ABSTRACT: Monitoring wastewater for SARS-CoV-2 from populations smaller than those served by wastewater treatment plants may help identify small spatial areas (subsewersheds) where COVID-19 infections are present. We sampled wastewater from three nested locations with different sized populations within the same sewer network at a university campus and quantified SARS-CoV-2 RNA using reverse transcriptase droplet digital polymerase chain reaction (PCR). SARS-CoV-2 RNA concentrations and/or concentrations normalized by PMMoV were positively associated with laboratory-confirmed COVID-19 cases for both the sewershed level and the subsewershed level. We also used an antigen-based assay to detect the nucleocapsid (N) antigen from SARS-CoV-2 in wastewater samples at the sewershed level. The N antigen was regularly detected at the sewershed level, but the results were not associated with either laboratory-confirmed COVID-19 cases or SARS-CoV-2 RNA concentrations. The results of this study indicate that wastewater monitoring based on quantification of SARS-CoV-2 RNA using PCR-based methods is associated with COVID-19 cases at multiple geographic scales within the subsewershed level and can serve to aid the public health response.

KEYWORDS: SARS-CoV-2, antigen assay, wastewater surveillance, campus monitoring, building level monitoring

1. INTRODUCTION

Many infectious agents are shed in human excreta and deposited into the waste stream; therefore, monitoring a composite biological sample may yield useful information about the health of the contributing population. In the past, concentrations of poliovirus, hepatitis A, and Salmonella measured in wastewater have been used to infer infections in the contributing population. Many people infected with SARS-CoV-2 shed the virus in feces, and during the COVID-19 pandemic, concentrations of SARS-CoV-2 RNA in wastewater influent and settled solids from wastewater treatment plants have been found to correlate strongly to laboratory-confirmed COVID-19 cases in their associated sewersheds. This approach has been increasingly utilized to guide pandemic response by larger entities such as state and local governments and smaller institutions such as colleges and universities, nursing homes, and prisons.

Samples from wastewater treatment plants can provide information about COVID-19 occurrence within a relatively large population. At this scale, wastewater samples are particularly useful when data on laboratory-confirmed COVID-19 cases are limited or unreliable or when monitoring for a rare event such as the introduction of a new variant into a community. Monitoring wastewater upstream from plants, at the subsewershed or building level, may help identify small spatial areas where infections are present among a smaller population to focus a more targeted public health response or investigate the sources of outbreaks. Much of the literature to date on wastewater monitoring has focused on implementing different monitoring approaches at the sewershed level, but smaller scale monitoring has also been successful.

Greenwald et al. found SARS-CoV-2 RNA concentrations correlated with incident COVID-19 in subsewershed wastewater from ~10^5 people and in prison wastewater with ~10^4 incarcerated individuals. Crowe et al. tested wastewater at urban K–12 schools with ~10^3 people and found detection of SARS-CoV-2 RNA was consistent with the presence of...
COVID-19 infections on campus as determined by saliva tests. Many smaller scale efforts have focused on detection of COVID-19 cases in individual buildings, particularly with the goal of identifying asymptomatic COVID-19 cases. Ahmed et al.9 showed that detection of SARS-CoV-2 RNA in aircraft wastewater can indicate the presence of onboard infections in flights with approximately 20–190 passengers, and wastewater monitoring has also been used to detect infections at nursing homes with 70–200 residents10 and even at correctional facilities.11 A number of studies with populations ranging from 70 to 300 people have illustrated how SARS-CoV-2 monitoring at university campuses might inform pandemic response.12−17 In more than one case, programs report detection of single asymptomatic COVID-19 cases in wastewater (as confirmed by diagnostic testing).13,18 While these studies consistently indicate that SARS-CoV-2 RNA in wastewater reflects the presence and rates of community infection, little has been done to compare nested sampling at different scales. Studies focused on small populations also differ in their approach to the interpretation of data and, in some cases, provide only qualitative assessments of agreement.

