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 monitoring of SARS-COV-2 RNA in wastewater in Brazil: A more responsive and economical approach

Ieda Carolina Mantovani Claro a, Aline Diniz Cabral a, Matheus Ribeiro Augusto a, Adriana Feliciano Alves Duran a, Melissa Cristina Pereira Graciosa a, Fernando Luiz Affonso Fonseca c, Marcia Aparecida Speranca b, Rodrigo de Freitas Bueno a, *

a Federal University of ABC. Center of Engineering, Modelling and Applied Social Sciences (CECS), Santo Andre, Sao Paulo, Brazil
b Federal University of ABC. Center for Natural and Human Sciences (CCNH), Sao Bernardo, Sao Paulo, Brazil
c Faculty of Medicine of ABC (FMABC), Department of Clinical Analysis, Santo Andre, Sao Paulo, Brazil

ARTICLE INFO

Keywords: COVID-19 Wastewater-based epidemiology SARS-CoV-2 Environmental surveillance Sewage Coronavirus

ABSTRACT

SARS-CoV-2, the novel Coronavirus, was first detected in Wuhan, China, in December 2019, and has since spread rapidly, causing millions of deaths worldwide. As in most countries of the world, in Brazil, the consequences of the COVID-19 pandemic have been catastrophic. Several studies have reported the fecal shedding of SARS-CoV-2 RNA titers from infected symptomatic and asymptomatic individuals. Therefore, the quantification of SARS-CoV-2 in wastewater can be used to track the virus spread in a population. In this study, samples of untreated wastewater were collected for 44 weeks at five sampling sites in the ABC Region (Sao Paulo, Brazil), in order to evaluate the SARS-CoV-2 occurrence in the sewerage system. SARS-CoV-2 RNA titers were detected throughout the period, and the concentration ranged from 2.7 to 7.7 log10 genome copies/L, with peaks in the last weeks of monitoring. Furthermore, we observed a positive correlation between the viral load in wastewater and the epidemiological/clinical data, with the former preceding the latter by approximately two weeks. The COVID-19 prevalence for each sampling site was estimated via Monte-Carlo simulation using the wastewater viral load. The mean predicted prevalence ranged 0.05 to 0.38%, slightly higher than reported (0.016 ± 0.005%) in the ABC Region for the same period. These results highlight the viability of the wastewater surveillance for COVID-19 infection monitoring in the largest urban agglomeration in South America. This approach can be especially useful for health agencies and public decision-makers in predicting SARS-CoV-2 outbreaks, as well as in local tracing of infection clusters.

1. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the etiological agent of Corona Virus Disease 2019 (COVID-19), was first identified in Wuhan, China, in December 2019. Since then, this novel Coronavirus has caused millions of deaths worldwide. The principal symptoms of COVID-19 are dry cough, fever, and difficulty in breathing and the main routes of transmission are through the spread of respiratory droplets, direct contact with infected individuals, and contaminated surfaces (WHO, 2021a).

According to WHO (2021b), until April 17th, 2021, Brazil has registered a total of 13,673,507 cases of COVID-19 with 361,884 deaths. On April 10th, 2021, the country reached the mark of 4,249 deaths, much higher than the peak of the “first wave” in July 2020 (1,595 deaths). Brazil is the country with more deaths reported in 24 hours and the second country with more deaths since the pandemic’s beginning. Brazil has administered 24,699,093 vaccine doses until March 17th, 2021, with 19,104,768 people having been vaccinated with at least one dose. However, this last one represents only 9.05% of all the population.

On March 17th, 2021, the state of Sao Paulo has a rate of bed occupancy of 66% for hospital ward and 85% for intensive care unit (ICU), a dangerous mark considering the increasing of cases each day. In the metropolitan region of Sao Paulo, where is located the ABC Region, the rate of bed occupancy is 69% for hospital ward and 83% for ICU (data source: https://www.seade.gov.br/coronavirus/). The severity of the situation highlights the need for alternative techniques for monitoring the virus.

Studies have shown that the SARS-CoV-2 is also shed in feces from
infected symptomatic, oligosymptomatic, and asymptomatic individuals (Kitajima et al., 2020). Wölfel et al. (2020) determined a shedding rate greater than 10^7 RNA copies/g feces one week after symptom onset. However, no viable (infective) viral particles were found based on cell cultures. On the other hand, other recent studies have verified the presence of viable particle viral in feces (Wang et al., 2020; Xiao et al., 2020).

Several studies have also shown the presence of SARS-CoV-2 in municipal wastewater samples, in The Netherlands (Medema et al., 2020), Spain (Randazzo et al., 2020), Australia (Ahmed et al., 2020a), USA (Wu et al., 2020), India (Kumar et al., 2020, 2021a), Brazil (Prado et al., 2021), among other countries. Randazzo et al. (2020), for example, detected RNA concentrations of 5.4 ± 0.2 log_{10} genome copies. L^{-1} on average in the Region of Murcia (Spain). Data from wastewater monitoring were compared to the historical series of cases of COVID-19, which showed that community members were shedding SARS-CoV-2 RNA titers before the first cases were reported by local epidemiological authorities. Kumar et al. (2021a) also observed the early occurrence of SARS-CoV-2 from wastewater data in Ahmedabad, Gujarat, India. The authors observed a correlation between the SARS-CoV-2 amount in wastewater and reported cases of COVID-19 with the former preceding the latter by 1 to 2 weeks. Although the fecal-oral transmission pathway has not been proven, monitoring of wastewater in the sewer network (sewer pipes) and municipal wastewater treatment plants (WWTPs) could support in predicting new SARS-CoV-2 outbreaks, as well as in the local tracing of infection clusters (Thompson et al., 2020).

