Correlation of SARS-CoV-2 RNA in wastewater with COVID-19 disease burden in sewersheds

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ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes the coronavirus disease (COVID-19), is shed in feces and the virus RNA is detectable in wastewater. A nine-week wastewater epidemiology study of ten wastewater facilities, serving 39% of the state of Utah or 1.26M individuals was conducted in April and May of 2020. COVID-19 cases were tabulated from within each sewershed boundary by public health partners. The virus was detectable in 61% of 126 unique wastewater samples. Urban sewersheds serving >100,000 individuals and tourist communities had higher detection frequencies of the virus RNA. An outbreak of COVID-19 across two positively communities

correlated with an increase in SARS-CoV-2 RNA in wastewater, while a decline in COVID-19 case counts preceded a decline in SARS-CoV-2 RNA. These result provide evidence of the utility of wastewater epidemiology to assist in public health responses to COVID-19.

KEYWORDS

SARS-CoV-2; COVID-19; wastewater epidemiology; disease burden; sewershed; interceptor

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the coronavirus disease (COVID-19). Within six months of the first reported case in Wuhan, China, this disease has been reported in more than 216 nations, areas or territories worldwide [1]. SARS-CoV-2 virions and viral ribonucleic acids (RNA) are detectable by molecular biology based methods in various patient samples including respiratory nasopharyngeal and oropharyngeal swabs [2], serum and tissues [3, 4]. Further, SARS-CoV-2 infects cells in the gastrointestinal tract, specifically glandular epithelial cells [3], and likely is responsible for early reports of 10% of COVID-19 hospital patients with gastrointestinal symptoms such as diarrhea, nausea, abdominal pain, and vomiting [5]. These glandular epithelial cells express angiotensin-converting enzyme 2 (ACE2), the cellular receptor for SARS-CoV-2 and SARS-CoV, also found in lung and oral mucosa [6, 7]. Detection of SARS-CoV-2 RNA in feces by molecular methods has been reported [3, 8, 9] from pre-symptomatic individuals 1-5 days before the onset of the clinical symptoms [5, 10], from individuals with mild symptoms [11] and for 7 to 11 days after symptoms have resolved in individuals who are no longer considered infectious [4, 5, 12, 13]. Concentrations of SARS-CoV-2 in feces of nine patients (15.3%) hospitalized with COVID-19 had 3.4 to 7.6 log RNA gene copies/mL feces (median 4.7 log RNA GC/mL feces) [14]. Further, patients presenting with gastrointestinal symptoms had a higher fecal RNA load (5.1 log GC/mL feces) compared to those without gastrointestinal symptoms (3.9 log GC/mL feces in 4 of 44 patients with COVID-19). While the virus is readily detectable in feces, a recent study reported no viable SARS-CoV-2 virus in feces, in spite of high viral RNA concentrations [15]. While there is ample evidence of the viral RNA in feces, there is disagreement regarding the presence of the viral RNA and virus in urine,

with some reporting detections of the RNA but not the virus itself [4], while others did not detect the virus in urine [16, 17].

Given the detection of SARS-CoV-2 RNA in feces, testing for SARS-CoV-2 RNA in sewersheds may allow for distributed monitoring of community disease burden [18] for the estimated 2.1 billion people living in 105,600 wastewater treatment plant (WWTP) districts worldwide [19]. This approach, termed wastewater epidemiology, has been widely used for monitoring for poliovirus eradication [20] and illicit drug use [21]. Building on reports of SARS-CoV RNA in 2004 in all untreated wastewater samples (10/10) and 30% (3/10) of disinfected wastewater samples in a Beijing, China hospital [22], several reports have documented the presence of SARS-CoV-2 in wastewater worldwide. Ahmed, et al reported variable SARS-CoV-2 RNA loads in treatment plant influent samples from three facilities in Australia, and recommended that collaboration with local health departments would be necessary to draw comparisons and generate useful data for monitoring the spread of the virus [23]. Three wastewater treatment plants (WWTPs) in France were sampled over a 7-week period and reported data that correlated with the country's nationwide lockdown [24]. Attempts to detect SARS-CoV-2 RNA in wastewater were also successful in Italy [25, 26], the Netherlands [27] and the United States [28, 29], and in some cases were shown to correlate with COVID-19 case counts. The maximum SARS-CoV-2 RNA concentrations reported in wastewater influent ranged from $1.2x10^3$ to $3.2x10^6$ gene copies/L in wastewater influent [23, 24, 25, 26, 28, 29].

Despite the widespread detection and interest in sewershed monitoring for SARS-CoV-2 RNA, there remains debate on how these data may be used and the extent of the methods utility in informing public health decisions. Possible suggested uses of the data include (1) direct correlation with disease burden, (2) disease trend analysis, (3) monitoring the efficacy of interventions in reducing disease in a community, or (4) new case identification in areas with no known cases of COVID-19 [18]. If the number of SARS-CoV-2 RNA gene copies in wastewater is correlated with the total number of COVID-19 positive individuals shedding viral RNA within a sewershed, this may give an indication of the total burden of disease in that population, beyond just those individuals identified through COVID-19 testing. If fact, increases or decreases in SARS-CoV-2 RNA in wastewater may indicate a change in the prevalence of shedders in a sewershed. Further, the change in wastewater may be observable before changes in the number of individuals who have tested positive as suggested by others [27, 28]. Finally, wastewater

epidemiology may provide insight in areas with low documented case counts where clinical testing is difficult.

Given the presence of viral RNA in feces and widespread detections in sewage, there is a need for rigorous studies over extended time periods in communities with and without confirmed COVID-19 cases to assess the relationship between SARS-CoV-2 RNA in wastewater and disease burden. Therefore, a nine-week SARS-COV-2 RNA sewershed monitoring study was conducted at ten wastewater facilities in Utah that served a range of urban (i.e., >100,000 individuals), medium sized (20,000 to 100,000 individuals) and rural communities (<20,000 individuals). Completion of this work during travel restrictions implemented by universities and municipalities and the total distance between facilities (i.e., > 500 km) required regional coordination and standardization of sample collection and analysis, multiple geographically dispersed testing laboratories, cross-laboratory validation of methods and early cooperation between academic and government personnel during project initiation. Finally, early sample collection during increasing and decreasing COVID-19 infection cases was required for understanding of trends in SARS-CoV-2 RNA loads and COVID-19 disease burden.

