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Letter

Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands

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ABSTRACT: In the current COVID-19 pandemic, a significant proportion of cases shed SARS-Coronavirus-2 (SARS-CoV-2) with their faeces. To determine if SARS-CoV-2 RNA was present in sewage during the emergence of COVID-19 in The Netherlands, sewage samples of six cities and the airport were tested using four qRT-PCR assays, three targeting the nucleocapsid gene (N1−N3) and one the envelope gene (E). No SARS-CoV-2 RNA was detected on February 6, 3 weeks before the first Dutch case was reported. On March 4/5, one or more gene fragments were detected in sewage of three sites, in concentrations of 2.6−30 gene copies per mL. In Amersfoort, N3 was detected in sewage 6 days before the first cases were reported. As the prevalence of COVID-19 in these cities increased in March, the RNA signal detected by each qRT-PCR assay increased, for N1−N3 up to 790−2200 gene copies per mL. This increase correlated significantly with the increase in reported COVID-19 prevalence. The detection of the virus RNA in sewage, even when the COVID-19 prevalence is low, and the correlation between concentration in sewage and reported prevalence of COVID-19, indicate that sewage surveillance could be a sensitive tool to monitor the circulation of the virus in the population.

INTRODUCTION

In December 2019, an outbreak of coronavirus respiratory disease (called COVID-19) was detected in Wuhan, China. The outbreak was caused by a new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The outbreak is now widespread, and WHO declared a pandemic on March 11, 2020, when the disease was reported in 114 countries.[1] The primary mode of transmission of SARS-Cov-2 is via respiratory droplets that people produce when they cough, sneeze, or exhale, and the virus may also be spread via fomites.[2] SARS-CoV-2 is 82% similar to the SARS coronavirus that caused an outbreak in 2003. Then, 16%−73% of patients with SARS were reported to have diarrhea in addition to respiratory symptoms,[3] and transmission of SARS through water droplets from faeces via air ventilation systems in Amoy Gardens in Hong Kong was reported.[4] Diarrhea is also reported in a significant proportion of the COVID-19 cases, and recent reports show that SARS-CoV-2 has been detected in stool samples of COVID-19 cases.[5−9] The shedding of SARS-CoV-2 was studied in a cluster of nine cases and was present at 10^7 RNA copies/stool swab of a gram faeces one week after symptom onset and decreased to 10^3 RNA copies/swab three weeks after symptom onset.[10] These authors could not detect infectious SARS-CoV-2 in stool samples with high RNA concentrations. Another study[11] reported that in two out of four stool samples with high SARS-CoV-2 RNA concentrations, infective SARS-CoV-2 was detected with cultures combined with electron microscopy. Although it is unlikely that wastewater will become an important transmission pathway for coronaviruses like SARS-CoV-2,[12] increasing circulation of the virus in the population will increase the virus load into the sewer systems of our cities. It is important to collect information about the occurrence and fate of this new virus in sewage to understand if they pose a health risk to workers exposed to wastewater. Moreover, sewage surveillance[13] of SARS-CoV-2 RNA could be a tool to monitor the circulation of COVID-19 in our communities. This could complement current clinical surveillance, which is underreporting the true number of people infected with SARS-CoV-2, as testing is limited to the COVID-19 patients with the most severe symptoms. Sewage surveillance could also serve as an early warning of (re)emergence of COVID-19 in cities, much like the sewage surveillance for the poliovirus that has been in place in The Netherlands and other countries for this purpose.[14] The objective of this investigation was to identify if SARS-CoV-2 RNA is present in domestic wastewater of cities and a main airport during the early stages of the COVID-

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Table 1. Results of Screening of SARS-CoV-2 Targets in 24 h Composite Samples of Incoming Wastewater at Different WWTP in The Netherlands 3 Weeks before and Approximately 1, 2.5, and 4 Weeks after the First COVID-19 Case Was Reported in The Netherlands (February 27, 2020)[a]

