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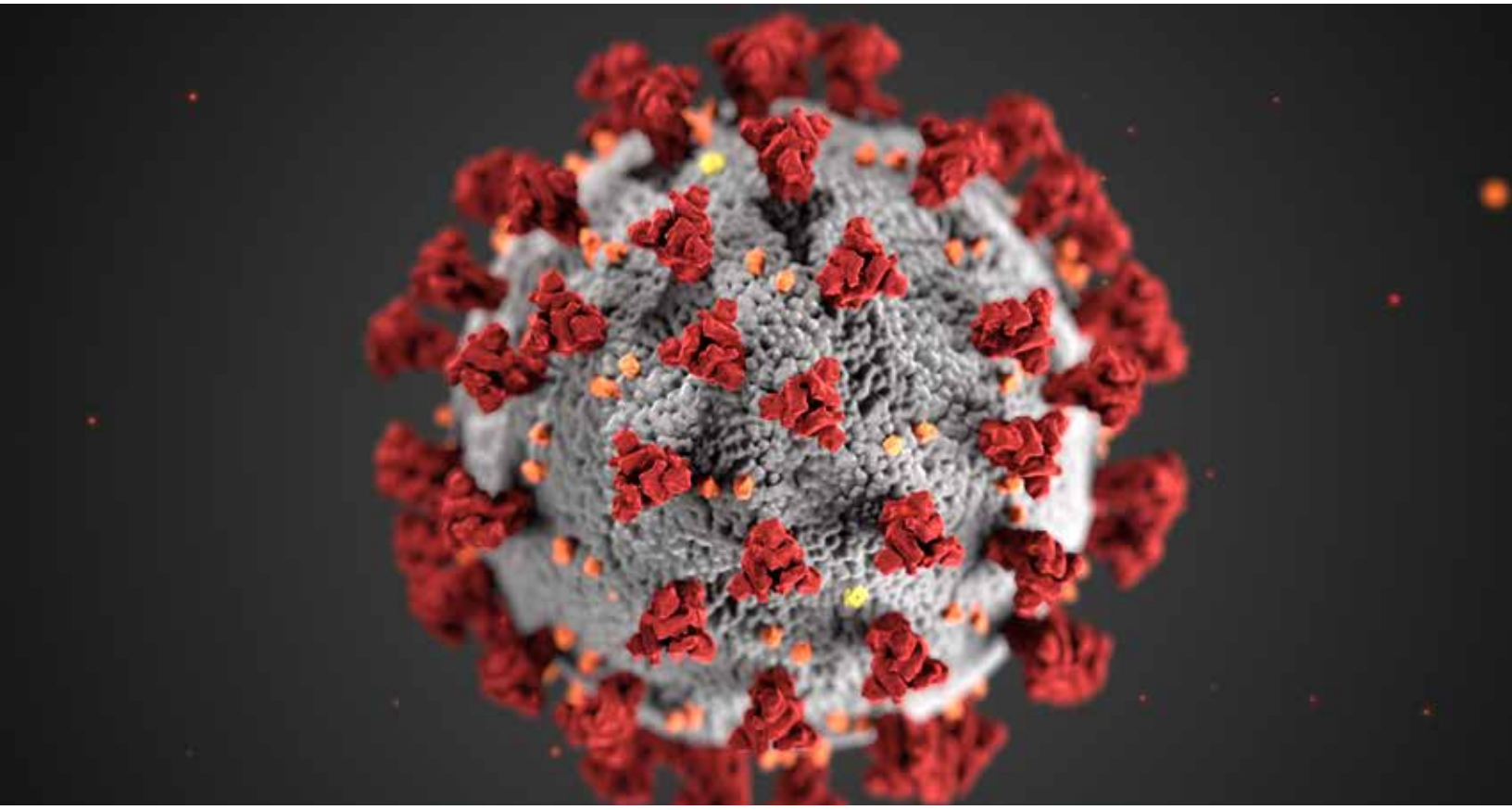

PROJECT NO.
5093

Understanding the Factors That Affect the Detection and Variability of SARS-CoV-2 in Wastewater

Understanding the Factors That Affect the Detection and Variability of SARS-CoV-2 in Wastewater

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Abstract and Benefits

Abstract:

Wastewater-based surveillance (WBS) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) represents a promising complement to clinical testing as a means of assessing COVID-19 trends within a community. The objective of this project is to understand how the wastewater sampling designs impact the quantifiable SARS-CoV-2 genome in both centralized and decentralized wastewater collection and treatment systems. SARS-CoV-2 genome concentrations were analyzed in primary clarifier sewage influents and primary sludge samples from centralized wastewater treatment plants (WWTPs). Study of the decentralized wastewater collection and treatment system for SARS-CoV-2 genome detection was carried out in septic systems that serve the public restrooms at Zuma Beach, Malibu, California. The results showed that 24-hour composite samples can best represent the trends of SARS-CoV-2 concentrations in centralized WWTPs. The primary sludge samples had nearly 10 times higher concentrations of the viral genome, suggesting that sludge testing could provide greater sensitivity for SARS-CoV-2 detection. The decentralized wastewater management system also has the potential to be used as the access point for WBS. The pumping and hauling services can be used to easily access samples if direct sampling from septic tanks is not feasible.

Benefits:

- SARS-CoV-2 genomes detected in wastewater tracked well with the trend of local COVID-19 cases, suggesting the value of WBS.
- 24-hour composite sampling could be used to represent the trend of daily SARS-CoV-2 concentrations in WWTPs.
- Primary sludge provides a higher sensitivity than primary effluent for detection of SARS-CoV-2 and is a viable option for WBS.
- The source and age of primary sludge may vary from plant to plant, which impacts the interpretation of SARS-CoV-2 results.
- Decentralized wastewater collection and treatment systems can be accessed for SARS-CoV-2 monitoring to track the spread of COVID-19.
- Monitoring of composite samples from sewer manholes may provide localized information regarding COVID-19 trends at the sub-community level.

Keywords: SARS-CoV-2, Centralized Wastewater Treatment Plant, Septic System, Sewer Manhole Sampling, Primary Influent, Primary Sludge; Grab Samples; Composite Samples

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Acronyms and Abbreviations

BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
COVID-19	Coronavirus disease 2019
ddPCR	Droplet digital polymerase chain reaction
GC	Genome copy
IRWD	Irvine Ranch Water District
JWPCP	Joint Water Pollution Control Plant
LACSD	Los Angeles County Sanitation Districts
LCWRP	La Cañada Water Reclamation Plant
MWRP	Michaelson Water Reclamation Plant
PCR	Polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SJCE	San Jose Creek East Water Reclamation Plant
TSS	Total suspended solids
WBS	Wastewater-based surveillance
WWTP	Wastewater treatment plant

Executive Summary

Wastewater-based surveillance (WBS) of SARS-CoV-2 represents a promising complement to clinical testing as a means of assessing COVID-19 trends within a community. This report presents the investigation of SARS-CoV-2 genome concentrations in centralized and decentralized wastewater collection and treatment systems over a 1-year period (2020-2021). Three centralized wastewater treatment plants (WWTP) representing sewersheds at different scales were selected to investigate the variability of SARS-CoV-2 concentrations in primary clarified sewage influent and primary sludge at each WWTP. SARS-CoV-2 genome concentrations were also compared in grab samples taken at different times of the day and in 24-hour flow-paced composite samples collected by autosamplers. SARS-CoV-2 genome concentrations in wastewater and sludge samples were analyzed using previously developed and cross-laboratory validated nucleic acid extraction and droplet digital PCR quantification methods. The results showed that the viral concentration in primary clarified influent was correlated with that in primary sludge. However, the primary sludge samples contain significantly higher concentrations of the virus, suggesting that direct monitoring of primary sludge may provide a higher sensitivity for detection of SARS-CoV-2 in centralized WWTPs without the need for sample concentration. Grab samples taken at random times of the day had a high variability of SARS-CoV-2 concentration. There was no clear pattern of SARS-CoV-2 concentration relative to daily sewage inflow or other wastewater quality parameters (TSS, COD, BOD, NH_3-N). However, the daily mean value from all grab samples collected during the day matched well with the value from the 24-hour composite sample. Therefore, the results suggest that 24-hour composite sampling should be used as a high priority sampling strategy in managing WBS to best use the available resources. Moreover, there was no relationship between routinely measured water quality parameters and SARS-CoV-2 concentrations identified in the study, which suggests that there is no substitution for direct quantification of SARS-CoV-2 in the WBS effort. Significant differences in wastewater inflow pattern and water quality parameters were observed between the WWTPs included in the study due to their distinct sewershed characteristics. However, the SARS-CoV-2 concentrations detected in wastewaters from two large plants both tracked well with the local Department of Public Health reported COVID-19 cases. These outcomes further suggest the value of WBS.

Until now, most WBS investigations have focused on centralized wastewater treatment facilities such as WWTPs. Decentralized wastewater treatment systems or onsite wastewater treatment systems such as septic systems are widely used in unsewered areas. Also, in developing countries it is common for human waste to be combined and compounded in holding tanks prior to periodic pumping of the contents into waste hauling vehicles or into treatment systems. However, few studies have been performed to monitor SARS-CoV-2 RNA in septic systems or in holding tanks. A study of SARS-CoV-2 detection was carried out in septic systems that serve high visitation public restrooms at Zuma Beach, Malibu, California. The supernatant and sludge layers of two primary tanks associated with two different public use restrooms were sampled at different depths of the septic tanks, bi-weekly over 6 months. SARS-CoV-2 RNA was detected in most samples. While the viral concentrations were positively correlated across the sludge and supernatant compartments, the viral concentrations were generally higher in the sludge layers than in the supernatants. The concentrations were positively and significantly correlated with TSS of the samples for all samples tested, suggesting attachment of the virus onto solids. Viral concentrations were also higher in the deeper supernatant or sludge layers than in the surface supernatant or sludge layers. While this apparent virus stratification may be due to the association with solids which themselves were stratified, further study would be required to determine the roles of other potential factors such as differential virus persistence in the various strata of solids and supernatant. A pumping and hauling study was also performed to determine if sampling of septic or holding tanks could be performed more conveniently according to the periodic tank service schedules, but without

introducing bias from sample decay *ex situ*. SARS-CoV-2 concentration in samples taken from the hauler truck tank represented the septage samples taken directly from the septic tank. However, SARS-CoV-2 concentrations declined during transport from the septic tank pumping site to the disposal site at a centralized WWTP. The results of this investigation suggest that decentralized systems have the potential to be used as access points for WBS. Further, pumping and hauling services can be used for easily acquiring samples, particularly if direct sampling from the septic tanks is infeasible.

WBS of SARS-CoV-2 in large centralized WWTPs and the public septic system like the Zuma Beach restrooms can offer a broad picture of the spread of COVID-19 in the larger community. However, to trace the viral signal back to a specific community or individuals will require sampling upstream of a specific sewershed at sub-community level, in order to relate the wastewater viral signal directly to a specific sub-community, neighborhood, or a specific building. A proof-of-concept study was carried in the sub-sewershed serving the University of California, Irvine (UCI) main campus, to investigate the feasibility of tracking SARS-CoV-2 in sewer manholes and relating the viral signal to the individual COVID-19 testing program at UCI. Between December 2020 and March 2021, weekly wastewater grab samples were collected from six manholes that serve different sizes of the UCI campus community, ranging from a few hundred to a few thousand people. In addition, manholes on the main sewer trunk line that directs sewage from the UCI collection system to the Irvine Range Water District's Michelson Wastewater Reclamation Plant (MWRP), in addition to the plant influent at MWRP, were also sampled on each sampling day. The results showed positive detection of SARS-CoV-2 during the December 2020 to January 2021, peak of the pandemic in the region. However, the expected intermittent sewer flow in a very small sewage collection area also implies that a single grab sample may miss the signal of SARS-CoV-2. Thus, a composite sample collected over time would improve the sampling quality, by eliminating the aleatory nature of grab sampling with unscheduled intermittent flow. Moreover, the viral signal detected in the local sewer manhole samples tracked well with the samples collected from main sewer trunk line and the influent of the WWTP at the downstream end of the sewershed. The trend of SARS-CoV-2 concentration in manhole samples tracked well with the UCI individual COVID-19 testing results and the case report from the local cities.

Future studies should develop rapid and more sensitive methods for SARS-CoV-2 monitoring in environmental samples. Higher sampling frequency and higher density of sampling sites are necessary to produce a more accurate picture of SARS-CoV-2 distribution in human wastewater and the spread of COVID-19. Also, future studies should address the threshold of minimum manhole flow rate to enable correlation between manhole grab samples and cases within the community served by the collection system upstream of the sampled manhole.

ES.1 Related WRF Research

- Environmental Persistence and Disinfection of the Lassa Virus and SARS-CoV-2 to Protect Worker and Public Safety (5029)
- Interlaboratory and Methods Assessment of the SARS-CoV-2 Genetic Signal in Wastewater (5089)

CHAPTER 1

SARS-CoV-2 in Centralized Southern California Wastewater Treatment Plants

1.1 Introduction

Understanding the spread of COVID-19 infection is critical in effectively responding to this pandemic. However, due to logistical and human factors involved in massive individual testing for COVID-19 and inconsistent reporting of results, generating reliable COVID-19 incidence and/or prevalence estimates in a community remains challenging. Wastewater-based surveillance (WBS) of SARS-CoV-2 represents a promising complement to clinical testing as a means of assessing COVID-19 trends and prevalence within a community (Ahmed et al., 2020; Kitajima et al., 2020; La Rosa et al., 2020). Unlike clinical testing data, which is susceptible to biases such as test availability and willingness of asymptomatic individuals to participate in the testing (Mizumoto et al. 2020; Nishiura et al. 2020), WBS yields a relatively unbiased community-scale viral load estimate for a wastewater treatment plant (WWTP) catchment population. Considering these benefits, there has been much support for WBS as a complementary strategy to clinical testing in response to the SARS-CoV-2 pandemic (United States Center for Disease Control 2022). Longitudinal WBS in major WWTPs has the potential to detect the resurgence of COVID-19 at the community level, which could facilitate the prioritization of communities for interventions.

However, wastewater varies significantly from one WWTP to another depending on the wastewater collection system and WWTP's service area. The urban sewer system, also known as the sewershed, is a complex network made up by trunk lines, tributaries and sub-sewersheds. WWTP is the final collection point of the sewershed. The properties of influent wastewater to a WWT are determined by: 1) source of the wastewater (i.e., proportion contribution of industrial, business, and residential discharge); 2) the size of the sewershed (aka service area, catchment, and population served); and much more. Because of such variability, it is necessary to design the sampling strategies that can capture the representative signal of SARS-CoV-2 in wastewater in order to use it as an inference for the community epidemic.

This chapter reports the investigation of the sampling strategies to capture SARS-CoV-2 signals in wastewater in centralized WWTPs. Specifically, primary clarified sewage influent and primary sludge samples were collected from three centralized WWTPs in Southern California and were monitored for SARS-CoV-2 viral genomes in grab samples, flow-paced composite samples, and sludge samples. The concentrations of SARS-CoV-2 in different types of samples were compared. The results of the study contribute to the understanding of the variability of the virus in WWTPs of metropolitan regions.

1.2 Materials and Methods

1.2.1 Selection of WWTPs for Wastewater Sample Collection

In the selection of WWTPs for sample collection, we considered the following sewershed criteria:

1. The sewersheds should represent diverse types of wastewater collection systems including both large centralized WWTPs and small sewer collection systems such that the outcomes of the research have broad implications for tracking the COVID-19 pandemic at different regional and community scales.
2. Sampling should follow a consistent plan to maximize comparability between different sets of results.

3. The samples collected from sewersheds should be able to be analyzed as soon as possible to avoid shipping, handling and preservation, which introduces additional variability and affect data quality.

Based on these criteria, we identified three centralized sewersheds in Southern California. Their geographic locations are shown in Figure 1-1. They are the Los Angeles County Sanitation Districts' (LACSD) Joint Water Pollution Control Plant (JWPCP), LACSD's San Jose Creek East Water Reclamation Plant (SJCE), and LACSD's La Cañada Water Reclamation Plant (LCWRP). A brief description of each sewershed is presented in Table 1-1.

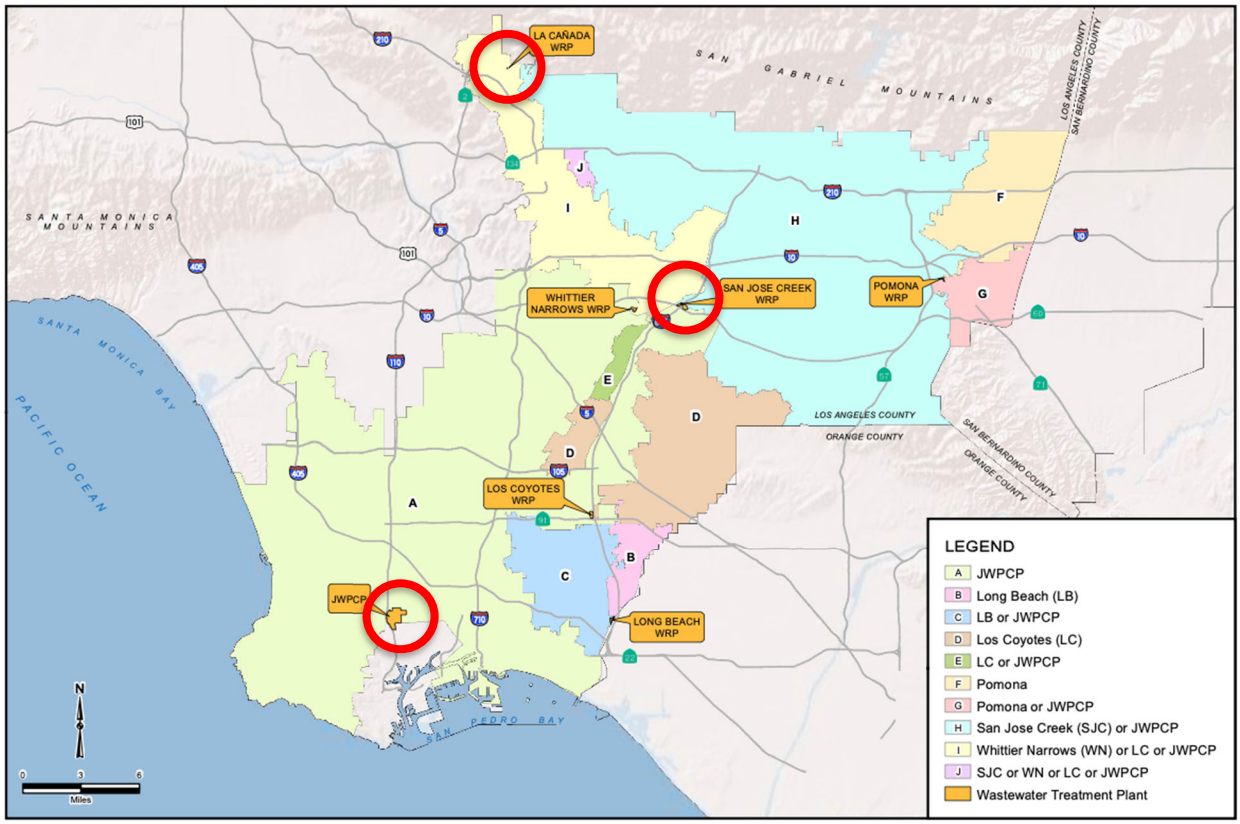


Figure 1-1. Three Southern California Sewersheds Sampled in the Study.

Table 1-1. Characteristics of the Sewersheds and WWTPs Investigated.

Name of the WWTPs	Description	Size Category
LACSD-Joint Water Pollution Control Plant (JWPCP)	Design capacity of 400 MGD serves over 4.9 million people	Large urban central plant with high industrial influent
LACSD-San Jose Creek East Water Reclamation Plant (SJCE)	Design capacity of 37.5 MGD serves ~1 million people	Large urban upstream plant with low industrial influent
LACSD-La Cañada Water Reclamation Plant (LCWRP)	Design capacity of 0.2 MGD serves the La Cañada Flintridge Country Club and 425 surrounding homes	Small community

1.2.2 Wastewater Sample Collection from WWTPs

The following sections give a brief description of each WWTP included in the study and the sampling scheme used for the analysis. In addition to treatment capacity, wastewater composition, size and

characteristics of the sewershed, each facility also differs in plant design and operation, which affected sampling strategy and procedures.

1.2.2.1 Joint Water Pollution Control Plant

JWPCP, with a capacity of 400 MGD, is one of the largest WWTPs in Southern California and provides service to approximately 4.8 million people. JWPCP is a centralized plant receiving local wastewater from municipal and industrial discharge as well as solids and occasionally diverted wastewater from LACSD's upstream water reclamation plants. Figure 1-2 is an aerial photograph of the facility showing the scale and urban setting of the plant.

Figure 1-2. Aerial Photograph of JWPCP.
Source: Courtesy of LACSD.

JWPCP has 6 grit chambers in total to settle primary solids. A total of 5 autosamplers are installed at the grit separation stage (labeled as A – E in Figure 1-3). Grit chambers 1, 2, 5, 6 have individually designated autosamplers. Grit chambers 3 and 4, which handle approximately half of the wastewater flow to the plant, have one autosampler sampling from their combined effluents (autosampler C in Figure 1-3).

