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Wastewater surveillance of SARS-CoV-2 at intra-city level demonstrated high resolution in tracking COVID-19 and calibration using chemical indicators

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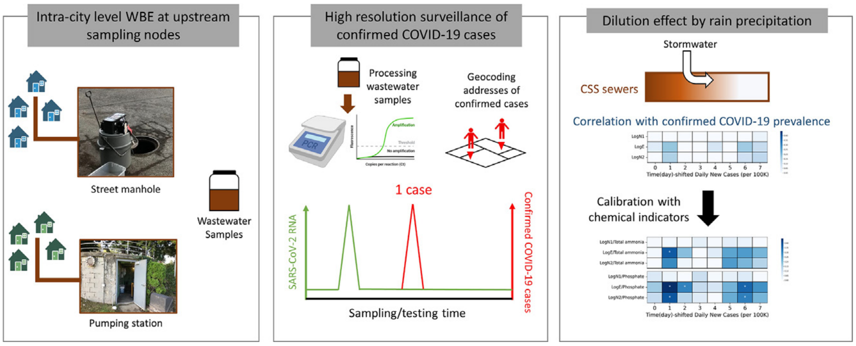
HIGHLIGHTS

- Wastewater surveillance at city subareas using geocoded address of COVID-19 cases
- High resolution surveillance of 1 confirmed case in subarea with 2580 populations
- Dilution effect of rain precipitation on the intra-city level surveillance
- Improved correlation with confirmed COVID-19 incidence using PO_4^{3-}-P and NH_4^+-N

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology has proven to be a supportive tool to better comprehend the dynamics of the COVID-19 pandemic. As the disease moves into endemic stage, the surveillance at wastewater sub-catchments such as pump station and manholes is providing a novel mechanism to examine the reemergence and to take measures that can prevent the spread. However, there is still a lack of understanding when it comes to wastewater-based epidemiology implementation at the smaller intra-city level for better granularity in data, and dilution effect of rain precipitation at pump stations. For this study, grab samples were collected from six areas of Seattle between March–October 2021. These sampling sites comprised five manholes and one pump station with population ranging from 2580 to 39,502 per manhole/pump station. The wastewater samples were analyzed for SARS-CoV-2 RNA concentrations, and we also obtained the daily COVID-19 cases (from individual clinical testing) for each corresponding sewershed, which ranged from 1 to 12 and the daily incidence varied between 3 and 64 per 100,000 of population. Rain precipitation lowered viral RNA levels and sensitivity of viral detection but wastewater total ammonia (NH_4^+-N) and phosphate (PO_4^{3-}-P) were shown as potential chemical indicators to calibrate/level out the dilution effect. These chemicals showed the potential in improving the wastewater surveillance capacity of COVID-19.

1. Introduction

The novel coronavirus disease 2019 (COVID-19) pandemic caused by the virus SARS-CoV-2 has led to millions of deaths worldwide and continues to pose a critical threat to global health. Wastewater-based epidemiology (WBE) has been applied to and demonstrated the viability of early

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detection of viral RNA in the wastewater in the early stage of COVID-19 outbreaks (Bivins et al., 2020). Most wastewater surveillance approaches include collection of samples from wastewater treatment plants (WWTP) which represent a large population, often an entire city (Graham et al., 2020; Li et al., 2021). However, COVID-19 outbreak typically occurs in few hotspots at intra-city level rather than in the entire city simultaneously (Miller et al., 2020). Yet little research has been devoted to evaluate sewer monitoring at pumping station and manhole targeting sewersheds of multiple residential units to enhance identification of COVID-19 hotspots on a finer level. While the viral RNA testing at building/facility level could show useful application of wastewater surveillance (Gibas et al., 2021), the implementation of such surveillance systems at targeted buildings across large areas could put a strain on budgets, resources, and staff. The wastewater surveillance at these levels could result in limited impact to prevention and control of the disease spread. Intra-city level of WBE with upstream sampling points can therefore be applied at different points within the surveillance system to enable different levels of granularity to present a subset of a larger population. This can be especially of interest if different areas in the same city exhibit different trends of COVID-19 infection.

Among a few in the literature, intra-city level WBE at upstream sampling points (street manholes and pump stations) were previously investigated; however, the WBE results were usually analyzed with clinical confirmed cases from the entire city or county, rather than those from the same intra-city level (Albastaki et al., 2021; Black et al., 2021; Li et al., 2022; Prado et al., 2021; Rafiee et al., 2021; Saguti et al., 2021; Yaniv et al., 2020). The relationship between SARS-CoV-2 RNA concentration in wastewaters and clinical confirmed COVID-19 cases in the sewershed of sampled wastewaters has been essential for performance investigation of WBE application. While the WBE studies at downstream locations could employ data of COVID-19 case at city and county level collected and reported by local governments, further processing of data screening would be needed to obtain the data of confirmed COVID-19 cases within sewersheds of intra-city level WBE. However, most studies of intra-city level WBE had relied on the reported cases at the upper level of combined multiple sewersheds (Prado et al., 2021), whole city (Yaniv et al., 2021), and national level (Albastaki et al., 2021; Rafiee et al., 2021). In some studies, the COVID-19 incidence was referred from reported hotspots (Li et al., 2022) or number of patients (Saguti et al., 2021) in the studied area, in which infected individuals might not necessarily shed viruses into collected samples. Among these studies, correlation between wastewater signals and disease incidence in communities could not be confirmed. Alternatively, the COVID-19 incidence in the intra-city level sewersheds can be obtained through screening confirmed cases for those residences with addresses within the sewersheds. Previously, those studies of the screened COVID-19 case data, nevertheless, were conducted with lack in focusing on sewersheds of 1000–10,000 population magnitudes (Greenwald et al., 2021b). Also, there are knowledge gaps in understanding the effect of rain precipitation on the relationship between WBE metrics and COVID-19 case at this fine level, which would promote the strengths in the disease surveillance at this level (Black et al., 2021; Weidhaas et al., 2021; Wilder et al., 2021).

