ABSTRACT: Wastewater based epidemiology (WBE) has emerged as a tool to track the spread of SARS-CoV-2. However, sampling at wastewater treatment plants (WWTPs) cannot identify transmission hotspots within a city. Here, we sought to understand the diurnal variations (24 h) in SARS-CoV-2 RNA titers at the neighborhood level, using pump stations that serve vulnerable communities (e.g., essential workers, more diverse communities). Hourly composite samples were collected from wastewater pump stations located in (i) a residential area and (ii) a shopping district. In the residential area, SARS-CoV-2 RNA concentration (N1, N2, and E assays) varied by up to 42-fold within a 24 h period. The highest viral load was observed between 5 and 7 am, when viral RNA was not diluted by stormwater. Normalizing peak concentrations during this time window with nutrient concentrations (N and P) enabled correcting for rainfall to connect sewage to clinical cases reported in the sewershed. Data from the shopping district pump station were inconsistent, probably due to the fluctuation of customers shopping at the mall. This work indicates pump stations serving the residential area offer a narrow time period of high signal intensity that could improve the sensitivity of WBE, and tracer compounds (N, P concentration) can be used to normalize SARS-CoV-2 signals during rainfall.

KEYWORDS: wastewater based epidemiology, SARS-CoV-2 RNA signal, pump station, neighborhood-scale monitoring, Covid-19, sewage
WBE studies have predominantly sampled WWTPs that collect from major fractions of a city\textsuperscript{1−4,15,16,18,20,22} or sampled single buildings of congregate living facilities.\textsuperscript{23−26} However, a revealing feature of the pandemic has been the differential impact of COVID-19 on socioeconomic groups,\textsuperscript{27,28} which could be more effectively observed at the neighborhood or census tract scale. For example, census tracts in Seattle (WA, US) that are less wealthy and more diverse correlate with higher rates of COVID-19.\textsuperscript{29,30} This links with national trends where Black, Indigenous, People of Color (BIPOC) are \textasciitilde 2X more likely to die from COVID-19 than White and Non-Hispanic people in the US\textsuperscript{31} due to a higher likelihood of being essential workers,\textsuperscript{32} living in a multigenerational home,\textsuperscript{33} and other risk burdens.\textsuperscript{34} Similarly, vaccine hesitancy is correlated with income and other demographics\textsuperscript{35} that could potentially be observed at the neighborhood level. Despite known vulnerabilities of specific communities within cities, the application of WBE at the neighborhood scale is lacking.

Monitoring pump stations, which collect wastewater from a small neighborhood, instead of WWTPs, which collect sewage from the whole city, could alleviate many of the limitations of WBE and capture neighborhood level trends in COVID-19. Pump station samples are retrieved closer to the source, which is less time for RNA to degrade. Further, if retention times in the sewer-shed are sufficiently low, pump station monitoring could temporally separate SARS-CoV-2 shedding activities (e.g., defecation) from diluting activities (e.g., bathing, washing dishes, laundry).\textsuperscript{36,37} Most importantly, pump station monitoring can be deployed more specifically to the neighborhoods that are vulnerable, which enables accounting for more local sewer characteristics (e.g., combined vs separated sewers) and generates more actionable data.

In this study, we sought to advance the methodology of pump station monitoring by testing two hypotheses. First, we hypothesize pump station RNA signals will be maximized at specific time windows each day due to the routine nature of RNA releasing behavior (e.g., defecation), as well as RNA diluting behaviors (e.g., bathing, laundry, or washing dishes). Second, we hypothesize SARS-CoV-2 quantities could be normalized using commonly observed chemicals in those time windows, which can account for variations between communities due to differences in water consumption and combined sewers. We test these hypotheses by conducting 24 h (diurnal) sampling at two pump stations, each serving a distinct neighborhood: (a) a low-density residential neighborhood (“Residential Area”) and (b) a primarily commercial shopping district with mixed residential sewer inflow (“Shopping district”). We measured nutrient concentration along with SARS-CoV-2 RNA across the diurnal cycle and corrected the signal for rainwater dilution using nutrient concentration.

2. MATERIAL AND METHODS

2.1. Site Description. Residential Area. The residential pump station collects sewage from a combined sewer system at the Lakewood residential area (Seattle, WA) (Figure 1a,b). Lakewood has a population of 2925 inhabitants and consists of 1242 households, of which 33.1% are single-person type and 43.6% are married/family type households.\textsuperscript{30} Lakewood is situated in the southeast area of Seattle, which is one of the most diverse neighborhoods in Seattle: 27% White, 26% Black, 10% Hispanic, and 31% Asian origin.\textsuperscript{33} The median age of residents in this neighborhood is 40−44 years, which is higher than the average age (34 years) of Seattle\textsuperscript{33,39}

Shopping District. The shopping district pump station collects sewage from a separated sewer system at the University Village shopping center (Seattle, WA) (Figure 1a,c) and an apartment complex primarily serving university students (occupancy 145). The district is located in northeast Seattle. The University Village shopping mall consists of over 120 restaurants and stores for books, apparel, groceries, furniture, jewelry, salons, etc., and is open from 11 am to 7 pm.

2.2. Sampling Procedure, RNA Extraction, qPCR, and Sanger Sequencing. An autosampler (5800 Refrigerated Sampler, Teledyne ISCO) was used to collect hourly samples across a 24 h diurnal cycle, where each hour was the aggregate sample of four 200 mL samples collected in 15 min intervals from the pump station wet-well. Samples were collected at the shopping district on November 8th, November 15th, and November 22nd, 2020. From the residential area, samples were collected on December 6th, December 13th, and December 20th, 2020. Samples were stored at 4 °C in the refrigerated
compartment of the autosampler until pickup. All analyses were performed within 8 h of sample pickup.