Wastewater surveillance is a methodology originally designed to monitor the use of illicit drugs in a community that now has been applied to COVID-19. Wastewater surveillance data could complement epidemiological/clinical data to provide a robust tool for monitoring the SARS-CoV-2 circulation (Daughton, 2020; Larsen and Wigginton, 2020; Medema et al., 2020). This methodology has been successfully used for predicting the outbreak of Aichi virus in The Netherlands (Lodder et al., 2013) and poliovirus in Israel (Brouwer et al., 2018), and for monitoring the antibiotic resistance on a global scale (Hendriksen et al., 2019).

Wastewater surveillance is interesting especially for emerging countries with high population density such as Brazil, India, Bangladesh, Pakistan, Sri Lanka, among other countries, whose clinical testing capabilities are limited (Bhattacharya et al., 2021). Wastewater provides an aggregated sample of an entire area or sub-basin, while clinical testing provides pooled individual data. In addition to reducing monitoring costs, wastewater surveillance provides more easily accessible data (Bivins et al., 2020b). Furthermore, this methodology allows for the tracking of asymptomatic and oligosymptomatic individuals, who are generally not detected during clinical surveillance (Bhattacharya et al., 2021; Bivins et al., 2020b; Kumar et al., 2021a; van der Voorn et al., 2021).

In Brazil, this methodology for tracking the virus spread has been used in the metropolitan region of Rio de Janeiro (Prado et al., 2021). As in other countries, the monitoring results have been successfully used as complementary data in the COVID-19 surveillance by the local authorities. However, as attested by Daughton (2020)(Daughton, 2020), the data published so far is insufficient for the wastewater surveillance implementation. There is still a need for a large time-series data of concentration of SARS-CoV-2 genetic RNA components in wastewater that combined with epidemiological data to prove the usefulness of wastewater surveillance (Kumar et al., 2021a). Furthermore, there are many epidemiological (shedding profile of infected individuals, among others) and methodological (sampling strategies and experimental methods) aspects to be elucidated (Zhu et al., 2021).

In this context, this study aimed to implement a low-cost wastewater surveillance methodology to monitor the SARS-CoV-2 circulation in low-income areas in ABC Region, Metropolitan Region of Sao Paulo, Brazil. This region has the largest urban agglomeration in South America. This methodology can support decision-making by local health agencies in combating the COVID-19 pandemic.

2. Material and Methods

2.1. Sampling sites and samples collection

Untreated wastewater from five points of the ABC Region, Sao Paulo, Brazil, was collected and analyzed for 44 weeks, between June 9th, 2020 and April 7th, 2021, for the SARS-CoV-2 RNA occurrence. All monitored points are shown in Fig. 1.

Additional information on sampling points is shown in Table 1. The sampling points represent the different income levels in the region. Besides, there are also differences in the level of urbanization, access to basic sanitation, and the flow of wastewater generated.

In the WWTPs (points 1 and 5), 24-hour composite sampling of 1000 mL (proportional to the hourly flow rate) was performed using a refrigerated Hach automatic sampler (model AWRS A950), with a storage temperature of 4°C. In the sewer pipes (points 2, 3, and 4), 4-hour semi-composite sampling (proportional to the hourly flow rate) was carried out using the same automatic sampler. These sampling strategies allow a greater representation of the wastewater characteristics. The sampling frequency was weekly at all monitoring sites.

2.2. Wastewater concentration and RNA extraction

Viral particles were concentrated by the precipitation method, as described by Wu et al. (2020). Briefly, 40 mL of samples were centrifuged at 8000g for 120 min at 4°C and the pellet was resuspended in 0.4 mL of 1x PBS (pH 7.2). For sample cleaning, 1 mL of acid phenol was added and mixed strongly (Cabral et al., 2020). An aqueous phase was then formed by centrifugation at 12,000g for 10 min and transferred to a microtube containing 0.3 mL of lysis buffer. RNA extraction was performed using the PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific), according to the manufacturer’s protocol. The concentration of RNA was measured in the Nanodrop Lite (Thermo Fisher Scientific) to assess the quality of the process of genetic material (nucleic acids) extraction. The enveloped bovine respiratory syncytial virus (BRSV – Inforce™ 3, Zoetis, US) was used for evaluated concentration methods recovery capacity.

2.3. Viral detection and quantification

The presence of SARS-CoV-2 genetic RNA components was determined using the 2019-nCoV TaqMan RT-PCR Kit (Norgen, Cat. TM67120) based on the assays and protocols developed by the Centers for Disease Control and Prevention (Cdc). The RT-qPCR was carried out following the manufacturer’s instructions, recommended standards, and positive controls in a Rotor-Gene Q (Qiagen) instrument. Each RNA extract was analyzed in duplicate. A calibration curve, showed in Fig. 2, was performed using the 2019-nCoV_N_Positive Control (Norgen, Cat. PC67102).

