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Longitudinal monitoring of SARS-CoV-2 in wastewater using viral genetic markers and the estimation of unconfirmed COVID-19 cases

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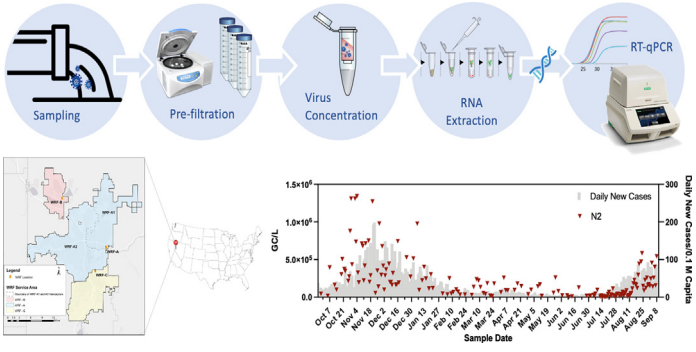
HIGHLIGHTS

- SARS-CoV-2 presence in wastewater was determined by genetic marker quantification.
- Two concentration peaks observed by SARS-CoV-2 wastewater monitoring between 2020 and 2021.
- Weekly average of SARS-CoV-2 and COVID-19 clinical data were strongly correlated.
- SARS-CoV-2 wastewater data can be used for a 7-day advanced warning of COVID-19 prevalence.
- SARS-CoV-2 wastewater levels can estimate the total unconfirmed COVID-19 cases.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, wastewater-based surveillance was carried out to establish the correlation between SARS-CoV-2 viral RNA concentrations in wastewater and the incidence of corona virus disease 2019 (COVID-19) from clinical testing. The influent wastewater of three major water reclamation facilities (WRFs) in Northern Nevada, serving a population of 390,750, was monitored for SARS-CoV-2 viral RNA gene markers, N1 and N2, from June 2020 through September 2021. A total of 614 samples were collected and analyzed. The SARS-CoV-2 concentrations in wastewater were observed to peak twice during the study period. A moderate correlation trend between coronavirus disease 2019 (COVID-19) incidence data from clinical testing and SARS-CoV-2 viral RNA concentrations in wastewater was observed (Spearman $r = 0.533$). This correlation improved when using weekly average SARS-CoV-2 marker concentrations of wastewater and clinical case data (Spearman $r = 0.790$), presumably by mitigating the inherent variability of the environmental dataset and the effects of clinical testing artifacts (e.g., reporting lags). The research also demonstrated the value of wastewater-based surveillance as an early warning signal for early detection of trends in COVID-19 incidence. This was accomplished by identifying that the reported clinical cases had a stronger correlation to SARS-CoV-2 wastewater monitoring data when they were estimated to lag 7-days behind the wastewater data. The results aided local decision makers in developing strategies to manage COVID-19 in the region and provide a framework for how wastewater-based surveillance can be applied across localities to enhance the public health monitoring of the ongoing pandemic.

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1. Introduction

The pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread worldwide. On March 11, 2020, the World Health Organization (WHO) designated coronavirus disease 2019 (COVID-19) as a pandemic. Throughout the pandemic, scientists in different fields made strides to participate in interdisciplinary research and multi-sector collaboration to strengthen the public health response, such as the vaccine and fast diagnostic test kit development, and the therapeutic strategies to mitigate the symptoms. One early need was the development of a reliable molecular assay for identifying SARS-CoV-2.

Throughout the COVID-19 pandemic, diagnostic surveillance has sometimes been limited due to a variety of obstacles, including limited availability of tests during periods of high demand (e.g., at the onset of the pandemic or during subsequent infection surges) or changes in public behavior over time. This highlights the need for alternative tools to monitor COVID-19 infection incidence or disease prevalence for local decision-making. Wastewater-based surveillance (WBS) has historically been used to monitor numerous diseases like norovirus-associated poliovirus (Deshpande et al., 2003), gastroenteritis, influenza A, and hepatitis A (Heijnen and Medema, 2011; Hellmér et al., 2014). WBS is now showing great potential as a complementary resource for public health response to COVID-19 (Ahmed et al., 2020a; Anand et al., 2021; Peccia et al., 2020; Polo et al., 2020), particularly since shedding of SARS-CoV-2 viral RNA is so common in the excreta of both symptomatic and asymptomatic virus carriers (Chen et al., 2020; Larsen and Wigginton, 2020; Lee et al., 2020; Michael-Kordatou et al., 2020; Wölfel et al., 2020; Zhang et al., 2020). The duration of the shedding through feces can be as long as 33 days, with a decrease shedding rate, ranging from 10^6 to 10^{12} gc/L, which is lower than some other infectious viruses, like MERS-CoV, and SARS-CoV-1 (Gupta et al., 2020; Jones et al., 2020; Wölfel et al., 2020). Nevertheless, more studies should be focused on developing countries, like sub-Saharan Africa, where sewer collection systems and sanitation are lacking (Pandey et al., 2021). The SARS-CoV-2 viral gene markers may be transported differently in water and wastewater systems and may be different with the developed countries (Sunkari et al., 2021). All those issues should not be ignored if we want to effectively address the application of WBS in the pandemic management.

Due to delayed onset of symptoms and lags in seeking/reporting of clinical tests, WBS can provide advanced warning of COVID-19 (re-)emergence within a community or changes in infection levels (Wu et al., 2020). For example, Kumar et al. (2021) observed that virus concentrations in wastewater were associated with one to two weeks advanced notice of COVID-19 cases in India. Similarly, Stadler et al. (2020) used a cross-correlation analysis to demonstrate that SARS-CoV-2 RNA markers in wastewater predicted the positivity rate of COVID-19 up to two weeks in advance in Houston, Texas. Betancourt et al. (2021) also used this approach to identify asymptomatic COVID-19 cases and potentially avert a larger outbreak within the whole university. Therefore, the monitoring of SARS-CoV-2 in wastewater may disclose predictive data on COVID-19 prevalence and incidence as an early warning in a community and needs to be studied further in more geographical regions of the world.