WBE applications currently encounter challenges in bias due to interference of rain precipitation. Municipal wastewater is typically collected by two types of sewer systems namely combined or separated. The separate sewer system (SSS) collects wastewater separately from stormwater. On the contrary, combined sewer system (CSS) collects wastewater and stormwater in the same piping network. In the partially separate system (PSS), a partition of stormwater such as those from roof gutters and downspouts were drained into sanitary sewers and mixed with sewage, whereas the rest of stormwater is drained into separated pipes for outfall. WBE from sampling at PSS and CSS would encounter interferences due to a dilution effect from stormwater inflows resulting in surveillance biases. In the United States, CSSs are very common, for example in 772 communities of Midwest, Southeast, and Pacific Northwest CSS are serving 40 million people (USEPA, 2004), and therefore it is important to better understand the dilution effect of precipitation events in WBE application. Water flow rate data has been used to calculate daily loads of SARS-CoV-2 RNA, showing correlations with clinical case prevalence (Agrawal et al., 2021; Markt et al., 2022; Weidhaas et al., 2021). However, the WBE at upstream sampling points (i.e. manholes) would have difficulty assessing the actual flow rate due to high variation of stormwater inflow and overflow systems. Also, there would be a financial barrier in obtaining expensive flow meters for use across many sampling points. Alternatively, co-quantification of fecal indicator DNA/RNA have been recommended for validating WBE (D'Aoust et al., 2021b; Li et al., 2021; Medema et al., 2020a; Wilder et al., 2021). In the meantime, previous studies pointed out the highly variable load in fecal samples at sampling points with smaller populations and resulted in ineffective use of fecal indicator normalization in WBE at this level (Feng et al., 2021; Nagarkar et al., 2022). It was noticed that the concentrations of fecal indicators used for the normalization SARS-CoV-2 typically showed broader range than the concentration range of chemical indicators (Symonds et al., 2015). Recent studies demonstrated similar temporal changes of chemical marker titers (i.e. ammonia, total nitrogen, and total phosphorus) and SARS-CoV-2 RNA concentrations (Amoah et al., 2022; Isaksson et al., 2022; Pons et al., 2020). Calibration of SARS-CoV-2 RNA concentrations in wastewater using chemical data could account for a dilution effect and improve the surveillance reflection (Sweetapple et al., 2021; Wade et al., 2022). In addition, previously, the diurnal studies at pump stations and demonstrated efficient approach to account for dilution effects by calibration of SARS-CoV-2 RNA concentrations in wastewaters using chemical markers (Nguyen Quoc et al., 2022).

In this study, a long-term wastewater SARS-CoV-2 surveillance measurement effort was conducted at intra-city level to six different regions in Seattle, King County, WA, USA from March 2021 to October 2021. Wastewater from manholes and a pump station was collected from different smaller sewersheds (with populations of ~2580 to 35,902) hence representing the trends of the pandemic with a finer level of granularity than at wastewater treatment plant level. Virus particles in the wastewater samples were captured using the PEG concentration method and SARS-CoV-2 RNA was quantified by three RT-qPCR assays (N1, N2 and E). COVID-19 incidence in communities was based on cases addressed to the studied area from data screening. Precipitation conditions were collected from weather stations nearby the six study sites. As the first objective, the relationship between SARS-CoV-2 concentrations in wastewaters and COVID-19 incidence in six intra-city WBE sites was assessed. Chemical indicators, including COD, NH_3-N, PO_4^{3-}-P and total suspended solid (TSS), were monitored for wastewater samples to analyze the changes in wastewater properties under different water precipitation conditions. As the second objective, the effect of precipitation on the WBE performance was studied by comparing the sensitivity of WBE with or without precipitation and the SARS-CoV2 signal was calibrated with chemical markers to decrease noise in the data set and improve the correlation to the clinical data set within the studied areas.

2. Material and methods

Study sites and wastewater sampling. This study investigated six different areas (Site1-Site6) located in Seattle (Fig. 1). Population size and land area varied across six areas in the range of 2580–39,502 (14,739 ± 6515) populations and of 38.8–2789 (771.9 ± 1054.3) acre, respectively as shown in Table 1. The population estimates in each sewershed were obtained by mapping census shapefiles corresponding to our sampled sewersheds, provided by the Small Area Estimates Program (SAEP) from the Department of Financial Management of Washington state, using ArcGIS software (Version 10.8.1, Esri, Redlands, CA). A total of 155 wastewater samples were collected from the six regions from March 2021 to October 2021, with 22–30 samples for each sampling site. These sewer systems were part of three different types of sewer collection systems, including CSS (Site 1), PSS (Site 2,4,5 and 6) and SSS (Site 3). Weather conditions, and amount in precipitation were monitored by three national weather service stations (Fig. 1 and Table 1). The sampling frequency was weekly during March to June 2021 and biweekly during July to October

