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Monitoring of SARS-CoV-2 in sewersheds with low COVID-19 cases using a passive sampling technique

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ABSTRACT

Monitoring SARS-CoV-2 RNA in sewer systems, upstream of a wastewater treatment plant, is an effective approach for understanding potential COVID-19 transmission in communities with higher spatial resolutions. Passive sampling devices provide a practical solution for frequent sampling within sewer networks where the use of autosamplers is not feasible. Currently, the design of upstream sampling is impeded by limited understanding of the fate of SARS-CoV-2 RNA in sewers and the sensitivity of passive samplers for the number of infected individuals in a catchment. In this study, passive samplers containing electronegative membranes were applied for at least 24-h continuous sampling in sewer systems. When monitoring SARS-CoV-2 along a trunk sewer pipe, we found RNA signals decreased proportionally to increasing dilutions, with non-detects occurring at the end of pipe. The passive sampling membranes were able to detect SARS-CoV-2 shed by >2 COVID-19 infection cases in 10,000 people. Moreover, upstream monitoring in multiple sewersheds using passive samplers identified the emergence of SARS-CoV-2 in wastewater one week ahead of clinical reporting and reflected the spatiotemporal spread of a COVID-19 cluster within a city. This study provides important information to guide the development of wastewater surveillance strategies at catchment and subcatchment levels using different sampling techniques.

1. Introduction

Wastewater surveillance is an effective and robust tool to understand the spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the population (Ahmed et al., 2020a; Wu et al., 2022). SARS-CoV-2 RNA fragments can be shed at the pre-symptomatic stage and detectable in wastewater days to weeks ahead of clinical testing, which enables early warning of incidence of coronavirus disease 2019 (COVID-19) (Medema et al., 2020; Saguti et al., 2021; Wu et al., 2022). In most surveillance programs, it is typical to collect composite wastewater samples at the wastewater treatment plant (WWTP) inlet, where autosamplers are readily available. For regions aiming to reduce SARS-CoV-2 transmission to near-zero levels, the limitation of WWTP-based sampling is that the results reveal the presence of viruses in the entire catchment with many thousands to millions of inhabitants, including RNA signals from hospitals and quarantine facilities. This thus hinders the early warning potential of wastewater surveillance to detect likely community transmission before more individuals become symptomatic and present for clinical testing. In regions where the society is living with COVID-19, holistic and evidence-based health data is required to inform response to the potentially surging caseload and unforeseen challenges such as new variants. Alongside testing and vaccine information, wastewater surveillance at both catchment and suburb levels can provide complementary information (CDC, 2022; Queensland Government, 2022; Victoria Government, 2022).

More recently, it is recognized that sampling in small sewersheds, namely the geographically small subcatchments, can be useful for mapping the spatiotemporal trends in the spread of COVID-19 at a finer resolution and assisting the planning of more localized public health responses. Monitoring sewersheds that exclude the inputs from known

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sources such as hospitals or quarantine facilities can enable the detection of disease emergence in local communities, narrow down source locations, and reduce uncertainties related to dilution and viral RNA decay in sewers (Ahmed et al., 2022b). To date, applications of upstream wastewater monitoring for SARS-CoV-2 have been carried out at schools and colleges (Barich and Slonczewski, 2021; Betancourt et al., 2021; Bivins et al., 2021; Gibas et al., 2021; Gutierrez et al., 2021; Liu et al., 2020), nursing facilities and hospitals (Davo et al., 2021; Goncalves et al., 2021; Jørgensen et al., 2020; Karthikeyan et al., 2021; Schang et al., 2021; Spurbeck et al., 2021), and in sewersheds at the intra-city scale (Albastaki et al., 2021; Saguti et al., 2021; Weidhaas et al., 2021; Wu et al., 2022). Wastewater COVID-19 monitoring in upstream catchments has been recognized as a strategy that promotes faster public health actions in the management of the epidemic (Black et al., 2021; Prado et al., 2020), e.g., to provide timely message to local residents and urge for mass clinical testing.

The major challenge for upstream monitoring is to collect representative samples at appropriate locations within the entire catchment. Small catchments and low case numbers could normally result in the short-term fluctuation of virus loads, leading to a great challenge of capturing as many toilet flushing events as possible to detect the sporadic pulses of SARS-CoV-2 RNA in wastewater. Detecting such pulses requires an increased sampling frequency to capture the shedding events of a small number of infected cases, preferably over 24-h period (Ahmed et al., 2021). Conventional autosamplers may not be sustainable for long-term upstream sampling due to their high expense, requirement of safe and secure space, and potential health and safety concern raising from residents, especially when considering that sewer manholes could be located near road, pedestrian, and in public areas. The use of passive samplers may be an alternative in this regard as they can capture and retain SARS-CoV-2 RNA in wastewater (Bivins et al., 2021; Hayes et al., 2021; Liu et al., 2020; Schang et al., 2021; Wang et al., 2022), and can be deployed within an enclosed manhole without visibility or accessibility to publics. Recent studies demonstrated that passive samplers containing electronegative membranes could achieve time-integrative sampling with continuous uptake of viruses over 48 h (Habtewold et al., 2022; Hayes et al., 2022; Li et al., 2022). The low investment and maintenance cost are also favorable properties for the application of passive sampling in upstream monitoring, which may require intensive sampling events (e.g., daily) at multiple locations (Harris-Lovet et al., 2021).

