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

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HIGHLIGHTS

• Nine-week study of ten wastewater treatment facilities covering 1.26 M people.
• SARS-CoV-2 (SC2) RNA was detectable in 61% of 126 samples.
• COVID-19 cases were geolocated within sewershed boundary sampled.
• COVID-19 outbreaks in two communities correlated with SC2 and informed response.
• Economic value of wastewater epidemiology was valued at $0.005 to $0.10 per capita.

GRAPHICAL ABSTRACT

Infiltration/Inflow? Sewershed characteristics? GIS population estimates? Community characteristics?

Refining wastewater epidemiology

Informing public health response

ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes the coronavirus disease (COVID-19), is shed in feces and the viral ribonucleic acid (RNA) is detectable in wastewater. A nine-week wastewater epidemiology study of ten wastewater facilities, serving 39% of the state of Utah or 1.26 M individuals was conducted in April and May of 2020. COVID-19 cases were tabulated from within each sewershed boundary. RNA from SARS-CoV-2 was detectable in 61% of 126 wastewater samples. Urban sewersheds serving >100,000 individuals and tourist communities had higher detection frequencies. An outbreak of COVID-19 across two communities correlated with an increase in wastewater SARS-CoV-2 RNA, while a decline in COVID-19 cases preceded a decline in RNA. SARS-CoV-2 RNA followed a first order decay rate in wastewater, while 90% of the RNA was present in the liquid phase of the influent. Infiltration and inflow, virus decay and sewershed characteristics should be considered during correlation analysis of SARS-CoV-2 with COVID-19 cases. These results provide evidence of the utility of wastewater epidemiology to assist in public health responses to COVID-19.

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1. 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 (WHO, 2020). 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 (Azzi et al., 2020), serum and tissues (Wang et al., 2020b; Xiao et al., 2020). Further, SARS-CoV-2 infects cells in the gastrointestinal tract, specifically glandular epithelial cells (Xiao et al., 2020), 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 (Wang et al., 2020a). 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 (Hamming et al., 2004; Xu et al., 2020a). Detection of SARS-CoV-2 RNA in feces by molecular methods has been reported (Wang et al., 2005a; Xiao et al., 2020; Zhang et al., 2020) from pre-symptomatic individuals 1–5 days before the onset of the clinical symptoms (Tang et al., 2020; Wang et al., 2020a), from individuals with mild symptoms (Wong et al., 2020) and in individuals 7 to 11 days after symptoms have resolved are no longer considered infectious (Chen et al., 2020; Wang et al., 2020a; Wang et al., 2020b; Wu et al., 2020b). Concentrations of SARS-CoV-2 RNA in feces of nine patients in two studies hospitalized with COVID-19 had 3 to 8 log viral RNA gene copies/mL feces (median 4.8 log RNA GC/mL feces) (Cheung et al., 2020; Wolfel et al., 2020). 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). One report (Zhang et al., 2020) reported measuring infectious SARS-CoV-2 virus in feces, confirming an earlier report that infectious SARS-CoV virus persisted in fecal samples (Wang et al., 2005a). Another study, however reported no viable SARS-CoV-2 virus in feces, in spite of high viral RNA concentrations (Wolfel et al., 2020). Viral RNA was detected in urine in one report (Wang et al., 2020b) but not in others (Paoli et al., 2020; Peng et al., 2020).

Given that SARS-CoV-2 RNA is detectable in feces, testing for SARS-CoV-2 RNA in sewersheds allows for distributed monitoring of community disease burden (Sims and Kaspryzk-Hordern, 2020) for the estimated 2.1 billion people living in 105,600 wastewater treatment plant (WWTP) districts worldwide (Hart and Halden, 2020). This approach, termed wastewater epidemiology, has been widely used for monitoring for poliovirus eradication (Lago et al., 2003), correlations between norovirus and public health status (Prevost et al., 2015), and illicit drug use (Kankaanpää et al., 2016). 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 (Wang et al., 2005b), several recent reports have documented the presence of SARS-CoV-2 RNA 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 (Ahmed et al., 2020a). 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 (Wurtzer et al., 2020). Attempts to detect SARS-CoV RNA in wastewater were also successful in Canada (D’Aoust et al., 2021a; D’Aoust et al., 2021b), Italy (La Rosa et al., 2020; Randazzo et al., 2020), the Netherlands (Medema et al., 2020), Germany (Westhaus et al., 2020), Japan (Hata et al., 2020), United Arab Emirates (Hasan et al., 2020) and the United States (Nemudryi et al., 2020; Wu et al., 2020a), 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 1.2 × 10^4 to 3.2 × 10^6 gene copies/L in wastewater influent (Ahmed et al., 2020a; La Rosa et al., 2020; Nemudryi et al., 2020; Randazzo et al., 2020; Wu et al., 2020a; Wurtzer et al., 2020).