			N1	N2	N3	E
Location	Date	Volume analyzed for each PCR assay (mL)	Genome copies (mL)			Cycle threshold (Ct)
A. Sampling round 1						
Amsterdam	7−2−2020	6.1	–	–	–	–
Den Haag	6−2−2020	6.0	–	–	–	–
Utrecht	5−2−2020	7.5	–	–	–	–
Apeldoorn	6−2−2020	6.8	–	–	–	–
Amersfoort	6−2−2020	6.4	–	–	–	–
Schiphol	7−2−2020	6.1	–	–	–	–
B. Sampling round 2						
Amsterdam	5−3−2020	2.25	–	–	–	–
Den Haag	4−3−2020	2.28	1.2×10^1	2.2×10^1	–	37.8
Den Haag	5−3−2020	2.21	–	–	–	–
Utrecht	5−3−2020	2.25	3.0×10^1	1.2×10^1	1.4×10^1	36.2
Apeldoorn	5−3−2020	2.73	–	–	–	–
Amersfoort	5−3−2020	2.04	–	–	6.6×10^0	–
Schiphol	5−3−2020	1.81	2.6×10^0	–	1.2×10^1	–
C. Sampling round 3						
Amsterdam	15−3−2020	0.83	1.2×10^2	2.7×10^2	2.6×10^2	32.6
Den Haag	15−3−2020	0.82	5.8×10^1	1.1×10^2	7.5×10^1	36.1
Den Haag	16−3−2020	0.77	5.7×10^1	4.2×10^2	2.6×10^2	34.5
Utrecht	15−3−2020	0.73	3.1×10^2	1.0×10^3	5.3×10^2	33.6
Apeldoorn	15−3−2020	0.70	–	9.1×10^1	8.0×10^0	39.2
Amersfoort	15−3−2020	0.84	1.1×10^2	1.8×10^2	1.1×10^2	35.6
Schiphol	15−3−2020	0.73	6.4×10^2	1.7×10^3	1.4×10^3	32.3
Tilburg	15−3−2020	0.74	6.1×10^2	1.9×10^3	1.5×10^3	32.3
Tilburg	16−3−2020	0.35	7.9×10^2	2.2×10^3	1.1×10^3	33.6
D. Sampling round 4						
Amsterdam	25−3−2020	2.51	6.6×10^2	1.4×10^3	1.8×10^3	29.9
Den Haag	no sample					
Utrecht 1	25−3−2020	2.48	6.5×10^2	1.2×10^3	1.7×10^3	29.9
Utrecht 2	25−3−2020	2.58	4.6×10^2	1.0×10^3	1.3×10^3	30.1
Apeldoorn	25−3−2020	2.48	2.6×10^1	5.9×10^1	1.8×10^2	33.3
Amersfoort	25−3−2020	2.44	8.2×10^1	2.3×10^2	4.7×10^2	32.1
Schiphol	25−3−2020	2.39	3.7×10^2	5.4×10^2	9.4×10^2	30.7
Tilburg	25−3−2020	2.59	2.7×10^2	4.3×10^2	6.6×10^2	31.0

[a]Recovery efficiency of culturable F-specific RNA phages by the concentration methods was 73 ± 50%, and Dengue virus internal control by RNA extraction and qRT-PCR was 30.4 ± 22.3%.

19 epidemic in The Netherlands. The SARS-CoV-2 RNA signal strength in wastewater at the inlet of the wastewater treatment plant was compared to the reported COVID-19 cases in the service area of that plant to obtain an indication of the sensitivity of sewage surveillance for SARS-CoV-2 RNA.

MATERIALS AND METHODS

Sewage Samples. Before the onset of the epidemic in The Netherlands, wastewater treatment plants (WWTP) were selected that serve two large- and three medium-sized cities and the main airport (Supporting Information, Tables S1, S2). The operators of the WWTP sampled a 24 h flow-dependent composite sample of 250 mL that was stored at 4 °C during sampling. Four rounds of samples were taken in February and March 2020 (Supporting Information, Table S2). The first sampling round turned out to be 3 weeks before the first COVID-19 case was recognized by the health surveillance system in The Netherlands, on February 27.[15] The second, third, and fourth sampling rounds were 1 week, 2.5 weeks, and 4 weeks into the epidemic in The Netherlands. As the epidemic progressed, a WWTP (Tilburg) in one of the most affected areas was included in the sampling scheme.