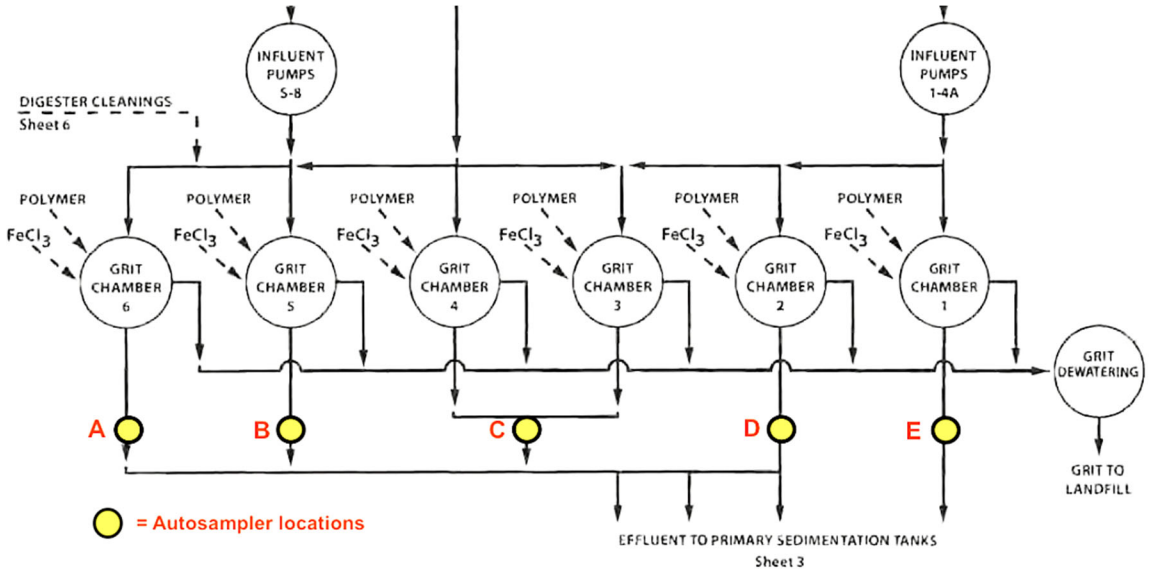


Figure 1-3. Autosampler Locations at the Primary Influent of the Grit Chambers.
Source: Courtesy of LACSD.

Two flow-paced 24-hour composite samples were collected on bi-weekly basis. The first composite sample was collected from autosampler C (grit chambers 3 & 4), which represents approximately half of the flow to JWPCP. The second composite sample, labeled "master-composite," was created manually from all autosamplers (A – E) in the lab using all 24-hour composites from each of the grit chamber autosamplers. Each grit chamber is designed differently and thus receives different flow rates throughout the year. Hence, the "master-composite" is prepared using volumes from each 24-hour composite that are proportional to the historic average flow rates of each grit chamber. Since different grit chambers also receive flow from different sewer trunk lines, the comparison between the two composite samples from the same plant could offer insight to the prevalence of SARS-CoV-2 in flows from different trunk lines (hence, from different service areas). A total of 14 samples (24-hour composites) were taken during the study period between Nov. 17, 2020, and Feb. 2, 2021. A total of 14 samples were taken during the study period, seven from autosampler C and seven more from the "master composite".

To compare SARS-CoV-2 in the composite samples with that of the grab samples, grab samples from sampling port at the autosampler C (grit chambers 3 & 4) were taken every 4 hours over a 24-hour period. These samples represent a snapshot of the SARS-CoV-2 concentrations in the main flow to the JWPCP. A total of 42 grab samples were taken during the study period.

1.2.2.2 San Jose Creek East Water Reclamation Plant

The SJCE plant is a regional plant with a designed capacity of 37.5 MGD and provides service to approximately 0.6 million people. The plant diverts a portion of the raw sewage influent during high flow to JWPCP at times and continuously discharges settled sludge to JWPCP. This plant is designed for water reclamation, and is not equipped with solids processing facilities. Therefore, this regional plant is hydraulically connected with JWPCP. However, the flow of sludge from SJCE to JWPCP varies daily, based on the operational needs. The aerial photograph of SJCE is shown in Figure 1-4. Most of the treated effluent from SJCE is reused across the local area for irrigation and other recycled water uses.

Figure 1-4. Aerial Photograph of LACSD's San Jose Creek East (SJCE) Plant.
The West Plant is in the foreground with the East in the background.
Source: Courtesy of LACSD.

The configuration of SJCE is simpler than that of JWPCP. A single 24-hour composite sample is sufficient to represent the plant influent. The grab samples were taken every four hours within a 24-hour period when the composite was collected. A total of 42 grab samples and 7 flow-paced composite samples were taken during the study period.

1.2.2.3 La Cañada Water Reclamation Plant

LCWRP is by far the smallest centralized facility sampled in this study. It provides service to roughly 425 surrounding homes in a golf club. The daily flow to the plant is highly variable, with the average design flow of around 200,000 gal/day (actual average flow was far less). Since the plant is located directly adjacent to the homes it serves, the wastewater from the household can reach the plant within minutes, which lessens the chance of viral decay in sewer transport. The treated effluent from the plant is stored in the local Country Club golf course lakes which is later used for irrigation of the golf course. The aerial photograph of LCWRP is shown in Figure 1-5.

Figure 1-5. Aerial Photograph of LCWRP.
A small community WWTP in California.
Source: Courtesy of LACSD.

LCWRP has no primary sedimentation. The raw influent is subjected to grinding before activated sludge treatment. There is no autosampler installed at LCWRP. Raw influent grab samples were taken after the influent grinding step. Secondary effluent and chlorinated secondary effluent were also taken at this plant to offer insight into this unique treatment process. A total of 15 samples were taken at the facility: five post grinding raw influent, five secondary effluent, and five disinfected secondary effluent.

1.2.3 Sludge Sample Collection from WWTPs

To compare the SARS-CoV-2 concentrations in wastewater samples with sludge samples, primary sludge from each of the centralized WWTPs were collected side-by-side with the primary influent wastewater samples. JWPCP used two wet wells, both of which were sampled during the study on a bi-weekly basis. The single wet well at SJCE was also sampled on the same schedule. The sludge samples were taken from the sampling port located at a sludge storage chamber every eight hours (per shift). The sludge residence time is estimated to be around two hours in the wet wells. A 24-hour composite sample was created on each sampling day by manually mixing grab samples taken every eight hours. A total of 30 sludge samples were taken from JWPCP (15 from each wet well) and 16 sludge samples were taken at SJCE. There is no primary sludge generated at LCWRP.

1.2.4 Sample Processing and Water Quality Data Collection

All samples were kept at 4°C during compositing, storage, and transport. The samples were transported by car on each sampling day, in the morning, from the WWTPs to UC Irvine lab. Immediately upon arrival to UCI lab, the samples were homogenized and sub-aliquoted (250 µL) to 1.5 ml sterile tubes containing 750 µL pre-aliquoted Zymo DNA/RNA Shield to prevent the degradation of nucleic acid. The preserved samples were either processed for RNA extraction using Zymo fecal RNA kit or stored at -80°C until extraction.

Water quality data associated with samples were retrieved from LACSD. The plant collected water quality data included wastewater flow rate, raw sludge flows to the wet wells, total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), and ammonia (NH_3-N) of primary influent. Since water quality data were not collected daily at each plant, the data collected on the date that was closest to the sampling date were used.

There is little information on the sludge samples from both WWTPs. TSS measurement of sludge samples were carried out at the UCI lab following the ASTM "Standard Test Method for Filterable Matter and Nonfilterable Matter in Water" (ASTM International 2018). Tests were conducted using a 1 mL volume of sludge stored in a -80°C freezer. The total samples analyzed for centralized wastewater treatment plants are summarized in Table 1-2.

Table 1-2. Number of Samples Collected from Centralized WWTPs.

Name	Grab Primary Influent	24-hour Flow-weighted Composite	Primary Sludge
JWPCP	42	14	30
SJCE	42	7	16
LCWRP	15	-	-
Total	**99**	**21**	**46**

1.2.5 Sample Analysis for SARS-CoV-2 Detection

The details of RNA extraction and SARS-CoV-2 ddPCR detection methods for wastewater are published in our manuscript (Song et al. 2021) and were calibrated during a WRF inter-laboratory methods comparison for SARS-CoV-2 detection (Pecson et al. 2021). The SARS-CoV-2 N2 gene was used as the indication of the viral concentration in wastewater. The final viral concentration in wastewater was calculated using: [(no of positive droplets ÷ cDNA vol) ÷ RNA to cDNA conversion factor] ÷ RNA concentration factor. The detection limit of ddPCR assay was set to 3 positive droplets per reaction based on a manual setting of the fluorescence threshold. Assuming 100% virus recovery, this converts to a 300 GC/mL quantification limit (LoQ) when 4 µl of cDNA were used in the ddPCR reaction. The lower limit of detection (LoD) was set at 150 GC/mL based background analysis.

The TaKara NecleoSpin RNA stool kit was used for extraction of primary sludge. The TaKara RNA stool kit is similar to the Zymo fecal RNA kit and the manufacturer's protocol was followed during the extraction procedures. Before adopting the new kit, a side-by-side comparison of SARS-CoV-2 genome quantification was carried using nine primary sludge samples seeded with known concentrations of heat-inactivated SARS-CoV-2 (Catalog No. NR-52350, BEI Resources, Manassas VA, USA). The results showed comparable outcomes between the two kits for extracting viral RNA for SARS-CoV-2 quantification (Table 1-3). The average recovery rate for each type of primary sludge sample was used in the final computation of SARS-CoV-2 in sludge samples.

Table 1-3. SARS-CoV-2 Recovery Efficiency in Seeded Primary Sludge Samples by the Zymo Fecal RNA Kit and the TaKara NecleoSpin RNA Stool Kit.

Seeded samples	Zymo Kit	TaKara Kit			Average of two kits
		Trial 1	Trial 2	Average	
SJCE sludge (n=3)	18.69%	16.00%	19.74%	17.87%	18.14%
JWPCP sludge (n=6)	37.31%	40.27%	32.51%	36.39%	36.69%

1.2.6 Statistical Analysis

Correlation analysis was performed using Pearson and Spearman correlation coefficients in MATLAB. Although both were considered, the Spearman correlation is most valuable due to the non-linear nature of environmental data. The Spearman correlation coefficient > 0.50 is considered moderate, and > 0.70 constitutes a high correlation. Any correlation coefficient value less than 0.5 is considered statistically insignificant (Schober and Schwarte, 2018). Paired t-tests were also performed to determine the degree to which the collected data sets were statistically different. $P \leq 0.05$ indicates a statistically significant difference.

1.3 Results and Discussion

1.3.1 Flow Variability and Water Quality

The patterns of wastewater daily influent flows to SJCE and JWPCP (collected every Tuesday for 7 weeks) are shown in Figure 1-6. There was a significant diurnal variability of wastewater inflow to the plants, and the peak and low flow differed in the two facilities. The lowest inflow to JWPCP occurred between 8 am – 12 pm each day and peaked in the late afternoon after 6 pm. The lowest inflow to SJCE occurred between 6 to 8 am each day and peaked at 10 am before becoming more stable. There were more day-to-day flow fluctuations at SJCE in comparison with JWPCP. This observed inflow pattern seems to reflect the difference between the regional (SJCE) and the centralized (JWPCP) WWTPs. The early dip of the inflow at the regional SJCE plant may reflect low water uses between 2 am – 4 am, assuming a wastewater travel time of 2 hours in the sewershed. The centralized JWPCP plant experiences a larger diurnal flow variation, and the timing of fluctuation was shifted by ~4 hours from the regional plant. The peak inflow after 6pm at JWPCP reflected the diversion of flows from other regional plants to the centralized facility, and thus the active daytime water uses in contribution sewersheds.

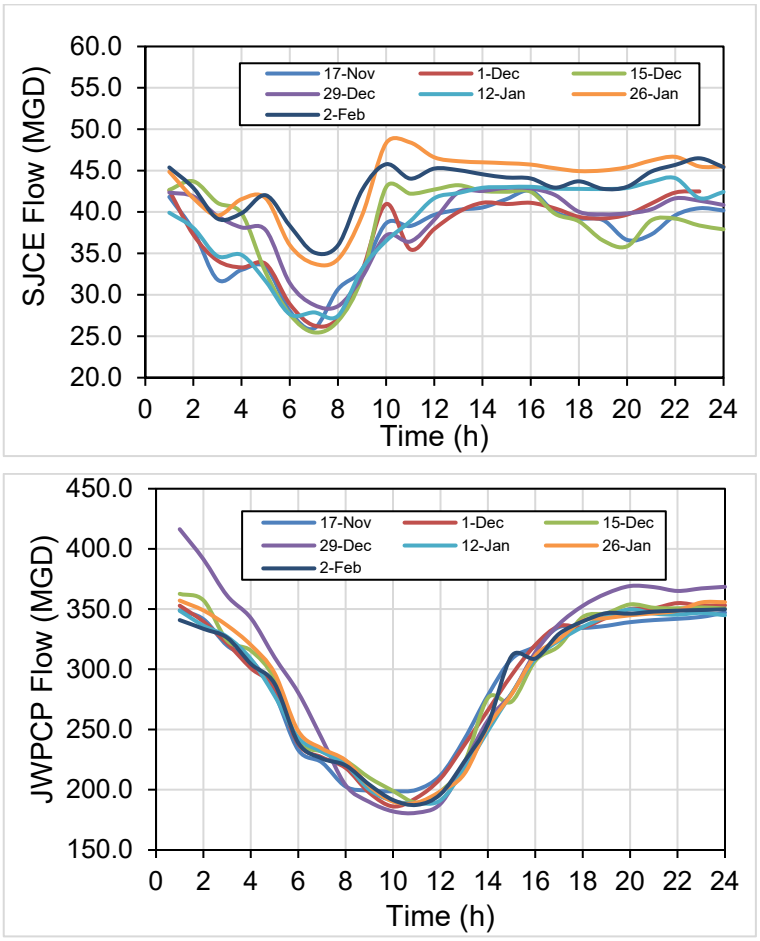


Figure 1-6. Daily Wastewater Influent Flow to SJCE and JWPCP during the Sampling Period.

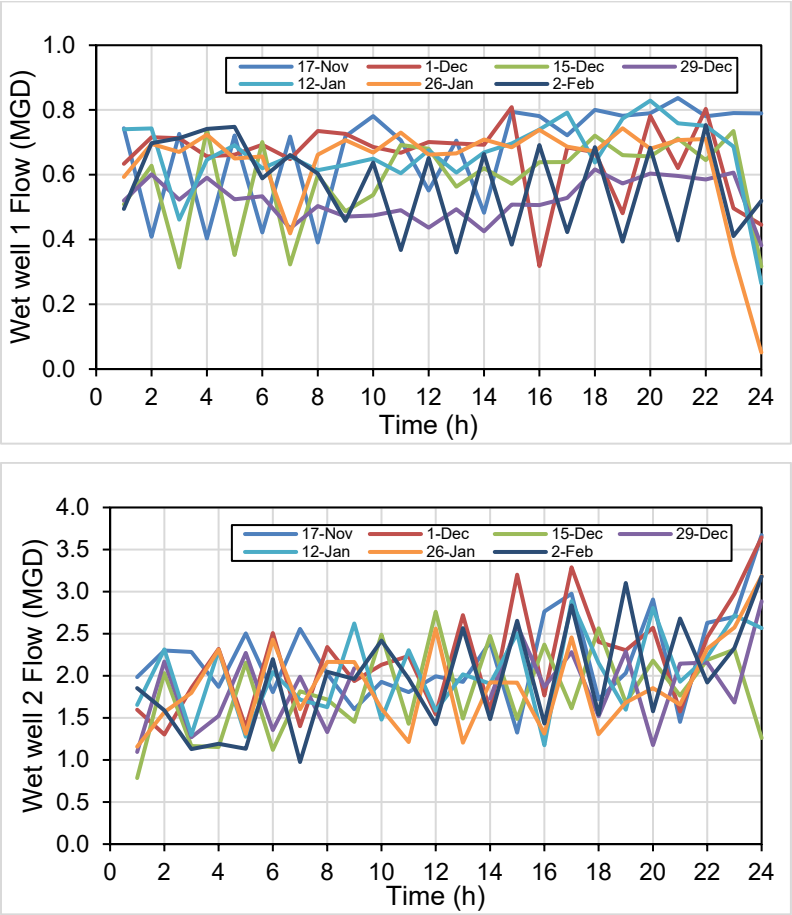
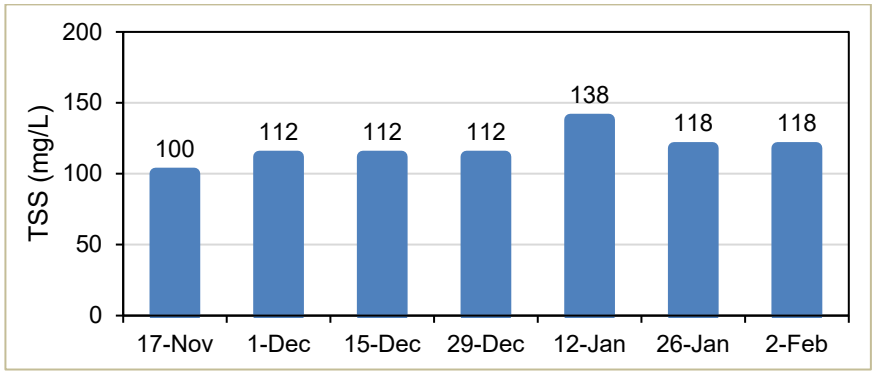
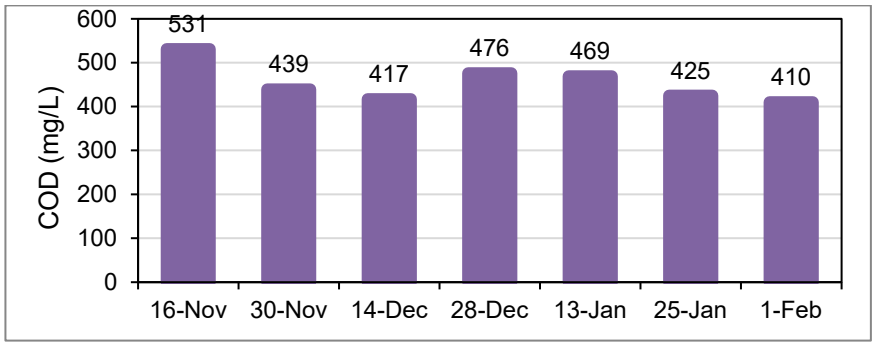


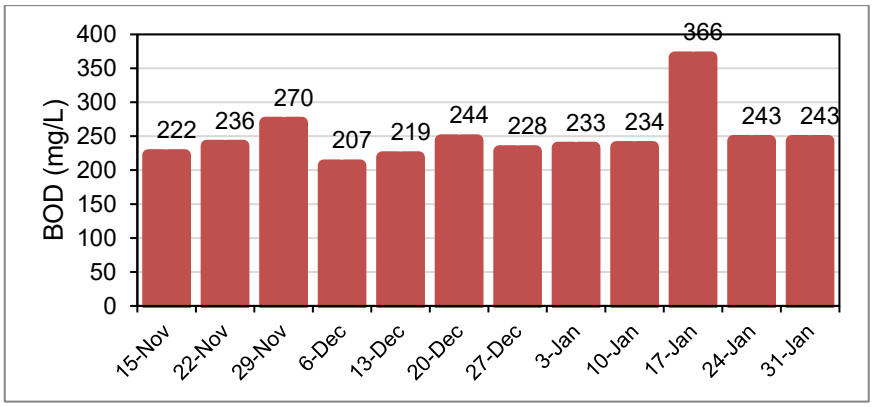
Figure 1-7. Daily Primary Sludge Flow to Wet Well 1 and Wet Well 2 at JWPCP during the Sampling Period.

The primary sludge flows to two wet wells at JWPCP are shown in Figure 1-7. Wet Well 2 was used as the main sludge settling well in the plant, which showed more than twice of the sludge flow that than that of well 1. The sludge flow was not measured at SJCE.

Figures 1-8 and 1-9 report water quality measurements of primary influent for SJCE and JWPCP, respectively. JWPCP's inflow had a significantly higher TSS (in the range of low 600 mg/L to mid-700 mg/L) in comparison with SJCE's inflow (in the range of 100 mg/L). This is because the wastewater collection and treatment infrastructure in the Greater Los Angeles Area (i.e., both LA City and County infrastructures) has the peculiar characteristic of being networks of centralized treatment plant (JWPCP) and upstream (SJCE) water reclamation facilities. The latter only treat the water for reclamation and discharge the settled solids in the collection system, to be treated by the centralized facility. Moreover, since the nitrification/denitrification is specifically required for water reclamation in the upstream facilities in this region, only a smaller fraction of nitrogen is embedded in the solids (nitrogen is predominantly in the ionic form) that are discharged downstream to the centralized facility. Accordingly, the COD and BOD of JWPCP's primary influent (Figure 1-9) were more than twice the concentrations in comparison with that of SJCE plant (Figure 1-8). However, the ammonia concentrations in the centralized plant (JWPCP) were not significantly higher than SJCE (Figure 1-8 and 1-9) because solids transferred from upstream plants were high in carbon but low in nitrogen.







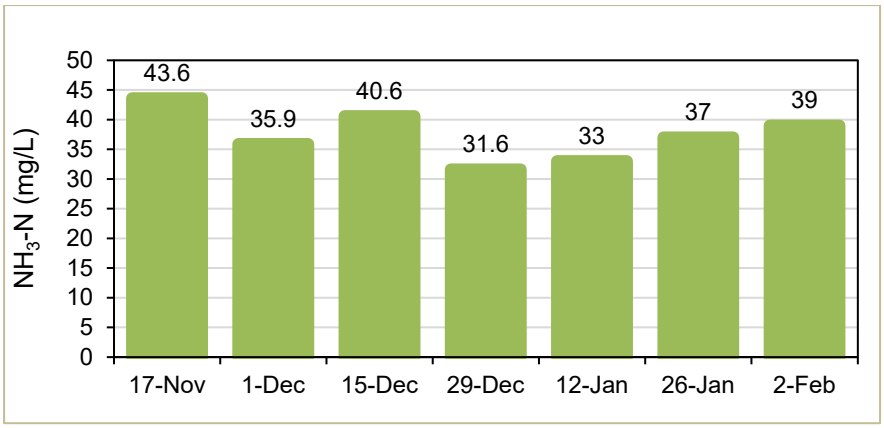


Figure 1-8. TSS, COD, BOD, and NH_3-N Concentrations in the Primary Influent Samples at SJCE.