Solids in the samples were settled for 30 min on the bench before 140 μL of the liquid (i.e., supernatant) from the top layer was used for direct RNA extraction. RNA extraction was performed in duplicate by using a QIAamp Viral RNA Mini Kit (Qiagen), following manufacturer’s instructions. The recovery of BCoV by this extraction method was 57% ± 13% (Section S1 in the Supporting Information (SI)).

Quantitative PCR (qPCR) for N1, N2, and E assays was performed in duplicate using 5 μL of QuantiNova Probe RT-PCR Kit (Qiagen), 4 μL of RNA extract, and additional reagents according to manufacturer’s protocol, amounting to 10 μL total reaction volume. qPCR was performed with a Roche LightCycler 96 Instrument (Roche). The qPCR thermal profiles started with 10 min of reverse-transcription at 45 °C, a 5 min heating step at 95 °C, followed by 40 cycles at 95 °C for 5 s (s), and annealing temperature 52 °C for 10 s and 55 °C for 10 s. Each sample was analyzed using two RT-qPCR replicates of each assay. Primers and probes are listed in Table S1. The nCoV-ALL-Control plasmids (Eurofins Genomics) in the range 6 × 10^7–1 × 10^8 copies per reaction were used for standard curves for the N1, N2, and E assay. All three assays have a limit of quantification (LOQ) of 6 × 10^1 copies per reaction. The limit of detection (LOD), at least 90% of replicates can be consistently detected, was 1.2 × 10^1 copies per reaction for N1, N2, and E assay. Positive and negative controls were included in every qPCR as quality controls. The amplification efficiencies of qPCR assays for N1, N2, and E assays were 99.4%, 100%, and 99.2%, respectively.

The qPCR products were purified by a MinElute PCR Purification Kit (Qiagen), purified cDNA PCR-products were sequenced with Sanger sequencing (Eurofins Genomics). The qPCR assay was externally validated against a CDC-based clinical assay performed in the University of Washington Department of Laboratory Medicine and Pathology Clinical Virology Laboratory using extracted samples (PMID 32269100).

2.3. Chemical Analysis. Sewage samples were filtered through a 0.2 μm nylon syringe filter (VWR) before chemical analysis. Ammonia and phosphate were measured spectrophotometrically at 660 and 880 nm wavelengths, respectively, using a Gallery Automated Photometric Analyzer, with
Figure 3. (a) Diurnal ammonia and (b) phosphate concentration at pump station 5 on December 6th, 2020, December 13th, 2020, and December 20th, 2020. Date was written in the format month/day/year.

2.4. Data Analysis. The correlation between log-transformed SARS-CoV-2 RNA titer (based on three targets N1, N2, and E assays) and nutrient concentrations was plotted using R. The biplot of PCA was performed using the generic functions autoplot() and prcomp() in R. The biplot was plotted on the basis the first two principal components. For PCA, the data frame included SARS-CoV-2 RNA titer, nutrients concentration, and flow rate from the residential pump station. Mean and confidence interval (significance level of 0.05) of SARS-CoV-2 genes concentration (gene copy/milliliter of sewage) were reported for diurnal samples.

Nutrient dilution is a reflection of flow rate, and nutrient concentration is easier to measure than flow rate; therefore, we focused on showing the normalization of SARS-CoV-2 by nutrient data. The data plotted in Figure 6 present the normalized log virus concentration and the phosphate concentration during the morning peak from 5–7 am according to eq 1. Data for the flow rates at the residential pump station were obtained with a flow rate sensor. The 24 h observation of rain precipitation at Seattle was obtained from the National Oceanic and atmospheric administration (noaa.gov).

\[
\text{normalized log (virus concentration)} = \frac{\log(\text{virus concentration})}{(\text{relative[nutrient] compared to December 6th})}
\] (1)

In which:

\[
\text{relative[nutrient] compared to December 6th} = \frac{[\text{nucleotide concentration in a given hour, mgP/L}]}{[\text{nucleotide concentration in the corresponding on December 6th, mgP/L}]
\] (2)

where [nutrient] is either the concentration of ammonia or phosphate from 5–7 am each investigated day.

3. RESULTS

3.1. Residential Area. 3.1.1. SARS-CoV-2 RNA Viral Load. Diurnal sampling revealed significant peaks in the quantity of SARS-CoV-2 RNA at varying times of day. RT-qPCR results from December 6th, 2020 showed that all three N1, N2, and E assays peaked at 2 and 5–7 am (Figure 2a). For N2 and E assay, the 2 and 5–7 am signals were in the range $10^3$–$5 \times 10^4$ gene copy/mL sewage (Ct values in the range 28–34), which was higher than the late afternoon and early evening peaks ($3 \times 10^2$–$1.4 \times 10^4$ gene copy/mL sewage; Ct values in the range 30–36). While all target genes showed similar trends across the day, calculated N1 values were 1–9 times higher than N2 and E assay throughout the day.

While COVID-19 cases increased steadily in the study area (Table S2, Supporting Information), viral signal did not follow this trend (Figure 2a–c). Instead, sewage flow rates were negatively correlated with signal intensity across these days (Figures 2d and 5c). This could indicate stormwater runoff diluted the viral signal in the sewage. For example, RT-qPCR results from December 13th, 2020 showed peak signals of all three assays (N1, N2, and E assays) during the 5–7 am period with $3 \times 10^2$–$8 \times 10^3$ gene copy/mL, which was about six times lower during the peak time 5–7 am than data from December 6th, 2020 (Figure 2b). Meanwhile, there was 0.20 in. of rain on December 13th, 2020, which was about six times the precipitation measured on December 6th, 2020 (0.03 in.) (Table S3).