At all scales, sewershed or subsewershed, wastewater surveillance for SARS-CoV-2 RNA monitoring has primarily used polymerase chain reaction (PCR)-based methods for quantification of SARS-CoV-2. However, other methods such as antigen-based detection methods may be considered for use in wastewater. SARS-CoV-2 antigen tests were approved by the U.S. Food and Drug Administration (FDA) for use on nasopharyngeal or nasal swab samples. These immunoassays detect the nucleocapsid (N) protein from SARS-CoV-2 in a clinical specimen. Pollock et al.19 demonstrated a strong correlation between antigen concentrations and RNA concentrations in clinical nasopharyngeal swab samples using the S-PLEX antigen assay.19 Previous work applied immunoassays to detect rotavirus antigens in urban and hospital sewage disposal systems, suggesting such methods may be used for other viruses.20 Antigen-based assays provide quick results and require minimal preanalytical steps that could provide a valuable alternative for monitoring SARS-CoV-2 in wastewater.

The goal of this study is to investigate how the scale of the area and size of the population being monitored via wastewater affects the utility of this approach to track community infection dynamics by monitoring multiple nested areas within a university campus. A second goal is to apply methods for measuring the SARS-CoV-2 N antigen in wastewater and investigate the relationship with SARS-CoV-2 RNA concentration and laboratory-confirmed COVID-19 cases. We tested wastewater from a small community (10 000 people) by collecting wastewater diverted from a large sewer main on campus, a sampling site representing a smaller group of people present in a multiple-building cluster, and a sampling site representing an even smaller group of people at a single building.

2. METHODS

2.1. Sampling Scheme. Raw wastewater samples were collected from within the Stanford University campus (Stanford, CA) from January 15 to April 25, 2021. Three nested locations within the same sewer network were selected to cover different spatial scales: (1) campus level, (2) multiple-building level, and (3) individual-building level. The sampling period included the first week of the new term through the middle of spring quarter. The full sampling schedule can be found in Table S1.

For the campus level (representing a small community, hereafter termed level 1), samples representing a large portion of campus were taken from a pilot scale wastewater treatment facility, the Codiga Resource Recovery Center (CR2C). CR2C treats approximately 0.5 million gallons per day (MGD) of wastewater and receives sewage from within the Stanford University campus (Figure 1). CR2C serves a small community of approximately 10 000 people and 181 buildings, including academic buildings and student and faculty housing. Sample collection consisted of 24 h composite samples from the influent line post-grit removal 4 days per week for the entire sampling period. The temperature-controlled autosampler was set at 4 °C and collected approximately 300 mL of sample every 30 min.

The second sampling location represents a cluster of student residence buildings (Building Cluster, representing multiple-building level monitoring and hereafter termed level 2). The Building Cluster drainage area is composed of approximately 500 people. All sewage from the Building Cluster drains to a manhole within the community served by CR2C, where an autosampler was installed and programmed to collect 24 h composite samples. The non-temperature-controlled autosampler collected samples every 15 min into a single container. Three 24 h composite samples were collected per week for 10 weeks from January 15 to March 26, 2021.

The third sampling location was a single student residence building (Residence Building, representing individual-building level monitoring and hereafter termed level 3) that housed approximately 200 people. This building was not represented in the Building Cluster (level 2) and did not contribute to those samples. Sewage from the Residence Building can be...
Table 1. Sampling Scheme Summary

| level               | area                                      | frequency (samples/week) | collection duration (weeks) | sample type                                                                 |
|---------------------|-------------------------------------------|--------------------------|-----------------------------|-----------------------------------------------------------------------------|
| level 1 (campus level) | Codiga Resource Recovery Center (CR2C)   | 4                        | 14                          | 24 h composite post-grit influent wastewater autosampler in influent line    |
| level 2 (multiple-building level) | Building Cluster                        | 3                        | 10                          | 24 h composite raw wastewater autosampler in one manhole                     |
| level 3 (individual-building level) | Residence Building                     | 3                        | 10                          | 24 h composite raw wastewater autosampler in two manholes                   |

