Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Long-term SARS-CoV-2 surveillance in the wastewater of Stockholm: What lessons can be learned from the Swedish perspective?

Mariel Perez-Zabaleta a,b, Amena Archerc, Kasra Khatamia,h, Mohammed Hakim Jafferalic, Prachi Nandy b, Merve Atasoy b, Madeleine Birgerssson c, Cecilia Williams c,1, Zeynep Cetecioglu a,b,†,1

a Department of Industrial Biotechnology, KTH Royal Institute of Technology, AlbaNova University Center, SE-10691 Stockholm, Sweden
b Department of Chemical Engineering, KTH Royal Institute of Technology, SE-10044, Sweden
c Department of Protein Science, KTH Royal Institute of Technology, Science for Life Laboratory, Solna, Sweden

HIGHLIGHTS

• SARS-CoV-2 measurements correlate significantly to positive COVID-19 cases.
• Viral RNA decay is lower when glycerol is added before storing samples at −80 °C.
• Omicron (BA.1, BA.2) replaced Delta as the predominant variant in the fourth wave.
• Normalization of SARS-CoV-2 to PMMoV improved statistical correlations.
• The different sampling protocols didn’t significantly impact the virus levels.

ABSTRACT

Wastewater-based epidemiology (WBE) can be used to track the spread of SARS-CoV-2 in a population. This study presents the learning outcomes from over two-year long monitoring of SARS-CoV-2 in Stockholm, Sweden. The three main wastewater treatment plants in Stockholm, with a total of six inlets, were monitored from April 2020 until June 2022 (in total 600 samples). This spans five major SARS-CoV-2 waves, where WBE data provided early warning signals for each wave. Further, the measured SARS-CoV-2 content in the wastewater correlated significantly with the level of positive COVID-19 tests (r = 0.86; p << 0.0001) measured by widespread testing of the population. Moreover, as a proof-of-concept, six SARS-CoV-2 variants of concern were monitored using hpPCR assay, demonstrating that variants can be traced through wastewater monitoring.

During this long-term surveillance, two sampling protocols, two RNA concentration/extraction methods, two calculation approaches, and normalization to the RNA virus Pepper mild mottle virus (PMMoV) were evaluated. In addition, a study of storage conditions was performed, demonstrating that the decay of viral RNA was significantly reduced upon the addition of glycerol to the wastewater before storage at −80 °C. Our results provide valuable information that can facilitate the incorporation of WBE as a prediction tool for possible future outbreaks of SARS-CoV-2 and preparations for future pandemics.

http://dx.doi.org/10.1016/j.scitotenv.2022.160023
Received 9 August 2022; Received in revised form 14 October 2022; Accepted 3 November 2022
Available online 8 November 2022
0048-9697/© 2022 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), known to cause Coronavirus Disease 2019 (COVID-19) has been a recent and major threat to public health. This virus was initially identified in December 2019 in Wuhan, China, and in March 2020, COVID-19 was categorized as a global pandemic by the World Health Organization (WHO, 2020). The first case of COVID-19 in Sweden was confirmed in the city of Jönköping on February 4, 2020, and by June 30, 2022, 2,52 million confirmed cases have been reported by the Swedish Public Health Agency. Stockholm, the capital and largest city of Sweden with a population of approximately 1.6 million in the urban area and 2.4 million in the metropolitan area. The metropolitan Stockholm area covers about a quarter (23%) of the Swedish population (10.4 million, 2020). Samples were received from three different municipal wastewater treatment plants (WWTP) in Stockholm: Bromma WWTP (Stockholm Vatten och Avfall) which treats wastewater from approximately 377,500 inhabitants, Henriksdal WWTP (Stockholm Vatten och Avfall) with 862,100 inhabitants, and Käppala WWTP (Käppala Association) with 700,000 inhabitants (Fig. S1 and S2, supplementary material). The wastewater samples were obtained from three inlets of the Bromma WWTP (corresponding to three regions: Hässelby, Riksby, and Järva), two inlets from Henriksdal WWTP (Sickla and Henriksdal), and the sole one inlet from Käppala WWTP. Approximately 500 mL of raw wastewater (taken before any biological or chemical treatment) from each WWTP were transported to the laboratory on ice.

During the monitored period, two sampling protocols were used. The first protocol was performed from the beginning of the study until week 36, 2021 (Table S1, supplementary material). In this protocol, an equal volume of flow-proportional-composite samples was collected each day for one week and stored at 4 °C before transferring to the laboratory. The second protocol was used from week 37, 2021, and onwards (Table S1, Fig. 1). Representative samples (500 mL) were taken over a period of 24 h from Monday to Tuesday every week, using a flow-proportional sampler (flow compensated samples). The sampling procedure was changed due to the recommendation of the European Union Commission made in March 2021 (European Commission, 2021). Further, at the beginning of the pandemic (week 16–33, 2020) and during the summer of 2021 (week 24 to week 32), sampling was performed every two weeks. Upon arrival at the laboratory, wastewater samples were kept at 4 °C until concentration and RNA extraction, which was usually performed the same or the next day.

For biobanking, the samples were initially stored at −20 °C from April 2020 until February 2021. From week 5, 2021, following results from the storage condition study (Section 3.7), glycerol (98 % v/v) was added to the fresh wastewater samples (1:3 ratio) and stored at −80 °C.

2. Materials and methods

2.1. Site information, wastewater sampling, and storage

Long-term monitoring of the wastewater of Stockholm was performed between April 2020 (week 16) to June 2022 (week 26). Stockholm is the capital and largest city of Sweden with a population of approximately 1.6 million in the urban area and 2.4 million in the metropolitan area. The metropolitan Stockholm area covers about a quarter (23%) of the Swedish population (10.4 million, 2020). Samples were received from three different municipal wastewater treatment plants (WWTP) in Stockholm: Bromma WWTP (Stockholm Vatten och Avfall) which treats wastewater from approximately 377,500 inhabitants, Henriksdal WWTP (Stockholm Vatten och Avfall) with 862,100 inhabitants, and Käppala WWTP (Käppala Association) with 700,000 inhabitants (Fig. S1 and S2, supplementary material). The wastewater samples were obtained from three inlets of the Bromma WWTP (corresponding to three regions: Hässelby, Riksby, and Järva), two inlets from Henriksdal WWTP (Sickla and Henriksdal), and the sole one inlet from Käppala WWTP. Approximately 500 mL of raw wastewater (taken before any biological or chemical treatment) from each WWTP were transported to the laboratory on ice.

During the monitored period, two sampling protocols were used. The first protocol was performed from the beginning of the study until week 36, 2021 (Table S1, supplementary material). In this protocol, an equal volume of flow-proportional-composite samples was collected each day for one week and stored at 4 °C before transferring to the laboratory. The second protocol was used from week 37, 2021, and onwards (Table S1, Fig. 1). Representative samples (500 mL) were taken over a period of 24 h from Monday to Tuesday every week, using a flow-proportional sampler (flow compensated samples). The sampling procedure was changed due to the recommendation of the European Union Commission made in March 2021 (European Commission, 2021). Further, at the beginning of the pandemic (week 16–33, 2020) and during the summer of 2021 (week 24 to week 32), sampling was performed every two weeks. Upon arrival at the laboratory, wastewater samples were kept at 4 °C until concentration and RNA extraction, which was usually performed the same or the next day.

For biobanking, the samples were initially stored at −20 °C from April 2020 until February 2021. From week 5, 2021, following results from the storage condition study (Section 3.7), glycerol (98 % v/v) was added to the fresh wastewater samples (1:3 ratio) and stored at −80 °C.