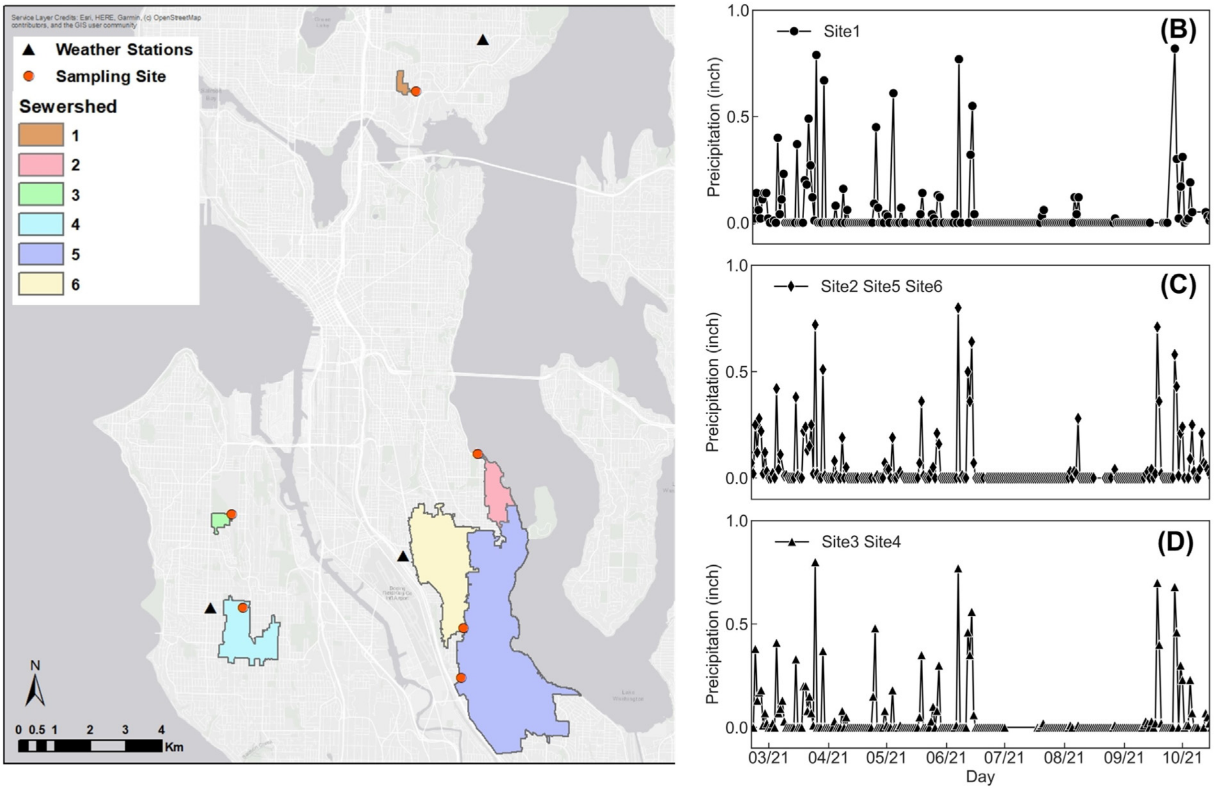

Fig. 1. Sampling sites and sewersheds for intra-city level WBE study in the Seattle city (A), King County, the State of Washington (WA) and daily precipitation of each sampling site during the sampling period (B–D). The location of sampling points (manholes and a pumping station) was indicated by orange circles, sewersheds of each site were indicated by line boundary, weather stations were indicated by black triangles. The daily precipitation was obtained from the weather stations nearby the sampling sites.

2021. The change was due to observed sporadic clinical case with the frequency longer than two weeks. GPS coordinates of a pump station or manholes were summarized in Table S1. For the Site 2, 200 mL of 8-hour composite grab samples were collected at a 30 min interval for an 8 h period during 4 AM-12 PM, using a 5800 refrigerated sampler (Teledyne ISCO, Lincoln, United states). 150 mL of grab samples were collected at each manhole (Site 1 and Site 3–6) during 6–8 AM at a 2 min interval for a 20 min period by GLS compact composite sampler (Teledyne ISCO, Lincoln, United states). The sampling time periods were selected according to a previous diurnal sampling campaign that the highest SARS-CoV-2 concentrations within 24-hour were observed during the morning hours (Nguyen Quoc et al., 2022). Samples were stored in ice during transportation and processed within 24 h or stored at −20 °C for processing within a week. All frozen samples experienced only one cycle of freeze-thaw at most. These storage conditions showed no significant decay of SARS-CoV-2 RNA comparing with fresh samples (Hokajärvi et al., 2021).

Sample processing and viral RNA extraction. To concentrate SARS-CoV-2 virus, 300 mL of the wastewater samples were settled for an hour to remove large size particles and potential PCR inhibitors in the wastewater (Sapula et al., 2021). 250 mL of the supernatant liquid layer was then processed to obtain the viral RNA using a polyethylene glycol (PEG) concentration method (Barril et al., 2021). 250 mL of the supernatant liquid was added with 10 % PEG6000 (W/V) and 0.3 M NaCl prior to stirring at 4 °C for 2 h. The mixture was centrifuged at 7500 ×*g* at 4 °C for 80 min using a Sorvall RC5B centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The pellet was resuspended in 1 mL phosphate buffered saline (PBS, pH 7.2) and incubated at room temperature for 1 h with occasional mixing. The PBS resuspension was centrifuged at 10,000 ×*g* for 20 min. 140 μL of supernatant was extracted for viral RNA using QIAamp®Viral RNA Mini Kit (Qiagen; Valencia, CA, USA) following the manufacturer's protocol. The bovine coronavirus (BCoV) (Zoetis, Kalamazoo, MI, USA) was spiked as an exogenous process control into the raw wastewater samples and used as the surrogate of SARS-CoV-2 in the processing to estimate the virus recoveries. Quantification of BCoV was achieved using modified qPCR assays of previously established M gene (Decaro et al., 2008).

RT-qPCR. Quantification of SARS-CoV-2 RNA was achieved by RT-qPCR using previously established N1 and N2 genes (CDC, 2020) and E gene assay (Corman et al., 2020). Details of primer and probe concentrations and thermocycling conditions were provided in the supplementary material (Tables S2 and S3). Calibration curves of the N1, N2 and E genes were constructed by triplicate qPCR with serials dilution of the nCoV-ALL-Control Plasmid (Eurofins Genomics, USA), in the range of 1.2 × 10

Table 1
The six areas selected as sampling sites for wastewater monitoring of SARS-CoV-2 in this study.

Site ID	Neighborhood[a]	Population	Land extent (Acre)	Sewage collection system	Weather service station ID	Samples	Sampling period
1	Ravenna Street	9468	38.8	Combined	US1WAKG0238	27	3/8/21–10/20/21
2	Lake Washington Blvd Street	4801	239.7	Partially separate	US1WAKG0200	30	3/6/21–10/17/21
3	Brandon Street	2580	53.3	Separated	US1WAKG0258	22	3/16/21–10/20/21
4	Elmgrove Street	13,338	479.6	Partially separate	US1WAKG0258	22	3/16/21–10/20/21
5	Norfolk Street	18,747	1030.7	Partially separate	US1WAKG0200	27	3/3/21–10/19/21
6	Cloverdale Street	39,502	2789.0	Partially separate	US1WAKG0200	27	3/4/21–10/19/21
					Total	155	

[a] Samples were collected from manhole for Site1, 3–6 and a pump station for Site 2.

to 1.2×10^5 copies per reaction. All RT-qPCR reactions in this study were run using Quantinova Probe RT-PCR Kits (Qiagen, USA) on a Roche Lightcycler 96® instrument (Roche, Germany). RT-qPCR assays of SARS-CoV-2 RNA quantification used in this study showed amplification efficiencies of 99.4 % (N1, R^2 of 0.99), 99.2 % (E, R^2 of 0.99), and 100 % (N2, R^2 of 0.99) (Fig. S1). The limit of quantification (LOQ) of all three assays was 1.2×10 copies per reaction. For wastewater samples, duplicates of qPCR reactions of each assay were performed and arithmetic means of Ct values were used for the subsequent calculation. Positive control, which was 1.2×10^5 copies of the nCoV-ALL-Control Plasmid, and negative control, which was nuclease-free water, were included in every qPCR run as quality controls.

Presence of PCR inhibitors in wastewater RNA extracts were assessed by spiking of controls following previous studies (Ahmed et al., 2018; Staley et al., 2012). 40 pg of Salmon Sperm DNA (Thermo Fisher Scientific, Waltham, MA) was spiked into RNA samples prior to qPCR reactions using Sketa22 qPCR assay (Haugland et al., 2005) (Tables S2 and S3). The inhibitor-free baseline was set by qPCR reactions of 40 pg of Salmon Sperm DNA in nuclease-free water, instead of wastewater RNA extracts. The RNA samples, which diverged >2 Ct value from the baseline were subjected to 10-fold serial dilutions prior to downstream qPCR analysis of SARS-CoV-2.

