Comparative Analysis of RNA-Extraction Approaches and Associated Influences on RT-qPCR of the SARS-CoV-2 RNA in a University Residence Hall and Quarantine Location

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ABSTRACT: Wastewater-based epidemiology (WBE) provides an early warning and trend analysis approach for determining the presence of COVID-19 in a community and complements clinical testing in assessing the population level, even as viral loads fluctuate. Here, we evaluate combinations of two wastewater concentration methods (i.e., ultrafiltration and composite supernatant—solid), four pre-RNA extraction modifications, and three nucleic acid extraction kits using two different wastewater sampling locations. These consisted of a quarantine facility containing clinically confirmed COVID-19-positive inhabitants and a university residence hall. Of the combinations examined, composite supernatant—solid with pre-RNA extraction consisting of water concentration and RNA/DNA shield performed the best in terms of speed and sensitivity. Further, of the three nucleic acid extraction kits examined, the most variability was associated with the Qiagen kit. Focusing on the quarantine facility, viral concentrations measured in wastewater were generally significantly related to positive clinical cases, with the relationship dependent on method, modification, kit, target, and normalization, although results were variable-dependent on individual time points (Kendall’s Tau-b (τ) = 0.17 to 0.6) or cumulatively (Kendall’s Tau-b (τ) = −0.048 to 1). These observations can support laboratories establishing protocols to perform wastewater surveillance and monitoring efforts for COVID-19.

KEYWORDS: wastewater-based epidemiology, SARS-CoV-2, wastewater concentration, nucleic acid extraction, correlation

1. INTRODUCTION

Wastewater-based epidemiology (WBE) is a form of environmental public health surveillance that has been frequently applied in the context of monitoring drug usage, including pharmaceuticals and illicit drugs, and their impact on wildlife (for examples, see refs 1–3) but has also been applied in the context of monitoring infectious disease outbreaks. The use of WBE as an environmental monitoring tool for diseases was recently reviewed4 and has been used in the environmental surveillance of polio from the 1980s. Within the context of the COVID-19 pandemic, the application of WBE received renewed attention, following reports that SARS-CoV-2 could be excreted in feces.5

First reported by The Netherlands,6 the application of WBE to track COVID-19 spread and outbreaks at the community level rapidly expanded to other countries including Australia,7 Japan,8 and the United States,9,10 with many recognizing, reviewing, and summarizing its application as a global collaborative to maximize contributions in the borderless fight against COVID-19.11–13 Surveillance and monitoring provide essential environmental public health services (https://www.cdc.gov/nceh/ehs/10-essential-services/index.html) and have been at the forefront of the global epidemic response to SARS-CoV-2, with wastewater representing an innovative technique with limited sociological bias, as well as few ethical issues due to the aggregated population sampling.14,15 Reports of WBE outperforming case numbers as a complementary tool to track pandemic trajectory,16 particularly when diagnostic testing is limited and/or ineffectively and inequitably delivered, has resulted in the increased use of this approach to support public health policies in the assessment, prioritization, and mitigation of viral outbreaks across population scales.14,17–20 Likewise, it has also been used in the allocation of testing resources, evaluation of possible irregularities in traditional surveillance, refinement

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of health messaging, and forecasting of clinical resource requirements at the community level. For example, Corchis-Scott et al.\textsuperscript{21} reported on the results of WBE testing that triggered mass testing at a dormitory, resulting in the identification of three B.1.1.7 variant positive cases (two of which were asymptomatic), and resulting in quick relocation to a quarantine facility in an effort to avert an outbreak on campus.

Most early studies of SARS-CoV-2 RNA in wastewater have focused on wastewater influent, where comprehensive individual PCR screening may not be possible. Yet early case detection, rapid infection control measures, and contact tracing are key in decreasing the transmission of COVID-19. Herein, the role of wastewater testing at smaller community levels is particularly important. Lee et al. (2021) reported on an ongoing study investigating the use of wastewater testing at a long-term care facility, where globally vulnerable populations with high morbidity and mortality have been particularly impacted by the pandemic. In the context of smaller communities, sampling at university dormitories represents a growing area of research and public health practice. Monitoring the upstream sewer network at the dormitory or building level allows for enhanced spatial resolution and enables more targeted surveillance and response efforts. Several studies have demonstrated the application of such an approach at universities,\textsuperscript{22−24} with Bivins and Bibby\textsuperscript{25} demonstrating its applicability both during and after mass vaccination on a college campus. On the basis of the wide diversity in reporting from 25 U.S.-based universities, Harris-Lovett et al.\textsuperscript{23} developed a conceptual framework for designing campus wastewater monitoring systems, although it was noted that further work is still required.

As the COVID-19 pandemic has evolved, it has dictated that the design of wastewater surveillance should be a collaborative learning and adaption process, whereby it is important to revisit whether initially identified information needs are being met and, if not, what can be adjusted to do so. In this respect, ongoing issues of supply chains of numerous laboratory consumables, especially those necessary for RNA extraction, remain a constant challenge. Consequently, various methods focused on wastewater RNA extraction have emerged that aim to make WBE more accessible. Initially, ultrafiltration methods were prominently reported in the literature;\textsuperscript{6,7,26} however, supply chain issues with these items quickly saw the emergence of other methods, some of which have been compared and contrasted.\textsuperscript{27−29} Importantly, while significant variation exists in concentration methods, targets, volumes, etc. (reviewed in ref\textsuperscript{14}), interlaboratory comparisons of various methods almost uniformly report that diverse methods are capable of producing reproducible results.\textsuperscript{30,31}

Figure 1. Experimental outline in the present study.

![Experimental outline in the present study.](https://doi.org/10.1021/acsestwater.1c00476)
Yet, surrogate enveloped viruses in wastewater have been shown to adsorb to the solid fraction of wastewater up to 26%, with a recent study identifying ~23% of the detected SARS-CoV-2 would be discarded during the debris-removal step. Further, a recent study identified that over 50% of the N1 target adsorbed to the wastewater solids fraction, with another study identifying that settle solids contain up to ~1000 times more SARS-CoV-2 RNA per mass basis. Additionally, recovery from ultrafiltration devices has also been reported at ~30% when seeded with mouse hepatitis virus (MHV) at the prefiltration step, suggesting significant possible underreporting based on the use of surrogate enveloped viruses. In this respect, emerging evidence supports the inclusion of solids in the liquid fraction as it performs better than those which exclude this fraction, with a recent meta-analysis also supporting this observation.

As part of optimizing procedures for upcoming WBE monitoring, we examined and compared the performance of four different nucleic acid stabilization methods (including no treatment) in addition to three nucleic acid extraction kits for the detection of SARS-CoV-2 RNA in wastewater at a university dormitory of unknown COVID-19 prevalence. To confirm and validate these results, the various pipelines were also assessed using samples collected in parallel from a quarantine facility containing clinically confirmed cases. We specifically aimed to identify viral stabilization solutions and kits that maximize RNA recovery in the form of Ct values from a given wastewater sample to further strengthen diagnostic protocols for the detection of SARS-CoV-2 in wastewater, even as levels fluctuate.

2. EXPERIMENTAL METHODS

2.1. Sample Collection. As part of diverse university responses to the COVID-19 pandemic, WBE was implemented in dormitory and quarantine locations. Composite dormitory wastewater samples (24 h) were collected once a week between January and March 2021 using an ISCO 6700 series automatic sampler (Teledyne ISCO, Lincoln, NE, U.S.A.), where wastewater was time-composited to a storage container in ice to maintain samples at or below 4 °C until collection. Thus, the assigned sample date to a 24-h composite sample was the date on which the sample was collected. On the basis of recommendations by the Research Coordination Network (RCN) for standardization of wastewater surveillance for SARS-CoV-2, associated study parameters can be found in Table S6 in the Supporting Information. Following collection, the composite samples were transported on ice to the laboratory for immediate processing.

As the experiment contains several steps and various factors under investigation, a schematic of the experiment is provided in Figure 1. For examination of the impact of prenucleic acid extraction modifications on RNA quantification from wastewater, one residential facility (referred to as “Dormitory A”) at Baylor University was chosen and sampled weekly over a period of January–March 2021. Because human viruses in sewage often show a nonhomogenous distribution, particularly when viral concentrations are low, a second facility containing PCR-positive inhabitants, referred to as “quarantine facility”, was sampled in parallel to confirm trends, although this facility served as an isolation facility predominantly rather than quarantine. The number of inhabitants of Dormitory A was ~539, while inhabitants of the quarantine facility varied based on the sampling week and ranged from 22 to 65.