2.2. SARS-CoV-2 concentration and RNA extraction

During the long-term monitoring, two different protocols were used for SARS-CoV-2 concentration and RNA extraction. The first protocol was performed from April 2020 to August 2021 (week 16,2020 to week 34,2021) (Table S1). In this protocol, 10 mL of wastewater was concentrated through double filtration on the same day the samples were received, as previously described by Jafferai et al. (2021). Briefly, wastewater samples were centrifuged for 30 min and then filtered two times through 10 kDa cut-off centrifugal ultrafilters (Sartorius). To increase the extraction efficacy, the concentrated municipal wastewater samples were spiked with 1 μL of 10 mg/mL yeast tRNA solution (ThermoFisher). Briefly, 3 volumes of Trizol LS reagent for liquid samples (Thermofisher) were added to 1 volume of concentrated wastewater. A volume of 0.2 mL chloroform (Sigma-Aldrich) was added for each mL of Trizol-wastewater mixture. Then, the aqueous phase was purified using miRNeasy Mini Kit (Qiagen, Chatsworth, CA) and 55 μL of RNA was obtained from each sample. The second protocol was performed from week 35, 2021 and onwards (Table S1, Fig. 1), and samples were processed the day after they were received. Viral total nucleic acid (TNA) was concentrated from raw wastewater samples using Maxwell RSC Enviro TNA Promega Kit, and RNA was extracted using Maxwell RSC Instrument (Promega Biotech AB, Sweden) following the manufacturer’s instructions. Briefly, a volume of 40 mL of wastewater was treated with a protease solution and centrifuged to remove precipitated proteins and solids present in the wastewater. Subsequently, the supernatant was filtrated and eluted to 500 μL using a column-based system, and then loaded into a cartridge provided by the kit. Maxwell RSC Pure Food GMO program was selected in the instrument software for automatic RNA extraction and eluted in 80 μL nuclease-free water. In both protocols, two independent replicates were analyzed for each sample and tap water was used as a negative control for viral concentration and RNA extraction steps.
2.3. RT-qPCR analysis

A reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed as previously described by Jafferali et al. (2021). The reaction was performed using SYBR Green one-step kit (Bio-Rad) according to the manufacturer’s instructions, with the modification of adding 2 μL of 4 mg/mL Bovine Serum Albumin (BSA) to reduce PCR inhibitors and enhance efficacy, for a final reaction volume of 20 μL. Primers (N3) targeting the Nucleocapsid gene (N-gene) were used for the quantification of SARS-CoV-2, FW: 5′-GGGAGCCTTGAATACACCAAAA-3′ and RV: 5′-TGTAGCACGATTGCAGCATTG-3′ (Medema et al., 2020). Pepper mild mottle virus (PMMoV) was also quantified in the samples and used for data normalization. The PMMoV primers used were FW: 5′-GAGTGGTTTGACCTTAACGTTTGA-3′ and RV: 5′-TTGTCGGTTGCAATGCAAGT-3′ (Ahmed et al., 2020).

Fig. 1. Long-term monitoring of SARS-CoV-2 in the Stockholm region. Three major plants in Stockholm were monitored, Käppala WWTP (700,000 inhabitants, purple bar), Bromma WWTP (377,500 inhabitants, pink bar) and Henriksdal WWTP (862,100 inhabitants, light-blue bar). SARS-CoV-2 content was expressed as the Total N-gene copy number per week, which has been normalized with PMMoV content (using PMMoV factor) and flow rate. Two or three biological replicates were analyzed for each data point. For the same monitoring period, the positive case numbers in Stockholm based on laboratory-confirmed PCR (yellow line/red dots) are plotted as well as the estimated case numbers based on SARS-CoV-2 measurements in wastewater (black interrupted line/green dots). Grey areas in the figure indicate the different waves in Stockholm during the monitoring of SARS-CoV-2. (A) SARS-CoV-2 measurements during the whole monitoring period (week 16, April 2020 to week 26, June 2022) (B) Zooming in on waves 1 and 2, reported data from week 16, 2020 until week 3, 2021. Two RNA methods and two sampling protocols were applied. In Fig. 1A, we present in which week the second protocol was applied in each case. In the samples denoted with ** in Fig. 1A, the Sickla inlet was not analyzed. Samples denoted with * in Fig. 1B were kept at −20 °C before analysis.
For each reaction, either 8 μL (N-gene detection) or 2 μL (PMMoV detection) was used as RNA template. RNA extracted with protocol 2 (from week 35, 2021), was diluted 1:2 because of the high RNA yield. Nuclease-free water and RNA extracted from tap water were included as negative controls for all qPCR reactions. RNA from inactivated cultured human SARS-CoV-2 (provided by the Public Health Agency of Sweden), SARS-CoV-2 DNA from a constructed plasmid (2019-nCoV N_Positive Control, IDT, Cat. 10,006,625), and a constructed plasmid containing the appropriate target for PMMoV (IDT, Custom MiniGene 25–500 bp) were used as positive controls. Standard curves were created using positive controls from IDT. Thermal cycling (50 °C 10 min, 95 °C 30 s, followed by 45 cycles of 95 °C 10 s, 60 °C for 30 s) was performed using the CFX96 Touch System (Bio-Rad). Melting curve detection (65 °C to 95 °C with an increment of 0.5 °C for 5 s) was analyzed for all included genes and compared to positive controls, to ensure specific amplification. Reactions were considered positive if the cycle threshold (Ct) was below 40 cycles with a single melting peak at the correct temperature. RT-qPCR inhibition studies and quality control tests of the reactions were performed as previously published (Jafferall et al., 2021).

### 2.4. Wastewater storage condition study

The impact of storage temperature, glycerol addition, and freeze-thaw cycles on the wastewater samples was investigated. For this purpose, samples were collected from the Hässelby inlet (Bromma WWTP) on February 2nd, 2021. Upon arrival at the laboratory, the samples were aliquoted into 50 mL Falcon tubes in triplicates for each condition (5) and time point (4) and stored at 4 °C, −20 °C, −80 °C, and −80 °C with the addition of glycerol (1:3 ratio). Samples were analyzed after 1 day (0 weeks), 1 week, 18 weeks, and 24 weeks (total 20 samples in triplicates, n = 60). The samples stored at −20 °C, and at −80 °C with and without the addition of glycerol, were further subjected to up to four freeze-thaw cycles (8 samples in triplicates, n = 24). One cycle (C1) specifies that the wastewater was thawed only once after freezing (regardless of the freezing temperature). Respectively, C2, C3 and C4 indicate two, three and four cycles of freezing and thawing (Table S2, supplementary material). Samples were concentrated using a double filtration method and RNA was extracted using miRNeasy Mini Kit (Qiagen, Chatsworth, CA). For the samples with glycerol, the initial volume taken in each filter was 13.5 mL (10 mL of wastewater and 3.5 mL of glycerol, considering the 1:3 ratio) to have the same initial volume of wastewater for all the samples.