Reactions were performed in a final volume of 20 μL, using 5 μL of the extracted RNA, 10 μL of 2x One-Step RT-PCR Master Mix, 1.5 μL of Primer-Probe Mix, and 3.5 μL of RNase free water. A series of positive (four 10-fold serial dilutions) and negative controls (extraction and PCR) were included for each RT-qPCR run by N1 and N2 genome target. A Calibration curve for N1 (y = -3.491x + 43.065; R2 = 0.996; Efficiency = 93.4%) and N2 (y = -3.723x + 44.750; R2 = 0.998; Efficiency = 85.6%) showed a linear dynamic. The limit of detection (LOD) was 10 genome copies for a Ct value of 39.28 ± 0.05 and 39.77 ± 0.58 for N1 and N2, respectively. The cycling program followed: cycle 1 – 50°C for 30 minutes; cycle 2 - 95°C for 3 minutes; cycle 3 - 45 °C for 15 seconds and 55°C for 30 seconds; cycle 4 - 45 °C for 15 seconds and 55°C for 30 seconds; cycle 5 - 95°C for 30 seconds; cycle 6 - 50°C for 30 seconds, as the probes contain FAM fluorescence. Cycle threshold (Ct) values were used to calculate GC/L in the original sample. Ct values lower than 40 were considered positive for SARS-CoV-2, as proposed previously (Medema et al., 2020; Wu et al., 2020). Inhibition test was performed on wastewater samples and no significant difference was
Fig. 1. Maps of the sampling location.
found between concentrated RNA and 10x diluted RNA. Thus, it was decided to use concentrated samples in all analyses.

BSRV RT-qPCR reactions were performed to evaluate the recovery capacity of the concentration methods, according to previous protocols described by Rajal et al. (2007) and Boxus et al. (2005). Recovery rates between 20 and 65% were obtained.

2.4. Prevalence estimation

The infected population in the ABC Region was estimated using the SARS-CoV-2 viral load measured in wastewater and other parameters, according to the following equation (Ahmed et al., 2020a; Saththasivam et al., 2021):

\[
\text{Infected population (N)} = \frac{C_{\text{RNA}} \times F}{\alpha \times \beta} \tag{1}
\]

Where:
- \(C_{\text{RNA}}\) = SARS-CoV-2 RNA concentration measured in wastewater samples (genome copies.L\(^{-1}\)).
- \(F\) = Wastewater volumetric flow rate (L.d\(^{-1}\)).
- \(\alpha\) = Fecal load (g.person\(^{-1}.d\(^{-1}\)).
- \(\beta\) = SARS-CoV-2 shedding rate by an infected individual (genome copies.g\(^{-1}\)).

In the WWTPs (points 1 and 5), the wastewater volumetric flow rate (F) was measured in loco, while for the sewer pipes points (points 2, 3, and 4), it was estimated from the per capita wastewater generation (160 L.person\(^{-1}.d\(^{-1}\)) and the contributing population (Table 1).

The daily fecal mass (\(\alpha\)) produced by humans from low-income countries usually ranges from 75 to 520 grams per person (with an average value of 149 \(\pm\) 95 g.person\(^{-1}.d\(^{-1}\)), according to Rose et al. (2015).

The SARS-CoV-2 shedding rate by an infected individual (\(\beta\)) usually ranges from \(6.3 \times 10^5\) to \(1.3 \times 10^8\) genome copies.g\(^{-1}\), according to Kitajima et al. (2020) and Gholipour et al. (2021).

Thus, the COVID-19 average prevalence was estimated for each sampling site considering the contributing population (Table 1), according to the following equation:

\[
\text{Predicted prevalence (\%) = } \frac{N}{\text{Contributing population}} \times 100 \tag{2}
\]

2.5. Statistical analysis

The one-way analysis of variance (ANOVA) was used to determine whether there were differences among the mean SARS-CoV-2 RNA concentrations of each sampling site, considering a significance level of 0.05.

The statistical Monte-Carlo approach was incorporated into the calculation of the prevalence estimate Equations 1 and (2), since parameters such as Fecal load (\(\alpha\)) and SARS-CoV-2 shedding rate (\(\beta\)) have large variation, which makes it difficult to interpret the results of the infected population (N) and, consequently, the predicted prevalence. Monte-Carlo simulation with 10,000 random samplings from each parameter/input of Equation 1 was implemented. The parameters values and their respective types of statistical distribution were shown in Table 2. The Monte-Carlo simulation summary was reported as mean and 95% confidence interval (CI) for each sampling point.

Statistical analysis was performed using Origin Pro, while the Monte-Carlo simulation was implemented in Microsoft Excel.

3. Results and Discussion

3.1. SARS-CoV-2 RNA occurrence in wastewater samples

A total of 220 untreated wastewater samples from five points of the...
ABC Region (São Paulo, Brazil) were analyzed between June 9th, 2020 and April 7th, 2021 (44 weeks) for the SARS-CoV-2 RNA occurrence. Samples with Ct (Cycle threshold) less than 40 were considered positive and had their concentrations determined (genome copies/sample volume), according to Medema et al. (2020) and Wu et al. (2020).

