Monitoring SARS-CoV-2 RNA in Wastewater with RT-qPCR and Chip-Based RT-dPCR: Sewershed-Level Trends and Relationships to COVID-19

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ABSTRACT: We evaluated the performance of reverse transcription quantitative PCR (uniplex and duplex RT-qPCR) and chip-based digital PCR (duplex RT-dPCR) using CDC N1 and CDC N2 assays for longitudinal monitoring of SARS-CoV-2 RNA in influent wastewater samples (n = 281) from three wastewater plants in Ohio from January 2021 to January 2022. Human fecal virus (PMMoV) and wastewater flow rate were used to normalize SARS-CoV-2 concentrations. SARS-CoV-2 measurements and COVID-19 cases were strongly correlated, but normalization effects on correlations varied between sewersheds. SARS-CoV-2 measurements by RT-qPCR were strongly correlated with 7-day moving average COVID-19 cases (average Spearman’s ρ = 0.58, p < 0.05). SARS-CoV-2 was detected more frequently in samples with duplex RT-dPCR than with duplex RT-qPCR during periods of low COVID-19 cases. Duplex and uniplex RT-qPCR N1 concentrations were more strongly correlated with cases (ρ = 0.62) than N2 (ρ = 0.52). RT-dPCR correlations (average ρ = 0.21) were weaker than those of RT-qPCR (average ρ = 0.58). We also share practical experience from establishing wastewater surveillance. Per sample, RT-qPCR had a lower cost ($6 vs $18) and sample turnaround time (3–4 h vs 7–9 h) than RT-dPCR. These findings reinforce selection and use of PCR-based wastewater surveillance tools.

KEYWORDS: COVID-19, SARS-CoV-2, wastewater surveillance, RT-qPCR, chip-based RT-dPCR, uniplex, duplex

1. INTRODUCTION

Wastewater monitoring is a promising tool for complementing clinical diagnoses of COVID-19 in community settings that provides early warning of community infection, near real-time monitoring of outbreaks, and cost-effective detection for pooled samples in communities, even with asymptomatic individuals.1–5 The typical workflow includes collection of grab or composite samples from wastewater treatment plants, virus concentration, RNA extraction, and detection or quantification of target genes in samples using molecular-based analytical methods (e.g., polymerase chain reaction, PCR). Since wastewater surveillance was used for monitoring COVID-19 from the start of the pandemic,6,5 methodologies have varied greatly.6,8–9 Numerous research groups have demonstrated that SARS-CoV-2 in wastewater reflects trends in community-level COVID-19 dynamics, and some reports observe that wastewater trends precede those of clinical reports.7,9 Molecular-based wastewater surveillance depends on many factors, such as molecular processing methods (e.g., concentration and extraction of viral RNA), biochemical and microbiological complexity of wastewater matrix that can cause inhibition of molecular analyses, residence time in the sewer system, composite sampling duration and frequency, upstream sample processing methods, and virus recovery.10–12

Molecular-based quantification tools available for wastewater surveillance include reverse transcription (RT) PCR techniques such as quantitative PCR (RT-qPCR), digital droplet PCR (RT-ddPCR), and chip-based digital PCR (RT-dPCR),13,14 each with different advantages and disadvantages. qPCR is a well-characterized technique that has been previously used for reliable detection and relative quantification of viral pathogens.15 Digital PCR is a relatively new PCR-based technology for detection and absolute quantification of viral pathogens.16 dPCR quantifies samples based on Poisson statistics of positive or negative signals of samples partitioned into many individual subreactions with few or no target sequences to limit primer and probe competition.19,20 dPCR platforms create thousands of partitions through the generation of water–oil emulsion droplets (droplet digital

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### Table 1. Summary of Wastewater Facility Operating Characteristics and Sewershed Population

| facility               | municipality       | county       | avg flow rate (m$^3$/d) | population served | total cases |
|------------------------|--------------------|--------------|--------------------------|-------------------|-------------|
| Beavercreek WRRF       | Beavercreek        | Greene       | 31 600                   | 47 000            | 7 344       |
| Oxford WWTP            | Oxford             | Butler       | 8 700                    | 21 300            | 3 856       |
| Tri-Cities North Regional WWTP | Tipp City, Vandalia, and Huber Heights | Montgomery | 37 600                   | 65 000            | 12 989      |

“Totals cases that meet the demographic criteria specified are newly reported for each date between 1/22/2020 to 1/2/2022. (OCWMN Dashboard). Approximate population data was obtained from each wastewater facility. The three wastewater collection systems are separate from stormwater systems.

PCR, ddPCR) or with microfluidic dPCR plates (i.e., chips) containing nanoliter volume wells.

