Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 22 No 6, 978 doi: 10.2166/wh.2024.303

Monitoring SARS-CoV-2 RNA in wastewater from a shared septic system and sub-sewershed sites to expand COVID-19 disease surveillance

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ABSTRACT

Wastewater-based epidemiology has expanded as a tool for collecting COVID-19 surveillance data, but there is limited information on the feasibility of this form of surveillance within decentralized wastewater systems (e.g., septic systems). This study assessed SARS-CoV-2 RNA concentrations in wastewater samples from a septic system servicing a mobile home park (66 households) and from two pumping stations serving a similarly sized (71 households) and a larger (1,000 households) neighborhood within a nearby sewershed over 35 weeks in 2020. Also, raw wastewater from a hospital in the same sewershed was sampled. The mobile home park samples had the highest detection frequency (39/39 days) and mean concentration of SARS-CoV-2 RNA (2.7×10^7 gene copies/person/day for the N1) among the four sampling sites. N1 gene and N2 gene copies were highly correlated across mobile home park samples (Pearson's $r = 0.93$, $p < 0.0001$). In the larger neighborhood, new COVID-19 cases were reported every week during the sampling period; however, we detected SARS-CoV-2 RNA in 12% of the corresponding wastewater samples. The results of this study suggest that sampling from decentralized wastewater infrastructure can be used for continuous monitoring of SARS-CoV-2 infections.

Key words: SARS-CoV-2, septic tanks, wastewater-based epidemiology

HIGHLIGHTS

- Monitoring of SARS-CoV-2 nucleic acid is feasible within shared septic systems.
- Wastewater-based epidemiology should be extended to small-scale decentralized systems to expand monitoring to populations that may experience health crises in unique ways.
- The effectiveness of sub-sewershed monitoring (e.g., neighborhood pump stations) for COVID-19 surveillance is influenced by the scale of populations being served.

GRAPHICAL ABSTRACT

INTRODUCTION

Monitoring Severe Acute Respiratory Syndrome CoronaVirus-2 (SARS-CoV-2) RNA concentrations in wastewater has been used to track Coronavirus Disease 2019 (COVID-19) infections in communities served by municipal wastewater treatment plants (WWTPs), COVID-19 quarantine facilities, and university dormitories in various locations worldwide, including Australia (Ahmed *et al.* 2020a, 2020b), France (Trottier *et al.* 2020), Italy (La Rosa *et al.* 2020), Japan (Kitajima *et al.* 2022), South Africa (Pillay *et al.* 2021), Spain (Randazzo *et al.* 2020b), and the United States (Ai *et al.* 2021; Betancourt *et al.* 2021; Wu *et al.* 2021; Al-Faliti *et al.* 2022; Cohen *et al.* 2022; Li *et al.* 2022; Yu *et al.* 2022; Grube *et al.* 2023; Kotlarz *et al.* 2023). During the initial months of the COVID-19 pandemic, there were two critical challenges to the public health response: the lack of clinical testing capacity and the unknown number of asymptomatic individuals (CDC 2020). Wastewater-based epidemiology (WBE) helped to address those issues on a community scale, providing an overview of the infection trend across a greater population than is captured through clinical testing. Thus, quantifying the viral load in a sample representing the larger community can provide early detection of disease spread, alerting health officials to take preventive measures before oversaturation of the healthcare system (Jahn *et al.* 2022; Vo *et al.* 2022).

According to the US Environmental Protection Agency (EPA), as of 2019, an estimated 21,718,000 households depend on decentralized septic systems to treat their wastewater (US EPA 2015; OW US EPA 2021). Among them, 52% had an annual income below the median household level (MHI) of $61,000, as reported in the 2017 American Housing Survey (AHS) (OW US EPA 2021). Living in economically deprived areas is linked to higher risks of heart disease, stroke, hypertension, and chronic conditions, some of which are associated with a higher risk of severe COVID-19 infection (Freedman *et al.* 2011; NCIRD 2022). If monitoring wastewater in these systems proves to be effective, it can significantly enhance the assessment of health of communities that are currently not covered by the Centers for Disease Control and Prevention National Wastewater Surveillance System (CDC NWSS), which relies on samples collected at the head of centralized wastewater treatment facilities (CDC 2023).

To date, there is not much information on the feasibility or usefulness of monitoring SARS-CoV-2 in small-scale, decentralized wastewater infrastructure. A recent study testing portable toilet wastewater from a California beach found average SARS-CoV-2 concentrations of 7.14×10^5 and 7.13×10^5 copies/mL for N1 gene and N2 gene, respectively (Li *et al.* 2023). Although the portable toilet contents are treated with dye, biocides, fragrances, and surfactants, the detection limit for SARS-CoV-2 RNA was sufficiently low (150 gene copies/mL) to allow for monitoring. Zhang *et al.* (2020a) detected SARS-CoV-2 RNA in 7 out of 9 effluent samples of septic tanks servicing temporary hospitals (cabin hospitals) for COVID-19 patients in Wuchang, China, despite the waste being treated with sodium hypochlorite (following the World Health Organization [WHO]-suggested guidelines for hospital wastewater disinfection). Similarly, Iwamoto *et al.* (2022) reported a mean N1 concentration of 3.1×10^6 copies/L of wastewater ($n = 3$) in the storage tank of a COVID-19 quarantine hospital septic system in Japan. Although all these studies have provided evidence of detecting the SARS-CoV-2 virus in decentralized systems in various settings, there is still an unanswered question as to whether continuous monitoring of septic systems for WBE is beneficial. This question is particularly relevant for the 60 million people relying on the decentralized septic systems in the United States who are currently not covered by CDC NWSS (US EPA 2015; CDC 2020).