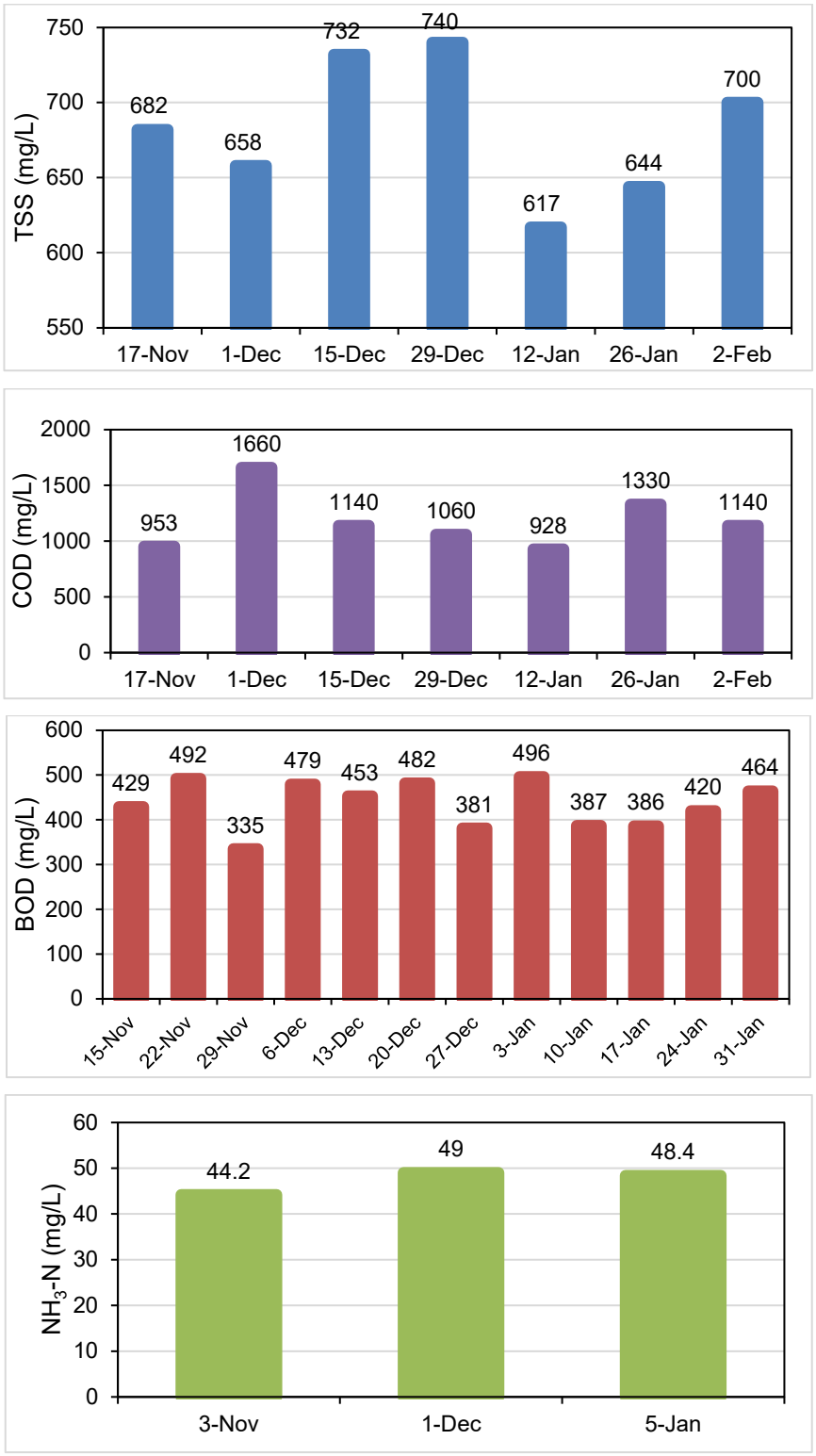


Figure 1-9. TSS, COD, BOD, and NH_3-N Concentrations in the Primary Influent Samples at JWPCP.

There is little information on the water quality of LCWRP. The three data points collected from the plant suggest the influent water quality in this plant was similar to that of SJCE, the regional plant (Figure 1-10). However, there were pulse elevations of COD, BOD, and ammonia on Dec. 10, 2020, reflecting the sporadic nature of the influent to the plant. Additional data points would be needed to better

understand the flow and water quality pattern of this WWTP. Secondary treatment effectively removed BOD to meet the discharge requirements (Figure 1-10).

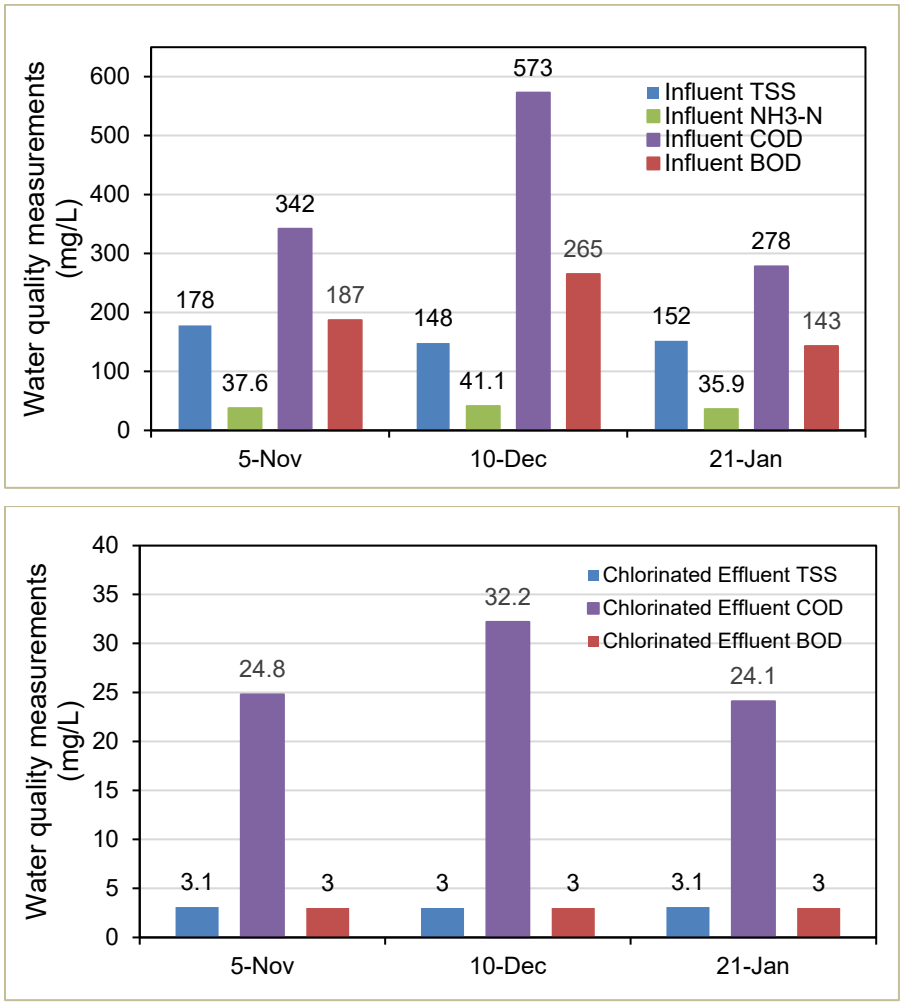


Figure 1-10. TSS, NH_3-N, COD, and BOD in the Wastewater Influent and the Chlorinated Effluent at LCWRP.
No NH_3-N measurements were made for the treated effluent.

1.3.2 Sludge TSS

The lipid outer layer of SARS-CoV-2 suggests the potential affinity of the virus to organic debris. Therefore, normalization of the viral concentration with the TSS concentration in the sludge may provide insight into the relationship between the viral concentration and the solids content of the sludge (Balboa et al., 2021). The TSS results from sludge samples are shown in Figure 1-11. The two Wet Wells in JWPCP was labeled as JWP_S1 and JWP_S2. The sub-digits designed sampling collection time, i.e., JWP_S1.1 denotes JWPCP Wet Well 1 first sludge sample. The higher TSS concentrations were observed in Wet Wells from JWPCP, reflecting the higher settleability of the solids in the wastewater influent. Elevated TSS concentrations were observed in the early December sludge samples at JWPCP but were stable around 25,000mg/L for most of the sampling period. The TSS concentrations at the SJCE plant sludge were below 25,000 mg/L for all sampling times. These values are slightly below the typical range of around 25,000-60,000 mg/L sludge TSS, according to Metcalf & Eddy (2014). This could be due to the measurement error from using a small volume of sludge sample stored for TSS analysis. These TSS results are used to normalize the SARS-CoV-2 virus concentrations by the solids content of the samples to understand the preference of virus to associate with liquid or solid fraction.

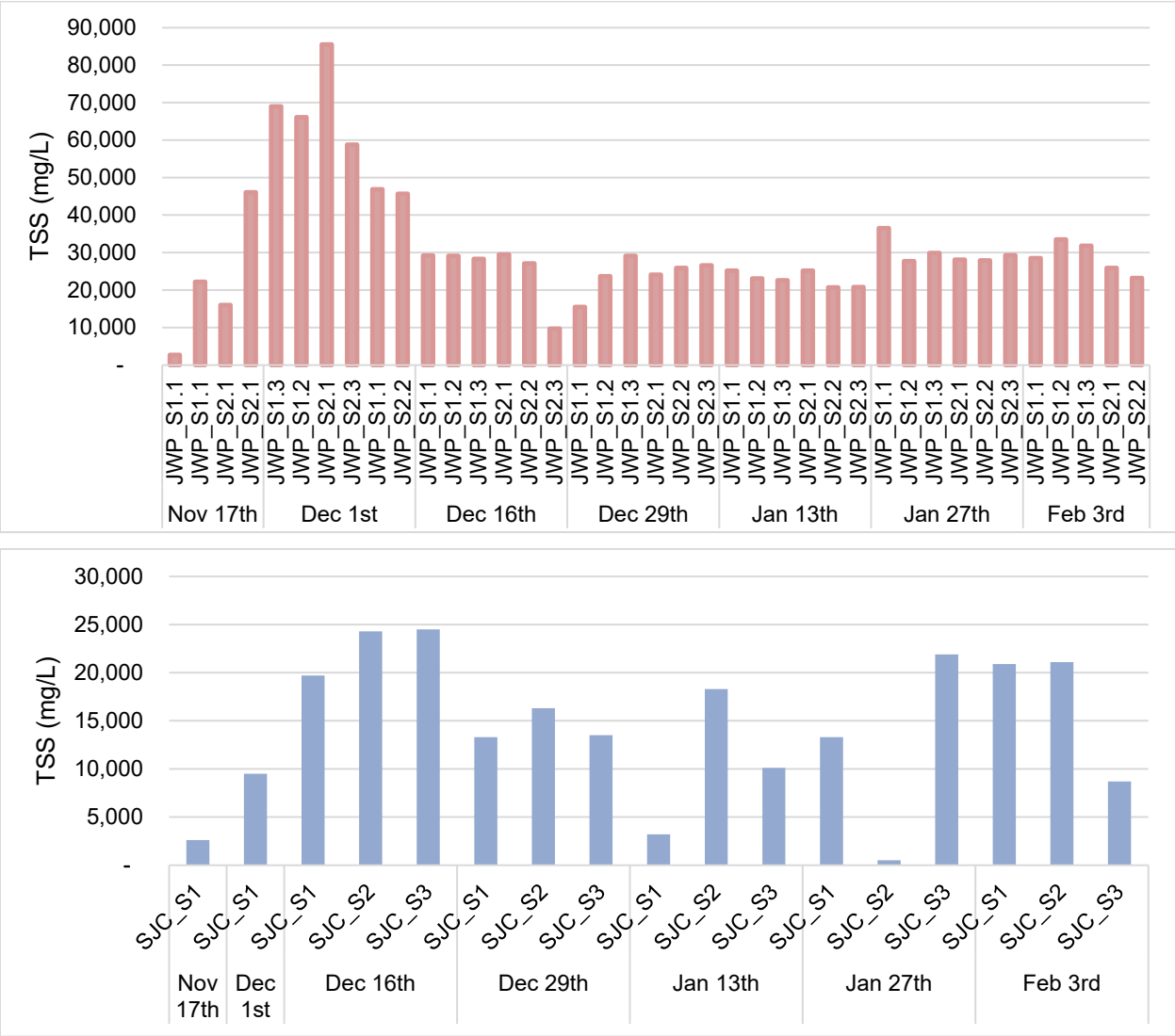


Figure 1-11. Sludge TSS Concentrations from Wells 1 and 2 of JWPCP and from the SJCE Samples.

1.3.3 SARS-CoV-2 in Primary Influent of Centralized WWTPs

The results of SARS-CoV-2 measurements in primary influent samples collected from the three WWTPs of the LACSD are presented both in tabulated table and graphic forms. The SARS-CoV-2 concentrations in SJCE varied from below detection in two samples collected on Jan. 12, 2021, to the highest of >4000 GC/mL in the December 2020 samples (Table 1-4). The variability of viral genome concentrations in grab samples taken every 4 hours within the 24-hour period (n=6/day) was also large (~8 folds difference in 25 to 75 percentile range), as seen in the wide spread of Box and Whisker plots in linear and logarithmic scales (Figure 1-12). A linear scale more distinctly shows the changes in virus concentrations detected over the study period. Nevertheless, similar trends were observed by both linear and logarithmic plots. Correlation analysis using the SARS-CoV-2 mean concentrations of six daily grab samples and SARS-CoV-2 in 24-hr flow-paced composite samples indicate positive correlations (Table 1-5) between the two data sets. The results suggest that composite samples could capture the overall trend of the viral concentration in wastewater, while the grab samples could capture the viral signal at some sampling times but miss the signal at other times. This episodic nature of SARS-CoV-2 signal over the 24-hour period maybe due to the pulse input of the municipal vs. industrial influent to WWTPs or the variability of wastewater inflow into the plants. The transport of the virus in the sewer system is likely in the plug

flow mode with minimal mixing along the trunkline, which resulting in the episodic signal at the influent of WWTPs. The relationships between viral signal and wastewater flow pattern and water quality parameters were further explored and are presented in Section 1.3.6. after presenting a comparison of the SARS-CoV-2 genomes detected in different sewersheds and using different sampling approaches.

Table 1-4. SARS-CoV-2 in Sewage Influent Collected by Grab Sampling and 24-Hour Composite from SJCE.

Sample ID	Sampling time/type	SARS-CoV-2 concentration (GC/mL)						
		11/17/20	12/01/20	12/15/20	12/29/20	1/12/21	1/26/21	2/2/21
SJC.3	3:00	277	564	631	1755	-*	1000	612
SJC.7	7:00	903	1114	389	984	499	1315	307
SJC.11	11:00	383	796	4151	4391	603	506	955
SJC.15	15:00	1385	347	4498	1070	1241	499	320
SJC.19	19:00	880	1035	1593	1340	554	498	315
SJC.23	23:00	576	1095	1372	2518	754	522	287
SJC.C24	Composite	278	2462	2674	1228	-	761	614

*Indicates no-call result in ddPCR analysis.

Table 1-5. Correlation Analysis of Daily Mean SARS-CoV-2 Genome Copies in Grab and in 24-Hour Composite Samples at SJCE.

Pearson		Spearman	
Corr.	p-value	Corr.	p-value
0.55428	0.25372	0.77143	0.0724

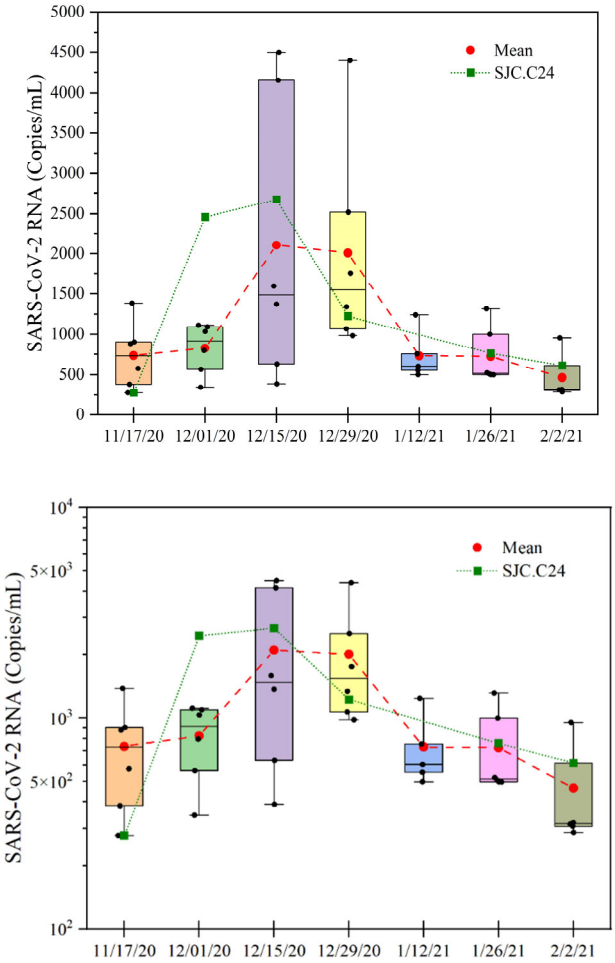


Figure 1-12. Comparison of SARS-CoV-2 Genome Copies Detected in Primary Influent by Grab and 24-Hour Composite Sampling at SJCE on Linear and Logarithmic Scales.

The Box and Whisker plots show SARS-CoV-2 genome copies in six grab samples taken every 4 hours within the 24-hour period. The red dots are the daily mean of SARS-CoV-2 genome copies of six grab samples. The green squares are SARS-CoV-2 genome copies in 24-hr composite samples.

Similarly, the concentrations of SARS-CoV-2 in the primary influent of JWPCP also varied over the study period (Table 1-6) but the range was less dramatic in comparison to SJCE. The JWPCP results are also plotted on both linear and logarithmic scales to see the trends in viral concentrations (Figure 1-13). The highest concentration was observed in late December 2020, which was consistent with the SJCE plant. The daily mean viral concentration of grab samples tracked well with those of the composite samples from the majority of the flow (autosampler C) to the plant and the “master composites” from all autosamplers to the plant. Statistically significant correlations were observed as seen in Table 1-7.

Table 1-6. SARS-CoV-2 Genome Concentration in Sewage Influent Collected by Grab and 24-Hour Composite Sampling from JWPCP.

Sample ID	Sampling time/type	SARS-CoV-2 concentration (GC/mL)						
		11/17/20	12/01/20	12/15/20	12/29/20	1/12/21	1/26/21	2/2/21
JWP.4	4:00	1373	937	1637	1761	1689	881	1242
JWP.8	8:00	745	1186	595	1992	1447	586	274
JWP.12	12:00	1556	913	1579	3365	1394	1036	831
JWP.16	16:00	2117	-	1623	2498	1402	278	849
JWP.20	20:00	1842	897	1412	1495	600	582	280
JWP.24	24:00	1327	978	1539	582	899	1770	273
JWP.C24	Composite 1*	1853	1026	1210	4132	1635	1529	893
JWP.IC24	Composite 2*	1606	1837	2062	3410	2020	310	269

*Composite 1 was made from the autosampler C to the plant corresponding to the trunklines, where grab samples were taken; Composite 2 (all autosamplers) included all of the inflow to the plant.

Table 1-7. Correlation Analysis of Daily Mean SARS-CoV-2 Genome Copies in Grab and in 24-Hour Composite Samples at JWPCP.

	Pearson		Spearman	
	Corr.	p-value	Corr.	p-value
JWP.C24	0.82773	0.02151	0.82143	0.02345
JWP.IC24	0.90057	0.00567	0.78571	0.03624

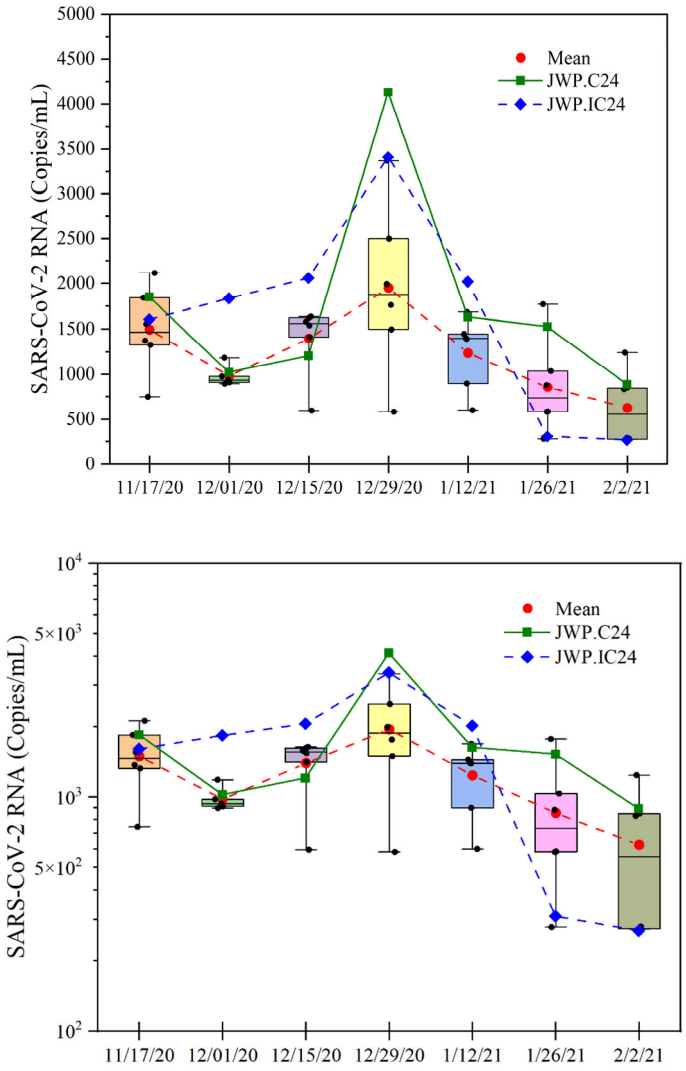


Figure 1-13. Comparison of SARS-CoV-2 Genome Copies Detected in Primary Influent by Grab and 24-Hour Composite Sampling at JWPCP on Linear and Logarithmic Scales.