RESULTS

Virus RNA detection frequency and correlation with COVID-19 case counts

During the nine-week study from April 1 to May 28 of 2020, SARS-CoV-2 RNA was detectable in wastewater influent to ten facilities in 61% of the 126 unique influent samples, not including replicates or sub-sewershed samples. All ten facilities had at least one detection of viral RNA during the study period (**Table 1**). Facilities in more urbanized areas that serve more than 100,000 people had higher detection frequencies (i.e., CVWRF 96%, TSSD 40%, SLCWRF 100%, and OWRF 82%) as compared to facilities serving smaller communities (i.e., HCWWTP 56%, TWWP 13%, PRWID 27% and LCCWWTP 50%). In contrast, two smaller cities differed from this pattern. First, MCWRF which serves the tourist destination of Moab, had a 60% frequency of detection but had a relatively low viral abundance of 22.1 ± 29.7 million viral gene copies/capita/day (average ± standard deviation, hereafter MVGC/cap/d). Similarly, ECWRF which serves the popular ski destination of Summit County, had a 91% detection frequency. ECWRF also had the second highest RNA abundances detected, averaging 153 ± 321 MVGC/cap/d, and is located in an area with the first reported detections of COVID-19 in Utah. Abundance of SARS-CoV-2 RNA in

wastewater from urban sewersheds and areas with higher COVID-19 caseloads (i.e., CVWRF, SLCWRF, and ECWRF) averaged 168 ± 183 MVGC/cap/d, compared to small sewersheds serving less than 100K people or in areas with lower COVID-19 caseloads (i.e., TSSD, LCCWWTP, OWRF, TWWTP, PRWID, MCWRF and HCWWTP) which averaged 24.9 ± 62.4 MVGC/cap/d. These trends were also observable in the untransformed data (i.e., viral gene copies/mL wastewater) that didn't account for flowrates and sewershed population. Specifically, we detected 390 ± 489 viral RNA GC/mL influent in urban centers or areas with higher COVID-19 caseloads compared to 66 ± 154 viral RNA GC/mL in more rural sewersheds or lower COVID-19 caseloads. Herein, RNA gene copes per mL wastewater were converted to MVGC/cap/d were reported without correcting for virus recovery efficiency (e.g., the loss of the virus during sample handling and extraction). Recent reports by others suggest the method used herein had a 26.7 ± 15.3% recovery efficiency for the murine hepatitis virus, a surrogate for SARS-CoV-2 [30]. While the exact load of the viral RNA in wastewater per capita is not known, the trends and concentrations measured were reproducible between labs and over time. The viral RNA was not detected in effluent samples evaluated herein (**Table 1**), although it may have been present below the detection limit of the assay. Other studies also reported an inability to detect the virus in the effluent of wastewater treatment plants which had detectable virus RNA in the influent [25].

Communities with higher confirmed COVID-19 caseloads tended to have higher SARS-CoV-2 MVGC/cap/d in wastewater. Specifically, over the reporting period Salt Lake County (served in part by CVWRF and SLCWRF) had 2443 confirmed COVID-19 cases, while Summit County (served in part by ECWRF) had 240 confirmed COVID-19 cases [31, 32]. In contrast, Grand and Carbon County in Utah reported only 12 and 23 cases, respectively over the study period. Grand County includes Moab and is served in part by MCWRF, and Carbon County includes Price and is served in part by PRWID [33]. In an effort to get a more refined picture of the relationship, if any, between SARS-CoV-2 viral concentrations in wastewater and COVID-19 disease burden, the new COVID-19 case counts reported to the Utah Department of Health were summed by week within the sewershed boundaries. Case rates were calculated based on the population living within each sewershed. These weekly case rates were then plotted against the weekly SARS-CoV-2 MVGC/cap/d in wastewater within each sewershed (**Fig. 2**). For

comparison, the daily SARS-CoV-2 MVGC/cap/d in all sewersheds were also plotted against the daily new COVID-19 cases in each sewershed (**Fig. 3A**).

Distinct trends in virus RNA abundance versus case counts were observable in a few sewersheds. First, SARS-CoV-2 wastewater loads in ECWRF decreased over the nine-week observation period, dropping from an average of 499 ± 938 to 138 ± 239 viral MVGC/cap/d, as the COVID-19 case rates dropped from 68.7 cases/100K (19 ± 21) to <5. Second, both HCWWTP and LCCWWTP wastewater viral loads increased sharply (109 and 101% increase) in the last three weeks of the study period concurrent with the increase in weekly COVID-19 case rates from 5 or less to > 252 cases/100K (22 to 252% increase) (**Fig. 2**). Significant correlations (Spearman, P<0.05) between SARS-CoV-2 RNA in wastewater and weekly case rates were found for LCCWWTP and HCWWTP (**Fig. 3B** and **3C**), but not for the other facilities. However, when a one-week lag was applied to the weekly COVID-19 case rates, the ECWRF virus RNA in wastewater did correlate with the COVID-19 case rates (**Fig. 3D**, Spearman correlation, ρ = 0.80, n = 8, P = 0.01). These results suggest that the increase in case counts may occur concurrently with or even precede the increase in SARS-CoV-2 RNA in wastewater, while the decline in SARS-CoV-2 RNA in wastewater may lag the decline in case counts. This temporal variation in RNA in wastewater versus case counts has been suggested by others [24, 25, 28, 34]. Further, the long term shedding of the SARS-CoV-2 after negative nasopharyngeal swabs may account for the detection of RNA in wastewater after the decline in case counts [35]. However, additional data would be needed to confirm these observations during outbreaks and during the decrease in case counts in a sewershed at multiple facilities. Understanding the temporal offset of virus RNA detected in wastewater with disease burden is an important factor to consider in designing a sampling regime to optimize the utility of this tool in an operational context at larger geographic scales.

Considerations on virus survival in sewersheds and sample handling

The data herein suggests that wastewater monitoring is useful for identifying new outbreaks of COVID-19 and confirming declining trends in infections. However, additional information is needed before SARS-CoV-2 RNA wastewater loads may be directly correlated with disease burden. Specifically, information is needed on: the rate and mass of virus RNA shedding in feces pre-, during and post-symptomatic COVID-19 phases; the virus survival and persistence in the sewer; the influence of facility

and sewershed-specific factors such as runoff or groundwater infiltration or the presence of hospitals caring for COVID-19 patients; and the effect of sample handling on the virus abundance estimation.

The quantity of SARS-CoV-2 introduced into a sewershed is generally expected to be proportional to the true number of cases in that area, both identified and unkown. To more accurately assess the number of infected individuals in a sewershed, an accurate estimate of the SARS-CoV-2 RNA gene copies per unit weight of feces is needed during all stages of the disease. Using the literature reported values for SARS-CoV-2 in feces (median 4.7 log RNA GC/mL feces) [14], the number of COVID-19 ill individuals within a sewershed was estimated herein over the study period by converting from GC/mL wastewater to SARS-CoV-2 shedding individuals and compared to the COVID-19 caseloads in the sewersheds. Overall, the estimated number of SARS-CoV-2 shedders in each sewershed was found to be linearly correlated with the cumulative diagnosed COVID-19 cases in a sewershed (linear regression, $R^2 = 0.81$, n = 10, $P < 0.001$) (**Fig. 4**). However, the daily estimated number of SARS-CoV-2 shedding individuals did not correlate with daily COVID-19 cases (Spearman correlation, $P > 0.05$). This lack of correlation at a finer temporal scale may be due to the variability in daily case counts reported to the Department of Health that are influenced by reporting lags due to weekends or holidays, test kit availability and processing rates, etc. Conversely, other biological and non-biological factors must be influencing the SARS-CoV-2 persistence or detection in the wastewater when the data is evaluated at a finer temporal scale. Therefore, rolling case counts are likely a better metric to compare to the SARS-CoV-2 RNA in wastewater.