Although previous studies have demonstrated the capability of passive sampling to detect SARS-CoV-2 RNA fragments in wastewater, systematic studies are required to understand the fate of SARS-CoV-2 in urban sewer systems and determine the detection sensitivity of the passive sampler together with the best practice for its full application to wastewater surveillance, including suggestions to select the optimal sampling sites with maximized coverage of inhabitants and minimized probability of false negative. To respond to this research need, our study aimed to understand the fate of SARS-CoV-2 during transportation in real sewer systems and determine the sensitivity of the passive sampler for detecting the number of COVID-19 infected cases in the monitoring areas. The performance of passive samplers in upstream settings was further assessed through a systematic upstream sampling program for SARS-CoV-2 in 11 individual sewersheds within a city (Brisbane, Australia) with low COVID-19 incidences during the study period. This study for the first time revealed the change and transport of SARS-CoV-2 in 11 individual sewersheds within a city (Brisbane, Australia) with low COVID-19 incidences during the study period. This study for the first time revealed the change and transport of SARS-CoV-2 in 11 individual sewersheds within a city (Brisbane, Australia) with low COVID-19 incidences during the study period.

2. Materials and methods

2.1. Passive sampling devices

This study used a Torpedo-style passive sampler, which has been applied previously to monitor wastewater for SARS-CoV-2 (Habtewold et al., 2022; Schang et al., 2021). The passive sampler contained two to three individual electronegative membranes (pore size 0.45 μm, diameter 47 mm; Cellulose Nitrate Filter, Sartorius, Germany) in a 3D-printed housing unit (Schang et al., 2021). The passive sampler and its deployment in a sewer manhole are showed in Supplementary Fig. S1.

2.2. Fate of SARS-CoV-2 in sewer systems and sensitivity of passive samplers

A study was designed to monitor the fate of SARS-CoV-2 RNA signals along the sewer pipes through 24-h passive sampling. A hospital with a COVID-19 ward that housed all diagnosed cases that arrived from interstate or abroad was considered as a positive control, being the single known source of SARS-CoV-2 in wastewater. During 2020 and most of 2021, Australia and specifically the State of Queensland pursued a “zero COVID-19 policy” where it was mandatory for anyone who entered the country (or the State) to undergo a 14-day quarantine period of isolation. If a quarantined person returned a clinically positive result for SARS-CoV-2, they would be transferred to a COVID-19 ward of a local hospital for treatment. This policy had been very successful in minimizing the number of COVID-19 infections in the State. During the study period, no COVID-19 cases were reported in the catchment outside of the hospital.

The manhole (labelled as Node 0, N0) that received wastewater from the COVID-19 ward together with its downstream 7 key manholes (N1 – N7) along a trunk sewer pipe (8.9 km from N0 to N7) were selected as the sampling sites (Fig. 1a). In the hospital, the COVID-19 ward was located in a separate building specifically connected to N0, while the majority of hospital effluent entered another trunk sewer line, thus the population movement due to hospital commuters was not considered. The selection of sampling sites was executed on QGIS (Version 3.4), which integrated Geographic Information System (GIS) visuals of sewer systems and demographic information at the mesh block level (the lowest geographic level of the Australian census). The site selection procedure and criteria are described in Supplementary material. The service area of a given sampling point Ni was a combination of the service areas of its upstream sampling point Ni-1 (and an area specific to Ni). Information on service area and population size of individual sampling sites is listed in Table 1. From N0 to N7, increase in wastewater flow rates was in line with the increment of population in the areas (Fig. 1b), suggesting that these subcatchments mainly received residential wastewater without significant infiltration or exfiltration in the sewer systems. The dilution factor α at Ni was determined by ratio of flow rates between N0 and Ni (Eq. (1)), representing the degree of virus gene copies (GC) in the original water pulse to be diluted at a downstream location.

\[ α_i = \frac{R_0}{R_i} \]

(1)

\( α_i \) is the dilution factor at the sampling point Ni. \( R_0 \) and \( R_i \) are the average daily flow rates (L/s) at the sampling point N0 and Ni, respectively.