The Box and Whisker plots show SARS-CoV-2 genome copies in six grab samples taken every 4 hours within the 24-hour period. The red dots are the daily mean of SARS-CoV-2 genome copies of six grab samples. The green squares are SARS-CoV-2 genome copies in 24-hour composite samples taken from the main sewer trunkline to the plant. The blue diamonds are SARS-CoV-2 genome copies in 24-hour composite samples taken from all trunklines to the plant.

The SARS-CoV-2 was not detected in the majority of the samples collected from the small community WWTP, LCWRP. Only one sample of post-grinding influent was above our LoQ (Table 1-8). The few sporadic fluorescent spots observed in the disinfected effluent and secondary effluent were likely the fluorescent background in the samples, which was below our 3 positive droplets per reaction threshold and below 300 GC/mL detection limit set in the assay methods. The result implies 1) there is a detection threshold of number of infected individuals among the total population served by a sewershed to result in a positive viral detection due to dilution effect; 2) grab sampling can miss the viral signal because it only captures a short plug of sewer flow to the treatment plant; 3) the result could also reflect the geographic spread of COVID-19 in the community indicating the Country Club community served by LCWRP has much lower infection rate in comparison with the large communities served by other LACSD

plants. Localized epidemiology records for the specific community and at the specific sampling time will help to confirm this result. It is currently beyond the scope of this study.

Table 1-8. SARS-CoV-2 Concentration in Grab Samples Collected from LCWRP.

Sample ID	SARS-CoV-2 concentration (GC/mL)				
	12/15/20	12/29/20	1/12/21	1/26/21	2/2/21
Influent Post-grinder	-	348	-	-	-
Secondary effluent	-	-	-	270*	230
Disinfected effluent	107	170	142	228	-

* The gray fonts indicate numbers below the LOQ but above LOD.

1.3.4 SARS-CoV-2 in Primary Sludge of Centralized WWTPs

Primary sludge samples had higher concentrations of SARS-CoV-2 in comparison with primary influent. The concentrations ranged between 6,000 and 91,853 GC/mL in grab samples collected from SJCE (Table 1-9 and Figure 1-14). A few samples were lost. These samples were previously frozen (transported to UCSB after collection) but thawed (during later shipment to UCI due to the delay of getting extraction kits at UCSC), suggested freezing and thawing cycles may have a significant impact on viral decay. Two additional samples collected on February 2, 2021, were also below the detection limit. These sludge samples appeared to have low solids content. To understand the viral signal in sludge samples, the SARS-CoV-2 genome concentration is also expressed as genome copies per milligram of TSS (Figure 1-15).

Table 1-9. SARS-CoV-2 Concentration in Primary Sludge Samples Collected by Grab Sampling Three Times per 24-Hour at SJCE.

Sample ID	Sampling time	SARS-CoV-2 concentration (GC/mL)						
		11/17/20	12/01/20*	12/15/20	12/29/20	1/12/21	1/26/21	2/2/21
SJC_S1	Afternoon	*	*	91853	50394	48694	6000	-
SJC_S2	Night	16203	*	15246	88056	18051	6819	16257
SJC_S3	Morning	*	13686	7515	66289	32316	6887	-

*Sample lost during transport.

Figure 1-14 illustrates the variability of SARS-CoV-2 concentration in grab sludge samples collected at different times of day from SJCE, on linear and logarithmic scales. The trends were similar for both plots. Three grab samples were taken at evenly spaced time intervals within the 24-hour period during each sampling day. A large concentration range was observed between afternoon sample and the morning sample collected on December 15, 2020 (>10 folds difference). Since variability in solids content of the sludge samples could affect the virus concentrations detected, normalizing the viral concentrations by solids content was carried out for each sample. The virus concentrations in the SJCE sludge normalized by TSS (genome copies/mg TSS) are shown in Figure 1-15, on linear and logarithmic scales, respectively. Normalization reduced the large concentration range for samples collected on December 15, 2020. The highest peak of SARS-CoV-2 shifted from 12/19 to 1/12, suggesting normalizing viral concentration by TSS could offer new insight to the viral distribution pattern in sludge.

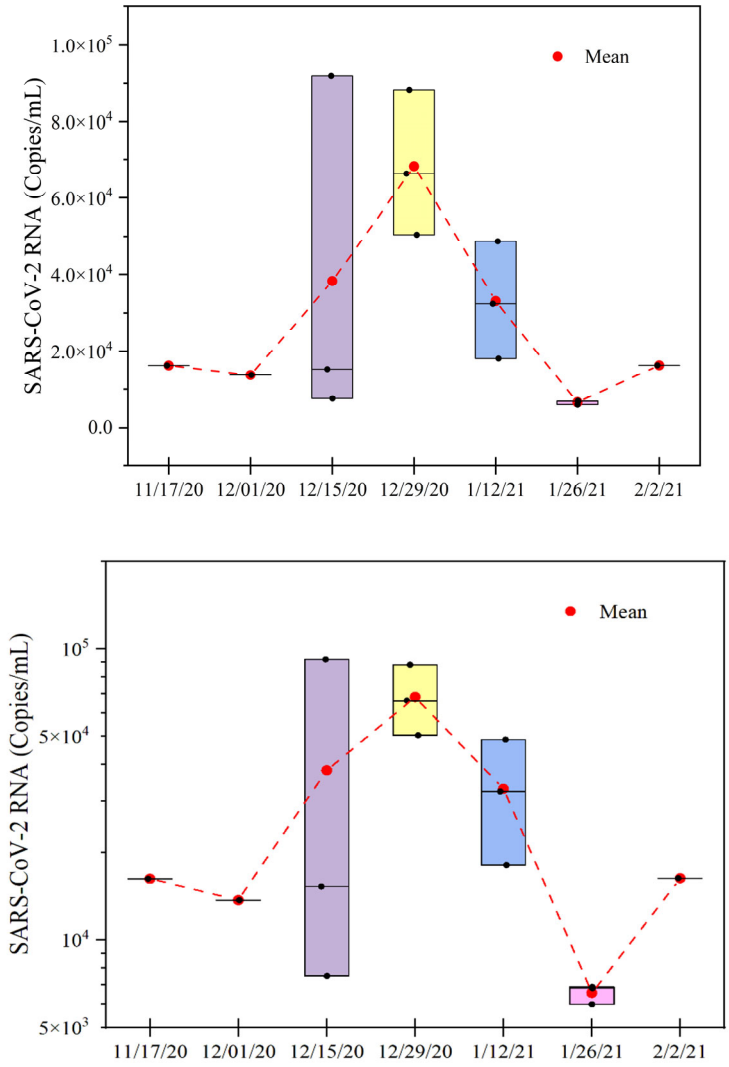


Figure 1-14. SARS-CoV-2 Concentration in Primary Sludge Samples Collected by Grab Sampling Three Times per 24-Hour at SJCE Plotted on Linear and Logarithmic Scales.

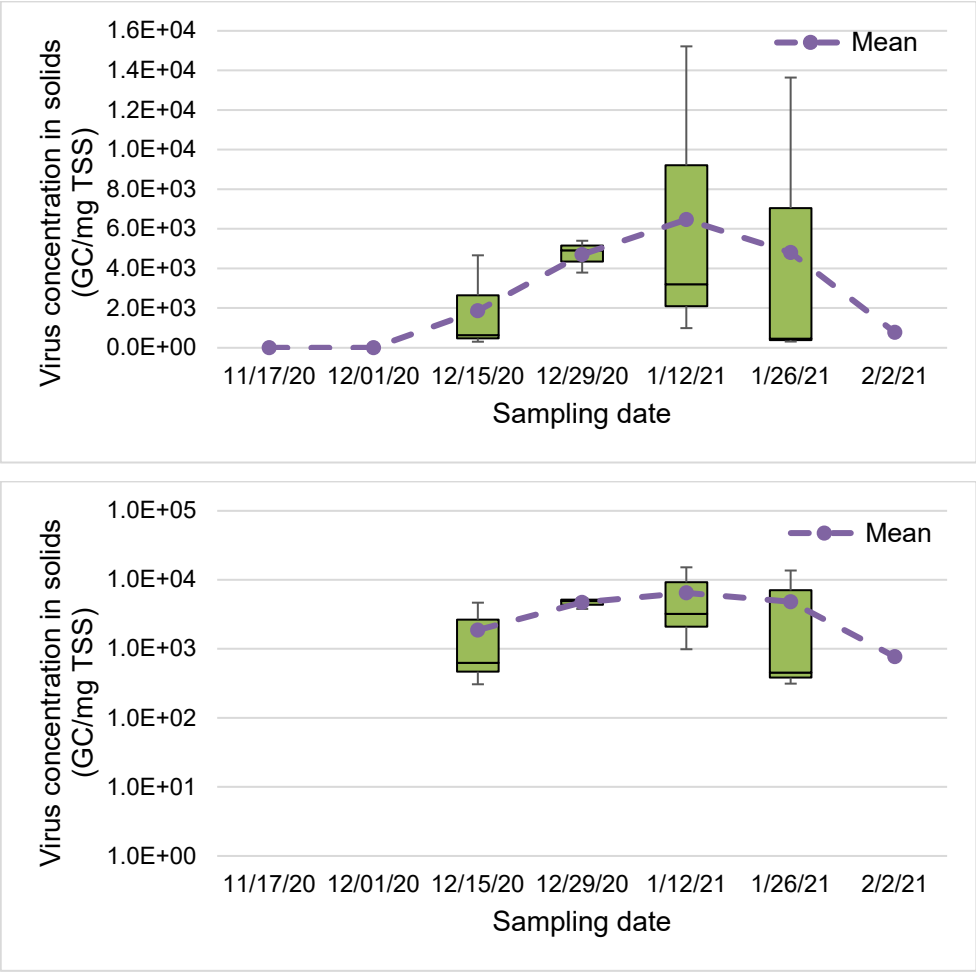


Figure 1-15. SARS-CoV-2 Concentration Normalized by TSS in Primary Sludge Samples Collected by Grab Sampling Three Times per 24-Hour at SJCE Plotted on Linear and Logarithmic Scales.

Similar observations were made with the primary sludge samples collected at JWPCP (Table 1-10). Three grab samples at evenly spaced time intervals were taken from two main wet wells at JWPCP over the 24-hour period at each sampling date (Figure 1-16). A paired T-test indicates no significant difference in the viral concentration among samples from two different wet wells ($p = 0.667$). Similar to the results from SJCE, the higher viral concentrations were also observed during late December 2020 and January 2021 (Figure 1-17). To account for the differences in varying solids content of our sludge samples, the virus concentration results for JWPCP normalized by TSS (genome copies/mg TSS) are also shown in Figures 1-18, on linear and logarithmic scales, respectively. For the linear Box and Whisker plots, the outlier concentrations were extreme due to low TSS and high virus concentrations.

Table 1-10. SARS-CoV-2 Concentrations in Grab Primary Sludge Samples Collected Three Times per 24-Hour from Two Wet Wells at JWPCP.

Sample ID*	Sampling Time/Type	SARS-CoV-2 concentration (GC/mL)						
		11/17/20	12/01/20	12/15/20	12/29/20	1/12/21	1/26/21	2/2/21
JWP_S1.1	Afternoon	_	9152	58968	44947	33596	15506	40904
JWP_S1.2	Night	8817	7652	90747	90466	18818	15495	28365
JWP_S1.3	Morning	_	11172	25939	14040	29089	14085	59839
JWP_S2.1	Afternoon	_	7968	14247	42677	17069	29093	27060
JWP_S2.2	Night	12448	19127	49388	96744	17705	11407	37507
JWP_S2.3	Morning	_	7629	59469	38387	63482	14087	12693

*S1 indicate sludge wet well 1 and S2 indicate sludge wet well 2.

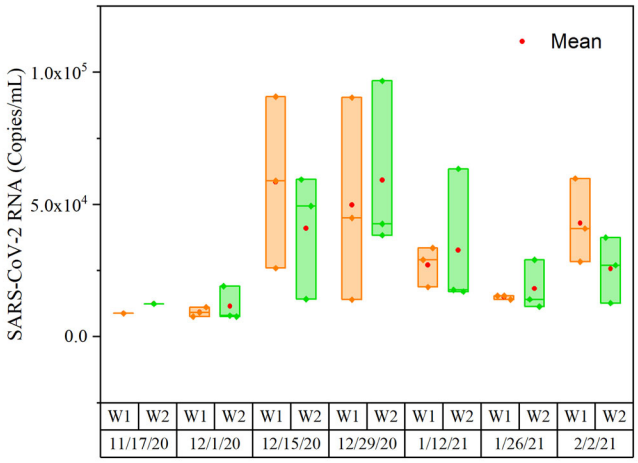


Figure 1-16. Comparison of SARS-CoV-2 Genome Copies in Daily Grab Primary Sludge Samples from JWPCP Wet Well 1 (W1, Orange) and Well 2 (W2, Green) Collected on the Same Sampling Date.
Three grab samples (roughly 8 hours apart) were taken from each wet well at each sampling date. Four samples collected on Nov. 17th, 2020, were lost during transport.

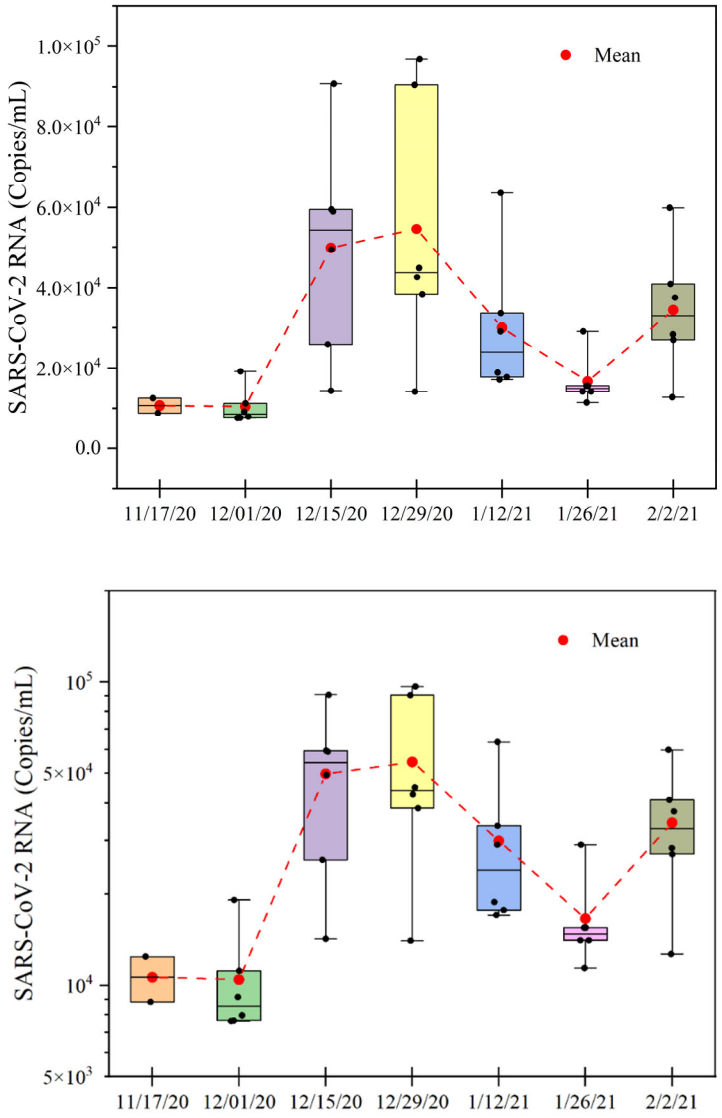


Figure 1-17. SARS-CoV-2 Genome Copies in Grab Primary Sludge Samples from Two Wet Wells Collected Three Times per 24-Hour at JWPCP Plotted on Linear and Logarithmic Scales.
The red dots are the mean of daily grab samples from both wells.

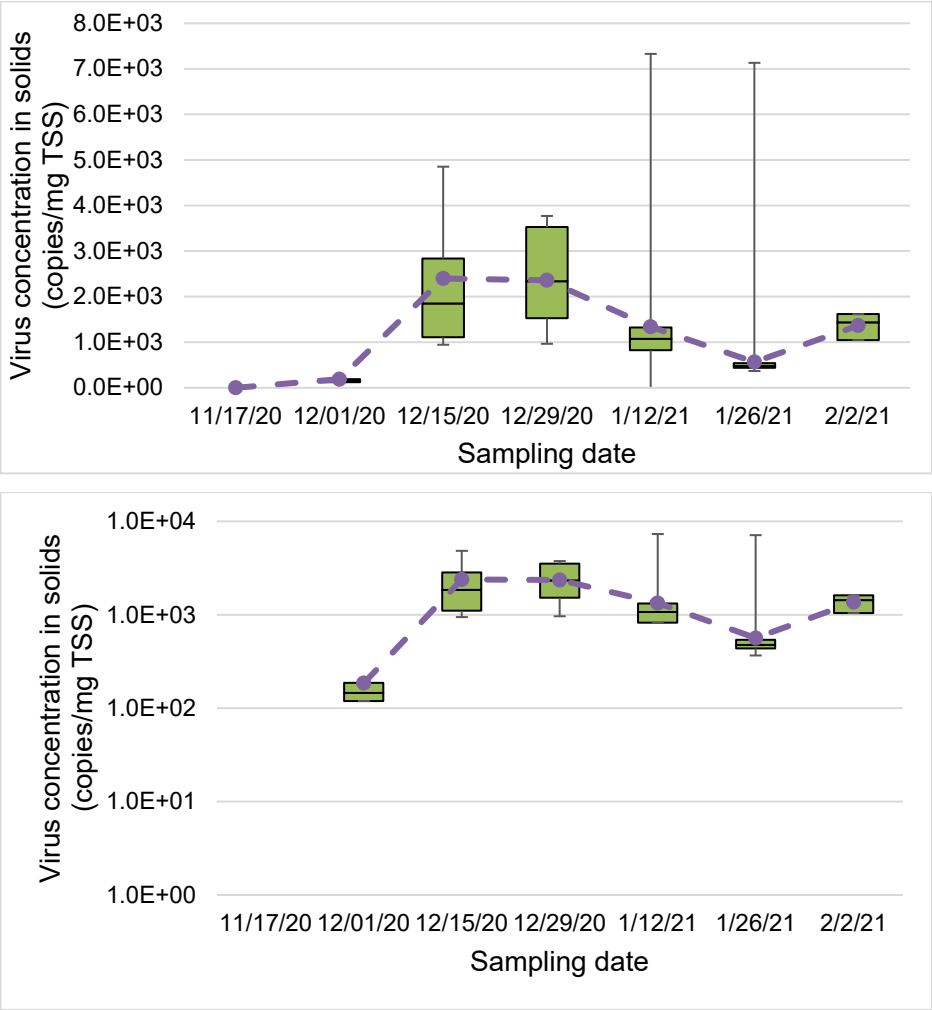


Figure 1-18. SARS-CoV-2 Concentration Normalized by TSS in Primary Sludge Samples Collected by Grab Sampling Three Times per 24-Hour at JWPCP, Plotted on Linear and Logarithmic Scales.

1.3.5 Relationship of SARS-CoV-2 in Wastewater and COVID-19 Case Reports

A similar trend of SARS-CoV-2 concentration in primary influent collected by 24-hr composite and the daily mean of primary sludge grab samples collected at SJCE is seen in Figure 1-19. Both the trends of SARS-CoV-2 in sewage and sludge matched well with that of 7-day average new COVID-19 cases reported by LA County Public Health during the study period (Figure 1-19). Correlation analysis showed daily mean grab sludge samples had the stronger correlation with COVID-19 cases than that with 24-hr composite sewage influent without considering the offset and lag times (Table 1-11). The 24-hr sewage composite seems to lead the reported cases and the mean of sludge samples (most obvious for 12/29). However, more frequent sampling is needed to better understand if sewage signal can be a leading indicator of reported cases. The mean sludge signal seems to match reported cases (as indicated by stronger correlation), which suggests daily viral load to the wastewater can be captured by grab sampling when samples are taken every six hours during the day. However, the high variability of SARS-CoV-2 concentration observed throughout the 24-hr period also indicates that reducing the frequency of sampling per day may miss the strong signal of viral load.