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 (Sims and Kaspryzk-Hordern, 2020). 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 provide an indication of the total burden of disease in that population, beyond just those individuals identified through COVID-19 testing. Increases or decreases in SARS-CoV-2 RNA in wastewater may indicate a change in the prevalence of shedders in a sewershed. Further, the changes in SARS-CoV-2 RNA concentration in wastewater may be observable before changes in the number of individuals who have tested positive as suggested by others (Medema et al., 2020; Nemudryi et al., 2020). Finally, wastewater monitoring may provide insight in areas with low documented case counts where clinical testing is difficult.

Given the presence of SARS-CoV-2 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 begun April 2020 at ten wastewater facilities in Utah that serve 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, sample collection during the early part of the pandemic with varying COVID-19 infection rates was required for understanding of trends in SARS-CoV-2 RNA loads and COVID-19 disease burden.

2. Materials and methods

2.1. Sample collection and handling

Ten wastewater treatment facilities were sampled during this study, which in combination treat wastewater generated by 1.26 M Utah residents or 39% of the total population of 3.2 M. The facilities are indicated in Fig. 1 and included: Central Valley Water Reclamation Facility (CVWRF), Hyrum City Wastewater Treatment Plant (HCCWWT), 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 (TWTP). Samples were collected from April 1 to May 28, 2020 and typically consisted of 1-L subsamples of a refrigerated 24-h 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-h 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 (MOID) and South...
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.