Numerous physical, chemical and biological factors could influence the persistence of viral RNA in wastewater. These factors include temperature, sunlight, ionic strength, presence of antiviral chemical constituents [36], solids content, residence time in the sewer, microbial antagonism [37, 38], and sampling methodology. In this study, we evaluated the effect of incubation of SARS-CoV-2 RNA containing wastewater at different temperatures on the loss of RNA over time (**Fig. 5**). Specifically, wastewater from three plants was evaluated to determine the loss of RNA during storage at 4°C and -80°C, and during transport in a sewer system at 10°C and 35°C. Overall the results indicate a first order decay rate of the viral RNA ranging from 0.09 to 0.12 hr^{-1} over the 22 to 24 hr at 4, 10 and 35°C. While the RNA was not

detectable after 6 hr at 35°C, the RNA was still detectable after 22 hr of incubation at 4 and 10°C and after one week at -80°C. The overall reduction in viral RNA during the storage or incubation periods was 67% at 10°C over 22 hr, 86.5 ± 0.5% at 4 °C over 24 hr and 92.4 ± 10.3% at -80°C over one week. These results suggest that the SARS-CoV-2 RNA may be more labile than previously reported coronaviruses. Specifically, previous research reported that SARS-CoV RNA could be measured by RT-PCR in domestic sewage for up to 14 days at 4°C but only 3 days at 20°C [22]. Similarly, enteric feline coronavirus and human coronavirus 229E took 2.5 to 3.5 d to decay 99.9% at 23°C [39]. Given that the wastewater residence time in some larger cities may be up to 13 hr [40], whereas smaller cities such as ECWRF had a 1 to 3-hr residence time, the potential for decay of the virus RNA should be considered when assessing virus loads. Before an accurate model of the likely number of COVID-19 infected individuals in a sewershed can be made, an understanding of the decay rate of the virus RNA in each sewer system is needed.

The effect of sample collection and handling on viral RNA concentrations is also needed to develop comparable relationships between wastewater samples and disease prevalence. An interlaboratory replicate analysis of spilt samples indicated comparability between the sample processing at the different labs (**Fig. 6**). This interlaboratory analysis suggests the mean among labs was 210 gene copies/mL wastewater (10 and 90% confidence intervals [CI] of 76 to 309 gene copies/mL) for CVWRF, 104 gene copies/mL wastewater (16 and 230 CI) for OWRF and 98 gene copies/mL wastewater (41 and 178 CI) for SLCWRF. These results suggest the data is comparable between labs with this sample handling and processing method.

Finally, the amount of SARS-CoV-2 RNA on wastewater influent solids, compared to the liquid was determined by quantifying the gene copies per g of solids and per g of liquid in eight samples. Overall, more viral SARS-CoV-2 RNA was detectable in the liquid phase (91 ± 12% by mass) of the wastewater influent compared to the RNA sorbed on the solids (9 ± 12% by mass). Others have reported the detection of the SARS-CoV-2 RNA on activated sludge in treatment plants [34, 41]. Given that most wastewater is near neutral pH and the SARS-CoV-2 spike proteins have estimated isoelectric points around 5.4 and 5.3 [42], the virion positive core is likely surrounded by negatively charged envelopes and spikes in these wastewaters. Moreover, RNA bases adenosine and cytidine can be protonated on N1 and

N3 atoms, respectively, with 3.8 and 4.3 solution pK_a [43, 44]. Therefore, the virus and viral RNA are likely to adsorb to activated sludge. However, due to variable return activated sludge wasting rates at facilities and periodic sludge bulking, using the virus RNA abundance in sludge to correlate with COVID-19 case rates may be difficult. In this study, we focused on wastewater influent, as it was the most comparable sample type between the ten facilities sampled, which varied from advanced mechanical plants to lagoon systems.

Refining sewershed sampling to aid public health interventions

To maximize the utility of wastewater monitoring for identifying regions of a city or larger geographic region for additional public health interventions, sample collection and analysis in smaller subunits may be helpful. In this study, we sampled sewer interceptors of sub-sewersheds on four separate occasions in the CVWRF service area to assess the (1) ability to quantify SARS-CoV-2 RNA in smaller areas (ranging in size from 9,682 people to 143,285 people) within a larger sewershed (total population of 515,484), (2) the effect of flow rate on SARS-CoV-2 gene copies/mL wastewater, and (3) the potential impact of inflow and infiltration (sewer I/I) the association between SARS-CoV-2 and case counts. Overall, it was found that sampling interceptors within a larger area did reveal finer resolution on COVID-19 disease burden. The sewer lines feeding into CVWRF showed significant variation in SARS-CoV-2 RNA abundance by city (**Fig. 7A** and **7B**). In some cases, the SARS-CoV-2 RNA abundance was greater than that measured at the influent to the treatment plant. As the travel time from the farthest lines feeding CVWRF can be several hours, the SARS-CoV-2 RNA may have decayed during the transit to the treatment plant influent collection point, thus, resulting in an apparent lower treatment plant influent virus concentration. The average temperature in the CVWRF influent and interceptor lines over the study ranged from 11 to 18^{o}C. Further, it was found that sewer systems with significant I/I, such as SSL which feeds CVWRF and had a significantly higher flow rate at 334 gallons per capital day (**Table 1**), had higher estimated SARS-CoV-2 MVGC/cap/d population than would be expected from the influent RNA GC/mL wastewater (**Fig. 8**). Thus, systems with high I/I and higher per capita flow were found to have higher RNA GC/cap/d after the effect of dilution due to I/I was considered. Accounting for wastewater sewer travel time, I/I, and other sewer-specific factors will be important in the data interpretation of a widespread wastewater epidemiology effort.

DISCUSSION

Given the trends in COVID-19 case load with the SARS-CoV-2 RNA abundance in wastewater there are several potential applications for using SARS-CoV-2 RNA wastewater data to inform public health interventions [45]. First, wastewater monitoring could be used to identify areas that may have a high number of active unidentified infections or where the number of COVID-19 infections are increasing above a predetermined action threshold, indicating an emerging infection hotspot [46]. Thus, wastewater SARS-CoV-2 RNA concentrations showing increasing trends may offer insights signaling the need to activate further clinical testing or other interventions in a particular area. Second, wastewater monitoring may indicate that the prevalence of COVID-19 in an area is non-existent, low or decreasing, provide a line of evidence that public health restrictions could be relaxed, as has been shown for poliovirus environmental surveillance studies [47]. In this case, the wastewater concentrations could provide assurance to the public and health officials if they fall below a predetermined action threshold and stay at that level for a period of time. This may be especially useful in areas where clinical testing is difficult to deploy such as isolated rural communities. Finally, wastewater monitoring could be used to assess the impact of public health precautionary restrictions or other interventions in areas where the SARS-CoV-2 RNA wastewater load indicated a change in trend over time.