Details of primer and probe concentrations and thermocycling conditions for the surrogate (BCoV) were provided in supplementary material (Tables S2 and S3). Calibration curves was constructed by serials dilution of the synthetic gene fragment of M gene (Twist Bioscience, USA), in the range of 1.5×10 to 1.5×10^5 copies per reaction. Nucleotide sequence of M gene was of a BCoV isolate, genome which was deposited on NCBI (Accession ID: MN982199.1). LOQ is 1.5×10 copies per reaction. The qPCR assay showed amplification efficiencies of 87.3 % (R^2 of 0.99) (Fig. S1). An average of 7.8 % of BCoV was spiked into wastewaters before solid settling were recovered (Fig. S2).

Analysis of wastewater parameters. Wastewater samples were filtered through 0.2 μm nylon filter (VWR, Radnor, PA) for analysis of total ammonia (NH_3-N), orthophosphate (PO_4^{3-}-P), and soluble chemical oxygen demand (sCOD). NH_3-N and PO_4^{3-}-P were analyzed via spectrophotometric measurements using Gallery™ Automated Photometric Analyzer and reagents from the manufacturer (Thermo Scientific, Waltham, MA) following the manufacturer's protocol. The analysis of total ammonia determines unionized ammonia and ionized ammonium and the parameter was referred as total ammonia (NH_3-N). sCOD measurement was achieved using COD Digestion Vials-Low Range (Hach, Loveland, CO), following the Standard Method 5220 D (WEF, 2005). Analysis of total suspended solids (TSS) was performed using well-mixed raw wastewater prior to solid settling following the standard method 2540 (Federation and Association, 2005). The water flow rate at the sewers of CSS, PSS, and SSS, could not represent actual wastewater flow due to unique variations (Fig. S4) of the stormwater inflows and proximity to the overflow systems and was therefore excluded from the analysis.

COVID-19 clinical data. Confirmed COVID-19 case number for each studied region was obtained by screening of public health information (PHI) and results of COVID-19 nasal swab tests. Public health information (PHI) and results of individuals taking COVID-19 nasal swab tests in Seattle was provided by the University of Washington Medical Center (UWMC) clinical laboratories. The laboratories received samples from testing sites around Seattle and the confirmed incidence numbers of the entire Seattle city. The UWMC laboratories posed sufficient test capacity in the six studied sewersheds with 2.6–7.9 test sites per 1000 residences, which was higher than the 0.7 sites per 1000 residence of the entire Seattle city average, implying high coverage of COVID-19 cases at the studied areas. The screening of UWMC laboratory data was achieved by identifying the cases which meet the criteria: (1) patients with positive and inconclusive test result; (2) patients with street addresses lying within sewersheds of the studied manholes and a pump station at the six sampling sites. These residential addresses lying within the sewersheds were identified and provided by collaborators from Seattle Public Utilities. Cases with the criteria were summed up to obtain daily confirmed COVID-19 case number. Use and handling of patient data was approved and authorized by the UW institutional review boards (IRB) committee. The data screening was performed by using adist function in Rstudio.

Wastewater collection systems and precipitation data. Collection systems of sampling sites were determined using the publicly available sewer maps on Seattle Public Utilities website (https://www.seattle.gov/utilities/construction-resources/water-and-sewer-map). The 24-hour precipitations at three weather stations (Table 1) were downloaded from the National Oceanic and Atmospheric Administration (NOAA) weather database (https://www.ncdc.noaa.gov/). Sampling point of site 2 is a pump station of Seattle Public Utilities installed with water flow sensor. The 24-hour precipitation from the weather station nearby site 2 showed significant correlation with water flow during sampling period at a pump station ($\rho = 0.48$, p-value <0.001).

Data analysis and visualization. Daily COVID-19 incidence was calculated using the daily confirmed COVID-19 case number and population estimates in six sewersheds. The incidence was reported as confirmed case number per 100 K population. Statistical analyses were performed under Jupyter notebook. The normal distribution of data was tested using Shapiro test. To investigate the effect of rain precipitation, samples were categorized using levels of 24-hour precipitation. The samples with precipitation were categorized further using presence of precipitation or percentile thresholds. Correlations between average daily concentrations of chemical indicators and TSS and population density were performed using Spearman's correlation analysis (ρ). The concentration of chemical indicators and TSS at each sampling site were averaged across the study period. Population density was the number of populations per acre of each sewershed area. The effect of rain precipitation on sensitivity of SARS-CoV-2 RNA detection was investigated using two COVID-19 incidence metrics within seven days following the day of wastewater sampling. The two metrics were the average daily incidence and the highest incidence. Cross-correlation tests between SARS-CoV-2 concentrations and time-shifted COVID-19 incidence for comparative analysis with and without calibration by chemical indicators were also achieved using Spearman's correlation analysis. Cross-correlation using Spearman's correlation analysis was previously performed to investigate SARS-CoV-2 gene concentration normalization by different indicators in WBE studies (Fahrenfeld et al., 2022; Maal-Bared et al., 2023). Data of undetected SARS-CoV-2 genes were replaced with detection limits prior to correlation analysis. Time period of calculating the COIVD-19 incidence metric in the sensitivity analysis and cross-correlation tests was seven days based on previously reported lead time of fewer than seven days (D'Aoust et al., 2021a; Jiang et al., 2022; Nemudryi et al., 2020; Peccia et al., 2020) and to avoid potential miscorrelation as described in the supplementary (Figs. S5–6). Comparison among factor groups were performed using One-way ANOVA with post-hoc Turkey HSD's test or non-parametric Kruskal-Wallis with post-hoc Dunn's tests or Wilcoxon tests. Paired samples comparison was performed using *t*-tests or Wilcoxon tests. The investigation of chemical calibration was performed on filtered dataset. The data filters included the number of positive genes and precipitation level. Statistical significance was determined at p-value of <0.05 and marginal significance at p-value of <0.15. Maps of sampling sites were generated with ArcGIS (Version 10.8.1, Esri, Redlands, CA). Data were visualized using the Seaborn package in Jupyter Notebook.

