Assessment of Concentration, Recovery, and Normalization of SARS-CoV-2 RNA from Two Wastewater Treatment Plants in Texas and Correlation with COVID-19 Cases in the Community

Kiran Kumar Vadde, Haya Al-Duroobi, Duc C. Phan, Arash Jafarzadeh, Sina V. Moghadam, Akanksha Matta, and Vikram Kapoor*

ABSTRACT: The purpose of this study was to conduct a correlative assessment of SARS-CoV-2 RNA concentrations in wastewater with COVID-19 cases and a systematic evaluation of the effect of using different virus concentration methods and recovery and normalization approaches. We measured SARS-CoV-2 RNA concentrations at two different wastewater treatment plants (WWTPs) in the Bexar County of Texas from October 2020 to May 2021 (32 weeks) using reverse transcription droplet digital PCR (RT-ddPCR). We evaluated three different adsorption–extraction (AE) based virus concentration methods (acidification, addition of MgCl₂, or without any pretreatment) using bovine coronavirus (BCoV) as surrogate virus and observed that the direct AE method showed the highest mean recovery. COVID-19 cases were correlated significantly with SARS-CoV-2 N1 concentrations in Salitrillo ($\rho = 0.75, p < 0.001$) and Martinez II ($\rho = 0.68, p < 0.001$) WWTPs, but normalizing to a spiked recovery control (BCoV) or a fecal marker (HF183) reduced correlations for both treatment plants. The results generated in this 32-week monitoring study will enable researchers to prioritize the virus recovery method and subsequent correlation studies for wastewater surveillance.

KEYWORDS: Wastewater-based epidemiology, SARS-CoV-2, bovine coronavirus, COVID-19, droplet digital PCR, Texas

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the etiologic agent of the ongoing pandemic of coronavirus disease 2019 (COVID-19), has continued to affect the world population ever since its first outbreak in December 2019.¹ Current approaches to community-wide health assessment are based primarily on clinical testing; however, wider access to testing for COVID-19 has so far been severely limited due to logistical difficulties and high costs. In this regard, with growing evidence of SARS-CoV-2 fecal shedding and the existence of viral RNA in domestic sewage, wastewater-based surveillance of SARS-CoV-2 RNA at the community level has gained attention to complement the clinical testing and efficiently monitor the transmission of COVID-19.²–⁵ Analysis of a wastewater sample for the presence of SARS-CoV-2 genes can be an effective and efficient way to test defined catchment areas. Wastewater-based testing can be used alongside clinical testing to provide cost-effective and objective measures of the presence of COVID-19 in the community.⁶,⁷ Wastewater surveillance affords a potential advantage that infected individuals contribute to the signal independent of health seeking behavior or access to health care. Because of the ability to observe viral shedding prior to onset of symptoms or even in the absence of symptoms, in some applications, wastewater surveillance has provided an early warning to a local or regional surge in COVID-19 cases.⁸¹⁰

Numerous studies based on wastewater surveillance of SARS-CoV-2 RNA have been applied to determine the variation in COVID-19 incidence at the community level, indicating the technical possibility for regular monitoring. This approach has the potential to generate results for a community at a comparatively lower cost than individual clinical testing, which could allow policymakers to recognize the hotspots of an outbreak and take proper interventions on time.¹⁶,¹⁸,¹⁹ While the association between COVID-19 cases and wastewater SARS-CoV-2 RNA concentrations is now being reported widely in the literature, most initial studies were carried out for a short sampling duration (from a few days to a month).⁹,¹⁰,²⁰,²¹ Recent reports¹⁴,¹⁶,²² have indicated that

Special Issue: Wastewater Surveillance and Community Pathogen Detection

Received: January 31, 2022
Revised: April 12, 2022
Accepted: May 4, 2022
studies carried out for a prolonged period can provide a comprehensive relationship between COVID-19 cases and the SARS-CoV-2 RNA concentrations in the wastewater of corresponding communities. There is a need for longer-term studies ranging from a few months up to a year to account for seasonal variability and periods of high and low COVID-19 case incidence; this information will be useful for establishing robust assessments of COVID-19 cases with SARS-CoV-2 concentrations in wastewater. Additionally, different virus concentration methods that were applied in several studies from different countries need to be evaluated due to the variability in the characteristics of wastewater and sampling strategies. Furthermore, studies on the effects of normalizing SARS-CoV-2 RNA concentrations to account for recovery efficiency or to the estimated amount of human fecal strength in the samples are still limited.22,23