### 2.5. Clinical COVID-19 case data

Epidemiological data on the number of positive COVID-19 cases in the studied areas were provided by the Public Health Agency in Sweden (Folkhälsomyndigheten) using the data portal “COVID-19 in Sweden at the regional level”. Here, the number of individuals that had tested positive for SARS-CoV-2 was published weekly per region (Swedish Public Health Agency, 2022). The positive number of cases reported in the statistics (called clinical cases in this study) is based on laboratory-confirmed cases by PCR throughout healthcare, volunteering testing, and sentinel sampling. Over time, there have been changes in the access to and recommendations for COVID-19 tests, affecting the number of laboratory-confirmed cases. However, except at the beginning of the pandemic (week 4 to week 16, 2020), and after week 6 of 2022, all individuals with symptoms in the population were encouraged to sign up for a PCR test, free of charge (including home delivery and pick up service in Stockholm), resulting in a large-scale testing effort. The number of clinical COVID-19 cases per WWTP uptake area was calculated by the sum of positive cases in the regions covered by each WWTP. The information on the regions was obtained from Stockholm Vatten och Avfall and Käppala WWTP (Figs. S1 and S2). The clinical positive case numbers relating to the total Stockholm area covered by the wastewater measurements were estimated by the sum of the case numbers of Bromma WWTP, Henriksdal WWTP, and Käppala WWTP.

### 2.6. Calculations

In this study, in order to adjust for variations in dilutions of wastewater and variations in the population, the SARS-CoV-2 levels were normalized to PMMoV levels. Following calculations of gene copy number detected per volume, two types of adjustments to PMMoV levels were applied. In the first approach, the weekly N-gene copy number per inlet was adjusted for variations in PMMoV levels per week (PMMoV factor). The second calculation presents the results as total N-gene copies per total PMMoV gene copies, per inlet.

#### 2.6.1. Weekly N-gene copy number normalized to PMMoV

Initially, Ct values of either the N-gene or PMMoV gene were converted to gene copy numbers per reaction (copies per 20 μL of reaction volume) using the corresponding standard curves. Then, the values were recalculated to gene copy number per mL of wastewater (C), by correcting for the respective dilutions of input RNA to PCR reaction (4:10 N-gene or 1:10 PMMoV), RNA elution volume, and initial wastewater sample volume (either 55 μL RNA extracted from 10 mL wastewater for method 1, or 80 μL RNA extracted from 40 mL for method 2). To calculate the total copy number per week, C was then multiplied by the flow rate data provided by the WWTPs. The average flow rate in m³/day was determined, then multiplied by 7 days and converted to mL to obtain the volume of wastewater per week in each inlet (sample point). These flow rates were used to convert C into N (N-gene copy number per week), or P (PMMoV gene copy number per week), Eq. (1). Thus, the N-gene copy number per week and PMMoV-gene copy number per week in each inlet (Henriksdal, Sickla, Hässelby, Järva, Riksby and Käppala) were obtained.

\[
N = \frac{C \times V}{W}
\]

PMMoV was then used to normalize the SARS-CoV-2 content in each inlet by dividing N (N-gene copy number/week) with the PMMoV factor, which was calculated using Eq. (2).

\[
PMMoV \text{ factor}_{\text{week }, y, \text{inlet } x} = \frac{P_{x, y}}{AvgP \cdot \text{PMMoV gene copy number per week}^{(x, y, \text{inlet } y)}}
\]

Where \( P_{x, y} \) correspond to the gene copies of PMMoV obtained in a specific week (x) and inlet (y), (e.g., week 1, 2021 and Henriksdal inlet); and \( AvgP \) to the numerical mean of PMMoV-gene copies/week of all the tested weeks in a specific inlet.

Then, N values were corrected by applying the PMMoV factor as is shown in Eq. (3).

\[
N_{\text{corrected}} = \frac{N_{(x, y, \text{inlet } y)}}{PMMoV \text{ factor}_{(x, y, \text{inlet } y)}}
\]

The total N-gene copy number per week in the Bromma region (Bromma WWTP) was calculated by summing \( N_{\text{corrected}} \) values from Hässelby, Järva and Riksby inlets. For Henriksdal WWTP, the values of Henriksdal inlet and Sickla inlet were used. Käppala WWTP has only one inlet. For the full Stockholm areas, the resulting three \( N_{\text{corrected}} \) values (Bromma, Henriksdal, Käppala WWTP) were summed.

#### 2.6.2. N-gene copy number per PMMoV-gene copy number

The second method used to express SARS-CoV-2 content is by the relation between N-gene and PMMoV-gene copy numbers. In this method, N (N-gene copy number per week) and P (PMMoV gene copy number per week) were calculated for each inlet. In order to determine the SARS-CoV-2 content in each inlet, a PMMoV factor was calculated using Eq. (2). Then, the N-gene copy number per PMMoV gene copy number was calculated using Eq. (3).
week) are calculated as before using Eq. (1). Then, N was dived by P to obtain the ratio N-gene copy number per PMMoV-gene copy number, Eq. (4).

\[
\frac{N - \text{gene copy number}}{\text{PMMoV} - \text{gene copy number}} = \frac{N_{x,y}}{P_{x,y}}
\]  

(4)

where \( N_{x,y} \) and \( P_{x,y} \) correspond to the gene copies of N-gene and PMMoV-gene, respectively, obtained in a specific week and inlet. Then, to obtain the N-gene copy number/PMMoV-gene copy number in the Bromma region (Bromma WWTP), the N values and P values, respectively, from H ässelby, Jårva, and Riksby inlets were summed and the ratio \( \frac{N_{\text{Bromma}}}{P_{\text{Bromma}}} \) was calculated. For Henriksdal WWTP, Henriksdal, Käppala WWTP) were summed and the ratio \( \frac{N_{\text{Stockholm}}}{P_{\text{Stockholm}}} \) was calculated.

2.6.3. Estimation of positive COVID-19 cases using WBE data

Large-scale testing of COVID-19 in Sweden ended after week 6 of 2022. To estimate the number of COVID-19 positive cases, previous records of clinical positive cases and corresponding SARS-CoV-2 content in the wastewater of the Stockholm region were plotted, and the resulting linear equation was used for the determination of positive cases (Fig. S3, supplementary material).

2.6.4. Statistical analyses

Standard deviations (s) were calculated between the biological replicates (duplicates or triplicates) by Eq. (5).

\[
s = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{n-1}}
\]  

(5)

Pearson correlations were performed to determine the correspondence between wastewater SARS-CoV-2 content and the number of positive clinical cases of COVID-19. p-values <0.05 were considered statistically significant. Statistical analyses were conducted in Prism version 9.4 (GraphPad Software, CA, USA). The variance of flow rates in the inlets was calculated using excel and presented as a percentage.

