Dispersion of SARS-CoV-2 RNA across a wastewater treatment plant and its workers

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Abstract

Within urban and suburban sewersheds, SARS-CoV-2 released through faeces is transported through sewage systems into municipal wastewater treatment plants (WWTPs). Studies have shown that viral RNA is detectable in untreated wastewater but not in WWTP effluent. In this study, we investigated treatment steps between the influent and final treated effluent to identify the point at which viral RNA is below detection. Additionally, we examined air surrounding high turbulence treatment steps to test for the presence of SARS-CoV-2 RNA in WWTP-generated bioaerosols. To examine potential worker exposure to SARS-CoV-2, WWTP workers were tested for the presence of viral RNA. The data show that despite high viral RNA concentration in the influent, SARS-CoV-2 RNA concentration decreased significantly ($p < 0.02$) in the main treatment steps and was below detection in the effluent. Additionally, SARS-CoV-2 RNA was below detection in air samples ($n = 42$), and the worker rate of infection was not significantly different ($p = 0.99$) from the rate of infection in the surrounding community. These results suggest that WWTP workers may have minimal exposure to SARS-CoV-2, WWTP generated bioaerosols, and that the WWTP successfully reduces the amount of viral RNA entering effluent receiving waters, providing a vital public health service to communities.

KEYWORDS
COVID-19, pandemic, public health, SARS-CoV-2, wastewater treatment plant

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19), a disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was officially labelled a global pandemic on 11 March 2020 and as of 3 November 2021 had resulted in 247 751 159 global cases and 5 017 214 deaths (COVID-19 Map, n.d., p. 19). SARS-CoV-2 is an enveloped, single-stranded RNA virus and is characterized by high infectivity, high asymptomatic ratio in the population and potential to cause severe health complications (Gonzalez et al., 2020). Based on the pathology of SARS-CoV-2 and its characteristic to cause severe respiratory syndrome among other respiratory illnesses, it is believed that the virus infects respiratory epithelial cells and is transmitted through respiratory droplets (Xiao et al., 2020b). The virus has also been shown to infect and replicate within the gastrointestinal tract, resulting in viral shedding through faeces along with a potential for faecal-respiratory transmission through aerosolized faeces (Hao Zhang et al., 2020; Xiao et al., 2020a).
Within urban and suburban sewersheds, SARS-CoV-2 released through faeces is transported through sewage systems into municipal wastewater treatment plants (WWTPs). Since the onset of the COVID-19 pandemic, studies and monitoring efforts have focused on examining viral RNA abundance in wastewater influent to better understand community-level viral transmission within the sewershed population (Ahmed et al., 2020; Hata & Honda, 2020; Kocamemi et al., 2020; Medema et al., 2020; Nemudryi et al., 2020; Wu et al., 2020; Wurtzer et al., 2020). While these studies have advanced our understanding of using a wastewater-based epidemiological approach as an additional viral mitigation strategy, fewer studies have examined the fate of SARS-CoV-2 RNA throughout the WWTP process (Balboa et al., 2021; Qu et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020). These studies showed that although the viral RNA was detectable and could remain in untreated wastewater for days to weeks, it largely partitioned into sludge and was not detected in treated effluent. It is important to note that detection of SARS-CoV-2 RNA markers does not infer viral infectivity as marker genes can exist as both genomic and subgenomic RNAs that may be protected from external degradation (Alexandersen et al., 2020). In this study, we examined SARS-CoV-2 RNA across the treatment steps between the raw wastewater influent and the final treated effluent in order to identify the point at which SARS-CoV-2 RNA markers are no longer detected. Determining how individual WWTP processes affect SARS-CoV-2 concentrations can identify regions across the WWTP environment with different levels of potential worker exposure, aiding in the development of process-specific risk mitigation strategies.