3. Results

3.1. Dynamic of wastewater SARS-CoV-2 and COVID-19 cases at the sampling sites

Clinical COVID-19 cases with addresses in each sewershed of the six intra-city sites were reported during the wastewater sampling period. Throughout the sampling period, low incidence of COVID-19 was observed in the study sites with reported daily COVID-19 cases of 1–12 and incidence of 3–64 per 100 K residences (Fig. 2). Corresponding with occurrence of

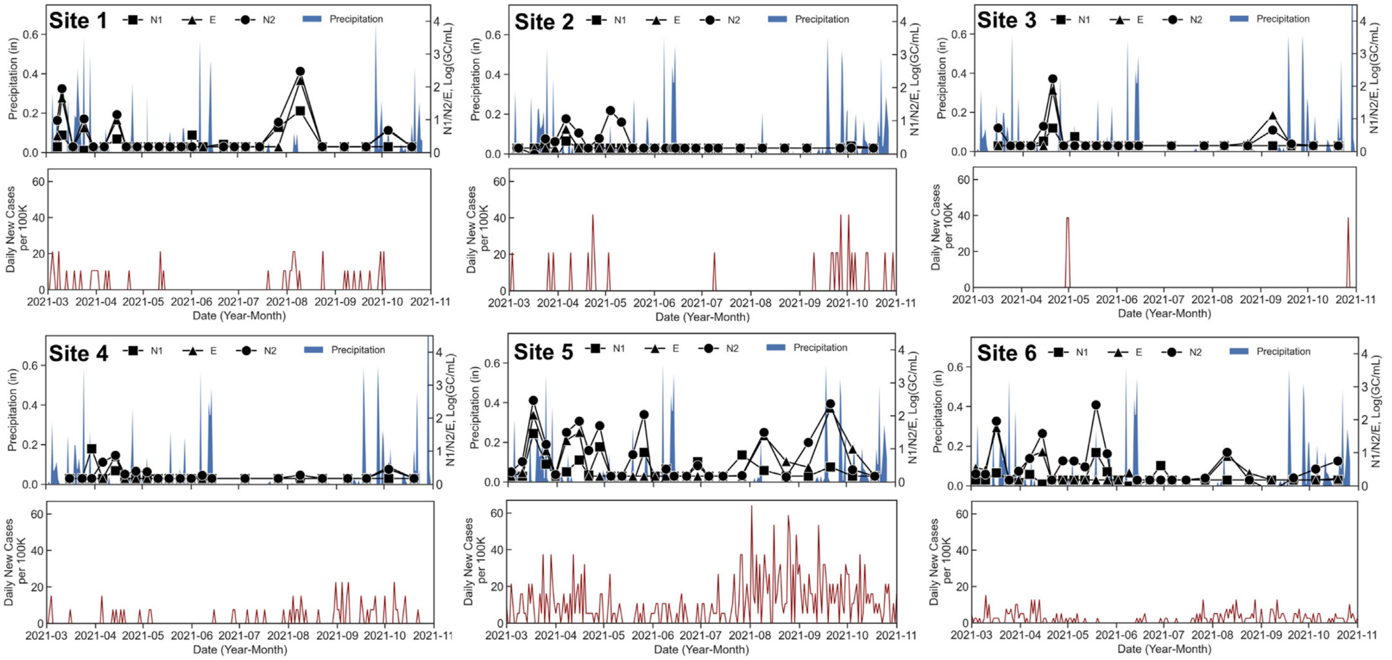

Fig. 2. SARS-CoV-2 RNA (log of N1, N2 and E) concentrations (in black color) and COVID-19 incidences (daily cases per 100 K residences) (in red color) during 03/2021–10/2021 at the six sampling sites. The RNA concentrations were presented with 24-hour precipitation data (in blue color) reported at weather stations nearby the sampling sites.

COVID-19 cases, positive detection by WBE of SARS-CoV-2 were observed in all three gene assays (N1, E, and N2) among the six study sites (Fig. 2) with detected concentrations ranging from 0.2 to 2.5 Log (GC/mL). Particularly, at site 3, the two occasions of SARS-CoV-2 RNA positive detections were prior to reports of single COVID-19 case, in May 2021 and in October 2021 (Fig. 2, Site 3). Incidence of COVID-19 increased in March–May 2021 and after July 2021, and more frequent positive detections of SARS-CoV-2 were observed accordingly at the similar period.

3.2. Effect of precipitation on wastewater chemical parameters

Among the measured parameters of wastewaters, PO_4^{3-}-P and NH_3-N were observed with potentials as human activity indicator rather than COD and TSS. In Fig. 3, across days with no dilution effect (i.e. no precipitation), PO_4^{3-}-P and NH_3-N concentration showed significant differences among the six sampling sites (One-Way ANOVA test, p-value 0.001 and 0.07 respectively). The average concentrations at sites 1, 3, and 4 were significantly higher concentrations than sites 2 and 5, being consistent with trends of population densities. The correlation analysis indicated positive correlation of these two chemicals with population density ($\rho = 0.54$ and 0.6) although only marginal significance was observed (p-value 0.11 and 0.15) (Table S4). The trend following population density was not observed with COD and TSS.

Precipitations were observed to dilute concentrations of wastewaters NH_3-N and PO_4^{3-}P but not on COD and TSS (Fig. 3). Concentrations of NH_3-N and PO_4^{3-}P were significantly different across different levels of precipitations at most sites including 1, 2, 5, and 6 (One-Way ANOVA test, p-value <0.076 and Kruskal-Wallis test, p-value <0.12). Concentrations of NH_3-N and PO_4^{3-}P of samples collected on days with precipitation were significantly lower than the samples on days with no precipitation (One-sided *t*-test, p-value <0.029 and Wilcoxon test, p-value 0.001). In average, NH_3-N concentrations of samples of up to 50th percentile, >50th percentile, and >75th percentile was 1.20, 1.18, and 2.52 times lower than days of no precipitation. Similarly, the PO_4^{3-}-P concentration was 1.09, 1.22, and 1.98 times lower. Such dilution effect was not observed with COD and TSS results. At site 3, which is the only separated sewer system in this study, no particular trend of concentrations was observed.

3.3. Effect of precipitation on the sensitivity of WBE of SARS-CoV-2

In this study, significant improvement in number of positive assays of SARS-CoV-2 detections following increased incidence of COVID-19 clinical ratio within following 7 days was observed (Kruskal-Wallis with Dunn test, p-value <0.05) (Fig. 4). On average across all samples with one positive SARS-CoV-2 assay, the detection was followed with the average daily incidence above 2 (Fig. 4) and the highest incidence above 6 within seven days (Fig. S3). Rain precipitation lowers the sensitivity of WBE, in which higher COVID-19 clinical caseloads are required to detect SARS-CoV-2 in the wastewater. Likewise, among samples with two to three detected assays, the average incidence within seven days following positive detections on days with precipitation showed a higher average value than the incidence following positive detections on days without precipitation (Fig. 4). Similarly, when the highest incidence within seven days was considered, the samples of precipitation showed higher level of incidence than the samples from days without precipitation among those with two detected assays (Fig. S3). The differences were not statistically significant, which might be due to influence of convoluted variations in disease etiology across studied regions during sampling with and without precipitation.