In this study, we used both RT-qPCR and chip-based RT-dPCR to quantify target genes after reverse transcription of viral RNA. Compared to qPCR, dPCR can reduce sample volume for analysis, is less impacted by matrix inhibition, achieves absolute quantification of nucleic acid targets without external standards, has greater repeatability, and is approaching the linear dynamic range of qPCR. Moreover, the ideal PCR technique for wastewater monitoring will have a wide dynamic range and low limit of detection (LOD). Additionally, PCR techniques can detect two or more gene targets within a single reaction (i.e., duplexing or multiplexing), which can benefit wastewater surveillance by improving sample throughput, increasing information output from less sample volume, minimizing variability in pipetting, and saving reagents.

Accurate quantification can be achieved by optimizing primer and probe sets for each target, but increasing number of targets can decrease multiplex assay performance (e.g., efficiency and limit of detection). Previously, researchers have employed both digital droplet PCR and chip-based dPCR for wastewater surveillance of SARS-CoV-2 RNA. However, few studies report the use of chip-based dPCR for wastewater monitoring. Multiplex RT-qPCR reactions have also been used for quantification of SARS-CoV-2 in wastewater surveillance. Recently, RT-qPCR and RT-dPCR platforms were compared for trace detection of SARS-CoV-2 RNA in wastewater. However, multiplex, chip-based RT-dPCR has not yet been evaluated for wastewater surveillance of SARS-CoV-2 RNA.

The objectives of our study were to compare duplex RT-qPCR assays to uniplex RT-qPCR and duplex RT-dPCR assays for quantification of SARS-CoV-2 in wastewater samples from three sewersheds in Ohio. We report the first use of the QuantStudio 3D Digital PCR System for nondroplet, chip-based RT-dPCR analyses of SARS-CoV-2 RNA in wastewater, and of duplex RT-dPCR analyses of SARS-CoV-2 RNA in wastewater. Additionally, utility of these methods for wastewater-based epidemiology (WBE) was compared across PCR platforms by correlation and regression analyses between SARS-CoV-2 RNA concentrations and COVID-19 cases. To compensate for sewershed variability, we normalized SARS-CoV-2 RNA concentrations with two methods: a common human fecal viral indicator, pepper mild mottle virus (PMMoV), and daily wastewater flow rates. We also compare pragmatic considerations (time, cost, etc.) for establishing wastewater surveillance procedures based on our ongoing contributions to the Ohio Coronavirus Wastewater Monitoring Network (OCWMN).  

#### 2. MATERIALS AND METHODS

##### 2.1. Wastewater Sample Collection and Processing

Composite wastewater samples (flow-proportionate, 24 h, $n = 281$) were collected by autosamplers at the influents of Beavercreek Water Resources Recovery Facility (WRRF) ($n = 95$), Oxford Wastewater Treatment Plant (WTTP) ($n = 93$), and Tri-Cities North Regional WWTP ($n = 93$) in Ohio from January 3, 2021 to January 2, 2022. Sewersheds were defined as the service boundaries containing the residences of confirmed COVID-19 cases. Population details and wastewater facility characteristics are summarized (Table 1). Each facility provided 1 L of 24 h composite, untreated wastewater influent starting on Sunday and Wednesday between 7 to 9 AM each day. Samples were shipped overnight on ice until switching to same-day courier delivery on 08/02/2021. Samples were immediately processed upon receipt at the lab.

Upon arrival at the lab, samples were inoculated with 500 μL of Bovine Coronavirus (BCoV) Cell Vaccine (Merck Animal Health, Product No. 1644) as RNA extraction positive control and for calculating recovery efficiency. After inoculation, samples were homogenized by orbital shaking at room temperature (180 rpm, 10 min). Viral particles, microorganisms, and nonfilterable solids were concentrated from inoculated wastewater samples on 0.45 μm pore size mixed cellulose ester filter membranes ( Pall, GN-6 Metrical) by vacuum filtration with sterile disposable volumetric filter funnels (VWR International, Product No. 28143-568). Duplicates of each sample from each sampling day were vacuum filtered to dryness within 1–2 h. The average filtered volume for all filtration replicates for Beavercreek, Oxford, and Tri-Cities was 187.5 ± 40.9 mL (n = 190), 140.5 ± 36.4 mL (n = 186), and 109.6 ± 19.4 mL (n = 186), respectively. For each day of sample processing, a filtration blank consisting of a dry filter membrane was processed in parallel to samples to detect contamination of extraction reagents. After filtration, membranes were transferred to PowerBead Tubes (QIAGEN, Griel 0.70 mm) with 1 mL of TRIzol Reagent (Invitrogen). RNA extraction was performed with slight modifications to manufacturer’s instructions (Supplemental Text S1). During routine monitoring, samples and blanks were stored at −80 °C before RT-qPCR analysis.

##### 2.2. Quantification of SARS-CoV-2 RNA, PMMoV, and BCoV by RT-qPCR

One-step RT-qPCR assays were used to quantify copies of SARS-CoV-2 N1 and N2 genes, PMMoV, and BCoV. Positive control DNA in plasmid form or gBlocks gene fragments were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA). Primer, probe, and positive control details are summarized in the Supporting Information, Table ST1. N gene RT-qPCR standards were prepared from 2019-nCoV-N. Positive Control nonlinearized, double-stranded DNA (dsDNA) plasmid with standard dilutions ranging from 2.0 × 10⁻¹ to 2.0 × 10⁵ copies/μL. PMMoV and BCoV standards were prepared from the same dsDNA gBlocks gene fragments containing both target sequences with standard dilutions ranging from 2.78 ×
10^{-2} to 2.78 × 10^{6} \text{ copies/μL}. Standard dilutions were stored at −80 °C and reused up to 3 times.