Sampling sub-sewersheds may allow for a more refined picture of infected individuals within a sewershed, and there is increasing interest in using this type of sampling in smaller communities or even buildings. For example, university dormitories, athletic facilities or retirement or nursing communities could be sampled. However, there are several concerns with using this analysis in sewer systems with increasingly lower flow rates. First, representative samples may require time or flow weighted composited samplers to catch what could be a rare signal from a few infected persons among potentially hundreds or thousands of individuals. As urine is not consistently reported as a source of the SARS-CoV-2 RNA [4, 16, 17], samples should be collected over an 8-hr or 24-hr time period on multiple occasions to enable representative sampling of virus RNA shedding in feces and detection of a rare infection. This sampling duration and frequency will be dependent on the type of facility sampled, where a residential facility might need a 24-hr period while a business might only feasibly be sampled over 8 to 10 working hours. Further,

not all sewer systems near buildings or athletic facilities have a good turbulent mixing point or lift station that would collect sufficient wastewater to increase the likelihood of detecting the rare virus from a few infected individuals. Therefore, caution must be used in designing sampling plans for smaller and smaller sewer systems with fewer infected individuals to avoid providing a false negative in a critical public health setting.

Nevertheless, the economic value in wastewater epidemiology for disease monitoring is significant. For example, in Utah, weekly sampling of wastewater treatment facilities greater than 1 million gallons/day will cost approximately $220 per sample and cover 79% of the population ($.005 per person/week). These data may provide community level surveillance and identification of emerging hotspots to help maximize the use of other limited public health resources such as targeted clinical testing. Utah also plans to sample targeted smaller rural facilities which increases the per sample cost to $525/sample for an additional 2% of the population. Nonetheless, the per capita cost of wastewater sampling even in rural areas ($0.10 per person per week) is substantially lower than clinical testing and can identify new areas of increasing disease prevalence. Further, it could provide confirmation of low levels of community infection in many areas of a large state. This information could reassure the public, support responsible reopening of local economies where appropriate and provide early warning of outbreaks.

MATERIALS AND METHODS

Sample collection and handling

Ten wastewater treatment facilities were sampled during this study, which in combination treat wastewater generated by 1.26M Utah residents or 39% of the total population of 3.2M. The facilities are indicated in **Fig. 1** and included: Central Valley Water Reclamation Facility (CVWRF), Hyrum City Wastewater Treatment Plant (HCWWTP), Logan City Corporation WWTP (LCCWWTP), Price River Water Improvement District (PRWID), Moab City WWTP (MCWWTP), Orem WRF (OWRF), Salt Lake City WRF (SLCWRF), Snyderville Basin Water Reclamation District-East Canyon Water Reclamation Facility (ECWRF), Timpanogos Special Service District (TSSD), and Tremonton WWTP (TWWTP). Samples were collected from April 1 to May 28, 2020 and typically consisted of 1-L subsamples of a

refrigerated 24-hr flow weighted composite sample. The only exceptions to this were the ECWRF samples that were grab samples from the grit chamber from April 1 to April 16 and a 6-hr flow weighted composite afterwards. The eight sewer interceptors of sub-sewersheds of CVWRF were sampled on April 13, 15 and 17 and on May 6. These interceptors collected wastewater from Cottonwood Improvement District (CID), Granger-Hunter Improvement District (GRA), Kearns Improvement District (KRN), Mount Olympus Improvement District East (MOIDE) and South (MOIDS), Murray City (MUR), South Salt Lake City (SSL), and Taylorsville-Bennion Improvement District (TAY). Wastewater utilities provided average flow rates for the time period of sample collection (**Table 1**).

To ensure limited personnel exposure to wastewater potentially containing infectious SARS-CoV-2, all samples were handled according to Institutional Biosafety Committee approved protocols, utility specific safety plans and US Department of Transportation Hazardous Materials Regulation (HMR; 49 C.F.R., Parts 171-180). After collection of the wastewater by the utility personnel, samples were transferred to non-sterile 1-L polypropylene collection bottles and the exterior of the bottle was bleached. The bottles were then transferred to secondary storage containers and transported at 4^{o}C within 1 to 8 hr to either the University of Utah, Utah State University or Brigham Young University. Herein, the labs will be referred to as lab 1, 2 or 3, respectively. Upon receipt in the laboratory the samples were immediately heated to 65^{o}C for a minimum of 1 hr in either a water bath or an incubator to inactivate SARS-CoV-2. [48]

After inactivation, samples were centrifuged at 4000xg for 20 minutes. Supernatant was then acidified to pH 3.0 to 3.5 with 1.0 N HCl. Acidification of the sample increased acidity of virus capsid proteins and virus RNA, which were then filtered through a negatively-charged mixed cellulose ester 0.45 uM membrane filters (Fisher Scientific, USA). [49] Following membrane filtration, the samples were placed in sterile 50 mL centrifuge tubes and frozen at -80^{o}C. Frozen and shattered filters were taken into the nucleic acid extraction protocols. To assess the number of virus gene copies per mass of solids, RNA from eight post centrifugation samples from CVWRF, LCCWWTP, ORWF and PRWID were also extracted. Finally, total suspended solids were determined in samples, in which, the virus gene copies per mass of solids was determined using Standard Methods [50].

Nucleic acid extraction

Extraction of the nucleic acids from the frozen and shattered filters followed previously published manual RNA extraction methods [51] or the RNEasy Power Water extraction kit (Qiagen, USA). Lab 1 and lab 2 used the manual extraction method, while lab 3 used the RNEasy kit. To assess the mass of virus RNA on solids compared to that suspended in solution, RNA from the wastewater solids recovered from the centrifugation step were also extracted using the same methods. Resulting RNA concentrations were quantified by a plate reader with a Take3 plate (BioTek, USA), nanodrop (ThermoScientific, USA) or fluorometer (Qubit, Invitrogen) and were diluted to working concentrations of 25 to 50 ng/µl.