Prior to downstream analysis, wastewater samples (200 mL) were centrifuged at 4 000g for 20 min at 4 °C to pellet solids. The resulting clarified supernatant (1) (~150 mL) was decanted into aliquots of 45 mL, and the resulting supernatant (~90 mL) was concentrated using Centricon Plus-70 centrifugal ultrafiltration (CeUF) devices, with a molecular weight cutoff of 30 kDa (Millipore, MA, U.S.A.). The residual volume of ~60 mL was discarded. Due to supply chain issues, recovery could only be estimated in some of the quarantine facility samples (n = 2). To characterize potential sample loss during the concentration process, matrix effects among the methods from the concentration steps were evaluated using a surrogate envelope virus, viz., attenuated bovine coronavirus (BCoV) vaccine (Calf-Guard, Zoetis, NJ, U.S.A.), that was shipped lyophilized was resuspended in 3 mL of RNase/DNase-free water on the sampling day and stored at 4 °C until use. This stock was also later used to evaluate loss during the RNA extraction process, viz., estimation of recovery in nonwastewater samples. To each wastewater sample, 1 mL of resuspended BCoV stock was spiked into the clarified supernatant volume of 90 mL prior to concentration. Ultrafiltration devices were pretreated to block virus adsorption using 2 mL of bovine serum albumin (1% w/v) in phosphate-buffered saline (PBS); 5 min at room temperature) and then washed with 70 mL of deionized (DI) water. Filtrate (which had been spiked with 1 mL of BCoV) was loaded on ultrafiltration devices in increments and centrifuged for 10–15 min at 3 500g (19 °C). The flow-through was discarded, and the samples were concentrated to a final volume of 10 mL, which was subsequently aliquoted into concentrate stocks of 200 μL for later RNA extraction. In parallel to the samples, BCoV was spiked into DI water and processed the same as the wastewater samples to act as the process control.

For composite supernatant—solid samples (2), a wastewater sample paired to the clarified supernatant (200 mL) was collected, and a 90 mL aliquot was subsampled. To this sample, 1 mL of BCoV was spiked, and the sample was centrifuged for 10–15 min at 3 500g (19 °C) to concentrate debris and settled solids. The clarified supernatant was subsequently discarded to leave a composite supernatant—solid stock with a volume of ~10 mL. To ensure ease of pipetting, this composite was homogenized using a BioSpec Tissue-Tearor (Laboratory Supply Network, U.S.A.) at 30 000 rpm in two intervals of 15 s. This homogenizer was chosen for the little heat produced during this process; it was washed between each sample with 10% HCl, 10% bleach, 70% ethanol, and DI water in duplicate (25 s each). As with the ultracentrifuged sample preparation, the steps were repeated with lab-grade water to calculate the recovery as a percentage of similarly treated controls to calculate the preprocessing and filtration loss.

2.2. Prenucleic Acid Extraction Modifications. Aliquots of the supernatant (1) and composite supernatant—solid samples (2) associated with the quarantine facility and the dormitory were further treated based on four pre-RNA extraction modifications as follows:

Modification 1 (no treatment): Aliquots of 200 μL of the 10 mL clarified supernatant/composite supernatant—solid samples were collected without any treatment.

Modification 2 (UC buffer): Seventy μL of urine-concentrating buffer (also referred to as water-concentrating buffer [R2042-1, Zymo, CA, U.S.A.]) was added to 1 mL of water concentrate/composite supernatant—solid samples and
incubated at room temperature for 10 min prior to centrifugation at 5,000g (room temperature) for 15 min. Thereafter, 870 μL of supernatant was removed, leaving behind ~200 μL of the concentrated sample for RNA extraction.

Modification 3 (RNA/DNA shield): To 200 μL of the water/composite supernatant—solid concentrate, RNA/DNA shield was added. For Zymo and the Qiagen kit, this resulted in the addition of 600 μL of RNA/DNA shield (R1100, Zymo, CA), while for the NEB kit, 200 μL of kit-specific (T2011L, New England Biolabs, MA, U.S.A.) RNA/DNA shield was added.

Modification 4 (UC buffer + RNA/DNA shield): Seventy μL of urine-concentrating buffer (also referred to as water-concentrating buffer [R2042-1, Zymo]) was added to 1 mL of water concentrate/composite supernatant—solid samples and incubated at room temperature for 10 min prior to centrifugation at 5,000g (room temperature) for 15 min. Thereafter, 870 μL of supernatant was removed, leaving behind 200 μL of concentrated sample. To this, RNA/DNA shield was added, as outlined for modification 3.

Each concentrated and modified sample was immediately frozen and stored at −80 °C prior to nucleic acid extraction and reverse transcription—quantitative polymerase chain reaction (RT-qPCR). Due to supply chain issues, samples were stored between 4 and 50 days prior to RNA extraction.

2.3. Nucleic Acid Extraction. Nucleic acids from the concentrated dormitory and quarantine facility wastewater and water/composite supernatant—solid (0.2 mL) were extracted with volumes modified to account for variations following prenucleic acid extraction modifications as follows.

For the Qiagen PowerViral DNA/RNA kit (28000-50, Qiagen, Maryland, U.S.A.), extraction was carried out according to the manufacturer’s guidelines with two modifications. (1) In step 2, 650 μL of PM1 stock was added to the samples, which were then vortexed at max speed (5 min room temperature, 2 min at 4 °C, and 3 min at room temperature. (2) In step 8, 200 μL of solution IRS was added to the samples to increase inhibitor removal. RNA was eluted in 100 μL, as per manufacturer’s guidelines.

For the ZYMO Environ Water RNA extraction kit (R2042, CA, U.S.A.), extractions were carried out according to the manufacturer’s guidelines with three modifications. (1) In step 3, samples were homogenized through vortexing at max speed for 5 min at room temperature. (2) In step 5, 700 μL of sample was transferred to the RNA purification step to increase viral capsid capture with later volumes adjusted to account for this increase. (3) In step 15, 35 μL of water was added directly to the column matrix, and nucleic acid was eluted via centrifugation from the column.

For the New England Biolabs (NEB) Monarch Total RNA miniprep kit (T2010S), extractions were carried out according to the manufacturer’s guidelines, including gDNA elimination and on column DNase treatment, with the following modifications. (1) For every 300 μL of sample mixture, 30 μL of Proteinase K reaction buffer and 15 μL of Proteinase K were added and incubated for 15 min at 55 °C, briefly vortexing every 5 min. (2) Samples were allowed to come to room temperature for 5 min prior to the addition of lysis buffer. (3) For RNA elution, 100 μL of RNAse/DNase-free water (preheated to 50 °C) was added to the spin column and incubated at room temperature for 1 min prior to elution. In the case of each kit, an extraction blank (negative control) was also included in both the ultrafiltered water and composite supernatant—solid batch extractions, and all RNA extractions were stored at −80 °C until further analysis by qPCR.

2.4. Variation in Extraction Recovery. It is well-established that viral recovery efficiency is critical in the study of COVID-19 in wastewater,41 with many methods reporting the reduction of RNA elution volume presumably to increase RNA yield. The manufacturer’s guidelines, specifically for the Qiagen PowerViral DNA/RNA kit and New England Biolabs (NEB) Monarch Total RNA miniprep kit, report explicitly that elution in smaller volumes will provide a higher concentration but lower overall yield. For both kits, elution in 100 μL is reported as ensuring efficient recovery of nucleic acids. However, explicit examination of changes in viral recovery as a function of nucleic acid elution volume are limited. To examine if changes in RNA elution volume could impact surrogate viral recovery, RNase/DNase-free water (175 μL) was spiked with 75 μL of the reconstituted attenuated bovine coronavirus (BCoV) vaccine (Calf-Guard, Zoetis) (section 2.1), and RNA was extracted according to the manufacturer’s guidelines as outlined in the previous section for the Qiagen PowerViral DNA/RNA kit (elution volumes: 100, 80, and 50 μL), Zymo Environ Water RNA extraction kit (elution volumes: 50, 35, 20, and 15 μL), and NEB Monarch Total RNA miniprep kit (elution volumes: 100, 80, 50, and 30 μL). Using this approach, the theoretical viral recovery of each kit can be assessed without the effects of varying environmental parameters such as the wastewater matrix. All viral nucleic acid extracts were stored at −80 °C until further analysis by qPCR. Further details on calculation of the recovery are outlined in the Supporting Information.

2.5. Viral Extraction, Detection, and Quantification. Because PCR inhibition is a frequently reported problem in WBE, preliminary experiments were carried out. To test for inhibition, nucleic acid from the quarantine facility was diluted at a 1:1 ratio (7 points) using the one-step RT-qPCR methodology whereby each reaction was run in duplicate consisting of a starting volume of 1 μL of RNA template (and thereafter 1 μL of diluant, 2.5 μL of Luna 4X Mastermix, 0.4 μL of bovine serum albumin (BSA) (final concentration 8 μg/mL, B9000S, NEB), and target to a final volume of 10 μL. Thermocycling conditions consisted of an initial single-cycle carryover prevention step of 30 s at 25 °C, reverse transcription for 10 min at 55 °C, initial denaturation for 1 min at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C and extension for 60 s at 60 °C. A linear range was identified with less-diluted samples having higher Ct values, in addition to increases in fluorescence intensity proportionate to the amount of starting material, suggesting no qPCR inhibition.