We also observed that, if it rains intermittently within the course of a day, the SARS-CoV-2 titers at different time points will be diluted disproportionately. For example, the flow rate on December 13th, 2020 surpassed the December 6th, 2020 flow rate at 3 am (Figure 2d), suggesting the rain picked up after 3 am. As a result, we found the SARS-CoV-2 titer in the 5–7 am window on December 13th, 2020 to be similar to the SARS-CoV-2 titer in the 2–3 am window of both December 6th and 13th, 2020 (Figure 2b).

RT-qPCR results from December 20th, 2020 did not show a peak for SARS-CoV-2 throughout the day. The copy number of all three genes was in the range $10^2$–$10^3$ gene copy/mL sewage, which was in the same range as the copy number after 8 am December 13th, 2020. The precipitation level was around 0.06 in. on December 20th, 2020; however, the precipitation level the day before, on December 19th, 2020, was 0.45 in. (Table S3). It is likely that the rain increased the water level in the groundwater and subsequently infiltrated into the sewage line and then diluted the sewage as such that the morning peak of SARS-CoV-2 did not show up anymore. This theory was
confirmed by a significant higher flow rate going through pump station 5 on December 20th, 2020 if compared to a day with the relative dry weather flow on December 6th, 2020 (Figure 2d).

To verify the fidelity of RT-qPCR reaction, we used Sanger sequencing to validate RT-qPCR products. Sanger sequencing confirmed that the RT-qPCR products were from SARS-CoV-2 RNA with 97% match with N gene and 99% match with E gene sequence from NCBI database (Section 3, SI). This verification is important as previous studies have reported amplification of unspecific products from sewage samples.44

3.1.2. Nutrient Concentration and its Correlation with SARS-CoV-2 RNA Viral Concentration. Nutrient concentrations (i.e., ammonium, phosphate) were monitored across the day to investigate potential correlations with SARS-CoV-2 RNA concentrations. Both nutrients followed the same trend, peaking around noon (Figure 3). The nutrient concentrations on December 13th, 2020 were lower than on December 6th, 2020 throughout the entire day and became more diluted after 3 am (Figure 3). This is in alignment with the decrease of SARS-CoV-2 RNA signal on December 13th, 2020 after 3am (Figure 2b). This event can be explained by a high sewage flow rate on December 13th, 2020, which picked up after 3 am (Figure 2d). Nutrient profiles and flow rates on December 20th, 2020 were diluted throughout the day and especially during the morning between 1 and 8 am (Figure 3) when SARS-CoV-2 peaked at dry weather flow. This was in alignment with the missing SARS-CoV-2 signal in the early morning hours of December 20th, 2020 (Figure 2c).

To investigate how SARS-CoV-2 RNA concentration clustered in the course of a day and potential correlations of virus titer with other parameters, a PCA biplot was performed on the dataset from December 6th to December 13th, 2020 when the peak in the early morning was shown (Figure 4). The dataset from December 20th, 2020 was not used for the PCA plot because it was heavily diluted throughout the day and thus interfering with the interaction of SARS-CoV-2 RNA concentration, nutrient loading, and flow rate (Figure S4, Supporting Information). The data for 5–7 am clustered in the PCA plot and distinguished from the rest of data (Figures 3 and 2). The 2–4 am values were merged with the rest of data in the PCA plot and cannot be differentiated (Figure 4). Ammonia and phosphate vectors were nearly perpendicular to the SARS-CoV-2 RNA titer vector, suggesting that nutrient concentrations were not correlated with SARS-CoV-2 RNA signals within a day (Figure 4). This was in alignment with previous observation where SARS-CoV-2 RNA concentration peaked at the lowest nutrient concentrations (Figures 3 and 2). Ammonia and phosphate vectors formed a small angle, indicating positive correlation between these parameters. The time (hour of a day, from 1 through 24 h) and SARS-CoV-2 RNA titer formed a large angle (close to 180°) (Figure 4), suggesting they were negatively correlated, which was in agreement with the observation in Figure 2 where SARS-CoV-2 RNA titer had a higher concentration earlier in the day.

Stormwater diluted both SARS-CoV-2 RNA concentration (in log-scale) and nutrient concentration during the morning peak 5–7 am window across different days (Figure 5). When plotting the flow rate of sewage versus the SARS-CoV-2 RNA titer during the 5–7 am window, we observed a negative correlation: the higher the flow rate, the lower the SARS-CoV-2 RNA concentration (Figure 5c), suggesting that the signal was diluted by stormwater (Figure 2). The flow rate increased on December 13th, 2020, which also led to a decrease in the SARS-CoV-2 RNA signal within the same day (Figure 5c). Since flow rate is hard to measure in many manholes or pump stations, we looked into other alternative features that can easily be collected and able to indicate the dilution by flow
rate. Nutrient concentration could be a good alternative parameter since it negatively correlated with flow rate (Figure S3, Supporting Information). In fact, the viral concentration in the morning peak 5−7 am window across several days showed a positive correlation with nutrient levels measured at that time frame (Figure 5a,b). This decrease of SARS-CoV-2 RNA signal happened during the period when rate of new COVID-19 cases increased from 42 to 91 new cases (Figure 6).

While Covid-19 cases in the pump station-associated neighborhood rose, we observed decreasing SARS-CoV-2 signal, due to stormwater dilution (Figure 6a). We could connect the SARS-CoV-2 signal to the Covid-19 cases by normalizing the log-transformed gene copy number of SARS-CoV-2 with nutrient concentrations (eqs 1 and 2) as well as the flow rate through the pump station (Figure 6, Table S4, Supporting Information). On the contrary, normalizing the absolute gene copy number concentration or calculating the virus load (= viral concentration × flow rate) did not show clear trends (Table S4, Supporting Information). The observation when dilution factor can only correct for log of virus concentration but not the absolute virus concentration suggests that stormwater dilution impacts SARS-CoV-2 signal more severely (by a factor of 10) than nutrient concentration.