While studies have identified SARS-CoV-2 RNA in liquid wastewater, very little is known about the potential emission and dispersal of the virus through WWTP-generated bioaerosols. Previous studies have observed the emission of other viruses from wastewater treatment facilities including the nonenveloped viruses: norovirus, human adenovirus, rotavirus and coliphages (Heinonen-Tanski et al., 2009; Masclaux et al., 2014; Pasalari et al., 2019). However, it has been shown that enveloped viruses, such as SARS-CoV-2, are more readily inactivated in wastewater and show an increased rate of adsorption to solids as compared to nonenveloped viruses (Ye et al., 2016). Additionally, using lab-scale aeration basins, Lin and Marr (2017) demonstrated the potential for aerosolization of viable viruses from wastewater and found decreased emission for an enveloped virus as compared to a nonenveloped virus. These studies suggest that while the aerosolization of SARS-CoV-2 during wastewater treatment is feasible, more knowledge is needed to determine the fate and transport of this enveloped virus throughout wastewater treatment processes. This knowledge is important for understanding potential respiratory routes of viral exposure for wastewater workers.

In this study, we examined SARS-CoV-2 RNA concentrations in liquid samples collected across the main processes of wastewater treatment at a medium-sized secondary treatment WWTP, air collected from treatment areas of greatest bioaerosol generation and SARS-CoV-2 abundance and from WWTP worker nasopharyngeal and saliva samples. Additionally, the study was conducted during periods of low and high COVID-19 case counts in the surrounding community to examine how viral abundance in the influent may influence viral distribution throughout the treatment process.

2 | MATERIALS AND METHODS

2.1 | WWTP location

The WWTP examined in this study is located in Columbia, South Carolina, USA, and serves approximately 60,000 customers over a sewershed spanning an area of 120 square miles with an estimated population size of 363,714. The average wastewater influent flow through the facility is 45 million gallons per day (MGD). Wastewater enters the site at a central location before being split across two treatment trains. The first step in the treatment process in each train involves the primary clarifiers, which settle and remove suspended solids while allowing the remaining wastewater to pass to the aeration basins. During the aeration step, the organics in the wastewater are broken down by microorganisms that are fueled by air that is injected into the treatment tanks through surface aerators or fine bubble diffusers. The aeration basins in train 2 are variable bottom-fed fine bubble diffuser treatment tanks, while the aeration basins in train 1 are complete mix basins run at low dissolved oxygen in order to facilitate simultaneous denitrification. Aeration at train 1 basins is supplied by four 75 HP surface aerators, each with an individual capacity of 247 lbs/O₂/HP/HR. The wastewater then moves to the secondary clarifiers before undergoing a chlorination and de-chlorination step. Finally, the treated wastewater from the two trains merges to a central effluent site before being discharged into the adjacent Congaree River.

2.2 | Wastewater liquid collection

To monitor community-level SARS-CoV-2 infection prevalence, 24 h composite influent samples were collected from the WWTP headworks prior to chemical or mechanical modification. Sample collection began on 20 April 2020 and is currently ongoing with samples collected twice per week (Sunday and Wednesday) by wastewater operators using an on-site refrigerated ISCO autosampler. SARS-CoV-2 RNA concentrations in these samples were used to monitor trends in community viral concentration and to identify the sampling timepoints for the specific studies outlined in this current work. To track SARS-CoV-2 RNA concentrations across the treatment processes, liquid samples were collected within the wastewater treatment facility during a period of low daily case counts as reported by the local health department for the sewershed catchment population (May 2020) and periods of increased daily case counts (September 2020 and December 2020). Since the WWTP splits the incoming sewage into two trains for treatment, both trains were tested to ensure accuracy. For SARS-CoV-2 tracking in liquid wastewater, samples were collected at sites representing sequential treatment processes across both treatment trains: Headworks (Influent), Train 1 Primary Clarifier (T1PC), Train 2 Primary Clarifier (T2PC), Train 1 Aeration Basin.
(T1AB), Train 2 Aeration Basin (T2AB), Train 1 Secondary Clarifier (T1SC), Train 2 Secondary Clarifier (T2SC) and Effluent (Figure 1). One litre grab samples were collected from each site midmorning (0800–1100) on 11 May, 21 September and 15 December 2020 and were stored on ice for transport back to the laboratory. All samples were processed within 2 h of sample collection. During the May sampling period, 81 L grab samples were collected but due to supply chain issues, only a single replicate was analysed for each site. During September and December sampling, 81 L grab samples were collected and each sample was split into three 250 ml replicates for processing.