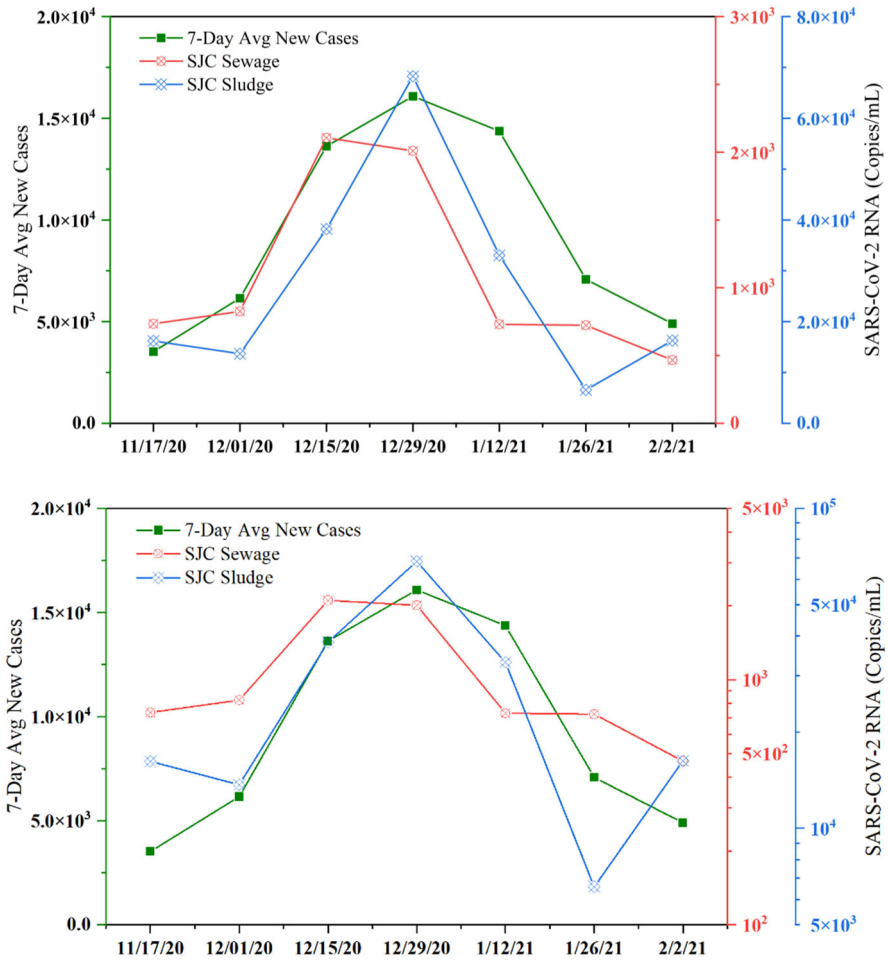


Figure 1-19. SARS-CoV-2 Genome Copies in 24-hr Composite Sewage Samples and Daily Mean Primary Sludge Grab Samples in SJCE Are Overlaid with 7-Day COVID-19 New Cases in LA County.
Sewage and Sludge Virus Concentrations are Plotted on Linear and Logarithmic Scales.

Table 1-11. Correlation Analysis of SARS-CoV-2 Genome Copies in Sewage and Sludge Samples from SJCE and 7-Day Average COVID-19 New Cases.

Sample ID	Pearson		Spearman	
	Corr.	p-value	Corr.	p-value
Daily mean grab sewage samples	0.73502	0.05982	0.42857	0.33737
24-hr sewage composite samples	0.47349	0.34284	0.71429	0.11079
Daily mean grab sludge samples	0.85227	0.01485	0.64286	0.11939

Similar to the observations at SJCE, correlation analysis also indicated a significant relationship between viral concentration in 24-hour sewage composite samples, daily mean grab sludge samples from JWPCP and 7-day average COVID-19 new cases in LA County (Table 1-12). This correlation is graphically shown in Figures 1-20, with linear and logarithmic scales. A different from the SJCE results is that sewage composite samples do not seem to lead the case reports, and sludge and sewage composite samples have similar correlations with the case reports. Since JWPCP is a central plant and receives discharged of solids from upstream plants, the average sludge age in the plant is older than the upstream facilities.

However, the complexity of connected sewer network challenges the determination of the age of sludge and even the age of wastewater inflow.

Table 1-12. Correlation Analysis of SARS-CoV-2 Genome Copies Detected in JWPCP and 7-Day Average New COVID-19 Cases in LA County.

Sample ID	Pearson		Spearman	
	Corr.	p-value	Corr.	p-value
Daily mean grab sewage influent samples	0.61504	0.14158	0.39286	0.38332
Main flow 24-hr sewage composite	0.56484	0.18644	0.42857	0.33737
Plant 24-hr sewage composite	0.72384	0.0659	0.78571	0.03624
Daily mean grab primary sludge samples	0.78603	0.03611	0.64286	0.11939

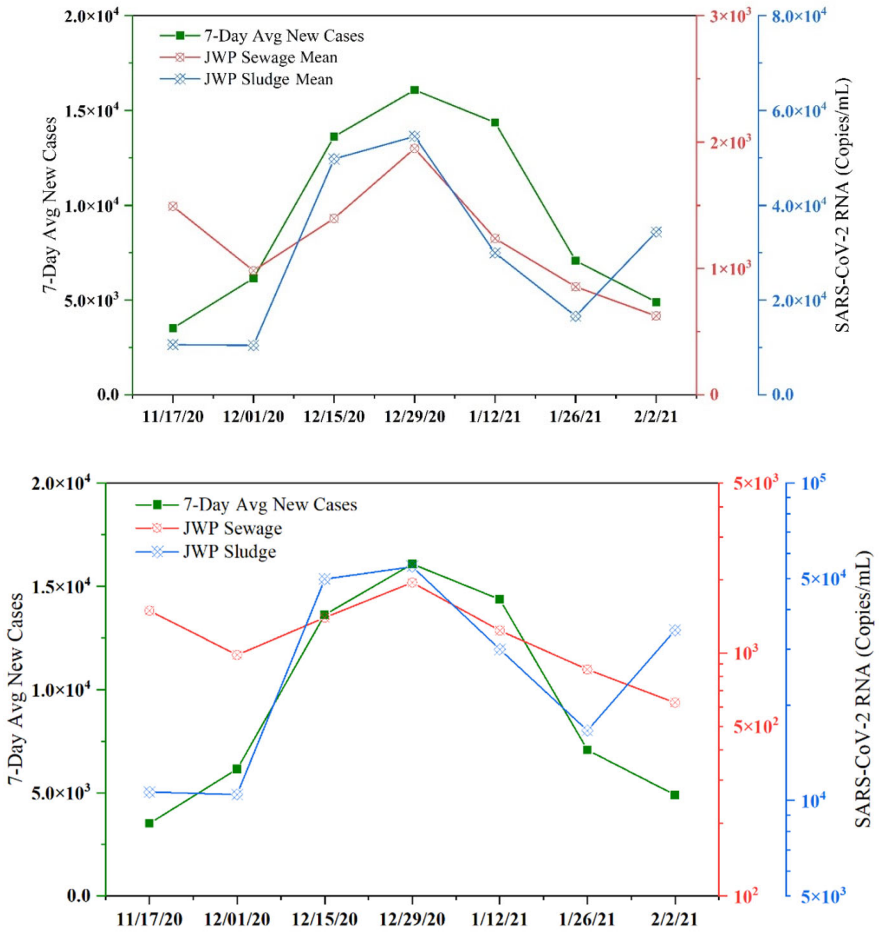


Figure 1-20. SARS-CoV-2 Genome Copies in 24-Hour Composite Sewage Samples and Daily Mean Primary Sludge Grab Samples at JWPCP Are Overlaid with 7-Day COVID-19 New Cases in LA County.
Sewage and sludge virus concentrations are plotted on linear and logarithmic scales.

1.3.6 Relationship of SARS-CoV-2 and Water Quality Parameters in Centralized Wastewater Treatment Systems

Figure 1-21 shows SARS-CoV-2 concentrations in daily grab samples plotted together with daily sewage inflow rate at SJC. These variables are plotted on a linear scale to view subtle changes and correlations of the viral concentrations in grab samples over the day. Correlation analysis indicated the average viral concentration collected from grab samples at a specific time of day had a positive but not statistically significant relationship (Table 1-13). The correlation on individual days was unclear (Table 1-13). It is important to mention that the correlation analysis performed here did not account for the residence time in the plant. According to the plant operator that the theoretical contact time in primary treatment is a little over 2.5 hours at 40 MGD. Further analysis could explore the correlation between flow and viral concentration with the incorporation of estimated primary treatment contact time.

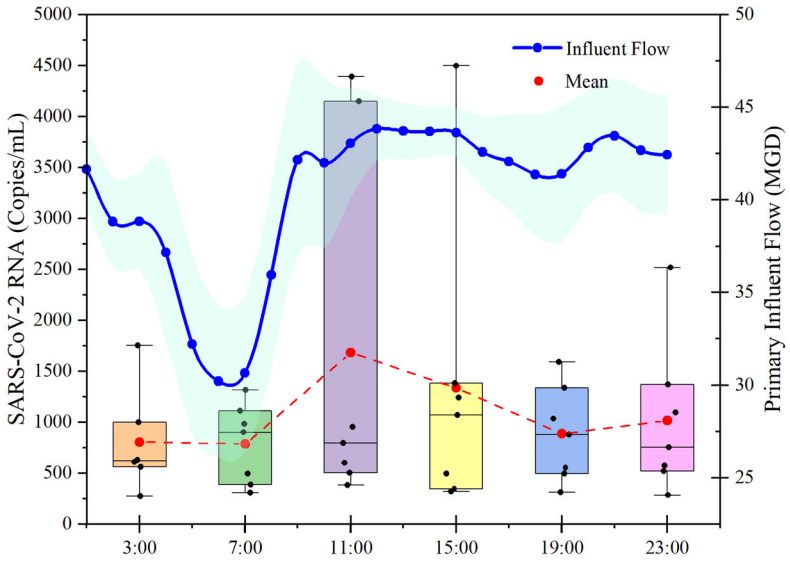


Figure 1-21. SARS-CoV-2 in Daily Grab Samples Plotted Together with Daily Sewage Inflow Rate at SJC.
The blue line is the average value of flow rate at time of the day, the shaded region around the blue line is the range of variability.

Table 1-13. Correlation Analysis of SARS-CoV-2 in Daily Grab Samples and Daily Sewage Inflow Rate at SJC.
Both the average viral concentration at ana specific time of the day and individual viral concentration collected on specific day were used in the correlation analysis.

	Pearson		Spearman	
	Corr.	p-value	Corr.	p-value
Avg.	0.62533	0.18427	0.82857	0.04156
11/17/20	0.07597	0.88626	0.25714	0.62279
12/01/20	-0.39759	0.43504	-0.48571	0.32872
12/15/20	0.70938	0.11442	0.82857	0.04156
12/29/20	0.41322	0.41545	0.31429	0.54409
1/12/21	0.56216	0.32397	na	na
1/26/21	-0.96471	0.00185	-0.6	0.208
2/2/21	0.18167	0.73049	0.02857	0.95715

The similar plot for JWPCP also shows the lowest daily sewage flow occurs in the late morning each day (Figure 1-22). The day-to-day flow rate in JWPCP was less variable as indicated by the narrow range (light blue band) of the flow curve. Again, no clear relationship between flow rate and viral concentration was observed at JWPCP (Table 1-14).

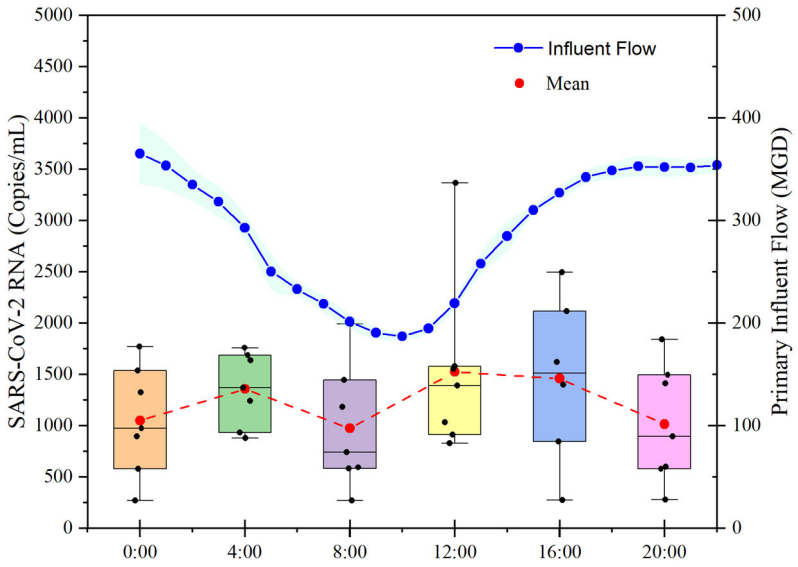


Figure 1-22. SARS-CoV-2 in Daily Grab Samples Plotted Together with Daily Sewage Inflow Rate at JWPCP.
The Blue Line Is the Average Value of Flow Rate at Time of The Day, and the Shaded Region Around the Blue Line Is the Range of Variability.

Table 1-14. Correlation Analysis of SARS-CoV-2 in Daily Grab Samples and Average Daily Sewage Inflow Rate at JWPCP.

Both the average viral concentration at the specific time of the day and individual viral concentration collected on a specific day were used in the correlation analysis.

JWPCP	Pearson		Spearman	
	Corr.	p-value	Corr.	p-value
Avg.	-0.15573	0.7683	-0.02857	0.95715
11/17/20	0.61161	0.19698	0.31429	0.54409
12/01/20	-0.56315	0.32292	-0.3	0.62384
12/15/20	0.59386	0.21393	0.08571	0.87174
12/29/20	-0.63094	0.17917	-0.71429	0.11079
1/12/21	-0.62608	0.18358	-0.65714	0.15617
1/26/21	0.21939	0.67619	0.08571	0.87174
2/2/21	-0.13879	0.79315	-0.25714	0.62279

To explore the relationship between water quality parameters of sewage influent and SARS-CoV-2 concentrations, the BOD, COD, TSS, and NH_3-N concentrations provided by SJCE and JWPCP were analyzed together with mean viral data of grab samples and composite samples on each sampling date. Since the viral sampling schedule was not synchronized with water quality measurements, the water quality data collected within 48 hours of viral sample collection time were used in the analysis. Correlation analysis indicates there is no clear relationship between the viral concentration and any of the water quality parameters included in the analysis (Tables 1-15 and 1-16). The water quality parameters are stable over time, while viral concentrations in wastewater were highly variable. This result suggests routine measured water quality parameters cannot be used to indicate SARS-CoV-2 concentration in the wastewater. Correlation analysis cannot be performed for samples collected from the LCWRP since only one positive sample was found.

Table 1-15. Correlation Analysis of SARS-CoV-2 Concentrations with Water Quality Parameters at SJCE.

Pearson correlation coefficient and p-values								
	TSS		Ammonia		BOD		COD	
	Corr.	p-value	Corr.	p -value	Corr.	p -value	Corr.	p -value
Mean Grab	-0.2869	0.5327	-0.1529	0.7435	-0.4831	0.2722	-0.0577	0.9022
24-hr Composite	-0.3438	0.4502	0.0528	0.9105	0.2406	0.6033	-0.4611	0.2977
Spearman correlation coefficient and p-values								
	TSS		Ammonia		BOD		COD	
	Corr.	p-value	Corr.	p -value	Corr.	p -value	Corr.	p -value
Mean Grab	-0.6736	0.0971	0.0000	1.0000	-0.5045	0.2482	0.2143	0.6445
24-hr Composite	-0.3930	0.3832	0.0000	1.0000	-0.0541	0.9084	-0.3571	0.4316

Table 1-16. Correlation Analysis of SARS-CoV-2 Concentrations with Water Quality Parameters at JWPCP.

Pearson correlation coefficient and p-values							
	TSS		NH_3-N	BOD		COD	
	Corr.	p -value	n/a	Corr.	p -value	Corr.	p -value
Mean Grab	0.4756	0.2807	n/a	-0.3173	0.4880	-0.3919	0.3845
24-hr Composite Influent ECG ¾	0.4284	0.3375	n/a	-0.2742	0.5518	-0.3607	0.4267
24-hr Composite Influent Raw Combined	0.4033	0.3697	n/a	-0.4897	0.2647	-0.2214	0.6332
Spearman correlation coefficient and p-values							
	TSS		NH_3-N	BOD		COD	
	Corr.	p -value	n/a	Corr.	p -value	Corr.	p -value
Mean Grab	0.4643	0.2939	n/a	-0.2857	0.5345	-0.5406	0.2103
24-hr Composite Influent ECG 3/4	0.1071	0.8192	n/a	-0.3571	0.4316	-0.6487	0.1150
24-hr Composite Influent Raw Combined	0.3929	0.3833	n/a	-0.4643	0.2939	-0.3063	0.5040

1.4 Conclusions

This study quantified the SARS-CoV-2 genome copy concentrations in the primary clarified influent and primary sludge samples from WWTPs representing different types of centralized sewersheds, including different size (service area), wastewater composition and wastewater travel time within the sewer networks. The results of the study have extended our knowledge in wastewater SARS-CoV-2 testing. The outcomes of the study offer insights into the future design of wastewater-based surveillance of SARS-CoV-2. The continuous measurements of SARS-CoV-2 in wastewater can provide long-term data on the dynamics of the virus in the community. These surveillance data in correlation with the sewershed characteristics showed a proof-of-concept of WBS in understanding the spread of disease. Together with clinical information, such as individual fecal shedding rate and duration of SARS-CoV-2 shedding among COVID-19 patients, these data can be used to assess key variables that can affect per-capita estimation of disease prevalence. Moreover, the wastewater monitoring results at the regional plant may have the potential to serve as a warning signal, should the resurgence of COVID-19 occur in the future years.

The following conclusions can be made from the results of this investigation:

- Significant variability of SARS-CoV-2 concentrations was found in sewer influent throughout a 24-hour period. The sewershed characteristics have significant impact on diurnal wastewater inflow. However, the complexity of sewer network (i.e., travel time in the sewer) together with solids and flow transferring practices used between treatment facilities investigated in this project further challenged the calculation of viral load in the service area. The situation could be resolved in a WWTP with a well-defined sewer collection and treatment system. A sewer network model (i.e., EPA SWMM) could be a useful tool to relate the SARS-CoV-2 concentration detected in WWTPs and viral loads from the community.
- Sewage composite samples appear to be more representative of daily SARS-CoV-2 concentration in wastewater at centralized WWTPs. For future studies, composite samples should be prioritized if resource limitation prevents the collection and analysis of multiple time-spaced samples throughout the 24-hour period.

- The small community plant, represented by LCWRP in this study, may be used to identify localized cases in the community. Installation of autosamplers at the plant can improve the coverage of the viral detection. Additional sampling efforts for an extensive period are needed to improve the confidence of the study outcomes. Community based epidemiological data also would further strengthen this conclusion, but the epidemiological data gathering is current beyond the scope of this study.
- The primary sludge has nearly one order of magnitude higher concentration of quantifiable SARS-CoV-2 than primary wastewater influent. Therefore, sludge samples may offer higher sensitivity for SARS-CoV-2 tracking in centralized wastewater facilities. The SARS-CoV-2 concentrations in primary influent and primary sludge were well correlated and both reflected the general trend of COVID-19 cases in the large community.
- Wastewater based surveillance at centralized facilities provides valuable insight into the epidemiological state of the community and can be used as a supplement to on-going clinical based epidemiological investigation.

CHAPTER 2

SARS-CoV-2 in a Decentralized Wastewater Treatment System

2.1 Introduction

Despite the success in detecting and quantifying SARS-CoV-2 in centralized WWTPs, a large portion of the country (> 20%, based on EPA estimates), and much of the developing world rely on small decentralized wastewater treatment systems to manage their sanitary needs (Massoud et al., 2009; U.S. EPA, 2002). Septic systems differ from conventional collection systems and WWTPs due to a longer-term waste storage times and stratified solid and liquid layers (Lusk et al., 2017; U.S. EPA, 2011; Withers et al., 2013). So far, little is known about the potential of using decentralized wastewater collection and treatment systems for wastewater-based surveillance of SARS-CoV-2.

Decentralized wastewater treatment systems or on-site wastewater treatment systems such as septic tanks are widely used in urban or suburban areas (U.S. EPA, 2002; Weiss et al. 2008; Diaz-Valbuena et al. 2011). The surveillance of septic wastewater particularly in communities served exclusively by septic systems might provide critically important information regarding the epidemiology of diseases such as COVID-19. However, monitoring of SARS-CoV-2 RNA and its temporal variance in septic tank systems has generally been overlooked in the literature. Further, in many rural areas of developing countries, wastewater may be held in tanks that function similarly to septic tanks yet are simply holding prior to transfer for further treatment. Studying septic tanks for SARS-CoV-2 is therefore relevant to how one might sample and understand SARS-CoV-2 in human waste holding tanks. In this chapter, we report investigations of septic tank systems of two public restrooms located in Zuma Beach, Malibu, CA for detection of the SARS-CoV-2 RNA concentrations. Specifically, we address: i) What is the feasibility of sampling from a community septic system to monitor SARS-CoV-2 in areas not served by centralized wastewater collection and treatment systems? and; ii) How and where should samples be collected from these systems? The results contribute to the understanding of the distribution of the SARS-CoV-2 RNA in the supernatant and sludge of septic tanks, the temporal variance, and the decay of the virus.

As part of the scope to determine how to sample on-site waste treatment systems—particularly in reference to translating the work to developing countries that commonly hold communal waste before pumping and hauling for treatment and disposal (Massoud et al., 2009)—this project also identified an information gap regarding sampling location in the septic tank-hauling-disposal pipeline. Specifically, we asked: i) What is the relationship of SARS-CoV-2 concentrations in the septic tanks to those after pumping from holding (septic, in this case) tanks, i.e. just prior to transport for further disposal? and ii) What is the persistence of the virus during the pump and haul process such that end-point sampling of hauled waste provides for a representative understanding of SARS-CoV-2 at the holding point? To address these questions, a sub-study was designed and performed to understand the relationship between SARS-CoV-2 concentrations in the septic system at Zuma Beach, the haul truck, and the discharge at the waste disposal site (JWPCP, Carson).