2.7. Identification of SARS-CoV-2 variants using hpPCR assay

The hpPCR analysis of RNA extracted from wastewater samples was performed using a custom kit assembled by APLEX Bio AB (Solna, Sweden). The kit comprises four steps: (1) a PCR primer mixture containing 8 pairs of forward and reverse primers (six pairs for different regions within the S1 and S2 domains of SARS-CoV-2 along with the N3 and PMMoV primers from Section 2.3); (2) 13 padlock probe (PLP) sequences (targeting key regions in the SARS-CoV-2 S1 and S2 domains as well as the N3 and PMMoV amplicons) allowing the distinction of Alpha, Beta, Gamma, Kappa, Delta, Omicron BA.1, Omicron BA.2, Omicron BA.2.12.1 and Omicron BA.4/BA.5 variants; (3) rolling circle amplification (RCA) and finally; (4) probing of RCA products using a proprietary highly multiplexed technology developed by APLEX Bio based on optically encoded nanoparticles. Primer sequences and viral genome sequences recognized by the PLPs are provided as supplementary information (Tables S3 and S4). Samples were measured in triplicate and the median value was considered as the assay output. Briefly, the PCR step was performed by adding 1 μL of extracted RNA to 9 μL of PCR master mix containing 0.15–0.8 μM of each of the 16 primers. The solution was then subjected to 10 min at 50 °C; 20 s at 95 °C and 45 cycles of 3 s at 95 °C and 30 s at 55 °C using a Bio-Rad T100 Thermal cycler. Then, PLP ligation was performed by combining 1 μL of the PCR amplicons with 19 μL of ligation master mix containing 10 pm-1 nM of each of the 13 PLPs, followed by heating at 60 °C for 30 min. RCA was performed by adding 10 μL of RCA master mix to the previous 20 μL solution followed by incubation at 37 °C for 60 min. The RCA products (RCPs) were diluted and immobilized (5 μL to each well) on a 75 × 25 mm glass slide with a standard 8 × 2 well array adaptor (both provided by APLEX Bio). One spot on each slide contains an internal control provided with the kit for readout filtering and normalization. After drying the spots in a convection oven at 37 °C for 10 min, the surface was treated with 50 μL of blocking solution for 10 min, washed three times with 100 μL of wash buffer, followed by the addition of 50 μL of labelling solution containing probes diluted in the hybridization buffer. The slide was then incubated for 60 min at 37 °C and washed four times with 100 μL of wash buffer. Finally, the washing solution was removed, the well array was disassembled from the glass slide and a cover slip was mounted on top of the spot array using 20 μL ProLong™ Diamond Antifade Mountant (Thermo Fisher). The slide was imaged in a Zeiss Axio Imager 2 Fluorescence microscope equipped with an external LED light source (Colibri 7), a 20 × /0.8 Papo objective, a sCMOS camera (ORCA-Fusion) and the following filter sets: 112HE multiband pass for far-red/near-infrared emission, 59HE multiband pass for green emission, 50SBF single bandpass for red emission and 112HE multiband pass for yellow emission. Each spot was imaged according to the instructions in the kit for recommended exposure time and z-stacking and the output images were quantified with a proprietary software from APLEX Bio.

3. Results

Surveillance of the wastewater in Stockholm, Sweden, was initiated in week 16 (13–19 April) 2020 and continued over a period of 26 months, until June 2022. During this period, wastewater samples from the three major WWTPs of the Stockholm region were monitored, representing a population of approximately two million people.

3.1. Long-term monitoring identifies the waves occurring in Stockholm

The long-term monitoring is illustrated in Fig. 1. Virus levels are presented as copy numbers of the N gene per week following normalization to PMMoV, along with the number of weekly positive cases as reported by the Swedish public health agency. The wastewater detection identified increased levels at each of the five full COVID-19 waves occurring in Stockholm during this time: June 2020, October 2020 to January 2021, February to May 2021, November 2021 to February 2022, and March to May 2022 (Fig. 1). A sixth wave started in Stockholm in June 2022, but only the first three weeks of this wave were included in this study.

Overall, the detected wastewater levels follow the spread as indicated by the clinical COVID-19 tests to a high degree. The detected levels were noticeably lower in waves 1 and 2 compared to the later waves (Fig. 1A). This is true also in relation to the number of infected cases (which were approximately equal in waves 2 and 3, Fig. 1A). The difference in detection level between waves 2 and 3 coincides with the SARS-CoV-2 variant Alpha emerging and likely reflects a difference in the virus shedding per infected person. Despite the difference in the detected levels of the virus in the wastewater, the measurements follow the pattern of clinical positive cases (Fig. 1A and B). In February 2022 (week 6), a large fraction of the Swedish population had been vaccinated (74 %), and Sweden ceased large-scale testing for COVID-19. Consequently, the measurements of the virus load in the wastewater could not be compared to the number of positive cases after this (including waves 5 and 6). Overall, our results show that the wastewater testing reflects the pandemic spread during this full 2-year surveillance period in the Stockholm area.

3.2. Wastewater measurement correlated significantly with clinical positive cases

To determine how accurately the wastewater measurements reflected the pandemic spread, statistical analyses were needed to determine the correlation of this data with the public data on the level of infection in the Swedish population. The best available data came from the widespread COVID-19 PCR testing offered to the Swedish population upon symptoms (until week 6, 2022). This would not detect all infected individuals but would likely capture the pattern of the spread well, including the peaks of
infection. The Pearson correlation performed between the positive case numbers in the Stockholm area during this period (until week 6, 2022) and the total gene copy number of SARS-CoV-2 (normalized to PMMoV) detected per week in the wastewater during wave 1 and 2 (Fig. 1B) revealed a significant positive correlation coefficient ($r$) of 0.84, with a confidence interval (CI) of 0.70–0.92 (Table 1). Similarly, the correlations for wave 3 and 4 were also significantly positive ($r = 0.88$, CI: 0.79–0.93, p-value <0.0001) (Table 1). Correlation and simple linear regression between the clinical positive Covid-19 cases and estimated positive COVID-19 cases in Stockholm are shown in Fig. 2.

Pearson correlations were also performed using the number of deaths and the number of patients in intensive care units (ICU) in Stockholm during the pandemic (Fig. S4), without applying time shifts. While the levels of SARS-CoV-2 in the wastewater and the number of deaths or the number of patients in the ICU did not significantly correlate for wave 1 (Fig. S4), they did so for wave 2 ($r = 0.83$, CI: 0.57–0.94, p-value <0.0001 with the number of deaths; $r = 0.88$, CI: 0.69–0.96, p-value <0.0001 and with the number of ICU patients). For wave 3, only the number of patients in the ICU ($r = 0.64$, CI: 0.21–0.86, p-value = 0.008) correlated, but not the number of deaths. No positive correlations were found after the 3rd wave, which coincided with increased levels of vaccinated persons and the emergence of Delta and Omicron variants.

Thus, we demonstrate that the long-term monitoring of the detected SARS-CoV-2 content in the wastewater strongly correlates to clinical data and could consistently follow the spread throughout the pandemic in the Stockholm area.

### 3.3. Identification of SARS-CoV-2 variants in wastewater using hpPCR

Although SARS-CoV-2 levels correlated significantly to the number of positive clinical COVID-19 cases during each respective wave, we detected considerably higher viral loads of SARS-CoV-2 in the wastewater in relation to positive clinical cases during wave 3 and onwards (Fig. 1). It is known that at the beginning of 2021, the variants B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma) appeared and became dominant. These variants increased the shedding and transmission (Sandoval Torrientes et al., 2021). Therefore, in addition to the viral load measurements, the identification of the prevalent viral variants at each wave of the pandemic provides additional information of major epidemiological relevance. Here, the identification of SARS-CoV-2 variants in the wastewater samples was performed using a novel hpPCR assay. Thirty-six representative samples, covering the five COVID-19 waves in Stockholm were analyzed by hpPCR. The presence of Alpha, Beta, Delta, Gamma, Kappa, and different Omicron (BA.1, BA.2, BA.4, BA.5, and BA.2.12.1) variants was investigated. Besides targeting the variant-specific regions, the SARS-CoV-2 N-gene and PMMoV were also measured simultaneously in each sample.

