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Acquired insights from the long-term surveillance of SARS-CoV-2 RNA for COVID-19 monitoring: The case of Monterrey Metropolitan Area (Mexico)

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ABSTRACT

Wastewater-based epidemiology offers a time- and cost-effective way to monitor SARS-CoV-2 spread in communities and therefore represents a complement to clinical testing. WBE applicability has been demonstrated in a number of cases over short-term periods as a method for tracking the prevalence of SARS-CoV-2 and an early-warning tool for predicting outbreaks in the population. This study reports SARS-CoV-2 viral loads from wastewater treatment plants (WWTPs) and hospitals over a 6-month period (June to December 2020). Results show that the overall range of viral load in positive tested samples was between 1.2×10^3 and 3.5×10^6 gene copies/l, unveiling that secondary-treated wastewaters mirrored the viral load of influents. The interpretation suggests that the viral titers found in three out of four WWTPs were associated to clinical COVID-19 surveillance indicators preceding 2–7 days the rise of reported clinical cases. The median wastewater detection rate of SARS-CoV-2 was one out of 14,300 reported new cases. Preliminary model estimates of prevalence ranged from 0.02 to 4.6% for the studied period. This comprehensive statistical and epidemiological analysis demonstrates that the applied wastewater-based approach to COVID-19 surveillance is in general consistent and feasible, although there is room for improvements.

1. Introduction

The emergence of SARS-CoV-2 and the resulting COVID-19 pandemic is causing tremendous impact on lives and economies. After the declaration of a global health emergency by the World Health Organization in March 2020 (WHO, 2020), many countries implemented a range of mitigation strategies to reduce the spread of the disease, including personal protection, social distancing, and lockdowns. Clinical testing using reverse-transcription polymerase chain reaction (RT-PCR) has been another effort aimed at establishing of a COVID-19 surveillance system (Noh et al., 2017; Shen et al., 2020; Tahamtan and Ardebili, 2020; Wan et al., 2016).

However, clinical testing was rapidly overwhelmed in many countries as SARS-CoV-2 spread for several reasons. One complication is the long incubation time of up to 14 days of COVID-19 infected people, which represents a non-diagnostic period, increasing the risk of spreading the virus (CDC, 2021). Another difficulty is the limited testing

capacity especially in underdeveloped and developing countries (Kevadiya et al., 2021; Ward et al., 2020). This situation has been exacerbated from the emergence of more transmissible genetic variants (Fontanet et al., 2021; Grubaugh et al., 2021; Wu et al., 2021). This had to consequence that only a fraction of infected people - the sickest and those looking for medical attention - had been captured by the health care system. For example, Mexico has conducted only 107 daily clinical tests per million people compared to USA or Spain with 3480 and 3354 clinical tests per million people, respectively (OurWorldInData.org).

Monitoring the occurrence of SARS-CoV-2 in wastewater, often referred to as wastewater-based epidemiology (WBE), offers an alternative to clinical testing for monitoring virus transmission in communities. WBE is not a new tool; it has been already used to monitor the community-level incidence of polio (Asghar et al., 2014), hepatitis A, dengue, and norovirus (Hellmér et al., 2014), as well as SARS-CoV (Sims and Kasprzyk-Hordern, 2020). Thus, WBE has been proposed as a complementary tool to clinical testing for population-wide surveillance

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of COVID-19 (Peccia et al., 2020; Ahmed et al., 2020a,b). A large number of studies have detected the presence of viral RNA in stool from COVID-19 patients, and there are several reports of viable virus identified in patient stool samples (e.g. Gao et al., 2020; Wang et al., 2020; Xiao et al., 2020; Zhang et al., 2020). There is also sufficient evidence that raw wastewaters and even environmental waters contain RNA fragments (Ahmed et al., 2020a; La Rosa et al., 2020; Medema et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Wu et al., 2020a; Wurtzer et al., 2020; Ihsanullah et al., 2021; Langone et al., 2021; Mahlknecht et al., 2021). These studies have shown the utility of viral RNA monitoring in municipal wastewater for SARS-CoV-2 infection surveillance at a community level.

Yet, uncertainty remains regarding the reproducibility of results across different case studies due to a lack of standardization of processes regarding sampling collection, concentration, RNA extraction and amplification, and the presence of compounds in environmental samples that inhibit RT-PCR (Ahmed et al., 2020b; Mahlknecht et al., 2021; Bivins et al., 2021; Cervantes-Avilés et al., 2021). Other prevailing questions are whether the viral concentration in wastewater reliably mirror the prevalence of the virus in a population and whether WBE is an effective early-warning tool for new outbreaks in the future. So far, most published WBE studies have covered only a short period of time (1 month) in the early stage of the spread of COVID-19 with a limited number of samples.

In this sense, we propose one of the first long-term WBE studies for tracking the prevalence of COVID-19 and an early-warning tool for predicting outbreaks in the population. This study includes a comprehensive statistical and epidemiological analysis of data obtained from wastewater samples of four wastewater treatment plants and four hospitals of a metropolitan area of an upper middle-income country. The number of samples along time contributed to elucidate factors that can

induce uncertainty on prevalence, with some trade-offs. It is one of the first studies which considers the simultaneous and systematic measurements of influents and effluents (before and after disinfection) of WWTPs, which may be useful for facilities and public health risks management.

Here, we tested wastewater from the Monterrey metropolitan area, Mexico, between June and December 2020, a time period that captures an expansion of disease within the community largely due to a surge in November. The objectives of this study were to: (a) detect and quantify the presence of SARS-CoV-2 virus and its temporal dynamics in raw and treated municipal wastewater from treatment plants and in effluents from hospitals; (b) explore whether these longitudinal data reflect and predict the number of infections in the population, considering uncertainty of independent variables such as viral load in wastewater, wastewater flow rate and daily viral load from stool production; and (c) investigate detection rates and accuracy of surveillance. Criticalities and future directions of WBE including, procedures, applicability, uncertainty factors, data analysis, among other relevant issues are addressed based on the experience of this and previous WBE studies. The larger goal of this scholarly exercise was to contribute to the acquired insights of the long-term monitoring of SARS-CoV-2 to the ongoing discussions on the cruxes of usefulness of WBE surveillance of COVID-19.