To evaluate the usefulness of WBE in septic systems, we monitored a shared septic system servicing a mobile home park (MHP) (66 households) over 22 weeks in 2020. We hypothesized that the concentration of SARS-CoV-2 RNA would be quantifiable in decentralized septic systems and SARS-CoV-2 RNA levels would correlate with reported COVID-19 case rates in the service area. Additionally, we received samples from two pumping stations serving two neighborhoods of different sizes (71 and 1,000 households) that are part of a large municipal sewerage network and from a hospital's discharged wastewater over 22–35 weeks in 2020. As our previous study by Kotlarz *et al.* (2023) established a modified method to isolate and quantify the SARS-CoV-2 N1 gene and N2 gene from wastewater collected from a centralized wastewater treatment plant within the same sewershed boundary, we adopted the method and applied it to samples from these four sites to understand its robustness within the sewershed boundary.

METHODS

Wastewater sampling sites and sample collection

Our project was initiated with Raleigh Water (The City of Raleigh Public Utilities Department) to test their existing wastewater sampling sites for COVID-19 monitoring. Within their sewershed boundary, utility personnel collected and sent us samples from two neighborhood's pump stations, a hospital's discharged wastewater, and influent and primary solids from a municipal wastewater treatment plant (Neuse River Resource Recovery Facility, NRRRF). NRRRF serves approximately 580,000 people and had an average treated wastewater flow of 48 million gallons per day in 2020. We published the primary study testing the municipal wastewater treatment plant's samples (Kotlarz *et al.* 2023).

However, NRRRF does not include communities served by decentralized wastewater treatment systems. An opportunity to understand how SARS-CoV-2 surveillance in septic systems differs from that in centralized treatment systems arose when the MHP septic system was deemed by North Carolina Department of Health and Human Services (NC DHHS) to be a suitable system for study. Additionally, we included samples from the neighborhoods, which can be considered as having 'similar demographics' due to their close proximity and comparable house prices. The inclusion of hospital wastewater samples diversified the sample types and strengthened the study.

From April to December 2020, wastewater samples were collected from two pumping stations that collected wastewater from two neighborhoods (71 households and 1,000 households, respectively) and a hospital (970-bed capacity). From July to December 2020, samples were collected from a community septic tank serving the MHP (66 households). The sampling

frequency and number of samples varied between locations depending on access to the sites and the availability of samples from the public utility (Table 1).

Grab samples of raw wastewater were collected from the four sites between 9:00 AM and 1:00 PM. Wastewater samples were collected directly from the wet well pump stations of the neighborhoods and the sewer line of the hospital (via a manhole) using a sample collection pole and sterile HDPE Nalgene bottles (100 or 500 mL) (Thermo Fisher Scientific, Waltham, MA). Samples were stored on ice in a cooler until transported back to North Carolina State University for processing within 2 h of collection.

The MHP wastewater system contained two septic tanks and a pump tank in series (Figure S1). The internal dimensions of the first septic tank were $20' \times 10' \times 8'$ (length × width × depth), resulting in a volume of 1,600 cubic feet (11,968 gallons). The second septic tank's effective volume was 3,770 gallons and the pump tank's effective volume was 7,480 gallons. Based on the average flow of 18,476 gallons per day for the sampling duration (July - December 2020), the hydraulic retention time (HRT) in the first and second tank was a combined 20.4 hours. After leaving the second tank, effluent water flowed to the pump tank, from where it was discharged to a recirculating gravel filter prior to final discharge to the drainfield. MHP wastewater samples were collected from the inlet of the second septic tank (Figure S2).

Water consumption and case data

Based on the 2020 data provided by the U.S. Census Bureau QuickFacts (2022), the MHP had 66 households with 149 residents (2.26 persons/household). Pumping station 1 (PS1, Neighborhood A) was receiving wastewater from 71 households and had an estimated 2.30 persons/household. Similarly, pumping station 2 (PS2, Neighborhood B) was receiving wastewater from 1,000 households and had an estimated 2.68 persons/household.

Monthly water consumption data for Neighborhoods A and B, daily average wastewater flow for Neighborhood B, and daily average flow for the MHP septic system were acquired from the NC DHHS and the City of Raleigh. Laboratory-confirmed COVID-19 case counts for sampling areas were acquired from NC DHHS. Neighborhoods A and B, and MHP case counts represent any reported cases within a month prior to the first day of wastewater sample collection in each site through one week after the sampling ended to account for fecal viral shedding leading or lagging case confirmation (Lamers *et al.* 2020; Pan *et al.* 2020; Wang *et al.* 2020b; Wu *et al.* 2020; Xiao *et al.* 2020).

The accurate reporting of COVID-19 cases may be compromised by limited testing infrastructure, potentially resulting in underreported data and an incomplete understanding of the true extent of the pandemic's impact in the beginning, which was reported by Finch & Hernández Finch (2020). In addition, many cases were asymptomatic, thereby contributing to the underreporting of the true number of cases. Kronbichler *et al.* (2020), summarizing 38 studies from December 1, 2019, through March 29, 2020, in PubMed that reported on asymptomatic COVID-19 cases, indicated that 9.2–69% of cases were underreported or undocumented. These findings were obtained from 38 studies that conducted predictive models and were published in PubMed. The 7-day average of laboratory confirmed COVID-19 hospitalized patients (patients may be hospitalized for COVID-19 or influenza) data were acquired from the U.S. Department of Health and Human Services (HHS).