First, we compared the calculated N-gene and PMMoV copy number generated by the hpPCR data with our previously generated data based on qPCR. The N and PMMoV genes were detected in all samples using hpPCR and showed a similar semi-quantitative output as the previous qPCR (Fig. S8). Next, we investigated the SARS-CoV-2 variants (Fig. 3). Although the Alpha variant would be expected in the wave 3, we did not detect any variant before week 35, 2021. This coincides with the implementation of the higher-yield RNA extraction protocol (Promega), suggesting that the detection of variants may require relatively higher RNA concentrations. The variants that then emerged from the analysis were first Delta appearing in week 37 and becoming dominant (nearly 100% of detected variants) by week 52, 2021. The Delta variant thus preceded wave 4 but did not appear to cause a wave on its own. However, after Omicron BA.1 appeared in week 49, quickly followed by BA.2 (week 51), wave 4 grew into the largest peak to date. Both Omicron variants became dominant by week 3,2022 at the peak of wave 4. While BA.1 disappeared by week 9, BA.2 remained during wave 5 and was still dominant by week 20,2022. Week 20 was the last sampling point for the hpPCR analysis which did not include wave 6, and at this time Omicron BA.4, BA.5, and BA.2.12.1 were not detected in the wastewater of Stockholm by hpPCR (Fig. 3). Thus, hpPCR could semi-quantitatively detect SARS-CoV-2 variants in wastewater samples.

### Table 1

| Statistical analyses between SARS-CoV-2 content in wastewater and positive clinical COVID-19 cases. The statistical analyses were performed using Pearson correlation. The Pearson correlation coefficient ($r$), the confidence interval (CI) and p-values are presented in the table. The relation was considered significant when P-values were lower or equal to 0.05. The statistical analyses are presented in three sections: (A) Statistical analyses per wave, where the data for the whole Stockholm area were evaluated. The first and second waves covered the period from week 16, 2020 until week 2, 2021. While the third and fourth waves were from week 3, 2021 until week 6, 2022. The analyzed data were normalized using the PMMoV factor (B) Normalized vs. non-normalized data from week 16, 2020 until week 6, 2022. (C) Statistical analyses per WWTP, where the data of each WWTP were evaluated and the results from week 16, 2020 until week 6, 2022 were considered for the analyses. The data from Bromma and Henrikaasl WWTP were normalized using the PMMoV factor method. For Käppala WWTP, two normalized methods (PMMoV factor and N3/PMMoV) were used and compared by statistical analyses.

| Pearson correlation | Statistical analyses per wave (Stockholm) | Normalized vs. non-normalized data (Stockholm) | Statistical analyses per WWTP |
|---------------------|-------------------------------------------|-----------------------------------------------|-------------------------------|
|                      | First and second waves | Third and fourth waves | Normalized Data | Non-normalized Data | Henrikslund WWTP | Bromma WWTP | Käppala WWTP (PMMoV factor) | Käppala WWTP (N3/PMMoV) |
| $r$                 | 0.84 | 0.88 | 0.86 | 0.84 | 0.78 | 0.85 | 0.89 | 0.88 |
| CI                  | 0.70–0.92 | 0.79–0.93 | 0.80–0.91 | 0.76–0.89 | 0.68–0.85 | 0.77–0.90 | 0.84–0.93 | 0.82–0.92 |
| Sample size         | 32 | 52 | 84 | 84 | 83 | 82 | 79 | 79 |
| p-value             | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Significant?        | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

Fig. 2. Correlation and simple linear regression between the clinical positive COVID-19 cases and estimated positive COVID-19 cases in Stockholm. The statistical analysis was performed using estimated positive cases and clinical positive cases from week 16, 2020 until week 6, 2022. The Pearson correlation coefficient ($r$) and the $r^2$ of the linear regression are presented in the figure.
3.4. SARS-CoV-2 surveillance as a tool to estimate the infection rate in a population

The need to estimate the positive cases in Stockholm became especially important after the massive COVID-19 testing ceased in February 2022. Therefore, we estimated the positive cases (number of infected people) using the previous correlation between wastewater measurements and clinical cases. Statistical analyses between the actual number of clinical positive cases in Stockholm and the estimated cases using the wastewater measurements revealed a significant positive Pearson correlation of 0.97, with a confidence interval of 0.95–0.98, and a p-value <0.0001 (Fig. 2). This strong correlation confirms the high potential of WBE as a tool to estimate the infection rate in a region. The positive case numbers and estimated case numbers between week 16, 2020 and week 6, 2022 gave a similar pattern as seen in Fig. 1A.

Next, we applied this to the period when large-scale testing was no longer available (following week 6), as plotted in Fig. 1. This shows that, although not inferred by any clinical data (testing numbers, ICU, or deaths), wave 5 (and 6) continued to be of substantial sizes and the virus continued circulating in the population.

3.5. Wastewater measurements provided early warnings

The viral levels in the wastewater have been reported to provide early warning for upcoming waves (Lastra et al., 2022; Medema et al., 2020). Indeed, before each wave, we noticed increased levels in the wastewater ahead of noticeable increases in clinical cases (Fig. 1). Ahead of wave 2, the wastewater measurements (weeks 38 and 39, 2020) indicated high levels of SARS-CoV-2. The low viral levels measured in the following weeks (40 to 44), were negatively affected due to the storage of the wastewater resulting in the degradation of viral RNA and were not representative. Wave 3 could be noted in the clinical COVID-19 testing from week 7, 2021 but an early warning was noticeable in the wastewater measurements already at week 3, 2021 (mainly in the Henriksdal area, Fig. 1B). Likewise, an increase ahead of wave 4 was detected in week 46, 2021 and a warning of its very high amplitude was indicated in week 49-2021, especially in the two regions of Henriksdal and Bromma (Fig. 1A). Wave 4 coincides with the emergence of yet another variant, Omicron (Fig. 1B), which caused a sudden increase in COVID-19 cases all over the world (Gaurav and Ramarao, 2022). Further waves are noted in the wastewater after population testing has ceased (wave 5 and onwards) providing evidence of continuous ongoing spread. We conclude that early warnings were detected for all waves in Stockholm, at least one week before large-scale clinical testing showed an increase.

3.6. PMMoV normalization in the surveillance of SARS-CoV-2

The normalization with respect to the population is an essential parameter to consider in order to provide more robust information. The population size of a specific area is a variable factor that can be difficult to predict since it depends for example on people working from home, commuting, industry activity, and tourism. However, the Pepper mild mottle virus (PMMoV) present in the wastewater can be assumed to reflect the size of the population at the moment of sampling, as it is the most abundant RNA virus in human feces and has been reported to be a good population indicator in wastewater samples (Kitajima et al., 2018; Zhang et al., 2006).

To study the effect of PMMoV normalization in the measured levels of SARS-CoV-2 in the wastewater, the Pearson correlation analysis was performed for both normalized and non-normalized data (Table 1, section B). For the data where PMMoV normalization was not applied (Fig. S5 and Table 1), the correlation was positive and significant but slightly lower ($r = 0.84$) compared to the normalized data ($r = 0.86$). However, this correlation is not accurate since without normalization to PMMoV, we cannot account for changes in the protocol. For example, the second RNA extraction method gave a considerably higher RNA yield for both N-gene and PMMoV, enabling better detection (Fig. S5).

To be noted, the data were also taking the flow-rate into account, by multiplying the gene copies per volume with total volume, and presented as total number of gene copies per week to account for the recorded variations due to precipitations, snowmelt, and groundwater inflow. The WWTPs and the different inlets had a variance percentage in the flow rates as high as 23.6% (Sickla inlet) or as low as 2.1% (Riksby inlet, Table S5). Thus, the flow rate is also an important parameter to consider for normalization.