Fig. 3 shows the temporal variation in SARS-CoV-2 concentration for the five sampling points for the entire monitoring period. The RT-qPCR N1 and N2 gene assays were analyzed for all wastewater samples. However, as shown in Fig. 3, there was a higher SARS-CoV-2 occurrence for the N1 target. The SARS-CoV-2 RNA was detected in 53% (116/220) and 34% (74/220) of wastewater samples, for N1 and N2 gene assays, respectively. The differences among N1 and N2 assays on wastewater samples were also reported by other recent studies (Medema et al., 2020; Randazzo et al., 2020; Wu et al., 2020). This could be associated with the different analytical sensitivity between the RT-qPCR gene assays (Randazzo et al., 2020). Furthermore, different

### Table 2

Monte-Carlo simulation parameters/input.

| Parameter/Input                                    | Unit | Distribution          | (Value) |
|---------------------------------------------------|------|-----------------------|---------|
| SARS-CoV-2 RNA concentration in wastewater samples ($C_{RNA}$) | genome copies/L⁻¹ | Point (Mean = Measured) | -        |
| Wastewater volumetric flow rate (F)               | L.d⁻¹ | Point (Mean = Table 1) | -        |
| Fecal load ($\alpha$)                             | g.person⁻¹ | Normal (Mean = 149, SD = 95) | -        |
| SARS-CoV-2 shedding rate ($\beta$)                | genome copies.g⁻¹ | Uniform (Min = 6.3 × 10⁵, Max = 1.3 × 10⁹) | -        |

*According to Rose et al. (2015).*

*According to Kitajima et al. (2020) and Gholipour et al. (2021).*

---

**Fig. 3.** Temporal variation in SARS-CoV-2 concentration for RT-qPCR N1 (A) and N2 (B) gene assays.
5.0. PCR reactions may not be identically susceptible to the inhibitory effects of the evaluated matrix (Bustin et al., 2009).

As in this study, Medema et al. (2020) verified a higher sensitivity for N1 gene assay, detecting SARS-CoV-2 RNA titers for a greater number of monitoring points even when clinical data indicated a low prevalence of 1 case per 100,000 inhabitants.

Kumar et al. (2021b) evaluated the efficacy of two different treatment processes for SARS-CoV-2 RNA removal, namely, conventional activated sludge and root zone treatment. The authors analyzed three different RT-qPCR gene assays: SARS-CoV-2N-genes (nucleocapsid protein), S-genes (spike glycoprotein), and ORF 1ab genes (polyprotein). The results indicated that, regardless of the treatment system, the N-gene was more stable than the others. ORF 1ab and S genes were more sensitive to treatment processes. According to the authors, this stability and persistence occur because the nucleocapsid protein (N-gene) is more protected in the viral structure.

As shown in Fig. 3, except for point 1, the SARS-CoV-2 occurrence in the first weeks was less frequent. However, from November onwards, the SARS-CoV-2 RNA titers were detected in most of the wastewater samples. Coincidentally, at that same time, there were signs of the beginning of a “second wave” and/or recurring local outbreaks of COVID-19 in Brazil (Sabino et al., 2021). In January, February, March, and April 2021, the SARS-CoV-2 RNA was detected in 82% (23/28), 89% (25/28), 82% (23/28), 67% (19/28), and 71% (20/28) of the wastewater samples from points 1, 2, 3, 4, and 5, respectively, clearly indicating the late spread of SARS-CoV-2 in the ABC Region. Fig. 4 shows the Box Plot of the SARS-CoV-2 viral load (N1 and N2 assays) per sampling point for the entire monitoring period.

There were no statistical differences among the mean SARS-CoV-2 RNA concentrations of each sampling point, as determined by the one-way analysis of variance (ANOVA), considering a significance level of 0.05. Also, no statistical differences were detected between the means of the RNA concentrations for N1 and N2 gene assays obtained in the present study and in other relevant works.

Table 3 shows the mean, minimum, and maximum SARS-CoV-2 RNA concentrations for N1 and N2 gene assays obtained in the present study and in other relevant works.

Naturally, there are differences between the viral loads measured in wastewater samples from the ABC Region and those found in other studies around the world. According to Prado et al. (2021), different factors can influence the viral load determination, including the circumstances of the COVID-19 pandemic experienced in each region. Besides, we believe that the specificities of the sewage and the sewer network in each region also affect the experimental determinations. In Brazil, sewage, and surface run-off (rainwater and stormwater) are collected separately. However, there is a high rate of clandestine connections to the sewer network, which promotes the dilution of sewage during rainy events. Furthermore, there are particularities of each research laboratory regarding sampling, concentration, and detection of the virus.

Although it is not possible to determine absolute virus concentrations in wastewater, the use of this methodology can be very effective in tracking the virus, predicting outbreaks, and monitoring the pandemic, as will be discussed in the next section.

The results of this work have been shared with federal government health and scientific research authorities through weekly reports. The monitoring of different sampling points enables localized action by the authorities in the most affected regions according to wastewater information.

3.2. Environmental surveillance

Fig. 5 shows the spread of the SARS-CoV-2 in the ABC region for different dates, considering the viral load measured (N1 assay) in the wastewater samples.

The heat maps were made using QGIS software. The color scale ranged from the highest (red color) to the lowest (white color) SARS-CoV-2 RNA concentration, considering the period analyzed. As shown in Fig. 5, the SARS-CoV-2 RNA concentration from all sampling sites increased gradually over time, indicating the spread of COVID-19 infection in the ABC Region. At the beginning of monitoring (June 2020), the amount of SARS-CoV-2 RNA titers in wastewater samples were much less expressive than those found in the last weeks. As previously shown, this behavior was also observed for the Ct results (Fig. 3).

The presentation of monitoring results through heat maps can be especially useful for health agencies since it allows the spatial analysis of the pandemic situation. Epidemiological/clinical data on COVID-19 in the ABC Region was obtained from the publicly available repository of the Federal University of ABC, “Onde tem coronavirus?” project (available at https://ondetemcoronavirus.ufabc.edu.br/).

Fig. 6 shows the new cases during the monitoring period normalized by each city’s population. The cumulative prevalence of COVID-19 (in percentage), considering all municipalities of the ABC Region, was also plotted.