2.3 | Wastewater bioaerosol collection

Similar to the viral tracking in the liquid, air sampling was conducted during timepoints of low (April/May 2020) and elevated (September 2020) daily case counts. Air samples were collected using SKC BioSampler liquid impingers that were placed in insulated tubs designed to maintain sample temperature at 4–10°C and minimize sample exposure to UV radiation during extended collection times. Each tub contained three BioSampler impingers in order to collect triplicate samples from each location. To assess potential viral release through bioaerosols generated as the raw sewage flows into the WWTP, air samples were first collected at three different locations of high liquid turbulence at the influent site approximately two meters directly above the liquid surface at the base and the top of the screw lift pumps (A1–A3) for 2 days and were operated for 4 h each day (Figure 1). In total, 24 samples were collected from the wastewater treatment headworks area across the study period. Air samples were also collected for 2 days from areas immediately adjacent to the primary clarifier (A4) and the surface-agitated aeration basin (A5) at 1.5 and 2 m directly above the surface of the water, respectively. Over the course of the study period, nine samples were collected at both the primary clarifier and aeration basin treatment tanks. During a previous study, we observed that each propeller at the train 1 surface aerators generated a consistent circular spray with a diameter of 17 m and a height of approximately 1 m above the liquid surface. The BioSampler impingers contained 20 ml of 0.5X phosphate buffered saline (PBS) as the sample collection buffer. The impingers were attached to BioLite high volume sonic flow pumps that provided an air flow of 12.5 L of air per minute per impinger, resulting in a total of 3000 L of air sampled for each replicate (9000 L per site) during 4 h of sampling. Each set of three impingers was checked periodically over the course of sampling and was amended with autoclaved deionized water as needed to maintain a constant volume of 20 ml. After sampling, the PBS solution containing the collected bioaerosols was transferred into 50 ml conical tubes and stored on ice for transport back to the laboratory.

2.4 | Wastewater and bioaerosol concentration, RNA extraction and SARS-CoV-2 quantification

The replicate liquid wastewater samples (250 ml) were homogenized for 10 min using Waring LB10S variable speed laboratory blenders (Waring Laboratory, Torrington, CT), and 50 ml aliquots was
centrifuged at 4577 x g for 20 min. The resulting pellets (approximately 180–300 mg) were stored at –80°C, and the supernatants (50 ml) were filtered through Millipore Amicon 30 kDa ultrafilters at 4000 x g until the sample volumes were concentrated to 400 μl. The bioaerosol samples did not require homogenization and were directly concentrated through Amicon 30 kDa ultrafilters until the sample volume was reduced to 400 μl. The concentrated sample volumes were transferred to 1.5 ml microcentrifuge tubes, and 200 μl was used for total nucleic acid extraction using the Qiagen AllPrep PowerViral DNA/RNA extraction kit following the manufacturer’s instructions. Extracted nucleic acids were eluted in 51 μl of RNase-free water and stored at –80°C. Nucleic acid quality and quantity were assessed via a NanoDrop 2000 spectrophotometer (ThermoScientific, Waltham, MA). Reverse transcription droplet digital PCR (RT-ddPCR) was used to prepare N1 and N2 CDC diagnostic assays (BioRad #10031279) using 5 μl of extracted nucleic acids (10–15 ng RNA) and the BioRad One-Step RT-ddPCR kit following the manufacturer’s instructions. The Exact Diagnostics SARS-CoV-2 control (manufactured with synthetic RNA transcripts containing the gene targets E, N, ORF1ab, RdRP, S) and nuclease-free water were used as positive and negative (no-template) controls, respectively, in all reaction plates in order to ensure proper assay performance. Approximately 10 000–20 000 droplets were generated for each sample using the BioRad Automated Droplet Generator, and droplets were thermal cycled for 40 cycles using a BioRad C1000 Touch thermal cycler with the following conditions: reverse transcription at 50°C for 60 min, enzyme activation at 95°C for 10 min, denaturation at 95°C for 30 s, annealing/extension at 55°C for 1 min, enzyme deactivation at 98°C for 10 min and a 4°C hold. Ramp rate was set to 2°C/s. Positive and negative droplets were quantified using a BioRad QX200 and samples containing more than 10 000 droplets were manually analysed using the QuantaSoft Pro program. The positive controls and no-template controls (NTC) were used to establish threshold positions during quantification. No positive droplets were observed in the NTC reactions across all RT-ddPCR runs. The number of SARS-CoV-2 genome copies per litre (GC/L) of wastewater was determined through dimensional analysis according to:

\[ C_1 = C_2 \times V_{\text{ran}} \times \frac{V_{\text{RNA eluted}}}{V_{\text{RNA per ran}}} \times \frac{V_{\text{concentrate1}}}{V_{\text{concentrate2}}} \times \frac{1}{V_{\text{influent}}} \times 1000 \]  