### Table 1

| Facility/type | AVG (SD) flow rates, MGD | Population served | AVG gal/capita/day | No. of samples/% positive | AVG (SD) of SARS-CoV-2, GC/mL | AVG (SD) of SARS-CoV-2, MGVGC/capita/day | AVG (SD) of daily new COVID-19 cases/100 K |
|--------------|--------------------------|------------------|-------------------|--------------------------|-----------------------------|----------------------------------------|------------------------------------------|
| CVWRF/INF   | 51.4 (0.7)               | 515,494          | 100               | 25 / 96                  | 479 (495)                   | 1810 (1871)                           | 8.2 (3.1)                               |
| CVWRF-CID/INT | 8.1 (0.5)              | 91,827           | 88                | 4 / 75                   | 129 (201)                   | 464 (737)                             | 4 (4)                                   |
| CVWRF-GRA/INT | 14.7 (0.2)             | 143,285          | 103               | 4 / 100                  | 1038 (1294)                 | 4087 (5115)                           | 16 (12)                                 |
| CVWRF-KRN/INF | 3.4 (0.6)              | 55,069           | 61                | 4 / 75                   | 48 (116)                    | 99 (237)                              | 13 (14)                                 |
| CVWRF-MOIDE/INF | 7.1 (0.4)            | 65,424           | 108               | 4 / 50                   | 460 (695)                   | 1972 (2984)                           | 5.9 (6.4)                               |
| CVWRF-MOIDS/INT | 6.1 (0.1)            | 47,820           | 129               | 4 / 100                  | 197 (241)                   | 942 (1141)                            | 9.1 (13)                                |
| CVWRF-MUR/INF | 3.7 (0.3)              | 35,394           | 104               | 4 / 75                   | 367 (398)                   | 1485 (1708)                           | 6.3 (6.3)                               |
| CVWRF-SSL/INF | 3.2 (0.2)              | 9882             | 334               | 4 / 100                  | 170 (160)                   | 2118 (1998)                           | 13 (12)                                 |
| CVWRF-TAY/INT | 4.7 (0.1)              | 66,993           | 70                | 4 / 75                   | 333 (365)                   | 884 (950)                             | 9.6 (8.7)                               |
| CVWRF/EFF    | NA                      | NA               | NA                | 1 / 0                    | BDL d                      | BDL                                   | NA                                       |
| SLCWRF/INF   | 31.6 (0.5)              | 209,645          | 151               | 10 / 100                 | 240 (303)                   | 1376 (1748)                           | 8.7 (4.0)                               |
| TSDD/INF     | 19.2 (2.4)              | 253,098          | 76                | 15 / 40                  | 23 (38)                    | 64 (113)                              | 2.4 (1.6)                               |
| LCCWWP/INF   | 15.4 (2.5)              | 94,005           | 164               | 10 / 50                  | 35 (82)                    | 240 (565)                             | 2.4 (6.3)                               |
| OWRF/INF     | 8.7 (0.7)               | 112,901          | 77                | 11 / 82                  | 111 (124)                  | 320 (368)                             | 5.4 (4.4)                               |
| ECWRF/INF    | 2.9 (0.6)               | 23,304           | 124               | 22 / 91                  | 314 (573)                  | 1534 (3210)                           | 4.7 (3.3)                               |
| ECWRF/EFF    | NA                      | NA               | NA                | 1 / 0                    | BDL                       | BDL                                   | NA                                       |
| TWWTP/INF    | 1.43 (0.25)             | 12,451           | 115               | 8 / 13                   | 0.6 (1.7)                  | 2.7 (8.2)                             | <5                                      |
| PRWID/INF    | 1.3 (0.1)               | 17,312           | 75                | 11 / 27                  | 86 (267)                   | 175 (525)                             | <5                                      |
| MCVRF/INF    | 1.13 (0.25)             | 9896             | 114               | 10 / 60                  | 52 (71)                    | 221 (297)                             | <5                                      |
| HHCWTP/INF   | 0.97 (0.09)             | 9095             | 106               | 9 / 56                   | 121 (273)                  | 531 (1244)                            | 4.8 (16)                                |

*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; TSDD = Timpanogos Special Service District; CH = Cedar Hills; SV = South Valley; VY = Vineyard; LCWWTP = 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; MCVRF = Moab City Wastewater Reclamation Facility; HHCWTP = Hyrum City Wastewater Treatment Plant.*

*Days with <5 new cases by sewershed or sub-sewershed were assumed to be 1 for averaging.*

*BDL = below estimated detection limits of 3 GC/mL.*

To ensure limited personnel exposure to wastewaters 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 wastewaters by the utility personnel, samples were transferred to non-sterile 1-L polypropylene collection bottles and the exterior of the bottle was bleached. The samples were then transferred to secondary storage containers and transported at 4 °C within 1 to 8 h 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 °C for a minimum of 1 h in either a water bath or an incubator to inactivate SARS-CoV-2 (Darnell and Taylor, 2006). Heat inactivation of all samples to achieve disinfection outlined in WHO guidelines was required by Institutional Biosafety Committees prior to processing handling wastewater for the protection of laboratory workers. Recent studies demonstrated that SARS-CoV-2 quantitation in replicate pasteurized and unpasteurized samples are not significantly different in samples that were not recovery corrected. In contrast the SARS-CoV-2 levels were slightly higher in pasteurized samples after recovery correction (Pecson et al., 2021).