3.2. Shopping District. 3.2.1. SARS-CoV-2 RNA Titer. RT-qPCR results showed that the N1, N2, and E assay were not consistently detected at the shopping district pump station (Figure 7). Unlike in the residential area, no notable peaks of SARS-CoV-2 RNA signal were observed; however, SARS-CoV-2 RNA concentrations increased toward the afternoon. On November 8th, 2020, the N1 and N2 concentrations were in the range $10^3$−$1.4 \times 10^4$ gene copies/mL sewage in the middle of the day (noon and 5 pm), which was about 3−4 times higher than the values detected in the early morning from 5−7 am ($4 \times 10^2$−$3 \times 10^3$ gene copies/mL sewage). On November 15th, 2020, the N1, N2, and E signals were in the range $1 \times 10^2$−$5 \times 10^2$ gene copies/mL sewage around noon but were not detected during the late afternoon. On November 22nd, 2020, N1, N2, and E concentrations were stronger in the middle of the day (3−6 pm, the signal was in the range $4 \times 10^2$−$1.4 \times 10^3$ gene copies/mL sewage).

3.2.2. Nutrient Loads. Nutrient (ammonia and phosphorus) concentrations peaked around noon and 5 pm (Figure 8). This nutrient trend matched with higher values in SARS-CoV-2 RNA viral signal in the afternoon. The nutrient profiles for three sampling days (November 8th, 2020, November 15th, 2020, and November 22nd, 2020) were similar. This was due to the fact that the influent to pump station 35 is a sanitary sewer system, which does not collect stormwater, and in addition, there was little to no rainfall (0.00−0.05 in.) during the sampling campaign (Table S2).

4. DISCUSSION

Previous projects that tested SARS-CoV-2 in the sewer relied on monitoring at wastewater treatment facilities, which typically serve an entire city. However, COVID-19 prevalence is not evenly distributed within a city and sampling at wastewater treatment plants can only provide a broad municipality-level view of the spread. COVID-19 disproportionately impacts ethnic minorities and low-income communities due to generally increased housing density, the likelihood of being an essential worker, and/or having no, or reduced, access to healthcare. To shed light on virus prevalence per census tract, random testing of individuals or pooling samples have been used to map geographical dynamics and occurrence of COVID-19. However, individual testing capacity was a limiting factor as infections rose, making neighborhood-scale sewer monitoring an appealing alternative. Monitoring SARS-CoV-2 RNA at pumping stations could provide an early warning for the resurgence of COVID-19 on a much finer scale compared to monitoring at WWTPs.
Furthermore, pump station monitoring data could be powerful to analyze geographical dynamics and occurrence of SARS-CoV-2 and how this links to demographic factors such as race, social status, and other factors. This could further our understanding of unequal impacts of COVID-19 on ethnic minorities, low-income communities, essential workers, people without healthcare, or people living in congregate facilities. Additionally, sewage monitoring at the WWTP-scale could

Figure 7. Estimated SARS-CoV-2 RNA viral titer in sewage determined by the N and E gene assays for pump station 5 that served the shopping mall: (a) November 8th, 2020, (b) November 15th, 2020, and (c) November 22nd, 2020. Error bars represent the standard deviation of replicate results.

Figure 8. Nutrient concentration at pump station 5 collecting sewage from a shopping mall.
overlook rapidly changing signals on the neighborhood-scale. Viral signals of, for instance, neighborhoods with localized outbreaks could become undetectable after mixing of the wastewater with that of neighborhoods having no COVID-19 cases. Sampling upstream in the sewer collection system is expected to provide greater sensitivity to changes in the COVID-19 prevalence with a corresponding greater dynam-icity in RNA quantity.16 In addition, signals at WWTP scale are impacted by dilution and virus degradation through longer residence times in the sewer.15,16 Our data showed that Ct values at the tested pump stations during the morning peak (5–7 am) were considerably higher than the Ct values reported during the same sampling period at WWTPs that received sewage from the residential pump station (Table S5). Similar high Ct values were also reported in other studies focusing on WWTP-scale monitoring.3 Therefore, sampling at the neighborhood-scale could be a powerful tool to improve detection, quantification, and sensitivity.

4.1. Diurnal (24 h) Variation of SARS-CoV-2 RNA Titer. Diurnal (24 h) sampling from the residential area showed the highest nutrient peak around noon when SARS-CoV-2 was lowest. Therefore, this nutrient load likely originated from activities like cooking, laundry, and cleaning (Figures 2 and 8). The morning peak of SARS-CoV-2 titers were found to be highest in our case study, which is similar to previous research.47 This peak could be associated with human behavior: 61.3% of 2000 people confirmed to defecate in the morning according to a poll conducted by Healthline news outlet 2017.48 During morning hours, nutrient concentrations were lowest, presumably because morning activities linked to human hygiene release lower amounts of nutrients and higher amounts of virus if compared to noon activities (e.g., cooking).

On the contrary, the diurnal sampling from the shopping district showed stronger SARS-CoV-2 RNA viral signal in the afternoon (Figure 6). As the mall opens at 11am, more customers visit in the afternoon, increasing the chance of virus shedding into the sewage from bathroom usage. Diurnal samples from the shopping district indicated that SARS-CoV-2 RNA viral signal were heavily influenced by the changing numbers of infected persons visiting the mall during the course of the day, thus explaining high variations in SARS-CoV-2 signals even without any dilution phenomena by rain events.