Three sampling campaigns were carried out in this study. In Campaign 1, eight passive samplers were deployed at N0 – N7, while passive samplers were deployed at six sampling sites (N0 – N4 and N6) in Campaign 2, and three sites (N0, N4 and N6) in Campaign 3. No precipitation had occurred in the catchment (separate sewer systems) during the study. The passive samplers were retrieved after 24 h, transported to the laboratory, stored at 4°C, and analyzed using reverse
transcription-polymerase chain reaction (RT-qPCR) within 24 h after collection. The number of GC of SARS-CoV-2 detected from each passive sampler ($N_{GC}$, GC per sampler) was the amount of viral RNA accumulated over 24-h sampling period. Additionally, a 24-h composite sample (500 mL) was collected during each sampling campaign by an automatic sampler at the inlet of WWTP, allowing for the comparison between performance of passive and automatic sampling techniques.

In this study, the sensitivity of passive samplers for detecting COVID-19 infected cases is determined by the lowest infection rate that could allow for a positive detection of SARS-CoV-2 RNA on membranes. The infection rate is dependent on the number of clinically diagnosed COVID-19 cases and the total number of residences in the service area of a sewer manhole where the passive sampler is deployed (Eq. (2)). The number of residence ($P$) in the service area of a given sewer manhole was collected from the Census population data. The diurnal population movement within the monitored subcatchments was not considered due to the lack of real-time population data. During the three sampling campaigns, the exact number of patients in the COVID-19 ward was 15, 13, and 25, respectively, resulting in the infection rates of 0.8 – 18.6 cases per 10,000 people at $N_0$ – $N_7$. In this study, we assumed that the virus shedding patterns of COVID-19 patients on the day of sampling could be represented by an average shedding rate. It was indicated that although virus shedding could have greater variance between infected patients, the associated uncertainty became limited for the estimated COVID-19 prevalence when there were more than 10 infection cases in a catchment (Li et al., 2021).

$$I_i = \frac{P_c}{P}$$

$I_i$ is the infection rate (case per 10,000 people). $P_c$ is the number of clinically diagnosed COVID-19 persons and $P$ is the total number of people residing in the entire service area of sampling point $N_i$.

2.3. Upstream sampling program for city-wide COVID-19 monitoring

From March 2021, an upstream sampling program applying passive sampling was undertaken in two large wastewater catchments (named

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**Table 1** Information on upstream sampling points and service areas monitored in this study.

| Sampling point | Population | Service area (km$^2$) |
|----------------|------------|-----------------------|
| N0             | 13,436     | 6.5                   |
| N1             | 22,133     | 9.7                   |
| N2             | 28,950     | 13.1                  |
| N3             | 44,836     | 24.0                  |
| N4             | 54,043     | 32.4                  |
| N5             | 57,971     | 43.5                  |
| N6             | 68,639     | 47.2                  |
| N7             | 187,692    | 163.4                 |

**Fig. 1.** (a) Geospatial topology of a trunk sewer pipe (the blank line), sampling points $N_0$ – $N_7$ and corresponding subcatchment areas monitored in the study. Sampling points are located at the end of the trunk pipe in each subcatchment; (b) Wastewater flow rate at $N_0$ – $N_7$ and cumulative population in individual subcatchments.
Catchment A and B, both with separate sewer systems) in Brisbane, Australia, as a part of Queensland’s Wastewater Surveillance Program for SARS-CoV-2. The number of residences is about 500,000 in Catchment A and 230,000 in Catchment B. In total 11 upstream sampling points were established for the routine wastewater monitoring with 7 sites (A1 – A7) covering approximately 30% of the total population in Catchment A and 4 sites (B1 – B4) covering approximately 70% of the total population in Catchment B. These sampling points were sitting on trunk sewer lines and the population size in individual service areas ranged from ~7,000 to 70,000 (Table 1). None of these upstream sewersheds had COVID-19 facilities such as quarantine hospitals or hotels.

On July 31, 2021, a public health alert was issued following the clinical confirmation of COVID-19 infections at local schools in Brisbane’s west, which triggered an immediate lockdown for Brisbane city (restricting travel beyond houses except for prescribed essential activity). Lockdown of the city was lifted on August 7 (7 days later) and the final case linked to the cluster was reported on August 23. As per the health department’s request, we immediately conducted a responsive upstream sampling program comprising daily 24-h sample collection coupled with intraday virus analysis and data reporting from July 31 to August 12. Five days after the lockdown was lifted, the sampling strategy was shifted to two consecutive deployments per week as a combination of 72-h (Mon-Wed) and 96-h (Wed-Sun) sampling, until no clinical case was reported and no virus was detected in wastewater (September 12). Information on the exposure sites (namely the hotspots where the confirmed COVID-19 cases had visited) was collected from the open contact tracing data source (Supplementary Table S1) (Queensland Government, 2022). During the upstream sampling program from the end of July to mid-September, no rain event had occurred in individual monitoring periods within the two catchments, except for a slight rainfall (1.4 mm precipitation in 24 h) on August 9.