SARS-CoV-2 RNA concentrations in wastewater have been shown to provide insights into the community infection trends that clinical testing may fail to obtain.11,12,16 Subsequently, wastewater-based surveillance of SARS-CoV-2 RNA has the potential to be implemented as a complementary tool to monitor the effectiveness of intervention strategies, such as mass vaccination campaigns, at the community level.7,24−26 It has been further reported that the virus concentration and extraction procedures could significantly affect the sensitivity of the analyses.27,28 Therefore, evaluation of the virus concentration methods at individual wastewater treatment plants (WWTPs) may be necessary to precisely understand the correlation between SARS-CoV-2 RNA concentration and COVID-19 prevalence.

The first case of COVID-19 in Bexar County, Texas, was reported in late March 2020 and increased to a cumulative total of 118,057 cases by end of the year in December 2020. To complement the clinical testing data and provide real-time community infection trends in Bexar County, we applied wastewater-based surveillance of SARS-CoV-2 RNA at a domestic WWTP in our previous 13-week pilot study.14 In the present study, we expanded our analyses to include one additional WWTP and evaluated the effect of using different virus concentration methods and recovery and normalization approaches. In this study, we compared SARS-CoV-2 RNA concentrations in wastewater influent of two different WWTPs in Bexar County, Texas, namely Salitrillo WWTP and Martinez II WWTP. We evaluated three different adsorption−extraction (AE) based virus concentration methods using bovine coronavirus (BCoV) as a surrogate virus. Subsequently, we applied the most recovery-efficient method to measure SARS-CoV-2 RNA using reverse transcription droplet digital PCR (RT-ddPCR) in weekly wastewater samples collected from October 2020 to May 2021, which captured a winter surge in 2020−2021 in COVID-19 cases and the initiation of COVID-19 vaccination in the city. In addition to quantifying two SARS-CoV-2 RNA targets (N1 and N2), we quantified coronavirus recovery and wastewater fecal strength in every sample using bovine coronavirus (BCoV) and human-associated Bacteroidales (HF183), respectively. This extensive data set allowed for a robust evaluation of recovery and normalization approaches during both low and high incidences of COVID-19. We used both raw and adjusted SARS-CoV-2 concentrations to correlate with COVID-19 case data in the community. The results presented here may enable the advancement of wastewater-based surveillance of SARS-CoV-2 as a valuable epidemiologic surveillance tool for tracking the spread and impact of COVID-19.

**MATERIALS AND METHODS**

**Wastewater Samples.** The inlets of two wastewater treatment plants (Salitrillo and Martinez II) operated by the San Antonio River Authority (SARA) in Bexar County, Texas, were sampled (Table 1). Martinez II and Salitrillo have separate sewer systems and serve approximately 10,000 and 17,000 connections, respectively, with permitted flows of 3.5 and 5.83 million gallons per day, respectively. In both cases, this was approximately 2−5% of the population of the county. As per the Texas Commission on Environmental Quality permit, the WWTPs do not appear to receive significant industrial wastewater contributions. Composite, time-based 24 h samples were collected from both plants using ISCO 3700 autosamplers (Teledyne ISCO, Lincoln, NE). Samples were taken weekly from October 2020 to May 2021, for a total of 32 weeks. Samples were stored on ice and delivered to the laboratory at the University of Texas at San Antonio (UTSA) and immediately concentrated upon delivery and frozen at −80 °C, followed by molecular analyses within 7 days. It has been reported that there was no significant decay in SARS-CoV-2 RNA concentrations when wastewater samples were stored at −80 °C; therefore our method should permit reliable quantification of SARS-CoV-2 RNA.