(Equation1)

where \( C_1 = \text{GC/L}, \) \( C_2 = \text{GC/ul} \) reported from the QuantaSoft Pro analysis, \( V_{\text{ran}} = \text{volume of PCR reaction (22 μl)}, \) \( V_{\text{RNA eluted}} = \text{amount of RNA eluted after extraction (51 μl)}, \) \( V_{\text{RNA per ran}} = \text{amount of RNA template added to PCR reaction (5 μl)}, \) \( V_{\text{concentrate1}} = \text{volume of concentrated wastewater (400 μl)}, \) \( V_{\text{concentrate2}} = \text{volume of concentrated wastewater used for RNA extraction (200 μl)} \) and \( V_{\text{influent}} = \text{volume of wastewater influent used for concentration (50 ml)}. \) To estimate viral recovery from wastewater using the ultrafiltration concentration method, bovine respiratory syncytial virus (BRSV) (Inforce 3, Zoetis Inc. Veterinary Services, ~80 million copies/ml) was spiked into representative wastewater samples as an internal control before homogenization. Spiked wastewater samples were processes as described above and analysed using RT-ddPCR with the BioRad BRSV assay (BioRad #10031279), and the percent viral recovery was calculated based on the initial spike concentration. Pepper Mild Mottle Virus (PMoV) is one of the most abundant RNA viruses in human faeces (Kitajima et al., 2018) and served as a faecal-indicator positive control for the air samples and validated the presence of nucleic acids extracted from WWTP-generated bioaerosols. A limit of blank (LOB) and a limit of detection (LOD) experiment were conducted for the ddPCR using both the wastewater and the air samples. Utilizing the nonparametric method, the LOB was found to be 0.827 copies per reaction. The theoretical LOD for the wastewater samples was 1.3 copies per reaction, which is similar to the findings by Gonzalez et al., 2020, and the LOD for the air samples was also 1.3 copies per reaction. After dimensional analysis, the theoretical LOD was found to be 520 copies per litre or 2.716 log10 copies per litre. Information on primers and probes used as well as concentrations and volumes of ddPCR reactions are listed in Tables S1 and S2, respectively. Other information as requested in the updated dMIQE guidelines are located in Table S3, and representative ddPCR plots are shown in Figures S1–S10. Additionally, a comparison between the RT-qPCR and RT-ddPCR technologies was conducted using the Exact Diagnostics SARS-CoV-2 standard at known concentrations (Figure S11).