2. Materials and methods

2.1. Description of main sanitation and health facilities in Monterrey

The Monterrey Metropolitan Area (MMA) is located in the northeastern part of Mexico and comprises 12 municipalities with 5.3 million inhabitants (INEGI, 2020). In 2020, the metropolitan area generated 336.97 hm^3 of wastewater. The wastewater sanitation service is

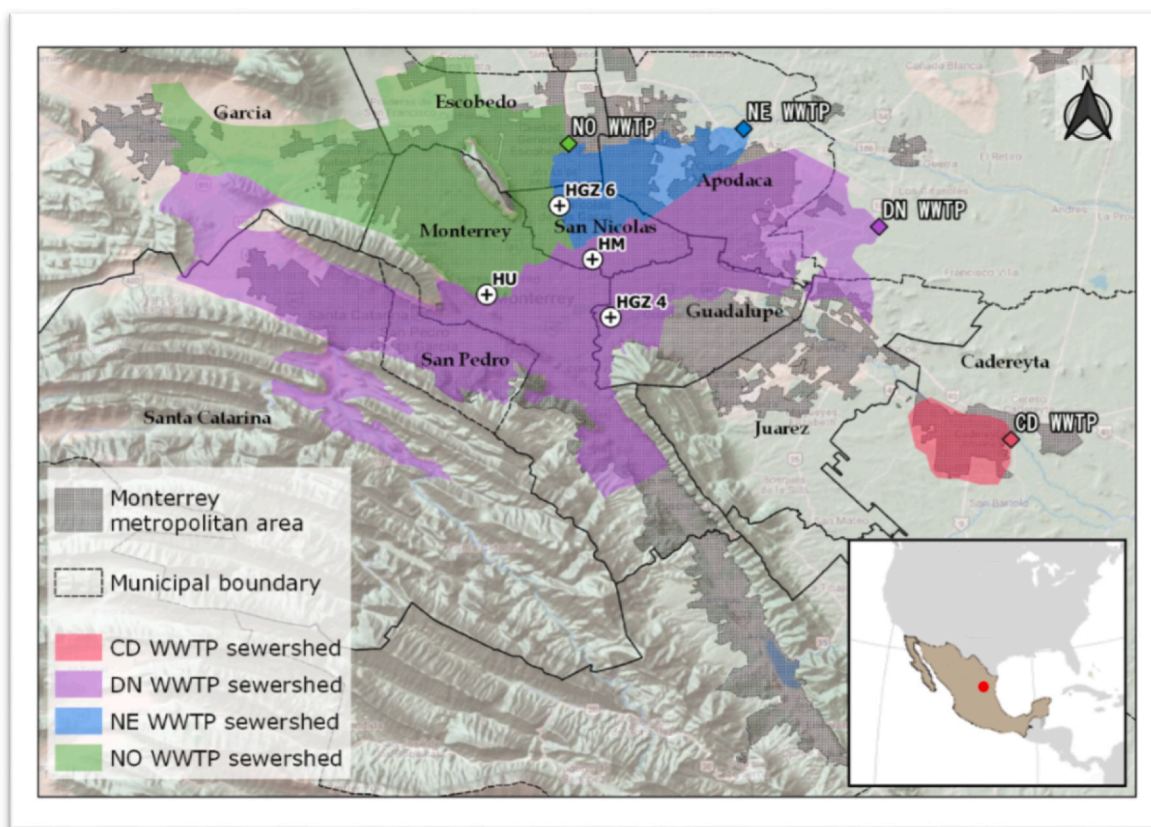**Fig. 1.** Location of the WWTPs and COVID-19 hospitals whose wastewater was analyzed to detect the presence of SARS-CoV-2. Note: DN WWTP = Dulces Nombres WWTP, NO WWTP = Norte WWTP; NE WWTP = Noreste WWTP; CD WWTP = Cadereyta WWTP. HGZ 4 = Hospital General de Zona 4; HGZ 6 = Hospital General de Zona 6; HM = Hospital Metropolitano; HU = Hospital Universitario. The sewershed areas have been provided by the water agency (SADM, 2021).

provided centrally by the local water agency *Servicios de Agua y Drenaje de Monterrey* (SADM). Fig. 1 shows the location and sewershed areas of the four main WWTPs that collect and treat wastewater of 72% of the MMA population (~3,812,000 residents). The sewershed areas do not align with geographic areas of municipalities, hence one WWTP may be serve more than one municipality.

Table 1 summarizes the characteristics of the four main wastewater treatment facilities in the MMA. The treatment process for Dulces Nombres and Norte WWTPs included pretreatment, primary treatment, secondary treatment using activated sludge and disinfection using chloride. Primary treatment is not included in the Noreste and Cadereyta WWTPs.

In response to the sudden surge of severe COVID-19 cases during March 2020, the state health authority increased the physical capacity by converting five hospitals into COVID-19 hospitals (Gobierno de Nuevo Leon, 2020). The most relevant of these were Hospital General de Zona 4 (HGZ 4). Hospital General de Zona 6 (HGZ 6), Hospital Metropolitano (HM), and Hospital Universitario (HU) (Fig. 1).

2.2. Wastewater sampling

Wastewater was sampled on a weekly basis of influents and effluents of the four WWTPs and effluents of the four hospitals (a total of 581 samples) between June 20 and December 20, 2020. A weekly sampling schedule was chosen in coordination with the water agency as a cost-effective and practical alternative compared to daily measurements.

At each of the WWTPs, 24-h composite samples were collected at the inlet (influent) and in secondary treated wastewater (effluent) before disinfection. Between June and mid-August, the weekly samples were collected on Fridays, Saturdays, and Sundays. To assess the variation between the three sampling days, a Kruskal-Wallis test was performed. The results revealed that there was no significant difference between the three sampling days ($p = 0.87$, Table S1). Starting in mid-August, the sampling program was reduced to one weekly composite sample (collecting on Sundays). In addition, effluents after disinfection were included in the sampling program to evaluate the effectivity of chlorine disinfection.

Single grab samples were collected from hospitals as raw sewage before discharge into the municipal sewer system. Studies suggest that 24-h composite sampling is more representative than grab sampling, however, it has been shown that grab samples are accurate to within an order of magnitude (Hillary et al., 2021). The sampling program for hospitals started with Hospital Universitario, Hospital General de Zona 4, and Hospital General de Zona 6. Hospital Metropolitano was included starting on August 29, 2020, after obtaining official permission. All samples were collected in 1-gallon bottles, transported to the laboratory, stored at 4 °C and analyzed as soon as possible (<48 h). COVID-19 protective measures and personal protection equipment were considered during the sampling campaign and analysis to ensure the safety of the involved personnel (Bain et al., 2020).