Table 1 | Characteristics of sub-sewershed wastewater sampling sites

Sample source	Site type	Service area population	Average monthly water consumption (million gallons)	Average daily flow (gallons/day)	Sampling duration	Sampling frequency	*N*
Mobile home park	Septic tank	66 Households	N/A	18,476	July 14–December 12, 2020	Twice a week	39
Neighborhood A	Pumping station	71 Households	0.25	N/A	April 14–September 15, 2020	Weekly	23
Neighborhood B	Pumping station	1,000 Households	3.25	164,985	April 14–September 15, 2020	Weekly	17
Hospital	Hospital discharge	970 Patient beds	N/A	N/A	April 14–December 12, 2020	Weekly	28

Sample processing and SARS-CoV-2 RNA analysis

Wastewater samples were aliquoted into two 50 mL tubes immediately after receiving them in the laboratory. A matrix recovery control of vaccine-strain bovine coronavirus (BCoV, Calf Guard, Zoetis, Parsippany, NJ, USA; concentration of ~3.7×10^4 copies/μL) was spiked into one of the aliquots before freezing them. This spike experiment allowed for examination of overall viral RNA recovery (through freezing, filtration, and extraction, Supplementary material, Figure S4). At the beginning of the study, methods for isolating and quantifying the virus from multiple wastewater sources were being optimized. Thus, the 50-mL tubes and the original Nalgene sample bottle with remaining wastewater were stored at −80 °C within 2 h of sample collection for future processing.

We followed the methods for processing wastewater that have been previously reported by Kotlarz *et al.* (2023). Briefly, the samples were thawed overnight at 4 °C and 40 mL of wastewater was treated with $MgCl_2$ (final concentration of 25 mM), followed by membrane filtration through 0.45 μm HA filters (Millipore, Burlington, MA) to concentrate the virus. Duplicate filtrations were performed on 30% of the samples from each site. We transferred the filter paper into a 5 mL screwcap tube. Viral RNA was extracted from the filters using a modified RNeasy 96 QIAcube HT (Qiagen, Hilden, Germany) protocol, and concentrated nucleic acid was eluted to a final volume of 100 μL (Kotlarz *et al.* 2023). The modified RNA extraction protocol is described in the Supplementary material. Total RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Concentrations of the N1 gene and N2 gene targets were enumerated using reverse-transcription droplet digital PCR (RT-ddPCR) with the Bio-Rad QX200 Droplet Digital PCR System (Kotlarz *et al.* 2023). Additional information on the RT-ddPCR assay design is provided in Supplementary material, Tables S1 and S2. Each sample was processed in triplicate wells where two wells were merged to measure the concentration of gene copies and a third well was used for an internal positive control-spike inhibition test (1 μL of SARS-CoV-2 standard, Bio-Rad/Exact Diagnostics, with a concentration of 9 copies/μL) (EDX 2020).

Horse fecal slurry collected pre-COVID-19 was used as a negative control for extraction, and both the N1 gene and N2 gene were not detected across all negative control samples. In addition, all ddPCR no-template controls using nuclease-free water did not result in detection of any of the targets (N1, N2, and BCoV) throughout the study. The ddPCR inhibition test (described in the following) using EDX SARS-CoV-2 standard identified inhibited samples from MHP ($n = 2$), Neighborhood A ($n = 1$), Neighborhood B ($n = 1$), and hospital ($n = 2$) from the collected samples, which were excluded from analysis (Supplementary material, Table S3).

Estimating viral RNA recovery

In addition to assessing overall BCoV recovery, we conducted step-by-step RNA recovery on a subset of samples to quantify losses attributed to the filtration and extraction processes. 50 μL (concentration ~ 3.7×10^4 copies/μL) of BCoV was dosed into a sample aliquot at each of the following steps: (i) immediately before filtration of the thawed sample, or (ii) after filtration (dosing onto the filter) but before beginning RNA extraction (Decaro *et al.* 2008; Kotlarz *et al.* 2023). These spike experiments allowed for the examination of viral RNA recovery through filtration and extraction, and through extraction alone (Supplementary material, Figure S4). We excluded the step-by-step investigation of RNA recovery for the MHP samples, as the overall BCoV recovery during the optimization period fell within the range of 26.7–65.7% reported by Ahmed *et al.* (2020c).

The limit of detection (LOD) was determined following Bio-Rad's recommendation of considering a sample positive when three positive droplets are detected in each reaction. The Poisson analysis to estimate absolute quantification further provided the LOD of 750 copies/L of wastewater for both N1 gene and N2 genes. Further, overall BCoV recovery from samples dosed prior to freezing determined the reduction factor (Table 2). This reduction factor was then used to establish the detection limit for a sample from a given site. In addition, we estimated the inhibition of the sample using the SARS-CoV-2 standard (Bio-Rad EDX SARS-CoV-2 synthetic RNA transcripts containing E, N, ORF1ab, RdRP and S Genes of SARS-CoV-2) as an internal spike in ddPCR wells to check for inhibition of SARS-CoV-2 gene markers during RT-ddPCR analysis. Every RT-ddPCR plate included triplicate positive controls and wells were merged for analysis (see Supplementary material for details of the process [Supplementary material, Figure S5]). We considered a sample as not having inhibition if the recovery of both the N1 gene and N2 gene targets was between 70 and 130% of the positive standard spike concentration (Kotlarz *et al.* 2023).