### 2.5 Wastewater worker SARS-CoV-2 monitoring

WWTP worker volunteers were examined every 2 weeks during April–September 2020 resulting in 11 participant sampling events. Each participant sampling consisted of a self-administered questionnaire that requested information on demographic characteristics, symptoms, possible exposures at work and outside of work, the average number of hours spent per day on facility grounds where exposure to wastewater might occur and the use of face masks in office spaces and in the community. The questionnaire was followed by the collection of a nasopharyngeal (NP) or saliva sample for SARS-CoV-2 testing. Over the course of the 6-month sampling period, 272 samples were obtained, which represented 42 of 50 (84%) of the WWTP workers. The COVID-19 status of all WWTP workers tested at off-site facilities was also collected and added to the case data. The sampled employees included administrative and management staff, laboratory personnel, wastewater operators, mechanics, electricians and engineers. Time spent in outside areas where wastewater bioaerosols might be generated was collected and varied by job. All questionnaires and worker sampling procedures were approved through the University of South Carolina Institutional Review Board (IRB #Pro00084695). RNA was extracted from participant NP samples using the Qiagen AllPrep PowerViral DNA/RNA extraction kit following the manufacturer’s instructions. Extracted nucleic acids were eluted in 51 μl of RNase-free water and stored at −80°C. Saliva samples were heat inactivated for 10 min at 70°C and used directly in RT-qPCR using the CDC 2019-nCoV N2 diagnostic panel and the Luna Universal Probe One-Step RT-qPCR kit (New England Biolabs; Ipswich, MA). Each RT-qPCR assay was performed in a 20 μl reaction composed of 1X Reaction Mix, 1X RT Enzyme Mix, 500 nM/125 nM IDT
2019-nCoV Combined Primer/Probe Mix (IDT, 2019-nCoV RUO kit, 500rxn) and 2.5 μl of nuclease-free water. Five microliters of NP extracted RNA or heat-inactivated saliva was used as a template per reaction, and all samples were run in duplicate. The RT-qPCR assay was amplified using the Applied Biosystem 7900HT Fast Real-Time PCR system with the following thermal cycling conditions: reverse transcription at 55°C for 10 min, initial denaturation at 95°C for 1 min, 45 cycles of denaturation at 95°C for 10 s and extension at 60°C for 1 min. Saliva collected from a confirmed positive clinical case containing high SARS-CoV-2 titre (Ct value 25) was used as a positive control for the saliva-based assays, the linearized IDT 2019-nCoV_N plasmid was used as a positive control for NP samples and nuclease-free water was used as a no-templated control. Controls were added in duplicate in each plate and were used for process validation and for threshold setting. The amplification of RNase P was used as an internal control to identify possible inhibition of amplification. For the RNase P assay, the Hs_RPP30 Positive Control plasmid (IDT, #10006626) was used as the positive control template. Samples were investigated solely for absence/presence of SARS-CoV-2 RNA, and both the NP and saliva samples were deemed “not detectable” if the RNase P successfully amplified but the N2 target did not amplify within 40 cycles as per the CDC 2019-nCoV N2 diagnostic panel guidelines.

2.6 | Statistical analysis

Statistical analyses were computed on log_{10} SARS-CoV-2 RNA copies per litre using RStudio (version 2022.02.0+443). Data normality was tested using the Shapiro–Wilk test which indicated that the data were not normally distributed (p-value range 7.718e-06 to 1.021e-05). Due to the lack of data normality, Kruskal–Wallis tests were used to analyse significant differences among treatment processes, and Dunn t-tests were used to assess differences between specific treatment processes (mean of train 1 and 2 processes). The Fisher’s exact test was used to compare the Covid-19 case rate in workers at the WWTP to the Covid-19 case rate within the sewershed population.

3 | RESULTS AND DISCUSSION

3.1 | Temporal variability in COVID-19 case counts and wastewater SARS-CoV-2 concentration

During the study period, more than 334 000 cases of COVID-19 and 4800 COVID-19 related deaths were documented across South Carolina with peaks in viral transmission occurring during holiday events (Memorial Day, Independence Day, New Year’s Eve, etc.) (South Carolina County-Level Data for COVID-19, n.d.). While the community-level wastewater monitoring was not the primary focus of the current study, it provided the baseline data on SARS-CoV-2 wastewater concentration and community COVID-19 status that helped guide the selection of timepoints for the liquid, air and participant sampling as outlined in Figure 2. The data indicate that the overall trends observed in SARS-CoV-2 RNA concentration in wastewater influent (Figure 2a) as part of our long-term temporal study are similar to trends in COVID-19 clinical cases identified within the surrounding community (Figure 2b). Throughout 2020, the peaks in case counts and wastewater concentration corresponded to times of increased social gatherings during United States holidays occurring in May, July, September and December. Mandatory mask ordinances were established in communities surrounding the tested WWTP during mid-July and corresponded to reduced case counts and wastewater SARS-CoV-2 RNA concentration. These trends are similar to other studies that have monitored SARS-CoV-2 RNA in untreated wastewater, further supporting the role of wastewater-based epidemiology (WBE) approaches for monitoring patterns in community viral