As shown in Fig. 6, although there is a wide variability of data, an upward trend of COVID-19 cases in the ABC Region can be observed, especially from November onwards. As previously discussed, the SARS-CoV-2 occurrences in wastewater samples showed the same behavior (Fig. 3).

Fig. 7 shows the SARS-CoV-2 viral load (N1 assay) in the five sampling points throughout the monitoring period.

Despite the relatively large variance, an upward trend in SARS-CoV-2
viral load (red arrow) was observed for the five monitoring points (Fig. 7), following the continuous increase of reported clinical cases (Fig. 6). Therefore, there is a correlation between the amount of SARS-CoV-2 in wastewater and the hospitalization data, as observed by other authors (Medema et al., 2020; Miyani et al., 2020; Prado et al., 2021; Saththasivam et al., 2021). However, few studies have reported long-term monitoring (over several months) of SARS-CoV-2 in wastewater (Prado et al., 2021; Saguti et al., 2021). A large dataset allows for more robust statistical analysis and, therefore, a more reliable model for wastewater surveillance (Duathlon, 2020).

Fig. 8 shows the SARS-CoV-2 amount (N1 assay) for samples of Point 1 (WWTP ABC) in relation to the number of new COVID-19 cases of ABC Region. The temporal delay between the SARS-CoV-2 RNA peaks (for wastewater samples) and clinical data are indicated with black arrows. Point 1 was chosen for this comparative analysis, since it receives most of the wastewater generated in the ABC region and, therefore, is the most representative monitoring point.

As observed in Fig. 8, there is a correlation between the SARS-CoV-2 amount variation in wastewater and the clinical data on COVID-19 with the former preceding the latter by approximately 14 days (two weeks). These results are consistent since the transmission of SARS-CoV-2 generally precedes the notification of a positive test by 2 to 3 weeks. This time interval corresponds to an incubation period between 2 and 14 days followed by clinical testing about a week after symptoms onset (Bi et al., 2020; Peccia et al., 2020; Saguti et al., 2021).

Other studies have observed the same behavior (Ahmed et al., 2021; La Rosa et al., 2020; Medema et al., 2020; Nemudryi et al., 2020; Peccia et al., 2020; Saguti et al., 2021). Saguti et al. (2021), for example, verified peaks of SARS-CoV-2 in wastewater samples from a WWTP in Sweden occurring 3 to 4 weeks before clinical notification. Ahmed et al. (2021), detected SARS-CoV-2 in wastewater samples from three WWTPs in Australia up to 3 weeks before the first reported clinical case. Peccia et al. (2020), observed a shorter delay of about a week when analyzing samples of primary sludge from a WWTP in USA.

In Florianopolis (Santa Catarina, Brazil), the viral genome of SARS-CoV-2 was detected in wastewater samples in November 2019, before the first case in the Americas was reported. The SARS-CoV-2 occurrence was confirmed by genome sequencing analysis. The mean concentration was 5.83 ± 0.12 log10 genome copies L−1, while the maximum and minimum values were 6.68 ± 0.02 and 5.49 ± 0.02 log10 genome copies L−1, respectively (Fongaro et al., 2021).

These results indicate that wastewater surveillance can be used successfully as an early warning system for monitoring COVID-19. This methodology allows verifying the increase or reduction in the number of active cases about 2 weeks in advance. In addition, since the monitoring can be regionalized by sewer sub-basins, control actions by health agencies can be directed to the infection and transmission clusters.

### 3.3. Prevalence estimate

The COVID-19 prevalence for each sampling site, shown in Table 4, was estimated using the SARS-CoV-2 RNA titers data (N1 assay) and other parameters Equations 1 and (2). The positive results (for SARS-CoV-2 occurrence) of all monitoring weeks were considered. The predicted prevalence of COVID-19, resulting from the Monte-Carlo simulation, was summarized in Table 4 as mean and 95% confidence interval (CI) (lower and upper).

The average prevalence (in percentage) in the ABC Region for the same period (June 9th, 2020 – April 7th, 2021) was 0.016 ± 0.005%. As shown in Table 4, the predicted values were much higher (about 10 times) than the observed COVID-19 prevalence (considering epidemiological/clinical data).

Wu et al. (2020), also estimated prevalence values (0.1 – 5%) higher than those reported (about 0.026%) in Massachusetts, USA.

Although the wastewater surveillance approach emerges as a promising and attractive tool for epidemiological surveillance, there are still some challenges to be considered. Some factors that influence the prevalence estimation model.

The municipal wastewater is a complex matrix with different chemical (organic and inorganic) and biological compounds. In general, it provides a lot of information about the habits and characteristics of a population. However, some of its constituents may contribute to the degradation of SARS-CoV-2 RNA (Mao et al., 2020; Zhu et al., 2021). Bivins et al. (2020b)(Bivins et al., (2020b)), observed a time for 90% reduction (T90) of viable SARS-CoV-2 in untreated wastewater about 26.2 days at 20 °C. Ahmed et al. (2020b)(Ahmed et al., (2020b)), verified a T90 of 20.4 and 12.6 days at 15 and 25 °C, respectively. As expected, the decay rate increases as the temperature also increase.