Data analysis

RNA gene copies for SARS-CoV-2 were normalized per μg of RNA extracted (Equation (1)) and per litre of wastewater filtered (Equation (2)). Viral genome copies/person/day (Equation (3)) was calculated for MHP samples using virus concentration

Table 2 | Recovery of the virus through freezing, filtration, and extraction using BCoV

Viral recovery stages	Mobile home park mean ± standard deviation	Neighborhood A mean ± standard deviation	Neighborhood B mean ± standard deviation	Hospital mean ± standard deviation
BCoV dosed before freezing	54 ± 20% ($n = 8$)	0% ($n = 5$)	0.20 ± 0.4% ($n = 5$)	2 ± 0.89% ($n = 5$)
BCoV dosed before filtration	–	12 ± 1.12% ($n = 4$)	5 ± 3.35% ($n = 4$)	1 ± 0.43% ($n = 4$)
BCoV dosed before extraction	–	24 ± 5.15% ($n = 4$)	17 ± 6.18% ($n = 4$)	5 ± 3.61% ($n = 5$)

per L of wastewater, L of wastewater discharged per day, and sewershed population following Weidhaas *et al.* (2021). Descriptive analysis was performed on the samples that were not inhibited.

$$\text{Gene copies}/\mu\text{g of RNA} = \frac{\frac{\text{viral gene copies}}{\mu\text{L of RNA extract}} \times \frac{1,000\text{ng}}{1\mu\text{g}}}{\frac{\text{amount of RNA in ng}}{\mu\text{L of RNA extract}}} \quad (1)$$

$$\text{Gene copies/L of wastewater} = \frac{\frac{\text{viral gene copies}}{\mu\text{L of RNA extract}} \times 100\mu\text{L}}{\frac{\text{L}}{1,000\text{ mL}} \times \text{filtration volume in mL}} \quad (2)$$

$$\text{Gene copies/person/day} = \frac{\frac{\text{viral gene copies}}{\text{L of wastewater}} \times \frac{\text{L of wastewater discharged}}{\text{day}}}{\text{sewershed population}} \quad (3)$$

We conducted Pearson's correlation and Student's *t*-test analyses on the log-transformed data using RStudio version 1.4.1106 (R Core Team 2022). The Shapiro-Wilks test was performed for the normality test, considering those with $p > 0.05$ as normal distributions. To assess the association between the confirmed COVID-19 cases and the concentration of SARS-CoV-2 in wastewater, a binary variable for the presence of confirmed cases within a cumulative 6-day window (encompassing 1 day before the sampling day, the day of sampling, and 4 days after) was employed following prior literature (Prasek *et al.* 2022). The selection of a 6-day time interval was based on fecal shedding estimates from previous studies, indicating maximum shedding just before symptom development, and approximately 6–8 days post-infection (Cavany *et al.* 2022; Petala *et al.* 2022). A Wilcoxon rank-sum test was performed on the log-transformed MHP data and the six-day window confirmed case data, with a significance level of $p < 0.05$.

RESULTS

BCoV recovery, RT-ddPCR inhibition, and process quality controls

Viral RNA recovery rate impacts the sensitivity of virus detection. BCoV recovery has been used to assess the SARS-CoV-2 RNA recovery, although it may not be a perfect surrogate (LaTurner *et al.* 2021; Guérin-Rechdaoui *et al.* 2022). Analysis of the BCoV recovery rates at various stages of sample processing (Table 2) showed that the MHP samples exhibited the highest average overall recovery of BCoV (dosed before sample freezing) at 54 ± 20% (mean ± standard deviation), which was substantially higher than the recovery rates for samples from the three other sites (two neighborhoods and the hospital). In contrast, both neighborhood pumping stations obtained an average BCoV recovery of <1% prior to freezing, while the hospital wastewater exhibited an average recovery rate of 2 ± 0.89%. After filtration, the average recovery rate improved to 24 and 17% for Neighborhood A and B, respectively (Supplementary material, Figure S4, method 2b, removing filtration bias). However, when dosed after filtration, there was minimal improvement in BCoV recovery rates for hospital wastewater, as the average recovery was 5 ± 3.61% of the dosing concentration.

An internal standard inhibition test using Exact Diagnostic SARS-CoV-2 Standard resulted in six inhibited samples across four sites. Subsequent 2x and 5x dilutions on the inhibited samples also yielded inhibited results. Although they were not inhibited at 10x dilution, the results were non-detect of the SARS-CoV-2 gene targets. Consequently, we excluded these

samples from the descriptive analysis. Additional details on the inhibition results are provided in Supplementary material, Table S3.

SARS-CoV-2 RNA detection frequencies and concentrations compared with reported COVID-19 cases

Shared septic system for MHP

Site-specific detection frequencies and normalized SARS-CoV-2 RNA concentrations in wastewater samples were determined (Table 3). The N1 gene was detected in all MHP samples ($n = 39$, mean N1 concentration = 86.0 copies/μg of RNA). The N2 gene was also detected in all samples, although generally at lower levels, with a mean concentration of 52.2 copies/μg of RNA. When normalized by population, the N1 gene mean concentration was 27.8 ± 27.8 million gene copies/person/day [MGC/person/d] (mean ± standard deviation) and the N2 mean concentration was 16.2 ± 15.7 MGC/person/d. N1 gene copies were highly correlated with N2 gene copies across MHP samples (Pearson's $r = 0.93$, $p < 0.0001$).

