sample location

Location (*n*)	N1	N2	E	N1 or N2	N1 or E	N2 or E	< MDL (*n* = 431)
A (435)	92% (89–95)	85% (81–88)	73% (68–77)	98% (96–99)	96% (94–98)	90% (87–93)	14%
B (546)	79% (74–83)	87% (82–90)	57% (52–63)	98% (95–99)	83% (78–87)	92% (88–94)	45%
C (4)	100% (43–100)	100% (43–100)	100% (43–100)	100% (43–100)	100% (43–100)	100% (43–100)	50%
D (237)	75% (68–81)	77% (70–83)	47% (39–55)	94% (89–97)	79% (72–85)	83% (77–88)	32%
E (48)	98% (88–100)	91% (79–97)	87% (74–94)	98% (88–100)	100% (91–101)	96% (85–100)	4%
F (181)	85% (78–90)	89% (82–93)	67% (59–74)	99% (95–100)	91% (85–95)	93% (87–96)	22%
Total (1,451)	85% (83–88)	85% (83–88)	64% (62–67)	98% (97–99)	90% (88–91)	91% (89–93)	29%

95% confidence intervals are shown in parentheses.

Table 2 | Frequency of positive samples in municipal and institutional sites* as well as the percent of samples below the minimum detection limit (MDL)

Site type (*n*)	N1	N2	E	N1 or N2	N1 or E	N2 or E	< MDL
Municipal (509)	93% (90–95)	86% (83–89)	75% (71–79)	98% (96–99)	97% (95–98)	91% (88–94)	12%
Institutional (942)	79% (75–82)	84% (81–87)	55% (51–59)	97% (95–98)	83% (80–86)	89% (87–92)	39%

*95% confidence intervals are shown in parentheses.

samples below the MDL, institutional sites had a much higher percentage of sites below the MDL (39%) when compared to their municipal counterparts (12%) (Table 2).

Degradation results

Visual analysis of the average decay rates of individual samples, combined with an estimated 95% CIs, indicated a high degree of overlap between individual samples and all three targets at both 25 and 35 °C (Supplementary material, Figures S2 and S3). Visual analysis of the pooled experimental data indicated a high degree of overlap between our three RNA targets, indicating that all targets degrade at approximately the same rate at both test temperatures (Figure 2). The average decay rates of pooled experiments at 25 °C for E, N1, and N2 were 0.0094, 0.0116, and 0.0117 h^{-1}, respectively (Table 3). The average decay rate of pooled samples at 35 °C were E, N1, and N2 were 0.0193, 0.0157, and 0.0134 h^{-1}, respectively (Table 4). Results of individual experiments, including decay rate k, half-life T_{50}, and time T_{90} to 90% decay, are provided in the supplemental material (Supplementary material, Tables S2 and S3).

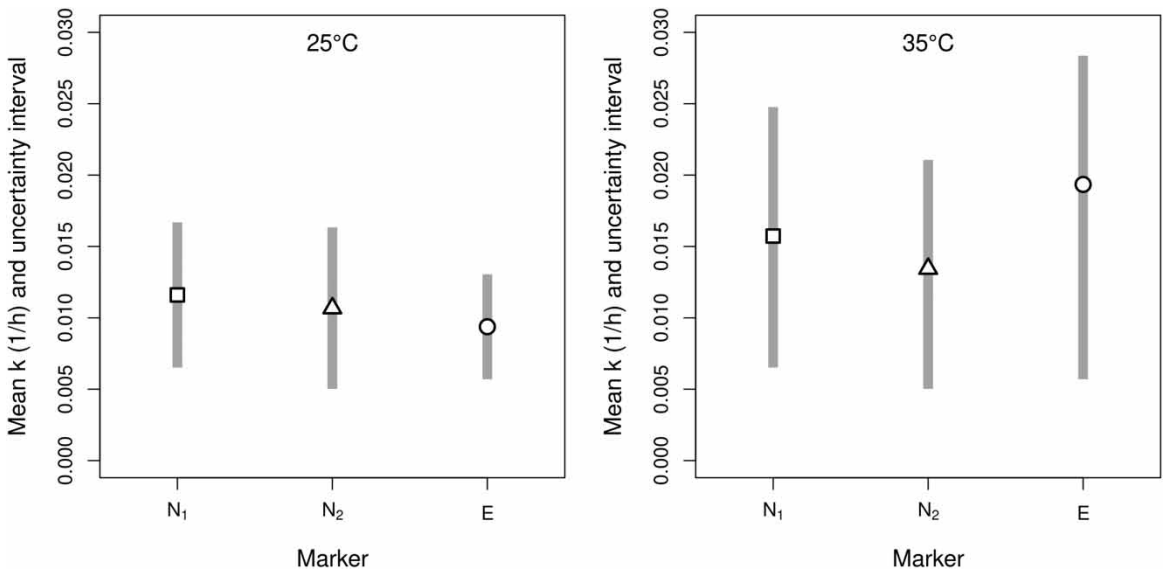


Figure 2 | Average decay rate for N1 (square), N2 (triangle), and E (circle) at 25 and 35 °C for six independent experiments. The average 95% confidence interval of all experiments is also plotted (shaded bar).