The concentrations of SARS-CoV-2 in wastewater can reflect the number of infected persons in a community, hence allowing the use of WBS as a tool to estimate total infectious cases (Ahmed et al., 2020a, 2020b; Gerrity et al., 2021; Gonzalez et al., 2020; Wurtzer et al., 2020). Recent literature includes efforts to describe correlations between SARS-CoV-2 viral RNA levels in wastewater and clinical case data, with the ultimate goal of developing predictive models to estimate COVID-19 incidence or prevalence (Ahmed et al., 2020a; Galani et al., 2022; Gerrity et al., 2021; Kaplan et al., 2020; Stadler et al., 2020). Based on a daily mass balance of viral RNA shedding into wastewater, Ahmed et al. (2020a) set up a predictive model to reflect the prevalence of COVID-19 within an Australian sewershed. Gerrity et al. (2021) also applied a mass-balance model coupled with COVID-19 incidence to rationalize observed wastewater SARS-CoV-2 concentrations in southern Nevada, USA. The correlations observed in these studies provide preliminary evidence that changes in viral concentrations in wastewater indicate changes in infection totals within communities.

Haak et al. (2022) used SARS-CoV-2 viral concentrations to identify spatially clustered patterns of viral RNA concentrations in neighborhood-scale sub-sewersheds in Reno-Sparks metropolitan area, Nevada, USA. They found that the SARS-CoV-2 concentration may reveal critical information about the patterns of disease spread and hot spots of disease. Based on this study, we continued to monitor SARS-CoV-2 concentration in the influents of untreated wastewater in the same study area. The objectives of this study were to 1) conduct long-term monitoring of SARS-CoV-2 viral marker concentrations in wastewater in the Reno-Sparks metropolitan area, Nevada, USA; 2) identify correlations between SARS-CoV-2 viral RNA signals in wastewater and COVID-19 incidence; 3) assess whether wastewater concentrations of SARS-CoV-2 provide a leading indicator of COVID-19 incidence, and 4) estimate the total number of infections in the community during the study period.

2. Methods and materials

2.1. Study area

The study area for this research was the Reno-Sparks metropolitan area in Nevada (NV), USA, which has a population of 390,000 and represents the main population center in northern Nevada. Influent wastewater to three water reclamation facilities (WRFs) was monitored from July 2020 to September 2021. WRF-A is the largest WRF in the region, with the capacity of 121,000 m^3/day (Lacroix et al., 2020). WRF-A receives wastewater through two interceptor sewer lines, one routed from the Sparks metropolitan area serving a population of 116,000 (indicated as WRF-A1) and a second routed from the Reno metropolitan area serving a population of 204,000 (indicated as WRF-A2). The flows from both interceptors were mixed directly in the main headworks of WRF-A, where wastewater flow was sustained at approximately 96,000 m^3/day during the sampling study period averagely. The influent of WRF-A consisted of approximately 50% domestic wastewater and 50% wastewater from industrial or commercial facilities. WRF-B is the smallest facility of the three sampling sites. As the facility serves 18,000 people, it manages approximately 4% of the total wastewater generated in the sampling region and receives mostly domestic wastewater. WRF-C is a biological suspended growth activated sludge process for nitrogen and organic carbon removal, serving approximately 52,000 people in south Reno. Fig. 1 shows the sewershed of the three WRFs and indicates the sampling locations.

2.2. Sample collection and pretreatment

Between 9:00 am to 12:00 noon, 1-L grab wastewater samples (N = 541) were collected after preliminary treatment at the headworks of the three facilities and were immediately transported on ice to the laboratory. After July 1, 2021, 24-h composite samples (N = 73) from WRF-A were collected and analyzed seven days per week. 1 L samples were taken every 2 h. The samples were heated in a water bath at 60 °C for 60 min for pathogen inactivation to ensure lab safety during sample processing and analysis (Kampf et al., 2020; Rabenau et al., 2005). After inactivation, we centrifuged the samples at 3000g for 15 min and filtered the supernatant sequentially through 1.5, 0.8, and 0.45-μm sterile membrane filters. This step was intended to remove debris and large particles, including eukaryotic and prokaryotic microorganisms. The resulting liquid sample was used to recover the virus and then analyze for RNA using reverse transcription and quantitative polymerase chain reaction (RT-qPCR).

2.3. Virus recovery, RNA extraction, and RT-qPCR analysis

A polyethylene glycol (PEG) precipitation method was initially employed to achieve virus concentration for SARS-CoV-2 viral RNA detection. The pH of the processed sample from Section 2.2 (total volume of 470 mL) was adjusted to 7.2 ± 0.1 by NaOH and H_3PO_4 and then the sample was