Although there was detection in 100% of samples, only ten COVID-19 cases were reported in the MHP during the 22-week study (from July through December, 2020), with two cases in the first week of November 2020 and eight cases in the second week of December 2020. When relating wastewater sample concentrations to the 6-day window of positive clinical case data, a positive association was found for the N1 gene target ($p = 0.05$) and for the N2 gene target ($p = 0.03$) using the Wilcoxon rank-sum test. The time series plot of the levels of N1 gene and N2 gene copies in MGC/person/d and the number of reported cases (Figure 1) shows higher levels of N1 gene and N2 gene in wastewater during both weeks when COVID-19 cases were reported, suggesting increased viral load was due to new confirmed cases in the community. Although no COVID-19 cases were reported in the MHP from August to October 2020, all wastewater samples collected during that time ($n = 23$) were positive for both N1 gene and N2 gene, and we observed an increase in N1 and N2 concentrations in August.

Neighborhood A

In Neighborhood A, which was of a similar size to the MHP, the SARS-CoV-2 N1 gene was rarely detected, even at times when there were reported COVID-19 cases in the area. We detected the N1 gene in only one of 23 wastewater samples collected. This sample from July 28, 2020 had an N1 concentration of 1.62×10^3 copies/L of wastewater. None of the samples had both N1 gene and N2 gene targets detected. There were three COVID-19 cases reported over the sampling period, including two cases reported on July 28, 2020. SARS-CoV-2 RNA was not detected in the first 15 samples collected between April 24, 2020 and July 21, 2020, a period when there were no reported cases in that area. Although there was one reported case on September 10, 2020, SARS-CoV-2 RNA was not detected in any samples collected 1 month before or 5 days after the reported case. Studies have reported a 40% detection rate of the virus in feces after 14 days of infection ($n = 86$) (Natarajan *et al.* 2022). However, it is important to note that our sampling at this site concluded on September 15, 2020.

Table 3 | Concentration (copies/L of wastewater and copies/μg of RNA) of SARS-CoV-2 RNA and its detection at sub-sewershed sampling sites (calculated among detected samples)

Sample source	Mobile home park	Neighborhood A	Neighborhood B	Hospital
No. of samples	39	23	17	28
prevalence of N1 detection	100%	4.5%	17%	25%
range N1 (Copies/L)	5.79×10^3–3.96×10^5	1.62×10^3	1.40×10^3–6.77×10^4	7.20×10^2–6.38×10^3
Mean N1 (Copies/L)	6.61×10^4	–[a]	2.35×10^4	2.22×10^3
Range N1 (Copies/μg of RNA)	1.65–679.86	2.64	1.68–114.95	4.18–37.84
Mean N1 (Copies/μg of RNA)	86.04	–[a]	30.33	12.38
Prevalence of N2 Detection	100%	0%	17%	21%
Range N2 (Copies/L)	4.84×10^3–2.34×10^5	0	1.40×10^3–2.53×10^4	6.77×10^2–3.09×10^3
Mean N2 (Copies/L)	3.85×10^4	–[a]	9.60×10^3	1.26×10^3
Range N2 (Copies/μg of RNA)	1.65–400.76	0	2.24–42.99	3.89–18.30
Mean N2 (Copies/μg of RNA)	52.18	–[a]	15.87	1.56

[a]Cell data are not available as only one sample from that site has been detected.