accessed using three manholes within CR2C’s sewershed, two of which access sewage exclusively from the building. Two autosamplers were installed and programmed to collect 24 h composite raw sewage samples from these two manholes in the same manner that was used for the Building Cluster sampling. These two samples were combined in equal volume proportions into a single sample representing the Residence Building. Three 24 h composite samples were collected per week. Sampling for this location lasted 10 weeks, from January 15 to March 26, 2021. Table 1 shows a summary of the sampling scheme.

2.2. PCR Preanalytical Processing. Upon collection, samples were stored for up to 1 week at 4 °C until processing and were processed together on a weekly basis with results available 3–4 days after the start of processing. The method evaluation in the piloting stages of the study showed that there was greater detection of SARS-CoV-2 RNA levels in samples kept at 4 °C than in those samples that underwent a freeze/thaw cycle. Each week one sample was chosen randomly and processed in replicate. Processing consisted of viral concentration, RNA extraction, and droplet digital PCR (ddPCR) for quantification. Samples were concentrated using a modified version of the PEG precipitation method described by Graham et al., adapted so that solids suspended in the wastewater were not removed from the wastewater, and each 40 mL sample was spiked directly with a bovine coronavirus (BCoV) vaccine strain (Calf-Guard Cattle Vaccine, PBS Animal Health, Saint Henry, OH). The method continued as described by Graham et al.; flocculation agents PEG 8000 and NaCl were added to each sample and left to incubate overnight. After incubation, samples were centrifuged at 20000g for 30 min. Twenty milliliters of supernatant was removed without disturbing the pellet of solids. The remaining 20 mL of sample in the tube was centrifuged at 20000g for 20 min. The supernatant was decanted until only a thin pellet of solids remained at the bottom of the tube, and 200 μL of 1X PBS was added to the pellet and vortexed for resuspension. This 200 μL concentrated sample was transferred to a bead tube for nucleic acid extraction steps. If the concentrated sample had a volume of >200 μL, then 200 μL was transferred to a bead tube and the total volume was recorded for use in dimensional analysis to calculate the final concentration of viral targets as done by Graham et al. The concentration steps that follow overnight incubation and the nucleic acid extraction steps were completed on the same day to avoid degradation of the samples and preserve low detection limits.

RNA was extracted from viral concentrates, BCoV material used to spike the samples, and an extraction blank using the Qiagen AllPrep PowerViral DNA/RNA kit and QiaCube Connect (Qiagen, Hilden, Germany) system with a final elution volume of 100 μL. After RNA extraction, samples were treated with Zymo OneStepPCR kit inhibitor removal columns (Zymo Research, CA). The eluted RNA was stored at −80 °C.

2.3. PCR Quantification. Samples were analyzed via one-step reverse transcriptase droplet digital PCR (RT-ddPCR) using Bio-Rad SARS-CoV-2 droplet digital PCR kits and a Bio-Rad QX200 AutoDG droplet digital PCR system (Bio-Rad, Hercules, CA). Full details of the assays are available in ref 4 and Table S2. Samples were analyzed for two SARS-CoV-2 targets (N1 and N2, run as a duplex assay), a spike in recovery control (BCoV), and an endogenous viral control [pepper mild mottle virus (PMMoV)] run as a duplex assay (B/P assay). For the N1/N2 plates, samples and positive controls were run in triplicate; for the B/P plates, samples and positive controls were run in duplicate. The SARS-CoV-2 positive control was RNA from a nasopharyngeal swab of a clinical patient from Stanford Hospital. The BCoV positive control used was extracted RNA from a reconstituted BCoV vaccine diluted in molecular grade water to 10^6 copies/mL, and the PMMoV positive control was a synthetic DNA ultramer [Integrated DNA Technologies (IDT), Coralville, IA]. RNA from the reconstituted BCoV vaccine used to spike the samples was also included on the B/P plate to determine the concentration spiked.