**Table 1. Details of WWTPs and the Average Water Quality Parameters of Wastewater Influent Samples Analyzed in This Study**

| WWTP            | connections served | permitted flow (MGD) | average flow (MGD) | pH    | DO (mg/L) | CBOD (mg/L) | TSS (mg/L) |
|-----------------|--------------------|----------------------|--------------------|-------|-----------|-------------|------------|
| Salitrillo      | >17,000            | 5.83                 | 3.96               | 7.8 ± 0.14 | 0.51 ± 0.34 | 168.3 ± 18.7 | 111.4 ± 5.09 |
| Martinez II     | >10,000            | 3.5                  | 2.12               | 7.7 ± 0.17 | 0.60 ± 0.22 | 40.9 ± 7.3  | 371.6 ± 286  |

*MGD = million gallons per day; SD = standard deviation; DO = dissolved oxygen; CBOD = carbonaceous biochemical oxygen demand; TSS = total suspended solids. Reported as mean ± SD.*
Table 2. List of Primers and Probes Used in This Study

| assay                              | primers and probe                                                                 |
|------------------------------------|-----------------------------------------------------------------------------------|
| bovine coronavirus (BCoV)          | F: CTGGAAGTTGTGGTTGAGTTT R: ATTATCGGCTAATACATAC T: PAM-CCTTCATAT-ZEN-CTATACATCA |
| SARS-CoV-2 (CDC N1)                | F: GACCCCAAATAACGCCGAAAT T: RTGGTTCAGCCCCAGATTCGGTCTG T: PAM-ACCCCGCATTAGCTTTGTTGACC-BHQ-1 |
| SARS-CoV-2 (CDC N2)                | F: TTACAAAAATTTGGCGCAGAA R: GCGGGCATTTCGGAAAGAA T: PAM-ACAATTGTCGCCCCAGGGCTTCAG-BHQ-1 |
| human-associated Bacteroidales (HF183) | F: ATCATGAGTTTCACATGTCGCG R: CGTACGAGTTGGAGCAGGTGT T: PAM-CTGAGAGGAGGTCACACCACATTGA-TAMRA |

After treatment, 200 mL aliquots (n = 2) from each of the wastewater samples were centrifuged at 30,000g for 15 min at 4 °C to pool solids, and the supernatant was passed through 0.45 μm pore size, 47 mm diameter electronegative membranes (GN-6 Membrane Micrel Membrane Disc Filter, Pall Laboratory). Both the pellet and membrane were stored at −80 °C until nucleic acid extraction.

Frozen membranes and pellets were transferred into screw-cap microcentrifuge tubes containing DNase and RNase free glass beads (PowerBead Tube, Qiagen, Hilden, Germany) and subjected to bead-beating for 45 s at maximum speed (PowerLyzer 24 Homogenizer, MO-BIO, Carlsbad, CA) in the presence of 500 μL of lysis buffer (Buffer RLT Plus; Qiagen GmbH, Hilden, Germany) containing β-mercaptoethanol (Sigma-Aldrich Co., St. Louis, MO). Samples were centrifuged at 21,130 g for 15 min at 4 °C, and the supernatant was then transferred to spin columns from the nucleic acid extraction kit. Total RNA and DNA were extracted simultaneously from the supernatant using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) and in combination with automated robot QIAcube Connect (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, and the resulting RNA and DNA were each eluted in 100 μL of RNase-free water. Negative extraction controls without a membrane were also processed. The concentration and purity of RNA and DNA was determined by using a NanoDrop One Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA extracts were stored at −80 °C and DNA extracts were stored at −20 °C until used in molecular assays.

RT-ddPCR. RNA extracts were used as templates in one-step reverse transcription droplet digital PCR (RT-ddPCR) assays using Bio-Rad QX200 Droplet Digital PCR System per the procedures detailed in the Supporting Information. SARS-CoV-2 RNA was measured in each sample using the CDC N1 and N2 assays (Table 2). Technical duplicates were run (two wells); wells were merged for data analysis. To test for PCR inhibition, each RNA extract was run both undiluted and at 10-fold dilution for both N1 and N2 assays. PCR inhibition tests resulted in target copies proportional to a 10-fold dilution relative to the undiluted RNA templates, suggesting that PCR inhibition did not interfere with the amplification efficiency. For samples near or below LOD, no detectable SARS-CoV-2 was observed for 10-fold dilutions. For each plate, a positive control and no-template controls (NTC) were run in duplicate. The positive control was the 2019-nCoV_N_ Positive Control containing the complete nucleocapsid gene from SARS-CoV-2 purchased from Integrated DNA Technologies (IDT, Coralville, IA). The ddPCR data was analyzed using the QuantaSoft Analysis Pro (Bio-Rad) software, and concentrations were calculated by either allowing the program to autoselect a threshold or by manual calling when the program was not able to autoselect the threshold. The required number of droplets for a sample with merged duplicate wells was at least 10,000. The limit of detection (LOD) and the limit of quantification (LOQ) were determined for the N1 and N2 assays (see Supporting Information). Average concentrations per reaction were converted to copies per liter of wastewater using dimensional analysis (Table S1). For method evaluation and RNA recovery efficiency, BCoV was quantified using RNA extracts and one-step RT-ddPCR assay targeting the bovine coronavirus gene (34 Table 2; see Supporting Information).