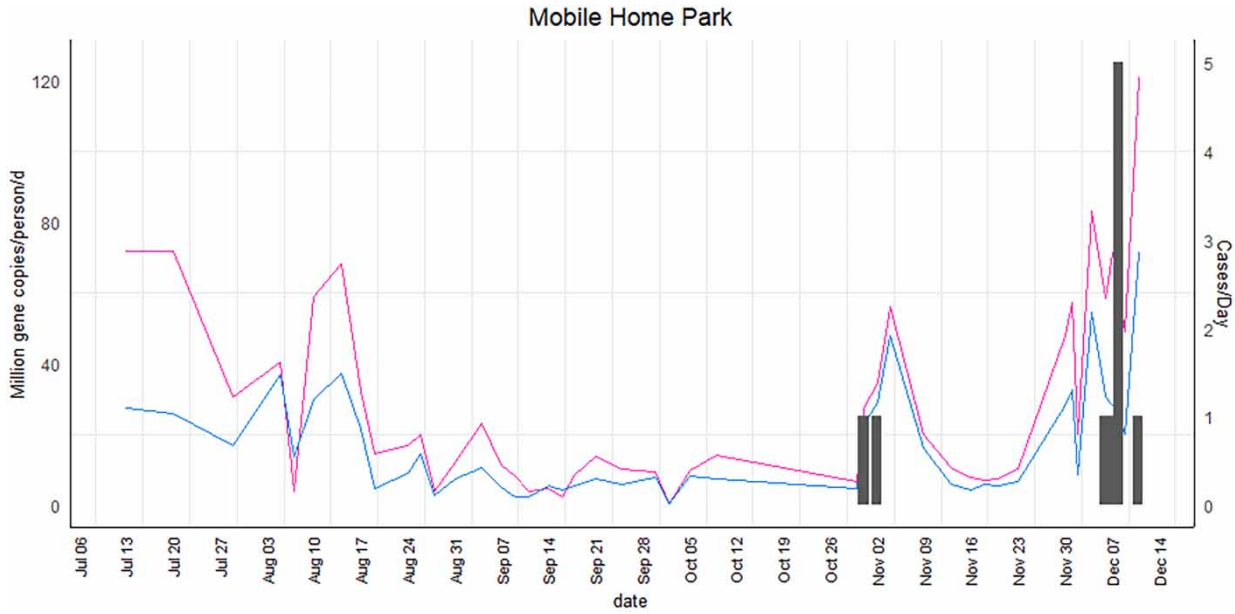

Figure 1 | Time series of N1 gene and N2 gene in MHP samples. The left *Y*-axis shows the viral gene copies in million/person/day; the right *Y*-axis shows the confirmed case/day. N1 gene levels are shown in pink, and N2 gene levels in blue. Gray columns show the number of confirmed cases.

Neighborhood B

In Neighborhood B, which had more households than the MHP, three out of 17 samples were positive for both N1 gene and N2 gene with mean concentrations of 0.50 MGC/person/d and 0.20 MGC/person/d, respectively. We started collecting samples in this neighborhood on April 24, 2020. There were no reported cases in Neighborhood B between April 24, 2020, and June 17, 2020 and wastewater samples were non-detect for N1 gene and N2 gene during that entire time. From June 17, 2020 to September 21, 2020, 16 cases were reported (two cases on June 25, 2020, and one case/day for the rest of the 14 reported cases); both SARS-CoV-2 N1 gene and N2 gene were detected in three of the 11 samples collected during that time.

Hospital discharge wastewater

We detected the N1 gene in seven of the 28 hospital wastewater samples and the N2 gene in six of the samples. Five samples had both the N1 gene and N2 gene targets detected. Supplementary material, Figure S6 illustrates the time series of SARS-CoV-2 virus concentrations in the hospital discharge wastewater alongside hospitalized patients with COVID-19. Qualitatively, N1 gene and N2 gene concentrations followed similar trends to the COVID-19 hospitalized counts. The average number of COVID-19 hospitalized patients over a 7-day period starting on July 31, 2020 through December 18, 2020 ranged from 22.4 to 105.6 patients with an average of 50.78 ± 20.05 (mean $\pm$ standard deviation) patients per week.

Water consumption and wastewater flow comparison

The interactive effects of water consumption, wastewater flow, and SARS-CoV-2 concentration in wastewater can have significant impacts on the accuracy of virus detection and, consequently, understanding of community transmission. Neighborhood B had the largest number of households among the two neighborhoods and MHP sites. Neighborhood B's average monthly water consumption rate was significantly higher than Neighborhood A's average monthly water consumption rate (*t*-test, $p < 0.0001$).

Because Neighborhood A was of a similar size to the MHP community, we compared Neighborhood A's daily wastewater flow to the MHP's flow data from April 1, 2020, to November 30, 2020 to evaluate the possible impact of flow on differences in viral concentrations between these two communities. Neighborhood A's average daily flow was 1.65×10^5 gallons, and MHP's average daily flow was 1.81×10^4 gallons, an order of magnitude lower than the pumping station. MHP's flow data

was inversely correlated with N1 gene and N2 gene concentrations in Pearson's correlation test, obtaining $r = -0.21$ for N1 and $r = -0.23$ for N2 ($p < 0.001$) suggesting that higher viral concentrations are measured during low flow situations.

DISCUSSION

As of December 2022, 1,090 sewersheds participate in the CDC's NWSS (CDC 2023). Samples are collected from either the head of the wastewater treatment plant or upstream in the sewage network. Sample collection at these points accounts for 54% of the U.S. population but does not cover households on a septic system or smaller communities on shared septic systems (e.g., mobile home communities) (CDC 2020). This study measured concentrations of SARS-CoV-2 in wastewater samples from a shared septic system servicing a MHP, two pumping stations servicing medium and large size neighborhoods and a hospital to compare viral concentrations among and within sites.

During the 21-week sampling period for the shared septic system, SARS-CoV-2 RNA concentrations ranged from 5.79×10^3 to 3.96×10^5 N1 gene copies/L of wastewater with a mean concentration of 6.61×10^4 copies/L. Iwamoto *et al.* (2022) studied the first storage tank of a septic system that collected wastewater from a 461-room COVID-19 quarantine facility in Japan and reported a mean N1 concentration (3.1×10^6 copies/L using RT-qPCR) two magnitudes higher than the concentration found in our study likely due to the higher viral load. In addition, all 39 samples from the shared septic system tested positive for both the N1 and N2 gene targets. The positivity rate for our study was similar to that from a previous study in the same city by Kotlarz *et al.* (2023) (comparing July–December, 2020 data), that sampled the influent from the centralized wastewater treatment plant.