For RNA extraction evaluation of samples, PMMoV, BCoV, and SARS-CoV-2 N1 and N2 (2019-nCoV CDC RUO kit, IDT, no. 10006713) were quantified using one-step RT-qPCR (Luna OneStep 4X mix with UDG, M3019, New England Biolabs), with sequences of primers and probes derived from prior studies (see Table S1). Each reaction was run in triplicate and consisted of 1 μL of RNA template, 2.5 μL of Mastermix, BSA (8 μg/mL, B9000S, NEB), and target to a final volume of 10 μL. For the development of a standard curve for estimation of viral RNA in wastewater that is hypothesized to correlate with infected individuals within a sampling community/unit, a 7-point serial dilution at a 1:2 ratio (starting volume = 1 μL) was carried out for each target (N1, N2, and PMMoV) using RNA
extracted from wastewater collected and concentrated from the quarantine location with known numbers of infected individuals.

Thermocycling conditions consisted of an initial single-cycle carryover prevention step of 30 s at 25 °C, reverse transcription for 10 min at 55 °C, initial denaturation for 1 min at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C and extension for 60 s at 60 °C. Samples, standards (for each target), and no-template controls (NTC) were run in duplicate on each plate and for each gene. Scal-HF (NEB) was used to generate a single cut to linearize the plasmid (IDT, 2017) standards (1:2 dilution, 7.8×1000 gc/µL). PMMoV standards (8 point, 1:2 dilution) were DNA ultramers from IDT and were diluted in DNase/RNase water to a final concentration of 2.8×10⁸ gc/µL. In addition, each PCR plate also contained an RNA extraction method blank whereby water was handled and processed similarly to the samples. No amplification of this sample was observed during the study.

To examine matrix effects among the various methods, paired samples containing similarly spiked BCoV in RNase/DNase-free water were run in parallel. Although not matrix-matched to the wastewater samples, this offers a process control to account for any loss or gain due to the matrixes used as the starting material, in addition to any variability caused by the various modifications. Recovery efficiency (%) was based on variation in the elution volume, and for each preprocessing step it was calculated as a percentage of the optimal elution volume recommended per kit. Further details are in the Supporting Information.

Concentrations of unknown samples were calculated based on primer-specific standard curves per plate. Samples were considered undetected if they did not amplify within 40 cycles. Copies of N1/N2 SARS-CoV-2 RNA and PMMoV were quantified by plotting the crossing threshold (Ct) to an external standard curve (described previously) using a linearized plasmid standard (IDT). The calibration curve showed a linear relationship (see Figure S2). Both the limit of detection (LOD) and limit of quantification (LOQ) were quantified as outlined previously using standard curves of each target (N1, N2, and PMMoV; n = 4). The limit of detection (LOD) is defined as the number of target copies per reaction that can be reliably detected with 95% confidence, which was ~8 gene copies (gc) per reaction for both N1 and N2. No-template controls (NTCs) and extraction blanks were negative for all targets in all methods. Assay efficiencies (qPCR) are presented in Figure S2, with the standard curve R² ranging from 0.8 to 0.99 and efficiency ranging from 80 to 116%, target-dependent.

PCR uncertainty is usually characterized by coefficient of variation (CV) and used to roughly compare the Ct dispersions due in part to Ct absolute values varying in a limited range. CV was calculated by dividing the arithmetic standard deviation by the mean and presenting it as a percentage, whereby a CV of 11–25% was deemed to have a good extraction efficiency, based on instrument performance specification cutoff (https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/gene-expression-analysis-real-time-pcr-information/precision-qpcr.html) and validated interlaboratory methods for GMO testing (European Commission Joint Research Centre, 2017).

Copies of targets were subsequently converted to concentrations per reaction (gc/mL, converted to gc/L for convention) based on dimensional analysis outlined in ref 9 for both SARS-CoV-2 targets and PMMoV. Normalization of the SARS-CoV-2 concentrations in wastewater was performed through the division of SARS-CoV-2 concentrations (gc/L) by the associated sample-specific PMMoV concentration (gc/L). RT-qPCR minimum information for publication of quantitative real-time PCR experiment (MIQE) documentation is detailed in the Supporting Information (Table S7).

**2.6. Data Analysis.** For each target quantified via qPCR, duplicate Ct values were determined through automatic thresholding on QuantiStudio 6 Design and Analysis Software (v1.3), with values imported into R for further analysis. Summary information (mean Ct, standard deviation, and coefficient of variation) and statistics were subsequently computed in RStudio using R (version 4.0.5, “shake and throw”), with plots produced using the package ggplot2. Statistical analysis was performed using both the raw crossing threshold (Ct) values and the data presented as the log 10 concentration (gc/L), due to variability in the formulas used for calculating viral load in wastewater. Data was tested for homogeneity of variance using the Levene test, while normality was assessed using the Shapiro–Wilk test, with the alpha set at 0.05.

Following tests for normality and variance, a two-way ANOVA with an interaction term was used to compare the different RNA extraction methods, solid removal versus solid use, RNA pretreatment modifications, and primer/probe target. A Tukey’s posthoc test was used to perform multiple pairwise comparisons, with p-value < 0.05 considered significant. For each of the method steps evaluated, the first independent variable was the form of the extraction (either ultrafiltered water or composite supernatant–solid), and the second independent variable was the applied modifications: no treatment, urine concentration buffer, DNA/RNA shield and urine concentration buffer, and DNA/RNA shield. The dependent variable was the SARS-CoV-2 concentration, with each target evaluated independently (raw) and following normalization with PMMoV.

Concordance or correlation between PCR positive cases and the wastewater data from the quarantine facility were calculated using the Kendall’s Tau-b coefficients, which is recommended for small research studies with n < 10, using both the target non-normalized gc/L and normalized (PMMoV) for each kit and modification. Estimated SARS-CoV-2 shedders in a sewershed have been reported as highly correlated with cumulative diagnosed COVID-19 cases in a sewershed (R² = 0.81). Furthermore, Lee et al. noted that summing the concentrations of wild-type COVID fragments in wastewater resulted in an improvement in correlation from 0.68 to 0.83. To examine this further, while recognizing that there was high variability in occupancy within the quarantine facility over the 4 sampling time points, data (sum of the total copies (gc/L), with and without normalization) are presented as the sum of the inhabitants and the individual and summed N1 and N2 quantified targets. As outlined previously, correlations were classified as weak (r < 0.3), moderate (0.3 < r < 0.5), or strong (r > 0.5).

### 3. RESULTS AND DISCUSSION

#### 3.1. Extraction Effectiveness of Nucleic Acid Methods

Many factors can differentially affect the quantification of
viral titers in wastewater, one of which is the recovery efficiency, with Pecson et al. recently reporting variations of several orders of magnitude across different methods, with values varying from 0.5 to 100%. To measure viral losses during the concentration step, available protocols have typically employed a proxy virus of a known titer, with bovine coronavirus (BCoV) reported frequently. Recovery can be measured at the point of sampling to capture degradation that occurs during or after sample storage or immediately before extraction, which will capture only losses associated with this step of the protocol. In the current study, we used BCoV to first establish any potential loss during the RNA extraction process across numerous kits and then later to evaluate the impact of the WBE sample matrix on estimates of viral load in addition to loss due to the preconcentration step.

To directly compare the potential viral recovery of each kit and specifically the RNA extraction process under unmodified conditions, BCoV was spiked in molecular-grade water and the relative percentage extraction efficiencies were determined using qPCR. For all three kits tested, recovery was calculated as a percentage of a theoretical known gene copies/molecules BCoV spike. As noted earlier, after confirming normality and homogeneous variance (n = 3 per kit, p > 0.05), data (Ct values) were statistically analyzed with a one-way ANOVA, with no significant difference observed between the Ct and the variable elution volume or among the nucleic extraction kits (n = 3, p > 0.05), although significant differences in recovery between the Zymo Environ Water RNA kit and the NEB total RNA miniprep kit were observed (n = 3, p < 0.05). The Qiagen PowerViral DNA/RNA kit (recovery range 12–29%, Figure S1A) was significantly different (n = 3, p < 0.05) from the NEB total RNA miniprep kit (Figure S1D) but not the Zymo Environ Water RNA kit (Figure S1C). Variation in Ct among the elution volumes examined per kit was minimal (<1 Ct), with the highest difference noted at the smaller volumes, which is summarized in Figure S1 for each kit (B, D, and F). Based on the lack of significant difference among kits, and elution volumes, subsequent results used the highest volume recommended by manufactures although a marginal increase in recovery can be obtained reducing elution volume based on the use of BCoV as a viral recovery surrogate.