The peak of SARS-CoV-2 RNA signal in the morning at the residential pump station is not expected to always show up at the WWTPs. Sewage from different neighborhoods will have different residence times to reach the WWTPs,49 likely spreading out the peak of SARS-CoV-2 RNA signal at the WWTPs. In addition, longer sewer lines may result in more degradation of SARS-CoV-2 RNA, reducing the viral signal to the baseline as seen in, for instance, Curtis et al. (2020).50 Furthermore, diurnal patterns can vary within WWTPs under different situations (lockdown, open phases). When most people work from home, e.g., during the lockdown for COVID-19 pandemic, WWTPs may have a noticeable peak similar to what was observed in the residential area. For instance, Bivins et al. (2021)47 detected high SARS-CoV-2 RNA titers during the morning and mid-day hours from the influent sewage in Northern Indiana, USA. However, as the economy opens back up, there will be more signals from offices and malls later in the day, potentially bringing the baseline to a higher value and lowering the intensity of the signal in the morning similar to Curtis et al. (2020)50 who reported lower SARS-CoV-2 RNA concentrations during the morning hours from the influent of WWTPs in Norfolk, VA, USA.

4.2. Impact of Rainwater Dilution on SARS-CoV-2 RNA Titer and Proposing Nutrient Concentration as Normalization Factor. Cities that receive a high amount of precipitation are exposed to a high dilution factor of the SARS-CoV2 signal in the sewer.51,52 Seattle is one of the ideal cities to test how rainfall impacts viral signals, due to the cities’ relatively high precipitation level,53 and our results show that heavy rain events lead to a dilution of the virus signal (Figure 2). Combined sewer systems (CSS) are expected to decrease concentrations and loads of human-associated indicators by an order of magnitude during heavy rainfall events if compared to a separate sewer system.23 The residential pump station is based on a CSS, hence explaining a lower SARS-CoV-2 RNA viral signal during high precipitation events (Figures 2 and 5c) despite increasing COVID-19 cases numbers during that sampling campaign (Table S2).

More than 700 cities across the US have a combined sewer system (USEPA 2014), which will similarly lead to the dilution of SARS-CoV-2 RNA.3 Some cities, like Seattle, have both combined and separate sewer systems. This highlights the importance of dilution corrections to appropriately compare and interpret the SARS-CoV-2 RNA signal from different neighborhoods and over time. This is especially problematic during wet seasons and might explain some results of studies that had difficulties to correlate SARS-CoV-2 signals to COVID-19 cases.53 Furthermore, the dilution of RNA is inconsistent across different neighborhoods due to varying water consumption across socioeconomic groups.53 Correcting the dilution of RNA may be further complicated by phased opening or closing of the economy causing fluctuations in commercial and industrial discharges within a sewer-shed. To make use of the RNA signal, marker compounds can be used to correlate the RNA signal to commonly discharged chemicals to normalize the RNA signal.54 Many researchers have used different tools to normalize the SARS-CoV-2 RNA signal to cope with the problem of dilution, for example, Westhaus et al. (2021)45 suggested to use the viral load (taking into account of the flow), D’Aoust et al. (2021)18 used the fecal indicator pepper mild mottle virus (PMMoV), while Green et al. (2020)55 used gut-virome cross-assembly phage (crAsphage). Alternative markers that are only associated with human excreting products are substances such as caffeine, cholesterol, and coprostanol (a cholesterol metabolite), but they are more costly to measure and require expensive equipment.56

Our study suggested that the nutrient data correlated well with the SARS-CoV-2 RNA titer when looking at a specific time window in the early morning hours (5–7 am) when SARS-CoV-2 signals were highest (Figure 5a,b). We were able to use phosphate and ammonia concentrations to correct log-transformed virus concentrations in this window (Figure 6), correlating virus titer to new Covid-19 cases in the sampled neighborhood. Therefore, nutrient data may be useful in normalizing the SARS-CoV-2 RNA titer, especially for upstream sampling locations (maintenance manholes, pump station) that are not equipped with a flow meter to track the flow rate.

4.3. Future Research Perspectives. The effectiveness of grab samples and daily composite samples for WBE was discussed in previous research, and daily composite samples were recommended over grab samples.22,57,58 However, if the grab sample is based on only a few samples taken sporadically
throughout the day\textsuperscript{17,50} then signals can vary tremendously as shown by our data (Figures 2 and 6) or even completely miss out the detection of SARS-CoV-2. On the contrary, a daily composite sample can be heavily impacted by intermittent rain throughout the day and thus might get unnecessarily dilution. We therefore suggest for future studies to identify the time during which SARS-CoV-2 RNA are highest and then focus on that window for future analyses. This method could advance WBE significantly by increasing the sensitivity (lower Ct signals), shortening the composite window, and reducing the time to result.