All RT-qPCR reactions were performed using Applied Biosystems QuantStudio 6 Flex Real Time PCR System (Waltham, MA) using automatic settings for the threshold and baseline. N1 and N2 uniplex and duplex RT-qPCR reaction mixtures contained 10 μL of qScript XLT 1-Step RT-qPCR ToughMix (Quantabio, Beverly, MA), 500 nM forward primer, 500 nM reverse primer, 125 nM probe, and 2 μL of template RNA, standard dilution, nontemplate control, or blanks. PMMoV and BCoV uniplex and duplex RT-qPCR mixtures contained 10 μL of qScript XLT 1-Step RT-qPCR ToughMix, 500 nM forward primer, 500 nM reverse primer, 125 nM probe, and 2 μL of template RNA, standard dilutions, nontemplate control, or blanks. UltraPure DNase/RNase-Free Distilled Water (Invitrogen, 10977-023) was used to dilute standards and template RNA and as nontemplate control (NTC). NTC and filtration blank extracts were quantified in triplicate for QA/QC. During routine monitoring, plates were set up by hand or by BrandTech Liquid Handling Station (BrandTech Scientific, Essex, CT). All plates that were reanalyzed were set up by hand. Due to spatial and temporal variability in wastewater strength, analysis was performed on RNA samples diluted to factors of 1:2, 1:5, 1:10, or 1:100 to minimize impact of inhibition on RT-qPCR analysis by the wastewater matrix while minimizing dilution impacts on detection limit.

RT-qPCR thermal cycling conditions for N1 and N2 were 50 °C × 10 min, 95 °C × 3 min, and 95 °C × 3 s followed by 55 °C for 30 s for 40–45 cycles. RT-qPCR thermal cycling conditions for PMMoV and BCoV were 50 °C × 10 min, 95 °C × 3 min, and 95 °C × 3 s followed by 60 °C for 60 s for 40–45 cycles. Total cycle numbers were 40 and 45 for uniplex and duplex reactions, respectively. The ramp rate was 1.6 °C/sec for all RT-qPCR reactions. Technical duplicates were analyzed for each biological duplicate, totaling four replicates per facility per sample date.

During a laboratory personnel training period (January 3, 2021 to February 17, 2021), RNA samples were analyzed for routine monitoring using uniplex RT-qPCR assays. The training period data were reported to the OCWMN and included here for trend visualization, but not included in statistical analyses. Both RT-qPCR and RT-dPCR were used to analyze the same RNA extracts, but not at the same time. Starting February 24, 2021, routine monitoring switched from uniplex to duplex RT-qPCR assays (N1 with N2, and PMMoV with BCoV) to increase sample throughput and decrease hands-on time. RNA samples analyzed during routine monitoring were stored at −80 °C and subsequently reanalyzed using either uniplex or duplex RT-qPCR and duplex RT-dPCR targeting N1 and N2. The MIQE Checklist is provided in Table ST2.

2.3. RT-qPCR Data Analysis. RT-qPCR standard curves for each instrument run were generated from linear regression of cycle threshold (CT) values versus log_{10} concentration of standard, and used to convert CT values into N1, N2, PMMoV, and BCoV copies/μL per reaction. Standard curves were manually inspected after each instrument run. As performed previously, 40 grossly false positives and false negatives of standard dilutions were omitted from the standard curve (n = 11 standard curve points for duplex N1 and N2 and n = 3 for duplex PMMoV and BCoV) if they were at least ±2 CT values from expected values, and standard curve parameters and concentrations were recalculated using the QuantStudio 6 software. During the entire routine monitoring period, potential contamination detected by amplification of nontemplate controls (NTC) was noted when reporting data to ODH and presented for trend visualization, but samples were not rerun nor included in subsequent statistical or WBE analyses reported here.

Copies/μL per well were converted to SARS-CoV-2 RNA copies/L (GC/L) in wastewater accounting for the following: dilution factor (e.g., 10 for 1:10 dilution of original RNA sample), reaction volume per well (20 μL), RNA template volume (2 μL), RNA elution volume (50 μL), and wastewater volume filtered. In the QuantStudio 6 software, we entered standard concentrations as dsDNA plasmid copies, so RT-qPCR concentrations for N1 and N2 were multiplied by 2 to correct for the 1-cycle difference for reverse transcription of single stranded SARS-CoV-2 RNA unknowns to obtain equivalent copies of target ssRNA. Concentrations of N1 or N2 were averaged across biological duplicates after averaging technical duplicates for each individual primer set. For N1 and N2, error was determined as the standard error between biological duplicates. Samples where at least one technical replicate was quantified were considered detects by RT-qPCR: CT > 40 (uniplex) or CT < 45 (duplex). Samples were considered nondetects when no amplification was observed for all replicates. PMMoV and BCoV concentrations were calculated as the average of biological duplicates after averaging technical triplicates.