After inactivation, samples were centrifuged at 4000 × g for 20 min to remove some of the solids and increase the amount of supernatant that could be filtered, similar to methods recommended by others (Ye et al., 2016). A total of 100 mL of 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 μm membrane filters (Fisher Scientific, USA) (Ahmed et al., 2015). Following membrane filtration, the filters that passed the 100 mL of wastewater were placed in sterile 50 mL centrifuge tubes and frozen at -80 °C. Frozen
and shattered filters were taken into the nucleic acid extraction protocols. Recent comparisons of 36 different SARS-CoV-2 wastewater detection methods among 27 laboratories, demonstrated that the methods used herein estimated SARS-CoV-2 GC/L levels that fell within the 10 to 90% range of all methods tested (Pecson et al., 2021). Further, the method detection limits for two of the labs herein that participated in this prior study were reported to be 3 GC/mL of wastewater. To assess the number of virus gene copies per mass of solids, RNA from the solids collected post centrifugation from CVWRF, LCCWWTP, ORWF and PRWID were also extracted and processed by directly placing the solids in bead beating tubes and proceeding as described in the next section. Finally, total suspended solids were determined in replicate samples from CVWRF, LCCWWTP, ORWF and PRWID using Standard Methods (Baird et al., 2017), so that the virus gene copies per mass of solids could be determined.

2.2. Nucleic acid extraction

Extraction of the nucleic acids from the frozen and shattered filters followed previously published manual RNA extraction methods (Griffiths et al., 2000) 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 to prevent RT-qPCR reaction inhibition due to excess template or inhibitors.

2.3. RT-qPCR

Determination of the number of viral gene copies per mL of wastewater was determined by reverse transcriptase quantitative polymerase chain reaction (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 1 × mastermix (either TaqPath™ 1-step RT-qPCR from ThermoFisher or qScript XLT One-step RT-qPCR from Quantabio), 1.5 mM N1 primer/probe mix, 1.5 mM 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 (CDC, 2020). Briefly, at lab 1 and lab 2 the thermocycler conditions were: an initial step of 25 °C for 2 min; 50 °C for 15 min; 95 °C for 2 min; and 45 cycles of denaturation at 95 °C for 3 s and annealing at 55 °C for 30 s. At lab 3 the thermocycler conditions were: an initial step of 50 °C for 10 min; 95 °C for 3 min; and 45 cycles of denaturation at 95 °C for 3 s and annealing at 55 °C for 30 s. Each RT-qPCR run included positive controls consisting of 2019-nCoV Negative 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 10 gene copies/5 μL RT-qPCR reaction and 93% efficiency, Lab 2 MDL of 10 gene copies/5 μL RT-qPCR reaction and 97% efficiency, Lab 3 MDL of 10 gene copies/5 μL RT-qPCR reaction and 98% efficiency. Dilution factors from the filtered sample volume, the RNA extraction procedure, and the RNA-containing sample volume per well in the RT-qPCR assay resulted in the calculated gene copy/mL of wastewater. Method detection limits of SARS-CoV-2 from wastewater, based on the recovery of spiked human beta coronavirus OC43 reported by two labs herein in other studies (Pecson et al., 2021) were <3 GC/mL or 1.2 ± 0.5 MVGC/cap/d for all facilities monitored.

2.4. Virus RNA signal decay during storage

To assess the influence of sewer travel time and sample storage conditions on the decay of SARS-CoV-2 RNA signal in wastewater several decay studies were conducted. Specifically, influent wastewater to CVWRF was collected in replicate non-sterile 250 mL PP bottles and transported to the laboratory. Upon arrival at the laboratory they were immediately incubated at 4, 10 and 35 °C for 1 to 22 h. Five bottles were incubated at each temperature. After the specified incubation period, one bottle at each temperature was sacrificed tested for SARS-CoV-2 using the methods presented above. Duplicate samples collected at the same time as the 250 mL samples were processed immediately and assumed to represent the initial concentration of virus in the incubated samples. To compare effects of refrigeration and freezing on the virus in wastewater, influent to LCCWWTP and HCWWTP was incubated in sterile centrifuge tubes at 4 °C and -80 °C for 6 h, 24 h and 7 d, respectively. After storage for the required time, frozen samples were then thawed and processed by the methods above. The virus RNA abundance in the incubated samples was compared to samples that were processed immediately upon arrival at the laboratory.

2.5. Cross lab validation study on split samples

To evaluate the reproducibility of SARS-CoV-2 RNA detection and quantitation in wastewater between labs, split samples from four utilities were shared among testing laboratories on three different sampling events. Each sample was filtered in singlet (n = 1) or triplicate (n = 10). RNA from each filter was extracted and then triplicate RT-qPCR was performed. RNA quality as measured by A260/280 ratios were suitable, although the RNA yields from the RNEasy Power Water kit were lower (96.3 ± 50 ng/μL) than the Griffiths et al. (2000) manual extraction method (1674 ± 432 ng/μL). Analysis of variance (ANOVA) or general linear modeling (GLM) of the reproducibility of the analysis was performed in SAS (ver. 9.4; SAS Institute, Inc., Cary, NC).