SARS-Coronavirus-2 RNA Detection and Quantification. Sample transport, concentration by ultrafiltration, extraction of viral RNA, and quantification of RNA with a quantitative real-time polymerase chain reaction and the quality assurance of these methods are detailed in the Supporting Information. Four primers/probe sets were used in this study: the N1−N3 set from the CDC[16] that each target a different region of the nucleocapsid (N) gene and the set against the envelope protein (E) gene from Corman et al.[17] to include targets against two separate SARS-CoV-2 genes (Supporting Information, Table S4).

COVID-19 Cases in WWTP Catchment Areas. To get an indication of the sensitivity of the monitoring of sewage, a proxy for the period prevalence of COVID-19 in the cities served by the WWTP sampled was created using (1) the cumulative number of COVID-19 cases reported per day from February 27 to March 26[15] for the cities that are served by each of the WWTP as the numerator and (2) the number of

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people served by each of the WWTP as the denominator. The number of inhabitants in the catchment of the WWTP was estimated from the design capacity (in inhabitant equivalents) of each of the WWTP and the average difference between design capacity and number of inhabitants in The Netherlands (Supporting Information, Table S2).

RESULTS AND DISCUSSION

Controls. The recovery of F-specific RNA phages by the purification and concentration steps was 73 ± 50% (n = 16). No trends were observed between the sample volume processed and phage recovery. The nonenveloped F-specific RNA phages may overestimate the recovery efficiency of the enveloped SARS-CoV-2; Ye et al.[18] showed that ultrafiltration recovered 55% of MS2 (a F-specific RNA phage) spiked to raw wastewater compared to 25% for the murine coronavirus. Model studies by these authors estimated that 26% of the murine coronavirus absorbed to wastewater solids compared to 6% for MS2, which would partially explain the lower recovery efficiency they observed for the coronavirus. This implies that a proportion of the SARS-CoV-2 virus particles may have been present in the particulate fraction that was removed by the centrifugation step. The recovery efficiency of the RNA extraction and qRT-PCR combined was evaluated in 16 samples with the internal control (IC) RNA (Supporting Information) and showed a recovery of 30.4 ± 22.3%. Also here, no trend was observed between the recovery and volume of sample processed. The slopes of the standard curves for the quantification of the N-gene assays were −3.29 ± 0.12 for N1, −3.48 ± 0.14 for N2, and −3.47 ± 0.11 for N3. Respective Y-intercept values were 42.44 ± 0.46, 43.88 ± 0.19, and 43.24 ± 0.57, and amplification efficiencies were 98.5 ± 1.3%, 94.3 ± 5.2%, and 94.3 ± 4.1% for the N1, N2, and N3 quantification, with a correlation coefficient of 0.997 ± 0.002, 0.992 ± 0.009, and 0.987 ± 0.015. The results of the qRT-PCR assays are presented without correcting for recovery efficiencies. Reliable quantification of SARS-CoV-2 with qRT-PCR in sewage will be required to make reliable COVID-19 surveillance via sewage feasible. Therefore, further development of controls to consistently monitor coronavirus recovery efficiency of the concentration and purification, RNA extraction, and qRT-PCR is of great importance. Here, nonenveloped F+ RNA phages were used, which may overestimate the recovery efficiency of enveloped viruses.[18] The analyses of an added quantified suspension of another coronavirus (such as Mouse Hepatitis Virus)[18] to the sewage samples can potentially be a feasible control to improve quantification. Reference materials for qRT-PCR quantification would allow both intra- and interlaboratory comparison of qRT-PCR results. Also, digital droplet PCR could aid in the quantification of SARS-CoV-2 in water, as shown for other RNA viruses.[19]