Next, using BCoV as a surrogate to calculate the impact of the sampling matrix among methods, sample processing, and filtration, the variation in effects was calculated as percentage differences based on the preconcentrating step and per extraction kit (n = 2 sampling time points per method). Importantly, to evaluate the impact of the sampling matrix, BCoV was treated in parallel to the samples, whereby BCoV was spiked in an equivalent volume of DI water, which was processed by ultrafiltration or by the methodology outlined for the composite supernatant–solid sample (e.g., 1 mL of BCoV was spiked into 90 mL of DI water). In this respect, potential adsorption to the filter or 50 mL falcon tube should be accounted for in the analysis, with recent studies pretreating filters with bovine serum albumin to block virus adsorption/loss of viral titers during concentration. The Ct values of the BCoV samples processed in DI water and run in parallel were assumed to represent 100% recovery, with the Ct of wastewater samples spiked with BCoV calculated as a percentage of this control. For spiked wastewater samples concentrated using ultrafiltration, the optimal recovery identified was ordered from highest recovery to lowest as follows: Qiagen PowerViral DNA/RNA kit (98 ± 1%), Zymo Environ Water RNA extraction (86 ± 21%), followed by NEB Total RNA miniprep kit (84 ± 16%). In contrast, the spiked composite samples had higher recoveries, with the optimal recovery ordered as follows: NEB Total RNA miniprep kit (103 ± 5%), Zymo Environ Water RNA extraction (95 ± 8%), followed by Qiagen PowerViral DNA/RNA kit (82 ± 3%). While unusual, other studies have also noted that DI water controls spiked with BCoV showed significantly different levels of recovery and attributed it to large differences in osmotic pressure, but equally possible is the influence of the sampling matrix on the final calculations. Increases in percentage differences of composite samples may be related to prior work that estimated ~15% adsorption of envelope viruses to wastewater solids under equilibrium conditions, with this hypothesis further supported in ongoing work. Finally, for surrogate viral recovery analysis, wastewater samples (n = 2) were processed using the same input volume of wastewater as outlined in sections 2.1 and 2.2 for both clarified supernatant and for the composite supernatant–solid sample method side by side. Further, all modifications and nucleic acid extractions were carried out side by side. Variability in recovery is presented in Table S2, with nondetects being more common in the ultrafiltered water samples. Focusing on the composite supernatant–solid sample, estimated BCoV recovery was markedly higher using the NEB total RNA miniprep kit across all modifications, followed by the Zymo Environ Water RNA kit, with the lowest recovery observed using the Qiagen PowerViral DNA/RNA kit. In the current study, marked variability was observed between replicates and between the two WBE sampling methods, although it is within the range of recovery previously reported in the literature. By varying the sampling volume, Juel et al. also observed wide recovery ranges that were dependent on the processing concentration methodology (<10–70%), associating this high variability with turbidity, which was also reported in other studies. Indeed, the high degree of recovery of gene copies between the clarified supernatant and a solids-based method has also been observed for N gene targets, with a solid base methodology reporting on average 100–1000 times higher concentrations in solids. Notably, while no significant difference was observed among the pre-RNA extraction modifications between the ultracentrifuged (clarified supernatant) and composite supernatant–solid for both the Zymo and Qiagen kits (with broadly comparable surrogate viral recovery), significant differences were observed between samples (clarified supernatant or composite supernatant–solid) for the NEB extracted samples (n = 2 sampling dates per modification per sample, p < 0.05) (Table S2). It is apparent that surrogate recovery is methodology/sample matrix-dependent, whereby a surrogate can behave similarly to another in one standard operating procedure (SOP) but differently in another. This has also been noted previously in other studies that compared multiple methods (N = 36) for quantifying SARS-CoV-2, with orders of magnitude difference noted. Indeed, recovery in general has been reported as highly variable. In early research, Medema et al. reported the highest recovery with the ultrafiltration device Centricon Plus-70 30 kDa, reaching 73% of the surrogate seeded F-specific RNA phages. By contrasting, using murine hepatitis virus as the recovery surrogate, Ahmed et al. identified recoveries of 56% and 28% for Amicon Ultra-15 30 kDa and Centricon Plus-70 10 kDa, respectively. Indeed, a recent review highlighted that there is no gold standard approach to measure or determine the stock concentration of
the proxy virus for estimation of recovery. Thus, a standardized approach for the interpretation of recovery controls is still lacking, although recommendations for how to incorporate recovery controls into WBE reporting is suggested. In this respect, it is important to consider several factors, including when and where in the process recovery rates are calculated, the spiking stock (i.e., different values may be obtained when the quantification is done directly from viral stocks versus standards, etc.), and the reagents/method being used (i.e., one-step or two-step RT-PCR versus ddPCR), some of which is discussed by Rusinòl et al. Indeed, the importance of where in the process spiking occurs is clearly outlined in the current study, whereby using BCoV to evaluate loss at the RNA extraction step resulted in increased recovery with low variability, compared to spiking at the preprocessing step, despite increasing volumes. While many studies have reported on the use of the primer probe set first outlined by Decaro et al., recently Juel et al. established an improvement in primer efficiency from a mean of 85−103% by adjusting the annealing temperature from 55 to 60 °C. Using the reconstituted BCoV stock as our standard for quantification of viral load in recovery, an efficiency of ~89% was observed. For the detection and quantification of SARS-CoV-2 RNA in wastewater, there are variations in the conducting of experiments with various assays, reagents, instruments, and data analysis, and lack of quantification standards could lead to errors in quantitative data reporting; similar findings could also potentially be observed in the recovery literature. The crossing threshold (Ct) is highly dependent on the amplification efficiency, which can vary among targets and samples, and is affected by dilution errors and hampered by properties of the standard, diluent, and calibration of equipment. To minimize these impacts, a recent study recommended the calculation of absolute quantification based on a single undiluted calibrator with a known target concentration and efficiency derived from amplification curves of the calibrator and unknown samples. The application of this method has not been evaluated in the context of the WBE; however, noting that variability of PCR efficiency among biological samples is likely and that the use of a mean efficiency per assay would lead to biases for samples with deviating PCR efficiencies, this may somewhat work to address the large degree of variability in efficiencies currently reported in the WBE literature, although variability in PCR efficiencies for BCoV is only assumed to be similar because it is less frequently reported.

3.2. Method Evaluation. The characteristics of wastewater collected from congregate living facilities such as universities, long-term care facilities, and correctional institutions are different from highly pooled wastewater treatment influent to a wastewater treatment plant (WWTP). This was clearly demonstrated by Corchis-Scott et al., who reported that PMMoV in residence hall wastewater varies in concentration across 4 orders of magnitude with a CV of 3%, contrasted with a more modest concentration variability of only 1 order of magnitude with a CV < 1% for five WWTPs. The primary concentration is a critical step in the WBE monitoring of COVID-19, with numerous studies recognizing its importance. In this respect, studies examining numerous methods explicitly recommended strategies to facilitate comparability. Available ultrafiltration methods, which suffer markedly from supply chain issues in addition to variable recovery largely dependent on the surrogate virus used, continue to play a role in WBE. While researchers appear to be moving to alternative methods, optimization of protocols has resulted in better efficiency in spike recovery and quantification of SARS-CoV-2. However, much of the recommendations and protocol optimizations are based on larger municipalities and not on WBE carried out on smaller communities that may require modifications. Furthermore, although studies have reported on increased SARS-COV-2 detection efficiencies using pellet/settled solids versus corresponding clarified influent samples, many studies continue to concentrate COVID-19 from presettled influent wastewater (e.g., ref 57). Importantly, if the maximum nucleic acid yield is required, then the pellet component must also be extracted, with studies establishing that 10−20% of SARs-CoV-2 is associated with the solid fraction, which may contribute to variability in translation to clinical data sets.

The effects of different preconcentrating methods, preenucleic extraction modifications, RNA purification methods, and final target quantifications were assessed over a 3−4-week period based on sampling at two facilities containing variable levels of clinically positive cases and variable population levels. Alongside the use of the quarantine facility site containing a population composed of PCR positive inhabitants at varying stages of shedding, Dormitory A was later confirmed to contain one clinically positive case (equivalent to 0.18%) for 2 of the 3 sampling events, with detection in the week where nobody tested positive likely due to shedding. Viral shedding of SARS-CoV-2 has been reported as highly variable, with viral shedding continuing well beyond 14 days, especially for certain groups such as long-term care residents or those living in less-affluent neighborhoods. On the basis of recent reports that it is highly likely that 10 actively shedding individuals can be reliably detected in a catchment area of 100 000 individuals, it was anticipated that all samples would result in positive detections.

3.2.1. Clarified Wastewater versus Composite. On the basis of each time point representing an n = 1 for each of the two sampling locations, percentage agreements based on positive detection of the N1 or N2 target in composite supernatant−solid samples samples were 95% and 83%, respectively. In contrast, clarified supernatant concentrated using ultrafiltration resulted in 75% and 73% agreement based on quantification using the N1 and N2 targets, respectively. Composition of wastewater contains a diverse range of PCR inhibitors including fats, proteins, and humic and fulvic acids, which can cause downstream processing problems during PCR. The availability of different commercial extraction kits for DNA/RNA has demonstrated sometimes variable efficiencies and consistencies when extracting from PCR inhibitor-rich samples, including wastewater and sediment. In this respect, clear trends emerged based on the common prenuclidean modifications and kits being used that are summarized in Tables S3 (ultrafiltration) and S4 (composite supernatant−solid). In general, SARS-CoV-2 was identified consistently in pellet samples, with a clear consistent improvement in detections using modification 4, pre-RNA extraction treatment (UC buffer + RNA/DNA shield), with all kits resulting in positive detections (100%) using the N1 target assay. In contrast, clarified water concentrated using ultrafiltration resulted in increased variability in detections, although similar modification 4 resulted in the most consistent and conclusive detections for both targets except for the PowerViral DNA/RNA kit (Qiagen), which resulted in detections of 43 and 71% for N1 and N2 target assays, respectively. While modification 4 resulted in equivalent 100%
conclusive detections for all kits using the composite supernatant−solid samples (N1 target), the Environ Water RNA extraction kit (Zymo) was the only kit that successfully identified SARS-CoV-2 in all samples preprocessed using the ultrafiltration method combined with modification 4 for both targets.