Table 3 | Average decay rate, half-life T_{50}, and time T_{90} to 90% decay for E, N1, and N2 at 25 °C for six independent experiments

Marker	k, h^{-1}	T_{50}, h	T_{90}, h
E	0.0094 (0.0057–0.0131)	102 (69–257)	338 (228–853)
N1	0.0116 (0.0065–0.0167)	71 (50–176)	236 (165–585)
N2	0.0117 (0.0050–0.0163)	74 (49–247)	246 (162–821)

Average values of confidence intervals for the six experiments are shown in parentheses.

Table 4 | Average decay rate, half-life, and T90 for E, N1 and N2 at 35 °C for six independent experiments

Marker	k, h^{-1}	T_{50}, h	T_{90}, h
E	0.0193 (0.0103–0.0284)	42 (29–80)	138 (95–266)
N1	0.0157 (0.0067–0.0248)	54 (35–172)	180 (115–570)
N2	0.0134 (0.0058–0.0211)	64 (40–276)	214 (133–917)

Average values of confidence intervals are shown in parentheses.

DISCUSSION

Our study focuses on the frequency of occurrence of three SARS-CoV-2 targets (N1, N2, and E) commonly used in WBE to monitor for SARS-CoV-2, and on the degradation rates of those targets over time. While a variety of previous studies have assessed the frequency of viral targets in wastewater, the majority of these studies are rather short in duration (<4 months), only focus on one or two markers, and have relatively low sample numbers (Randazzo *et al.* 2020; Ahmed *et al.* 2021; Baldovin *et al.* 2021). When assessing all targets over a large number of diverse sites, the data show that the E target is observed less frequently compared to N1 and N2. When assessing detections across all three markers, N1 and N2 account for 98% of the reportable signal, meaning that including E as a third target added only 2% to SARS-CoV-2 detections. This finding provides a strong rationale for removing the E target from future analyses to reduce cost and analysis time while still maintaining high sensitivity for detecting SARS-CoV-2. By analyzing just two viral targets, a duplexed analysis can be employed, saving significant time and reducing the usage of expensive reagents. We do not recommend using a single viral target, as this will increase the false-negative rate, regardless of which target is used. The use of a duplex assay with N1 and N2 balances the competing needs of high detectability and low cost while providing actionable results more rapidly than existing methods allow.

Unlike previous degradation studies, the present study incorporates the three most common WBE targets (N1, N2, and E) and measures their frequency and degradation rates with true environmental samples instead of spiked samples. We believe this approach provides a better approximation to typical real-world conditions, especially given that SARS-CoV-2 gene targets in wastewater have been documented to be associated with solids, which may affect their stability and degradation rates (Peccia *et al.* 2020; Graham *et al.* 2021).

As mentioned in the introduction, because the E target was detected with significantly lower frequency, it was initially hypothesized that the E gene target degrades faster than the N1 and N2 gene targets. The E gene target also generally had a lower signal than the N gene targets. However, degradation experiments show that all the targets have similar first-order degradation rate constants. A potential explanation for the lower frequency of E is that this target may be expressed at a lower rate or have fewer copies present per virus (Valadan *et al.* 2022). Additional potential reasons are that the E target may remain bound to viral proteins, may be lost in the clean-up procedure, or may drop out as a result of an SNP (Artesi *et al.* 2020; Chavarria-Miró *et al.* 2021).

Results from this study did not indicate a strong temperature dependence. These results show agreement with similar studies assessing the degradation of Sars-CoV-2 viral targets or similar-structured viruses (Ahmed *et al.* 2020b; Bivins *et al.* 2020a; McCall *et al.* 2022). Ahmed *et al.* (2020b) investigated only the N1 target and found that its degradation rate constant at 25 °C was 0.183 d^{-1} (0.008 h^{-1}). McCall *et al.* (2022) investigated both N1 at 'room temperature' (roughly 20 °C but not controlled) and found higher first-order decay constants of 0.84 d^{-1} (0.035 h^{-1}). Bivins *et al.* (2020a) investigated the degradation of E at 'room temperature' (roughly 20 °C but not controlled) and found a first-order rate constant of 0.67 d^{-1} (0.028 h^{-1}). We found a slightly higher value for N1 and a slightly lower value for E, at 0.2784 d^{-1} for N1 and 0.2256 d^{-1} for E. Our values fall in between the rates for N1 and are ~30% lower for E compared to literature values. These differences may be attributed to variations in matrices and slight differences in methodologies and lead us to believe that these values are consistent with previous studies.