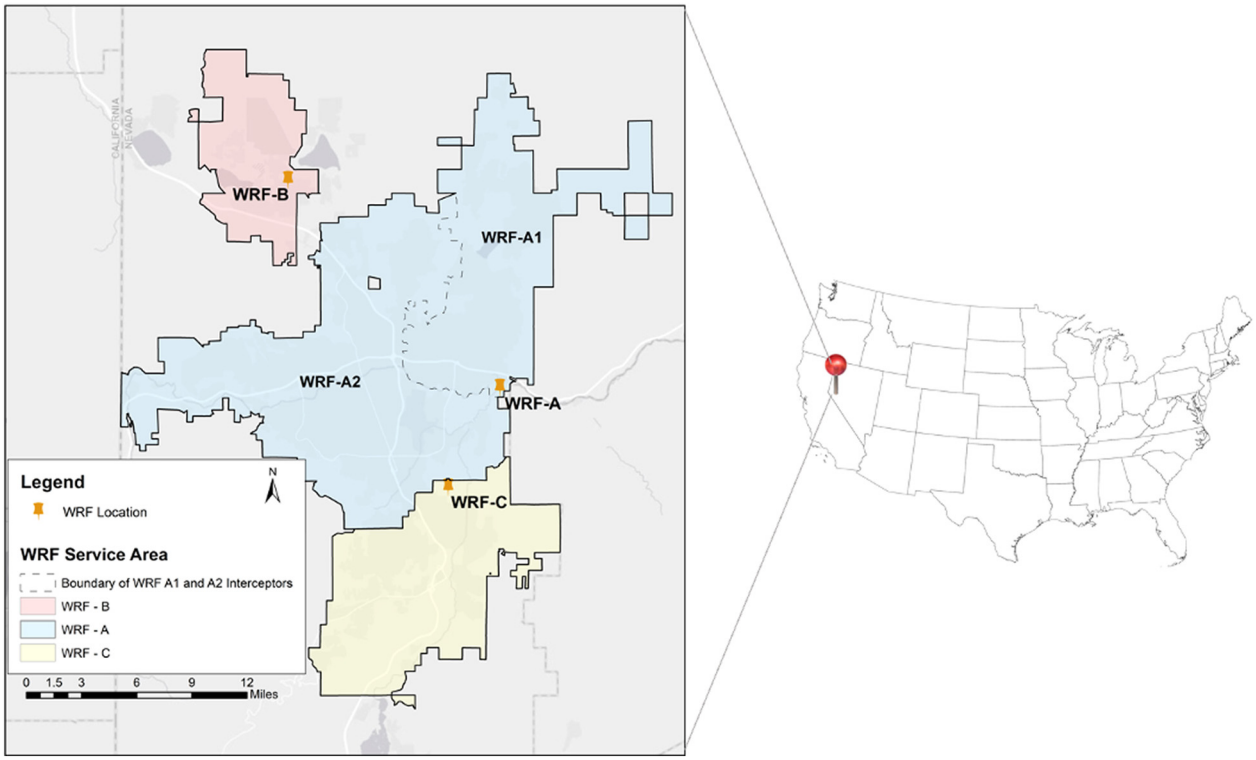

Fig. 1. Sampling locations and respective sewershed catchment areas in the Reno-Sparks metropolitan area, NV, USA.

amended with PEG 8000 [10% (*w/v*), Fisher Scientific, Pittsburgh, PA, USA] and sodium chloride (0.5 M NaCl) and mixed thoroughly. The samples were incubated overnight at 4 °C under mild shaking in dark conditions. The second day, the incubated samples were centrifuged at 12,000*g* for 30 min at 4 °C, and the resulting supernatant was discarded. The remaining pellet was resuspended in 3 mL TRIzol (Fisher Scientific, Pittsburgh, PA, USA) and then aliquoted into 1.5-mL sterile centrifuge tubes and stored at −80 °C until downstream analysis. After October 16, 2020, the virus recovery method was changed to ultrafiltration using 30–60 mL of processed sample from Section 2.2 and 100 kDa Amicon® Ultra-15 Centrifugal Filter Units (Millipore Sigma, St. Louis, MO, USA) according to Gharoon et al. (2021). This process was repeated multiple times to achieve a total processed volume of 30 to 60 mL, depending on the expected SARS-CoV-2 concentration (i.e., we processed 60 mL samples to reach a high concentration factor of the samples with low virus levels, otherwise processed 30 mL when the virus concentrations were expected high); this resulted in ~500 μL of concentrate in each cartridge. The concentration factors (CF) were calculated for each sample according to Eq. (1). The viral concentrates were typically stored at −80 °C until downstream analysis unless they were analyzed the same day.

$$CF = \frac{Processed\ volume\ of\ wastewater}{Final\ volume\ of\ concentrated\ sample} \quad (1)$$

We used the AllPrep PowerViral DNA/RNA kit (QIAGEN, Inc., Germantown, MD, USA) to extract total RNA from the concentrated samples according to the user's manual. RT-qPCR was conducted on the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Briefly, the reaction contained 5 μL 4× Reliance One-Step Multiplex Supermix (BioRad, Hercules, CA, USA), 5 μL of the total genomic RNA template, probes (0.4 μL, 0.2 μM) and primers (0.4 μL, 0.4 μM each) in a total volume of 20 μL. RT-qPCR was carried out according to the following program: reverse transcription at 50 °C for 10 min, denaturation at 95 °C for 10 min, followed by 45 cycles of 3 s denaturation at 95 °C, 30 s annealing/extension, and plate read at 60 °C. The threshold cycle (Ct) was determined using the default algorithm in CFX Manager Software (BioRad, Hercules, CA, USA). As per US CDC recommendation, the RT-qPCR assay used N1 and N2 primers and probes (Table S1). Positive and non-template controls were included in each run. Field and RNA extraction blanks were included monthly. Calibration curves were generated with 10-fold serial dilutions of SARS-CoV-2 positive control (IDT, Coralville, IA, USA) in the range from 200,000 to 2 gc/μL. Correlation coefficients (R^2) > 0.99 were obtained for all calibration curves, with 90% to 110% amplification efficiencies. The limit of detection (LoD) was determined to be 2 gc/μL, which was the lowest concentration after serial dilution of the positive controls that showed positive amplification in more than 50% of RT-qPCR reactions. Equivalent sample volumes (ESV) were calculated according to Gerrity et al. (2021) shown as Eq. (2), which is the actual volume of the wastewater sample that can be used to reflect the virus concentrations from RT-qPCR to the concentrations in wastewater. Sample concentrations were dividing the virus concentration (gc/reaction) by the corresponding ESV for each assay.

$$ESV\ (mL) = (0.005/0.02) \times (0.05/0.2) \times (1/CF) \quad (2)$$

Volume of RNA template for N1/N2 assays

Volume of RNA elution after RNA extraction

Volume of RT-qPCR reaction

Volume of concentrated sample used for RNA extraction

2.4. SARS-CoV-2 recovery efficiency, quality control, and PCR inhibition

Pepper mild mottle virus (PMMoV) was analyzed as a process control because it is an endogenous wastewater constituent, specifically an RNA virus, and can be used to validate the overall processing, concentration, and analysis pipeline. PMMoV was quantified according to the SARS-CoV-