Modeling the associated risks of infectious virus-laden aerosols (norovirus and adenovirus) can be used to inform health safety protocols.\textsuperscript{57,58} While SARS-CoV-2 RNA is relatively stable for a few days in aquatic environments, infectious SARS-CoV-2 was shown to decay rapidly in wastewater, river, and seawater.\textsuperscript{59−63} Therefore, modeling the existence of SARS-CoV-2 RNA in wastewater does not confirm the presence of infectious SARS-CoV-2 and the risk of infection for Covid-19 from wastewater. However, there are likely similar peaks in the wastewater for other virus-laden aerosols during a certain time of the day, which may be used in the future for quantitative microbial risk assessment of aerosols at WWTPs or pump stations.\textsuperscript{60}

5. CONCLUSION

- In this case study, SARS-CoV-2 RNA titers in the sewage vary strongly throughout the day, with residential area shedding highest titers in the morning whereas in the shopping mall the signals were more likely to be detected in the afternoon.
- Pump stations have a unique time window for optimal sampling, which is likely influenced by the activities in the sewer-shed and the short source-to-sample point distance. Focusing the collection of composite samples on a narrow time window during which SARS-CoV-2 signal is highest helps to increase sensitivity and shortens sample procedures, which will greatly benefit WBE.
- For the residential area in this case study, a strong correlation between SARS-CoV-2 RNA titer and nutrient concentrations was found within the time window of high RNA concentrations, indicating the potential of simple tracers to correct for dilution.
- SARS-CoV-2 RNA concentrations were diluted both by rainwater and infiltration of groundwater into the sewer. We also found the intermittently rain dilute SARS-CoV-2 signal differently within a day as an example for samples on December 13th, 2020 mentioned in Section 3.1.1. This suggested that the hydrodynamic characteristics of the sampling site need to be understood and taken into account.
- We obtained a better correlation of SARS-CoV-2 signal and Covid-19 cases after normalizing the log of SARS-CoV-2 gene copy number to the ammonia and phosphate concentrations as well as the flow rate of composite samples collected during the hours of highest SARS-CoV-2 RNA prevalence (likely due to defecation and personal hygiene activities in the morning hours).
- This case study is the first to demonstrate neighborhood-scale sewage surveillance, for both a fully residential and commercial area, showing the potential to increase the geographical resolution of sewage surveillance.
- Since the SARS-CoV-2 RNA profile fluctuates during the day at the pump station, we suggest characterizing the sampling site diurnally should be done prior to monitoring for a better understanding of the pattern and how dilution might affect the SARS-CoV-2 RNA profile. We would like to call for more research to take on that approach.

## ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00016.

Discussions of method for RNA recovery, qPCR inhibition control, and Sanger sequencing of RT-qPCR products, figures of Sanger sequencing results and alignment, Ct values RT-qPCR results of SARS-CoV-2 at wastewater treatment plants and at the residential pump station during the peak hours, correlation between flowrate and ammonia and phosphate in the morning, and PCA biplot by first two principal components, and tables of primers and probes for RT-qPCR, new positive cases tested in the Lakewood area at the pump station served during December 2020, precipitation in Seattle during November and December 2020, different normalization methods, and Ct values of RT-qPCR results of SARS-CoV-2 at wastewater treatment plants and at the residential pump station during the peak hours (PDF)

## AUTHOR INFORMATION

**Corresponding Author**

Bao Nguyen Quoc — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States; orcid.org/0000-0002-8415-2698; Email: quocbao@uw.edu

**Authors**

Prakit Saingam — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States

Raymond RedCorn — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States

John A. Carter — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States

Tanisha Jain — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States

Pieter Candry — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States; orcid.org/0000-0002-2477-2916

Meghan Gattuso — Seattle Public Utilities, Seattle, Washington 98124, United States

Meei-Li W. Huang — Dept of Laboratory Medicine and Pathology, University of Washington, Seattle, Washington 98105, United States

Alexander L. Greninger — Dept of Laboratory Medicine and Pathology, University of Washington, Seattle, Washington 98105, United States
John Scott Meschke — Department of Environmental & Occupational Health Sciences, University of Washington, Seattle, Washington 98105, United States  
Andrew Bryan — Dept of Laboratory Medicine and Pathology, University of Washington, Seattle, Washington 98105, United States  
Mari K. H. Winkler — Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98105, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acsestwater.2c00016

Author Contributions
2 B.N.Q. and P.S. equally contributed to this work.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to thank the AXA Research for funding this project (CPO00054476). The authors express their appreciation to Amy Minichillo (Environmental Science Team Manager, SPU) who facilitated the cooperation with Seattle Public Utilities. The authors are grateful to Anthony Russell — Crew Chief, Scott Helmbrecht, Uipa Antonio, Jerome Mika, Angela Meadows, and Ceci Caldwell of Seattle Public Utilities. The authors acknowledge Sam Bryson (postdoc at Winkler Science of The Total Environment, USA) for assisting in the gathering of samples. The authors acknowledge Sam Bryson (postdoc at Winkler’s lab) for valuable discussion and idea of sampling at the pump station. The authors appreciate the help and data provided by Sarah Philo and other lab members from Meschke lab (University of Washington).