2.2 Materials and Methods

2.2.1 Study Site - Zuma Beach Restrooms

Zuma Beach is a 1.8-mile-long popular recreational beach located in Malibu, CA. Monthly visitation to the Beach in 2019 ranged from 20,000 (February) to 1,000,000 (July) people. There are 9 restroom facilities at Zuma Beach (Figure 2-1), and the monthly restroom users in 2019 ranged from 2,000 (February) to 100,000 (July) people, hence approximately 10% of visitors use the restroom during their Zuma Beach visit. Two restrooms were sampled at Zuma Beach during this study, Restroom #1 and Restroom #9. Restroom #1 includes 6 showers, 9 toilets, 8 sinks, and 3 urinals. The volume of the primary tank and the recirculation tank are 15,000 and 10,000 gallons, respectively. Post-treatment of the septage includes filter pods, chlorination and dechlorination, UV, and a 1600 square foot leach field. The total depth of the supernatant and sludge in the primary tank was between 2 ft and 4 feet at the time of sampling; the sludge depth ranged from 4 to 9 inches. This was a relatively small depth of sludge owing to reduced restroom use in 2020. In contrast, the total depth (~9.5 feet) and the sludge depth (~2 feet) were much deeper in restroom #9. Restroom #9 has a slightly different design than restroom #1 (newer installation), but still contains 6 showers, 9 toilets, 8 sinks, and 3 urinals. The average flow rate from the primary septic tank to the recirculation tank in restroom #1 could not be accurately determined due to the system malfunction; the average flow rate to the leach field in restroom #9 was 548 gallons per day during the period January-December 2020.

Figure 2-1. The Location Map of Zuma Beach Restrooms #1 and #9.
Source: Map data from Google Earth.

2.2.2 Sample Collection at Zuma Beach Restrooms

The supernatant and/or sludge samples were collected from Zuma Beach restroom #1 eight (8) times and from restroom #9 twelve (12) times (every other week) from December 2020 to May 2021 (Table 2-1). This sampling design focused on understanding the temporal and spatial variations of SARS-CoV-2 concentrations in the system. Sludge samples were collected from the bottom of the primary tank at the port close to the middle of the tank using a Sludge Judge® (Nasco Co.), a sludge measuring tool, comprised of a 10-foot long pole with bailer on one end, and with depth markings). Supernatant samples were collected from the mid-point of depth (i.e., liquid between the scum layer and the sludge) at the same location using the same sludge tool. The total depth and approximate sludge depth were recorded during each sampling event. Additionally, on March 9th and 22nd, 2021, supernatant from the top and middle depths, and sludge samples from the top and bottom of the sludge layer were also collected. These samples were collected to enable understanding of the stratification of the virus across

the depth profiles of the supernatant and the sludge, separately. The system at restroom #9 was pumped on March 22nd, 2021. Before the last two sampling events, restroom #9 was closed due to a system malfunction. Thus, on April 20th, 2021, only supernatant samples from the middle and bottom depths were collected, given that the sludge depth was too shallow to sample. The men's and women's counts of usage at restroom #9 were recorded using OmniCounter-ProA (Traf-Sys Co.) a people counting system from March 22nd and Jan 26th, 2021, respectively.

2.2.3 Sample Collection during Pumping and Hauling

A pumping and hauling study was initiated at restroom #9 on March 22nd, 2021 (Figure 2-2). Four types of samples were collected at Zuma Beach restroom #9, including sludge and supernatant prior to pumping, mixed samples (mixture of supernatant and sludge) from the pumping truck immediately after pumping of the primary tank, and mixed samples (mixture of supernatant and sludge) from the truck prior to discharge at JWPCP (Figure 2-3). Decay of the virus during hauling was evaluated by comparing SARS-CoV-2 concentrations in mixed samples (collected from the truck just after pumping) to those in samples collected just prior to discharge, as well as using positive controls (sludge samples spiked with heat-inactivated SARS-CoV-2) that were prepared using aliquots of septage collected from the truck immediately after pumping.

Figure 2-2. Pumping at Zuma Beach Restroom #9 on March 22, 2021.

Figure 2-3. Discharge of Septage from Zuma Beach Restroom #9 at JWPCP on March 22, 2021.

In total, 12 sludge and supernatant samples were collected before pumping, including 3 supernatant samples each from the top and middle depths, and 3 sludge samples each from the top and bottom of the sludge layer. After pumping, 6 additional samples were collected directly from the truck, including 3 mixed samples just after pumping at Zuma Beach and 3 just prior to discharge at JWPCP. The mixed samples in the truck prior to discharge were collected from the access hatches on the top of the truck: one from the rear, one from the middle, and one from the front of the truck. Additionally, heat-inactivated SARS-CoV-2 was spiked into 6 replicate septage subsamples prepared using one of the truck samples collected immediately after pumping. Three spiked samples were retained on wet ice and the other 3 spiked samples were kept at ambient temperature (~70 °F in the dark) during the period between septage pumping and discharge at JWPCP.

2.2.4 Sample Processing and Analyses

The supernatant and sludge samples collected from Zuma Beach restrooms were transported on ice to UCSB within 3 hours of sampling. Some of the pumping and hauling samples were transported on ice to UCSB within 6 hours of sampling. Total suspended solids (TSS) measurements were carried out for all supernatant and sludge samples based on ASTM standard methods. Meanwhile, approx. 2-3 mL supernatant and sludge samples were mixed with 3 volumes of DNA/RNA Shield (Zymo Co.) and stored at -80 °C until RNA extraction. The RNeasy PowerSoil Total RNA Kit (QIAGEN) was used for the RNA extraction of supernatant and sludge samples, and the wet weights of supernatant and sludge samples used for extraction were recorded. The extracted RNA was stored at -80 °C until analysis. The Bio-Rad SARS-CoV-2 ddPCR Kit including the One-Step RT-ddPCR Advanced Kit for Probes Supermix and 2019-nCoV CDC ddPCR Triplex Probe Assay was used for the detection of SARS-CoV-2 virus. The detection was performed using droplet digital PCR (ddPCR) on a Bio-Rad QX200 dd PCR system (Hercules, CA). The reaction mixture was made for droplet generation using the Droplet Generator with droplet generation oil. Generated droplets were ddPCR amplified, including a positive control from ATCC (VR-1986HK) and a

negative (no template) control. Fluorescence measurement was performed with the QX200 Droplet Reader and analyzed using the QuantaSoft software following the instructions with the ddPCR detection kit. All samples were analyzed in triplicate. Samples with two or more positive replicates were considered positive and averaged; samples with one or no replicates amplifying were considered not detected (ND).

2.3 Results and Discussion

2.3.1 SARS-CoV-2 in Zuma Beach Restrooms #1 and #9

The sampling details including total depth and approximate sludge depth in restrooms #1 and #9, as well as the men's and women's count of usage at restroom #9 are listed in Table 2-1. The TSS of the supernatant and sludge samples from Zuma Beach restroom #9 are shown in Figure 2-4. SARS-CoV-2 N1 and N2 gene concentrations measured in supernatant and sludge samples of Restroom #1 are shown in Figure 2-5. For most sampling dates, the sludge samples contained higher concentrations of N1 and N2 genes than the supernatant samples. The highest concentrations of N1 and N2 genes were recorded in sludge sample collected on Dec. 1st, 2020, subsequent concentrations of the virus were significantly decreased.

Table 2-1. The Total Depth and Approximate Sludge Depth in Restrooms #1 and #9, as well as the Men's and Women's Count of Usage at Restroom #9[a].

Sampling Event	Date	Restroom #1		Restroom #9			
		Total Depth (inches)	Sludge Depth (inches)	Total Depth (inches)	Sludge Depth (inches)	Men's Count	Women's Count
TEST	10/7/2020	36	4	-	-	-	-
1	12/1/2020	24	9	108	24	-	-
2	12/15/2020	24	6	108	12	-	-
3	12/29/2020	30	8	108	6	-	-
4	1/12/2021	24	8	108	6	-	installed
5	1/26/2021	24	10	108	4	-	5158
6	2/9/2021	24	6	108	8	-	2715
7	2/23/2021	24	8	108	10	-	3001
8	3/9/2021	-	-	108	10	installed	3297
9	3/22/2021	-	-	108	8	2862	2177
10	4/6/2021	-	-	108	4	5530	6182
11	4/20/2021	-	-	96	0	locked	locked
12	5/4/2021	-	-	117	6	locked	locked

[a] Each person that uses the restroom results in a count of 2 (one when entering and one when leaving). The numbers in the count columns indicate the original total counts before being adjusted for 2 per person.

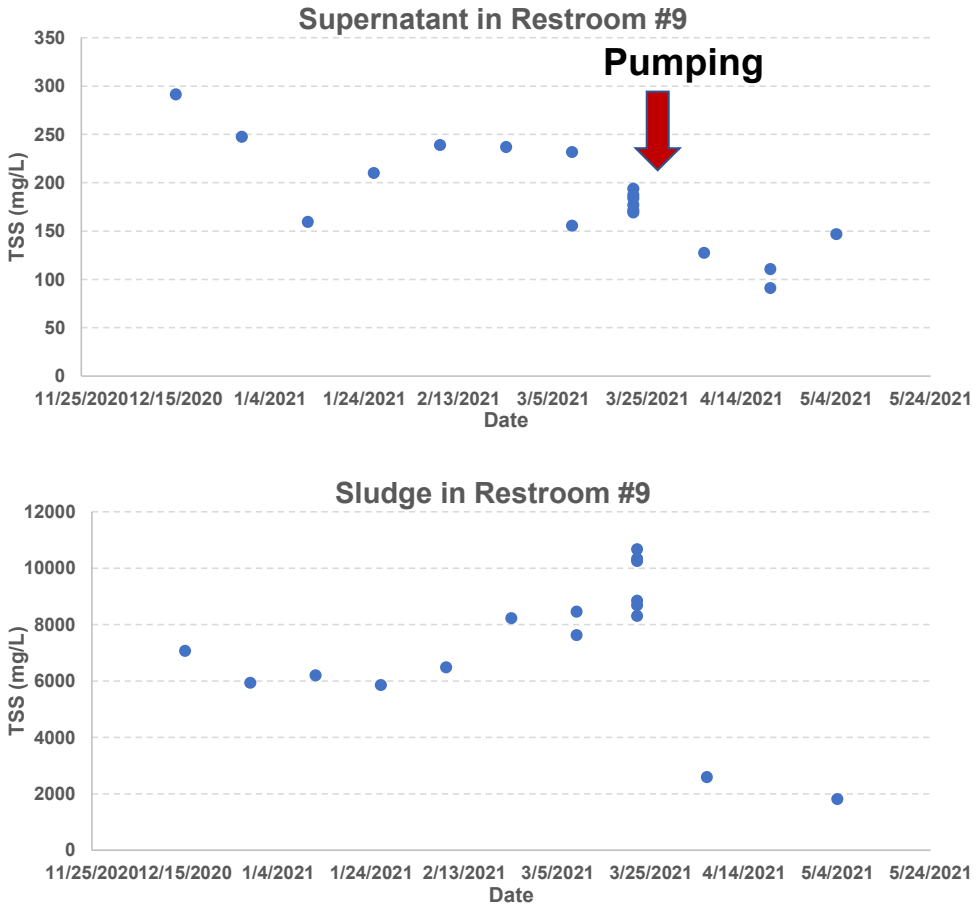


Figure 2-4. TSS Values of Supernatant and Sludge Samples Collected in Restroom #9.
Pumping of Restroom #9 Occurred on March 22, 2021.

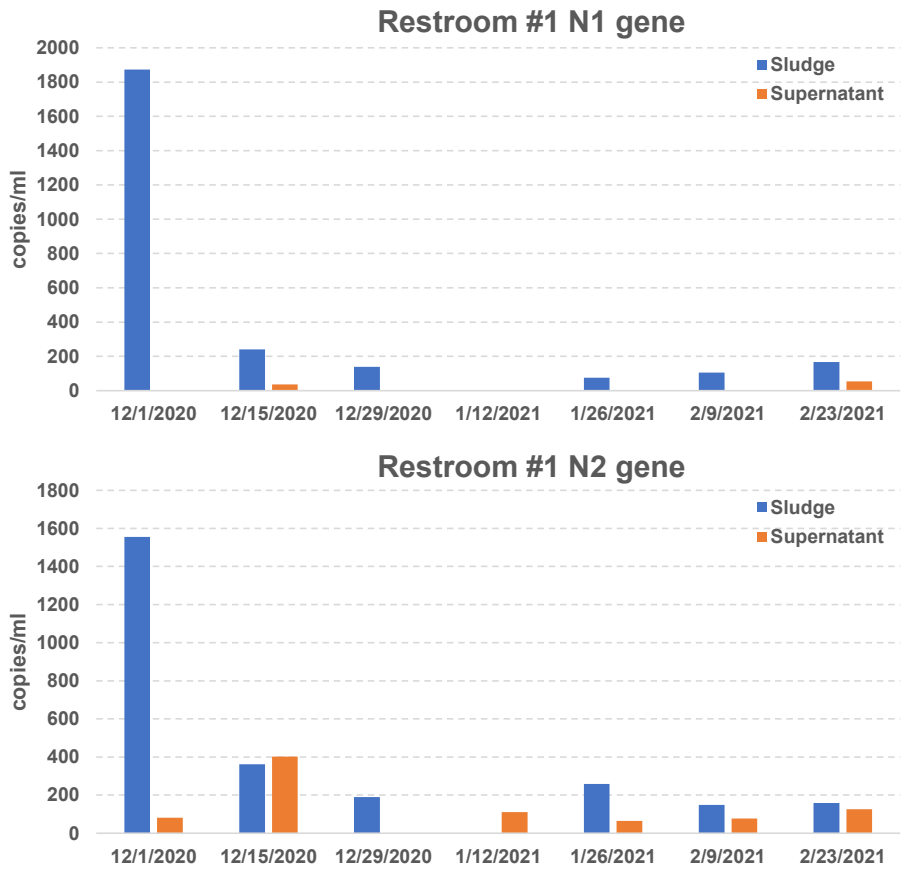


Figure 2-5. SARS-CoV-2 N1 and N2 Gene Concentrations in Supernatant and Sludge Samples of Restroom #1.

The N1 and N2 gene concentrations measured in supernatant and sludge samples of Restroom #9 are shown in Figure 2-6. For almost all sampling dates, the sludge samples contained significantly higher concentrations of N1 and N2 genes than the supernatant samples (Wilcoxon test, both p=0.0003). For sludge samples collected at multiple depths on Mar 9th and Mar 22nd, 2021, the bottom sludge samples contained significantly higher concentrations of N1 and N2 genes than the corresponding top sludge samples. Similarly, supernatant samples collected from the mid depth layer contained significantly higher concentrations of N1 and N2 genes than the corresponding supernatant samples from the top/scum layer on Mar 9th and Mar 22nd, 2021. The N2 gene concentrations were similar between the middle and bottom layers on Apr 20th, 2020.

Overall, when combining Restroom #1 and #9 samples together, the concentrations of both N1 and N2 genes showed significant correlations between sludge and corresponding supernatant samples (Spearman correlation test, r_s = 0.63 and 0.49, p<0.001 and p=0.016, respectively for N1 and N2 genes). Additionally, the concentrations of both N1 and N2 genes in all sludge and supernatant samples showed strong correlations with corresponding TSS values (Spearman correlation test, r_s = 0.68 and 0.51, both p<0.001, respectively for N1 and N2 genes).

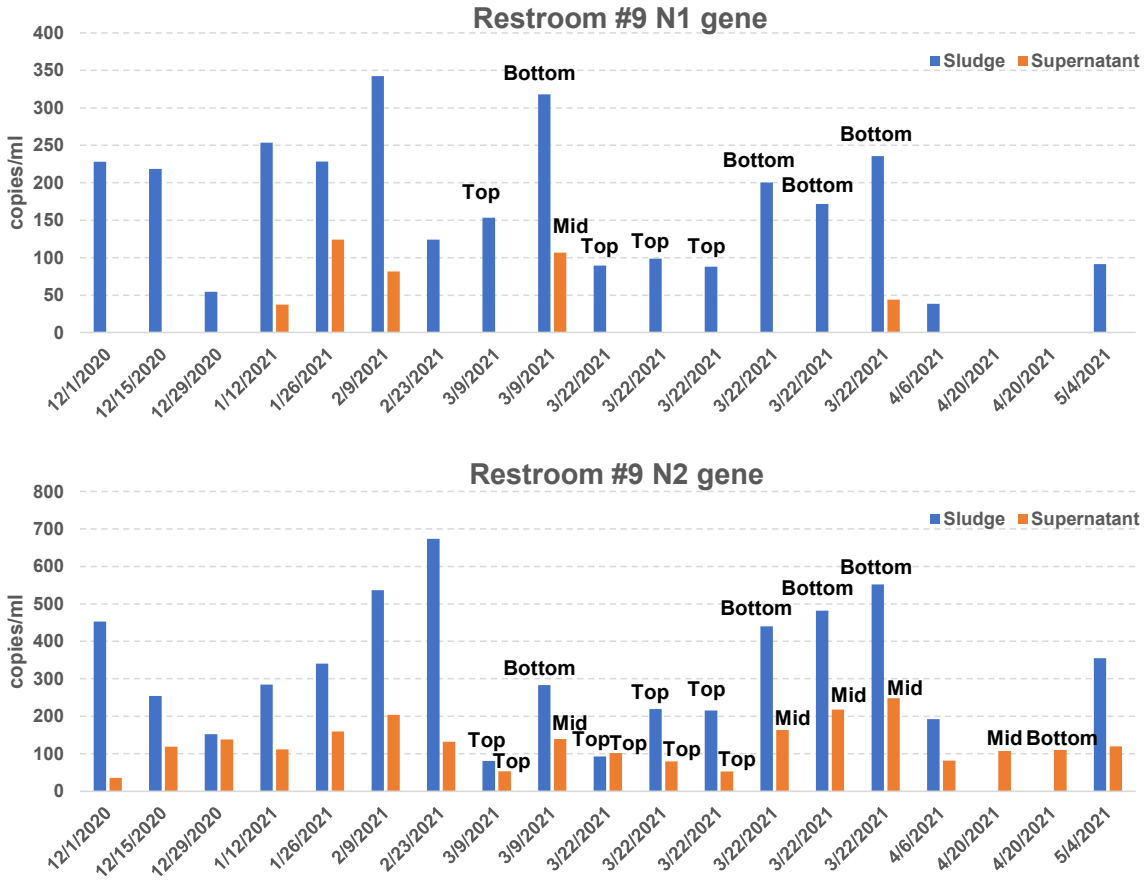


Figure 2-6. SARS-CoV-2 N1 and N2 Concentrations in Supernatant and Sludge Samples of Restroom #9.
The depth of sludge samples (top or bottom) collected on Mar 9 and Mar 22, 2021, is marked on the top of each column. Similarly, the depth of supernatant samples (top or middle) collected on Mar 9 and Mar 22, 2021, is marked on the top of each column. Sludge samples were not available on Apr 20th, and the depth of supernatant samples (middle or bottom) collected on Apr 20th is marked on the column.

2.3.2 Pumping and Hauling Study

The concentrations of SARS-CoV-2 N1 and N2 genes were measured for three mixed samples collected from the pumping truck just after pumping and for three mixed samples collected prior to discharge at the Joint Water Pollution Control Plant (JWPCP) in Los Angeles County on March 22nd, 2021 (Figure 2-7). The average concentration of the N1 gene was highest in sludge samples (1.47×10^2 copies/ml), followed by mixed samples from the truck just after pumping (8.78×10^1 copies/ml), mixture samples from the truck just before discharge (2.97×10^1 copies/ml), and supernatant samples (7.3 copies/ml). The same trend was also observed for the N2 gene, with the average concentration of 3.33×10^2, 2.29×10^2, 1.89×10^2, and 1.44×10^2 copies/mL, respectively for sludge, mixture samples after pumping, mixture samples before discharge, and supernatant samples.

For positive control samples spiked with the same amount of heat-inactivated SARS-CoV-2 and prepared using aliquots of mixed septage collected from the truck just after pumping, N1 and N2 genes were also detected (Figure 2-8). The average concentrations of N1 and N2 genes in triplicate samples stored on ice during transportation from Zuma Beach to JWPCP were 7.14×10^5 and 7.13×10^5 copies/ml, respectively. In contrast, the average concentrations of N1 and N2 genes in triplicate samples kept at ambient temperature until discharge at JWPCP were 5.57×10^5 and 3.97×10^5 copies/ml, respectively, clearly indicating the decay of virus in the mixture under ambient temperature during transportation.

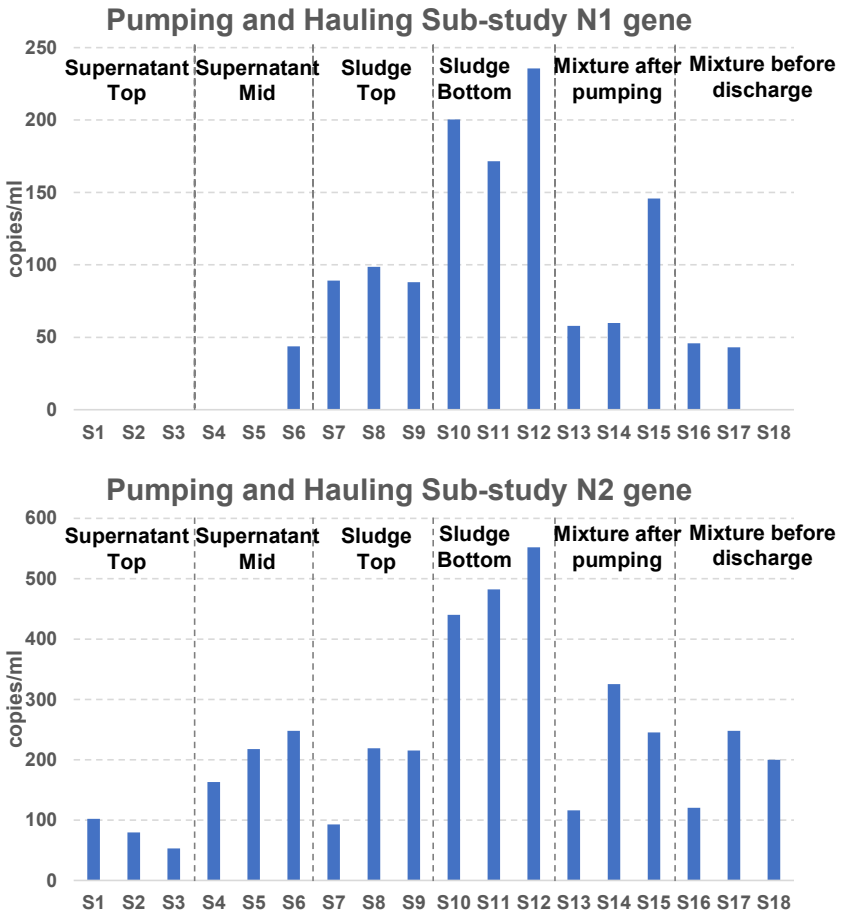


Figure 2-7. SARS-CoV-2 N1 and N2 Gene Concentrations in Supernatant, Sludge, and Mixture Samples of Restroom #9 during the Pumping and Hauling Study on March 22, 2021.