2.3. Laboratory methods

2.3.1. RNA and DNA extraction

We used previously reported protocols to extract nucleic acids form the wastewater samples and determine the concentration of SARS-CoV-2 by RT-qPCR (Mahlknecht et al., 2021). RNA extraction was performed using the QIAamp® Viral RNA Mini Kit (Qiagen, USA), while DNA extraction was performed with the QIAamp® DNA Mini Kit (Qiagen, USA).

First, wastewater samples were thermally inactivated at 95 °C for 5 min. A volume of 500 µL of the inactivated water sample was centrifuged for 10 min at $1500 \times g$. Then, a volume of 140 µL of the supernatant was added to a mix containing 0.56 µL of Buffer AVL solution (Qiagen, USA) and 5.6 µL of carrier RNA-AVE solution (Qiagen, USA) in a 1.5 mL microcentrifuge tube. This suspension was vortexed for 15s, incubated at room temperature (22–25 °C) for 10 min and centrifuged to remove drops from the interior of the lid. Next, 560 µL of ethanol (96–100%) was added to the sample and mixed by pulse-vortexing for 15 s.

The solution obtained (~630 µL) was filtered through a QIAamp Mini column (Qiagen, USA) to retain the nucleic acids originally present in the sample. The solution was loaded into the column contained in a 2 mL collection tube, the cap of the tube was closed, and the tube with the column was centrifuged at $6000 \times g$ (8000 rpm) for 1 min.

After this first centrifugation, the QIAamp Mini column was loaded into a clean 2 mL collection tube, and the filtrate was discarded. In the first rinsing step, 500 µL of 96% ethanol was loaded into the column contained in the 2 mL collection tube, the cap of the tube was closed, and the tube with the column was spun again $6000 \times g$ (8000 rpm) for 1 min. Following these two centrifugation steps, 500 µL of buffer AW1 (Qiagen, USA) was added to the QIAamp Mini column, the cap of the container tube was closed, and the tube with the column was centrifuged at $6000 \times g$ (8000 rpm) for 1 min. As before, the QIAamp Mini column was placed into a clean 2 mL collection tube, and the filtrate was discarded. In a fourth centrifugation cycle, a QIAamp Mini column was added to 500 µL buffer AW2 (Qiagen, USA), the cap of the container tube was closed, and the tube with the column was centrifuged at high speed ($20,000 \times g$; 14,000 rpm) for 3 min.

The QIAamp Mini column was placed in a clean 1.5 mL microcentrifuge tube and the filtrate was discarded. In a fifth centrifugation cycle, 60 µL buffer AVE (Qiagen, USA) equilibrated to room temperature was added to the QIAamp Mini column, the cap of the container tube was closed, and the tube with the column was centrifuged at a high speed ($6000 \times g$; 8000 rpm) for 1 min.

For the DNA extraction, 500 µL of the water sample was centrifuged for 5 min at $5000 \times g$; 400 µl of the centrifuge supernatant were discarded. The remaining 100 µL was added to 20 µL of proteinase K solution and 80 µL of buffer ATL (Qiagen, USA), vortexed, and incubated at 56 °C for at least 1 h. The solution obtained was filtered through a QIAamp Mini column (Qiagen, USA) to retain the nucleic acids originally present in the sample. The remainder of the extraction protocol was analogous to that previously described.

2.3.2. RNA and DNA amplification

In each amplification reaction we used two sets of primers (commonly referred to as N1 and N2) to amplify RNA segments of SARS-CoV-2. These primers targeted to sequences that encode the N protein of SARS-CoV-2. The sequences of these primers, extensively used for the identification of SARS-CoV-2 in human samples (González-González et al., 2020; Nalla et al., 2020) and wastewater (Haramoto et al., 2020; Medema et al., 2020; Nemudryi et al., 2020a; Peccia et al., 2020; Randazzo et al., 2020; Sherchan et al., 2020; Wu et al., 2020a), are shown in Table S2.

Table 1
Main characteristics of the four selected WWTPs of MMA (SADM, 2021).

WWTP	Treatment	Sewershed area (km^2)	Design capacity (l/s)	Treated volume in 2020 (l/s)	Served population (hab)
Dulces Nombres	Primary and secondary	504	7500	6780	1,808,190
Norte	Primary and secondary	277	4000	2520	1,201,965
Noreste	Secondary	75	1875	1230	734,646
Cadereyta	Secondary	36	250	190	68,111

2.3.3. RT-qPCR assays

Quantitative amplification was conducted in a quantitative PCR thermal cycle (Rotor gene Q 5plex, Qiagen, Germany) using the QuantiNova® SYBR® Green RT-PCR and QuantiNova® SYBR® Green PCR Kits (Qiagen, USA). For the amplification of SARS-CoV-2 RNA sequences, the amplification mix (final volume of 20 μL) consisted of 10 μL of 2 × QuantiNova Syber Green RT-Master Mix, 0.2 μL of QN SYBR Green RT-Mix, 1 μL of 10 × primer mix (0.5 μM final concentration), and 8.8 μL of RNA extract. The amplification cycle consisted of 10 min of reverse transcription at 50 °C and 2 min of amplification activation at 95 °C, followed by 40 iterative cycles of denaturation for 5 s at 95 °C and combined annealing and extension for 10 s at 60 °C.

A calibration curve was constructed to establish the conversion between Ct values and equivalent gene copies per milliliter (gc/mL). For this purpose, we used commercial synthetic genetic material that contained the complete N gene from SARS-CoV-2 (Integrated DNA Technologies, Iowa, USA). Samples containing different concentrations of synthetic nucleic acids of SARS-CoV-2, in the range of 10^2 to 10^5 genome copies mL^{-1} were prepared by successive dilutions from stocks. This plasmid has been used before as a positive control in amplification assays of SARS-CoV-2 genetic material (González-González et al., 2020). The estimated lower limit of detection was ~1 copy/reaction of the N gene of SARS-CoV-2 per mL of water.

2.4. Monitoring of COVID-19 cases and interpretation

The Mexican government database (https://datos.covid-19.conacyt.mx/) was consulted for estimation of the daily records of the COVID-19 confirmed cases and deaths for Monterrey municipalities during 2020. Weekly averages (7-day moving average) were calculated to smooth out the impacts of random, and short-term fluctuations. The figures of hospitalized patients in the selected hospitals were provided by the state health authority (SSNL-Secretaria de Salud de Nuevo Leon, 2021). Pearson and Spearman correlations were tested to find associations between viral loads of wastewaters in WWTPs/hospitals and clinical cases/hospital admissions. A non-parametric Kruskal-Wallis was applied to compare influent and effluent loads before and after chlorination (Table S3).