REFERENCES
(1) Medema, G.; Heijnen, L.; Elsinga, G.; Italiaander, R.; Brouwer, A. Presence of SARS-CoV-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in the Netherlands. Environ. Sci. Technol. Lett. 2020, 7 (7), 511–516.
(2) Peccia, J.; Zulli, A.; Brackney, D. E.; Grubbaugh, N. D.; Kaplan, E. H.; Casanovas-Massana, A.; Ko, A. I.; Malik, A. A.; Wang, D.; Wang, M.; Warren, J. L.; Weinberger, D. M.; Arnold, W.; Omer, S. B. Measurement of SARS-CoV-2 RNA in Wastewater Tracks Community Infection Dynamics. Nat. Biotechnol. 2020, 38 (10), 1164–1167.
(3) Wu, F.; Zhang, J.; Xiao, A.; Gu, X.; Lee, W. L.; Armas, F.; Kaufman, K.; Hanage, W.; Matus, M.; Ghaeli, N.; Endo, N.; Duvallet, C.; Poyet, M.; Moniz, K.; Washburne, A. D.; Erickson, T. B.; Chai, P. R.; Thompson, J.; Alm, E. J. SARS-CoV-2 Titers in Wastewater Are Higher than Expected from Clinically Confirmed Cases. mSystems 2020, 5 (4), e00614.
(4) Shcheran, S. P.; Shahin, S.; Ward, L. M.; Tandukar, S.; Aw, T. G.; Schmitz, B.; Ahmed, W.; Kitajima, M. First Detection of SARS-CoV-2 RNA in Wastewater in North America: A Study in Louisiana, USA. Science of The Total Environment 2020, 743, 140621.
(5) Gonzalez, R.; Curtis, K.; Bibvs, A.; Bibby, K.; Weir, M. H.; Yetka, K.; Thompson, H.; Keeling, D.; Mitchell, J.; Gonzalez, D. COVID-19 Surveillance in Southeastern Virginia Using Wastewater-Based Epidemiology. Water Res. 2020, 186, 116296.
(6) Crites-Chrop, A.; Kentor, R. S.; Olan, M. R.; Whitney, O. N.; Al-Shayeb, B.; Lou, Y. C.; Flamholz, A.; Kennedy, L. C.; Greenwald, H.; Hinkle, A.; Hetzel, J.; Spitzer, S.; Koble, J.; Tan, A.; Hyde, F.; Schroth, G.; Kuersten, S.; Banfield, J. F.; Nelson, K. L. Genome Sequencing of Sewage Detects Regionally Prevalent SARS-CoV-2 Variants. mBio 2021, 12 (1), 1–9.
(7) Fontenele, R. S.; Kraberger, S.; Hadfield, J.; Driver, E. M.; Bowes, D.; Holland, L. A.; Faleye, T. O. C.; Adhikari, S.; Kumar, R.; Inchausti, R.; Holmes, W. K.; Deitrick, S.; Brown, P.; Duty, D.; Smith, T.; Bhatnagar, A.; Yeager, R. A.; Holm, R. H.; Reitzenstein, N. H.; von Weller, E.; Dixon, K.; Constantine, T.; Wilson, M. A.; Lim, E. S.; Jiang, X.; Halden, R. U.; Scotch, M.; Varansi, A. High-Throughput Sequencing of SARS-CoV-2 in Wastewater Provides Insights into Circulating Variants. medRxiv, in press, 2021.
(8) Matrajt, G.; Naughton, B.; Bandopadhyay, A. S.; Meschke, J. S. A Review of the Most Commonly Used Methods for Sample Collection in Environmental Surveillance of Poliovirus. Clinical Infectious Diseases 2018, 67, S90–S97.
(9) Ranta, J.; Hovi, T.; Arjas, E. Poliovirus Surveillance by Examining Sewage Water Specimens: Studies on Detection Probability Using Simulation Models. Risk Anal. 2001, 21 (6), 1087–1096.
(10) Hewitt, J.; Leonard, M.; Greening, G. E.; Lewis, G. D. Influence of Wastewater Treatment Process and the Population Size on Human Virus Profiles in Wastewater. Water Res. 2011, 45 (18), 6267–6276.
(11) Nordgren, J.; Matussek, A.; Mattsson, A.; Svensson, L.; Lindgren, P. E. Prevalence of Norovirus and Factors Influencing Virus Concentrations during One Year in a Full-Scale Wastewater Treatment Plant. Water Res. 2009, 43 (4), 1117–1125.
(12) Ye, Y.; Ellenberg, R. M.; Graham, K. E.; Wigginton, K. R. Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater. Environ. Sci. Technol. 2016, 50 (10), S077–S085.
(13) UC Merced. COVIDPoops19. https://ucmerced.maps.arcgis.com/apps/dashboards/c77814e5a5bb4aeeb58d31afee389082 (accessed 2021-11-16).
(14) Naughton, C. C.; Roman, F. A.; Grace, A.; Alvarado, F.; Tariqi, A. Q.; Deeming, M. A.; Bibby, K.; Bibvs, A.; Rose, J. B.; Medema, G.; Ahmed, W.; Katisvelis, P.; Allan, V.; Sinclair, R.; Zhang, Y.; Kinyua, M. N. Show Us the Data: Global COVID-19 Wastewater Monitoring Efforts, Equity, and Gaps. medRxiv, in press, 2021.
(15) Ahmed, W.; Bertsch, P. M.; Bibby, K.; Haramoto, E.; Hewitt, J.; Huygens, F.; Gyawali, P.; Korajkic, A.; Riddell, S.; Sherchan, S. P.; et al. Decay of SARS-CoV-2 and Surrogate Murine Hepatitis Virus RNA in Untreated Wastewater to Inform Application in Wastewater-Based Epidemiology. Environmental Research 2020, 191, 110092.
(16) Weidhaas, J.; Aanderud, Z. T.; Roper, D. K.; VanDerslice, J.; Gaddis, E. B.; Ostermiller, J.; Hoffman, K.; Jamal, R.; Heck, P.; Zhang, Y.; Torgersen, K.; Laan, J. V.; LaCrosse, N. Correlation of SARS-CoV-2 RNA in Wastewater with COVID-19 Disease Burden in Sewersheds. Science of The Total Environment 2021, 775, 145790.
(17) Kapo, K. E.; Paschka, M.; Yamshi, R.; Sebasky, M.; McDonough, K. Estimation of U.S. Sewer Residence Time Distributions for National-Scale Risk Assessment of Down-the-Drain Chemicals. Science of The Total Environment 2017, 603–604, 445–452.
(18) D’Aoust, P. M.; Mercier, E.; Montpetit, D.; Jia, J. J.; Alexandrov, I.; Nealut, N.; Biau, A. T.; Mayne, J.; Zhang, X.; Alain, T.; Langlois, M. A.; Servos, M. R.; MacKenzie, M.; Figyes, D.; MacKenzie, A. E.; Graber, T. E.; Delattolle, R. Quantitative Analysis of SARS-CoV-2 RNA from Wastewater Solids in Communities with Low COVID-19 Incidence and Prevalence. Water Res. 2021, 188, 116560.
(19) Philo, S. E.; Keim, E. K.; Swanstrom, R.; Onf. A. Q. W.; Burnor, E. A.; Kossik, A. L.; Harrison, J. C.; Demekhe, B. A.; Zhou, N. A.; Beck, N. B.; Shirai, J. H.; Meschke, J. S. A Comparison of SARS-CoV-2 Wastewater Concentration Methods for Environmental Surveillance. Science of The Total Environment 2021, 760, 144215.
(20) Medema, G.; Been, F.; Heijnen, L.; Petterson, S. Implementation of Environmental Surveillance for SARS-CoV-2 Virus to Support Public Health Decisions: Opportunities and Challenges. Current Opinion in Environmental Science & Health 2020, 17, 49–71.
(21) Bibvs, A.; Kaya, D.; Bibby, K.; Simpson, S. L.; Bustin, S. A.; Shanks, O. C.; Ahmed, W. Variability in RT-QPCR Assay Parameters Indicates Unreliable SARS-CoV-2 RNA Quantification for Waste-Water Surveillance. Water Res. 2021, 203, 117516.
Curtis, K.; Keeling, D.; Yetka, K.; Larson, A.; Gonzalez, R. Wastewater SARS-CoV-2 Concentration and Loading Variability from Grab and 24-h Composite Samples. *medRxiv*, in press, 2020.