Samples were processed for N1/N2 and B/P undiluted and at a 1:10 dilution. SARS-CoV-2 positive controls were processed at a 1:10 dilution, and BCoV positive controls (the vaccine used to spike samples) at 1:100 and 1:1000 dilutions. A summary of the setup for both ddPCR plates can be found in Table S3. Replicate wells were merged for data analysis and thresholded manually using QuantaSoft and QuantaSoft Analysis Pro (Bio-Rad) as described in ref 4 and as instructed in the Bio-Rad SARS-CoV-2 ddPCR Kit Instructions for Use (ref 12013743). Merged wells were required to have at least 10 000 droplets. To be scored as positive for a target, three or more positive droplets were required in the merged wells. Dimensional analysis was used to convert concentrations per reaction to copies per volume of the original sample.

2.4. SARS-CoV-2 Nucleocapsid (N) Antigen Detection. The first 10 weeks of samples collected for level 1 (campus level) were also processed using an antigen-based assay. Samples for all 10 weeks were assayed at the same time and stored at 4 °C prior to analysis. To determine the best way to prepare samples, four samples were assayed both by utilizing the whole sample without centrifugation and by utilizing the supernatant after centrifugation to remove solids, and results...
were compared (Figure S1). The full set of samples was prepared by transferring 1 mL of each sample into a microcentrifuge tube and centrifuging at 16,000 rcf for 3 min. Clarified samples were then assayed undiluted and as 1:10 and 1:100 dilutions.

SARS-CoV-2 nucleocapsid (N) antigen (hereafter termed N antigen) was detected in the clarified raw wastewater samples using S-PLEX SARS-CoV-2 N Kits [Meso Scale Diagnostics (MSD), Rockville, MD]. First, S-PLEX 96-well SECTOR plates were coated with biotinylated capture antibodies and incubated for 1 h at room temperature (RT) or overnight at 4 °C. Plates were then blocked using the MSD blocking solution, and 25 μL of the sample was immediately added to each well. A seven-point calibration curve (ranging from 1.09 × 10^6 to 2.66 × 10^7 fg/L) and a negative control consisting of assay diluent were run in duplicate on each plate. Following sample incubation at RT for 1.5 h, the detection antibody was added (MSD TURBO-BOOST Detection Antibody) and incubated for 1 h at RT. An enhancement step was performed by using the MSD S-PLEX Enhance solution with a 30 min incubation at RT. Subsequently, the detection solution (TURBO-TAG Detection Solution) was added. After incubation for 1 h at 27 °C and addition of MSD GOLD Read Buffer B, the plates were read using a MESA QuickPlex SQ 120 Reader.

All incubations were performed in a plate shaker at 700 rpm. Each incubation step was followed by a washing step, consisting of three washes in MSD Tris Wash Buffer using the BioTek 405 Select automated 96-well plate washer (BioTek, Winooski, VT). The raw signal was converted to a concentration per volume of wastewater based on linear regression to the calibration curve.

2.5. COVID-19 Case Data. Data for laboratory-confirmed COVID-19 cases (hereafter termed COVID-19 cases) were provided by Stanford University from their system for tracking COVID-19 among students. COVID-19 case counts are a function of test report date, which is reported to lag 10–24 h behind sample collection. During the period of the study, students living and working on campus were required to participate in asymptomatic screening with PCR-based tests once or twice per week. Students were also tested when presenting COVID-19 symptoms, and both types of testing results were included in the Stanford data set with each case counted only once. COVID-19 cases were aggregated at each level on the basis of whether the location of their residence or quarantine location was within the sewershed (delimited using a GIS shape file), within the building cluster, or in a wing of the building that was sampled, respectively. These data do not include nonstudents residing in the sewershed. The Codiga sewershed includes housing for faculty and staff; individuals were not represented in the data. Data provided by the state through data use agreements were also considered to identify COVID-19 cases in the faculty housing areas of campus for which testing was not mandated; none were identified (details in the Supporting Information). This work is covered by Stanford Institutional Review Board (IRB) approval (IRB-59746) for human subject research.