With the success of passive sampling in small-scale sewersheds monitoring, it was adopted as one of the validated sampling approaches in the wastewater surveillance program for COVID-19. Since August 2021 to now, passive samplers have been dispatched to statewide regional catchments as a routine monitoring strategy with two consecutive deployments per week. The number of residences in the regional catchments ranged from 100 to 11,000. With the State Borders reopening in December 2021, the passive samplers were used to indicate the emergence and transmission of SARS-CoV-2 in remote communities. In individual catchments, the first detection of SARS-CoV-2 by wastewater and clinal testing was compared to evaluate the sensitivity of passive sampling technique to low incidence of COVID-19.

2.4. Virus concentration and RNA extraction

For each passive sampler, SARS-CoV-2 RNA was extracted directly from two electronegative membranes for RT-qPCR analysis as previously described (Ahmed et al., 2020a; Schang et al., 2021). RNA was extracted using the RNeasy® PowerWater Kit (Cat. No. 14700-50-NF) (Qiagen, Valencia, CA). In brief, a 990 μL of buffer PM1 and 10 μL of beta-Mercaptoethanol (Sigma-Aldrich) were added into each bead beating tube. Bead beating tubes were homogenized using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at conditions 3 × 15 s at 10,000 rpm at a 10 s interval. Tubes were further centrifuged at 4,000 g for 5 min to pelleting the filter debris and beads. 600-700 μL of sample lysate from the bead beating tube was further processed as per the manufacturer’s recommendations to obtain a final elution volume of 100 μL of RNA.

2.5. Inhibition assessment

After homogenization of membranes, known quantities (~2 × 10⁴ GC) of murine hepatitis virus (MHV) were seeded into bead beating tube containing the lysate as an inhibition process control. The same quantity of MHV suspension was also added to a distilled water extraction control (same volume of lysate) and subjected to extraction. The presence of PCR inhibition in nucleic acid samples extracted from wastewater was assessed using an MHV RT-PCR assay (Besselsen et al., 2002). The reference PCR quantification cycle (Cq) values obtained for MHV seeded into distilled water for all methods were compared with the Cq values of the MHV seeded into wastewater lysate to obtain information on potential RT-qPCR inhibition. If the Cq value resulting from the sample was > 2 Cq difference from the reference Cq value for the distilled water control, the sample was considered inhibited (Ahmed et al., 2020b; Ahmed et al., 2018).

2.6. PCR analysis

Previously published RT-PCR and RT-qPCR assays were used for the analysis of MHV and SARS-CoV-2 (information on primers and probes in Supplementary Table S2). US CDC N1 RT-qPCR mixture contained 5 μL of Supermix, 2019-nCoV Kit (500 nM of forward primer, 500 nM of reverse primer and 125 nM of probe) (Catalogue No. 10066606), and 5 μL of template RNA. For each qPCR run, a series of standard (5 × 10⁶ to 5 GC/reaction) prepared from gamma-irradiated SARS-CoV-2 and no template controls (n = 3) were included. Gamma-irradiated SARS-CoV-2 was used to prepare the standard after determining the concentration in stock using digital PCR (dPCR) as described elsewhere (Ahmed et al., 2022a). The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All RT-qPCR reactions were performed in triplicate (performance characteristics in Supplementary Table S3). GC numbers/reaction was converted to GC per passive sampler where two membranes were extracted and analyzed for each passive sampler.

2.7. RT-qPCR ALOD

The RT-qPCR assay limit of detection (ALOD) was determined in a concurrent study (Ahmed et al., 2022a). Briefly, gamma-irradiated SARS-CoV-2 were diluted (6 × 10⁶ to 0.6 GC/reaction) and analyzed using RT-qPCR. At each dilution, 15 replicates were analyzed. The 95% ALOD was defined by fitting an exponential survival model to the proportion of PCR replicates positive at each step along the dilution series (Verbyla et al., 2016).

2.8. Quality control and data analysis

An extraction negative control was included for each batch of nucleic acid extraction to ensure no carryover contamination occurred during extraction. No carryover contamination was observed in extraction negative control. To minimize potential qPCR and RT-qPCR contamination, nucleic acid extraction and PCR setup were performed in separate laboratories. For RT-qPCR, samples were considered positive (SARS-CoV-2 detected) if amplification was observed in at least one of the three replicates within 45 cycles. Samples were considered quantifiable with concentrations above the ALOD.