While the reported case frequency was low (10 cases in 22 weeks) in the MHP, there are three possible reasons for the consistent presence of SARS-CoV-2 RNA in the septic tank: (i) continued unreported infection or asymptomatic case presence in the community (Ahmed *et al.* 2020a; Lodder & de Roda Husman 2020; Wu *et al.* 2020; Wannigama *et al.* 2021), (ii) persistence of the virus in septic tank wastewater for a prolonged period of time (Wang *et al.* 2005; Ahmed *et al.* 2020c; Bivins *et al.* 2020) and (iii) shedding of the virus through feces during the pre-symptomatic and post-infection period (Parasa *et al.* 2020; Wu *et al.* 2020; Natarajan *et al.* 2022). Studies reported lack of education, health insurance complications, and distrust of healthcare providers in low-income communities created barriers to seeking healthcare, which might result in fewer reported cases in the MHP (Lazar & Davenport 2018). In addition, underreported case counts could have resulted from less availability of testing in under-resourced communities, which also was reported by the Lerner Center for Public Health Promotion at Syracuse University (Monnat & Cheng 2020). Monnat & Cheng (2020) also observed a reduction in COVID-19 testing with an increase in the poverty level among states and a higher percentage of black populations governed.

Published viral recovery efficiency studies conducted with wastewater samples have used a variety of methods and have yielded a wide range of recovery rates but, in general, viral recoveries for wastewater from septic tanks are higher than recoveries from municipal influent wastewater. The septic tank study conducted by Hong *et al.* (2021) reported a recovery efficiency of 10 – 73% using murine norovirus (MNV) and RT-qPCR for wastewater samples from a hospital in Jeddah, Saudi Arabia. Despite having different wastewater storage (4 °C), viral concentrations, and extraction method, our recovery rate was comparable. Iwamoto *et al.*'s (2022) septic tank study observed a significant lower viral recovery in pre-filtered samples compared to non-filtered samples (paired *t*-test, $p < 0.05$) using murine hepatitis virus (MHV) as the surrogate virus. They used polyethylene glycol (PEG) precipitation and achieved <20% viral RNA recovery in pre-filtered samples compared to our study's 54%. A more direct comparison can be made between our study and Feng *et al.* (2021), since both used BCoV as a surrogate virus and performed similar $MgCl_2$ treatment before membrane filtration, and used RT-ddPCR for quantitation. We observed higher recovery of 54 ± 20% ($n = 8$) in our septic tank study compared to Feng *et al.*'s (2021) recovery of 4.9 ± 4.2% ($n = 106$) (mean ± SD). However, they collected wastewater samples from the head of a centralized water resource recovery facility (WRRF). Our previous study by Kotlarz *et al.* (2023) also collected samples at the wastewater plant influent, and yielded similar recoveries to those of Feng *et al.* (2021). These results indicate that several techniques yielded consistent outcomes for the same sampling sites (e.g., wastewater influent to a centralized WRRF), but results can vary depending on the site despite following similar methods. Moreover, viral RNA concentrations may vary between the RT-qPCR and RT-ddPCR platforms. In one study, RT-ddPCR demonstrated a significantly higher detection rate for CDC's SARS-CoV-2 N1 gene and N2 gene assays (Ahmed *et al.* 2022). Limited studies have utilized the RT-ddPCR platform on samples collected from septic tanks. We compared our results with any studies of septic tanks and sewerlines; although

the comparison may be impacted by different quantitation approaches used. We recommend performing site-specific method optimization for higher viral recovery prior to implementing large scale wastewater processing.

To expand wastewater surveillance of septic systems, further evaluation of sample collection and processing strategies is warranted. Insights from studies of centralized systems can possibly be applied to septic systems, but continued method optimization should improve the analytical sensitivity of tests. Sampling methods for virus detection in wastewater vary from 24-h composite samples to single grab samples during peak flow, with varying collection times and volumes (Polo *et al.* 2020; Randazzo *et al.* 2020a; La Rosa *et al.* 2021; Westhaus *et al.* 2021). Grab samples and 24-h composite samples showed similar concentrations in a study by Curtis *et al.* (2021), while Bivins *et al.* (2021) found higher viral loads in grab samples collected between midday and early evening. Our sampling approach potentially captured the evening period of the previous day (14-h composite sample from first tank HRT) which may have supported the high positivity rate throughout the sampling period. Thus, for septic tanks with two or more tanks in series, grab sample collection from the inlet of the second tank may be sufficient. However, some uncertainty remains regarding the removal and decay of SARS-CoV-2 in the first septic tank. Hong *et al.* (2021) reported 0.3-log-$_{10}$ decay of the SARS-CoV-2 N1 gene in the rooftop biological activated sludge tank compared to the underground septic tank; however, N2 gene abundance was increased by 44%. Thus, for septic tank systems, sample collection prior to the first septic tank would best capture real-time concentration data and remove concerns related to the virus persisting within the tanks. Composite sampling could also be beneficial in these systems. George *et al.* (2022) conducted a study comparing samples from various scales (building, neighborhood, city block, and wastewater treatment plant influent) to evaluate the effect of catchment sizes on grab versus composite sampling. In smaller scaled systems (~280 persons), 24-h composite sampling revealed a concentration increase of 2 $\log_{10}$ units in SARS-CoV-2 virus compared to grab samples, while in medium (2,200 persons) to large scale (48,000 persons), the difference was not significant. Augusto *et al.* (2022) found 24-h composite sample (proportional to the hourly flow rate) from both large (1,400,000 persons) and medium (2,320 persons) scales was not significantly different from grab samples collected from 8 AM to 10 AM. For smaller scale (<1,000 person) composite sampling, we recommend time-weighted sampling unless hourly flow rates are significantly different throughout the day. In this case, flow-weighted sampling would be ideal.