2 RT-qPCR Kit for wastewater (Promega, Madison, WI, USA). For the samples that did not yield a positive amplification by RT-qPCR for SARS-CoV-2 but yielded a detectable amplification of PMMoV, the concentration of SARS-CoV-2 was considered below the LoD in the wastewater. Human coronavirus OC43 strain (HCoV-OC43) was used as a surrogate to study virus recovery because of its enveloped structure, which is similar to SARS-CoV-2 (Brant et al., 2021). 100 μL of HCoV-OC43 strain were spiked into 180 mL wastewater, followed by the Amicon ultrafiltration method (Section 2.3). HCoV-OC43 was quantified according to the method by Uppal et al. (2021) (for details, see Tables S2 and S3). The recovery rate was calculated according to Eq. (3). The recovery study was investigated by PEG precipitation, following the same method.

$$Recovery\ rate = \frac{HCoV\_OC43\ recovered}{HCoV\_OC43\ spiked} \bullet 100\% \quad (3)$$

Inhibition was also assessed for virus concentration and RT-qPCR, as inhibition can lead to underestimation of SARS-CoV-2 gene markers during RT-qPCR analysis. 100 μL of HCoV-OC43 was spiked into sterile 1× phosphate-buffered saline (PBS), and then the sample was concentrated with the Amicon ultrafiltration method to assess recovery in a clean matrix. RNA eluates were also diluted 10- and 100-fold in duplicate or triplicate, and ΔCt was calculated to check the presence of PCR inhibitors, according to Li et al. (2020).

2.5. Metadata collection and wastewater quality analyses

Metadata were collected and recorded during each sampling event, including sample date, time, temperature, and flowrate. Wastewater quality parameters such as dissolved oxygen (DO), electrical conductivity (EC), and pH were measured immediately onsite during sampling, using a HACH portable meter and probes (HQ40D) (Hach, Loveland, CO, USA). Other parameters, including total suspended solids (TSS), total dissolved solids (TDS), ammonia-N (NH_4-N), total Kjeldahl nitrogen (TKN), biochemical oxygen demand (BOD), chemical oxygen demand (COD), turbidity, and UV254 absorbance were measured in the laboratory using standard methods (Rice et al., 2017).

2.6. Data analysis

SARS-CoV-2 concentrations of N1 and N2 showed similar trends in the monitoring period, and were highly correlated. However, in this study, N1 was not monitored during one month because of an unknown contamination of N1 in RT-qPCR assay since March 2021. Therefore, in this paper, we use the concentration of N2 gene to show the results and conduct data analysis. To decrease data variability due to sampling, virus concentration, and the RT-qPCR assay, weekly average concentration data were calculated and used to assess correlations between SARS-CoV-2 concentrations and daily new cases of COVID-19. Nonparametric Spearman r correlation coefficients were calculated with GraphPad Prism 9 Software (Graphpad Inc., San Diego, CA). Clinical COVID-19 case data were normalized by the population in each WRF sewershed and reported as cases per 100,000 people.

The approach for estimating infection totals from wastewater surveillance data was based on (Gerrity et al., 2021). The model assumes an initial viral shedding rate of $10^{8.9}$ gc/g-feces—decreasing exponentially over 25 days—and a feces production rate of 126 g-feces/day/person, resulting in a total viral load of 1.0×10^{11} gc/infection. Infection estimates can then be calculated by integrating the SARS-CoV-2 wastewater concentration data over the monitoring period to calculate the total viral load to the WRF (in gc) and then dividing by the total viral load per infection.

3. Results and discussion

3.1. Virus recovery and qPCR inhibition

The recovery of HCoV-OC43 in untreated wastewater by Amicon filtration was 24 ± 2% (N = 9), whereas it was 22 ± 10% in spiked PBS (N = 9). No significant difference was observed between the wastewater and the PBS (Mann-Whitney test, p = 0.85). Therefore, the wastewater matrices were not considered a source of virus loss during sample processing and concentration. With respect to inhibition, ΔCt values were approximately 3.33 for 1× versus 10×-diluted RNA eluate and 4.08 for 10×-diluted versus 100×-diluted RNA eluate. PCR inhibition is often identified as a factor when ΔCt is <2.3 between 10-fold dilutions (Jennings et al., 2020). Therefore, PCR inhibition was assumed to be negligible for this study. 10 samples that were processed by PEG precipitation were included in our dataset and the recovery rate by PEG precipitation was 65.06% (N = 3).

3.2. SARS-CoV-2 RNA presence in untreated wastewater in Washoe County, NV, USA

The first confirmed case of COVID-19 in Washoe County, NV, USA, was reported on March 5, 2020, and the number of confirmed cases rose to 58,000 with 789 deaths by September 13, 2021 (i.e., the end of this study). Through September 2020, the 7-day average for daily new confirmed COVID-19 cases in Washoe County (i.e., the Reno-Sparks metropolitan area) reached a maximum of 39 per 100,000 people, but that number surged to 111 per 100,000 people in last week of November 2020. The case load then declined over the next three months, remained relatively stable through the spring and early summer, but then surged again in the summer and fall with the introduction of the Delta variant, reaching a maximum of 95 per 100,000 people in September 2021.

As noted earlier, there was a possibility that clinical testing capacity might decrease over time, so early in the pandemic, WBS of SARS-CoV-2 was proposed as a complementary resource for decision-making (Peccia et al., 2020). SARS-CoV-2 viral RNA was confirmed in the region's wastewater samples, specifically in 85.8% of 611 samples collected from the three WRFs over 15 months. Table 1 and Tables S2 - S4 present the summary of SARS-CoV-2 wastewater monitoring results and wastewater quality. During the sampling campaign, the gene copy concentrations ranged from 2.76×10^3 to 3.86×10^6 gc/L (Table 1).