SARS-CoV-2 RNA in Sewage Samples. The results of the samples of February 6, 2020, 3 weeks before the first case was reported in The Netherlands on February 27, showed no positive signals for primer sets N1−N3 and E (Table 1A). The samples of March 4 and 5, 1 week into the epidemic, showed a positive signal for all primer/probe sets in sewage of one of the six WWTP sampled (Utrecht) at 14−30 gene copies (gc)/mL. Den Haag produced a signal with three qRT-PCR assays at 12−22 gc/mL on March 4 but was negative again on March 5 (Table 1b). Schiphol was positive for N1 and N3 (2.6−12 gc/mL) and Amersfoort for N3 (6.6 gc/mL). March 15 and 16, all qRT-PCR targets produced a signal at each of the seven WWTP sampled (8−2200 gc/mL), except for N1 in WWTP Apeldoorn (Table 1c). On March 25, all sewage samples were positive for each of the assays at 26−1800 gc/mL (Table 1D). High resolution electrophoresis with a bioanalyzer confirmed that the length of the PCR products match the length of the PCR target gene fragments. The three N-gene assays ideally yield similar quantitative results. The N2 and N3 assays produced RNA concentrations that differed by only 0.19 ± 0.12 log10 gc/mL, while N1 deviated more (difference N1−N2, 0.37 ± 0.16 log10 gc/mL; N1−N3, 0.40 ± 0.23 log10 gc/mL). For clinical samples, the U.S. FDA reported the sensitivity of the primer/probe sets of N1 = N3 > N2 on SARS-CoV-2 RNA.[20] Looking at the samples of March 4 and 5, when signal strength was low, the N1 and N3 assays each picked up SARS-CoV-2 RNA in three WWTP and N2 in two WWTP.

Sewage Surveillance of COVID-19. The cumulative number of reported COVID-19 cases in each of the cities served by the WWTP (Figure 1) shows how the number of reported cases increased at different rates in each of the cities as the epidemic spread. Tilburg reported the most cases, followed by Utrecht and Amsterdam, the latter particularly from March 18 onward. The number of cases at the airport could not be estimated, as the number of COVID-19 cases were not reported for this denominator.

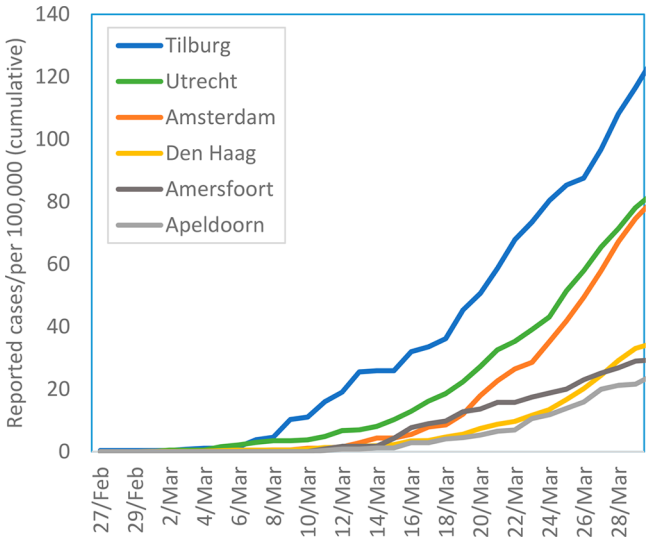

Figure 1. Cumulative prevalence of reported COVID-19 cases in the cities that are served by the WWTP from February 27−March 29, 2020.

The concentration of SARS-CoV-2 RNA in sewage of each of the cities was compared to the reported cumulative prevalence of COVID-19 in the study period in the same city. Virus shedding in stools of cases with mild or severe symptoms was reported to continue for 3 to 4 weeks after symptom onset, and virus RNA concentration in these stools were higher in weeks three and four than in week two.[21] Therefore, it was considered appropriate to use the cumulative prevalence per city for this four week study period. This provided a rough estimate of the cumulative prevalence of COVID-19 since the service areas of the WWTP do not precisely overlap with the city boundaries. The SARS-CoV-2 RNA concentration as measured in the WWTP inlets for each of the monitoring days was plotted against the cumulative