Interestingly, regardless of viral concentration method, preconcentration modifications, and nucleic acid extraction kits, decreased sensitivity and reproducibility were observed between the two CDC target assays. For composite supernatant−solid samples pretreated with modification 4, SARS-CoV-2 was successfully identified in all samples using the N1 assay, compared to 71–100% for the N2 assay (kit dependent), with this variability even more pronounced for ultrafiltration concentrated samples. This appears to agree with other campus studies that singly used the N1 target assay to quantify viral loads.61 Using the optimized modification 4 data set, we further explored the impact of concentration step and nucleic extraction kit on the resulting Ct value and subsequent quantification of viral load, a central component of WBE surveillance. Focusing on the N1 target assay, comparable Ct values were broadly obtained regardless of ultrafiltration concentration (Figure 2A) or using the composite supernatant−solid samples (Figure 2C), with composite supernatant−solid samples frequently reporting lower Ct values and more positive detects. While Ct values are on average broadly comparable for similar samples when samples have been preconcentrated using ultrafiltration, it is interesting to note the ∼2 Ct difference for paired samples, with the Zymo kit on
average reporting lower Ct values with the trend similarly observed in composite samples. This is likely linked to the smaller elution volume used (∼15–30 μL) compared to the typical elution volume recommended for the other kits (100 μL), although for some sampling time points, equivalent Ct values were observed using the NEB kit and higher elution volumes. Likewise, there is a substantial difference in the viral concentration quantified between the ultrafiltration method (Figure 2B) and the composite supernatant–solid sample (Figure 2D), although comparable trends are visible irrespective of nucleic acid extraction method. Following normalization with PMMoV, less variability and more reproducibility were observed in the composite supernatant–solid samples using the N1 and N2 target assays (Figure 3C and D), respectively, compared to the ultrafiltered samples (Figure 3A and B). Higher SARS-CoV-2 detection frequencies and quantifiable viral load were consistently observed using the composite supernatant–solid sample methods regardless of nucleic acid extraction kits, compared to the clarified supernatant, with gene copy per liter (following normalization) ∼100 times higher in composite supernatant–solid samples, irrespective of target assay used (Figure 3). This is in agreement with prior literature, which noted that there seems to be a trend in N1 solid concentrations that had viral levels ∼100 times higher in settled solids using the polyethylene glycol (PEG) precipitation method, although the current methodology identified that viral loads of N2 were only 5 times higher, compared to the 1000 reported previously. While it is important to note differences among the studies, the emergence of a consistent trend in the use of N1 targets, despite differences in community structure and various other methods, is consistent with the literature.

3.2.2. Relationship between COVID RNA Targets and Confirmed Cases. Unlike typical research studies that correlate SARS-CoV-2 viral concentrations in wastewater to daily per capita cases aggregated across locations or rolling 5–7-day averages consisting of an unknown population of asymptomatic and symptomatic COVID-19 carriers, in the current study we had a unique opportunity to examine the relationship in viral load to a population of known clinically confirmed cases. In this respect, the influences of sample type, pre-RNA modifications, and nucleic acid extraction kits could be examined and compared for a quarantine location. These correlations and potential relationships were studied based on serial dilutions of each sampling time point, which were examined individually to confirm linearity within this group and then summed due to the fact that estimated shedders in a sewershed are related to cumulative diagnosed cases but are

![Figure 3. RT-PCR results for N1 target assay (A, C) and N2 target assay (B, D) for ultrafiltration concentrated water samples (green and orange) and composite supernatant–solid samples (blue and yellow) with normalization using the fecal normalization control PMMoV. Viral concentrations, reported as gene copy per liter (log10 scale), were measured using a one-step RT-PCR and an RNA volume of 1 μL. Samples are separated based on sample location, viz., Dormitory A with variable viral loads and the quarantine facility containing a population consisting of primarily (>95%) clinically positive COVID-19 cases. Importantly, there are clear scale differences between the two concentration methodologies and with normalized target gc/L being almost 100 times more in composite supernatant–solid versus ultrafiltered for both targets investigated.](https://doi.org/10.1021/acsestwater.1c00476)
Table 1. Matrix of Correlations Based on Variation in Initial Sample Type, Nucleic Acid Extraction Kit, pre-RNA Extraction Modifications, and the Final Target Used to Calculate Concordance, Without Being Normalized

| sample type                  | kit   | modification | N1          | N2          | N1 + N2     |
|------------------------------|-------|-------------|-------------|-------------|-------------|
| composite supernatant—solid  | Qiagen| 1           | 1           | 0.8         | 1           |
|                              | Zymo  | 1           | 0.71        | 0.9         | 0.81        |
|                              | Neb    | 1           | 0.9         | 0.9         | 1           |
|                              | Qiagen| 2           | 0.9         | 1           | 0.9         |
|                              | Zymo  | 2           | 1           | 0.9         | 1           |
|                              | Neb    | 2           | 0.9         | 1           | 0.9         |
|                              | Qiagen| 3           | 0.71        | 0.81        | 0.43        |
|                              | Zymo  | 3           | 1           | 0.9         | 1           |
|                              | Neb    | 3           | 1           | 0.9         | 1           |
|                              | Qiagen| 4           | 0.9         | 0.9         | 0.9         |
|                              | Zymo  | 4           | 1           | 0.9         | 0.9         |
|                              | Neb    | 4           | 1           | 0.9         | 0.9         |
| ultrafiltered water          | Qiagen| 1           | 0           | 0.6         | 0.14        |
|                              | Zymo  | 1           | 0.9         | 0.9         | 0.9         |
|                              | Neb    | 1           | 0           | 0.9         | 0.9         |
|                              | Qiagen| 2           | 1           | 0           | 0           |
|                              | Zymo  | 2           | 1           | 0           | 0           |
|                              | Neb    | 2           | 0.9         | 0.9         | 0           |
|                              | Qiagen| 3           | 0.8         | 0.87        | 0.87        |
|                              | Zymo  | 3           | 0.9         | 0.9         | 0.9         |
|                              | Neb    | 3           | 1           | 0           | 0           |
|                              | Qiagen| 4           | 0.87        | 0.81        | 0.87        |
|                              | Zymo  | 4           | 1           | 0           | 0           |
|                              | Neb    | 4           | 0.73        | 0.06        | 0.048       |

aWhile the use of the N1 assay for both sample types and kits typically results in highly significant correlations, the use of the N2 assay has significantly more variability. Further, values that have been normalized to PMMoV prior to concordance typically result in marked decreases in correlation and significance. To view correlation plots, including those following normalization, see Figures S6–S13. bNon-significant result.

not correlated with daily covid cases. A positive and statistically significant (Kendall’s Tau, p < 0.05) relationship between N1 and N2 and COVID-19 clinical cases was observed, regardless of any of the tested variables (Table 1), although linear regressions ranging from 0.71 to 1 were typically associated with the N1 target assay and composite supernatant—solid sample preparation.

Furthermore, following the improvement in relationships between RNA and clinical cases using the combined concentration of wild-type and mutant targets (B.1.1.7), the impact of the summation of gene copy per liter for N1 and N2 targets per time point was also investigated. Generally, these correlations varied depending on the sample type (ultrafiltered or composite supernatant—solid), nucleic acid extraction method, and modifications, as summarized in Table 1, which thus highlights the need to further understand the implications of method selection at various stages of WBE in characterizing SARS-CoV-2 RNA concentrations in wastewater. Individual Kendall correlations based on nucleic acid extraction kit and pre-RNA modifications for composite supernatant—solid samples, with and without normalization, for the N1 and N2 assays are available in the Supporting Information (Figures S6–S13). The increased strong relationship between SARS-CoV-2 WBE data and COVID-19 prevalence is further supported by other studies showing similar correlations. As noted earlier, WBE research has typically focused on correlating wastewater measures with clinical cases of COVID-19 within large municipalities. In recent studies, daily estimated numbers of SARS-CoV-2 shedding individuals estimated through WBE did not correlate with daily COVID-19 cases, which was attributed to potential reporting lags, test kit availability, processing rates, etc. Improvements in correlation have been observed when external factors such as inlet flow rate and rain were considered. Further, a recent meta-analysis identified 9 explanatory variables that could explain up to 50% of the observed variation, including testing coverage, SARS-CoV-2 concentration (recovery corrected or not), water temperature, air temperature, population size, water usage pattern, precipitation/rainfall, and sampling technique, although factors contributing to the remaining 50% of variation still need to be identified.