qPCR. DNA extracts were used as templates to measure the concentration of HF183 marker (Table 2) in wastewater samples. The qPCR assay for HF183 was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using iTaq Universal Probes Supermix (Bio-Rad). The reaction mixtures (25 μL) contained 12.5 μL of supermix, 300 nM of each primer (forward and reverse), 100 nM of probe, and 2 μL of DNA template. The thermal cycling profile included 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The qPCR data were analyzed using Bio-Rad’s CFX Manager Software (version 3.1). Standard curves were run in duplicate for each qPCR plate by using serially diluted plasmid standards purchased from Integrated DNA Technologies (IDT, Skokie, IL) containing the sequences for the targeted gene. Each standard curve was generated from at least six 10-fold plasmid dilutions in duplicate. The percent amplification efficiencies were calculated by the instrument manufacturer’s instructions (Bio-Rad). Controls containing no template were used to check for cross contamination. Undiluted and 10-fold diluted templates were tested to determine the absence of PCR inhibition in the samples.

COVID-19 Clinical Data. Clinical testing data for COVID-19 within the Bexar County were obtained from the City of San Antonio’s COVID-19 Open Data repository (https://covid19data.sanantonio.gov/). The daily positive cases include COVID-19 cases reported on each date, and the weekly infection rate is the percentage of positive COVID-19 tests in the last 7 days.

Data Analysis. All statistical analyses and graphs were completed in GraphPad Prism, version 9.3.1 (LaJolla, CA). The wastewater virus RNA concentrations were reported as copies per liter of wastewater. Paired and unpaired t tests were
used to compare groups after confirming that data were normally distributed. Spearman’s rank correlation coefficients were used to determine the relationship between the SARS-CoV-2 wastewater concentration and its association with COVID-19 weekly infection rate. Correlations were also examined using SARS-CoV-2 wastewater concentrations normalized to BCoV recovery rate and HF183 concentration. Normalization was performed using wastewater N1/N2 concentrations divided by BCoV recovery rate or HF183 concentration. The raw RT-ddPCR data have been provided in Tables S2 and S3, examples of positive and negative results for each assay are listed in Table S4, and a Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) checklist has been completed in Table S5.

### RESULTS

**Evaluation of Adsorption-Extraction Based Virus Concentration Methods.** The BCoV RNA recovery efficiency (%) for the six wastewater (influent) samples subjected to three different adsorption–extraction (AE) viral concentration methods is given in Figure 1. The mean recoveries for the three methods ranged from 10.2% to 16% (Table S6). The AE-direct (without pretreatment) method had the highest mean recovery of 16.0% ± 13.4%, followed by the AE-MgCl₂ treatment method with 11.8% ± 9.1% mean recovery. The AE-acid treatment method had the lowest mean BCoV recovery (10.2% ± 9.7%) among the three viral concentration methods. Although the BCoV recovery rates were not significantly different among the methods analyzed (p > 0.05), the AE-direct method was selected for the virus concentration in the wastewater samples for this study because of its highest mean recovery in these initial assessments.

**Spiked BCoV Recovery and Fecal Strength of Monitored Wastewater Samples.** During this 32-week study, the mean recovery of spiked BCoV in wastewater samples collected from Salitrillo and Martinez II WWTPs was 26.5% ± 18.4% and 34.7% ± 30.1%, respectively. The BCoV recovery rates were not consistent and varied from 0.1% to 79% in this study (Figure S1A). Although the recovery rate for the wastewater samples collected from Martinez II WWTP was generally higher compared to that from the Salitrillo WWTP, the values were not statistically significant (t-test, p > 0.05). The HF183 human fecal marker was measured to evaluate the temporal dynamics and magnitude of fecal inputs at the two WWTPs. The average concentration of HF183 marker in the wastewater samples of Salitrillo and Martinez II WWTPs was $1.04 \times 10^7$ and $1.31 \times 10^8$ copies/L, respectively, and a significant difference in the concentrations was observed between the two WWTPs (t-test, p < 0.05). However, the concentrations were relatively consistent across the samples of individual WWTPs, indicating stable fecal strength of the samples (Figure S1B).