FIGURE 2 Sampling intervals cover periods of low, medium and high COVID-19 infection. (a) SARS-CoV-2 RNA copies per litre of wastewater influent collected at the Columbia wastewater treatment plant (WWTP) and (b) reported daily clinical case counts of COVID-19 for the Columbia WWTP catchment population between April and December of 2020. Shaded areas represented weeks of liquid and air sampling. Vertical dotted lines represent the 11 weeks of WWTP worker sampling. Created with tableau and biorender.
transmission (Ahmed et al., 2020; Gonzalez et al., 2020; Haramoto et al., 2020; Medema et al., 2020; Randazzo et al., 2020; Wu et al., 2020). For examining SARS-CoV-2 across treatment processes, liquid wastewater samples were collected at three timepoints that represented both low and high case counts in the surrounding sewershed catchment population. The average viral concentration in the WWTP composite influent samples across the three timepoints ranged from approximately 6000 RNA copies per litre in May to approximately 80 000 RNA copies per litre in December, which highlighted the increase in community viral transmission over the three sampling events, and agreed with studies that utilized a similar ultrafiltration method for sample processing (Nemudryi et al., 2020; Wu et al., 2020).

3.2 | SARS-CoV-2 concentration across treatment processes

Overall, the concentration of SARS-CoV-2 RNA N1 and N2 markers significantly decreased throughout the treatment process (Figure 3a–c and Table 1). The May sampling event occurred during a period of low COVID-19 case counts observed in the catchment population, which translated to low viral RNA concentration in the wastewater influent (3.95 and 3.76 log_{10} RNA copies per litre for N1 and N2, respectively; Figure 3a). From the WWTP headworks (influent), the wastewater is divided between two treatment trains and enters the primary clarifier treatment process. Viral RNA concentration decreased only slightly to 3.75 and 3.44 log_{10} RNA copies per litre for N1 and N2, respectively in the train 1 primary clarifiers (T1PC) and to 3.29 and 3.41 log_{10} RNA copies per litre for N1 and N2, respectively, in the train 2 primary clarifiers (T2PC). SARS-CoV-2 RNA was not detected in subsequent trains 1 and 2 aeration basins (T1AB and T2AB) and secondary clarifiers (T1SC and T2SC) or the final treated effluent during May sampling. A limitation of May sampling is that due to critical supply chain shortages, we were unable to process the required number of replicates for statistical validation of this time-point. However, this limitation was resolved before the September and December sampling, allowing statistical testing of those data. Following several holidays, September showed increased COVID-19 cases in the catchment population, resulting in higher viral abundance in the influent (4.08 and 4.10 log_{10} RNA copies per litre for N1 and N2, respectively; Figure 3b) as compared with May. No significant decreases in viral RNA were observed from the influent to the primary clarifiers during September (N1: \( p = 1 \); N2: \( p = 0.87 \); Table 1). However, significant decreases were observed in N1 marker RNA (\( p = 0.02 \)) from the influent to the aeration basins and in N1 and N2 marker RNA from the influent to the secondary clarifiers (N1: \( p = 0.02 \); N2: \( p = 0.03 \)). No SARS-CoV-2 RNA was detected in the

![Figure 3](image-url)
effluent during September sampling. The December timepoint occurred during the highest peak in COVID-19 clinical cases and wastewater viral RNA concentration. Trends in viral reduction at this timepoint were similar to those observed in September, albeit a significant reduction in viral RNA was not observed until the secondary clarifier process (Figure 3c and Table 1). The high abundance observed in T2PC was a result of a highly concentrated biological replicate with elevated viral RNA. No SARS-CoV-2 RNA was detected in the secondary clarifiers and effluent during this timepoint. The Exact Diagnostics SARS-CoV-2 control amplified successfully for each timepoint, and the lack of amplification of the negative control (nuclease-free water) confirmed the absence of cross-contamination.