RT-qPCR uniplex and duplex assay limits of detection (ALOD) were determined by diluting and analyzing IDT dsDNA plasmid control. Data analysis for ALOD was performed by fitting a Logit regression to the observed proportion of positive replicates at each concentration of the dilution series. More details and R Script for calculating LOD are provided in Supporting Information, Text S2. To estimate overall RT-qPCR method sensitivity to losses during sample processing, method LOD (MLOD) and method limit of quantification (MLOQ) were calculated as a function of ALOD and ALOQ, respectively, by accounting for template volume per well, RNA elution volume, and filtration volume. We assumed a typical filtration volume of 150 mL, based on the average of all filtration volumes. Samples that were detects but less than ALOD were considered Below LOD. Logit tables are provided in Table ST3.

Viral recovery efficiency was calculated for each facility using BCoV as viral surrogate to evaluate consistency of sample processing and RNA extraction, as described in Text S3. Between January 3, 2021 and January 2, 2022, BCoV recovery efficiency was calculated for 206 of 281 total samples across the three facilities; 75 samples were excluded from BCoV assays with contamination. BCoV recovery ranged from 0.34 to 90.65% for all facilities (Figure S1). Mean recoveries for Beavercreek, Oxford, and Tri-Cities were 15.6% (n = 76), 20.5% (n = 74), and 18.6% (n = 74), respectively. Wastewater data in this study are not corrected for recovery efficiency.

2.4. SARS-CoV-2 RNA quantification by RT-dPCR and data analysis. Two-step duplex RT-dPCR assays for N1 and N2 were performed. Reverse transcription of extracted RNA template was performed using the iScript cDNA Synthesis Kit (Bio-Rad) with 4 μL of 5x iScript Reaction Mix, 1 μL of iScript Reverse Transcriptase, 2 μL of RNA template, and nuclease free water in 20 μL of reaction volume. Undiluted wastewater RNA extracts were used for RT-dPCR analysis. Thermal
cycling (Bio-Rad S1000) for reverse transcription was 25 °C for 5 min (priming), 46 °C for 20 min (reverse transcription), and 95 °C for 1 min (RT inactivation). cDNA was immediately analyzed by dPCR or stored at 4 °C until quantification. Each biological replicate was assayed once with chip partitions resulting in up to 20 000 pseudotechnical replicates.

RT-dPCR was performed using the QuantStudio 3D Digital PCR System (Applied Biosystems, Forest City, CA) and 20 000 partition QuantStudio 3D digital PCR v2 chips (Applied Biosystems, Catalog No. A26316). N1 and N2 primer and probe concentrations for RT-dPCR were the same as for RT-qPCR (Table ST1), but total reaction volume was decreased from 20 to 15 μL. The RT-dPCR reaction mixture was composed of 7.5 μL of QuantStudio 3D Digital PCR Mastermix v2 (Applied Biosystems), 500 nM each of forward and reverse primers, 125 nM probes, 3 μL of cDNA template, and nuclease free water to bring the total volume to 15 μL. 14.5 μL of reaction mixture was loaded onto a chip using the QuantStudio 3D Digital PCR Chip Loader (Applied Biosystems, Catalog No. 4482592) according to manufacturer instructions. Thermal cycling conditions were 96 °C × 10 min, 60 °C × 2 min, and 98 °C × 30 s for 40 cycles. One NTC chip was included in each instrument run.

Chips were imaged using the QuantStudio 3D Digital PCR system (Application version 3.1.6-PRC-build18; Algorithm version 4.4.10). Chips not imaged immediately were held no longer than 24 h at 10 °C before imaging. All chips were uploaded to and analyzed using cloud-based QuantStudio 3D AnalysisSuite Software. The number of partitions ranged from 8251 to 18232 with a mean ± standard deviation (SD) of 17775 ± 915. RT-dPCR assays were analyzed using automatic settings for fluorescence thresholds for both VIC and FAM channels. Quantities were expressed as copies/μL of reaction mixture, and then converted to SARS-CoV-2 RNA concentrations per liter of wastewater (GC/L). The standard error of means for biological duplicates was calculated for each sample. Each chip (n = 468, includes wastewater samples and positive