Chleborad, A. F. *Preliminary Evaluation of a Precipitation Threshold for Anticipating the Occurrence of Landslides in the Seattle, Washington, Area - Open-File Report*; US Geological Survey, 2003; p 39.

Olds, H. T.; Corsi, S. R.; Dila, D. K.; Halmo, K. M.; Bootsma, M. J.; McLellan, S. L. High Levels of Sewage Contamination Released from Urban Areas after Storm Events: A Quantitative Survey with Sewage Specific Bacterial Indicators. *PLOS Medicine* 2018, 15 (7), e1002614.

Willis, R. M.; Stewart, R. A.; Giurco, D. P.; Talebpour, M. R.; Mousavinejad, A. End Use Water Consumption in Households: Impact of Socio-Demographic Factors and Efficient Devices. *Journal of Cleaner Production* 2013, 60, 107−115.

Li, X.; Zhang, S.; Shi, J.; Luby, S. P.; Jiang, G. Uncertainties in Estimating SARS-CoV-2 Prevalence by Wastewater-Based Epidemiology. *Chem. Eng. J.* 2021, 415, 129039.

Green, H.; Wilder, M.; Collins, M.; Fenty, A.; Gentile, K.; Kmush, B. L.; Zeng, T.; Middleton, F. A.; Larsen, D. A. Quantification of SARS-CoV-2 and Cross-Assembly Phage (CrAssphage) from Wastewater to Monitor Coronavirus Transmission within Communities. *medRxiv*, in press, 2020.

Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. *Environ. Sci. Technol.* 2002, 36 (6), 1202−1211.

Pasalari, H.; Ataei-Pirkooh, A.; Aminikhah, M.; Jafari, A. J.; Farzadkia, M. Assessment of Airborne Enteric Viruses Emitted from Wastewater Treatment Plant: Atmospheric Dispersion Model, Quantitative Microbial Risk Assessment, Disease Burden. *Environ. Pollut.* 2019, 253, 464−473.

Carducci, A.; Donzelli, G.; Cioni, L.; Federigi, I.; Lombardi, R.; Verani, M. Quantitative Microbial Risk Assessment for Workers Exposed to Bioaerosol in Wastewater Treatment Plants Aimed at the Choice and Setup of Safety Measures. *Int. J. Environ. Res. Public Health* 2018, 15 (7), 1490.

Foladori, P.; Cutrupi, F.; Segata, N.; Manara, S.; Pinto, F.; Malpei, F.; Bruni, L.; la Rosa, G. SARS-CoV-2 from Faeces to Wastewater Treatment: What Do We Know? A Review. *Science of The Total Environment* 2020, 743, 140444.

Zaneti, R. N.; Girardi, V.; Spilki, F. R.; Mena, K.; Westphalen, A. P. C.; da Costa Colares, E. R.; Pozzebon, A. G.; Etchepare, R. G. Quantitative Microbial Risk Assessment of SARS-CoV-2 for Workers in Wastewater Treatment Plants. *Science of The Total Environment* 2021, 754, 142163.

Bivins, A.; Greaves, J.; Fischer, R.; Kwe, C. Y.; Ahmed, W.; Kitajima, M.; Munster, V. J.; Bibby, K. Persistence of SARS-CoV-2 in Water and Wastewater. *Environ. Sci. Technol. Lett.* 2020, 7 (12), 937−942.

de Oliveira, L. C.; Torres-Franco, A. F.; Lopes, B. C.; Santos, B. S. Á. d. s.; Costa, E. A.; Costa, M. S.; Reis, M. T. P.; Melo, M. C.; Polizzi, R. B.; Teixeira, M. M.; Mota, C. R. Viability of SARS-CoV-2 in River Water and Wastewater at Different Temperatures and Solids Content. *Water Res.* 2021, 195, 117002.

Sala-Comorera, L.; Reynolds, L. J.; Martin, N. A.; O’Sullivan, J. J.; Meijer, W. G.; Fletcher, N. F. Decay of Infectious SARS-CoV-2 and Surrogates in Aquatic Environments. *Water Res.* 2021, 201, 117090.