The incidence rate of daily new cases was calculated using reports of new clinical cases in the metropolitan area normalized by the population size of the metropolitan area. The total virus load of SARS-CoV-2 in wastewater (gc/day) was calculated by multiplying SARS-CoV-2 concentration by the daily average influent flow reported for each WWTP (Table S4). The number of new cases weighted by population was calculated as metropolitan new cases multiplied with sewershed area and divided by metropolitan population.

2.5. Prevalence of SARS-CoV-2 infection

The prevalence of SARS-CoV-2 infection within a catchment was estimated by employing a mass balance approach using the total number of viral RNA copies in wastewater for a given day, as measured in wastewater by RT-qPCR, and the number of SARS-CoV-2 RNA copies shed in stool by an infected individual (Ahmed et al., 2020a) (Eq. (1)):

$$Persons\ infected = \frac{(viral\ load\ in\ wastewater)*(wastewater\ flow\ rate)}{(daily\ stool\ production)*(viral\ load\ in\ the\ stool)} \quad (1)$$

The viral load in wastewater as gene copies per liter (gc/L) was determined by the RT-qPCR analyses performed on each sample. The daily wastewater flow rate (L/day) was obtained by direct flow readings at each WWTP (SADM, 2021). The daily stool production (g stool/person-day) was simulated as a normal distribution with a mean of 149 and standard deviation of 95, with data reported for high-income countries (Rose et al., 2015). Monterrey is one of the wealthiest cities in Mexico qualifying as a high-income area. Finally, the viral load in stool (gc/g of feces) in log_{10} was modeled as a log-normal distribution

from 5.79 to 8.11 (Lescure et al., 2020; Wölfel et al., 2020). To account for the variability of the variables in Eq. (1), a Monte Carlo simulation was conducted using Oracle Crystal Ball (version 11.1.2.4850 Oracle©) Excel add-on. The Monte Carlo simulation is an iterative mathematical technique that is used to approximate the likelihood of outcomes by running thousands of "trial" scenarios. The technique numerically quantifies and graphically depicts potential results (along with their associated likelihoods) based on provided uncertain inputs.

3. Results

3.1. Temporal dynamics of SARS-CoV-2 in wastewater

We collected and processed 422 wastewater samples from four municipal WWTPs and 159 effluent samples from four hospitals. Fig. 2 summarizes the number of samples and results of analytical detection. The share of positive detected samples from influent, effluent and disinfected effluent was similar for all treatment facilities (35.0 ± 4.8%). The percent of positive samples collected from hospital wastewater was similar (35.5 ± 9.5%) with a larger variation.

The results of viral loads in wastewater facilities were compared to the COVID-19 clinical cases and deaths in the MMA over time (Fig. 3). From the longitudinal data, we observed that the dynamics of the viral titers replicates the behavior of the clinical cases in the MMA, i.e. following the trends of the reported clinical cases. However, the association is not always consistent. The lowest viral loads of positive tested samples found were on the order of 10^3 gc/L and were observed during June, when daily new cases of COVID-19 averaged 347. During July and August, daily cases of COVID-19 increased to an average of 538, while viral loads in the wastewater also increased during these months, being on the order of 10^4 gc/L. In September, although daily cases of COVID-19 showed a decrease compared to July and August, viral loads showed a spike in concentration of 10^6 gc/L. For the month of October, daily cases of COVID-19 increased to an average of 576 cases per day, and viral loads were on the order of 10^4. In November, the maximum values were reached, with an average of 714 COVID-19 cases per day and viral loads in wastewater in order of 10^5 and 10^6 gc/L. Finally, during December, viral loads in the wastewater were in order of 10^3, while daily cases averaged 548.

The temporal trend of the SARS-CoV-2 concentrations in hospital effluents were also associated with clinical cases. However, only a weak correlation with hospitalizations of COVID patients was observed (Fig. 4).

In June, an average of 19 hospitalized COVID patients per day were reported, and viral loads on the order of 10^5 gc/L were detected. During July and August, the number of patients decreased to an average of 12 per day and viral loads in the wastewater also showed a decrease, being on the order of 10^3-10^4 gc/L. In September, viral loads increased to 10^4-10^5 gc/L, while the daily average number of hospitalized patients was 15. In October, there was an increase in the number of patients in the hospitals, averaging 20, and in this month the highest viral load was reached, which was on the order of 10^5-10^6 gc/L. During November, the average number of hospitalized patients remained at 20, while the viral loads decreased to 10^4-10^5 gc/L. Finally, during December, the daily average number of patients were 25 and the viral loads were in the order of 10^3-10^4 gc/L.