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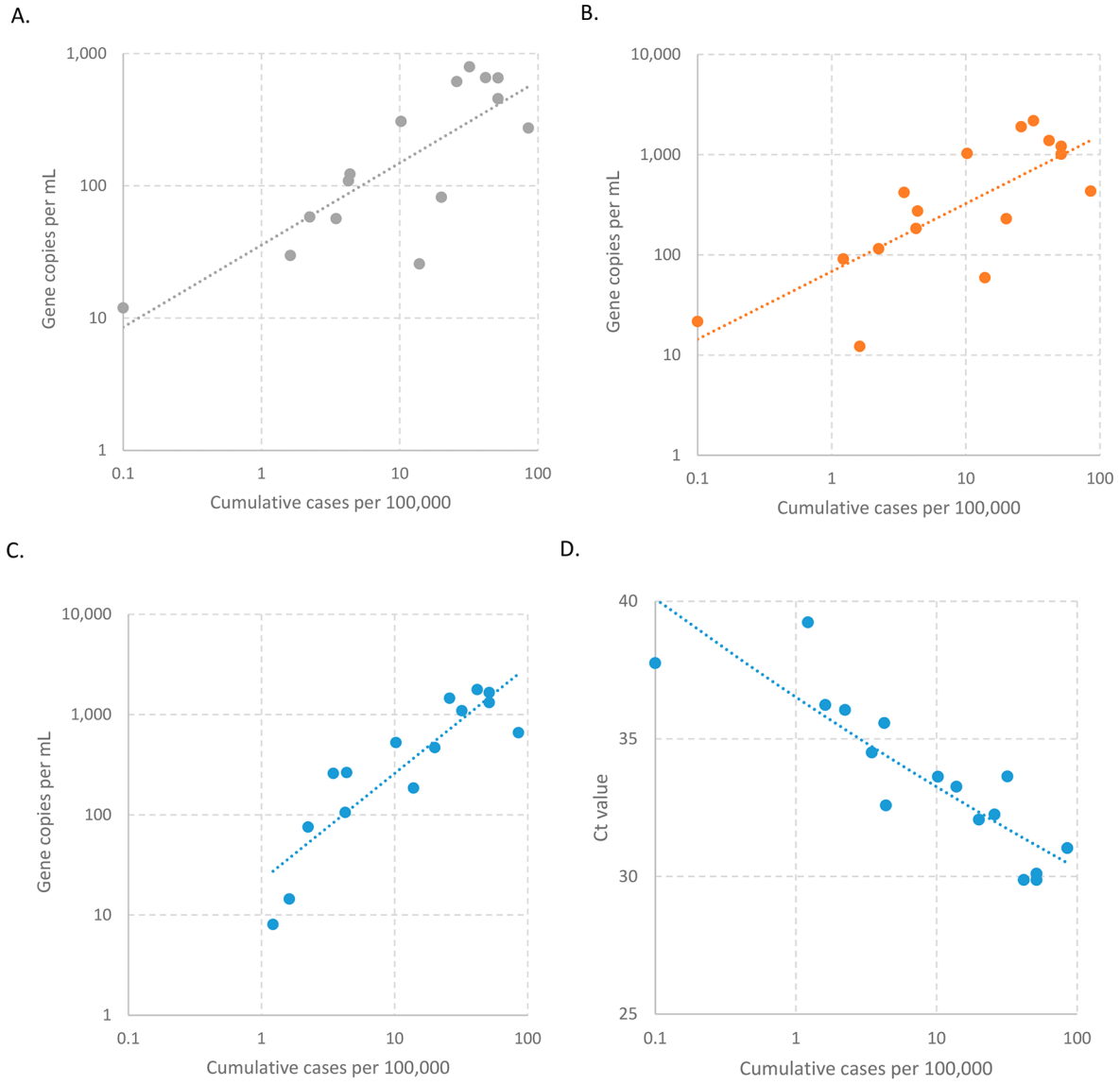

Figure 2. Concentration of SARS-CoV-2 RNA in sewage as determined by the N gene assays in gene copies/mL, N1 (A), N2 (B), and N3 (C), and the E gene assay in cycle threshold (Ct-value; D) against the cumulative number of reported COVID-19 cases in the sewer catchment for all WWTP combined.