Even though wastewater monitoring started in July 2020, approximately three months after the first reported COVID-19 case in Washoe County, few samples had detectable levels of N1 or N2 through September 2020 (40.3% detection frequency from July–September 2020, for both N1 and N2 genes, N = 47). Starting in October 2020, SARS-CoV-2 viral RNA concentrations in wastewater increased, which aligned with trends in daily new reported cases in Washoe County (Figs. 2 and 3). This confirmed that SARS-CoV-2 wastewater monitoring could be a complementary tool for documenting COVID-19 trends in the community.

SARS-CoV-2 viral RNA concentrations in wastewater were then compared with daily new cases per 100,000 people in each sewershed. In Fig. 2 (a), wastewater concentrations of N2 at WRF-A are plotted against daily new COVID-19 cases per 100,000 people, confirming that COVID-19 infections were increasing in early October and that the peak occurred one week after Thanksgiving Day (November 26, 2020). These data suggest that holidays (i.e., Halloween (October 30, 2020) and Thanksgiving (November 26, 2020) Holidays) led to a steady increase in COVID-19 infections and SARS-CoV-2 viral RNA concentrations in wastewater, likely due to group celebrations and increased social contact during these periods (Mehta et al., 2021). After the Thanksgiving holiday (November 26, 2020), COVID-19 infections and SARS-COV-2 viral RNA concentrations started to decrease, presumably due to implementation of stringent pandemic mitigation measures by local authorities. This observation is in agreement with the study by (Mehta et al., 2021). This led to low daily case loads and wastewater concentrations through the spring and early summer of 2021. In fact, from May through the second week of July 2021, most wastewater samples failed to amplify due to low SARS-CoV-2 viral RNA concentration in the wastewater. However, due to the circulation of the Delta variant, the second wave of COVID-19 infections and SARS-CoV-2 concentration in wastewater occurred in the middle of July (Yu et al., 2021).

Table 1
Summary of SARS-CoV-2 surveillance in three water reclamation facilities.

Facility	Service Population	Flowrate ($\times 10^3$ m^3/Day)	SARS-CoV-2 Detection Frequency	Wastewater Generation Coefficient (m^3/capita/d)	SARS-CoV-2 Concentration Range (gc/L)
WRF-A	319,939	94.2	89.5% (N = 218)	0.294	2.76×10^3– 2.36×10^6
WRF-A1	115,792	47.0	86.2% (N = 131)	0.406	6.12×10^3– 3.86×10^6
WRF-A2	204,147	47.2	86.2% (N = 131)	0.231	4.62×10^3– 3.23×10^6
WRF-B	18,808	6.47	77.6% (N = 67)	0.344	3.85×10^3– 2.82×10^6
WRF-C	52,003	12.4	79.7% (N = 64)	0.238	3.72×10^3– 1.02×10^6

Interestingly, we observed lower viral RNA concentrations relative to daily new COVID-19 cases in the second COVID-19 wave (i.e., summer 2021) than in the first wave (i.e., fall 2020), particularly with respect to the peak concentrations observed. One possible reason is that we changed the sampling strategy from grab samples to 24-h composite samples (N = 73), which may cause more 'averaging' of the wastewater throughout the day and, hence, attenuate peaks in SARS-CoV-2 wastewater concentrations (Ahmed et al., 2020b). Another possible explanation is that more businesses were open in 2021 than in 2020, and those businesses may have contributed more commercial wastewater to WRF-A, thereby causing dilution of domestic wastewater. A third reason may be related to the Delta variant and its effects on symptoms and viral shedding. Specifically, since diarrhea is not a common symptom of COVID-19 caused by the Delta variant sequences, virus shedding may have decreased, at least via feces. Only one in 24 COVID-19 patients with the delta variant sequence showed the diarrhea symptom according to a clinical study in Poland (Mazur-Panasiuk et al., 2021). The symptom of diarrhea can cause a 3-fold higher SARS-CoV-2 shedding rate in the feces (Zhang et al., 2021). Therefore, we presume the Delta variant may cause a decrease in the virus shedding rate, even though the shedding rate is agnostic.

The vaccination rate is another important factor that affects the SARS-CoV-2 shedding and transmission in wastewater. According to Bivins and Bibby (2021), mass vaccination caused the decrease of SARS-CoV-2 shedding rate among the population of a college campus. Until September 13, 2021, 49.2% of the population were fully vaccinated in Nevada, which means less shedding of SARS-CoV-2 was possible during 2021, and WBS can be used as a tool to evaluate of the effectiveness of vaccination (Ai et al., 2021).

We also conducted a sub-sewershed analysis for WRF-A. The WRF-A1 interceptor showed minimal viral levels until the beginning of October when daily new reported cases increased in the study area, consistent with the overall results for WRF-A. The peak concentration of 9.28×10^5 gc/L was observed near the Thanksgiving holiday (November 26, 2020, Fig. 3 (a)), again consistent with WRF-A. In contrast, for the WRF-A2 interceptor, we observed a peak concentration of 5.46×10^5 gc/L (Fig. 3 (b)) near the Halloween holiday weekend (October 31, 2020). As this peak occurred approximately one month before that of WRF-A1, it was necessary to assess differences in population that might contribute to the discrepancy in peak concentrations and clinical cases between the two sub-sewersheds. In particular, WRF-A1 has a more stable residential population in comparison with WRF-A2, which has a more transient university/student-dominated population. Therefore, it might be expected for the Thanksgiving holiday (November 26, 2020) to cause a surge in cases and wastewater concentrations for WRF-A1 and at least a short-term decrease in cases and wastewater concentrations for WRF-A2 when students leave campus for the long weekend. In contrast, social events during Halloween (October 30, 2020) might have a more significant impact on WRF-A2, which is a highly commercial community in the study area. According to the local university records, COVID-19 cases among active students increased in two weeks after the Halloween holiday from 9 cases on October 28, 2020 to 30 cases on November 6, 2020, suggesting the significance of Halloween gatherings among students and younger populations, as opposed to other areas with mostly single-family homes. Sub-sewershed monitoring at WRF-A1 and WRF-A2 ended on July 20, 2021, immediately prior to the Delta surge.