3.2. Detection rate and accuracy of wastewater surveillance

It is possible to investigate the detection rate and accuracy of wastewater surveillance by comparing wastewater detection data to clinically reported case counts from the health authority (Wu et al., 2021). The reported daily incidence of COVID-19 cases is used to calculate the percentage of positive wastewater samples for different incidence rates. The detection of virus titers increases exponentially with clinical incidence rate. At a detection rate of 80%, the clinical
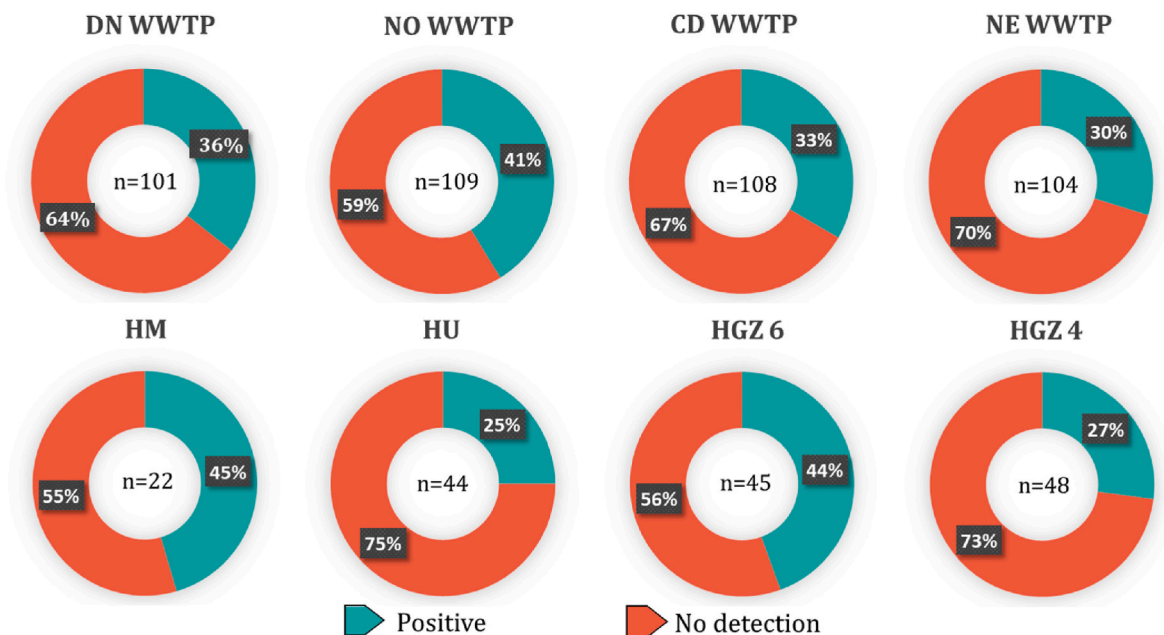
Fig. 2. Results of detection of SARS-CoV-2 RNA in wastewater samples from WWTPs (above) and hospitals (below), indicating the distribution of the total samples taken (n) and the share of positive tested samples. Note: The classification is done based on the lower detection limit. DN WWTP = Dulces Nombres WWTP, NO WWTP = Norte WWTP; NE WWTP = Noreste WWTP; CD WWTP = Cadereyta WWTP. HGZ 4 = Hospital General de Zona 4; HGZ 6 = Hospital General de Zona 6; HM = Hospital Metropolitano; HU = Hospital Universitario.
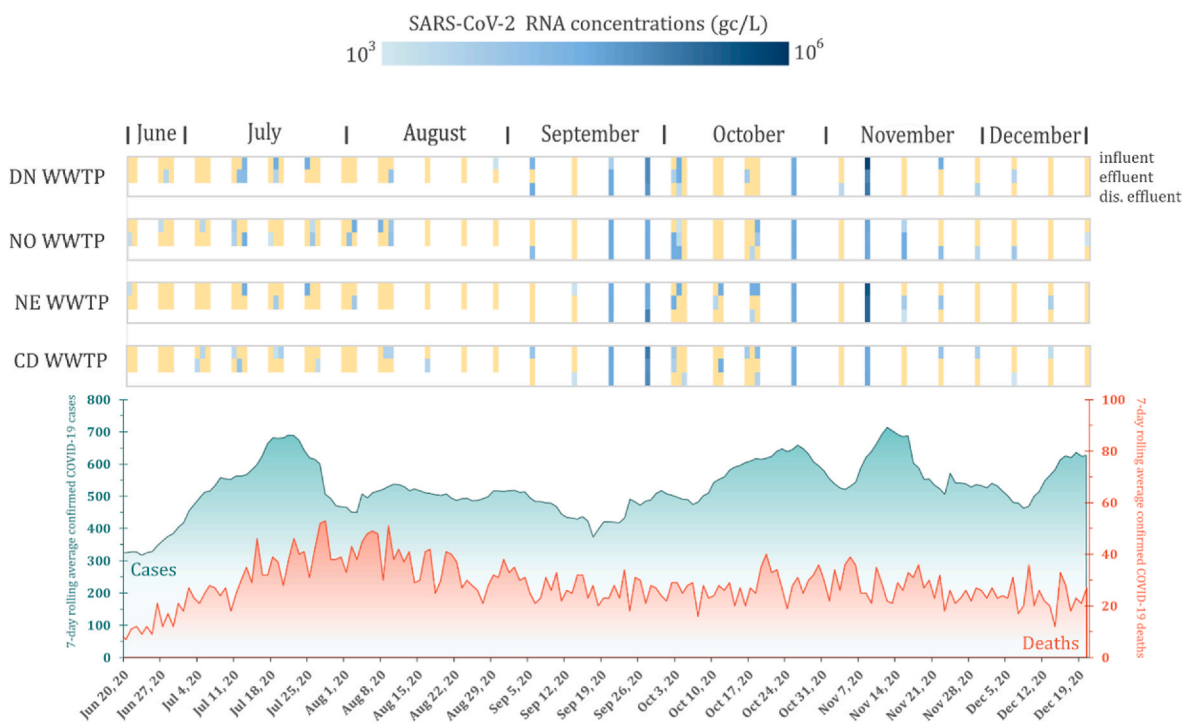

Fig. 3. Temporal trends of the occurrence of SARS-CoV-2 RNA in wastewater samples from WWTPs of the MMA compared to 7-day rolling average confirmed COVID-19 cases (green) and deaths (red). Note: The blue bar indicates a positive tested wastewater sample, and the intensity of the color refers to the viral load level. A yellow bar means that the wastewater sample was tested negative. The samples are plotted in the three categories: influent, effluents, and disinfected effluents. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

incidence lies on the order of 8 cases per 100,000 people. For all positive wastewater samples at the metropolitan level, the incidence rates of daily new cases ranged from 3.6 to 9.9 cases per 100,000 people (median 7.0). This means that the surveillance was capable to detect SARS-COV-2 for one new reported case out of ~14,300 people. It must

be noted that this number does not consider unreported infections.