2.6. Statistical Analysis. Statistical analysis was completed using RStudio version 1.4.1717. Daily COVID-19 cases associated with each sampling level were smoothed to a three-day centered running average to match with wastewater samples. A three-day centered running average was selected to account for the uncertainty in the case start date affiliated with a new COVID-19 case and to complement the sampling frequency of three or four times per week to capture the new cases that were reported around a wastewater collection day. All references to COVID-19 cases in statistical analysis refer to the three-day centered running average. SARS-CoV-2 RNA and N antigen concentration data were tested for normality using the Shapiro–Wilk test. Data for N1 and N2 gene concentrations and N antigen were not normally distributed (details in the Supporting Information). Kendall’s τ was used to test hypotheses of association between wastewater measurements and COVID-19 case data. For the association between RNA concentration and COVID-19 cases, a bootstrapping method for Kendall’s τ was utilized to determine the median Kendall’s τ and empirical p value from 1000 resamplings between the upper and lower confidence intervals to incorporate error as done by Wolfe et al. When a wastewater sample was below the lowest detectable concentration (LDC), the upper confidence interval was replaced with the LDC for bootstrapping and the lower replaced with zero. The correlation coefficient between RNA concentrations normalized by PMMoV and COVID-19 cases was also determined using the bootstrapping method.

3. RESULTS

3.1. Summary of Wastewater Measurements. Concentrations of SARS-CoV-2 RNA across all samples ranged from not detected (ND) to 2.35 × 10^4 gene copies (gc)/L for N1 and from ND to 3.32 × 10^4 gc/L for N2. The lowest detectable concentration (LDC, assuming three positive droplets across replicate wells) for N1 and N2 was 2.20 × 10^2 gc/L. N antigen concentrations (measured for only level 1) ranged from ND to 1.23 × 10^6 fg/L. The lowest detectable concentration for N antigen was 7.66 × 10^5 fg/L. Positive and negative controls were positive and negative, respectively, as expected. Recovery of BCoV using the PEG method was >0% for all samples but was not utilized beyond this given the challenges with spiked recovery controls in wastewater. The median recovery across all samples was 18.7% (n = 127), and additional recovery information can be found in the Supporting Information (Figure S2). Samples would be omitted from the statistical analysis if they had 0% recovery and <10^5 gc/L PMMoV, all samples met the criteria for inclusion. The median PMMoV was 2.79 × 10^6 gc/L across all samples (n = 127, min = 1.28 × 10^5 gc/L, max = 1.17 × 10^8 gc/L, level 1 median = 3.01 × 10^5 gc/L, level 2 median = 1.29 × 10^6 gc/L, level 3 median = 2.80 × 10^7 gc/L) (Figure S3). Wastewater data are available publicly at the Stanford Digital Repository; results below are reported as suggested in the EMMI guidelines, and MIQE reporting details are included in Table S5 and Figures S4 and S5.