RT-qPCR

Determination of the number of viral gene copies per mL of wastewater was determined by RT-qPCR. Primers and probes used for this study included the N1 and N2 primers and probe mix (2019-nCoV RUO, Integrated DNA Technologies, USA). Each 20 µl RT-qPCR reaction included 1X mastermix (either TaqPath™ 1-step RT-qPCR from Thermofisher or qScript XLT One-step RT-qPCR from Quantabio), 1.5 µM N1 primer/probe mix, 1.5 µM N2 primer/probe mix, 5 µl of template RNA at 25 to 50 ng/µl and PCR grade water. Thermocyclers used for the RT-qPCR included a QuantStudio 3 (ThermoFisher Scientific, USA) at lab 1 and lab 2 and a Quantabio (USA) at lab 3. The thermocycler conditions were used without modification from the CDC guidance [52]. Briefly, at lab 1 and lab 2 the thermocycler conditions were: an initial step of 25°C for 2 minutes; 50°C for 15 minutes; 95°C for 2 minutes; and 45 cycles of denaturation at 95°C for 3 seconds and annealing at 55°C for 30 seconds. At lab 3 the thermocycler conditions were: an initial step of 50°C for 10 minutes; 95°C for 3 minutes; and 45 cycles of denaturation at 95°C for 3 seconds and annealing at 55°C for 30 seconds. Each RT-qPCR run included positive controls consisting of 2019-nCoV_N_Positive Control (Integrated DNA Technologies, USA, hereafter positive control) and negative amplification controls consisting of 5 µl of PCR grade water. RT-qPCR assays were run in singlet (week 1 and 2) or triplicate (week 3 to 9). Virus concentrations were determined by comparing Ct values of samples against an assay-specific standard curve from a dilution of the positive control. Standard curves were made using a six-fold dilution of the positive control and the minimum detection limits (MDL) per RT-qPCR reaction and reaction efficiencies were as follows: Lab 1 MDL of 2 gene copies/µL RT-qPCR reaction and 93% efficiency, Lab 2 MDL of 0.7 gene copies/µL RT-qPCR reaction and 97% efficiency, Lab 3 MDL of 2 gene copies/µL RT-qPCR reaction and 98% efficiency. Dilution

359 factors from the filtered sample volume, the RNA extraction procedure, and the RNA-containing sample
360 volume per well in the RT-qPCR assay resulted in the calculated gene copy/mL of wastewater.

361 **Virus RNA signal decay during storage**

362 To assess the influence of sewer travel time and sample storage conditions on the decay of SARS-CoV-2
363 RNA signal in wastewater several decay studies were conducted. Specifically, influent wastewater to
364 CVWRF was incubated in non-sterile 250 mL PP bottles at 4, 10 and 35^{o}C without inactivation for 1 to 22
365 hr until processing by the methods presented above. Replicate samples collected at the same time as the
366 250 mL samples were processed immediately and assumed to represent the initial concentration of virus
367 in the incubated samples. To compare effects of refrigeration and freezing on the virus in wastewater,
368 influent to LCCWWTP and HCWWTP was incubated in sterile centrifuge tubes at 4^{o}C and -80^{o}C without
369 inactivation for 6 hr, 24 hr and 7 d, respectively. After storage for the required time, frozen samples were
370 then thawed and processed by the methods above and the virus RNA abundance was compared to
371 samples that were processed immediately upon arrival at the laboratory.

372 **Cross lab validation study on split samples**

373 To evaluate the reproducibility of SARS-CoV-2 RNA detection and quantitation in wastewater between
374 labs, split samples from four utilities were shared among testing laboratories on three different sampling
375 events. Each sample was filtered in singlet or triplicate, RNA from each filter was extracted and then
376 triplicate RT-qPCR was performed. Analysis of variance (ANOVA) or general linear modeling (GLM) of
377 the reproducibility of the analysis was performed in SAS (ver. 9.4; SAS Institute, Inc., Cary, NC).

378 **GIS census population overlay with sewershed maps and COVID cases**

379 To determine the area served by the individual treatment plants, sewershed polygons were either
380 provided by the individual utilities or were extracted from city boundaries. Polygons of adjacent cities were
381 clipped as necessary to prevent overlapping boundaries in the GIS shapefiles. These shapefiles were
382 used to define the sewersheds served by each utility. Populations served by each utility were estimated
383 by geocoding addresses for 3.2M current residents of Utah and summing the number of individuals
384 whose residence fell within each sewershed. These data were provided by the Utah Population
385 Database, a collection of administrative data compiled from vital statistics, driver license, voter registration

and healthcare claims provided by the State of Utah [53]. Over 97% of the provided addresses were geocoded with high confidence to the street segment or address point. COVID-19 daily and weekly counts of new cases were provided by the Utah Department of Health within the specific sewershed polygons provided to the Department of Health. If less than 5 cases were present in a sewershed during the specified time period, the data were suppressed and listed as <5 cases to protect privacy and avoid identifiability. For these time periods and sewersheds, it was assumed that one individual in the sewershed was ill with COVID-19 and the case load per 100,000 individuals was estimated.

Statistical methods and data management

The million viral gene copies per capita per day in a sewershed were estimated by multiplying (gene copies/L wastewater) X (L wastewater influent/day) X (1/sewershed population). Converting GC/mL wastewater to ill individuals in a sewershed was estimated by: (gene copies/L wastewater) X (L wastewater influent/d) X (mL feces/$10^{4.7}$ SARS-CoV-2 GC) X 1/(500 mL feces/d/person) X (1/sewershed population) X (1/0.26 recovery). Feces defecation rates were assumed to be at the lower range of feces defecation for individuals suffering from gastroenteritis, which are reported to range from 500 to 6,000 mL/day/person [54]. The SARS-CoV-2 virus recovery percentages by the membrane filtration methods used herein were assumed to be similar to those reported by others for MHV (i.e., 26%) [30]. Spearman correlations of MVGC/cap/d with COVID-19 case counts and linear regressions were calculated using SAS (ver. 9.4; SAS Institute, Inc., Cary, NC).

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AUTHOR CONTRIBUTIONS

J.W. initiated the project, directed the method development and sample analysis at UofU, interpreted the data and drafted the manuscript; **Z.A.** directed the sample analysis at BYU, contributed to data analysis and edited the manuscript; **D.K.R.** directed the sample analysis at USU, contributed to data analysis and edited the manuscript; **J.V.** contributed to research planning and coordination, provided population data for sewersheds, contributed to data analysis and interpretation; **E.G.** aided in overall project planning, directed the coordination of sample collection and data management, contributed to data analysis, and edited the manuscript; **J.O.** organized and coordinated sample collection and delivery, and aided in data analysis; **K.H.** coordinated sample collection and delivery, interactions with facilities and project planning; **R.J.** conducted the initial method development, conducted sampling and writing of the manuscript; **P.H**. aided in project planning, coordination and interpretation of data in sewersheds; **Y.Z.** completed the correlation and trend analysis; **K.T.** conducted sampling and analysis at BYU and aided in writing the manuscript; **J.V.L.** aided in GIS and data visualization and interpretation; **N.L.** aided in project planning and coordination with public health departments, provided COVID-19 case count data by sewershed, aided in data interpretation and edited the manuscript.

COMPETING INTERESTS

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

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Table 1. Facilities sampled organized by average flows (MGD), populations served and observed average (AVG) and standard deviation (SD) of SARS-COV-2 in the influent, effluent and sub-sewershed samples.