3. Results

3.1. Assay performance characteristics and QA/QC

The RT-qPCR standard curves prepared from gamma-irradiated SARS-CoV-2 had a linear dynamic range of quantification from 5 × 10⁶ to 5 GC/reaction (1 × 10 to 1 GC/μL). The correlation coefficients (r²), efficiency, slopes, Y-intercept of the standard curve were 0.999, 99.6%, -3.382, and 38.89 (Supplementary Table S3) were within the prescribed range of MIQE guidelines (Bustin et al., 2009). The ALODs for the RT-qPCR assays were between 9.50 GC/reaction (1.9 GC/μL of RNA). No RT-qPCR inhibition was identified as all wastewater RNA samples were within the 2-Cq values of the reference Cq value.
3.2. Fate of SARS-CoV-2 RNA in sewer networks

In this study, positive signals of SARS-CoV-2 RNA were detected from 15 out of 17 passive samples (7/8, 5/6, and 3/3 in Campaign 1, 2, and 3, respectively). The highest GC numbers of SARS-CoV-2 RNA were observed at the first two sampling sites (N0 and N1) with 256 – 1267 GC per sampler during the three sampling campaigns (Fig. 2a). SARS-CoV-2 RNA was still detectable at N5 and N6 in Campaign 1 (Cq values of 40.5 and 40.7) but RT-qPCR results were below ALOD. The two non-detect samples were collected from the last two sampling points, i.e., N6 in Campaign 2 and N7 in Campaign 1. In Campaign 3 when there was a greater number of patients in the COVID-19 ward (25 cases), viral signals could still be detected at N6 with 403 GC per sampler.

The amount of SARS-CoV-2 RNA accumulated on membranes in passive samplers decreased along the sewer pipes, in line with the dilution factors of the number of SARS-CoV-2 RNA on membranes was proportional to the dilution factors (αi) with a significant correlation coefficient (Pearson’s r = 0.84; Spearman’s r = 0.95; p < 0.05) (Fig. 2b). The normalized numbers of SARS-CoV-2 RNA per sampler by dilution factors αi showed relative consistency between the passive samplers deployed in Campaign 2 and 3 (Supplementary Fig. S1). This suggested that dilution played a dominated role in the dissipation of viral signals in sewer pipes compared to the potential RNA decay.

3.3. Correlation between SARS-CoV-2 RNA detected by passive sampling and infection rates

Using the above results, a positive relationship was identified between the numbers of SARS-CoV-2 RNA detected by 24-h passive sampling and the infection rates in sewersheds with a strong correlation (Pearson’s r = 0.73, p < 0.01; Spearman’s r = 0.73, p < 0.01; R2 = 0.55; more correlation analysis is provided as Supplementary data) (Fig. 3). The lowest infection rate that allowed for a positive detection of SARS-CoV-2 by the passive sampler was 2.2/10,000. This suggested that when there were more than two COVID-19 cases among 10,000 people, passive sampler had potential to detect the presence SARS-CoV-2 in wastewater, and such positive signal could provide early warning of virus transmission in the community. In parallel with the passive sampling in upstream sewersheds, three 24-h time-weighted wastewater samples were collected at the downstream WWTP using an autosampler during the three sampling campaigns, respectively. Two composite wastewater samples had positive detection of SARS-CoV-2 during Campaign 2 and 3 (1,758 and 2,102 GC/L, respectively), while the composite sample collected in Campaign 1 had the non-detectable viral signal. The infection rates within the entire catchment were 3.0, 2.6, and 5.0/100,000 during sampling Campaigns 1, 2, and 3, respectively, which indicated the sensitivity of 24-h time-weighted sampling for the COVID-19 cases at the catchment level.

3.4. Monitoring spread of COVID-19 via upstream sampling

During the upstream sampling program for COVID-19, 295 passive samples were collected from the 11 sampling sites (A1 – A7 and B1 – B4) from April 9 to September 15, 2021, and 60 samples (20%) were positive for SARS-CoV-2. Among the 11 monitoring sewersheds, 5 areas (A1, A3, A4, B1, and B4) had multiple detections of SARS-CoV-2 (6 – 21 times), the other 4 areas (A2, A6, B2, and B3) only had positive detection once, while virus signal had never been detected in 2 areas (A5 and A7) (Supplementary Fig. S3).

In subcatchment A1, the first positive passive sample for SARS-CoV-2 RNA detected in wastewater was reported on July 22, one week ahead of the first public health alert (July 31) about a potential COVID-19 cluster originating from schools and university in that area (Fig. 4). We postulated that SARS-CoV-2 RNA was initially shed from patient zero and five close contacts who stayed in the monitoring area of A1 before further transmission happened. Therefore, the first SARS-CoV-2 detection on July 22 at A1 could be attributed to these six cases, resulting in an infection rate of 2.9 cases/10,000 in A1 area (Fig. S4). QGIS map indicated that the schools and university campus were located near the subcatchment boundaries of A1 and B1 (Fig. 4). The retrospective contact tracing information also indicated the intensive exposure sites in A1 and B1 areas since July 24 to July 31. Detailed information on geography information and contact tracing statistics is provided in Supplementary materials.