A weak positive correlation was found between the incidence of daily new cases at the metropolitan level, and the wastewater viral titers at the sewershed level (Fig. 5a). The catchment size may influence the probability of SARS-CoV-2 detection in wastewater samples. This can be
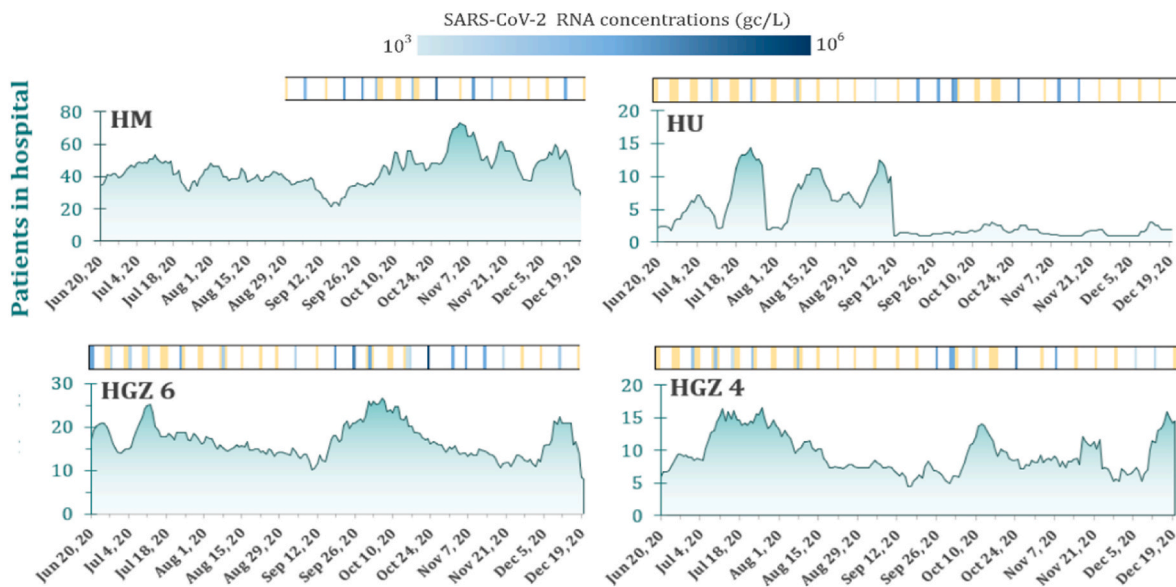
Fig. 4. Temporal trend of the presence of SARS-CoV-2 RNA in wastewater samples from hospitals in the MMA, related to daily hospitalized COVID-19 patients. Note: A blue bar indicates a positive wastewater sample, and the intensity of the color refers to the viral concentration. A yellow bar means that the sample was negative tested. Note that the access of patients to HU hospital was reduced after September 12, 2020. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
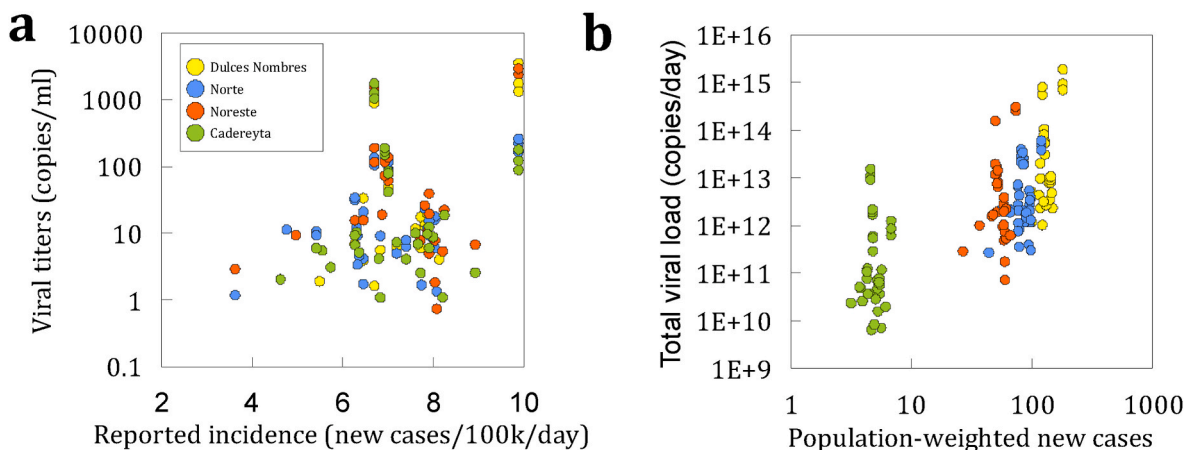

Fig. 5. (a) Association between viral titers in wastewater samples and the reported daily incidence rate in each sampled wastewater facility. **(b)** Association between the total viral load and estimated new cases in each of the catchment areas.

evaluated by analyzing the detection rate of positive samples from MMA with equal daily incidence. Fig. 5b demonstrates that the detection rate is positively correlated with the population size served by each wastewater facility (Dulces Nombres > Norte > Noreste > Cadereyta). This means that positive results from SARS-CoV-2 detection are over-represented by large wastewater facilities in comparison to smaller facilities considering the same incidence rate (F. Wu et al., 2020b; 2021).

A comparison of wastewater results with reported daily new clinical cases indicates that for all days with new clinical cases (n = 391), 34% were detected in wastewater. This low accuracy is probably due to the implemented sampling campaign among other factors as discussed below. Of the remaining samples (n = 259), 48% exhibited incidence rates below the median of all samples (7.0 cases per 100,000 people).

4. Discussion

4.1. SARS-CoV-2 in influents and treated wastewater of WWTPs

The SARS-CoV-2 viral RNA concentration of positive tested samples fluctuated between 10^3 and 10^6 gc/L. In general, the range of viral loads (gc/L) found in MMA is similar to that reported in other studies, i.e. F. Wu et al. (2020b): 5.7×10^4-3.0×10^5; Wurtzer et al. (2020): 5.1×10^4-3.1×10^6; Hata et al. (2021): 1.2×10^4-3.5×10^4; Hillary et al. (2021): 1.2×10^4-1.5×10^5; Xu et al. (2021): 0.5×10^3-1.9×10^6; Yaqub et al. (2020): 1.9×10^3-3.5×10^7. A few studies show higher loads, e.g. Yaniv et al. (2021): 3.2×10^6-3.7×10^7; Gonzalez et al. (2020): 1.7×10^6-4.6×10^8; Peccia et al. (2020): 1.7×10^6-4.6×10^8; and one study a lower range, i.e. Kumar et al. (2021): 2.1×10-6.7×10^3.

The detection of SARS-CoV-2 titers in treated wastewaters from WWTPs before and after disinfection of MMA were directly related to the viral loads in the influents, especially when the influents showed a

concentration equal to or higher than 10^4 gc/L. The viral loads found in treated wastewater remain on the same order of magnitude as those found in the influents (Figure S1). Considering all four WWTPs, in the occasions where only influents and corresponding secondary-treated wastewater before chlorination was measured in parallel (n = 69; June–August 2020), 16 influents and 16 secondary-treated wastewaters tested positive. Similarly, on the occasions where the influent and, treated wastewater before and after disinfection were measured simultaneously (n = 83; September–December 2020), 32 influents, 32 secondary-treated wastewaters before chlorination and 35 secondary-treated wastewater after chlorination tested positive. In addition, the non-parametric Kruskal-Wallis test confirms that the medians of all influent and secondary-treated wastewater loads before and after chlorination were statistically similar.