**SARS-CoV-2 Viral Detection and Quantification in Wastewater Samples.** The detection frequency of SARS-CoV-2 RNA in the influent samples of two WWTPs collected on a weekly basis between October 2020 to May 2021 is presented in Table 3. A total of 64 wastewater samples were tested in this study and the SARS-CoV-2 N1 and N2 gene amplicons were detected in 62 (96.8%) samples. The concentrations of SARS-CoV-2 RNA targets in the remaining 2 samples were below the limit of detection. The complete raw data for SARS-CoV-2 N1 and N2 RT-ddPCR assays tested on these 64 wastewater samples is provided in Supporting Information (Tables S2 and S3). For the 32 samples collected from Salitrillo WWTP, 31 (96.8%) samples were positive for the SARS-CoV-2 N1 assay. Among these positive samples, only 27 (84.3%) samples were in the quantifiable range with concentrations ranging from 387 to 12 800 copies/L of wastewater.

Table 3. SARS-CoV-2 Detection Frequency in Wastewater Samples Collected from Two WWTPs in Bexar County, Texas

| WWTP       | sampling event | sample type       | no. of positive samples/total samples tested<sup>a</sup> | no. of quantifiable positive samples/total samples tested<sup>b</sup> |
|------------|----------------|-------------------|---------------------------------------------------------|-------------------------------------------------|
| Salitrillo | weekly         | 24 h composite    | 31/32                                                   | 27/32                                            |
| Martinez II| weekly         | 24 h composite    | 31/32                                                   | 27/32                                            |

<sup>a</sup>Based on limit of detection.  
<sup>b</sup>Based on limit of quantification.
wastewater. For the SARS-CoV-2 N2 assay, 31 (96.8%) samples were positive but only 22 (68.7%) samples were quantifiable with concentrations ranging from 604 to 11 400 copies/L of wastewater (Figure 2A). The SARS-CoV-2 N1 and N2 concentrations showed a significant positive correlation (Spearman’s rank correlation coefficient = 0.87; \( p < 0.001 \)), and their highest concentrations were detected in the sample collected on December 8, 2020.

For Martinez II WWTP, 31 (96.8%) out of 32 wastewater samples analyzed were positive for SARS-CoV-2 N1 and N2 RT-ddPCR assays. However, the SARS-CoV-2 N1 gene amplicon was quantifiable in only 27 (84.3%) samples with concentrations ranging from 445 to 62 000 copies/L of wastewater (Figure 2B). Similarly, the SARS-CoV-2 N2 gene amplicon was quantifiable in 25 (78.1%) samples with concentrations ranging from 366 to 73 500 copies/L. A significant positive correlation (Spearman’s rank correlation coefficient = 0.92; \( p < 0.0001 \)) was observed between the SARS-CoV-2 N1 and N2 concentrations, and the sample collected on December 22, 2020, had the highest concentrations. Although Martinez II WWTP is comparatively smaller and serves less population, the concentrations of SARS-CoV-2 N1 and N2 gene amplicons were relatively higher compared to the samples of Salitrillo WWTP.

Among the two RT-ddPCR assays tested here, the SARS-CoV2 N1 assay showed higher sensitivity compared to the SARS-CoV-2 N2 assay. The N1 assay was quantifiable in 54 (84.3%) out of the total 64 wastewater samples, whereas the N2 assay was quantifiable in only 47 (73.4%) samples. Therefore, based on the results, the concentrations of SARS-CoV-2 N1 targets were selected for further correlation studies.

### Correlation of COVID-19 Clinical Data with Raw and Adjusted SARS-CoV-2 RNA Concentrations

The clinical COVID-19 daily cases reported for the sewershed area (Bexar County, Texas) were retrieved from the City of San Antonio’s COVID-19 Open Data repository. During this 32-week study...
that was carried out from October 2020 to May 2021, the daily positive cases at Bexar County were relatively high in December 2020 and January 2021, with the highest daily cases on January 10, 2021 (Figure 3A). We quantitatively