Both wastewater and clinical testing results indicated that the COVID-19 cluster in Brisbane’s west emerged at A1 and B1 and subsequently spread to other areas of the city. Positive signals were continuously detected by passive samplers deployed at A1 for up to 7 weeks (July 22 – September 8), with SARS-CoV-2 signals ranging from <ALOD to 1.3 × 105 GC per sampler (Fig. 4). Signals of SARS-CoV-2 were observed until 2 weeks after the last clinically diagnosed case linked to the cluster was reported. In subcatchment B1, SARS-CoV-2 signals in wastewater lasted for 4 weeks (August 2 to September 1), with a similar temporal pattern to that in A1. However, the accumulation of GC during

![Fig. 2.](image-url) (a) Numbers of SARS-CoV-2 RNA (gene count, GC per sampler) detected from passive samplers during the three sampling campaigns and the dilution factors (α) at sampling points N0 – N7; (b) Correlation between the changes of wastewater flow rates (Ri/R0, x-axis) and viral RNA numbers (N0C/N0C0, y-axis) compared to initials. Data is fitted by a linear regression with 95% confidence intervals.
Fig. 3. SARS-CoV-2 gene copies (GC) of passive samples and infection rates at corresponding upstream sampling sites during the virus fate study. Data is fitted with a linear regression with 95% CI. A zoom-in window shows the distribution of positive/negative samples at low COVID-19 incidences.

Fig. 4. Timeline and abundance of SARS-CoV-2 RNA (gene count, log GC) detected by continuous passive sampling at ‘hotspot’ subcatchments A1 and B1 during the upstream monitoring of a COVID-19 cluster originating from these areas (July to September 2021). Grey cells indicate no detect or where sample was non-available (NA when indicated).
the 24-h sampling period at B1 (4.0 × 10^3 ± 4.1 × 10^3 GC per sampler) was lower than that at A1 (2.9 × 10^3 ± 4.0 × 10^3 GC per sampler), which could be explained by the higher dilution in the larger service area of B1 compared to the small subcatchment of A1. Community transmission might have occurred at the end of July when SARS-CoV-2 spread to suburbs beyond A1 and B1, corroborated by the reported COVID-19 exposure sites in other subcatchments (Supplementary Table S1). The relationship between the two time-serial datasets, namely the daily wastewater testing results and clinically diagnosed daily new cases (from July 30 to August 23), was assessed using cross correlation. The estimated lag values suggested that wastewater data could lead or be behind clinical data by three days, according to the dominant lags of -3 (ACF = -0.6) and 3 (ACF = 0.7). However, it should be noted that the cross correlation could be subject to the low number of cases (<150 people associated with the cluster) and a small time window of monitoring (<1 month) in this study. For example, this could be highly dependent on when individuals within the cluster developed symptoms and presented for clinical testing.

In mid-September, a positive signal for SARS-CoV-2 was detected at B3, which was associated with a new cluster originated from a school in B3 area located at Brisbane’s south. The school was shut down after patient zero tested positive for SARS-CoV-2 on September 10. Within one week following the school shutdown, a total of 5 students tested positive. We assumed the shedding of SARS-CoV-2 RNA started during the pre-symptomatic period, when students attended school as usual. Therefore, we assumed that the positive detection of RNA fragments in September 12 was correlated with the 5 students attending the school in B3, and hence the infection rate that could enable a positive detection by passive sampling was approximately 1.6 cases/10,000 (Fig. S4).

With the State Border re-opening in December 2021, SARS-CoV-2 was spread with travelers to inner State areas after a few months of zero COVID-19 period, as confirmed by both clinical and wastewater testing. In 12 regional catchments with routine passive monitoring, the first detection of SARS-CoV-2 in wastewater was reported around mid of December 2021 to mid of January 2022, with 22 passive samples tested positive. According to the clinical record, the emergence of COVID-19 in those remote communities varied between 1 case among 5,000 people and 23 cases among 1,800 people, leading to the infection rates of 0.2–127.8 cases/10,000 people (Fig. S4). The detection of SARS-CoV-2 by passive samplers was ahead of or at the same time with clinical confirmation of the first few COVID-19 cases in local areas. Therefore, wastewater surveillance in regional catchments by passive sampling demonstrated the sensitivity of passive samplers to a small number of COVID-19 cases at a low infection rate (>2 cases/10,000 people).

4. Discussions

4.1. Factors on change of SARS-CoV-2 signals in sewer systems

Dilution of virus signals by sewer flows is one of the most important factors that could lead to ‘false negative’ for SARS-CoV-2 wastewater surveillance, especially when disease prevalence is low in a large catchment with huge dilution capacity (Ahmed et al., 2022b). In our sensitivity study, the signals for SARS-CoV-2 were reduced when large volumes of wastewater merged into sewer systems. The viral fragments shed by a limited number of COVID-19 patients were highly dissipated and not detected at the far end of the sewer pipes, where flow rates increased by 5.5 – 13.4 times than that at the near-source sampling point. The impact of dilution on the detectability of SARS-CoV-2 in wastewater has been underlined by an earlier wastewater surveillance study (Liu et al., 2020).