Facility/Type [a]	AVG (SD) flow rates, MGD	Population served	AVG gal/capita/day	No. of samples/ % positive	AVG (SD) of SARS-CoV-2, GC/L [b]	AVG (SD) of SARS-CoV-2, MVGC/ capita/day	AVG (SD) of daily new COVID-19 cases/100K [c]
CVWRF/INF	51.4 (0.7)	515494	100	25 / 96	479 (495)	1810 (1871)	8.2 (3.1)
CVWRF-CID/INT	8.1 (0.5)	91827	88	4 / 75	129 (201)	464 (737)	4 (4)
CVWRF-GRA/INT	14.7 (0.2)	143285	103	4 / 100	1038 (1294)	4087 (5115)	16 (12)
CVWRF-KRN/INT	3.4 (0.6)	55069	61	4 / 75	48 (116)	99 (237)	13 (14)
CVWRF-MOIDE/INT	7.1 (0.4)	65424	108	4 / 50	460 (695)	1972 (2984)	5.9 (6.4)
CVWRF-MOIDS/INT	6.1 (0.1)	47820	129	4 / 100	197 (241)	942 (1141)	9.1 (13)
CVWRF-MUR/INT	3.7 (0.3)	35394	104	4 / 75	367 (398)	1485 (1708)	6.3 (6.3)
CVWRF-SSL/INT	3.2 (0.2)	9682	334	4 / 100	170 (160)	2118 (1998)	13 (12)
CVWRF-TAY/INT	4.7 (0.1)	66993	70	4 / 75	333 (365)	884 (950)	9.6 (8.7)
CVWRF/EFF	NA	NA	NA	1 / 0	ND [d]	ND	NA
SLCWRF/INF	31.6 (0.5)	209645	151	10 / 100	240 (303)	1376 (1748)	8.7 (4.0)
TSSD/INF	19.2 (2.4)	253098	76	15 / 40	23 (38)	64 (113)	2.4 (1.6)
LCCWWTP/INF	15.4 (2.5)	94005	164	10 / 50	35 (82)	240 (565)	2.4 (6.9)
OWRF/INF	8.7 (0.7)	112901	77	11 / 82	111 (124)	320 (368)	5.4 (4.4)
ECWRF/INF	2.9 (0.6)	23304	124	22 / 91	314 (573)	1534 (3210)	4.7 (8.3)
ECWRF/EFF	NA	NA	NA	1 / 0	ND	ND	NA

TWWTP/INF	1.43 (0.25)	12451	115	8 / 13	0.6 (1.7)	2.7 (8.2)	<5
PRWID/INF	1.3 (0.1)	17312	75	11 / 27	86 (267)	175 (525)	<5
MCWRF/INF	1.13 (0.25)	9896	114	10 / 60	52 (71)	221 (297)	<5
HCWWTP/INF	0.97 (0.09)	9095	106	9 / 56	121 (273)	531 (1244)	4.8 (16)

[a] INF = influent; INT = interceptor sample from sub-sewershed; EFF = effluent; CVWRF = Central Valley Water Reclamation Facility; CID = Cottonwood Improvement District; GRA = Granger-Hunter; KRN = Kearns; MOIDE = Mount Olympus Improvement District South; MOIDS = Mount Olympus Improvement District South; MUR = Murray; SSL = South Salt Lake City; TAY = Taylorsville-Bennion; SLCWRF = Salt Lake City Wastewater Reclamation Facility; TSSD = Timpanogos Special Service District; CH = Cedar Hills; SV = South Valley; VY = Vineyard; LCCWWTP = Logan City Corporation Wastewater Treatment Plant, OWRF = Orem Wastewater Reclamation Facility; ECWRF = East Canyon Water Reclamation Facility; TWWTP = Tremonton Wastewater Treatment Plant; PRWID = Price River Water Improvement District; MCWRF = Moab City Wastewater Reclamation Facility; HCWWTP = Hyrum City Wastewater Treatment Plant

[b] any ND samples in raw wastewater were assumed to be 0 for estimation of averages and standard deviations

[c] days with <5 new cases by sewershed or sub-sewershed were assumed to be 1 for averaging

[d] ND = not detected

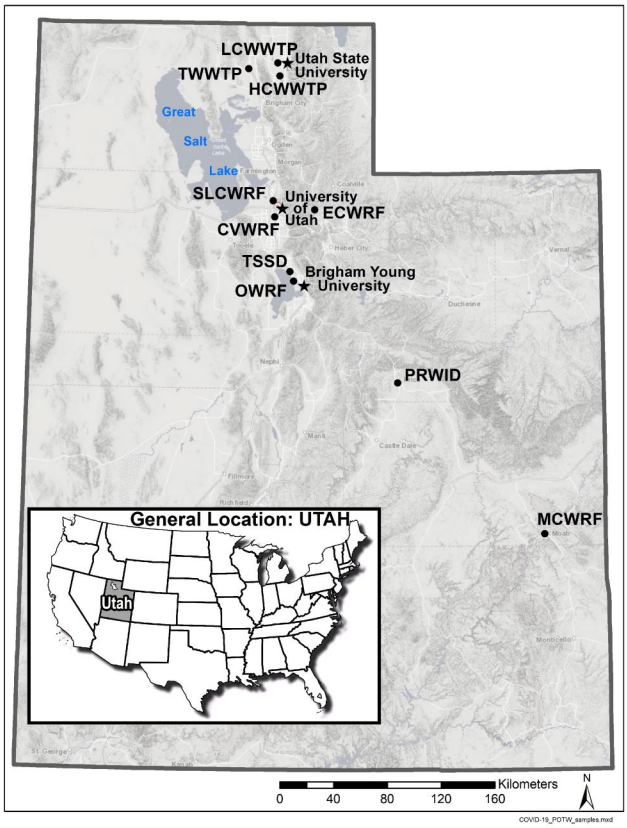

Fig. 1 Location of wastewater treatment plants sampled during this study, representing 1.26M individuals or 39% of Utah's population.