Figure 3. Comparison of raw and adjusted (based on BCoV recovery and HF183 concentrations) SARS-CoV-2 N1 concentrations in wastewater samples to the clinical COVID-19 cases in the sewershed area (Bexar County, Texas). (A) Daily and weekly average of new COVID-19 cases reported in Bexar County, Texas. (B) Raw and adjusted SARS-CoV-2 N1 concentrations in wastewater samples of Salitrillo WWTP. (C) Raw and adjusted SARS-CoV-2 N1 concentrations in wastewater samples of Martinez II WWTP.
comparing SARS-CoV-2 RNA concentrations in wastewater at both WWTPs with publicly reported data on COVID-19 cases. SARS-CoV-2 RNA N1 levels at both treatment plants exhibited both increasing and decreasing trends over the course of the study period (Figure 3B,C). Wastewater samples collected during the winter surge in COVID-19 cases (November 2020 to January 2021) in COVID-19 cases showed higher SARS-CoV-2 RNA concentrations as compared to other periods. Notably, the SARS-CoV-2 RNA levels decreased from February 2021 to May 2021, which corresponded well with the decline in daily positive COVID-19 cases and the initiation of vaccination in the study area.

Using the 7-day average of clinical COVID-19 cases reported on the wastewater sample collection date, the correlation between SARS-CoV-2 N1 concentrations in wastewater samples and the weekly moving average of reported COVID-19 cases in the sewershed area was determined. The correlation studies were performed with the raw and adjusted (BCoV and HF183) SARS-CoV-2 N1 concentrations to analyze the impact of normalization. A significant positive correlation was observed between the weekly average COVID-19 cases and the raw SARS-CoV-2 N1 wastewater concentrations of Salitrillo (Spearman rank correlation coefficient, $\rho = 0.75, p < 0.001$) and Martinez II (Spearman rank correlation coefficient, $\rho = 0.68, p < 0.001$) WWTPs, indicating the concentrations of SARS-CoV-2 RNA in these WWTPs were following the trend of COVID-19 clinical data (Figure 3). However, when analyzed with normalized data, the correlation was reduced between SARS-CoV-2 N1 concentrations and reported cases (Figure S2). When the SARS-CoV-2 N1 concentrations were adjusted to BCoV recovery, the correlation was slightly reduced for Salitrillo ($\rho = 0.72, p < 0.001$) and Martinez II ($\rho = 0.61, p < 0.001$) WWTPs. When analyzed with HF183 normalized data, the correlation was weak between the weekly average cases and SARS-CoV-2 N1 concentrations of Salitrillo WWTP ($\rho = 0.39, p < 0.001$). For Martinez II WWTP, the HF183 normalized SARS-CoV-2 N1 concentrations showed very weak correlation ($\rho = 0.16, p > 0.05$) with weekly average cases and results were not significant, indicating that normalization with HF183 may not be a useful strategy for adjusting wastewater SARS-CoV-2 RNA concentrations at the current monitored WWTPs.

### DISCUSSION

The wastewater surveillance of SARS-CoV-2 has emerged as an important tool in monitoring the ongoing global COVID-19 pandemic by providing reliable and timely community-level public health information. However, recent findings indicated discrepancies in the relationship of SARS-CoV-2 concentrations with the COVID-19 prevalence among different sewersheds and suggested a comprehensive evaluation of the overall approach is necessary at the individual sites to implement the wastewater surveillance of SARS-CoV-2. In this regard, this study was systematically carried out to evaluate the effective virus concentration method that is suitable to detect SARS-CoV-2 in the wastewater samples from the two WWTPs in Bexar County, Texas, and to determine the correlation of SARS-CoV-2 RNA concentrations with the COVID-19 prevalence in the study area.