2.6. GIS census population overlay with sewershed maps and COVID cases

To determine the area served by the individual treatment plants, sewershed polygons were either provided by the individual utilities or were extracted from city boundaries. Polygons of adjacent cities were clipped as necessary to prevent overlapping boundaries in the GIS shapefiles. These shapefiles were used to define the sewersheds served by each utility. Populations served by each utility were estimated by geocoding addresses for 3.2 M current residents of Utah and summing the number of individuals whose residence fell within each sewershed. These data were provided by the Utah Population Database, a collection of administrative data compiled from vital statistics, driver license, voter registration and healthcare claims provided by the State of Utah (UPDB, 2020). 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.

2.7. 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
influential/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/10^17 SARS-CoV-2 GC) X (1/500 mL feces/d/person) X (1/sewershed population) X (1/0.26 recovery). Feces defection rates were assumed to be at the lower range of feces defection for individuals suffering from gastroenteritis, which are reported to range from 500 to 6000 mL/day/person (Spindelboeck et al., 2017). 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%) (Ahmed et al., 2020b) or greater (i.e., 90%) as reported for human beta coronavirus OC43 (Pecson et al., 2021). 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).

3. Results and discussion

3.1. 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-influent samples. All ten facilities had at least one detection of viral RNA during the study period (Table 1). Facilities in areas that serve more than 100,000 people had higher detection frequencies (i.e., CVWRF 96%, TSSD 40%, SLCWWF 100%, and OWRF 82%, see Table 1 for facility abbreviations) as compared to facilities serving smaller communities (i.e., HCCWWTP 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 highest frequency for the murine hepatitis virus, a surrogate for SARS-CoV-2 (Randazzo et al., 2020).

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 SLCWWF) had 2443 confirmed COVID-19 cases, while Summit County (served in part by ECWRF) had 240 confirmed COVID-19 cases (Anonymous, 2020b; Anonymous, 2020c). 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 (Anonymous, 2020a). 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 weekly number of COVID-19 cases reported to the Utah Department of Health that occurred within each sewershed boundary was determined based on the geocoded residential addresses of the cases. 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 (i.e., those serving Park City [ECWRF], Hyrum [HCWWT] and Logan [LCCWWTP]). 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/100 K (19 ± 21) to <5. Second, both HCCWWTP and LCCWWTP wastewater viral loads increased sharply (109 and 101% increase) in the last three weeks of the study period just prior to increases in weekly COVID-19 case rates from 5 or less to >252 cases/100 K (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 HCCWWTP (Fig. 3B and C), 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, p = 0.80, n = 8, P = 0.01). This week lag did not result in a significant correlation in the facilities with outbreaks (LCCWWTP and HCWWTP) nor the other facilities besides ECWRF. These correlations with LCCWWTP, HCWWTP, and ECWRF suggest that the increase in case counts may occur concurrently or 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 (Nemudryi et al., 2020; Peccia et al., 2020; Randazzo et al., 2020; Wurzler et al., 2020). 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 (Xu et al., 2020b). 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. It should be noted that the new COVID-19 cases reported per week by sewershed likely were an underestimate as asymptomatic or pre-symptomatic individuals may have been shedding SARS-CoV-2 in the sewershed and not have been reported as
COVID-19 positive to health departments. Studies in Utah have suggested asymptomatic COVID-19 positives were prevalent in 2–5% of the pediatric and adult population (Laws et al., 2020; Waltenburg et al., 2020). However, a recent review of the literature reported a range of pre-symptomatic or asymptomatic COVID-19 cases averaging 43 ± 28% (Meyerowitz et al., 2020). Asymptomatic or pre-symptomatic individuals shedding SARS-CoV-2 may have influenced the correlation evaluation herein.
3.2. 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 actual number of infected individuals in that area – those that have and have not been clinically identified. To more accurately assess the number of infected individuals in a sewershed, an 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 RNA in feces (median 4.7 log RNA GC/mL feces) (Cheung et al., 2020), 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. Further, it is important to note that the Department of Health case counts are tied to a residential address, yet ill individuals may be residing or working in locations outside the expected sewershed or may be present in hospitals not in the same sewershed as their residence. In our study area’s the number of hospital beds accounted for only 0.8 ± 0.5% of the total sewershed population. Further, only a subset of these beds would have been occupied by patients hospitalized for COVID-19. 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.