Figure 1. Log₁₀ transformed SARS-CoV-2 RNA wastewater concentrations (left axis, data points) and 7-day moving average cases (right axis, black line) for three sewersheds in Ohio (Beavercreek, Oxford, and Tri-Cities) using uniplex and duplex RT-qPCR between January 2021 to January 2022. Wastewater data from the training period before 2/24/2021 (shaded gray) were not included in statistical analyses but were reported to OCWMN. Points represent mean concentrations of biological replicates after averaging technical duplicates and error bars represent standard error (SE) of mean biological replicate concentrations. The dashed and dotted lines represent the MLOD for uniplex and duplex N1 and N2 assays, respectively. Points at zero were samples with no amplification across four technical replicates. Points below the MLOD were considered quantified but below LOD. Points without error bars did not have sufficient data to calculate SE of mean. Some error bars are smaller than the symbols.
and negative controls) was inspected during QA/QC by two individuals. Chips with <10,000 partitions ($n = 11$) or poor imaging (e.g., due to leaking, bubbles) ($n = 7$) were omitted from data analysis and not reanalyzed due to time constraint. Manual thresholds were used to replace automatic thresholds of chips ($n = 43$) when automatic thresholds were inconsistent with other chips of the same run. Manual thresholds for FAM and VIC channels were determined for NTC chips of each run by adding three times the full-width half-maximum to the fluorescence value at the peak. As performed previously, seven replicates of NTC were used to calculate the 95% limit of blank for FAM (N1) and VIC (N2) channels to be $0.849 \pm 0.385$ and $0.626 \pm 0.138$ gene copies per microliter of reaction mixture (mean $\pm$ SD based on upper 95% confidence interval values exported from QuantStudio). Similar to the MLOD for RT-qPCR, the limit of blank value was converted by appropriate processing volumes (e.g., template volumes, master mix, RT, filtration, elution). The dMIQE Checklist is provided in Table ST4. Example positive and NTC chips are shown in Figure S2.

2.5. Statistical Analyses of Relationships between SARS-CoV-2 in Wastewater and COVID-19 Cases. For each sewersheds, relationships between SARS-CoV-2 RNA data and COVID-19 cases were evaluated through correlation and linear regression analyses for data from January 3, 2021 to January 2, 2022. Total number of COVID-19 cases and daily influent wastewater flow rate (i.e., flow rate to the same facility within 1 day) were obtained from the Ohio Department of Health COVID-19 Dashboard. The 7-day moving average for COVID-19 cases was calculated and used for correlation analyses because COVID-19 cases were not reported for each sampling dates for each sewersheds. Spearman correlation analyses were performed between 7-day moving average cases and (1) SARS-CoV-2 RNA concentrations, (2) concentrations normalized by the associated PMMoV concentrations, and (3) concentrations normalized for daily influent wastewater flow rate (i.e., daily load) to evaluate which wastewater based metric and quantification technique exhibited the strongest correlation and therefore potential to predict COVID-19 cases. COVID-19 cases were also normalized by population in each

Figure 2. Log$_{10}$ transformed SARS-CoV-2 RNA wastewater concentrations (left axis, data points) and 7-day moving average cases (right axis, black line) for three sewersheds in Ohio (Beavercreek, Oxford, and Tri-Cities) measured using RT-dPCR and RT-qPCR (repeated from Figure 1) from January 2021 to January 2022. The dotted line represents the MLOD for duplex N1 and N2 assay. RT-qPCR points below the MLOD were considered quantified but below LOD. RT-dPCR data points are means of biological duplicates with variable pseudoreplicates per chip. Points at zero were samples with no amplification across two biological replicates for RT-dPCR. Points without error bars did not have sufficient data to calculate the SE of mean. Some error bars are smaller than the symbols.
3. RESULTS

3.1. RT-qPCR Assay Performance. We compared performance of uniplex and duplex RT-qPCR assays targeting N1 and N2. For RT-qPCR, mean efficiencies for uniplex and duplex reaction standard curves for all targets were within the acceptable range for PCR amplification efficiency (90–110%), but average reaction efficiencies for all targets quantified by uniplex RT-qPCR were higher than duplex RT-qPCR (Table ST5). Mean y-intercepts for uniplex and duplex N1 (32.7 to 35.5) and N2 (30.9 to 34.8) standard curves were slightly lower than other reports of N1 (36.1 to 42.5) and N2 (37.8 to 53.5) generated using the same IDT positive control material. Summary statistics of standard curve parameters for uniplex and duplex RT-qPCR reactions for N1, N2, PMMoV, and BCoV (excluding training period and contaminated data as described in Methods) are provided in Figure S3 and Table ST5. For runs with NTC amplification (n = 4 duplex RT-qPCR N1 and N2; n = 1 uniplex N2), data were discarded and not included in further analysis, and samples were not reanalyzed. RT-qPCR ALODs determined by logit regression were 1.09 and 1.24 copies/μL for N1 and N2 uniplex and duplex reactions, respectively (Figure S4) and MLODs were $4.15 \times 10^3$ and $3.65 \times 10^3$ copies/μL wastewater, respectively. Additionally, RT-qPCR ALODs were 0.32 and 1.38 copies/μL for duplex PMMoV and BCoV, respectively (Figure S4). Determination and values for ALOQ and MLOQ are summarized (Table ST6).