The concentration of virus is a very important step for the wastewater surveillance of SARS-COV-2, particularly to detect low quantities of viral RNA when the clinical cases are minimal in the community. Various virus concentration methods, including ultracentrifugation, poly(ethylene glycol) (PEG) precipitation, electronegative membrane filtration, adsorption–extraction, and ultrafiltration have been used to concentrate SARS-CoV-2 in wastewater. In the present study, three different adsorption–extraction based virus concentration methods, which have been previously used elsewhere to recover surrogate viruses, were evaluated for their ability to recover BCoV in wastewater samples collected from the WWTPs of Bexar County, Texas. Among the three adsorption–extraction based methods, AE-direct (without pretreatment) showed the highest BCoV recovery in tested wastewater samples. Ahmed et al. (2020) reported that AE-MgCl$_2$ and AE-direct methods had optimal recovery rates for murine hepatitis virus as a surrogate for SARS-CoV-2 concentration in the wastewater samples and also indicated that the AE-MgCl$_2$ method had a slightly higher mean recovery (65.7%) than the AE-direct method (60.5%), differing from the results of our study. However, the authors used 50 mL aliquots of single bulk wastewater sample to evaluate these virus concentration methods, while our study was performed on six different influent samples. The variation observed in our study could be due to differences in characteristics of wastewater and larger treatment volumes processed in our study. A recent study reported similar results for AE-direct method and indicated that electronegative membranes are ideal for the concentration of SARS-CoV-2 in large volume influent samples. Therefore, the AE-direct method of virus concentration was applied to the wastewater samples of this study, and the BCoV recovery rates for these samples ranged from 0.1% to 79%. Feng et al. (2021) reported a similar difference in the BCoV recovery rates (0.89% to 28%) among samples collected from different WWTPs. These findings suggest that a variety of methods are capable of producing reproducible results, though the same SOP or laboratory should be selected to track SARS-CoV-2 trends at a given facility.

During this 32-week study (October 2020 to May 2021), the SARS-CoV-2 N1 and N2 gene amplicons were detected in 62 out of 64 wastewater samples collected from Salitrillo and Martinez-II WWTPs. The concentrations of SARS-CoV-2 RNA in the wastewater samples of two WWTPs varied from $10^2$ to $10^4$ orders of magnitude and followed the trend of COVID-19 clinical cases in the area (Bexar County, Texas), including the capture of November to January surge. Our observations were consistent with other studies in the USA that captured a similar surge in November 2020. Among the SARS-CoV-2 N1 and N2 assays, the N1 assay showed higher sensitivity as it quantified SARS-CoV-2 RNA in more wastewater samples (84.3%) than the N2 assay. Previous reports indicated that the SARS-CoV-2 N1 assay outperformed N2 and other assays targeting M and RdRP genes. Chavarria-Miro et al. (2021) reported that the SARS-CoV-2 N1 assay was able to detect targets in wastewater more consistently than the SARS-CoV-2 N2 assay. Therefore, because of high sensitivity and a positive correlation with the N2 gene targets, SARS-CoV-2 N1 RNA concentrations were selected for further correlation studies.

The raw SARS-CoV-2 N1 concentrations of the wastewater samples collected from Salitrillo and Martinez II WWTPs showed a strong correlation to the weekly average COVID-19 cases in the study area, similar to previous reports for other regions. In this study, the wastewater sample collection was conducted on a weekly basis; however, other studies have
suggested at least 2–3 samples per week for more robust tracking of the virus.\textsuperscript{22,23} During evaluation, the mean recovery efficiency of the AE-direct concentration method was around 16%; however, we obtained much higher values for remaining monitored samples that varied considerably from sample to sample and requires further evaluation. Previous studies recommended the normalization of SARS-CoV-2 RNA concentrations to wastewater characteristics such as fecal strength (human fecal markers like HF183, PMMoV, and crAssphage), per capita, and recovery efficiencies, although these reports indicated mixed results for correlation studies using normalized or recovery-adjusted data.\textsuperscript{8,23,46} Some studies\textsuperscript{40,47} indicated that recovery rate and fecal strength-based normalization had increased the correlation with the clinical data, while other studies\textsuperscript{22,48} reported that correcting with BCoV recovery and normalizing to HF183 reduced correlations of N1/N2 wastewater measurements to COVID-19 cases. Therefore, to assess the impact of recovery rate and fecal strength, the SARS-CoV-2 N1 concentrations in the current study were normalized with the BCoV recovery rates and HF183 copies and compared with the clinical data. A slight reduction in correlation was observed between clinical data and BCoV recovery corrected SARS-CoV-2 N1 concentrations, while HF183 normalization yielded a weaker correlation. The weaker correlation observed between clinical data and HF183 normalized SARS-CoV-2 N1 concentrations in our study indicates that there could be a higher variation in fecal markers and SARS-CoV-2 concentrations in the monitored wastewater samples.\textsuperscript{22}