In evaluating Fig. 4 it can be seen that the estimated wastewater SARS-CoV-2 concentration compared to case counts is 0.78:1. This suggests that the estimated sum of SARS-CoV-2 shedders is less than the sum of confirmed cases during the study period. While theoretically this would be a 1:1 ratio, there are several possible explanations for the discrepancy including (1) not all COVID-19 individuals shed the virus in their feces and the length and duration of shedding may vary between individuals, (2) weekly or bi-weekly wastewater sampling compared to daily case counts underestimating RNA abundance, (3) decay of the RNA signal in wastewater, or (4) inefficiencies in the sample processing method. Further, research in fecal shedding of the SARS-CoV-2 RNA in symptomatic and asymptomatic COVID-19 infected individuals is required to address some of these uncertainties.

Numerous physical, chemical and biological factors may influence the persistence of viral RNA in wastewater. These factors include temperature, sunlight, ionic strength, presence of antiviral chemical constituents (Nannou et al., 2020), solids content, residence time in the sewer, microbial antagonism (Boehm et al., 2019; Tennant et al., 1994) 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. Initial concentrations of the virus RNA in the wastewater used for the decay studies ranged from 135 to 953 gene copies per mL of wastewater. Overall the results indicate a first order decay rate of the viral RNA ranging from 0.09 to 0.12 h⁻¹ over the 22 to 24 h at 4, 10 and 35 °C. While the RNA was not detectable after 6 h at 35 °C, the RNA was still detectable after 22 h 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 h, 86.5 ± 0.5% at 4 °C over 24 h and 92.4 ± 10.3% at -80 °C over one week. These results presented herein on the effect of temperature, indicate that the SARS-CoV-2 RNA is quite labile in wastewater as suggested by others based on studies of SARS-CoV and surrogate coronaviruses. Reported decay rates for SARS-CoV and surrogate coronaviruses in unpasteurized wastewater at 23 °C range from 0.02 to 0.143 h⁻¹ (Gundy et al., 2008; Hart and Halden, 2020; Ye et al., 2016), well within the range reported herein. Similarly, the inactivation of MHV was reported to be 0.048 h⁻¹ at 4 °C, 0.059 h⁻¹ at 10 °C and 0.142 h⁻¹ at 25 °C. Others 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 (Wang et al., 2005b). These prior studies corroborate our findings of loss of SARS-CoV-2 RNA in wastewater. Recent reports of the decay rates of gamma irradiated SARS-CoV-2 spiked into untreated wastewater were 0.0035 h⁻¹ at 4 °C and 0.012 h⁻¹ at 37 °C (Ahmed et al., 2020c). Use of gamma irradiated SARS-CoV-2 may not necessarily represent the mixture of SARS-CoV-2 likely present in wastewater that could be detected by RT-qPCR. Specifically, SARS-CoV-2 shed with feces may contain intact virus, capsid compromised virus and free nucleic acids. Therefore, it is likely the more rapid decay of SARS-CoV-2 RNA measured by RT-qPCR in the studies herein reflect the decay of all three of these expected viral RNA sources which would more typically be found in naturally contaminated wastewater. Given that the wastewater residence time in some larger cities may be up to 13 h (Kapo et al., 2017), whereas smaller cities such as ECWRF had a 1 to 3-h 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 split 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. There was no significant difference in means among the three labs in the CVWRF and SLCWRF samples, but one of three filters from lab 1 and lab 2 did differ from the lab 3 results for the OWRF sample. This variation between filters was used to refine sample processing to include multiple filters for all wastewater samples processed in each of the labs. These results suggest the data when using replicate filters and RT-qPCR assays 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 influent solids (9 ± 12% by mass). This finding is similar to that reported for MHV, where 70% of the enveloped virus was associated with the wastewater liquids at equilibrium (Ye et al., 2016). A recent study showed that the removal of solids prior to SARS-CoV-2 quantitation did not systematically impact recovery corrected SARS-CoV-2 estimated concentrations. Specifically, as reported in Pecson et al., (2021), the methods used herein on five replicates of two influent samples from treatment plants in California, showed that solids removal reduced the average SARS-CoV-2 concentrations by 15%. Interestingly, some methods that removed solids prior to ultrafiltration showed a higher concentration of recovery corrected SARS-CoV-2 than ultrafiltration without solids removal (Pecson et al., 2021). Others have reported the detection of the SARS-CoV-2 RNA on activated sludge in treatment plants (Kocamemi et al., 2020; Peccia et al., 2020). Given that most wastewater is near neutral pH and the SARS-CoV-2 spike proteins and cytidine can be protonated on N1 and N3 atoms, respectively, with 3.8 and 4.3 solution pKa (Bink et al., 2002; Izatt et al., 1971), 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 pKa (Bink et al., 2002; Izatt et al., 1971). 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.