Samples S1-S3 were replicated supernatant samples collected from the top/scum layer; S4-S6 were replicated supernatant samples collected from the middle layer; S7-S9 were replicated sludge samples collected from the top layer of sludge; S10-S12 were replicated sludge samples collected from bottom layer; S13-S15 were replicated mixture samples collected just after pumping; S16-S18 were replicated mixture samples collected prior to discharge at the JWPCP in Los Angeles County on March 22nd, 2021. The results of supernatant and sludge samples (S1-S12) are also presented in Figure 2-6.

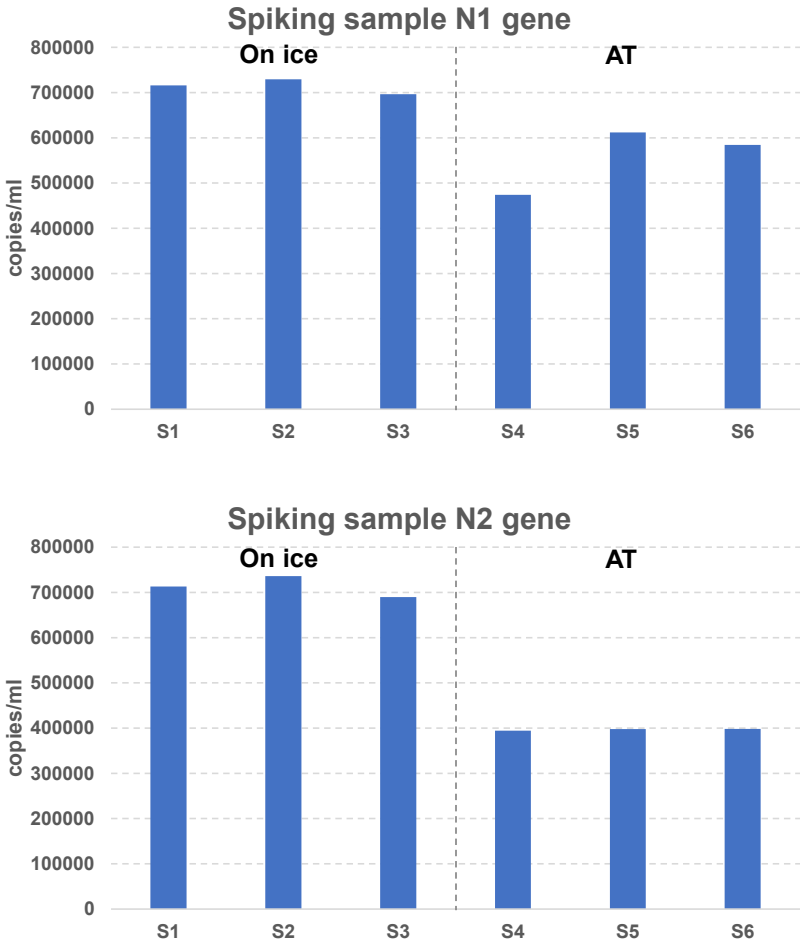


Figure 2-8. SARS-CoV-2 N1 and N2 Gene Concentrations in Mixed Septage Samples Spiked with Heat Inactivated SARS-CoV-2.

Samples S1-S3 were triplicate samples put immediately on ice, and samples S4-S6 were kept at ambient temperature (~70 ºF in the dark) for approximately 2 h until discharge.

2.4 Conclusions

In this investigation of septic system-based SARS-CoV-2, the results indicate that the decentralized system harbors SARS-CoV-2 in the primary tank supernatant and sludge compartments. Although the concentrations of SARS-CoV-2 were positively correlated across supernatant and sludge compartments, the sludge layer contained relatively higher concentrations of SARS-CoV-2 than the supernatant layer, suggesting that sampling of sludge can provide more sensitivity for the target detection. The depth profiles of SARS-CoV-2 RNA in both supernatant and sludge indicated that more abundant virus existed in deep layers compared to shallow layers, implying that the virus is likely existing in association with solids in the septic system. This is also supported by strong correlations between the viral concentrations in all sludge and supernatant samples with TSS values.

Pumping and hauling operations can be used as the sample access point for investigation of SARS-CoV-2 in septic systems. However, SARS-CoV-2 decays during transport from septic tank to discharge point, with first order decay rate coefficients estimated to range from 0.09 to 0.29 h^{-1}.

The results of this study contribute to the understanding of the presence and behavior of SARS-CoV-2 in decentralized wastewater collection points such as septic systems, and suggest how to implement appropriate strategies for sampling and assessing SARS-CoV-2 in such systems.

CHAPTER 3

Tracking SARS-CoV-2 in Sewers via Manhole Sampling

3.1 Introduction

Monitoring SARS-CoV-2 in large centralized WWTPs and the public septic system like the Zuma Beach restrooms can offer a broad picture of the spread of COVID-19 in a large community. However, to trace the viral signal back to a specific community or individuals will require sampling upstream of a specific sewershed at the sub-community level, in order to relate the wastewater viral signal directly to a specific sub-community, a neighborhood, or a specific building (Calle et al., 2021; Larson et al., 2020). The University of California, Irvine (UCI) sub-sewershed presents a unique opportunity to investigate the feasibility of tracking SARS-CoV-2 in sewer manholes and relating the viral signal to the individual COVID-19 testing program at UC Irvine.

The UC Irvine sanitary sewer system comprises of over 15,000 linear feet of collection pipe. Sanitary sewage is collected from campus buildings that house administration, classroom, and research facilities, and includes discharges from student dorms and extensive faculty housing at University Hills. The UCI sanitary sewer system is a sub-sewershed of the Irvine Ranch Water District (IRWD) sewershed. The sewer flow from most of the campus feeds by gravity to the IRWD Michelson Water Reclamation Plant (MWRP) through several main junctions.

Since Fall 2020, UCI has implemented a massive individual COVID-19 testing program, which requires all students and staff on campus to be tested weekly. The individual testing program conducts ~2000 tests/day to screen all people regardless of symptoms. The results from this massive testing offer the opportunity to correlate the individual testing results with wastewater monitoring on campus (Karthikeyan et al., 2021). In this chapter, we report the investigation of SARS-CoV-2 in sewer manholes on and around UCI campus and their relationship to asymptomatic testing results. Specifically, we address: i) Can we detect SARS-CoV-2 from sewage manholes that are carrying human waste on campus? ii) How does the SARS-CoV-2 concentrations correlate with known on-campus disease records?

3.2 Materials and Methods

3.2.1 Description of Sampling Sites

Figure 3-1 shows a section of the IRWD MWRP sewershed. Three stars on the map indicate the location of MWRP, the manhole Z, which covers the southern sub-sewershed that comprises the main flow from the UC Irvine campus, and the lift station X, where anti-odor chemicals ($FeCl_2$ or bioorganic additive) were dosed to the sewer line. The insert in Figure 3-1 marks the locations of sewer manholes sampled in the UCI sewershed. Manholes (MH) A, B, C, D, E, and F are identified on the insert map.

The sewer service areas of UCI MH A – F are presented in Figure 3-2, and are providing sewer collection service from a few hundred to a few thousand residents. MHA has the largest service area including both UCI research and administration buildings (area shaded in yellow), and sewer flow generated by student dorms from MH B, D, E, F. MH F only includes a single building. MH D and E serve graduate housing complex with a limited number of residents during the study period. MH C serves a portion of the University Hills faculty residential community, where faculty and staff families of diverse age groups reside.

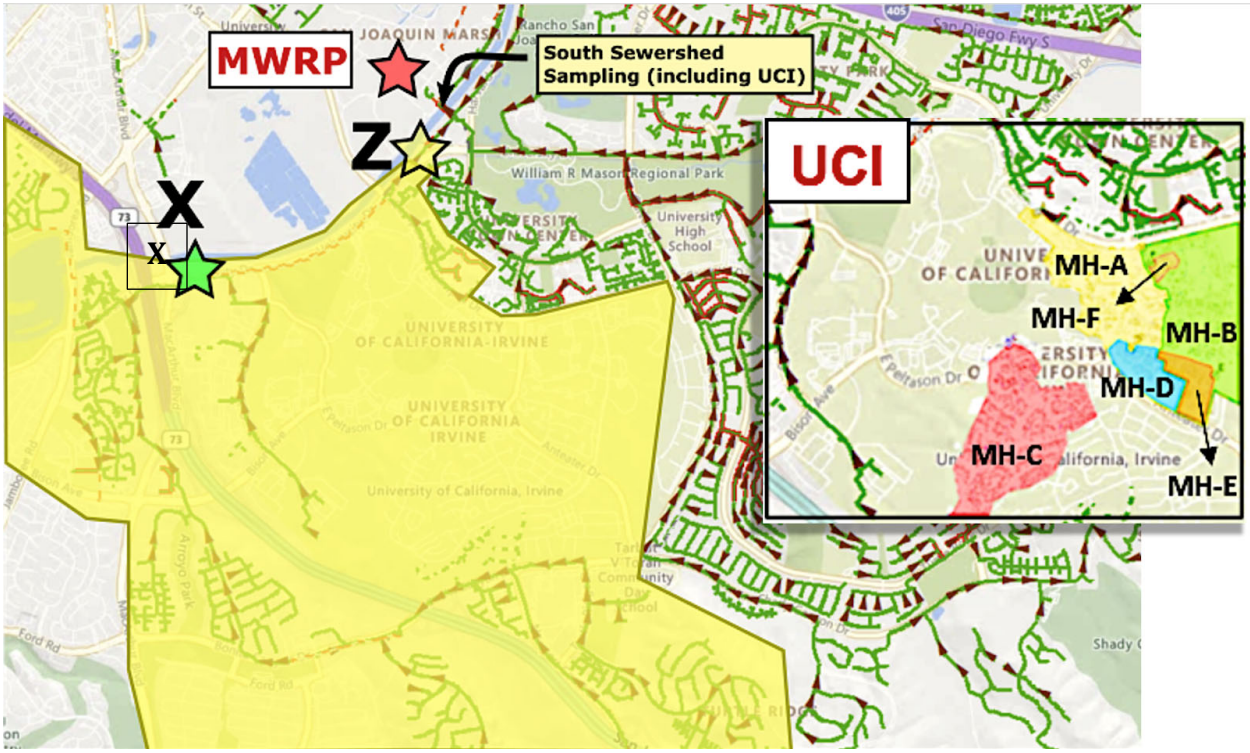


Figure 3-1. Map of a Section of IRWD Sewershed.
The stars indicate sampling points of two manholes on the two major trunk lines that are upstream of MWRP influent. The yellow section indicates UCI sewershed and the inset on the right shows UCI manholes sampled during this project (MH-A to MH F).

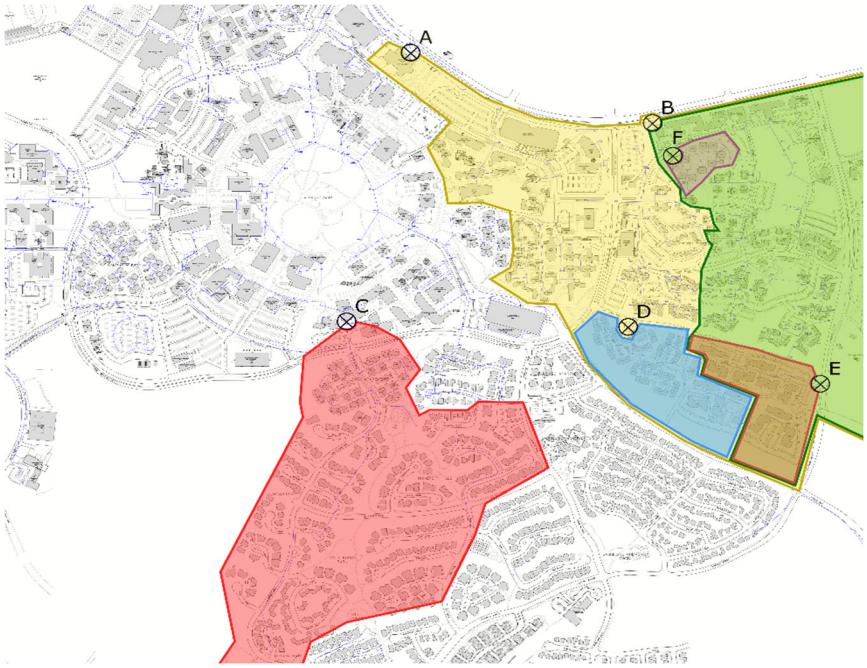


Figure 3-2. Detailed Map Showing Color-Coded Sewer Manhole Service Area on UCI Campus.
Manhole A collects combined flow from B, C, D, E, and F in addition to its own service area (yellow). Manhole B collects combined flow from E, F and green color-coded area.

3.2.2 Sample Collection

Sewer samples were collected weekly between December 2020 and March 2021 from the MWRP influent, manhole Z, the lift station X influent and effluent with the support provided by the staff from IRWD (Figure 3-3). On each sampling day, grab samples were taken using a sampling bottle attached to a metal weight. Grab samples were also retrieved at the influent of the MWRP on each day of manhole sampling.

Figure 3-3. Images of a Sampling Event at the Lift Station; Left: the Influent (left), Right: Effluent.

The sampling program for UCI manholes was performed from January 5, 2021, to March 23, 2021. A total of 62 manhole samples were collected from the UCI campus. Access to MH A – F were supported by the UCI facilities staff. The same sampling methods were carried by the UCI team (Figure 3-4).

Figure 3-4. Pictures of a Sampling Event at UCI-MH-D.

3.2.3 Water Quality and SARS-CoV-2 Analysis

During the sampling event, flow rates at the manholes were measured using the Marsh-McBirney Model 201 Portable Flowmeter, as shown in Figure 3-5. The flowmeter uses an electromagnetic sensor to measure the mean velocity of an open channel. The sensor is unidirectional; hence, a flag was constructed to provide alignment of the sensor with the flow. The sensor and flag assembly was mounted at the end of a telescopic pole used to reach the flow from above the manhole (Figure 3-6).

The readout of the mean velocity was then displayed on a digital display as feet per second, using a 30-second time-average.

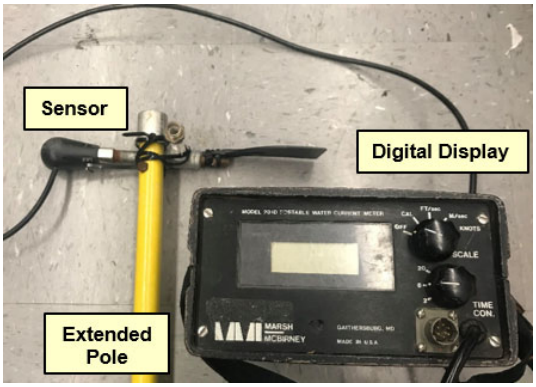


Figure 3-5. Marsh-McBirney Model 201 Flo-Mate.
The sensor was Attached to the end of a Telescopic Pole with a Directional Flag.

The flow in the manhole was calculated by using the measured mean velocity, the depth of the flow at the time of profile, and the inside diameter of the pipe, as shown in the Equation 3-1:

$$Q = K * D^2 * \overline{U} \qquad \text{(Equation 3-1)}$$

Where Q = flow rate (cfs), K = flow unit multiplier, D^2 = diameter squared (ft^2), $\overline{U}$ = mean velocity (ft/s). The flow unit multiplier was identified from the ratio of the depth of flow (L) in inches and the inside diameter (D) in inches. The depth of the flow was measured by wading a cardboard coupon attached to the end of the extended pole (Figure 3-6). The obtained flowrates from February 23, 2021, to March 23, 2021, are shown in Figure 3-7.

Figure 3-6. Pictures of a Sampling Event When Flowrate Was Measured at the UCI-MH-A Site.

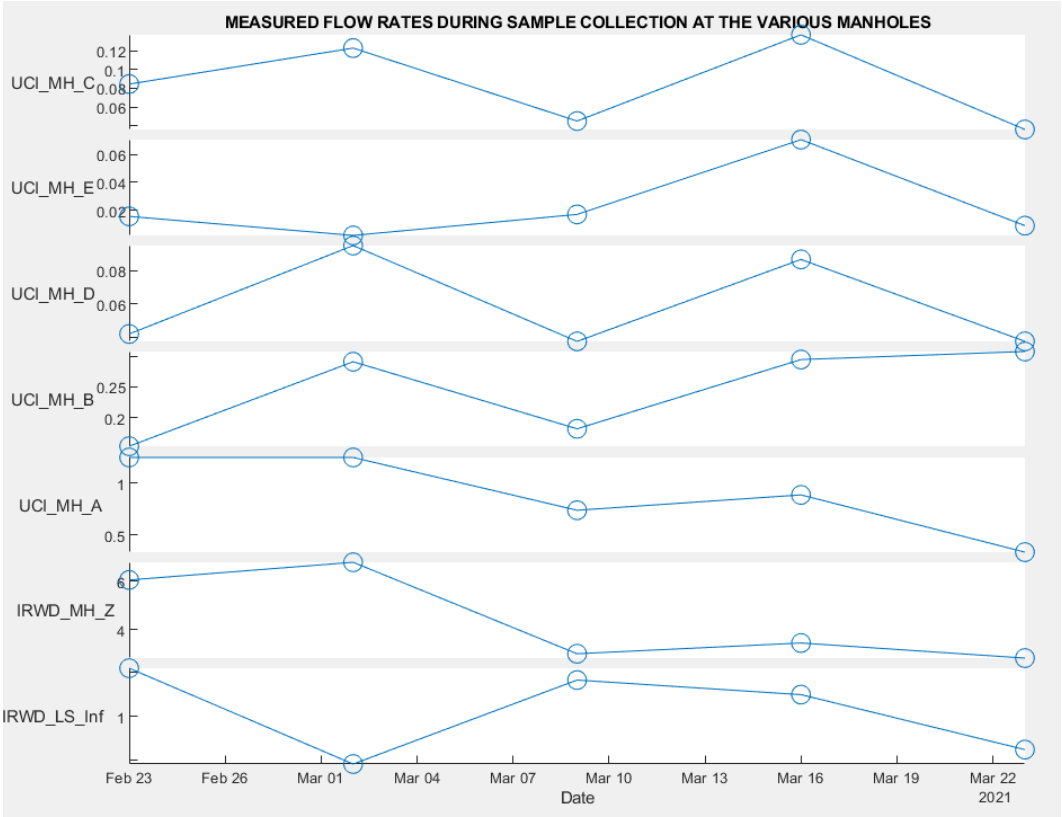


Figure 3-7. Measured Flow Rates during Sample Collection at the Various Manholes (cfs).

Water samples were analyzed for TSS, COD, and NH_3-N following standard methods. The sample process, nucleic acid extraction, and ddPCR assay for SARS-CoV-2 were carried out at the UC Irvine Lab following the same protocol reported in Chapter 1 of this report.

3.3 Results and Discussion

3.3.1 Water Quality of Sewer Manhole Samples

Figure 3-8 shows the TSS, COD, and NH_3-N concentrations in UCI manhole samples. The water quality parameters were overlayed with the MH service area. The sewer flow direction is indicated using gray arrows. Manhole F data were not included in the plot due to limited measurements (2 sampling events only). Larger variability of water quality parameters was observed in manhole samples collected from the smaller service area, indicating the sporadic nature of flow when the resident numbers were low. The variability is more clearly observed in Box and Whiskers Plot together with data from the manhole samples from the main trunkline downstream of the sewershed (Figure 3-9). As expected, the water quality signals were more stable as the sewerage travelled downstream in the sewershed. This is due to more equalization of flow and concentrations when more tributaries are adding flow to the collection channel being sampled. The higher variability of the water quality constituents was observed for manholes D and E, which were associated with the two smallest sub-sewersheds on the UCI campus. This variability of water quality is caused by the episodic sewer flow in a small service area. However, the median values of the water quality parameters from manhole samples were not significantly different from those in the main trunkline and influent samples at MWRP (Figure 3-9).