### Table 3

| Country      | City, state, or region | RT-qPCR targetregion | RNA concentration(Log10 genome copies. L−1) | Reference                           |
|--------------|------------------------|----------------------|------------------------------------------|------------------------------------|
| Brazil       | ABC Region:            | N1                   | 5.1 ± 1.0                                | Present study                      |
|              | Point 1                | N2                   | 5.5 ± 1.3                                |                                    |
|              | Point 2                | N1                   | 5.6 ± 1.1                                |                                    |
|              |                        | N2                   | 5.8 ± 1.1                                |                                    |
|              | Point 3                | N1                   | 5.7 ± 1.2                                |                                    |
|              |                        | N2                   | 5.9 ± 1.2                                |                                    |
|              | Point 4                | N1                   | 6.0 ± 1.3                                |                                    |
|              |                        | N2                   | 6.4 ± 1.2                                |                                    |
|              | Point 5                | N1                   | 5.3 ± 1.1                                |                                    |
|              |                        | N2                   | 5.6 ± 1.2                                |                                    |
| India        | Ahmedabad              | ORF1ab               | NA                                       | Kumar et al., (2021a)              |
|              |                        | S gene               | NA                                       |                                    |
|              |                        | N gene               | NA                                       |                                    |
| Spain        | Region of Murcia       | N1                   | 5.1 ± 0.3                                | Randazzo et al. (2020)             |
| USA          | Massachusetts          | N1                   | 5.5 ± 0.2                                |                                    |
|              |                        | N2                   | 5.5 ± 0.3                                |                                    |
|              |                        | N3                   | 5.5 ± 0.3                                |                                    |
| Virginia     | N1                   | NA                   | 4.4                                      | Wu et al. (2020)                   |
|              |                        | N2                   | 4.8                                      |                                    |
|              |                        | N3                   | 4.5                                      | Gonzales et al. (2020)             |
| France       | Paris                 | E gene               | 2.0                                      | Wurtzler et al. (2020)             |
|              |                        | NA                   | 2.0                                      |                                    |

NA: Data not available.
Although the wastewater residence times in the sewer network are not so long, the effect of degradation must be considered, especially in warm climate regions (Zhu et al., 2021). Furthermore, sampling sites that represent large areas and basins, such as the WWTP points, are more susceptible to the degradation effect of the viral genome during wastewater travel.

There are discrepancies regarding viral shedding by humans. Some studies have indicated that only 60–70% of infected individuals shed SARS-CoV-2 RNA titers (Saguti et al., 2021; Wu et al., 2020; Zheng et al., 2020). On the other hand, other studies have already proved that
asymptomatic and undiagnosed carriers also shedding SARS-CoV-2 RNA titers (Chavarria-Miró et al., 2021; Jiang et al., 2020; Kitajima et al., 2020). There are also discrepancies in the viral load excreted by an infected individual. Wölfel et al. (2020) observed a shedding rate between 2.56 and 7.67 log_{10} genome copies.g feces^{-1}. The shedding rates reported by Kitajima et al. (2020) range from 5.79 to 8.11 log_{10} genome copies.g feces^{-1}. There is still a great variation in the mass of feces eliminated by each infected individual, as previously discussed.

Another important aspect is the time that an infected individual remains to eliminate the SARS-CoV-2 RNA titers. Zheng et al. (2020) observed that the SARS-CoV-2 duration in feces samples is significantly longer than in serum and respiratory samples. The average duration of the virus in feces samples was 22 days (interquartile range from 17 to 31 days), while in respiratory and serum samples were 18 and 16 days, respectively. Thus, an infected individual remains to eliminate the virus even when it is no longer considered an active case.

In addition to the above-mentioned factors, there are still limitations and uncertainty in the sampling strategies, and in the methods of concentration, extraction, and quantification of SARS-CoV-2 RNA (Daughton, 2020; Zhu et al., 2021).

Therefore, an absolute comparison between the observed COVID-19 prevalence and the SARS-CoV-2 RNA concentrations in wastewater is significantly complex. On other hand, as also attested by Medema et al. (2020), the epidemiological surveillance based on relative changes in SARS-CoV-2 RNA titers from wastewater samples of sewer pipes and WWTP over time can be used as an additional monitoring tool capable of providing an early warning of SARS-CoV-2 circulation.
4. Conclusions

In this study, a long-term monitoring of SARS-CoV-2 occurrence in wastewater samples was performed. Five sampling sites in the ABC Region (São Paulo, Brazil) were evaluated. We observed a positive correlation between the viral load in wastewater samples and the epidemiological/clinical data, with the former preceding the latter by approximately two weeks. The long-term monitoring (44 weeks) allowed to assess different moments of the pandemic in the studied area, such as the increase in cases of COVID-19 in the first months of 2021, the worst period of the pandemic in the ABC Region.

The findings of this study indicate that wastewater surveillance can provide a powerful tool to complement the monitoring of COVID-19 infection in Brazil, supporting in the predicting of SARS-CoV-2 outbreaks, as well as in the local tracing of infection clusters. The wastewater surveillance is especially useful for health agencies and public decision-makers and should be incorporated in the country’s epidemiological surveillance and public policy development. The spread of the virus can be monitored in large communities from a few wastewater samples, reducing analytical costs, which is especially important for emerging and low-income countries like Brazil. Furthermore, this methodology allows for the tracking of asymptomatic and oligosymptomatic individuals, who are generally not detected during clinical surveillance.

This research is a step towards the improvement of wastewater surveillance to track COVID-19. The evolution of wastewater surveillance must be one of the legacies of the COVID-19 pandemic. This unfortunate moment made it possible to implement the methodology, at least at the research level, in different parts of the world. It is expected that the methodology will be effectively used for monitoring and predicting new events like this one.