3.2. Relationship between Wastewater Measurements and COVID-19 Case Data. 3.2.1. Level 1 (campus level). A total of 45 COVID-19 cases occurred among students residing in the larger subsewershed area during the study, and a maximum of three positive COVID-19 cases occurred on a single day. COVID-19 cases were reported on 34.8% of wastewater sample collection days (n = 66) for level 1. For wastewater, 23 of 66 of samples were above the LDC for N1 and 17 of 66 above the LDC for N2 (Table 2). The median detected concentration was 1.68 × 10^3 gc/L for N1 and 1.57 × 10^4 gc/L for N2 (n = 66, min detected N1 = 4.97 × 10^2 gc/L, max N1 = 2.03 × 10^4 gc/L, min detected N2 = 6.06 × 10^3 gc/L, max N2 = 3.31 × 10^4 gc/L), and they were highest at the beginning and end of the sampling period (Figure 2). Kendall’s
The correlation coefficients between gene concentrations and COVID-19 cases at level 1 were not significantly different from 0 for N1 ($\tau = 0.08$, $p = 7.30 \times 10^{-2}$) and positive and significant for N2 ($\tau = 0.15$, $p = 8.00 \times 10^{-3}$). Normalizing N1 and N2 gene concentrations by PMMoV concentrations did not substantially change the correlation coefficients (Table S4).

3.2.2. Level 2 (multiple-building level). During the study, one COVID-19 case was recorded among residents in the level 2 building cluster. For wastewater, 4 of 29 samples were above the LDC for N1 and 1 of 29 was above the LDC for N2 (Table 2). The median detected concentration was $1.07 \times 10^4$ gc/L for N1 ($n = 29$, min detected N1 = $9.90 \times 10^2$ gc/L, max N1 = $1.58 \times 10^4$ gc/L), and that for the only sample detected for N2 was $1.15 \times 10^4$ gc/L, with the highest concentrations identified at the beginning of the sampling period (Figure 2). Kendall’s $\tau$ correlation coefficients between gene concentrations and COVID-19 cases at level 2 were positive and significant for N1 ($\tau = 0.34$, $p = 0$) but not significantly different from 0 for N2 ($\tau = -0.01$, $p = 5.05 \times 10^{-1}$). Normalizing N1 and N2 gene concentrations by PMMoV concentrations did not substantially change the correlation coefficients (Table S4).

3.2.3. Level 3 (individual-building level). A total of four COVID-19 cases were reported in the building during the study, and a maximum of two COVID-19 cases occurred on a single day. COVID-19 cases were reported on 6.25% of sample collection days ($n = 32$) for level 3. For wastewater, 10 of 32 samples were above the LDC for N1 and 7 of 32 were above the LDC for N2 (Table 2). The median detected concentration was $5.11 \times 10^3$ gc/L for N1 and $6.75 \times 10^3$ gc/L for N2 ($n = 32$, min detected N1 = $6.34 \times 10^2$ gc/L, max N1 = $2.35 \times 10^4$ gc/L, min detected N2 = $1.06 \times 10^3$ gc/L, max N2 = $3.32 \times 10^4$ gc/L), with the highest concentrations present at the beginning of the sampling period (Figure 2). Kendall’s $\tau$ correlation coefficients between gene concentrations and COVID-19 cases at level 3 were not significantly

| level       | total no. of COVID-19 cases during the study time frame | days with COVID-19 cases/total days of study | positive samples/total no. of samples | positive samples/total no. of samples | positive samples/total no. of samples |
|-------------|--------------------------------------------------------|------------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| level 1     | 45                                                     | 23/66                                    | 23/66                               | 17/66                               | –                                   |
| level 2     | 1                                                      | 1/29                                     | 4/29                                | 1/29                                | –                                   |
| level 3     | 4                                                      | 2/32                                     | 10/32                               | 7/32                                | –                                   |
| level 1 (antigen only) | 16                                                     | 16/44                                    | –                                   | –                                   | 16/44                               |

Table 2. Laboratory-Confirmed COVID-19 Cases and Numbers of Positive Detections in Wastewater Measurements

Figure 2. Time series for level 1 (left), level 2 (center), and level 3 (right) time series. COVID-19 cases at each level (top) and corresponding N1 (middle) and N2 (bottom) RNA gene concentrations. Error bars represent the standard deviation as the total error reported by the ddPCR instrument. Dashed lines in N1 and N2 graphs represent the LDC.