In the current study, an important component of the increased correlation likely resulted from the use of a quarantine facility consisting of a population that tested positive using PCR that week and thus were actively shedding individuals. This facility was used specifically to interrogate the hypothesis that target assay, sample processing including modifications, and nucleic acid extraction kit could potentially influence correlations. As previously noted, a variety of methods are capable of producing reproducible results, and this study supports this observation. Reported correlations of clinically positive cases and WBE in the literature are varied, ranging from −0.2 to 0.87, with variations in factors including assay target, locations, and normalization likely playing a role in variability. Notably, examination of the impact of normalization on concordance in the current study using the PMMoV fecal indicator resulted in weaker associations. Although varying sample preparation and quantification, this trend has also been observed previously, whereby normal-
PMMoV has been detected in tap water, which may contribute agreed with the raw concentration data, minimizing the utility observed that data normalized by an endogenous control recovery yields.55 Higher volumes of in from values as low as 1 mL.31,48 Independent of what volume the lower end, studies are successfully isolating genetic signal inhibitors. While the volume concentrated in this study is on scale. In this respect, consistent data-reporting guidelines have standardize data reporting in the research community to treat and processing methods generally giving comparable results, there is still a lack of standardization or consensus in the calculation of viral gene copies in a sewershed, a particularly important characteristic in comparing viral loads between communities. As WBE has progressed, so too have the calculations. In the initial days of the pandemic, the literature simply reported variation in Ct values, which later began to account for dilution (e.g., weather, concentrating steps, nucleic acid extraction, etc.), community population variation, etc. With the increased frequency of studies reporting on WBE in communities globally, it is particularly difficult to compare among different methods and communities when gc/L is calculated differently in every study, and raw data (viz., Ct) is typically not reported.

In the current study, we compared two other methods of quantification reported in the literature that do not implicitly account for dilution in the PCR reaction.48,73 Concentration factors were calculated for each nucleic acid extraction kit (Table S5A–C) before calculating N1 genetic copies based on the quarantine facility. The reproducibility of the quantification methods within each sample type (viz., ultrafiltration or composite supernatant—solid) was evaluated (Figure S5). Irrespective of sample type, samples extracted using the Qiagen nucleic acid extraction kit were consistently lower than those in the other kits examined. Furthermore, genetic copies of SARS-CoV-2 (via N1 assay) were broadly comparable among the concentration factor methodologies. It should be noted that the dimensional analysis outlined previously9 was originally based on a calculation of the concentration in solids (gc/kg dry mass) or clarified water samples and has been recently updated to link SARS-CoV-2 RNA in settled solids to inputs from infected individuals based on feces and approximations of fecal shedding rates.67 The approach undertaken in the current study is therefore new, in that contains both viral fragments from the water and the solid fraction in the same sample, and the tentative examination of different quantification methods has highlighted the need for further investigations. While trends are broadly the same, the establishment of consistent calculation steps from copies per reaction to final gene copy per liter in wastewater will allow for a more harmonized approach in the future, although this may not be easy given that the genetic signal has been reported to be impacted by sample location/community, type, and time of collection. This may also be further complicated by the existence of several forms of SARS-CoV-2 RNA in wastewater in the form of infectious particles, noninfectious particles/ribonucleoprotein complexes, and free/unprotected viral RNA,75 in addition to SARS-CoV-2 variant genotypes with variable abundance,76 which may or may not be equally detectable based on current
assays. Our observations highlight the need for interlaboratory comparison of SARS-CoV-2 methodology, in terms of not just sample collection but also processing and/or analysis.73

4. CONCLUSIONS

Economical costs, as well as supply chain issues, have been a major determinant in the establishment and implementation of WBE at various facilities, with studies reporting evaluation of recovery, costs, and throughout of different concentrations methods for SARS-CoV-2 being particularly beneficial for communities in the process of setting up (e.g., ref 72). Observations from the current study suggest that comparable results are achievable using either the Zymo or NEB kit, especially when paired with a preconcentration step using UC buffer. Ongoing work is identifying whether scaling the UC buffer to work with larger volumes is feasible. Additionally, more research needs to be done to determine the best process controls (recovery and normalization) when using this composite supernatant—solid approach. Using UC buffer on composite supernatant—solid samples followed by RNA/DNA solid represents a powerful alternative with increased signal efficiency, whereby 6–8 sites can be concentrated and viral RNA can be isolated for RT-PCR within 3–5 h of sample arrival in the laboratory (e.g., ~1 h 15 min for incubation and concentrating followed by 2 h (NEB) or 3 h (Zymo) for nucleic acid extraction). This is particularly important, in that combining a low-cost composite sampler with a method that quantifies a SARS-CoV-2 signal in wastewater within 6 h has previously enabled actionable and timely response data delivery, enabling speedy interventions that minimize outbreaks.19

The findings of the present study indicate that, while the signal is detectable in the clarified supernatant on college campus communities, the use of the composite supernatant—solid represents a powerful alternative with increased signal and reproducibility while also using minimally impacted consumables. Likewise, the combined influence of modifications and nucleic acid extraction on target sensitivity highlights the need to further understand the implications of method selection (concentrating and extraction) for characterizing SARS-CoV-2 RNA concentrations in wastewater. It is also important to note that, while this study has been limited to a small community level with populations in the hundreds, it is possible that larger municipalities may also benefit from this approach that enables more frequent sampling, which could benefit in tracking smaller outbreak clusters and allowing early interventions. Ultimately, for building level, subsewershed, or sewershed WBE surveillance, this study demonstrates methods to concentrate SARS-Cov-2 RNA for WBE that are comparable, economical, and feasible to implement at various community levels.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.1c00476.

Additional experimental details including sampling location characteristics, MIQE of experiment, and individual correlations based on nucleic acid extraction kits (PDF)

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Notes
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REFERENCES

(1) Ort, C.; Nuijs, A. L. N.; Berset, J.-D.; Blijsema, L.; Castiglioni, S.; Covaci, A.; Voogt, P.; Emke, E.; Fatta-Kassinos, D.; Griffiths, P.; Hernández, F.; González-Marínó, I.; Grabic, R.; Kasprzyk-Hordern, B.; Mastroianni, N.; Mierjohann, A.; Nefau, T.; Ostman, M.; Pico, Y.; Racamonde, I.; Reid, M.; Slobodnik, J.; Terzic, S.; Thomaidis, N.; Thomas, K. V. Spatial Differences and Temporal Changes in Illicit Drug Use in Europe Quantified by Wastewater Analysis. Addiction 2014, 109 (8), 1338–1352.
(2) Horký, P.; Grubic, R.; Grabová, K.; Brooks, B. W.; Douda, K.; Slávik, O.; Hubená, P.; Sancho Santos, E. M.; Randák, T. Methamphetamine Pollution Elicits Addiction in Wild Fish. Journal of Experimental Biology 2021, 224 (13), No. jeb242145.
(3) Montgomery, A. B.; Bowers, I.; Subedi, B. Trends in Substance Use in Two United States Communities during Early COVID-19 Lockdowns Based on Wastewater Analysis. Environ. Sci. Technol. Lett. 2021, 8 (10), 890–896.
(4) Sims, N.; Kasprzyk-Hordern, B. Future Perspectives of Wastewater-Based Epidemiology: Monitoring Infectious Disease
Surveillance for Monitoring COVID-19.

D. L. Wastewater and Public Health: The Potential of Wastewater Spread and Resistance to the Community Level.

ACS ES&T Water pubs.acs.org/estwater

Julian, T. R.; Kohn, T. Wastewater Monitoring Outperforms Case & Mental Science Y.; Gao, T.; Bulat, R.; Craik, S.; Hrudey, S. E.; Ohinmaa, A.; Population-Based Health Management.

Making Waves: Wastewater Surveillance of SARS-CoV-2 for Outbreak.

V. B.; Haines, M. B.; Girones, R.; Ng, L. C.; Alm, E. J.; Wuertz, S. Detection of SARS-CoV-2 in Wastewater in Japan during a COVID-19 Outbreak.

Maximize Contributions in the Fight Against COVID-19.

R. J.; Nilsson, D.; Noble, R. T.; van Nuijs, A.; Peccia, J.; Perkins, T. Islam, Md. T.; Jones, D. L.; Kasprzyk-Hordern, B.; Kitajima, M.; Greenland, L.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K. Armstrong, N.; Kim, S.; Yamahara, K. M.; Sassoubre, L. M.; Mendoza−L.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K. Armstrong, N.; Kim, S.; Yamahara, K. M.; Sassoubre, L. M.; Mendoza−L.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K.

A. Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands. Environ. Sci. Technol. Lett. 2020, 7 (7), 511−516.

(17) Lee, B. E.; Sikora, C.; Faulder, D.; Rising, E.; Little, L. A.; Qiu, Y.; Gao, T.; Bulat, R.; Craik, S.; Hrudey, S. E.; Ohinmaa, A.; Estabrooks, C. A.; Gingras, A.-C.; Charlton, C.; Kim, J.; Wood, H.; Robinson, A.; Kanji, J. N.; Zelayas, N.; O’Brien, S. F.; Drews, S.; Pang, X.-L. Early Warning and Rapid Public Health Response to Prevent COVID-19 Outbreaks in Long-Term Care Facilities (LTCF) by Monitoring SARS-CoV-2 RNA in LTFC Site-Specific Sewage Samples and Assessment of Antibodies Response in This Population: Prospective Study Protocol. BMJ. Open 2021, 11 (8), No. e052282.