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Acknowledgments

The authors would like to acknowledge the financial support from the following Brazilian institutions: Brazilian National Council of Scientific and Technological Development (CNPq) in partnership with Ministry of Science, Technology, Innovations and Communications (MCTIC), and Ministry of Health (MS), Secretariat of Science, Technology, Innovation and Strategic Inputs – Decit/SCTIE 07/2020 (Research to cope with COVID-19, its consequences and other severe acute respiratory syndromes – Process Number 402432/2020-7).

References

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, 138764 https://doi.org/10.1016/j.scitotenv.2020.138764.

Ahmed, W., Bertsch, P.M., Bibby, K., Haramoto, E., Hewitt, J., Huygen, F., Gysawati, P., Korajkic, A., Riddell, S., Sherchan, S.P., Simpson, S.L., Sirikanchana, K., Symonds, E. M., Verhagen, R., Vasen, S.S., Kitajima, M., Bivins, A., 2020b. Decay of SARS-CoV-2 and surrogate murine 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., 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.

Bhattacharya, P., Kumar, M., Islam, Md.T., Hasque, R., Chakraborty, S., Ahmad, A., Niazi, N.K., Cretiogioli, Z., Nilsson, D., Ijiunulana, J., van der Vooen, T.,
Jiang, X., Luo, M., Zou, Z., Wang, X., Chen, C., Qiu, J., 2020. Asymptomatic SARS-CoV-2 Gholipour, S., Mohammadi, F., Nikaeen, M., Shamsizadeh, Z., Khazeni, A., Sahbaei, Z., Daughton, C.G., 2020. Wastewater surveillance for population-wide Covid-19: The Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Brouwer, A.F., Eisenberg, J.N.S., Pomeroy, C.D., Shulman, L.M., Hindiyeh, M., Manor, Y., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The Proc. Natl. Acad. Sci 115, E10625 outbreak in Rahat, Israel, based on modeling of environmental surveillance data. Palascik, P., Fonseca, F.L.A., Speranca, M.A., Bueno, R.de F., 2020. Standardization of the detection of SARS-CoV-2 and support public health policies at municipal level in Brazil. Water Res 191, 116810. https://doi.org/10.1016/j.watres.2021.116810. Rajal, V.B., McSwain, B., Thompson, D., Leutenegger, C., Kildare, B., Wuertz, S., 2007. Assessment of the potential for the use of SARS-CoV-2 RNA in wastewater as a surrogate for the quantification of viruses from water samples. Water Res 41, 1411-1422. https://doi.org/10.1016/j.watres.2006.12.034. Randazzo, W., Trachado, F., Cuervas-Ferrando, E., Simion, P., Allende, A., Sanchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res 181, 115942. https://doi.org/10.1016/j.watres.2020.115942. Rose, J., Parker, A., Jefferson, B., Cartmell, E., 2015. The Characterization of Feaces and Urine: A Review of the literature to Inform Advanced Treatment Technology. Crit. Rev. Env. Sci. Technol. 45, 1827–1879. https://doi.org/10.1080/10643389.2014.1000761. Sabino, E.C., Buss, L.F., Carneiro, M.P.S., Prete, C.A., Crispim, M.A.E., Fraiji, N.A., Pereira, R.H.M., Parag, K.V., da Silva Peixoto, P., Kraemer, M.U.G., Oiwak, M.K., Salomon, T., Cucumba, Z.M., Castro, M.C., de Souza Santos, A.A., Nascimento, V.H., Pereira, H.S., Ferguson, N.M., Pyhäs, O.G., Kucharski, A., Busch, M.P., Dye, C, Faria, N.R., 2021. Resurgence of COVID-19 in Manaus, Brazil, despite high seroprevalence. Lancet 397, 452-455. https://doi.org/10.1016/S0140-6736(21)00183-5. Saguth, M., Magni, E., Enache, L., Churupi, M., Johansson, A., Lumley, D., Davidson, F., Dotevall, L., Mattsson, A., Trybala, E., Lagging, M., Lindh, M., Gislén, M., Breitzka, T., Nyström, K., Norder, H., 2021. Surveillance of wastewater revealed peaks of SARS-CoV-2 preceding those of hospitalized patients with COVID-19. Water Res 189, 116620. https://doi.org/10.1016/j.watres.2021.116620. Sinhababu, A.M., Pathan, A.K., Ben Amor, S., El-Malah, S.S., Gomez, T.A., Jabbar, K.A., Remanan, R., Nemudryi, A., Nemudryia, A., Wiegand, T., Surya, K., Buyukyoruk, M., Cicha, C., Vanderson, K.K., Wilkinson, R., Wiedenheft, B., 2020. Temporal Detection and Phylogenetic Assessment of SARS-CoV-2 in Municipal Wastewater. Cell Reports Med Techol. Lett. 7, 511-516. https://doi.org/10.1016/j.coesh.2020.04.006. Prado, T., Fumian, T.M., Mannuccio, C.F., Resende, P.C., Motta, F.C., Eppinghaus, A.L.P., Chagas do Vale, V.H., Braz, R.M.S., de Andrade, J.d.S., Maranhão, A.G., Magno, M.P., 2020. Wastewater-based epidemiology: a useful tool to track disease outbreaks. Curr. Opin. Environ. Sci. Heal. 17, 1-7. https://doi.org/10.1016/j.cosesh.2020.03.013. Ahmed, K.M., 2021. Prevalence of SARS-CoV-2 in Communities Through Wastewater Surveillance—A Case Study Approach for Estimating Disease Burden.Curr. Pollut. Rep. 7, 160–166. https://doi.org/10.1007/s41467-021-00174-8. Bi, Q., Wu, Yongsheng, Mei, S., Ye, C., Zou, X., Zhang, Z., Liu, X., Wei, L., Truelove, S.A., Zhang, T., Guo, W., Cheng, C., Tang, X., Wu, X., Wu, Y., Sun, B., Huang, S., Sun, Y., Zhang, J., Ma, T., Gu, X., Wang, J., Feng, T., 2020. Epidemiology and transmission of COVID-19 in 391 cases and 1286 of their close contacts in Shenzhen, China: a retrospective cohort study. Lancet Infect. Dis. 20, 911–919. https://doi.org/10.1016/j.lindh.534.2020.06.018. 10.3201/eid1908.130312. Mao, K., Zhang, K., Du, W., Ali, W., Feng, X., Zhang, H., 2020. The potential of wastewater-based epidemiology as surveillance and early warning of infectious disease outbreaks. Curr. Opin. Environ. Sci. Heal. 17, 1-7. https://doi.org/10.1016/j.coesh.2020.04.006. Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. 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. Env. Sci. Technol. Lett. 7, 511–516. https://doi.org/10.1021/acs.estlett.0c00357. Miyuni, B., Fonolli, X., Norton, J., Mehrour, A., Xargoraki, I., 2020. SARS-CoV-2 in Dakar Wastewater. J. Environ. Eng. 146, 0602000416. https://doi.org/10.1061/ASCE EE.0002-0466.0001980. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611-622. https://doi.org/10.1373/clinchem.2008.121958. Chavarria-Miro, G., Anfruns-Estrada, E., Martinez-Velazquez, A., Vazquez-Poriero, M., Guix, S., Paraira, M., Galofre, S., Bancel, G., Pinto, R.M., Bosch, A., 2021. Time Evolution of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in Wastewater during the First Pandemic Wave of COVID-19 in the Metropolitan Area of Barcelona. Spain. Appl. Environ. Microbiol. 87, 1–9. https://doi.org/10.1128/AEM.02779-20. Davidsson, F., Dotevall, L., Mattsson, A., Trybala, E., Lagging, M., Lindh, M., Håkanson, S.O., Göran, P., Ekeberg, F., Anfruns-Estrada, E., Martínez-Velázquez, A., Vázquez-Poriero, M., Guix, S., Paraira, M., Galofre, S., Bancel, G., Pinto, R.M., Bosch, A., 2021. Time Evolution of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in Wastewater during the First Pandemic Wave of COVID-19 in the Metropolitan Area of Barcelona. Spain. Appl. Environ. Microbiol. 87, 1–9. https://doi.org/10.1128/AEM.02779-20. August 2021. Water Res 203 (2021) 117534
WHO, 2021a. Coronavirus disease (COVID-19) pandemic [WWW Document]. World Heal. Organ. URL https://www.who.int/emergencies/diseases/novel-coronavirus-2019.