3.4. Calibration of WBE of SARS-CoV-2 using changes in wastewater chemical indicators

From the results above, the changes in NH_4^+-N and PO_4^{3-}-P concentrations showed clear precipitation-based dilution effects on the wastewater and therefore both parameters can be used to calibrate the signal of SARS-CoV-2 after precipitation events. Improved correlation between the COVID-19 incidence and wastewater-based surveillance of SARS-CoV-2 was observed after calibration of SARS-CoV-2 concentrations to NH_4^+-N and PO_4^{3-}-P concentrations, compared with that without calibration (Fig. 5). Improvement of correlations by calibration of SARS-CoV-2 to chemical indicators (NH_4^+-N and PO_4^{3-}-P) were investigated among positive samples based on three criteria, including samples which were positive with two or more RT-qPCR assays (A) from any sampling days regardless of precipitation conditions (Fig. 5A); (B) from sampling days with

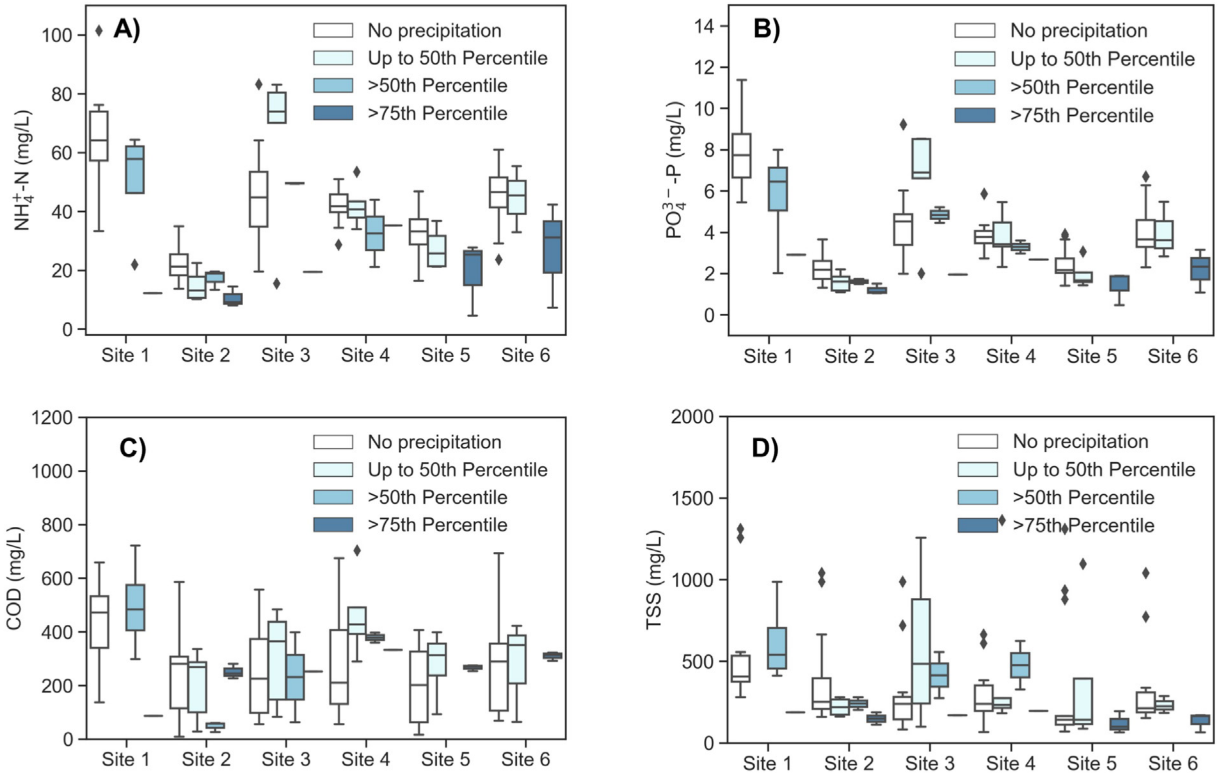

Fig. 3. Concentrations of NH_4^+-N (A), PO_4^{3-}-P (B), COD (C) and TSS (D) in six sampling sites. The samples were categorized based on presence of precipitation, samples on days with no precipitation ("No precipitation"), samples on days with precipitation level up to 50th Percentile of all samples ("Up to 50th Percentile"), samples on days with precipitation level more than 50th Percentile and up to 75th Percentile (">50th Percentile"), and samples on days with precipitation level above 75th Percentile (">75th Percentile").

precipitation (Fig. 5B) and (C) from sampling days with 0.1 in. (50th percentile) or more daily precipitation (Fig. 5C). For the criteria (A), all three genes calibrated with NH_4^+-N and PO_4^{3-}-P showed significantly higher correlation coefficients than the concentration without calibrations (paired one-sided *t*-test, p-value <0.03), in which PO_4^{3-}-P showed higher values than NH_4^+-N (paired one-sided t-test, p-value <0.001). The averaged and highest spearman's coefficient for N1 were 0.02 and 0.10 for the calibrated data, compared with the −0.04 and 0.03 for the noncalibrated data, respectively (Fig. 5A). The averaged and highest spearman's coefficient for N2 were 0.13 and 0.38 for the normalized data, compared with the −0.02 and 0.14 for the noncalibrated data, respectively (Fig. 5A). The averaged and highest spearman's coefficient for E was 0.20 and 0.45 for the calibrated data, compared with the 0.05 and 0.18 for the noncalibrated data, respectively (Fig. 5A). For the criteria (B), positive samples with considering the precipitation, the averaged spearman's coefficient was 0.03, 0.10, and 0.25 after calibration, compared with 0.01, 0.01, and 0.18 without calibration for N1, N2, and E assay respectively. Among those, the sustained significant correlation improvement was observed with N1 and N2 genes (paired one-sided *t*-test, p-value <0.05 and paired one-sided Wilcoxon-test, p-value <0.015) (Fig. 5B). For the criteria (C), positive samples with considering the precipitation level more than 50th percentile, the averaged spearman's coefficient was 0.28, 0.40, and 0.45 after calibration, compared with 0.13, 0.18, and 0.29 without calibration for N1, N2, and E assay respectively. The sustained significant correlation improvement was observed with N1 and E genes (paired one-sided Wilcoxon-test, p-value <0.006) (Fig. 5C).