(18) Prado, T.; Fuman, T. M.; Mannarino, C. F.; Resende, P. C.; Motta, F. C.; Eppinghaus, A. L. F.; Chagas do Vale, V. H.; Braz, R. M. S.; de Andrade, J. da S. R.; Maranhão, A. G.; Miagostovich, M. P. Wastewater-Based Epidemiology as a Useful Tool to Track SARS-CoV-2 and Support Public Health Policies at Municipal Level in Brazil. Water Res. 2021, 191, 116810.

(5) Wu, Y.; Guo, C.; Tang, L.; Hong, Z.; Zhou, J.; Dong, X.; Yin, H.; Xiao, Q.; Tang, Y.; Qu, X.; Kuang, L.; Fang, X.; Mishra, N.; Lu, J.; Shan, H.; Jiang, G.; Huang, X. Prolonged Presence of SARS-CoV-2 Viral RNA in Fecal Samples. Lancet Gastroenterol Hepatology 2020, S (5), 434−435.

(6) Medema, G.; Heijnen, L.; Elsinga, G.; Italiaander, R.; Brouwer, A. Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands. Environ. Sci. Technol. Lett. 2020, 7 (7), 511−516.

(7) Ahmed, W.; Angel, N.; Edson, J.; Bibby, K.; Bibvis, A.; O’Brien, J. W.; Choi, P. M.; Kitajima, M.; Simpson, S. L.; Li, J.; Tscharke, B.; Verhagen, R.; Smith, W. J. M.; Zaug, J.; Dierens, L.; Hugenholtz, P.; Thomas, K. V.; Mueller, J. F. First Confirmed Detection of SARS-CoV-2 in Untreated Wastewater in Australia: A Proof of Concept for the Wastewater Surveillance of COVID-19 in the Community. Science of The Total Environment 2020, 728, 138764.

(8) Hata, A.; Hara-Yamamura, H.; Meuchi, Y.; Imai, S.; Honda, R. Detection of SARS-CoV-2 in Wastewater in Japan during a COVID-19 Outbreak. Sci. Total Environ. 2021, 758, 143578.

(9) Graham, K. E.; Loeb, S. K.; Wolfe, M. K.; Catoe, D.; Sinnott-Armstrong, N.; Kim, S.; Yamahara, K. M.; Sassoubre, L. M.; Mendoza−Grijalva, L. M.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K. R.; Boehm, A. B. SARS-CoV-2 RNA in Wastewater Settled Solids Is Associated with COVID-19 Cases in a Large Urban Sewershed. Environ. Sci. Technol. 2021, 55 (1), 488−498.

(10) Scheran, S. P.; Shahin, S.; Ward, L. M.; Tandukar, S.; Aw, T. G.; Schmitz, B.; Ahmad, W.; Kitajima, M. First Detection of SARS-CoV-2 RNA in Wastewater in North America: A Study in Louisiana. USA. Science of The Total Environment 2020, 743, 140621.

(11) Bibvis, A.; North, D.; Ahmad, A.; Ahmad, W.; Alm, E.; Been, F.; Bhattacharya, P.; Bijlsma, L.; Boehm, A. B.; Brown, J.; Buttiglieri, C.; Calvoro, G.; Cardavo, L.; Carducci, A.; Castiglioni, S.; Cecetioglu Guroz, U.; Chakraborty, S.; Costa, F.; Curcio, S.; de los Reyes, F. L.; Delgado Vela, J.; Farkas, K.; Fernandez-Casi, X.; Gerba, C.; Gerrity, D.; Girone, R.; Gonzalez, R.; Haramoto, E.; Harris, A.; Holden, P. A.; Islam, Md. T.; Jones, D. L.; Kasparyk-Hordern, B.; Kitajima, M.; Kotlarz, N.; Kumar, M.; Kuroda, K.; La Rosa, G.; Malpe, F.; Maute, M.; McLellan, S. L.; Medema, G.; Meschke, J. S.; Mueller, J.; Newton, R. J.; Nilsson, D.; Noble, R. T.; van Nuuij, A.; Peccia, J.; Perkins, T. A.; Picking, A. J.; Rose, J.; Sanchez, G.; Smith, A.; Stadler, L.; Stauber, G.; Thomas, K.; van der Voord, T.; Wigginton, K.; Zhu, K.; Bibby, K. Wastewater-Based Epidemiology: Global Collaborative to Maximize Contributions in the Fight Against COVID-19. Environ. Sci. Technol. 2020, 54 (13), 7754−7757.

(12) Larsen, D. A.; Wigginton, K. R. Tracking COVID-19 with Wastewater. Nat. Biotechnol. 2020, 38 (10), 11151−11153.

(13) Tran, H. N.; Le, G. T.; Nguyen, D. T.; Juang, R.-S.; Rinklebe, J.; Bhatnagar, A.; Lima, E. C.; Iqbal, H. M. N.; Sarmah, A. K.; Chao, H.-P. SARS-CoV-2 Coronavirus in Water and Wastewater: A Critical Review about Presence and Concern. Environ. Res. 2021, 193, 110265.

(14) Farkas, K.; Hillary, L. S.; Malham, S. K.; McDonald, J. E.; Jones, D. L. Wastewater and Public Health: The Potential of Wastewater Surveillance for Monitoring COVID-19. Current Opinion in Environmental Science & Health 2020, 17, 14−20.

(15) Thompson, J. R.; Nanchariah, Y. V.; Gu, X.; Lee, W. L.; Rajal, V. B.; Haines, M. B.; Girone, R.; Ng, L. C.; Alm, E. J.; Wuertes, S. Making Waves: Wastewater Surveillance of SARS-CoV-2 for Population-Based Health Management. Water Res. 2020, 184, 116181.

(16) Fernandez-Cass, X.; Scheidegger, A.; Bänziger, C.; Cariti, F.; Tuñas Corzon, A.; Ganansanandamoorthy, P.; Lemaitre, J. C.; Ort, C.; Julian, T. R.; Kohn, T. Wastewater Monitoring Outperforms Case Numbers as a Tool to Track COVID-19 Incidence Dynamics When Test Positivity Rates Are High. Water Res. 2021, 200, 117252.

(17) Lee, B. E.; Sikora, C.; Faulder, D.; Rising, E.; Little, L. A.; Qiu, Y.; Gao, T.; Bulat, R.; Craik, S.; Hrudey, S. E.; Ohinmaa, A.; Estabrooks, C. A.; Gingras, A.-C.; Charlton, C.; Kim, J.; Wood, H.; Robinson, A.; Kanji, J. N.; Zelayas, N.; O’Brien, S. F.; Drews, S.; Pang, X.-L. Early Warning and Rapid Public Health Response to Prevent COVID-19 Outbreaks in Long-Term Care Facilities (LTCF) by Monitoring SARS-CoV-2 RNA in LTFC Site-Specific Sewage Samples and Assessment of Antibodies Response in This Population: Prospective Study Protocol. BMJ. Open 2021, 11 (8), No. e052282.

(18) Prado, T.; Fuman, T. M.; Mannarino, C. F.; Resende, P. C.; Motta, F. C.; Eppinghaus, A. L. F.; Chagas do Vale, V. H.; Braz, R. M. S.; de Andrade, J. da S. R.; Maranhão, A. G.; Miagostovich, M. P. Wastewater-Based Epidemiology as a Useful Tool to Track SARS-CoV-2 and Support Public Health Policies at Municipal Level in Brazil. Water Res. 2021, 191, 116810.
Hepatitis Virus, a Surrogate for SARS-CoV-2 from Untreated Wastewater. *Sci. Total Environ.* 2020, 739, 139960.

(28) Barril, P. A.; Pianciola, L. A.; Mazzeo, M.; Ouisset, M. J.; Jaureguiberry, M. V.; Alessandrello, M.; Sánchez, G.; Oteiza, J. M. Evaluation of Viral Concentration Methods for SARS-CoV-2 Recovery from Wastewaters. *Sci. Total Environ.* 2021, 756, 144105.

(29) Lu, D.; Huang, Z.; Luo, J.; Zhang, X.; Sha, S. Primary Concentration – The Critical Step in Implementing the Wastewater Based Epidemiology for the COVID-19 Pandemic: A Mini-Review. *Science of The Total Environment* 2021, 107, 218–229.

(31) SARS-CoV-2 Interlaboratory Consortium. Reproducibility and Sensitivity of 36 Methods to Quantify the SARS-CoV-2 Genetic Signal in Raw Wastewater: Findings from an Interlaboratory Methods Evaluation in the U.S. *Environ. Sci. Water Res. Technol.* 2021, 7, 504.