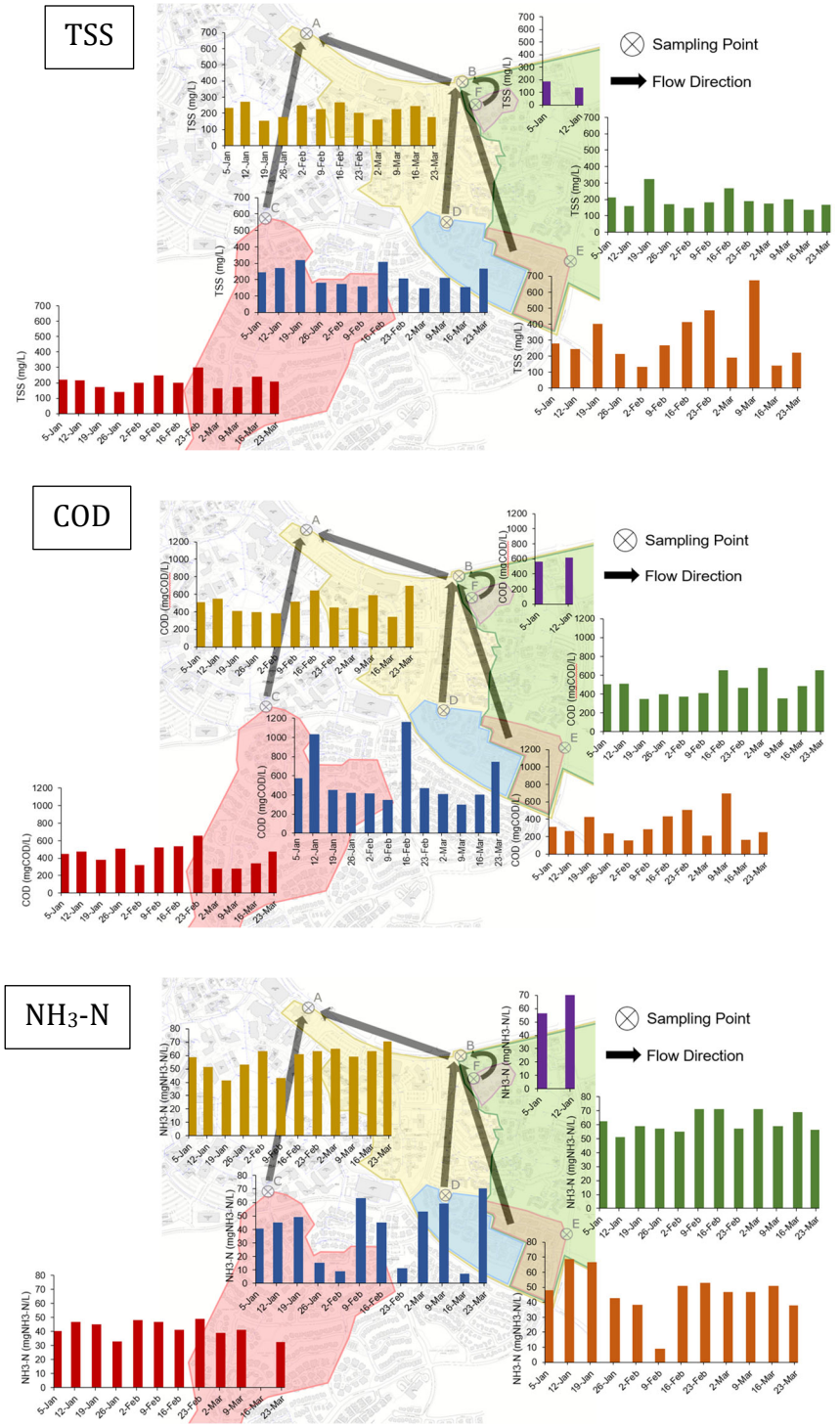


Figure 3-8. Water Quality Measurements in Samples Collected from Sewer Manholes on UCI Campus between Jan 5 and March 23.

Manholes are labeled from A to F, which corresponds to each colored collection area.

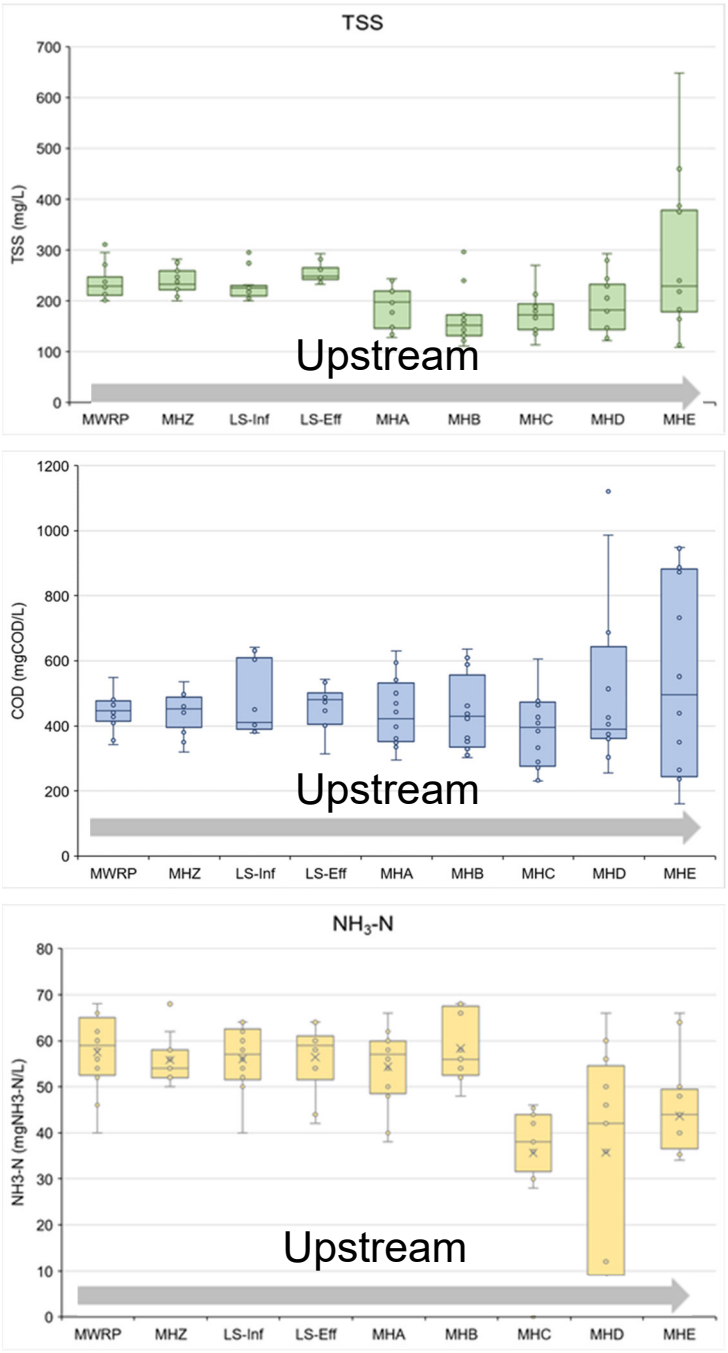


Figure 3-9. Comparison of Water Quality Parameters in Samples Collected from UCI Manholes A to E, the Downstream Manhole Z, Lift Station Influent (LS-Inf) and Effluent Manholes (LS-Eff), and Raw Sewage Influent at the MWRP Treatment Facility.

3.3.2 SARS-CoV-2 in IRWD Influent, Manhole Samples, and Relationship to COVID-19 Cases

SARS-CoV-2 genomes were detected in four of the six manholes sampled on the UC Irvine campus (Table 3-1). The virus was most frequently found in manhole A (MHA) that covers the largest sewer collection area of the UCI campus, followed by manhole C (MHC) that covers the second largest service area on the UCI campus. There was no positive detection in manhole D and one below quantitative threshold signal

in manhole E. In comparison with the downstream manhole Z, the SARS-CoV-2 concentrations were lower in general (Table 3-1).

The lift station influent (LS-Inf) and effluent (LS-Eff) manholes were sampled to investigate the impact of anti-odor chemical dosing on viral stability. Significantly higher viral concentrations were found in the influent manhole before chemical addition than in the effluent manhole after addition (Table 3-1 and Figure 3-10) suggesting chemical dosed for odor control reduces the detectable level of virus. However, there were limited numbers of positive samples since the clinical COVID-19 cases dropped dramatically in February in the sewershed (Irvine and Newport Beach). The viral signal was below the quantification limit after late February 2021 (Figure 3-10). The viral signal in grab samples of sewage influent at MWRP tracked well with the COVID-19 cases reported by the Public Health department for the Cities of Irvine and Newport Beach in the sewershed (Figure 3-10). This result suggests manhole sampling could be validate approach to identify the community spread of COVID-19.

Table 3-1. SARS-CoV-2 Genomes in Sewer Manhole Samples within the MWRP Sewershed.

Sample ID	% Detect.	SARS-CoV-2 concentration (GC/mL)									
		12/15 /20	1/5 /21	1/12 /21	1/19 /21	1/26 /21	2/9 /21	2/23 /21	3/9 /21	3/23 /21	Mean
MHA	75%	na*	425	391	413	197	194	-**	187	-	301±119
MHB	25%	na	180	-	-	223	-	-	-	-	202±30
MHC	37.5%	na	132	598	-	-	355	-	-	-	362±233
MHD	0%	na	-	-	-	-	-	-	-	-	
MHE	12.5%	na	-	107	-	-	-	-	-	-	
MHZ	75%	837	714	603		306	-	273	-	240	496±256
LS-Inf	50%	na		1896		1457	1061	-	-	-	1471±418
LS-Eff	83.3%	na		1099		661	561	-	252	257	566±349
MWRP	71.4%	1120		817		704	486	-	263	-	678±326

**Sample was not collected; **Below LOD*

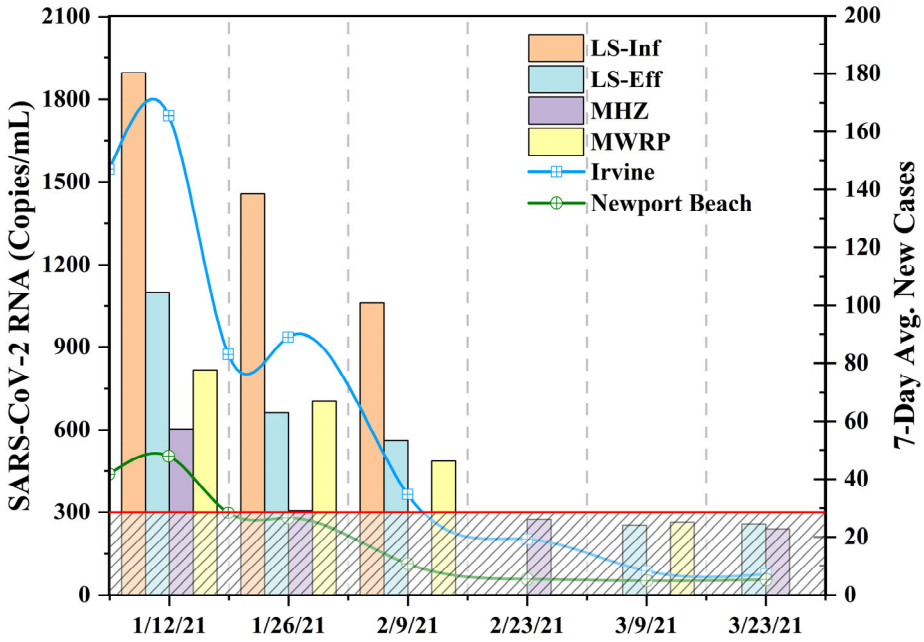


Figure 3-10. SARS-CoV-2 Concentration in Sewer Manhole Samples Collected at Lift Station Influent (LS-Inf) and Effluent (LS-Eff), Downstream Manhole Z (MHZ), and Influent of MWRP.
The redline indicates the LOQ of the assay. The viral concentrations (GC/ml of liquid, bar graph) are overlaid by 7-day average new COVID-19 cases reported for the Cities of Irvine and Newport Beach, the main service areas of the MWRP treatment facility.

Despite the very limited-service area of UC Irvine manholes and the low concentration of SARS-CoV-2 detected in the manhole samples, the viral concentrations in manhole samples also tracked with the trend of UC Irvine campus-based symptomatic and non-symptomatic COVID-19 testing results (Figure 3-11). It is important to note that the UCI testing program was mandatory for all students residing on campus dorms and included both symptomatic and non-symptomatic cases. However, residents at University Hills community (faculty and family), which has a much larger population than the combined population in student dorms, classrooms and office builds during the COVID-19 campus shutdown, were not included in the mandatory testing program. The fact that the on-campus individual testing results matched well with the trend of viral concentration in sewers may be a reflection of overall trend of the COVID-19 dynamics beyond UCI campus. Unfortunately, the decline of COVID-19 cases limited the number of positive detections and the statistical power of the outcomes.

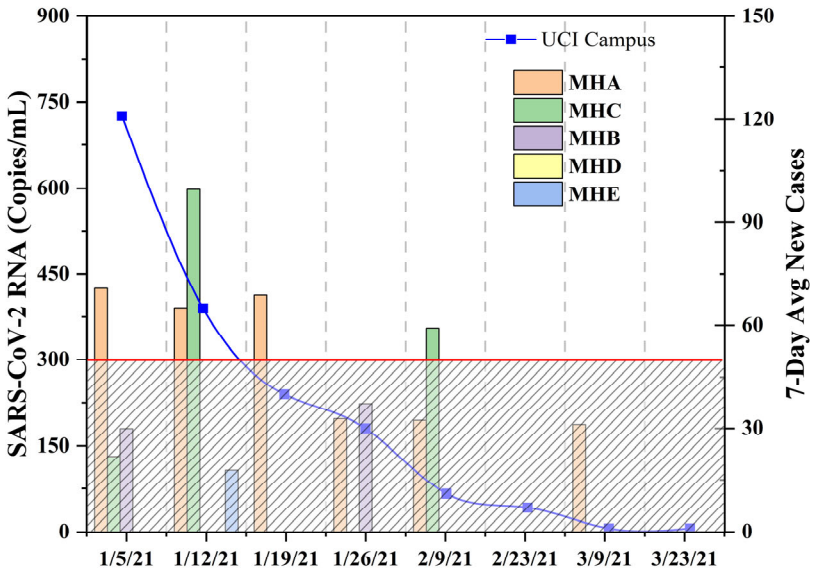


Figure 3-11. SARS-CoV-2 Genomes in Sewer Manhole Samples Collected on the UC Irvine Campus.
The Redline Indicates LOQ of the Assay.
The viral concentrations (bar graph) are overlaid by 7-day average new COVID-19 cases on the UC Irvine campus based on-site symptomatic and non-symptomatic testing.

3.4 Conclusions

The results of this study provide a proof-of-concept for the potential application of WBS in sewer manhole samples for tracking the spread of COVID-19 in the sewer service community. SARS-CoV-2 concentrations were detected in sewer manhole samples collecting wastewater from a few hundred to a few thousand residents without the need of a separated viral concentration step. However, the episodic nature of sewer flows in a very small sewage collection area also implies that a single grab sample may miss the signal of SARS-CoV-2 because the virus is likely traveling in the sewer as a plug flow. Composite sampling should improve the representativeness of samples within the service area. In addition, composite sampling is less labor-intensive, since it requires less oversight at the sampling sites during collection.

The viral signal detected in the local sewer manhole samples tracked well with the samples collected from main sewer trunk line samples and the influent of the WWTP, at the downstream end of the sewershed. Chemical treatment along the sewer trunk lines, such as chemical addition for odor control, can significantly alter the viral concentration in the liquid phase, putatively aggregating viruses with solids. The SARS-CoV-2 decay rate in the sewer lines was not investigated here but could potentially contribute to correlating the relationship between virus count at the treatment plant influent station with the viral count distributed upstream throughout the collection system. Towards this goal, it is important to have a transport model for the sewershed, which can be used to back calculate the SARS-CoV-2 shedding rate in a community and COVID-19 cases.

The challenges in sampling manholes are evident when attempting to quantify flow rates that are meaningful to correlate the sample concentrations with the case numbers in the sub-sewershed upstream of the sampled manhole. Future studies need to address what is the threshold above which projections on flow rate expected from tributaries can be quantified. The integration of spatially and

temporally distributed sampling plans and hydraulic-reactive models for collection systems should be entertained.

Future studies should also develop rapid and more sensitive methods for SARS-CoV-2 monitoring in environmental samples, or rapid surrogate measurements. Higher sampling frequency and denser sampling sites are necessary to produce a more accurate picture of SARS-CoV-2 distribution in human wastewater and the spread of COVID-19. Also, future studies should address the threshold of minimum manhole flow rate to enable correlation between manhole grab samples and cases within the community served by the collection system upstream of the sampled manhole.

References

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K.V., and Mueller, J.F. 2020. "First Confirmed Detection of SARS-CoV-2 in Untreated Wastewater in Australia: A Proof of Concept for the Wastewater Surveillance of COVID-19 in the Community." *Sci Total Environ*. 728(138764).

ASTM International. 2018. *ASTM D5907-18: Standard Test Methods for Filterable Matter (Total Dissolved Solids) and Nonfilterable Matter (Total Suspended Solids) in Water*. https://www.astm.org/d5907-18.html.

Balboa, S., Mauricio-Iglesias, M., Rodriguez, S., Martínez-Lamas, L., Vasallo, F.J., Regueiro, B., and Lema, J.M. 2021. "The Fate of SARS-COV-2 in WWTPS Points Out the Sludge Line as a Suitable Spot for Detection of COVID-19." *Science of the Total Environment*, *772*. https://doi.org/10.1016/j.scitotenv.2021.145268.

Calle, E., Martínez, D., Brugués-i-Pujolràs, R., Farreras, M., Saló-Grau, J., Pueyo-Ros, J., and Corominas, L. 2021. "Optimal Selection of Monitoring Sites in Cities for SARS-CoV-2 Surveillance in Sewage Networks." *Environment International*, *157*. https://doi.org/10.1016/j.envint.2021.106768.

Diaz-Valbuena, L.R., Leverenz, H.L., Cappa, C.D., Tchobanoglous, G., Horwath, W.R., and Darby, J.L. 2011. "Methane, Carbon Dioxide, and Nitrous Oxide Emissions from Septic Tank Systems." *Environ Sci Technol*. 45: 2741–47.

Karthikeyan, S., Nguyen, A., McDonald, D., Zong, Y., Ronquillo, N., Ren, J., Zou, J., Farmer, S., Humphrey, G., Henderson, D., Javidi, T., Messer, K., Anderson, C., Schooley, R., Martin, N. K., and Knight, R. 2021. "High-Throughput Wastewater SARS-CoV-2 Detection Enables Forecasting of Community Infection Dynamics in San Diego County." *mSystems*. 6: 793–814. https://doi.org/10.1128/mSystems.00045-21.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., and Rose J.B. 2020. "SARS-CoV-2 in Wastewater: State of the Knowledge and Research Needs." *Sci Total Environ*. 15 (739): 139076.

La Rosa, G., Bonadonna, L., Lucentini, L., Kenmoe, S., and Suffredini, E. 2020. "Coronavirus in Water Environments: Occurrence, Persistence and Concentration Methods - A Scoping Review." *Water Res*, 179: 115899.

Larson, R.C., Berman, O., and Nourinejad, M. 2020. "Sampling Manholes to Home in on SARS-CoV-2 Infections." *PLoS ONE*, *15* (10 October). https://doi.org/10.1371/journal.pone.0240007.

Lusk, M.G., Toor, G.S., Yang, Y.Y., Mechtensimer, S., De, M., and Obreza, T.A. 2017. "A Review of the Fate and Transport of Nitrogen, Phosphorus, Pathogens, and Trace Organic Chemicals in Septic Systems." *Crit. Rev. Environ. Sci. Technol*., 47: 455-541.

Massoud, M.A., Tarhini, A., and Nasr, J.A. 2009. "Decentralized Approaches to Wastewater Treatment and Management: Applicability in Developing Countries." *J Environ Manage*. 90 (1): 652-9.

Metcalf & Eddy, Inc. 2014. *Wastewater Engineering: Treatment and Resource Recovery*, 5th ed. New York: Mc-Graw-Hill.

Mizumoto, K., Kagaya, K., Zarebski, A., and Chowell, G. 2020. "Estimating the Asymptomatic Proportion of Coronavirus Disease 2019 (COVID-19) Cases on Board the Diamond Princess Cruise Ship, Yokohama, Japan, 2020." *Euro Surveill*. 25 (10).

Nishiura, H., Kobayashi, T., Miyama, T., Suzuki, A., Jung, S.M., Hayashi, K., Kinoshita, R., Yang, Y., Yuan, B., Akhmetzhanov, A.R., and Linton, N.M. 2020. "Estimation of the Asymptomatic Ratio of Novel Coronavirus Infections (COVID-19)." *Int J Infect Dis*. 94: 154-155.

Pecson, B.M., Darby, E., Haas, C.N., Amha, Y.M., Bartolo, M., Danielson, R., Dearborn, Y., Di Giovanni, G., Ferguson, C., Fevig, S., Gaddis, E., Gray, D., Lukasik, G., Mull, B., Olivas, L., Olivieri, A., and Qu, Y. 2021. "Reproducibility and Sensitivity of 36 Methods to Quantify the SARS-CoV-2 Genetic Signal in Raw Wastewater: Findings from an Interlaboratory Methods Evaluation in the U.S." *Environmental Science: Water Research & Technology*. doi: 10.1039/D0EW00946F.

Schober, P., and Schwarte, L.A. 2018. "Correlation Coefficients: Appropriate Use and Interpretation." *Anesthesia and Analgesia*, 126 (5): 1763–1768. https://doi.org/10.1213/ANE.0000000000002864.

Song, Z., Reinke, R. Hoxsey, M., Jackson, J., Krikorian, E., Melitas, N., Rosso, D., and Jiang, S. 2021. "Detection of SARS-CoV-2 in Wastewater: Community Variability, Temporal Dynamics, and Genotype Diversity." *ACS ES&T Water*. https://pubs.acs.org/doi/10.1021/acsestwater.1c00119.

United States Center for Disease Control. 2022. "National Wastewater Surveillance System (NWSS)." https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/wastewater-surveillance.html.

U.S. EPA (U.S. Environmental Protection Agency). 2002. *Onsite Wastewater Treatment Systems Manual*. EPA 625/R-00/008. U. S. Environmental Protection Agency, Office of Research and Development.

U.S. EPA (U.S. Environmental Protection Agency). 2011. *A Homeowner's Guide to Septic Systems*. http://www.epa.gov/owm/septic/pubs/homeowner_guide_long.pdf.

Weiss, P., Eveborn, D., Karrman, E., and Gustafsson, J.P. 2008. "Environmental Systems Analysis of Four On-Site Wastewater Treatment Options." *Resour. Conserv. Recy.,* 52: 1153–61.

Withers, P.J.A., Jordan, P., May, L., Jarvie, H.P., and Deal, N.E. 2013. "Do Septic Tank Systems Pose a Hidden Threat to Water Quality?" *Front. Ecol. Environ.*, 12 (2): 123-130.

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