In this study, the SARS-CoV-2 wastewater surveillance data was determined for a small sewershed region served by two WWTPs and comprising 2–5% of the population of Bexar County, while the COVID-19 case data used was reported for the entire Bexar County. The COVID-19 testing data at the scale of the sewershed regions was not available; and the minor variation in the trend or presence of low concentrations of SARS-CoV-2 RNA at WWTPs compared to cases reported could have been related to disease incidence in other parts of the Bexar County. Thus, wastewater-based epidemiology is particularly useful for tracking disease incidence in small communities, where public testing cannot currently provide real time and comprehensive information about the health of a community due to logistical difficulties and high cost. The limitations of public testing are compounded in low-resource settings and disadvantaged populations where testing every individual is impractical, slow, and cost prohibitive.\textsuperscript{19,49} Therefore, wastewater surveillance may provide more comprehensive data sets covering whole population and accounting for spatial and temporal variability.

Overall, the results of this study suggest that wastewater surveillance of SARS-CoV-2 and other disease biomarkers may provide an alternative and straightforward means of deducing the spread of the disease within the community. We suggest that in epidemiological studies, the use of these methods could provide complementary information. Some of the biggest advantages of using wastewater surveillance for any pathogen or metabolite are that this does not rely on health-seeking behavior, is not limited by health care access issues, and in the case of pathogens might be useful in the presymptomatic and early symptomatic stages if shedding is occurring. Because of this, wastewater surveillance can provide data for public health decision making that is more robust and potentially timelier than current public health data streams.

## CONCLUSIONS

Overall, the findings from this study suggest that wastewater surveillance of SARS-CoV-2 is an effective tool to determine trends in COVID-19 prevalence and provide complementary data to clinical testing. Virus concentration methods based on direct adsorption–extraction using electronegative membranes are effective for concentrating SARS-CoV-2 from wastewater without any pretreatment of samples such as acidification or addition of buffers. RNA can be directly extracted from the filters using commercially available kits. Adjusting the SARS-CoV-2 concentrations using recovery estimates or normalization using fecal markers may not be suitable for inferring wastewater influent data in general and should be assessed for applicability at individual WWTPs. Nonetheless, it is important to maintain a consistent workflow in order to compare results across samples.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00054.

Detailed procedure for RT-ddPCR, RNA recovery efficiency, dimensional analysis for SARS-CoV-2 wastewater concentration calculations, RT-ddPCR raw data, examples of positive and negative RT-ddPCR results, digital MIQE checklist, BCoV recovery efficiency for different virus concentration methods, spiked BCoV recovery estimates and HF183 concentrations, and heatmaps showing raw and adjusted SARS-CoV-2 N1 correlations with COVID-19 cases (PDF)

## AUTHOR INFORMATION

### Corresponding Author

Vikram Kapoor — School of Civil & Environmental Engineering, and Construction Management, University of Texas at San Antonio, San Antonio, Texas 78249, United States; orcid.org/0000-0002-7159-0883; Email: vikram.kapoor@utsa.edu

### Authors

Kiran Kumar Vadde — School of Civil & Environmental Engineering, and Construction Management, University of Texas at San Antonio, San Antonio, Texas 78249, United States

Haya Al-Duroobi — School of Civil & Environmental Engineering, and Construction Management, University of Texas at San Antonio, San Antonio, Texas 78249, United States

Duc C. Phan — School of Civil & Environmental Engineering, and Construction Management, University of Texas at San Antonio, San Antonio, Texas 78249, United States; Present Address: US Salinity Laboratory, USDA-ARS, Riverside, CA 92507, United States; orcid.org/0000-0001-8124-4039

Arash Jafarzadeh — School of Civil & Environmental Engineering, and Construction Management, University of Texas at San Antonio, San Antonio, Texas 78249, United States

Sina V. Moghadam — School of Civil & Environmental Engineering, and Construction Management, University of Texas at San Antonio, San Antonio, Texas 78249, United States
Author Contributions
K.K.V. and H.A. contributed equally to this work. H.A. and V.K. optimized the methods, H.A. and D.C.P. performed the measurements, and K.K.V. processed the experimental data. Statistical analyses were performed by K.K.V. and V.K., and D.C.P., S.V.M., A.J., and A.M. helped in sampling and measurements. K.K.V. and V.K. drafted the manuscript with support from H.A. and D.C.P.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This research was supported in part by the Health Collaborative and the City of San Antonio. We thank the San Antonio River Authority for providing access to wastewater samples. We also thank the 2019-nCoV WBE Slack community for providing guidance and resources that helped shape this study.

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