3.6.1. Normalization approaches: Käppala WWTP as a case study

Two different calculation approaches were compared using PMMoV as a normalization factor. The effectiveness of these approaches and the correlations to data of clinical positive cases were evaluated for Käppala. This WWTP has only one inlet and measured levels can be directly compared to the population and clinical cases within its region. The first approach, here named PMMoV factor, adjusts for variations in PMMoV levels for each week (Section 2.6.1). The benefit of this method is that once this correction is applied to the data for each inlet, the values can be added together to reflect the total amount of SARS-CoV-2 per WWTP or per Stockholm area (see Fig. 1). The second approach, named N-gene/PMMoV, normalizes the data by dividing the number of N-gene copies by the number of PMMoV copies (see Section 2.6.2). In this approach, once the ratio between the two genes is calculated (N-gene copies per PMMoV copies), the values cannot be added together for the different areas.
As it can be observed in Fig. 4, both normalization approaches showed similar trends, with only minor differences in the overall pattern. The Pearson correlation analysis between these two approaches and the clinical data of positive COVID-19 cases showed similar values for $r$, confidence interval and p-values (Table 1). The statistical analysis demonstrated that both calculation approaches for normalization provide reliable information. Thus, to combine data from different inlets/areas of interest, as in Fig. 1, the PMMoV factor calculations can be used. If the data is limited to one area, as in Fig. 4, either of the two approaches can be selected.

3.7. SARS-CoV-2 surveillance can identify risk areas

As shown above, normalization of the data to flow rate (sample dilution) and PMMoV (population variation) can reduce bias in the provided information. After normalization, factors such as infection rate or the effect of a new variant can be analyzed. The Pearson correlation between clinical cases and SARS-COV-2 content in the other two WWTPs: Henriksdal ($r = 0.78$) and Bromma ($r = 0.85$) were both significant (Table 1). For the Henriksdal WWTP, comparing the levels between the two inlets...
(Henriksdal and Sickla), we noted that one (Sickla) had higher levels of SARS-CoV-2 for nearly every wave (Fig. 5, Fig. S6). This might indicate a higher infection rate and more infected people in the Sickla area. Similarly, for the Bromma WWTP, Järva had substantially higher levels of SARS-CoV-2 than Hässelby and Riksby (Fig. 6, Fig. S7). It is to be noted that although the Hässelby inlet covers a higher population and a bigger area compared to Riksby, the SARS-CoV-2 levels were similar in these two areas after normalization, supporting the efficacy of the normalization methods (Fig. 6, Fig. S7). Having independent and normalized data from the inlets of the WWTPs thus enables comparisons and a deeper analysis of the population in specific areas.

3.8. Storage conditions impact the quality of the data

At the beginning of the pandemic (between weeks 16 and 20,2020) and for a period during the wave 2 (between weeks 40 and 44,2020) the study suffered a delay in the delivery of filters and reagents, and the wastewater had to be stored at −20 °C before processing. Following the delayed viral extraction and analysis, substantially lower values of SARS-CoV-2 was recorded compared to data points before and after storage (Fig. 1B). We hypothesized that this poor recovery was due to degradation of the viral RNA during the storage. Therefore, we investigated the impact of different storage conditions (wastewater samples) on the viral RNA stability in order to identify the optimal storage condition. For this purpose, wastewater samples from one and the same time point and inlet (Hässelby) were aliquoted and stored at 4 °C, −20 °C, and with and without the addition of glycerol for −80 °C storage. Respective aliquots were analyzed after 1 day (week 0), 1 week, 18 weeks, and 24 weeks of storage.

Samples kept at 4 °C showed a strong decrease in SARS-CoV-2 content after 1 week of storage and the virus was not detectable (<0.1 gene copies) after 18 weeks (Fig. S9A). Freezing the samples at either −20 °C or −80 °C, however, did not improve the detection. The number of N-gene copies after freezing (1 week) and subsequent thawing were even lower than those detected after storage in the refrigerator (4 °C) for the same time (Fig. S9A). After longer freezing periods (18 weeks and 24 weeks at −20 °C and −80 °C), SARS-CoV-2 was no longer detectable (Fig. S9A).

However, when glycerol was added, no significant reduction was observed after 18 weeks of storage (−80 °C, C1, 4.73 N-gene copy numbers) compared to the sample analyzed at week 0 (4.42 N-gene copy numbers) (Fig. 7A). To be noted, for each additional freeze-thawing cycle, the amount of SARS-CoV-2 decreased also in the sample stored at −80 °C with glycerol (Fig. 7A). After 24 weeks, the samples stored at −80 °C with glycerol, exhibited a 70 % decrease of the detectable N-gene copy number after one freeze-thaw cycle but there was no significant further decrease in these samples after undergoing two, three and four freeze-thawing cycles (Fig. 7A).

The detection of PMMoV RNA, which is substantially more abundant in wastewater samples, was also analyzed in this study. Similarly, to the analysis of SARS-CoV-2, we noted a decrease following extended storage at 4 °C and the best quality (up to 18 weeks storage at −80 °C) was achieved following the addition of glycerol (Fig. S9B and S9C). However, after prolonged storage (24 weeks), samples kept at −20 °C, −80 °C, and −80 °C with glycerol presented similar results (Fig. S9D). The impact of freeze-thaw cycles on PMMoV detection was not as substantial as in the case of the quantification of N-gene (Fig. 7). Overall, the levels of PMMoV were maintained at −20 °C and −80 °C, without glycerol and after repeated freeze-thawing, far better than the SARS-CoV-2 levels were.

This storage condition study demonstrates the possibility of storing wastewater samples for later SARS-CoV-2 detection by adding glycerol before storage at −80 °C. This reduces RNA damage or loss, which is critically important for reliable measurements. The samples should not, however, be stored for >18 weeks to have results comparable to freshly analyzed samples. The negative impact of keeping the samples at −20 °C or −80 °C without glycerol and the freeze-thaw cycles was detrimental for the detection of SARS-CoV-2, but not for PMMoV.

![Fig. 5. Surveillance of SARS-CoV-2 in Henriksdal WWTP. SARS-CoV-2 content in Henriksdal WWTP was the sum of the Sickla inlet (blue bar) and Henriksdal inlet (light-blue bar). SARS-CoV-2 content was expressed as the Total N-gene copy number per week, which has been normalized using the PMMoV-factor approach and flow rate. (*) No Sickla sample from week 22 to week 26, 2022. Two or three biological replicates were analyzed for each data point. For the same monitoring period, the positive case numbers based on laboratory-confirmed PCR (yellow line/red dots) in the Sickla and Henriksdal areas are plotted.](image-url)
4. Discussion

Few studies have monitored SARS-CoV-2 in wastewater over long periods of time. One study in Brazil presented 10-month data, while another study in Germany showed 6-month data (Agrawal et al., 2021; Claro et al., 2021). Our current study presents over two years (26 months) of monitoring six inlets (total 596 samples), which is a sufficient number and time frame to draw robust conclusions about factors influencing SARS-CoV-2 wastewater surveillance, unlike other recently published studies where the average number of analyzed samples was only around 10 (Hamouda et al., 2021).

During this long-term study, WBE generated early warnings for each of the 5 major waves. A sudden increase of SARS-CoV-2 content in the wastewater was detected before each wave, and before positive clinical case numbers indicated an increase of spread. Wastewater measurements, in general, are quicker than testing of individual patients, as there is no lag time between infection and testing, and because asymptomatic people are included. Asymptomatic people can account for as many as 45% of COVID-19 cases, depending on the population and the variant (Walsh et al., 2020).