Because this study was initiated early in the COVID-19 pandemic, ideal wastewater sample storage conditions for assessment of SARS-CoV-2 were unknown. Freeze-thaw cycles of wastewater samples decreased detection of the N1 gene by as much as 36% (Islam *et al.* 2022) and by 7.2-fold (Huge *et al.* 2022). Juel *et al.* (2021) mentioned higher turbidity as one of the reasons for lower recovery of the viral RNA. Storing raw wastewater samples at −80 °C before further processing likely resulted in substantial decay of the SARS-CoV-2 RNA target. Recent studies suggested that storage of wastewater samples at 4 °C is ideal for SARS-CoV-2 monitoring, with negligible decay observed after 9–18 days of storage (Markt *et al.* 2021; Simpson *et al.* 2021; Beattie *et al.* 2022). The recovery of BCoV after freezing and thawing indicated a 46 – 100% virus decay across all our sampling sites. We observed, based on BCoV recovery, that samples from Neighborhoods A and B were more impacted by sample storage and processing than septic tank samples, which was potentially due to the constituents within wastewater that may vary among sites. Simpson *et al.* (2021) (using pepper mild mottle virus) and Jafferali *et al.* (2021) (using BCoV) observed similar disparities in virus decay among sites when stored at −80°C. Moreover, the low detection frequency in Neighborhood A and B may have been due to the timing of wastewater sample collection, as a Seattle study found peak detection hours to be between 5 AM and 7 AM (peak hour) in pumping stations, while our samples were collected between 9 AM and 1 PM (Nguyen Quoc *et al.* 2022). Since their study found low flow during peak hours and higher flow after 8 AM, it is possible that our samples were diluted by higher flow. We observed a significant inverse relationship ($p < 0.0001$) between viral concentration and wastewater flow at MHP. However, we cannot simply apply this relationship to the pumping stations, as the storage times were much lower in the pumping stations (<1 h) compared to the septic tank at MHP (14 h).

We detected both the N1 gene and N2 gene in 18% of the hospital wastewater samples, a substantially lower detection rate than in the MHP samples. However, it was comparable to other studies of hospital wastewater from across the world (Wang *et al.* 2020a; Zhang *et al.* 2020a; Karami *et al.* 2022; Tandukar *et al.* 2022), and similar to Gonçalves *et al.* (2021) detections of 13.3% and 26.7% from 15 untreated hospital wastewater samples in Slovenia using two different concentration methods. While studying hospital wastewater for 32 weeks through continuous monitoring in three hospitals in Portugal, Monteiro *et al.* (2022) observed detections ranging from 24 to 85%, varying by sites. The authors noted moderate to no correlation with the number of patients admitted to the hospitals. Composite sampling at one site had higher detection than grab samples at the other two sites. Our grab sampling at the hospital site may have resulted in lower detections despite the continuous

presence of confirmed and symptomatic COVID-19 patients at the hospital. Moreover, our analysis suggested that during times of high hospital admissions, SARS-CoV-2 was detected in the wastewater and contributed to the overall viral load in the centralized wastewater system. Previous literature has highlighted the presence of concentrated constituents such as pharmaceutically active compounds (analgesics and antibiotics), general and multidrug-resistant bacteria, and enteric viruses unique to hospital wastewater (Zhang *et al.* 2020b; Majumder *et al.* 2021), necessitating their treatment before discharge into municipal wastewater treatment facilities to comply with the Clean Water Act (1972) and its updated 2002 version (Kumari *et al.* 2020). The pre-treatment process potentially influenced both the virus's persistence and the recovery of BCoV during sample storage and processing; however, SARS-CoV-2 was detected in some samples. The variable RNA recovery across the sites in our study indicate a potential challenge of wastewater surveillance implemented in decentralized systems. Further improvements are needed in the isolation of viral RNA from neighborhood and hospital sewerline wastewater samples to develop a robust method for wastewater surveillance, eliminating site-wise variations.

In the United States, approximately 21 million households depend on a shared septic system (OW US EPA 2021), but little research has been conducted on their potential as a tool for disease surveillance across the world (Zhang *et al.* 2020a; Hong *et al.* 2021; Iwamoto *et al.* 2022; Amin *et al.* 2023). Septic systems may serve populations with lower average household income levels and, therefore, septic system surveillance may offer opportunities to capture populations that have limited access to healthcare (Zhang *et al.* 2020a; Mattioli *et al.* 2021; OW US EPA 2021). Identifying approaches to expand access to and optimize sampling from these systems is necessary. Overall, our study highlighted the importance of understanding the dynamics of SARS-CoV-2 concentrations in shared septic systems and the need for further investigation to optimize sampling strategies in such systems for effective monitoring. This information can be crucial in developing targeted public health interventions to control the spread of the virus in communities relying on decentralized sanitation systems. By expanding our monitoring effort and enhancing our ability to detect the virus, we can identify and respond more effectively to disease outbreaks, particularly in smaller, under-resourced communities that may play a key role in monitoring programs. Ultimately, this can lead to better public health outcomes and help prevent the spread of infectious diseases.

ACKNOWLEDGEMENTS

We would like to acknowledge the support of Jeremy Lowe and Daniel Cockson for sample transport and collection. We also appreciate the support of Sean Daly in the laboratory.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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First received 10 October 2023; accepted in revised form 21 February 2024. Available online 12 April 2024