Degradation experimental design methodologies differ considerably. Most current studies spiked samples with SARs-CoV-2 from clinical samples or from reference laboratories. These samples were typically heat treated or irradiated to limit the risk of infection. Studies by Ahmed *et al.* (2020b) and Sala-Comorera *et al.* (2021) used SARS-CoV-2 clinical strains provided and inactivated them prior to use. Bivins *et al.* (2020a) used both a high and low viral titer isolated from a clinical patient to identify the decay rates of a viable virus. While these are reasonable starting points for degradation studies, the experiments do not

replicate the sewage collection system. Once the virus is excreted into sewage, in addition to potential inactivation, there are many mechanisms that could accelerate or slow 'apparent' degradation rates. It is likely that the SARS-CoV-2 virus shed will include both intact viruses and viral fragments (Choudhury *et al.* 2021). In addition to how viruses were acquired, most studies have analyzed one or two SARS-CoV-2 targets, typically N1 and N2, along with surrogate viruses that are thought to behave similarly to SARS-CoV-2 (e.g., PMMOV, BCoV, and MHV) but are safer to handle and monitor in-lab settings.

Sewer systems consist of numerous pipes of different sizes and lengths that collect wastewater from consumers and transport it to the wastewater treatment plant. The volumetric flow, velocity, and travel time are a function of many factors including population, social behaviors, seasonality, and weather events. Travel times for different sewage packets can be computed for the shortest path and a standard flow velocity corresponding to the collection system pipe diameter. For each sewershed site, mean travel times can be estimated and with the average temperature can be used to calculate the half-life of the virus. Consequently, for most sewage collection systems, degradation of the target RNA will be small (3.3 h median residence time for the USA), and results from the present and past studies indicate minimal degradation causing minimal impact in the quantification of RNA viral signal (Kapo *et al.* 2017; Ahmed *et al.* 2020b; Bivins *et al.* 2020a; McCall *et al.* 2022; Yang *et al.* 2022; Babler *et al.* 2023). These values may serve in future modeling efforts to measure the numbers of infected individuals in a population from wastewater signals for SARS-CoV-2. Additionally, these degradation values could aid in the creation of a framework that could be rapidly implemented for future outbreaks. Our results indicate that these gene targets degrade rather slowly at 25 or 35 °C. Additionally, average wastewater has a temperature of around 15.6 °C, with a range of 7–38 °C, implying the markers will degrade even slower *in situ* (Lerch & Heinz 2011; Nagpal *et al.* 2021).

CONCLUSIONS

The use of WBE in monitoring SARS-CoV-2 has helped mitigate the impact of this disease on participating communities. While there are many different RNA proteins that can be targeted and quantified, it is important to select the optimum combination of targets to provide the most accurate information possible to stakeholders as quickly as possible and at a reasonable cost. This study analyzed the expression of three commonly used RNA targets to determine the best combinations. Results indicate that the best dual combination of markers at both municipal and institutional sites was N1/N2 and that this combination performed much better than any single marker. In addition to frequency, we also looked at degradation rates in environmental samples at several target temperatures as a potential explanation for the difference in gene expression quantified in our study. Results indicate that all markers degrade at a similar rate at both tested temperatures (25 and 35 °C). In addition, the apparent rate was relatively slow. For example, half-life T_{50} was greater than 40 h for all three markers, which is much longer than the average travel times to the collection points for our municipal and institutional sites. This finding suggests that differences in degradation rates were not the reason for the observed differences in the viral signal. The average first-order decay constants provided could aid in future modeling efforts aimed at estimating the number of individuals infected using just WBE information.

ACKNOWLEDGEMENTS

We wish to thank the members of Hesco for sample collection for all sites in this study and AQUASIGHT LLC for support with logistics and project support. We would also like to thank the Michigan Department of Health and Human Services for providing the funding for this project 32501 (MDHHS), and Christine Laba, Emily Hunawill, Michael Vu, and Nicholas Castle for their contributions to the degradation study and monitoring.

DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 12 December 2022; accepted in revised form 25 March 2023. Available online 10 April 2023