(32) Ahmed, W.; Simpson, S. L.; Bertsch, P. M.; Bibby, K.; Bivins, A.; Blackall, L. L.; Boill-Mas, S.; Bosch, A.; Brandão, J.; Choi, P. M.; Ciesielski, M.; Donner, E.; D’Souza, N.; Famletiner, A. H.; Gerrity, D.; Gonzalez, R.; Griffith, J. F.; Gyawali, P.; Haas, C. N.; Hamilton, K. A.; Hapuarachchi, H. C.; Harwood, V. J.; Haque, R.; Jackson, G.; Khan, S. J.; Khan, W.; Kitajima, M.; Korajkic, A.; La Rosa, G.; Layton, B. A.; Lipp, E.; McCellan, S.; McMinn, B.; Medema, G.; Metcalf, S.; Meijer, W. G.; Mueller, J. F.; Murphy, H.; Naughton, C. C.; Noble, R. T.; Payappat, S.; Petter, S.; Pitkänen, T.; Rajal, V. B.; Reyneke, B.; Roman, F. A.; Rose, J. B.; Rusinol, M.; Sadowsky, M. J.; Salama Comorera, L.; Setoh, Y. X.; Sherchan, S.; Sirikanchana, K.; Smith, W.; Steele, J.; Samburg, R.; Symonds, E. M.; Thai, P.; Thomas, K. V.; Tynan, J.; Toze, S.; Thompson, J.; Whiteley, A. S.; Wong, J. C. C.; Sano, D.; Wuertz, S.; Xagoraraki, I.; Zhang, Q.; Zimmer-Faust, A. G.; Shanks, O. C. Minimizing Errors in RT-PCR Detection and Quantification of SARS-CoV-2 RNA for Wastewater Surveillance. *Sci. Total Environ.* 2022, 805, 149877.

(33) Ahmed, W.; Simpson, S. L.; Bertsch, P. M.; Bibby, K.; Bivins, A.; Blackall, L. L.; Boill-Mas, S.; Bosch, A.; Brandão, J.; Choi, P. M.; Ciesielski, M.; Donner, E.; D’Souza, N.; Famletiner, A. H.; Gerrity, D.; Gonzalez, R.; Griffith, J. F.; Gyawali, P.; Haas, C. N.; Hamilton, K. A.; Hapuarachchi, H. C.; Harwood, V. J.; Haque, R.; Jackson, G.; Khan, S. J.; Khan, W.; Kitajima, M.; Korajkic, A.; La Rosa, G.; Layton, B. A.; Lipp, E.; McCellan, S.; McMinn, B.; Medema, G.; Metcalf, S.; Meijer, W. G.; Mueller, J. F.; Murphy, H.; Naughton, C. C.; Noble, R. T.; Payappat, S.; Petter, S.; Pitkänen, T.; Rajal, V. B.; Reyneke, B.; Roman, F. A.; Rose, J. B.; Rusinol, M.; Sadowsky, M. J.; Sala Comorera, L.; Setoh, Y. X.; Sherchan, S.; Sirikanchana, K.; Smith, W.; Steele, J.; Samburg, R.; Symonds, E. M.; Thai, P.; Thomas, K. V.; Tynan, J.; Toze, S.; Thompson, J.; Whiteley, A. S.; Wong, J. C. C.; Sano, D.; Wuertz, S.; Xagoraraki, I.; Zhang, Q.; Zimmer-Faust, A. G.; Shanks, O. C. Minimizing Errors in RT-PCR Detection and Quantification of SARS-CoV-2 RNA for Wastewater Surveillance. *Sci. Total Environ.* 2022, 805, 149877.

(34) Whitney, O. N.; Kennedy, L. C.; Fan, V. B.; Hinkle, A.; Kantor, R.; Greenwald, H.; Crits-Christoph, A.; Al-Shayeb, B.; Chaplin, M.; Maurer, A. C.; Tjian, R.; Nelson, K. L. Tools for Interpretation of Wastewater Based Epidemiology: Long-Term Monitoring of 10 WWTP in France Reveals the Importance of the Sampling Context. *Sci. Total Environ.* 2021, 84, 1997–2013.

(35) Mantilla-Calderon, D.; Huang, K. K.; Li, A.; Chibwe, K.; Yu, X.; Ye, Y.; Liu, L.; Ling, F. Meta-Analyses on SARS-CoV-2 Viral Titters in Wastewater and Their Correlations to Epidemiological Indicators. *MedRxiv* 2022, DOI: 10.1101/2022.02.14.22270937.

(36) Ye, Y.; Ellenberg, R. M.; Graham, K. E.; Wigginton, K. R. Survivalivity, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater. *Environ. Sci. Technol.* 2016, 50 (10), 5077–5085.

(37) Lazuka, A.; Arnal, C.; Soyeux, E.; Sampson, M.; Lepeuple, A.-S.; Deleuze, Y.; Pouradier Duteil, S.; Lacroix, S. COVID-19 Wastewater Based Epidemiology: Long-Term Monitoring of 10 WWTP in France Reveals the Importance of the Sampling Context. *Water Sci. Technol.* 2021, 84, 1–14.

(38) Martin, J.; Klapsa, D.; Wilton, T.; Zambon, M.; Bentley, E.; Bujaki, E.; Fritzsche, M.; Mate, R.; Majumdar, M. Tracking SARS-CoV-2 in Sewage: Evidence of Changes in Virus Variant Prevalence during COVID-19 Pandemic. *Viruses* 2020, 12 (10), 1144.

(39) Agrawal, S.; Orscher, L.; Lackner, S. Long-Term Monitoring of SARS-CoV-2 RNA in Wastewater of the Frankfurt Metropolitan Area in Southern Germany. *Sci Rep.* 2021, 11 (1), 5372.

(40) Klymus, K. E.; Merkes, C. M.; Allison, M. J.; Goldberg, C. S.; Helbing, C. C.; Hunter, M. E.; Jackson, C. A.; Lance, R. F.; Mangan, A. M.; Monroe, E. M.; Piaggio, A. J.; Stokdyk, J. P.; Wilson, C. C.; Richter, C. A. Reporting the Limits of Detection and Quantification for Environmental DNA Assays. *Environmental DNA* 2020, 2 (3), 271–282.

(41) Larionov, A.; Krause, A.; Miller, W. A Standard Curve Based Method for Relative Real Time PCR Data Processing. *BMC Bioinformatics* 2005, 6, 62.

(42) R Development Core Team. R: A Language and Environment for Statistical Computing: R Foundation for Statistical Computing: Vienna, Austria, 2013.

(43) Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis; Use R!*; Springer: New York, 2009.

(44) Lee, W. L.; McElroy, K. A.; Arms, F.; Imakaev, M.; Gu, X.; Duvetale, C.; Chandra, F.; Chen, H.; Leifels, M.; Mendola, S.; Floyd-O’Sullivan, R.; Powell, M. M.; Wilson, S. T.; Wu, F.; Xiao, A.; Moniz, K.; Ghazi, N.; Matus, M.; Thompson, J.; Alm, E. J. Quantitative Detection of SARS-CoV-2 B.1.1.7 Variant in Wastewater by Allele-Specific RT-qPCR. *medRxiv* 2021, DOI: 10.1101/2021.03.28.21254404.

(45) Greenwald, H. D.; Kennedy, L. C.; Hinkle, A.; Whitney, O. N.; Fan, V. B.; Crits-Christoph, A.; Harris-Lovett, S.; Flamholz, A. I.; Al-Shayeb, B.; Liao, L. D.; Seyers, M.; Brown, D.; Chakrabarti, A. R.; Dow, J.; Frost, D.; Koekemoer, M.; Lynch, C.; Sarkar, P.; White, E.; Kantor, R.; Nelson, K. L. Tools for Interpretation of Wastewater SARS-CoV-2 Temporal and Spatial Trends Demonstrated with Data Collected in the San Francisco Bay Area. *Water Research X* 2021, 12, 100111.

(46) LaTumer, Z. W.; Zong, D. M.; Kalavapalle, P.; Gamas, K. R.; Terwilliger, A.; Crosby, T.; Ali, P.; Avadhana, V.; Santos, H. H.; Weensner, K.; Hopkins, L.; Piedra, P. A.; Maresso, A. W.; Stadler, L. B. Evaluating Recovery, Cost, and Throughput of Different Concentration Methods for SARS-CoV-2 Wastewater-Based Epidemiology. *Water Res.* 2021, 197, 117043.

(47) Juell, M. A. I.; Stark, N.; Nicolosi, B.; Lontai, J.; Lambirth, K.; Schluter, J.; Gibas, C.; Munir, M. Performance Evaluation of Virus Concentration Methods for Implementing SARS-CoV-2 Wastewater Based Epidemiology Emphasizing Quick Data Turnaround. *Sci. Total Environ.* 2021, 801, 149656.

(48) Kantor, R. S.; Nelson, K. L.; Greenwald, H. D.; Kennedy, L. C. Challenges in Measuring the Recovery of SARS-CoV-2 from Wastewater. *Environ. Sci. Technol.* 2021, 55 (6), 3514–3519.