3.2. Quantification of SARS-CoV-2 RNA Concentrations in Wastewater by RT-dPCR. During the study period, N1 and N2 SARS-CoV-2 RNA targets were detected in wastewater influents from three wastewater facilities using both uniplex and duplex RT-qPCR assays (Figure 1). SARS-CoV-2 RNA N1 and N2 concentrations were relatively high in January 2021, decreased from February 2021 to May 2021, and stabilized in the summer months. Wastewater concentrations increased again in August 2021 into October 2021 and continued to increase through January 2022 as new variants emerged. Nondetects were frequent from April 2021 to July 2021 and coincided with low COVID-19 cases. Despite the differences in sewershed population (Table 1), unique and changing composition of wastewater (PMMoV concentrations for each wastewater facility are summarized in Figure S5), and variable dynamics of COVID-19 infection in the population, the overall magnitude and trend of wastewater concentrations were similar between the three facilities (Figure 1). More detects were observed for N1 than N2 for both assays, and for uniplex over duplex assays for both targets (Table ST7).

3.3. Quantification of SARS-CoV-2 RNA Concentrations in Wastewater by RT-dPCR. SARS-CoV-2 RNA concentrations in the three sewersheds quantified by duplex RT-dPCR ranged from $7.6 \times 10^3$ to $3.14 \times 10^4$ and $1.28 \times 10^3$ to $3.31 \times 10^4$ copies/L wastewater for N1 and N2, respectively, and were between one to four orders of magnitude lower than concentrations determined by duplex RT-qPCR (Figure 2). Between April 2021 and July 2021, detection of SARS-CoV-2 RNA by RT-dPCR was more likely than by RT-qPCR. Disparity in the SARS-CoV-2 RNA concentrations quantified by RT-qPCR versus RT-dPCR varied throughout the year. The MLODs calculated from the limit of blank for RT-dPCR for FAM (N1) and VIC (N2) channels were $7.08 \times 10^3$ and $5.21 \times 10^3$ copies/L wastewater, respectively. The duplex RT-dPCR assays for N1 and N2 were able to detect SARS-CoV-2 at about the same rate; about 74% of all sample concentrations were quantified but below the N1 and N2 method limit of blanks.

3.4. Relationships between SARS-CoV-2 RNA Measurements and COVID-19 Cases. Spearman’s correlation was used to quantify the association and linear relationships between wastewater measurements and COVID-19 cases, summarized in Figure S6. When considering RT-qPCR data for all individual sewersheds, significant positive correlations were observed between 7-day moving averages of COVID-19 cases and SARS-CoV-2 RNA concentrations. Significant correlations (Spearman) were noted with * p-values <0.05, ** < 0.01, *** < 0.001, and **** <0.0001, respectively. Correlation analyses were not performed between RT-dPCR data and PMMoV Normalized Concentrations.

Figure 3. Spearman correlations between wastewater data and 7-day moving average COVID-19 cases. Concentrations = SARS-CoV-2 RNA concentrations (copies/L wastewater) quantified using RT-qPCR or RT-dPCR (uniplex or duplex CDC N1 and CDC N2); Loads = SARS-CoV-2 RNA loading rate (copies/day); PMMoV Normalized Concentrations = SARS-CoV-2 RNA concentrations divided by PMMoV concentrations quantified using duplex RT-qPCR (copies N gene/copies PMMoV). Significant correlations (Spearman) were noted with *, **, ***, and **** for p-values <0.05, < 0.01, < 0.001, and <0.0001, respectively.
cases and either SARS-CoV-2 RNA concentrations, SARS-CoV-2 RNA load, and PMMoV normalized SARS-CoV-2 RNA concentrations (Figure 3), indicating strong association of the concentration of SARS-CoV-2 RNA in wastewater with COVID-19 cases. Normalizing cases by population (7-day moving average cases per 100,000 population, Figure S7) yielded the same correlations (Figure S6). Normalizing by PMMoV concentration only slightly improved correlations for Beavercreek (maximum increase in $\rho = 0.17$) (Figure S6C). We only observed an increase in COVID-19 correlation with load compared with concentration for Oxford (Figure S6D). When considering RT-dPCR data, however, only cases correlated with N2 concentration in Oxford ($\rho = 0.32$), SARS-CoV-2 load in Oxford (average $\rho$ for N1 and N2 = 0.37), SARS-CoV-2 load in Tri-Cities (average $\rho$ for N1 and N2 = 0.33), and SARS-CoV-2 concentration in Tri-Cities (average $\rho$ for N1 and N2 = 0.34) were significant ($p < 0.05$). Across all PCR assays and 7-day moving averages, correlations were greater for Tri-Cities (average $\rho = 0.67$) than other sewersheds (Beavercreek and Oxford each had average $\rho = 0.53$). For 7-day moving averages of cases across sewersheds, RT-qPCR uniplex N1 exhibited the strongest association (average $\rho = 0.67$), followed by RT-qPCR duplex N1 (average $\rho = 0.59$), RT-qPCR uniplex N2 (average $\rho = 0.57$), and RT-qPCR duplex N2 (average $\rho = 0.48$).