The wastewater surveillance of SARS-CoV-2 in the capital of Sweden, Stockholm, represents an interesting case to analyze and compare since Sweden, unlike most other countries, did not apply strict lockdowns during the pandemic. The health authorities issued recommendations for social distancing, restricted traveling, and working from home but there were no penalties if not adhered to (Born et al., 2020). Facemasks were never mandatory, merely recommended when no social distance could be kept, and never adopted by the general population. The wastewater data is independent of factors such as testing recommendations, adherence to testing recommendations, or the level of symptoms a person may or may not experience. Thus, it presents a unique opportunity to gain an unbiased overview of the pandemic. For example, comparing waves 2, 3, and 4, it can be
noticed that the lengths of the waves were almost the same, between 12 and 14 weeks. In the absence of lockdowns, a decrease in SARS-CoV-2 levels was noticed in each wave after around 7 weeks (Fig. 1). In France, a decrease in the virus content was observed after 4 weeks of lockdown but the study did not show a complete decrease in the levels of SARS-CoV-2 in wastewater (Wurtzer et al., 2020). A study in the UK investigated the impact of a national lockdown and showed that the imposed measure reduced the infection rates in three of the six studied locations (Hillary et al., 2021). In the first year of the pandemic (2020), Sweden had one of the highest infection rates (as measured by positive clinical tests/population) in Europe but the number of deaths per 100,000 population was lower than in countries where stronger measures were imposed on the population such as in Belgium, the Netherlands, and Italy (Born et al., 2020). By week 6, Sweden lifted all its recommendations, COVID-19 testing was no longer offered, and the population returned to pre-pandemic behavior (e.g., keeping distance, working from home, traveling). During this period (February to June 2022), the number of deaths and ICU patients decreased (Fig. 54), but wastewater analysis showed that SARS-CoV-2 continued to circulate, and high infection rates returned in waves (Fig. 1). The lack of ICU cases and death was thus due to other causes, such as less virulent strains or a high immunity in the population.

To investigate virus variants, we used hpPCR in this study. This allowed time-efficient analysis of the samples while retaining single nucleotide specificity and could be suitable for routine analysis of SARS-CoV-2 variants in wastewater samples instead of the more time-consuming next-generation sequencing. hpPCR rapidly detected the emergence fractions of the Delta and Omicron (BA.1, BA.2) variants in the wastewater. The impact of vaccination rate and immunity on the different SARS-CoV-2 strains was noticeable in this study. Following wave 3, an estimated 12 % (based on positive cases) of the population had immunity from previous infections, and the fraction that was vaccinated with at least one dose increased from 38.3 % (week 21, May) to 71.5 % (week 45, November), with 67.2 % having received two doses and 8.4 % three doses (Covid19dataportal and SciLifeLab, 2022). During this time, only low levels of SARS-CoV-2 were observed in the wastewater (week 21 to week 45, 2021). Delta, while increasing in the rest of Europe during spring (most prevalent in London, UK, in May 2021,McCrone et al., 2022) and summer, was not detected in the Stockholm wastewater until September (week 37, Fig. 5). Furthermore, it took until November (6 months after wave 3 had ended) that a slight increase in wastewater levels (week 46) or patient cases (week 47) were noticeable. The wastewater levels and patient cases peaked at a record level, however, once Omicron (BA.1. emerging in week 47) took over (week 52, 2021 to week 3, 2022). The ICU cases increased during wave 4 (BA.1 dominant variant), although much less than in the previous peaks. Interestingly, ICU cases nor deaths increased during wave 5, when only Omicron BA.2. circulated. Notably, after the public testing stopped in Sweden, the wastewater measurement provided information about another wave and showed remaining ongoing transmissions within the population. The extensive data provided in this study was used to estimate the positive COVID-19 cases in Stockholm after the massive testing in Sweden was stopped (Figs. 1 and 2).

It is to be noted, that our analysis of wastewater levels and correlation to clinical cases, ICU, or deaths were not time-shifted. The death rate was clearly lagging with several weeks compared to wastewater levels (Fig. 54). However, although WBE provided early warnings for each wave, overall, it correlated well with clinical cases and did not show an apparent lag (Fig. 1). This is contrary to other reports that suggested a shifting time of up to 16 days forward due to a time-delayed trend (Ho et al., 2022). This may be dependent on the health infrastructure of the city or country, such as how fast test results are reported.

Furthermore, the provided unbiased information on SARS-CoV-2 surveillance of each inlet and WWTP in this study enabled a deeper analysis of specific areas of Stockholm. This is exemplified by the noticeably larger spread in certain areas (Sickla and Järva). Several social factors such as income, the predominant type of work in the area (health, manufacture, office), or the prevalence of tourism can have a direct impact on the infection rates. This study provides data that can be used for social studies to determine the most important social factors that can cause higher infections in a population.

Finally, during this long-term monitoring, several technical parameters were evaluated. This included two different sampling protocols, two different RNA extraction/concentration methods, with and without normalization to PMMoV, different calculations, and different storage conditions. The two different sampling protocols (passive sampling of wastewater during 24 h or composite sampling of the full week) generated data that equally well correlated to clinical cases (Pearson correlations were around r = 0.86 in both cases). In theory, the week-long sampling (composite sampling) would be more representative as samples are taken every day. However, this sampling would also suffer from more degradation, as we show that storage of wastewater for one week at 4 °C can significantly reduce the levels of detectable RNA (Fig. 59). Passive sampling (24 h), on the other hand, is faster and the results can be presented more rapidly. Recent studies support that passive sampling improves the detection of low RNA levels compared to composite sampling (Bivins et al., 2022; Li et al., 2022; Schang et al., 2021).

Concerning the RNA extraction/concentration methods, higher RNA concentrations were obtained with the second protocol using Promega kit but this did not affect the provided information, including correlation to clinical cases (r = 0.86 using either protocol), since the data was normalized to PMMoV levels (i.e., the higher concentrations of SARS-CoV-2 were equaled by higher PMMoV concentrations). However, the higher yield achieved with the second protocol substantially improved the ability to detect very low levels and increased the precision of the measurements. Higher RNA concentrations and yields using this extraction method have also been reported by other research groups (Alamin et al., 2022; Isaksson et al., 2022; Mondal et al., 2021).

When the data of different RNA extraction/concentration methods are to be compared, normalization (here to the PMMoV) becomes crucial, as exemplified in this study. While Feng et al. (2021), found that normalization to Bovine coronavirus (BCoV), human Bacteroides HFI83, or PMMoV had little effect, results obtained by Wolfe et al. (2021) showed an improved correlation when the data were normalized to PMMoV levels. In our study, the normalization of PMMoV and flow rate showed positive effects on the correlations (Table 1) and enabled comparisons between different methods and different areas (inlets, WWTP, and regions).