In our study, the flow-normalized gene counts were similar at different sampling sites along the sewer line. As the change of viral GC in sewers was a combined result of physical dilution and potentially sorption and biodegradation processes, the spatial and temporal consistency of the flow-normalized gene counts indicated that dilution was the dominant factor influencing the fate of SARS-CoV-2 RNA, compared to the less influence of RNA decay in sewers. This also implied the stability of SARS-CoV-2 RNA, which led to limited RNA decay over time in the monitored sewer pipe (<5 h considering the minimal self-cleaning flow velocity of 0.6 m s⁻¹). To date, there is still limited study about the fate of SARS-CoV-2 RNA in real sewer systems, and the available studies about RNA stability in raw wastewater (i.e., in-sample stability) reported mixed findings. Some observed the persistence of SARS-CoV-2 RNA for a few days (Bivins et al., 2020; Wurzler et al., 2020), while another study reported the first-order decay of virus RNA with half-lives from 4 h to 17 h at 4, 10, and 35°C (Weidhaas et al., 2021). Further investigations on the association between viruses and the solid fractions in sewer systems such as suspended solids, biofilms, and sediments are required to better understand factors for the fate of viruses in sewers which could potentially lead to false positives or negatives. Another issue that could affect the amount of GC captured by passive samplers is ‘ragging’ due to the accumulation of cloths, tissues, etc., around the samplers deployed in wastewater, which was observed occasionally at sampling site N4. For the passive samplers experienced ragging, SARS-CoV-2 RNA was still detectable but the number of GC attached on the passive sampling membranes could have been affected. Future research is needed to understand the potential impact of ragging on the uptake of viruses on membranes and optimize the sampler design to minimize ragging occurrence.

4.2. Sensitivity of passive samplers compared to other wastewater sampling techniques for SARS-CoV-2

A range of wastewater sampling techniques, such as autosamplers, passive samplers and grab sampling, has been applied to monitor SARS-CoV-2 RNA at community scales ranging from buildings (e.g., hospitals, schools, and campuses) to city areas. Data on detection limits of those sampling techniques is available or can be estimated from literature as summarized in Table 2. Based on data form and preference, Table 2 provides different metrics for the sensitivity of each sampling technique for SARS-CoV-2 detection (e.g., Cq values, GC/reaction, and GC/L) and infection rates per population size (that we normalized to per 10,000 people).

Sensitivity of wastewater surveillance approach for COVID-19 incidences varied between different sampling techniques. For studies using autosamplers, it was possible to detect a single COVID-19 case for building-level monitoring, e.g., in wastewater from university dormitories with 415 residents (0.24%) (Karthikeyan et al., 2021), or 150 – 200 students (0.5%) (Gibas et al., 2021). Monitoring of hospital wastewater using autosamplers could detect positive signals of SARS-CoV-2 when only 1 – 2 COVID-19 patients were hospitalized (Goncalves et al., 2021; Karthikeyan et al., 2021; Spurbeck et al., 2021). A study summarized the estimated probabilities of SARS-CoV-2 detection in wastewater accounting for different COVID-19 incidences in a catchment, time since onset, and distances from the source to the sampling points, suggesting the probability of detection as 10% with one infected case and 60% with 5 cases within 5 km and 1 week of onset (Black et al., 2021).

In studies where use of an autosampler was not feasible, grab sampling was applied for building-level wastewater monitoring (Albastaki et al., 2021; Betancourt et al., 2021; Davo et al., 2021). Taking grab samples in the early morning during the peak flow periods was able to detect a single COVID-19 case among 311 students (0.32%) in a university dorm (Betancourt et al., 2021) or in a nursing home with 165 people (0.61%) (Davo et al., 2021). However, grab samples could only provide a snapshot of the presence of viruses in wastewater at the time of sampling with higher probability of false negative than 24-h aggregated sampling methods. A discordant ratio of 13.5% between grab wastewater samples and clinical tests was reported (Betancourt et al., 2021). It was concluded that the capture of SARS-CoV-2 RNA by grab sampling was less likely to allow for a positive detection when there was a small
proportion of viral shedders in the population (Ahmed et al., 2022b).

A range of newly designed passive samplers are now being applied as a simple and practical tool for frequent monitoring in sewer networks. Liu et al. (2020) used 24–72 h deployment of Moore-swabs to monitor wastewater from campuses and hospitals, which was able to detect SARS-CoV-2 RNA originating from 1 – 2 COVID-19 cases in a building (Liu et al., 2020). Similarly, the 3-h deployment of tampon swabs was able to detect SARS-COV RNA in wastewater from university halls that contained 1 – 2 COVID-19 cases (Bivins et al., 2021). The passive samplers used in our study showed higher sensitivity, being able to detect more than 2 COVID-19 cases among 10,000 residents in a catchment.

This sensitivity was further validated by the application of passive samplers for upstream monitoring of the spread of COVID-19 clusters in a city, where approximately 5 – 6 initial infected cases in sewersheds with ~20,000 – 32,000 residents could be identified, corresponding to the infection rates of 2 – 3 cases per 10,000. Results of these studies demonstrated that passive sampling could be a reliable and cost-effective method to identify a few COVID-19 incidences related to a small group of population.