Normalizing viral concentrations by wastewater flow (i.e., viral load) generally increased the correlation with 7-day moving average cases, as with Oxford, but did not for Tri-Cities and Beavercreek. Normalizing concentrations by PMMoV resulted in slight improvements in correlation coefficients for Tri-Cities and Beavercreek, while Oxford correlations decreased (Figure 3).

The results of the nonparametric Spearman’s rank correlation indicate strength of the direct relationships between all three measures of SARS-CoV-2. Goodness of fit and magnitudes of the relationships were determined through linear regressions of 7-day moving average cases versus viral concentrations, viral loads, and PMMoV normalized viral concentrations. Slopes of linear regressions indicate the predictive relationship between COVID-19 and log$_{10}$ SARS-CoV-2. The slopes for Tri-Cities were generally greater than those for Beavercreek and Oxford (Figure S6), suggesting that the ability of WBE to detect changes in COVID-19 increases as population increases. The fit for Tri-Cities RT-qPCR N1 data was consistently high for viral concentration ($R^2 = 0.47$) and viral load ($R^2 = 0.4$); PMMoV normalized concentrations and population normalized 7-day moving average cases yielded similar linear regression fits. Other fits ($R^2$) ranged from negligible to 0.38. For RT-dPCR, fits ($R^2$) were all less than 0.13, and mainly negligible.

4. DISCUSSION

4.1. Wastewater Matrix Impacts on Molecular Analyses for Wastewater-Based Epidemiology. Each wastewater facility reported varying quantity and composition of industrial wastewater streams. Industrial activities accounted for approximately 0.4, 9.8, and 0.3% of Beavercreek, Oxford, and Tri-Cities wastewater, respectively, and included metal finish, chemical etching, food production, pharmaceutical, and electrical processing waste. Temporal variation in wastewater may have impacted variability in BCov recovery (average 18.2%), which was slightly greater than one report (4.8%); but aligned with other reports (16.8 to 53.2%). Larger populations can also contribute to greater dietary variation that may contribute to fecal strength and human fecal markers (e.g., PMMoV). RT-qPCR nondetects from May 2021 to July 2021 could have been impacted by low virus recovery efficiency (Figure S1) coupled with low case counts (Figure 1), resulting in potential false negatives. After August 2, 2021, the switch from overnight shipment to same-day courier service decreased time between sampling to processing from 24 to 28 h to 4–5 h, minimizing any potential impacts of RNA degradation in wastewater. All RT-dPCR analyses were performed after RT-qPCR analyses, so RNA sample degradation during long-term storage at $-80\, ^\circ C$ (4–10 months) and unavoidable freeze thaw cycles (2–3 cycles) may contribute to variations and uncertainty in quantification. Additionally, RNA sample storage times were longer than 1 month, but RNA stability was not verified. Varying dilution of RNA (e.g., 1:10 or 1:100) to minimize PCR inhibition as wastewater quality changed temporally, and potential subsampling errors from pipetting, partitioning, and analyzing dilutions of low concentration samples may have also impacted recovery and quantification.

4.2. Comparison of RT-qPCR and RT-dPCR for Wastewater Monitoring. We compared duplex RT-qPCR and RT-dPCR assays for N1 and N2, but uniplex comparisons between the platforms were not performed due to reagent cost, supply chain issues, and time constraints. Although primer and probe concentrations and template volume were consistent, reverse transcription was one-step for RT-qPCR and two-step for RT-dPCR due to unavailability of one-step dPCR kits during study design.

PCR platform could impact measured SARS-CoV-2 concentrations due to differences between quantification methods and limits of detection. Calculated ALOD values for N1 and N2 uniplex and duplex RT-qPCR assays were 21.8 and 24.8 copies/reaction, which are around 6–7 times greater than the probabilistic ALOD determined by the Poisson distribution (3 copies/reaction) and ~20 times greater than the theoretical ALOD (1 copy/reaction). Our ALODs for uniplex and duplex N2 are comparable to the uniplex RT-qPCR N2 ALOD (26.7 copies/reaction) but our N1 ALOD is higher than the N1 ALOD value (9.5 copies/reaction) previously reported. Both our uniplex and duplex N1 and N2 ALODs are slightly greater than previous reports of MOLODs for triplex RT-qPCR (1 copy/μL). Our RT-qPCR LOD values suggest suboptimal reaction efficiency, and may have contributed to frequent nondetects. Although modeling ALOD is limited since it is based on probability rather than detection of fixed quantities, MLOD indicates the overall method capability by accounting for processing steps. Increasing filtered volume to decrease MLOD could increase the concentration of inhibitors in samples and increase sample turnaround time (e.g., due to filter clogging). The high limit of blank (LOB) determined for NTCs by RT-dPCR decreased the ability to distinguish SARS-CoV-2. Using 2-step RT-dPCR could have also decreased detection of SARS-CoV-2 due to extra dilution. The natures of RT-qPCR and RT-dPCR as relative and absolute quantification methods also contributed to the differences in quantification and limits of detection. We also evaluated practical considerations of using RT-qPCR and chip-based RT-dPCR for long-term wastewater surveillance. An important similarity between platforms was importance of operator knowledge. During training (January 2021 and February 2021), RT-qPCR efficiencies improved

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from <50% to >90%, incidents of contamination decreased, and sample turnaround time decreased. Prior knowledge of viral concentrations from running RT-qPCR before RT-dPCR minimized wasted runs for samples outside the dPCR dynamic range, but using dPCR alone may require analysis of multiple dilutions.