3.3. 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 9682 people to 143,285 people) within a larger
nursing communities could be sampled. However, there are several
For example, university dormitories, athletic facilities or retirement or
in using this type of sampling in smaller communities or even buildings.
infected individuals within a sewershed, and there is increasing interest
pretation of a widespread wastewater epidemiology effort.
I/I, and other sewer-specific factors will be important in the data inter-
cations for using SARS-CoV-2 RNA wastewater data to inform public
health interventions (Daughton, 2020). First, wastewater monitoring
flow rate on SARS-CoV-2 RNA abundance by city (Fig. 7A and B). In some cases, the
SARS-CoV-2 RNA abundance was greater than that measured at the in-
fluential 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 in
point, thus, resulting in an apparent lower treatment plant in

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, \( P = 0.023 \), \( F = 3.31 \)). SLCWRF: No significant difference in means among filters (GLM, \( P = 0.024 \), \( F = 4.51 \)).

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 bur-
den. The sewer lines feeding into CVWRF showed significant variation in
SARS-CoV-2 RNA abundance by city (Fig. 6A and B). In some cases, the
SARS-CoV-2 RNA abundance was greater than that measured at the in-
fluential 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 °C. Further,
accounting for wastewater sewer travel time, I/I, and other sewer-specific factors will be important in the data inter-
pretation of a widespread wastewater epidemiology effort.

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

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).
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 (Brouwer et al., 2018). 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.

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 ($0.005 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. Estimated costs for individual clinical testing by nasopharyngeal swabs vary from $10 to $15 per test (Esin et al., 2020; Won et al., 2020). While this cost could be lowered by batched clinical testing (i.e., multiple individual swab tests are pooled for analysis), current reports suggest pooling only 5 individual samples at a time (Abdalhamid et al., 2020). A further use of wastewater epidemiology is for providing confirmation of low levels of community infection in many areas of a large state. The lack of detection of the SARS-CoV-2 RNA in wastewater should be used as one among other lines of evidence that COVID-19 ill individuals are not present in a sewershed. A lack of detection of the virus in wastewater could be a false negative for a variety of reasons including (1) the RNA is present below the detection limit of the assay, (2) the wastewater sampling scheme failed to collect feces and wastewater contributed by the COVID-19 ill individuals or (3) decay of the RNA occurred during transport in the sewershed to the sample collection point. Cumulatively, however, this report and others suggest that a lack of detection of SARS-CoV-2 RNA in a sewershed likely suggests a low prevalence of COVID-19 cases. This information could reassure the public, support responsible reopening of local economies where appropriate and provide early warning of outbreaks.

CRediT authorship contribution statement

J.W. initiated the project, directed the method development and sample analysis at UoU, 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 data 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 sewersheds, aided in data interpretation and edited the manuscript.

Declaration of competing interest

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