Moreover, our storage condition study demonstrated substantial RNA decay of SARS-CoV-2 and PMMoV during wastewater storage. Adding glycerol before freezing (−80 °C), however, allowed excellent conditions for storage for up to 18 weeks, which is useful information including for biobank purposes. Although a previous study did not show a significant effect on the detection of SARS-CoV-2 following 18 weeks of storage at −20 °C (Isaksson et al., 2022), our study clearly showed that samples should not be stored at −20 °C or −80 °C unless glycerol is added, and that this storage should not extend 18 weeks. Unexpectedly, PMMoV detection increased following 18 weeks of storage compared to 0 or 1 week (Fig. 7b). This might be due to the release of PMMoV RNA from solid materials (Kitajima et al., 2018). Other studies have also shown RNA decay of SARS-CoV-2 but studied shorter periods and mainly for transport purposes. Weidhaas et al. (2021) investigated the RNA decay at 4 °C, 10 °C, 35 °C, and −80 °C for a maximum of one week, in which the overall recovery of viral RNA was 86.5 ± 0.5 % at 4 °C after 24 h and 92.4 ± 10.3 % at −80 °C after one week. Ahmed et al. (2020b) studied the RNA decay at 4 °C, 15 °C, 25 °C, and 37 °C, and showed that the RNA loss is higher at higher storage temperatures. Another study used commercially synthetic nucleic acid fragments of SARS-CoV-2 to investigate the RNA decay at −80 °C and the effect of freeze-thaw cycles, showing that inexpensive cryopreservatives such as glycerol together with PBS buffer produce a high level of stability and sensitivity in the RT-qPCR quantification of the synthetic SARS-CoV-2 RNA (Holohan et al., 2021). To the extent of our knowledge, this is the first study that investigates the effect of glycerol and freeze-thaw cycles on the quantification of SARS-CoV-2 and PMMoV in wastewater samples during longer periods and lower temperatures (−20 °C and −80 °C).
Here, the RT-qPCR detection method used with N3-primers which have been shown to be among the most sensitive. According to Hamouda et al. (2021), the sensitivity of the primers was in the order of N-Sarbeco>N3>N2>N1>E, which agrees with another study that found N3 and N2 primers the most sensitive among ten tested primer sets (which did not include the N-Sarbeco set) (Jung et al., 2020). A third study showed a similar detection of SARS-CoV-2 with N3 and N1 primers, which both performed better than 5 primers (Navarro et al., 2021). Further, our assay used SYBR green technology and not TaqMan. While the inclusion of a TaqMan probe for detection reduces the risk of analyzing unspecific amplicons, the inclusion of melting curve analysis along with positive controls can enable the identification of unspecific amplicons with high certainty also when SYBR green technology is used. In our SARS-CoV-2 surveillance, using N3 primers and SYBR green, we noted unspecific amplification in rare cases. Those samples were discarded from the analysis.

The slightly lower correlation noted for waves 1 and 2 (r = 0.84) compared to waves 3 and 4 (r = 0.88) may be due to the mentioned storage and delivery problems at the beginning of the pandemic resulting in lower yields. Also, to be noted, pandemic-related limitations in deliveries of reagents restricted the wastewater testing during some weeks of the first and the second waves (weeks 16 to 20 and 40 to 44 from 2020). At these times, samples were stored at –20 °C for extended week-long periods (indicated by * in Fig. 1B), resulting in lower yields of both SARS-CoV-2 and PMMoV levels due to degradation (as demonstrated in our storage condition study, Section 3.7).

Overall, the result of our approach strongly correlates to the number of diagnosed COVID-19 positive cases (r = 0.86) and mirrored clinical cases for all WWTPs regions and the Stockholm area, demonstrating the accuracy of the assay.

5. Concluding remarks

The interest in WBE increased during the COVID-19 pandemic and is now expected to have an important role as the countries reduce the budget for clinical COVID-19 testing. On February 9th, 2022, Sweden limited public testing of individuals to the healthcare sector or elderly care staff. In this situation, monitoring SARS-CoV-2 in wastewater can be an exceptionally valuable tool to estimate the spread, anticipate and further control of COVID-19 waves, complementing clinical data, and preventing risks to vulnerable populations. WBE is also a cheaper and more efficient option, as large populations can be tested simultaneously, and the testing includes asymptomatic cases. Our study clearly shows the feasibility of tracking spread in a population during a longer time period, the ability to provide early warnings, and the accuracy of the method. The data generated here is published and updated weekly on a COVID-19 data portal (SciLifeLab Data Centre, 2022), and has become an important tool to allow both the healthcare sector, authorities, and the Swedish population to track changes in SARS-CoV-2 content in the wastewater. This study reports here the analyses of data collected during a 26-month period of the COVID-19 pandemic for six different wastewater inlets of Stockholm area, which can be used to draw more robust conclusions about the feasibility and accuracy of such a monitoring. Technical parameters relating to sampling protocols, RNA concentration/extraction methods and normalization/calculation approaches, and storage conditions were evaluated and discussed. Both RNA-isolation methods generated equally accurate data and no significant difference between the protocols was found. However, one protocol generated a higher yield enabling lower variations at low levels. Also, both tested sampling protocols and normalization/calculation methods worked well with different strengths and limitations. However, the selected combination of RNA isolation and sampling protocols was more efficient in terms of time and sensitivity threshold. Furthermore, hPqPCR showed the potential to expand the WBE capacity in Sweden by allowing highly multiplexed viral variant analysis on a routine basis, providing higher sensitivity and faster turn-around (within one workday). This monitoring includes a constant improvement of the methods with normalization towards PMMoV and we have demonstrated that wastewater samples can be stored for up to weeks, if kept at ~80 °C with glycerol added, and still generate similar results to the ones obtained with fresh wastewater samples.

Funding

This research was supported by SciLifeLab, Pandemic Laboratory Preparedness, the Knut and Alice Wallenberg Foundation (KAW 2020.0182, 2020.0241), the Region Stockholm (RS2020-0754; RS2022-0044), the Swedish Research Council (2017-01658, 2018-06169), WaterCenter@KTH, and the KTH Life Science platform.

CRediT authorship contribution statement

MPZ: Investigation, methodology, validation, formal analysis, visualization, and writing-original draft. AA, MHJ and MB: Investigation, methodology, visualization. KK: Investigation, methodology and draft writing. PN, MA: Investigation, methodology. ZC and CW: Conceptualization, methodology, supervision, funding acquisition.

All authors revised the manuscript and approved the final version.

Data availability

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.

Acknowledgements

The authors would like to thank the Science for Life Laboratories Environmental Virus Profile Platform, Swedish Environmental Epidemiology Center (SEEC), the Kippala Association, Stockholm Vatten och Avfall, the Public Health Agency of Sweden, and the Stockholm Region. Additionally, the authors acknowledge SEEC collaborators for project management and scientific discussion on surveillance in the broader Swedish context. The authors also would like to thank APLEX Bio AB for hPCR analyses, Wei Wang for her support in the laboratory, and Anders F. Andersson and Isaac Owusu-Agyeman for commenting on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160023.

References

Agrawal, S., Orschler, L., Lackner, S., 2021. Long-term monitoring of SARS-CoV-2 RNA in wastewater of the Frankfurt metropolitan area in southern Germany. Sci. Rep. 11, 5372. https://doi.org/10.1038/s41598-021-84914-2.

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O’Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J., Tscharke, B., Verhagen, R., Smith, W.J.M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K.V., Mueller, J.F., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728. https://doi.org/10.1016/j.scitotenv.2020.138764.

Ahmed, W., Bertsch, P.M., Bibby, K., Haramoto, E., Hewitt, J., Huygens, F., Gywali, P., Korajkic, A., Riddell, S., Shcherban, S.P., Simpson, S.L., Sirikanchara, K., Symonds, E.M., Verhagen, R., Vasan, S.S., Kitajima, M., Bivins, A., 2020b. Decay of SARS-CoV-2 and surrogate marine hepatitis virus RNA in untreated wastewater to inform application in wastewater-based epidemiology. Environ. Res. 191, 110092. https://doi.org/10.1016/J.ENVRES.2020.110092.

Ahmed, W., Tscharke, B., Bertsch, P.M., Bibby, K., Bivins, A., Choi, P., Clarke, L., Dwyer, J., Edson, J., Nguyen, T.M.H., O’Brien, J.W., Simpson, S.L., Sherman, P., Thomas, K.V., Verhagen, R., Zaugg, J., Mueller, J.F., 2021. SARS-CoV-2 RNA monitoring in wastewater as a potential early warning system for COVID-19 transmission in the community: a temporal case study. Sci. Total Environ. 761, 144216. https://doi.org/10.1016/j.scitotenv.2020.144216.