### 4.3. Limitations of this study and future works

We acknowledge that there are uncertainties in this study related to recovery efficiency, viral RNA decay, viral shedding, flow conditions, etc. that could affect the sensitivity of passive sampling membranes for the COVID-19 infection rates. The stability of SARS-CoV-2 RNA on passive sampling membranes is not yet understood, which requires further studies to investigate the interactions between electronegative membranes and enveloped viruses. Viral RNA shedding in stool could contribute a major uncertainty to the results of this study due to the potentially high variance between infected individuals and throughout the time course of infection (Wolfel et al., 2020). Additionally, findings of this study were determined based on the performance of passive sampling membranes for viral RNA in the study (RT-qPCR unless otherwise stated).

### 4.4. Implication on upstream and downstream wastewater surveillance

Upstream sampling can enable the early identification of a small number of infected persons in a small sewershed, i.e., the low-case setting, before potential community transmissions occur. As SARS-CoV-2 RNA could be shed at the pre-symptomatic stage, wastewater surveillance has the potential to capture those RNA signals and trigger early warning prior to mass clinical testing. In our upstream sampling
program for city-wide COVID-19 monitoring, the first positive wastewater sample linked to a COVID-19 cluster was determined 1 week ahead of the clinical confirmation. In previous studies about the building-level wastewater monitoring at university dorms, hospitals, and clinic facilities, the positive detection of SARS-CoV-2 in wastewater has been found to precede the clinical confirmation by 1–2 weeks (Davo et al., 2021; Saguti et al., 2021) or up to 3 weeks (Barich and Slonczewski, 2021).

Based on the findings of this study, we provide some suggestions of building up wastewater surveillance for COVID-19 in small, medium, and large catchments using a combination of automatic and passive sampling methods.

• In a large urban catchment with known viral sources (e.g., quarantine hospitals and hotels), wastewater monitoring at upstream sewer systems could be considered as a more reliable approach for detecting potential community transmission. Manholes with a service area of 5,000–25,000 inhabitants could be suitable sampling sites, considering passive samplers could be sensitive to over 2 COVID-19 incidences among 10,000 people in a sewershed.

• In a medium-sized catchment (e.g., 25,000–200,000 people) without known viral source, wastewater monitoring at WWTP influents using autosamplers can be adopted as the routine strategy. The detection of positive signals of SARS-CoV-2 in an influent sample could be used to immediately trigger upstream sampling to monitor the spread of disease in different parts of a catchment and locate hotspot areas.

• In small-sized catchments such as at towns in regional areas with a small number of residents (e.g., hundreds to thousands), passive sampling can be applied as an efficient method that is sensitive to a few infection cases who might reside or travel in the catchments. As demonstrated in this study, the sampling strategy of two consecutive deployments (e.g., 72 h + 96 h, or with higher deployment frequency) will provide a full-time disease surveillance for a small catchment with sufficient sensitivity. The simple passive sampling device is of particular importance to regional and remote areas where clinical resource is limited.

• The deployment duration of a passive sampler can range from hours to days, depending on the potential threat of the virus circulating in local communities. The commonly applied sampling strategy is 24-h deployment (daily or weekly depending on local needs). For longer deployment, SARS-CoV-2 signals could be detected with 3–4 days deployment, because of the time-integrative uptake of viruses on membranes. However, the impact of long-term exposure on the uptake, retention, and stability of viral fragments on membranes requires further investigation.

5. Conclusion

Passive sampling in upstream sewer systems enables the isolation of subcatchment regions for a more granular picture of the COVID-19 situation as well as the early warning of disease emergence in local communities when deployed at appropriate locations. Through the systematic studies of membrane-loaded passive samplers for monitoring SARS-CoV-2 RNA in sewer systems, we found that:

• Dilution is an important factor for the dissipation of virus signals in sewer systems and could lead to false negatives. The decrease of SARS-CoV-2 RNA GC on passive samplers was proportional to in flow rates.

• The accumulation of SARS-CoV-2 RNA on passive sampling membranes over 24-h continuous sampling was positively correlated with the infection rates in monitored areas. The membranes provided an effective and sensitive passive sampling material for SARS-CoV-2 monitoring with a sensitivity to more than 2 COVID-19 cases per 10,000 people in a catchment.

• Systematic upstream sampling revealed the spatiotemporal changes of SARS-CoV-2 concentrations in different sewersheds and the spread of COVID-19 over time within a city. Positive signals of SARS-CoV-2 RNA were detected in an upstream subcatchment one week ahead of the public health alert of a new COVID-19 cluster. In this hotspot area, wastewater samples remained positive for up to 7 weeks, which could cover the entire viral shedding period of individuals from pre-symptom, symptomatic period to full recovery.

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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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118481.

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