These results shed new light on the findings reported in other recent studies. Haramoto et al. (2020) found a viral concentration of 2.4×10^3 gc/L in one out of five samples of secondary-treated wastewater before chlorination during the outbreak, which contrasted with the corresponding influent that had no detectable RNA. The viral load of treated wastewater after chlorination was not measured. They concluded that the contrasting results were due to the use of varying sample sizes and neglection of hydraulic retention time by applying simultaneous measurements. Randazzo et al. (2020) reported that 35 out of 42 samples from influents and two out of 18 secondary-treated samples were positive (2.5×10^5 gc/L), while all 12 tertiary-treated (chlorination and UV) samples tested negative. Sherchan et al. (2020) found that none of the secondary treated (n = 4) and final effluent samples (n = 4) tested positive for SARS-CoV-2 RNA indicating the removal of SARSCoV-2 RNA during wastewater treatment to undetectable level. Nasseri et al. (2021) studied chlorination and UV treated wastewater and found that samples from influents from all WWTPs were positive (n = 4), while two out of four chlorinated-treated samples were positive for viral presence, and all four UV-treated samples negative. They concluded that UV disinfection was more effective than chlorine disinfection. From this, it can be concluded that although there exist differences between the implemented studies, genetic material in wastewater can persist throughout the stages of WWTPs.

The discrepancies between studies could be due to a different level of effectivity of treatment processes or different sampling volumes, use of different primers and distinct analytical sensitivity among the assays. The detection of possible false positive samples using RT-qPCR has been shown for example in Haramoto et al. (2020) and Randazzo et al. (2020). In our case, influents were sampled as composite samples and effluents as grab samples. Regarding the first argument, it is expected that during the primary treatment stage, the adsorption of viral particles onto the coarse suspended solids accompanied by gravitational settling is the major mechanism for virus removal (Balboa et al., 2021; Saawarn and Hait, 2021). In our case, Noreste and Cadereyta WWTPs have no primary treatment stage which would explain part of the viral load in the secondary-treated wastewater. In the secondary stage, the removal of SARS-CoV-2 during the biological treatment of wastewater of the WWTPs is likely to be governed by operating parameters such as hydraulic and biological solids retention times, temperature, and pH. Higher hydraulic retention times and temperatures favor removal of SARS-CoV-2 (Bivins et al., 2020; Bogler et al., 2020), as well as the accumulation of genetic material in such basins. In terms of disinfection techniques, chlorination and UV irradiation have been used to effectively remove the viruses from wastewater. However, the presence of organic material (e.g. colloids) in secondary treated effluents may represent a barrier against disinfection of SARS-CoV-2 (Saawarn and Hait, 2021; L. Wang et al., 2005). More research is needed to assess the removal of SARS-CoV-2 in the various stages of conventional WWTPs. The treated effluent reaches surface water bodies and groundwater, and thereby may post a public health risk. However, the environmental detection of RNA from SARS-CoV-2 alone does not substantiate risk of infection (Bivins et al., 2020; Mahlknecht et al., 2021), and the

infectivity of positive SARS-CoV-2 water samples must be assessed to conclusively determine risk.

Overall, the elevated viral load in secondary-treated wastewater from WWTPs in MMA is not reflected in receiving rivers. These waters showed concentrations three orders of magnitude lower than wastewater effluents which suggests that dilution effects in the surface waters (Mahlknecht et al., 2021) are sufficiently important to minimize public health risk.

4.2. Correlation of RNA concentration with COVID-19 related cases

One of the main aims of WBE is the possibility of detecting spikes of infection in the population even before the clinical cases are recorded by testing. Here, a correlation test between SARS-CoV-2 viral RNA concentrations in wastewater from the WWTPs and reported COVID-19 clinical cases and hospitalized patients in the MMA was performed. We applied a distributed lag from the day of corresponding reported cases (Fig. 6a). The concentrations of SARS-CoV-2 viral RNA in the influent of Dulces Nombres WWTP revealed a strong correlation (r = 0.63, p = 0.051) between days 2 and 6 before the clinical cases were reported. Similarly, correlations were found in the Norte and Noreste WWTPs. In the Norte WWTP, data showed a strong association (r = 0.60, p = 0.055) within a range of 2–5 days before the report of clinical cases, while in the Noreste WWTP a moderate correlation (r = 0.58, p = 0.062) within 2–7 days before clinical cases was observed. In general, data suggests that viral RNA concentrations in WWTPs from the MMA precede COVID-19 cases by 2–7 days. These results are in agreement with recent studies. Hillary et al. (2021), Giraud-Billoud et al. (2021) and Peccia et al. (2020) found that wastewater RNA concentration leads clinical cases by 2–5 days, 3–6 days and 6–8 days, respectively.

In addition, Fig. 6a suggests that the correlation curves for the three mentioned WWTPs are similar and that these WWTPs are sensitive to the number of infected individuals at a population-wide level in the MMA. As for Cadereyta WWTP, no significant correlation was found in the same period. The difference is probably because this plant is relatively small in comparison to the others (serving between 10.8- and 26.5-times less population) and follows a different dynamic given the high variability of its fluxes.

The concentration of SARS-CoV-2 in wastewater from all four WWTPs correlates with figures from hospitalized patients (Fig. 6b), although in this case the correlation was weak. Dulces Nombres and Noreste WWTP had a correlation coefficient of 0.38 (p = 0.17) and 0.44 (p = 0.15), and 0.46 (p = 0.14) and 0.42 (p = 0.17), respectively, each at an offset of 4 and 7 days. Cadereyta WWTP showed a correlation of 0.37 (p = 0.14) with an offset of 3 days. The correlation coefficient of Norte WWTP (0.27) is even lower than the others with an offset of 4 day. In general, the correlation shape between Dulces Nombres and Noreste WWTPs show an "M" form (Fig. 6b), while Norte and Cadereyta WWTPs exhibit points of negative correlation. In summary, the range of correlation indicates that viral RNA is 3–7 days ahead of hospital admissions.

WBE for COVID-19 surveillance has been implemented in several cities to detect spikes of infection in the population ahead of clinical records. Table 2 shows a selection of studies who examined this early warning capability. The results show a large variation in the time lag from the increase of cases regarding to the concentration in the wastewater.