These data show that over the course of the three sampling events which were representative of periods of low, medium and high COVID-19 case counts in the community, the overall pattern of viral mitigation through the wastewater treatment process remained consistent. Viral RNA abundance decreased throughout the treatment process and was below detection in the final effluent, suggesting decreasing potential worker exposure to SARS-CoV-2 across the treatment process and low risk of viral transfer into effluent receiving waters. Our results are consistent with other studies that detected SARS-CoV-2 RNA in wastewater influent but not effluent (Abu Ali et al., 2021; Haramoto et al., 2020). Comparison of viral RNA concentration at different treatment stages showed that the greatest reductions in concentration occurred following the primary clarification treatment stage. This is the first step in biosolid removal and viral adsorption to solids and subsequent removal from the liquid treatment train may account for this reduction (Balboa et al., 2021; Graham et al., 2021). The decrease in the viral RNA signal may also be attributed to the presence of antagonist microorganisms that can inactivate viruses via extracellular enzymatic activity or degradation of viral RNA (Casanova et al., 2009; Gundy et al., 2008; Ye et al., 2016). While the WWTP examined in this study is typical of a secondary treatment facility in a medium-sized urban environment, it is possible that other WWTP sites with different characteristics (e.g., catchment population demographics, hydraulic retention time and treatment technology) may show different trends in viral mitigation. In addition, this study consisted of three sampling events with limited sample size; it is possible that the viral mitigation trends observed in this study may change with increased sampling over more time points.

### 3.3 SARS-CoV-2 concentration in bioaerosols

Despite being a potential exposure pathway for WWTP workers, SARS-CoV-2 transfer from liquid to air through WWTP-generated bioaerosols has not been published to our knowledge. Given our previous data showing the detection of SARS-CoV-2 RNA markers in wastewater influent and the early stages of the treatment process, we also examined the presence of viral markers in bioaerosols generated during the treatment process. Air sampling occurred during periods of low (April/May 2020) and high (September 2020) community viral transmission as indicated by clinical case counts and wastewater influent SARS-CoV-2 RNA concentration. Replica air samples (9000 L total) were collected across multiple days from areas immediately adjacent to the headworks receiving raw wastewater influent, the primary clarifiers and the aeration basin. Specific impinger locations were chosen to be immediately above and downwind from the most turbulent areas within these treatment processes to increase the likelihood of capturing WWTP-generated bioaerosols. To verify that wastewater aerosols generated at each process were being captured using the liquid impingers, the abundance of PMMoV was also examined in each sample. PMMoV is a nonenveloped single-stranded positive-sense RNA virus that is one of the most abundant viruses in human faeces and is often used as a wastewater indicator (Kitajima et al., 2018). While PMMoV was detected in all air samples, SARS-CoV-2 RNA was not detected in any air samples collected throughout the different sampling timepoints (Table 2). These results are similar to other studies that have observed greater rates of wastewater emission of nonenveloped viruses as compared to enveloped viruses (Heinonen-Tanski,

### Table 1 Treatment process comparison of SARS-CoV-2 RNA concentration utilizing N1/N2 assays during September and December 2020 timepoints

| Treatment process comparison | September | December |
|------------------------------|-----------|----------|
|                              | N1 (p-value) | N2 (p-value) | N1 (p-value) | N2 (p-value) |
| Influent-PC                  | 1          | 0.87     | 1            | 1           |
| Influent-AB                  | 0.02       | 0.06     | 0.62         | 0.74        |
| Influent-SC                  | 0.02       | 0.03     | 0.04         | 0.03        |
| Influent-effluent            | 7.84e-03   | 3.82e-03 | 0.04         | 0.03        |
| PC-AB                        | 0.09       | 0.46     | 0.59         | 0.74        |
| PC-SC                        | 0.09       | 0.2      | 9.27e-03     | 0.02        |
| PC-effluent                  | 0.03       | 0.04     | 9.27e-03     | 0.02        |
| AB-SC                        | 1          | 0.96     | 0.38         | 0.31        |
| AB-effluent                  | 1          | 0.87     | 0.38         | 0.31        |
| SC-effluent                  | 1          | 0.96     | 1            | 1           |

Note: Means of trains 1 and 2 were used, and p-values were derived from Dunn t-tests following Kruskal–Wallis tests for significant differences. Bold values indicate significant difference.