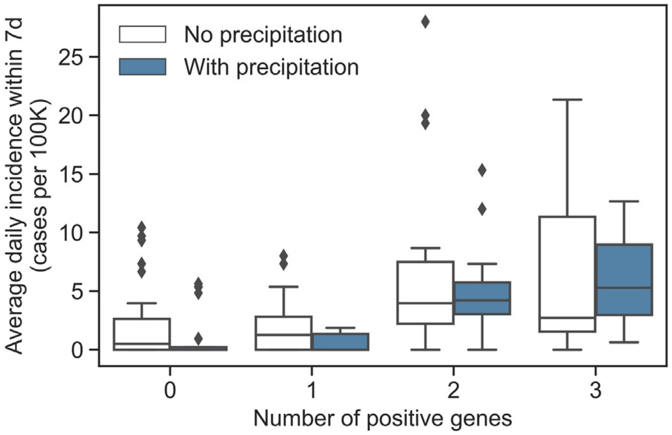

Fig. 4. Average daily COVID-19 incidence within 7-days lag periods of sampling days with precipitation and with no precipitation.

4. Discussion

Intra-city level wastewater surveillance of SARS-CoV-2 and its sensitivity. Wastewater surveillance at WWTP has been a major approach for surveillance of COVID-19 (Gutierrez et al., 2021), likely due to the practice being readily incorporated with the procedure at the facilities i.e. sample accessing point, collection, and staff. However, the surveillance reflects prevalence of disease only at city level, which introduces challenges in prevention and control of infectious disease spread. A previous study pointed out that dynamics and timing of total new daily COVID-19 incidence within entire cities did not necessarily align with the trends observed in subareas such as counties (Thomas et al., 2020). As COVID-19 disease transitions into an endemic and people rely more on home testing kits, the major goal of wastewater surveillance is to identify hotspots in order to timely prevent and control the spread. Wastewater surveillance with higher resolution at intra-city level is essential for the surveillance of COVID-19 moving forward. In this study, the wastewater surveillance of SARS-CoV-2

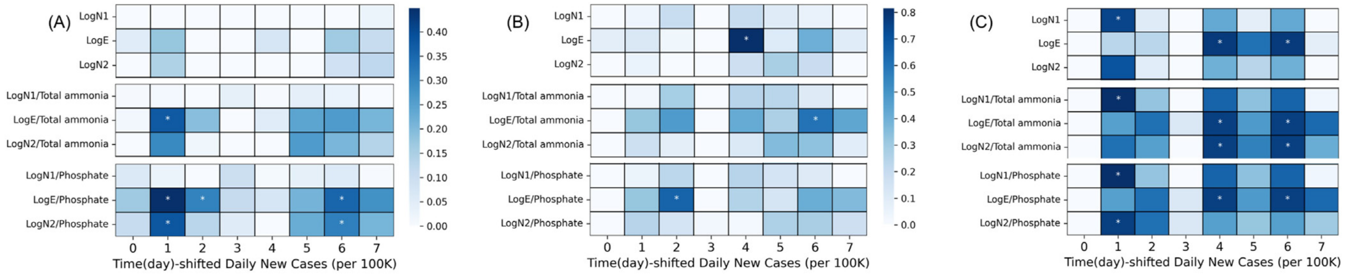

Fig. 5. Spearman's correlation between SARS-CoV-2 gene copies in wastewaters from six sites and the time shifted clinical COVID-19 cases (0–7 days shifted). The correlation was performed with only samples of two or more positive genes (A), with only samples of two or more positive genes and with precipitation (B), with only samples of two or more positive genes and precipitation level of >0.1 in. (C). The significant correlation was marker with * (p-value <0.05).

RNA from upstream points as manholes and a pump station in Seattle effectively reflected heterogeneity in COVID-19 incidence across the city with effective sensitivity.

Previously, a study of wastewater surveillance of COVID-19 to monitor trend of an entire city of Seattle was conducted at three WWTPs during March–July 2020 (Philo et al., 2021). The temporal trend of viral RNA concentrations was like the clinical COVID-19 incidence reported by local government and surveillance at all three WWTPs did not show major difference. While the collective clinical data by city governments can reasonably be used for comparing the trends with large level wastewater surveillance, the screening processing of clinical data is needed to validate wastewater surveillance at intra-city levels. Like previous studies at upstream sampling points (Black et al., 2021; Greenwald et al., 2021a; Weidhaas et al., 2021; Wilder et al., 2021), the case number of COVID-19 in this study corresponded to individuals living in addresses with sewers linked to studied manholes and a pump station and their fecal shedding would contribute to detected viral RNA in collected wastewaters. In contrast to the previous study at three WWTPs in Seattle, concentrations of SARS-CoV-2 RNA of this study here showed variations in timing of peaks across six studied sites, which depicted heterogeneous dynamic of COVID-19 among subareas in Seattle (Fig. 2). The detections of SARS-CoV-2 RNA well reflected trend of mapped confirmed COVID-19 cases living within the sewersheds. The peaks of clinical cases linked to a site were found to deviate from the total number of the city, which peaked in the second week of April 2021 and the second week of August 2021. Wastewater surveillance of SARS-CoV-2 RNA at intra-city level in previous studies also demonstrated similar reflection of community prevalence of COVID-19 (Black et al., 2021; Greenwald et al., 2021a; Weidhaas et al., 2021; Wilder et al., 2021).

Sampling level is considered as a factor affecting the results of WBE (George et al., 2022). A major challenge for intra-city level surveillance is the low case numbers could normally result in the short-term fluctuation of virus loads, leading to more difficulties of capturing sporadic pulses of SARS-CoV-2 RNA than that at a city level with much higher incidences (Li et al., 2022), highlighting the need for more studies at intra-city level. Detection of SARS-CoV-2 RNA in wastewaters was observed to occur prior to spikes in clinical cases (Nemudryi et al., 2020), which is influenced by various factors including disease etiology and delay in seeking testing and medical care as discussed in a previous study (Olesen et al., 2021). In this study, detection frequency of SARS-CoV-2 RNA across three PCR assays were positively correlated to incidence reported within seven days following the sampling of wastewater. Among the sampling that showed one positive assay, range of the averaged and highest daily COVID-19 incidence within the following seven days were 0–8 (arithmetic mean = 2) and 0–22 (arithmetic mean = 6) per 100 K residences (Figs. 4 and S3). A previous study at similar intra-city level showed the positive detection of SARS-CoV-2 RNA in wastewater when the weekly incidence rate ranged around 3.75–59.5 per 100 K residences (Wilder et al., 2021).