prevalence observed on that day in that city (Figure 2). For each of the qRT-PCR assays, an increase in the SARS-CoV-2 RNA concentration was observed as the number of reported COVID-19 cases increased. Combining data from all cities and all monitoring days, the slope of the increase was 0.62 log10 gene copies per log10 reported cases (standard error (SE) = 0.12; R^2 = 0.66, p < 0.01, Figure 2A) for N1, 0.68 log10 gene copies per log10 reported cases (SE = 0.15; R^2 = 0.59, p < 0.05, Figure 2B) for N2, and 1.07 log10 gene copies per log10 reported cases (SE = 0.15; R^2 = 0.79, p < 0.001, Figure 2C) for N3. The slopes of N1 and N2 were impacted by the RNA concentrations at the prevalence of 0.1 case per 100,000, where N3 did not produce a signal. For the E gene assay, no gene copy concentrations were available. The cumulative prevalence was plotted against the observed cycle threshold value; the slope was −3.17 Ct value per log10 reported cases (SE = 0.46; R^2 = 0.77, p < 0.001; Figure 2D). Even though there is a clear and significant correlation for each of the qRT-PCR assays, there is scatter in the combined data of all the WWTP. This may be caused by variations in virus RNA recovery in the detection method and variations in RNA concentration in wastewater, as well as by variations in policies for virus testing in clinical samples between cities. Changes in testing policy over the duration of the study has likely reduced the slopes in Figure 2. Each week the epidemic progressed, more people were tested. Not enough data were available to normalize the reported prevalence by the number of tests per week. The absence of positive qRT-PCR assays in sewage samples 3 weeks before COVID-19 was first reported in The Netherlands, the detection of fragments of two genes of SARS-CoV-2 in sewage of multiple WWTP in a temporal pattern that aligns with the emergence of the epidemic in The Netherlands, and the observed significant correlation between the RNA concentration in sewage and the cumulative prevalence of COVID-19 provide compelling evidence that SARS-CoV-2 RNA is detected in sewage. It also indicates that sewage surveillance of SARS-CoV-2 RNA could be a tool for monitoring trends in COVID-19 prevalence in cities, which

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is very valuable information to support social distancing policies. To substantiate this relation between virus RNA concentration in sewage and COVID-19 prevalence, more precise quantification of virus RNA in sewage and of COVID-19 cases in the cities is needed, and more data are needed per city and at different cities and towns. For the E gene assay, translation to gene copy per mL is underway. The development of the SARS-CoV-2 RNA concentration in sewage will be monitored as the epidemic progresses to determine if sewage surveillance continues to follow the epicurve.

The detection of N3 in WWTP Amersfoort on March 5, when no cases had been reported in Amersfoort, suggests virus circulation in the population before COVID-19 cases are reported through the health surveillance system. The first two cases in Amersfoort were reported on March 11. So, the sewage signaled virus circulation in Amersfoort 6 days before the first cases were noted. Figure 2 shows that the N and E assays started to produce signals in sewage samples when the observed COVID-19 prevalence was around or even below 1.0 case in 100,000 people. Given the roughness of the prevalence estimates, these numbers are indicative but do indicate that sewage surveillance with the method used in this study is sensitive.

Other authors have reported on SARS-CoV-2 RNA in city sewage.[22−24] In Brisbane, 0.019−0.12 gene copies per mL were detected,[23] and Wu et al.[24] reported 10−240 gene copies per mL in Boston. Ahmed et al.[23] estimated that the concentrations in sewage matched with the prevalence in Brisbane, but Wu et al.[24] suggested that their concentrations implied a much higher COVID-19 prevalence (0.1%−5%) than reported (0.026%). However, an absolute comparison between prevalence of people infected with SARS-CoV-2 and SARS-CoV-2 RNA concentration in sewage may prove difficult since the reported prevalence depends heavily on the policy and method of clinical testing. The testing policy in The Netherlands focused on people with a travel history to Hubei or Italy, people with severe symptoms, and healthcare workers. A study among healthcare workers in The Netherlands indicated that SARS-CoV-2 was already circulating undetected in the community a week prior to February 27, when the first COVID-19 case was reported, suggesting that there is a high prevalence of mild COVID-19 in the community.[25] Recent serological surveys in The Netherlands and elsewhere show that the percentage of people that have been infected with COVID-19 is much higher than reported through clinical surveillance and is in the range of 1%−14%.[26] While absolute estimates of COVID-19 prevalence based on SARS-CoV-2 RNA concentrations in sewage are complex, our data suggest that surveillance of relative changes in SARS-CoV-2 RNA concentrations at the inlet of WWTP over time can serve as a sensitive tool for early warning for increasing virus circulation in the population.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.0c00357.

Methods: Sample processing, RNA extraction, real-time RT-PCR, virus concentration control, RT-PCR controls. Table S1: Sampling dates and cumulative number of COVID-19 cases in The Netherlands, as reported through the health surveillance system. Table S2: Wastewater treatment plants and population served. Table S3: Volumes processed in RNA extraction per site and date. Table S4: Primer-probe sets. References. (PDF)

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Notes

The authors declare no competing financial interest.

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