Abbreviations: AB, aeration basin; PC, primary clarifier; SC, secondary clarifier.
et al., 2009; Lin & Marr, 2017; Masclaux et al., 2014; Pasalari et al., 2019; Ye et al., 2016) and suggest that the risk of SARS-CoV-2 exposure through WWTP-generated bioaerosols is minimal during routine WWTP worker outside exposure times. While our study aimed to replicate a potential WWTP worker exposure scenario with low air flow rate and across an average 4 h outside exposure time, given that the theoretical LOD for SARS-CoV-2 RNA in air samples is 520 copies or 2.716 log10 copies, it is possible that sampling with higher flow rate over a longer period of time may enhance SARS-CoV-2 RNA marker detection in air samples. However, a study conducted comparing a variety of samplers showed that low flow rate samplers measured airborne viral concentrations more accurately than high flow rate samplers with extended sampling times (Raynor et al., 2021). Another potential caveat to this study is that due to a limited number of liquid impingers and pumps, it was not possible to collect air samples from nonwastewater impacted areas to validate the lack of PMMoV in background air controls. However, in a previous study, PMMoV was not detected in air samples collected near an inland lake located approximately 21 km from the WWTP examined in this study, indicating a potential low presence of PMMoV in background air as compared to air collected immediately above wastewater treatment processes.

### 3.4 WWTP worker infection rates

The completed worker questionnaires indicated possible outside exposure times ranging from 30 min to 12 h daily with a majority (64%) of participants spending 5–10 h on facility grounds. Workers were required to wear facemasks indoors as part of a mandatory COVID-19 mitigation strategy, but outside tasks involved the use of standard industry PPE practices with no COVID-specific adaptations except that face masks were required for persons working within 6 ft of other workers. During the 11 participant sampling events spanning 6 months of varying concentrations of SARS-CoV-2 RNA in the wastewater, no viral amplification was observed in worker NP or saliva samples (Table S4). The successful amplification of the internal RNase P control, demonstrating the lack of PCR inhibition, along with the absence of N2 amplification in these samples confirmed the negative participant SARS-CoV-2 test results throughout the study period. However, one worker who was not included in the study did receive a positive SARS-CoV-2 test result at an outside testing facility. This case was confirmed through contact tracing to have resulted from off-site community exposure. During the same testing period, 10 915 confirmed COVID-19 cases were identified in the surrounding sewershed population, based on ZIP code-level data provided by the South Carolina Department of Health and Environmental Control. When comparing the case rate in workers at the WWTP (one case among 43 workers [0.2%]) to the case rate within the sewershed population (10 915 cases among 363 714 residents [0.3%]), Fisher’s exact test indicated no significant difference ($p = 0.99$) in COVID-19 incidence between workers at the WWTP and the general population. The lack of a significant difference between COVID-19 case rates in WWTP workers and the case rate observed in the surrounding sewershed community suggests that SARS-CoV-2 transmission from wastewater and wastewater bioaerosols to wastewater workers may be minimal when appropriate worker protections, including job-specific PPE, are in place.

### 4 CONCLUSIONS

In this work, we used the quantification of viral RNA markers to demonstrate the efficacy of the WWTP in the removal of SARS-CoV-2. Across three timepoints and varying levels of viral load in the influent,
the WWTP in this study successfully reduced SARS-CoV-2 RNA markers to below detectable levels before the treated water exited the WWTP, thus suggesting minimal potential release of SARS-CoV-2 into effluent-receiving waters, some of which see heavy recreational use. The significant decrease in SARS-CoV-2 RNA abundance during the treatment process also suggests a reduced likelihood of exposure to the virus for the wastewater workers, although additional experiments are necessary to assess infectivity risk to workers. Additionally, the bioaerosol experiment which was designed to replicate potential collective exposure during a typical shift showed that airborne exposure to SARS-CoV-2 is minimal. Infection rates of WWTP workers were similar to the infection rates of the surrounding community. While sampling with a higher flow rate over a longer period of time may identify SARS-CoV-2 RNA in bioaerosols, literature shows that a lower flow rate measures airborne viral concentrations more accurately. Additional cell culture experiments could be used in future studies to examine the infectivity of SARS-CoV-2 in wastewater and air samples, as the detection of SARS-CoV-2 RNA markers does not infer infectivity (Alexandersen et al., 2020). Although a single WWTP site was examined in this study, it is a widely used secondary treatment design, and the results will be relevant to similar facilities located globally.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
COVID-19 clinical daily case count data are accessible through the South Carolina Department of Health and Environmental Control. All other data are provided in the supporting information data set and tables.

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