θ correlation coefficients between gene concentrations and COVID-19 cases at level 1 were not significantly different from 0 for N1 ($\tau = 0.08$, $p = 7.30 \times 10^{-2}$) and positive and significant for N2 ($\tau = 0.15$, $p = 8.00 \times 10^{-3}$). Normalizing N1 and N2 gene concentrations by PMMoV concentrations did not substantially change the correlation coefficients (Table S4).
different from 0 for N1 (τ = −0.01, p = 5.16 × 10⁻¹) and not significantly different from 0 for N2 (τ = 0.04, p = 3.42 × 10⁻¹). Normalizing N1 and N2 gene concentrations by PMMoV concentrations did not substantially change the correlation coefficients, but results for N2 became statistically significant when normalized by PMMoV (τ = 0.14, p = 3.70 × 10⁻²) (Table S4).

3.2.4. N Antigen Data. Samples collected during the first 10 weeks of the study were analyzed for N antigen concentration. A total of 16 COVID-19 cases occurred among students residing in the community during the study, and a maximum of three positive COVID-19 cases occurred on a single day. COVID-19 cases were reported on 36.3% of sample collection days for level 1 N antigen testing (n = 44). For wastewater, 16 of 44 samples were above the LDC for the N antigen (Table 2). The median detected concentrations for the N antigen was 1.86 × 10⁵ fg/L (n = 44, min detected = 7.66 × 10⁴ fg/L, max = 1.23 × 10⁶ fg/L) and were highest at the end of the sampling period (Figure 3). There was no significant association between N antigen concentrations and COVID-19 cases (τ = −0.049, p = 7.04 × 10⁻¹) (Table S4) or between N antigen concentrations and N1 and N2 concentrations (for N1, τ = −0.013 and p = 9.17 × 10⁻¹; for N2, τ = −0.069 and p = 5.97 × 10⁻¹).

4. DISCUSSION

This study implemented wastewater monitoring for SARS-CoV-2 RNA in catchment areas with small, nested populations (ranging from approximately 100 to 10000 people) and a small number of COVID-19 cases (maximum of three COVID-19 cases per day in the 10 000-person sewershed) at a university campus using samples of 24 h composite wastewater. This study was performed alongside a COVID-19 clinical screening program for students that provided thorough and frequent case data that included laboratory-confirmed asymptomatic cases. The correlation analysis between COVID-19 case data and wastewater monitoring data was thus deemed appropriate due to the robust clinical testing program in place, although this may not always be the best way to assess the performance of wastewater programs given that many COVID-19 cases may not be captured by clinical testing. SARS-CoV-2 was detected using two methods (PCR-based and antigen-based); however, results from the antigen-based method were not associated with laboratory-confirmed COVID-19 cases. SARS-CoV-2 RNA was detected in wastewater and associated with COVID-19 cases at all levels: campus level, multiple-building level, and individual-building level.

These results are consistent with those reported by other studies indicating that concentrations of SARS-CoV-2 RNA in wastewater are associated with COVID-19 cases at the sewershed level. Normalizing N1 and N2 gene targets ranged between ND and 3.31 × 10⁴ gc/L and are also consistent with concentrations found in studies that have comparable sample collection methods and COVID-19 incidence rates and studies with similar preanalytical methods and sewershed sizes. While association significance varied between N1 and N2, we did not observe a pattern associated with this difference, and previous work has found that the N1 assay is more sensitive than the N2 assay once concentration levels are near the limit of detection. In this study, normalizing SARS-CoV-2 RNA concentrations by PMMoV RNA concentrations did not substantially change the association between COVID-19 cases and wastewater measurements. This is in contrast to results reported by Scott et al. and D’Aoust et al. that normalization by this biomarker improved the association between wastewater and incidence data, although other studies concur with our results and did not find this improvement when normalizing with PMMoV. One consideration is that the applicability of this type of normalization might be situational. Wolfe et al. showed that normalizing by PMMoV allows for comparison of measurements made using diverse methods as it likely corrects for changes in recovery across the methods. Simpson et al. show that normalizing by PMMoV may also aid in controlling for RNA degradation in samples stored for different lengths of time. Therefore, normalizing by PMMoV may be the most useful when comparing measurements across studies or methods and serve as an internal process control.