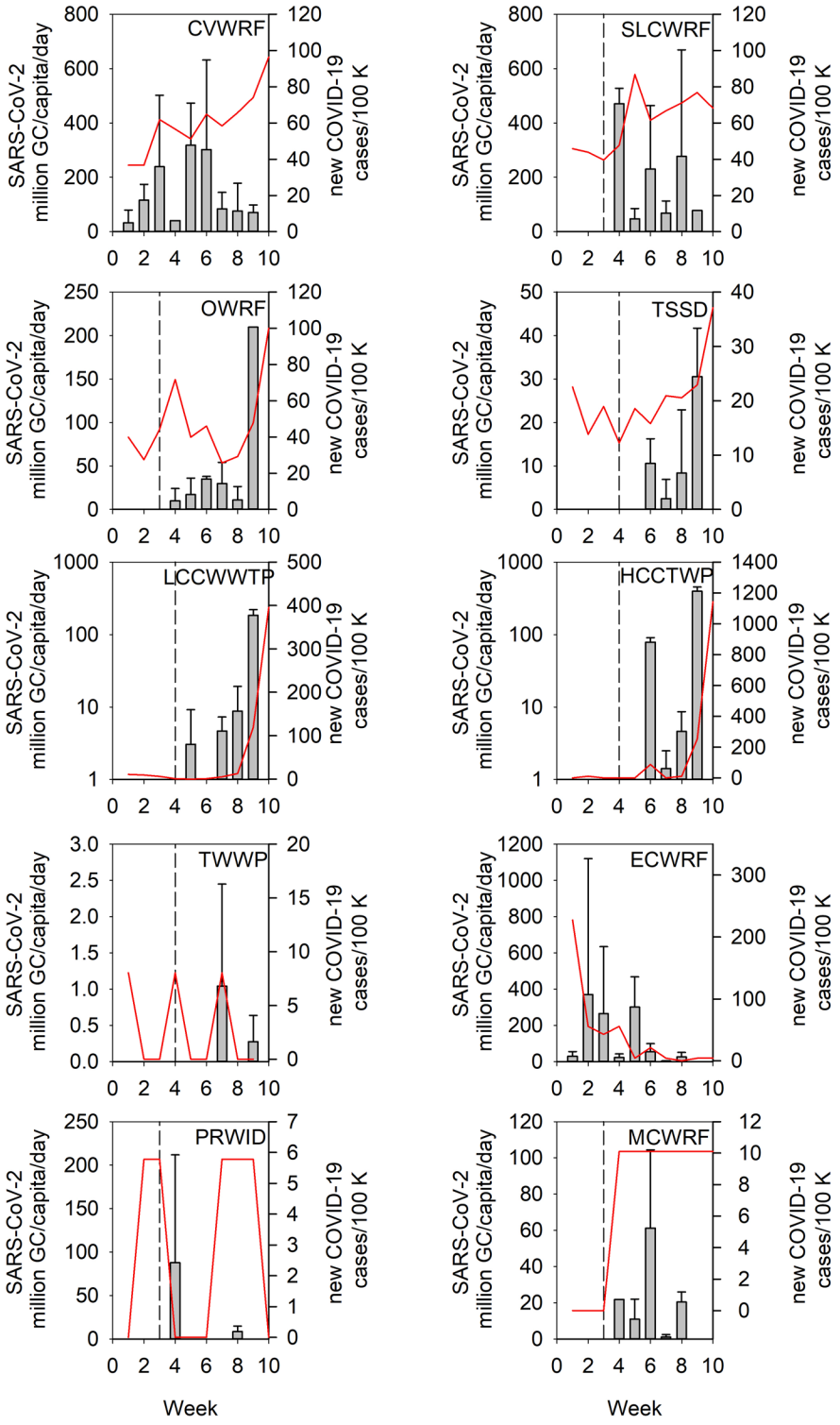

Fig. 2 Average and standard deviation of SARS-CoV-2 million viral GC/capita/day in wastewater (bars) compared to weekly COVID-19 case rate per 100,000 (red lines). Vertical dashed lines indicate the first week of sampling.

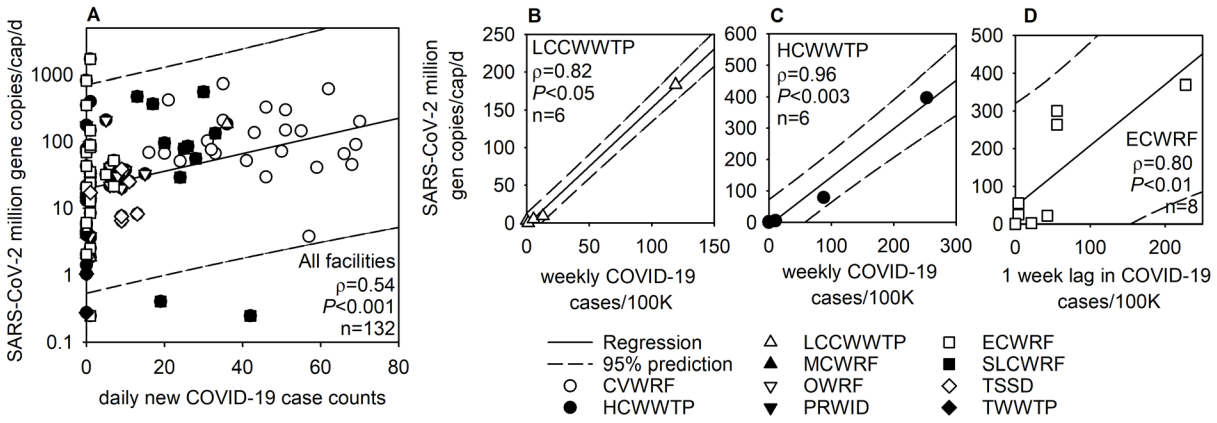

Fig. 3 Correlations between daily (plot A) or weekly (plots B, C, D) COVID-19 cases or cases/100K and SARS-CoV-2 million viral gene copies/capita/day in a sewershed. Plot D shows the 1-week lag in COVID-19 cases in ECWRF compared to the prior week SARS-CoV-2 RNA. Spearman correlations and 95% prediction intervals (dashed line) on the linear regressions (solid line) are shown in each figure.

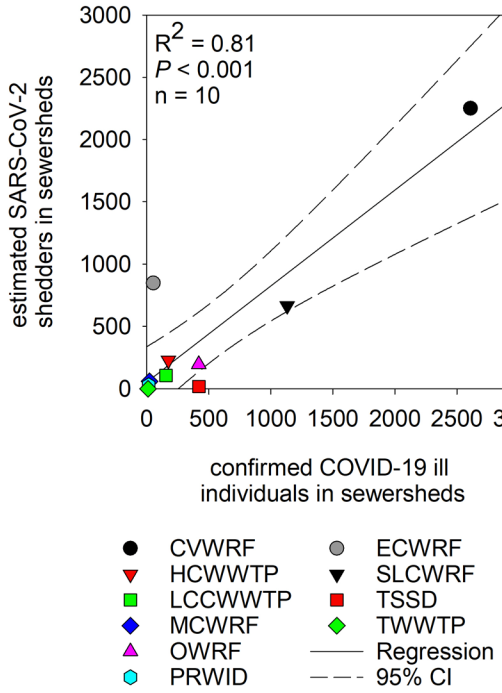

Fig. 4 Estimated sum of SARS-CoV-2 shedding individuals in sewersheds over the study period compared to the sum of confirmed COVID-19 cases.

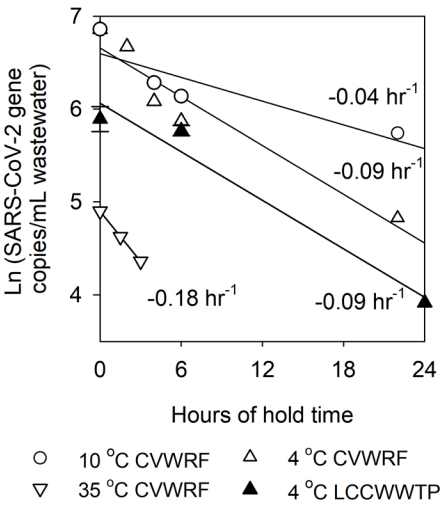

Fig. 5 Observed decay (symbols) of SARS-CoV-2 RNA in wastewater during storage at 4, 10 or 35°C and predicted first order decay rates (lines). Virus signal was not detectable after 12 hr of storage at 35°C. The grey area indicates the data used to estimate the first order decay rates. Correlation coefficients exceeded 95%.