The strength of wastewater surveillance is its capability to capture re-emergence of COVID-19 and guide the public health measures to promptly prevent the spread when there are still only a few cases in the community. The surveillance at the level of whole cities previously showed that detection of SARS-CoV-2 RNA was observed when there were <10 cases per 100 K residences (Hata et al., 2021; Hewitt et al., 2022; Medema et al., 2020b). Although this indicated the sensitive detection, there are challenges for public health agencies to control the spread in a timely manner due to large areas of surveillance coverage. In the meantime, highly useful application of wastewater surveillance was observed at building/facility level, in which positive detection of SARS-CoV-2 prevented spread of COIVD-19 from a single asymptomatic case within 150–200 residences (Gibas et al., 2021). However, setting up the surveillance systems at buildings across large area would cause challenges in budgets, resources, staffs, and implementation. The wastewater surveillance at these levels could result in limited impact to prevention and control of the disease spread. In this study, we showed effective application of wastewater surveillance at intra-city levels, in which presence of 1–12 clinical cases of COVID-19 in communities of 2580–39,502 could be alerted from positive detections of SARS-CoV-2 RNA (Fig. 2). At site 3 of total of 2580 residences, a single COVID-19 case was reported during a two-month period during the study and the positive detection of SARS-CoV-2 RNA was followed with the reported of both incidences of single cases delineating high level of sensitivity when sampled at intra-city level.

Use of chemical indicators for calibration of precipitation effect. Rainfall-derived inflow and infiltration into sewers would have dilution effect on concentrations of SARS-CoV-2 RNA in wastewaters and hence, surveillance of COVID-19 prevalence in the community. The effect is more prevalent in the CSS and PSS, which sanitary sewers and storm drain sewers are interconnected, although sewer cracks and illegal connections also causes the inflows to the separate sewer system (Staufer et al., 2012). The dilution effect to intra-city level WBE of COVID-19 or other infectious diseases is inevitable. In a previous study, across three diurnal sampling campaigns at two pump stations in Seattle, the rain precipitation was demonstrated to cause decreasing trends of SARS-CoV-2 RNA concentrations in wastewaters although the incidence of COVID-19 in communities were comparable throughout (Nguyen Quoc et al., 2022).

In this study, we demonstrated the effect of rain precipitation on WBE of COVID-19 using samples from manholes and a pump station in Seattle throughout seven-month period. Frequency of positive PCR assays of SARS-CoV-2 RNA was shown significantly influenced by reported COVID-19 incidence. Among the samples showing two and all three positive assays, we observed that averaged COVID-19 incidence within seven days following the sample collection on days with rain precipitation sampling was higher than the collection on days without rain precipitation (Fig. 4), implying potential misleading interpretation of incidence using SARS-CoV-2 concentrations influenced by the dilution effect.

While approaches to calculation of viral loads using flow rate or calibrations using biological indicators of fecal matter have been recommended to account for the dilution effect, their application might be less certain due to reported variation of efficiency following different surveillance system settings (Nagarkar et al., 2022; Zhan et al., 2022). Related cost has been a major challenge and bottleneck to establish or maintaining the WBE as a tool to secure public health (Safford et al., 2022). Tracking the flow rates at the upstream sampling points as manhole, which would need advance and expensive sensors, as well as additional molecular-based analyses of biological indicator would increase the capitol costs and hence, introduce more challenges. It is vital that alternative indicators should be made available to support WBE application.

NH_4^+-N and PO_4^{3-}-P, which are common in municipal wastewaters (USEPA, 2009), are derived from human metabolites which are excreted at the same time as human pathogens hence providing a means for correlation analysis. The breakdown of nitrogen and phosphorus molecules in food diet produces NH_4^+-N and PO_4^{3-}-P products, which are then excreted in urine (Pradhan et al., 2017). In our study, dilution effect of rain precipitation on NH_4^+-N and PO_4^{3-}-P concentrations were strongly demonstrated across all the sites of CSS or PSS (Fig. 3). After the rainwater induced dilution of the wastewater the calibration with NH_4^+-N and PO_4^{3-}-P concentrations showed improvement on surveillance of COVID-19 incidence (Fig. 5). The cross-correlation analysis showed high correlation coefficients between viral concentration and shifted COVID-19 incidence at multiple time points (1, 2, 4, and 6 day) (Fig. 5). Variation of correlation coefficient across time shifting periods without the calibration was consistent with the variation after the calibration. More importantly, the calibrated SARS-CoV-2 gene concentrations using NH_4^+-N and PO_4^{3-}-P concentrations showed improvements of correlations with the time-shifted clinical COVID-19 incidence as proven by a significant increase of the Spearman's correlation coefficient (p-value <0.05), highlighting the advantage of using chemical indicators to account for the dilution effect. Previously, a diurnal sampling study at two pump stations demonstrated the calibrations of SARS-CoV-2 RNA concentrations using NH_4^+-N and PO_4^{3-}-P could improve correlation to the ratio of reported COVID-19 cases (Nguyen Quoc et al., 2022). The results from our study have assured the improvements through a seven-month long study and across six sites of manhole sampling and one pump station, showing that chemical indicators can be used to correct sewer derived SARS-COV-2 signals for rainwater dilution as such that these signals better match COVID- 19 caseloads.

5. Conclusions

Overall, this study provided insights of intra-city level wastewater surveillance of COVID-19, which is essential to secure public health in the post-pandemic period. High vaccination rates, COVID-19 endemicity, and public reliance on ‘at-home testing kits’ contributes to heterogeneous dynamics of the disease across geographical area, in which intra-city level WBE could support identification of disease reemergence at certain hotspots to promptly control and prevent the spread of the disease. Also, the alternative population indicator as NH_4^+-N and PO_4^{3-}-P, affordable and convenient analyses of which have been increasingly diverse and available, could respond to demanding application of WBE. The knowledge could also be used to guide the WBE application toward other infectious diseases such as monkeypox and reemergent polio.

CRediT authorship contribution statement

Prakit Saingam: Methodology, Investigation, Validation, Data curation, Formal analysis, Visualization, Writing-original draft, review & editing. **Bo Li:** Conceptualization, Data curation, Formal analysis, Writing-original draft. **Bao Nguyen Quoc:** Methodology. **Tanisha Jain:** Resources, Methodology. **Andrew Bryan:** Resources. **Mari K. H. Winkler:** Funding acquisition, Supervision, Writing - review & editing, Project administration. **Prakit Saingam** and **Bo Li** contributed equally to this work.

Data availability

If the requested data can be shared, the data will be made available on request.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161467.

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