The WRF-B and WRF-C sewersheds captured wastewater from smaller-sized populations in Washoe County, NV, USA (Table 1, Fig. S1). The first peak observed after Thanksgiving in 2020 in both sewersheds. The second peak started after July 14, 2021, in the sewershed of WRFB. However, no apparent peak was observed during the same sampling time in WRFC, even though more samples were above the detection rate in the WRF-C sewershed between May and June 2021. Although trends observed in these areas may not have captured the incidence/prevalence of COVID-19 in the metropolitan area's community-at-large, they provided insights into disease spread in more suburban communities. Both WRF-B and WRF-C exhibited similar trends in viral RNA concentration and daily new cases per 100,000 people throughout the duration of the study.

3.3. SARS-CoV-2 concentration in wastewater correlated to the daily new reported cases of COVID-19

According to the longitudinal analyses, SARS-CoV-2 wastewater concentrations appeared to exhibit correlations with daily new COVID-19 cases in the community. However, the experimental methods, including sampling method, concentration method, and RT-qPCR method, can introduce variability because wastewater testing is stochastic and also involves a complex matrix. Therefore, inherent variability, due to differences in

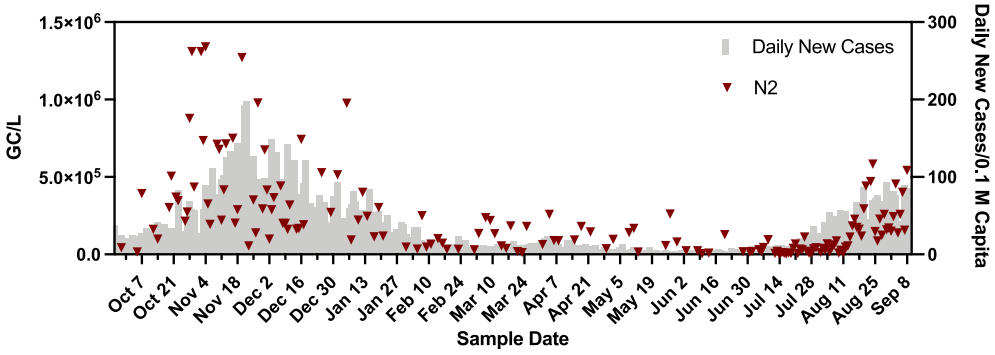

Fig. 2. Longitudinal analysis of SARS-CoV-2 monitoring in WRF-A wastewater from September 2020 through September 2021. The dark red marks display the SARS-CoV-2 concentration of N2 as gc/L. The gray bar show the daily new cases of the sampling day; data were retrieved from Washoe County Health District COVID-19 Dashboard (COVID-19 (Novel Coronavirus)).

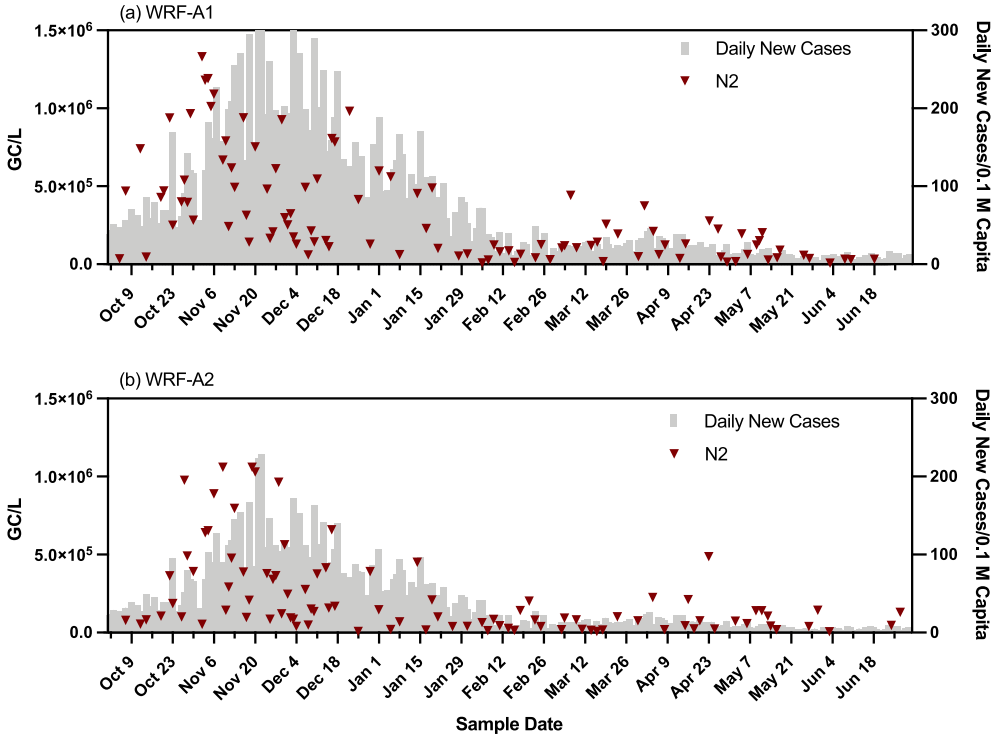

Fig. 3. Longitudinal analysis of SARS-CoV-2 monitoring in WRF-A1 and WRF-A2 wastewater of between September 25, 2020, and June 27, 2021. The dark red marks display the SARS-CoV-2 concentration of N2 as gc/L. The gray bar show the daily new cases of the sampling day; data were retrieved from Washoe County Health District COVID-19 Dashboard (COVID-19 (Novel Coronavirus)).

sampling time, precipitation and temperature, between samples may cause uncertainty when using SARS-CoV-2 monitoring as a complementary tool for public health decision-making (Bivins et al., 2021; Curtis et al., 2020). So, it is important to mitigate the effects of this variability when interpreting WBS data. For most of this study (July 2020 to June 2021), grab samples ($N = 538$) were collected and analyzed three to five times per week during the weekdays. To account for variability, we calculated 7-day averages (from Sunday to Saturday) of both SARS-CoV-2 concentrations and clinical case data. We then used nonparametric Spearman r correlation coefficients to evaluate if the two datasets were correlated. The results are shown in Table 2.