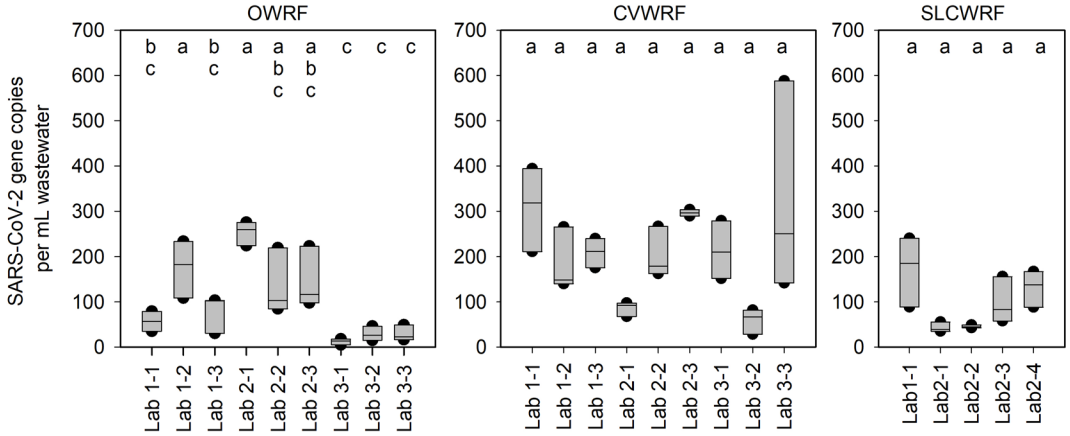

Fig. 6 Intra laboratory comparison of replicate filter extractions and triplicate qPCR assays. Bonferroni grouping of least square means (alpha = 0.05). Mean estimated gene copies/mL by filter, with the same letter are not significantly different. OWRF: significant difference in means among filters (GLM, $P < 0.001$, F = 9.52). CVWRF: No significant difference in means among filters (GLM, $P = 0.023$, F = 3.1). SLCWRF: No significant difference in means among filters (GLM, $P = 0.024$, F = 4.51).

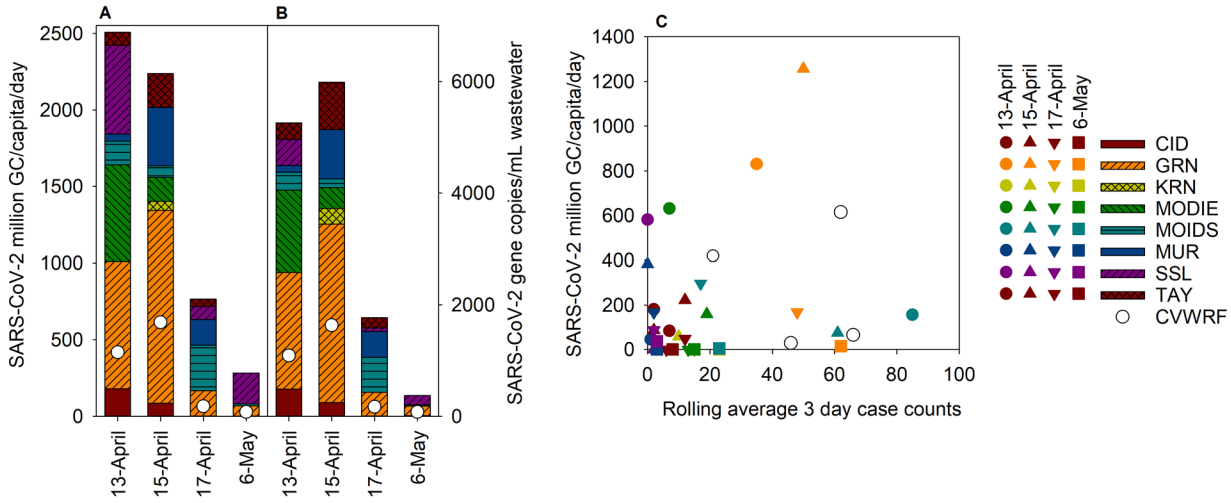

Fig.7 SARS-CoV-2 RNA in the sewer interceptors feeding CVWRF influenced by flow rates (plot A) and without considering flow rates (plot B), and correlation between SARS-CoV-2 million viral gene copies/capita/day in wastewater as compared to rolling 3-day average COVID-19 case counts in the cities contributing to CVWRF (plot C).

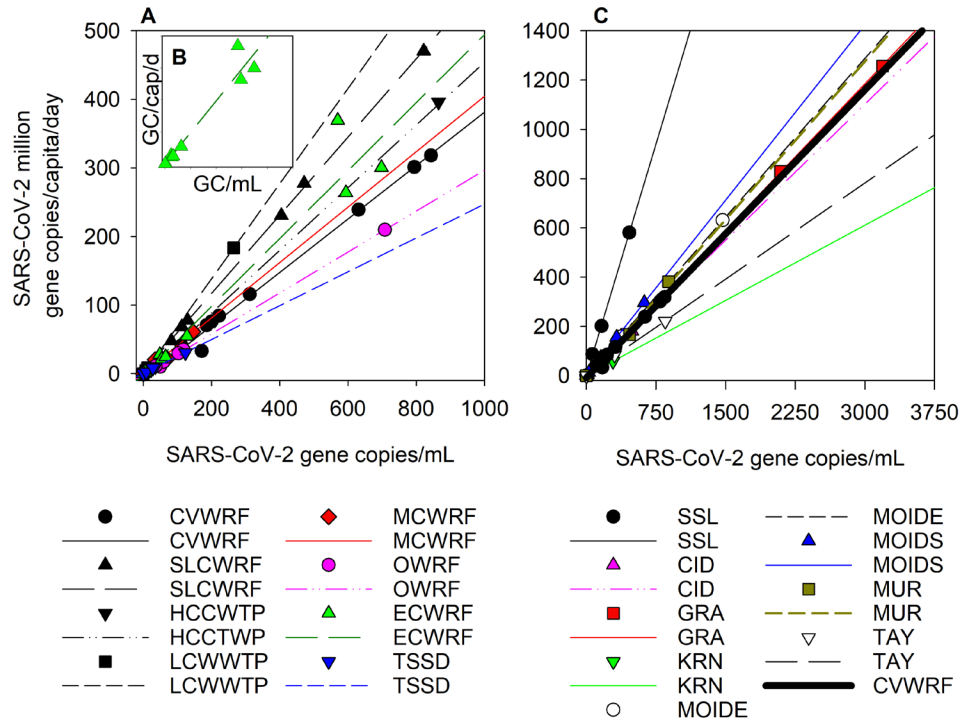

Fig 8. Effect of flow rates on relationship between SARS-CoV-2 million viral gene copies/capita/day or gene copies/mL wastewater. Plot A: eight facilities sampled with more than two detections of the virus RNA. Inset B: variation in the ECWRF flows. Plot C: sub-sewersheds feeding CVWRF as compared to the influent into the main plant (bold black line).