To the best of our knowledge, this is the first study to report SARS-CoV-2 N antigen detection in wastewater. The antigen-based assay was used to detect the nucleocapsid (N) protein from SARS-CoV-2, which may be found from viral capsids that are intact or not intact. Antigen-based assays are attractive because they require very minimal preanalytical processing and results are available quickly. While the N antigen was regularly detected at the sewershed level, the results were not associated with either COVID-19 cases or SARS-CoV-2 RNA concentrations.

Although higher antigen concentrations were observed during a no-case period, we do not believe that this corresponds to cases that were unaccounted for, as the clinical

![Figure 3. Level 1 time series. COVID-19 cases associated with CRC2 sewershed (top) and N antigen concentration (bottom).](https://doi.org/10.1021/acsestwater.2c00050)
testing program included asymptomatic screening. Thus, we conclude that these differences are likely due to fate, transport, and storage considerations. Preliminary testing suggested that the protein was detectable in wastewater after storage, but it is possible that this target is less stable in wastewater than expected. We note that all samples were processed together at the end of the sampling period (stored for approximately 10 weeks) and concentrations increase toward the end of the study period. Further analysis is needed to determine whether decay of the N antigen is significant in wastewater on this timescale and if these methods can be optimized for improved detection.

Despite COVID-19 case data that included asymptomatic screening, a significant limitation of this study is that COVID-19 case data reflected only the student population and did not include faculty or staff living or working in each catchment area. It is possible that additional COVID-19 cases that were not counted may have contributed to the wastewater, including staff living or working in the buildings, students who did not participate in testing, or guests of students. It is also possible that individuals with COVID-19 may continue to shed SARS-CoV-2 RNA that is detectable in wastewater even after they have cleared their respiratory disease. Another limitation present in the study was that the autosamplers, at times, were clogged by tissue paper or malfunctioned, and this resulted in failure to collect some sample points and potentially prevented the collection of a true 24 h composite sample (Table S1).

5. CONCLUSIONS

SARS-CoV-2 RNA concentrations and/or concentrations normalized by PMMoV were positively associated with COVID-19 case data for the campus, multiple-building, and individual-building level, suggesting that wastewater monitoring may be a useful tool for tracking and investigating outbreaks at any of these levels. The choice of level at which to sample may therefore depend on both the primary purposes of monitoring and the resources available for testing. Building cluster and building level testing may be primarily useful for investigating the sources of outbreaks and for buildings that have a consistent and known population (e.g., a dormitory or school). Although the study began during a significant surge in COVID-19 cases in the area, relatively few COVID-19 cases were identified on the smaller monitoring scales (building cluster and building levels). If many buildings need to be independently monitored to cover the population of interest, this level of monitoring becomes very resource intensive. Recent research shows that composite sampling, as was done for this study, is important for capturing signals that reflect COVID-19 cases at this smaller scale. If building level monitoring is required, passive sampling approaches may be considered to achieve sensitive sampling at a more attainable price point while sacrificing the ability to quantify the target. By contrast, monitoring at the small community level (such as campus level) may allow for effective monitoring of COVID-19 cases at a scale that is large enough to provide a more sustainable option for stakeholders while also allowing for a targeted response. The small community represented at the highest level of sampling in this study still represents only a portion of the university campus, and multiple sampling points of a similar size can be used in the future to monitor disease occurrence in parts of a larger community such as a city.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00050.

Additional details about methods, COVID-19 incidence data, and results (Tables S1–S5 and Figures S1–S5) (PDF)

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Notes

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