The results show that using weekly averages does in fact decrease variability and improve correlations between viral wastewater concentrations and clinical case data,. For example, the Spearman r correlation coefficient was 0.533 for the daily results and 0.790 for the weekly averages for WRF-A. Improved correlations were also observed in the WRF-A1 and WRF-A2 sub-sewersheds (Table 2). Ai et al., (2021) have applied 7-day moving average of clinical cases and found that it had a strong correlation with SARS-CoV-2 concentrations. They developed a quadratic polynomial model between those two. In our study, we calculated the weekly average of both wastewater monitoring data and clinical data, which can be beneficial to smooth the environmental data, and can provide guidance for future studies.

Table 2
Evaluation of nonparametric Spearman r correlation coefficients between SARS-CoV-2 concentrations in wastewater and daily new COVID-19 cases. All the data were based on weekly average, from Sunday to Saturday.

	SARS-CoV-2 concentration versus clinical cases on the same sampling day	7-day advance SARS-CoV-2 concentration versus clinical cases
WRF-A (Daily)	0.533	0.550
WRF-A (Weekly average)	0.790	0.793
WRF-A1 (Daily)	0.595	0.635
WRF-A1 (Weekly average)	0.695	0.689
WRF-A2 (Daily)	0.428	0.403
WRF-A2 (Weekly average)	0.502	0.485
WRF-B (Daily)	0.615	0.232
WRF-B (Weekly average)	0.602	0.606
WRF-C (Daily)	0.353	0.248
WRF-C (Weekly average)	0.472	0.415

The correlations of SARS-CoV-2 concentrations and the clinical cases data of WRF-B and WRF-C sewersheds were shown in Table S6. A strong correlation was observed for WRF-B (Spearman r was 0.615 for the daily results and was 0.602 for the weekly average), which serviced a largely domestic sewershed with stable influent flows during the study period. WRFC, which serviced a larger sewershed with more commercial and industrial users, was observed to have weak correlations between the SARS-CoV-2 concentrations with clinical cases (Spearman r was 0.353) for daily results and moderate correlations (Spearman r was 0.472) for weekly results. The weekly average did not improve the correlation much because of the lower sampling frequency at those two sewersheds. We sampled twice per week in 2020 and only sampled once per week in 2021. The slight improvement in correlations may be attributed to the average clinical cases, which may decrease the variability.

3.4. SARS-CoV-2 wastewater monitoring as an early-warning of COVID-19 in a community

One major objective of this study was to evaluate if SARS-CoV-2 can be used as a complementary tool for early warning of COVID-19 outbreaks in the community. To accomplish this, we once again determined Spearman correlation coefficients between SARS-CoV-2 wastewater concentrations and daily new COVID-19 cases, but the concentration data were associated with case data reported 7 days later. We found that SARS-CoV-2 wastewater concentrations were still correlated with the lagged clinical data, but the correlations did not consistently improve after the adjustment (Table 2). For example, the Spearman r for WRF-A increased from 0.533 to 0.550 (daily data) and from 0.790 to 0.793 (7-day average data) after adjusting

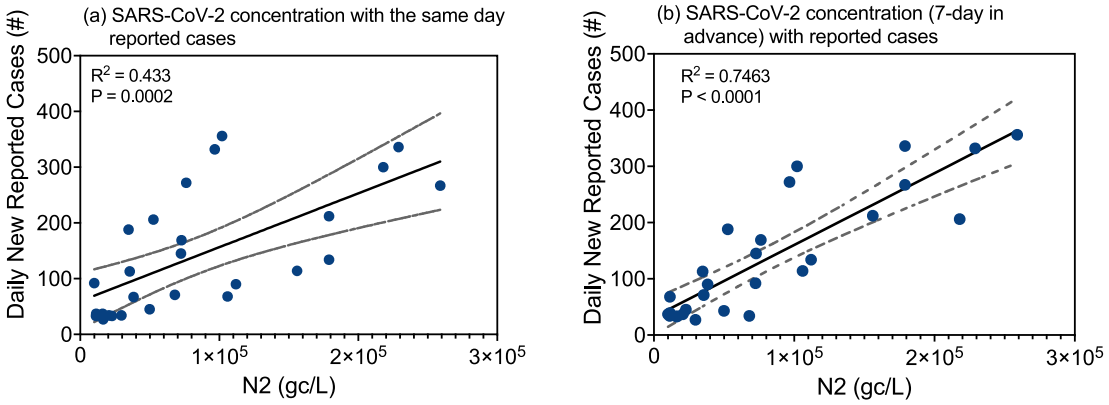

Fig. 4. Comparison of the relationship between SARS-CoV-2 wastewater concentration, with or without modification for 7-day advanced notice, and daily new COVID-19 cases. The raw data for SARS-CoV-2 wastewater concentrations and daily new COVID-19 cases were based on 7-day averages.

for the 7-day lag. While the Spearman r for WRF-A1 increased from 0.595 to 0.635 for the daily data, the correlation coefficient decreased from 0.695 to 0.689 for the 7-day average data. The Spearman r correlation coefficients for WRF-A2 both decreased after accounting for the 7-day lag. However, WRF-A1 is a more residential sewershed whereas WRF-A2 is more commercial, which suggests that commercial contributions may dampen correlations between viral concentrations and clinical cases. In WRF-B and WRF-C sewersheds, the Spearman r increased from 0.602 to 0.606 (7-day average data) for WRF-B but decreased from 0.472 to 0.415 (7-day average data) for WRF-C after adjusting for the 7-day lag. However, the correlations of daily results for both WRFs were weak (Spearman r was 0.232 for WRF-B and 0.248 for WRFC). Therefore, SARS-CoV-2 wastewater concentrations can be used as a 7-day advanced predictor of COVID-19 case trends, but the 7-day lag has a more muted impact relative to the effect of calculating 7-day averages for the two datasets.