WHO, 2021b. Coronavirus (COVID-19) Dashboard [WWW Document]. World Heal. Organ. URL https://covid19.who.int/.

Wolfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M.A., Niemeyer, D., Jones, T.C., Vollmar, P., Rothe, C., Hoeßer, M., Bleicker, T., Brünink, S., Schneider, J., Ehlmann, R., Ziegler, K., Drosten, C., Wendtner, C., 2020. Virological assessment of hospitalized patients with COVID-19. Nature 581, 465–469. https://doi.org/10.1038/s41586-020-2196-x.

Wu, F., Zhang, J., Xiao, A., Gu, X., Lee, W.L., Armas, F., Kaufman, K., Hanage, W., Matus, M., Ghazi, N., Endo, N., Duvallet, C., Poyet, M., Moniz, K., Washburne, A.D., Erickson, T.B., Chai, P.R., Thompson, J., Alm, E.J., 2020. SARS-CoV-2 Titers in Wastewater Are Higher than Expected from Clinically Confirmed Cases. mSystems 5, 1–9. https://doi.org/10.1128/mSystems.00614-20.

Wurtzer, S., Marechal, V., Moucheil, J., Maday, Y., Teyssou, R., Richard, E., Almayrac, J., Moulin, L., 2020. Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, Greater Paris, France, 5 March to 23 April 2020. Eurosurveillance 25. https://doi.org/10.2807/1560-7917.ES.2020.25.50.2000776.

Xiao, F., Tang, M., Zheng, X., Liu, Y., Li, X., Shan, H., 2020. Evidence for Gastrointestinal Infection of SARS-CoV-2. Gastroenterology 158, 1831–1833.e3. https://doi.org/10.1053/j.gastro.2020.02.055.

Zheng, S., Fan, J., Yu, F., Feng, B., Lou, B., Zou, Q., Xie, G., Lin, S., Wang, R., Yang, X., Chen, W., Wang, Q., Zhang, D., Liu, Y., Gong, R., Ma, Z., Lu, S., Xiao, Y., Gu, Y., Zhang, J., Yao, H., Xu, K., Lu, X., Wei, G., Zhou, J., Fang, Q., Cai, H., Qiu, Y., Sheng, J., Chen, Y., Liang, T., 2020. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. BMJ 369, m1443. https://doi.org/10.1136/bmj.m1443.

Zhu, Y., Oishi, W., Maruo, C., Saito, M., Chen, R., Kitajima, M., Sano, D., 2021. Early warning of COVID-19 via wastewater-based epidemiology: potential and bottlenecks. Sci. Total Environ. 767, 145124. https://doi.org/10.1016/j.scitotenv.2021.145124.