Although both platforms required similar capital investment and space in the laboratory, sample processing time, sample throughput, and material and reagent costs favored RT-qPCR, as summarized in Table S79. After filtration and RNA extraction the day samples were received, sample turnaround times for RT-qPCR and RT-dPCR were 3–4 and 7–9 h, respectively (Table ST10). Although multiple runs of RT-qPCR but not RT-dPCR could be performed in a typical workday, dPCR requires no time preparing standards. However, newer chip-based dPCR systems with automated chip loading decrease hands-on time and variability, and increase efficiency and throughput. The separate RT step for RT-dPCR took extra time and may introduce error and contamination, but transcribing RNA to cDNA can minimize RNA degradation from freezing and thawing and provides a stopping point to batch samples and reduce hands-on time. RT-dPCR turnaround time is similar to RT-dPCR.31 The cost per sample for RT-dPCR ($17) is about 3 times the cost per sample for RT-qPCR ($6). Weekly analysis of technical duplicates of 9 samples at one dilution would cost $60 per plate using RT-qPCR (including technical replicates of samples, an 8-point standard curve, and NTC) and $300 per instrument run using RT-dPCR (including NTC).

4.3. Impacts of Quantification on Relationships between Wastewater Measurements and COVID-19 for Tracking Public Health Trends. Use of exogenous control materials has an impact on quantification of SARS-CoV-2 in wastewater samples.21 During routine monitoring by duplex RT-qPCR, our results were generally an order of magnitude greater than other laboratories in the OCWMN. When we compared measured to reported concentrations of various SARS-CoV-2 standard materials (IDT dsDNA plasmid control, and Promega linearized dsDNA quantification standard, and ssRNA positive control; Text S4, Table S8, and Figure S8), the IDT control overestimated by approximately 1 order of magnitude, similar to 1–2 orders difference reported by Chik et al. (2021). Overestimation could be problematic in public health decisions requiring absolute concentrations or when comparing between laboratories, but impacts can be minimized by only monitoring trends in sewersheds monitored by one laboratory (as with the OCWMN). For RT-dPCR, the measured concentration of Promega dsDNA and ssRNA was the same order of magnitude as the reported concentration. Quantification of the IDT plasmid control showed that the measured concentration was less than half of the reported concentration, confirming that using the IDT control for RT-qPCR is overestimating the sample concentrations (Text S4 and Table ST8).

Although SARS-CoV-2 RNA wastewater measurements strongly associate with community level COVID-19 dynamics (Figure 1), the utility of WBE can vary between sewersheds13,34 due to differences in viral shedding rates, temporal fluctuations in wastewater flow, and composition, and analytical technique (e.g., dPCR vs qPCR) (Figure 2). Several factors impacting WBE include sampling period, impacts of genome mutations on primer specificity, prominence of variants with different infectivity, shedding rates, duration, persistence in wastewater,38,39 robustness of and access to clinical testing programs and resources, and level of asymptomatic individuals. To smooth data gaps and account for shedding differences, we applied a 7-day moving average to cases. Different moving averages or lag correlation analyses may improve WBE predictions.13,40 Normalizing SARS-CoV-2 with PMMoV RNA concentrations has different impacts, as previous studies report little or no improvement on correlation13 but may control variability of SARS-CoV-2 measurements by accounting for dilution of fecal material.40

5. CONCLUSION

This study demonstrates the effectiveness of uniplex and duplex RT-qPCR and duplex RT-dPCR for wastewater surveillance over 12 months. SARS-CoV-2 N gene concentrations were correlated with COVID-19 cases. Normalization by PMMoV and by wastewater flow rates had varying effects on the correlation between SARS-CoV-2 and COVID-19 based on sewershed. We demonstrated for the first time the use of duplex chip-based RT-dPCR for wastewater surveillance and compared it to duplex RT-qPCR. In addition to other practical trade-offs, the RT-dPCR assay detected SARS-CoV-2 in more samples than the RT-qPCR assay during periods with low COVID-19 cases, but RT-dPCR costs more per sample and required greater sample turnaround time than RT-qPCR. This study shows promise for duplex PCR for cost-efficient, multigarget wastewater surveillance to enable wastewater-based epidemiology, a public health tool that will continue to be useful for monitoring community level trends of pathogens including SARS-CoV-2 as variants emerge.

ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00055.

Additional experimental details, materials, methods, minimum information reporting requirements for qPCR and dPCR experiments (MIQE and dMIQE checklists), R script used to calculate LOD, tables containing summary of results, RT-qPCR standard curve parameters, results of correlation and linear regression analyses (PDF)

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Notes
The authors declare no competing financial interest.

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