To further investigate the role of SARS-CoV-2 wastewater data as a leading predictor of daily new COVID-19 cases, we also conducted simple linear regressions (Fig. 4, Table S6). In this case, SARS-CoV-2 concentration in wastewater was the independent variable and the daily new COVID-19 case count was the dependent variable. We found that SARS-CoV-2 concentrations with 7-day advanced notice decreased variability in the estimation of daily new COVID-19 cases, increasing the R^2 value from 0.433 to 0.746.

3.5. Use SARS-CoV-2 concentration in wastewater to estimate the infected population in a community

Finally, wastewater surveillance data for three WRFs were used to derive total infection estimates for the region (Table 3). After integrating the SARS-CoV-2 wastewater concentration data for WRF-A from July 2020 through September 2021 and accounting for flow rate, it was estimated that 1.09×10^{16} gene copies were shed into the wastewater in three WRFs. The wastewater-derived estimate for total infections during that timeframe was 108,382 (28% of the service population), whereas there were 54,772 confirmed cases (14% of the population) according to clinical data posted on the Washoe County COVID-19 Dashboard (Washoe County Health District, 2021). Therefore, there were 53,610 asymptomatic or unconfirmed cases in the study area, which constitutes 14% of the service population (Table 3). The results from this study clearly suggest that clinical data underestimate actual infection totals. Interestingly, in the sewersheds of WRF-A and WRFB, the results produced similar of estimations for the ratio of asymptomatic or unconfirmed cases. This may because those two sewersheds are largely residential, whereas WRF-C sewershed has higher level of commercial activity. The Reno international airport is located in WRF-C sewershed and can cause a relatively high level of population mobility and tend to have more non-resident population in this area. Those reasons can contribute more difficulty when estimate COVID-19 cases using SARS-CoV-2 monitoring data in this sewershed.

4. Conclusions

This represents the first study to monitor untreated wastewater in the Reno-Sparks metropolitan area, Nevada, USA, for SARS-CoV-2 viral RNA. This study demonstrates that wastewater surveillance can be a complementary tool for understanding overall COVID-19 incidence/prevalence, predicting future COVID-19 cases, and aiding decision makers in managing the pandemic at the local or regional level.

SARS-CoV-2 viral RNA concentrations in local wastewater accurately reflected daily new COVID-19 case trends from September 2020 through September 2021, specifically capturing two infection surges in northern Nevada. The first peak occurred in the last week of November 2020, which was after the Thanksgiving holiday; the second peak occurred in the late summer and early fall of 2021, primarily due to spread of the highly infectious Delta variant. These peaks were successfully captured in the SARS-CoV-2 wastewater concentration datasets as well, even highlighting subtle

Table 3
Summary of total estimated COVID-19 cases in the sewersheds of three monitored water reclamation facilities since July 2020, to September 2021.

	Results			
	WRF-A	WRF-B	WRF-C	Total
Total gene copies shed in wastewater during monitoring period (gc)	$(9.49 \pm 0.76) \times 10^{15}$	$(5.62 \pm 0.45) \times 10^{14}$	$(7.99 \pm 0.63) \times 10^{14}$	$(1.09 \pm 0.08) \times 10^{16}$
Estimated total infection cases by wastewater monitoring (#)	94,782 ± 7583	5613 ± 449	7987 ± 638	108,382 ± 8670
Confirmed infections during monitoring period (Clinical data from WCHD, #)	44,996	2645	7131	54,772
Service population in sewershed (#)	319,939	18,808	52,003	390,750
Estimated infection ratio by wastewater monitoring (%)	30%	30%	15%	28%
Estimated persons of asymptomatic or unconfirmed cases (#)	49,786 ± 3982	2968 ± 237	856 ± 68	53,610 ± 4288
Estimated ratio of asymptomatic or unconfirmed cases (#)	16.0%	15.8%	2%	14%

Note: SARS-CoV-2 shedding per infected individual (gc/person) is 1.0×10^{11}, according to the shedding rate of $10^{8.9}$ gc/g of feces from virus carriers and 126 g of feces per day per person.

temporal differences at the sub-sewershed level. Vaccination rates may have an effect on the SARS-CoV-2 shedding rate (i.e., decrease in the shedding rate), although future study is needed.

A strong correlation between SARS-CoV-2 concentrations and clinical cases was confirmed according to nonparametric Spearman r correlation coefficients. Using weekly averages rather than daily data decreased inherent variability related to sampling, sample processing, and RT-qPCR analysis methods, thereby improving the correlations. Furthermore, correlations were also observed when using wastewater concentrations as a 7-day advanced predictor of COVID-19 cases, even leading to improve linear regressions. Finally, wastewater-derived infection estimates highlighted a discrepancy between total and confirmed COVID-19 cases, which demonstrates the value of WBS as a tool for public health officials.

CRediT authorship contribution statement

Lin Li: Methodology, Investigation, Data curation, Writing - original draft. **Lauren Mazurowski:** Investigation, Validation, Writing - original draft. **Aimee Dewan:** Validation, Investigation. **Madeline Carine**: Validation, Investigation. **Laura Haak:** Investigation, Validation, Visualization, Draft review and editing. **Tatiana Guarin:** Methodology, Investigation, Validation, Draft review and editing. **Daniel Gerrity:** Methodology, Validation, Draft review and editing. **Casey Mentzer:** Methodology, Investigation, Data curation. **Krishna Pagilla:** Project conceptualization, Funding acquisition, Supervision, Project administration, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.152958.

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