This study suggests a lead time of 2–7 days between the increase of viral load in raw wastewater and the increase of clinical cases by reporting date. Hillary et al. (2021), Giraud-Billoud et al. (2021), Peccia et al. (2020), Nemudryi et al. (2020a,b) and Lastra et al. (2022) reported similar results, indicating a lead time within a week. Other studies imply that the lead time is greater, preceding the increase one to two weeks (Claro et al., 2021; Kumar et al., 2021) or even by three weeks (Robotto et al., 2022). In contrast, Ai et al. (2021) found that wastewater viral loads do not show a lead time in correlation with COVID-19 cases and correlated well when compared on a day-to-day basis, however, the
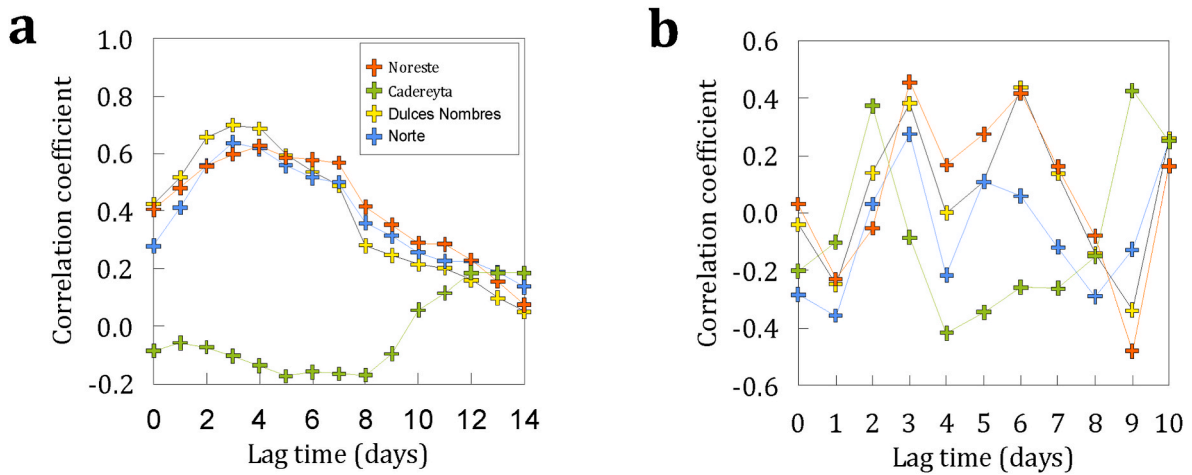
Fig. 6. (b) Pearson correlation test between SARS-CoV-2 viral RNA concentrations in wastewater from the influent of the WWTPs and COVID-19 reported cases in the MMA; (b) and wastewater from influent of the WWTPs and hospitalized patients in MMA.

Table 2

Results from recent literature on estimation of time lag between SARS-CoV-2 viral loads in wastewater and COVID-19 reported cases.

Reference	Country	Treatment facilities and served population	Monitoring time and number of samples	Sample periodicity and sample type	Concentration and quantification methods	Genes analyzed	Correlation method	Correlated with	Time lag/ Lead time
Ai et al. (2021)	USA	9 WWTPs 1.49 million	5.5 months 250 samples	Twice a week 24-h composite	Flocculation and ultrafiltration RT-ddPCR	N1, N2, E	Pearson Spearman	Symptom start date	0 days
Kumar et al. (2021)	India	4 WWTPs	2 months 43 samples	Weekly and fortnightly Grab samples	Centrifugal concentrators and filtration RT-PCR	ORF1ab, N, S	Time series	Clinical test date	7–14 days
Peccia et al. (2020)	USA	1 WWTP 0.2 million	2.5 months 73 samples	Daily grab samples (?)	Isolation RT-qPCR	N1, N2	Poisson regression models	Reporting date	6–8 days
Nemudryi et al. (2020a)	USA	1 WWTP 49 thousand	2.5 months 17 samples	Daily 24-h composite	Ultrafiltration RT-qPCR	N1, N2	Pearson	Clinical test date	2–4 days
Hillary et al. (2021)	United Kingdom	6 WWTPs 3 million	3.5 months 90 samples	Weekly Grab and 24-h composite	Centrifugal concentrators and ultrafiltration RT-qPCR	N1, E	Spearman	Clinical test date	2–5 days
Claro et al. (2021)	Brazil	2 WWTPs 1.4 million	10 months 220 samples	Weekly 4-h and 24-h composite	Precipitation RT-qPCR	N1, N2	Time series	Reporting date	14 days
Giraud-Billoud et al. (2021)	Argentina	2 WWTPs 1.2 million	7 months 46 samples	Weekly and fortnightly grab samples	polyethylene glycol and adsorption-flocculation RT-qPCR	N1, N2	Pearson	Reporting date	3–6 days
(Robotto et al. (2022)	Italy	4 WWTPs 1.7 million	11 months 500 samples	Bimonthly and weekly grab samples and 24-h composite samples	concentration/ extraction/ purification RT-qPCR	N1, N2, E	Regression analyses	Reporting date	7 to 21
Feng et al. (2021)	USA	12 WWTP 1.65 million	5 months 418 samples	1–2 days per week 24-h composite	Filtration RT-ddPCR	N1, N2	Cross-correlation	Symptom start date	−2 to +3 days
Lastra et al. (2022)	Spain	289 sampling points in sewer network 6.5 million	11 months (on-going)	Weekly Grab samples	Not reported	Not reported	Time series	Reporting date	3–11 days
This study	Mexico	4 WWTPs 3.8 million	6 months 584 samples	Weekly 24-h composite (WWTPs) and Grab samples (hospitals)	Extraction RT-qPCR	N1, N2	Pearson	Reporting date	2–7 days

correlation was made with the symptom onset date and were improved when compared with the 5-day rolling average of reported cases. Feng et al. (2021) also conclude that there were high correlations without lag. The correlations slightly improved as the case data were moved forward in time (positive values), this means clinical cases increased at the same time or a few days prior to increases in wastewater; however, the optimal time frames were different for each WWTP (−2 to +3 days).

Nemudryi et al. (2020a) argue that the detection of SARS-CoV-2 RNA titers in wastewater could closely overlap with the virological laboratory test dates, with SARS-CoV-2 RNA detectable in the wastewater 5–8 days after infection. As hospitalization/clinical testing generally occurs between 3 and 9 days after the onset of the symptoms, there is a possibility for these events to coincide. In general, lead times of wastewater tests may differ from WWTP to WWTP depending on location, sewershed area

and population, sampling strategy or environmental temperature, among many other factors, as wastewater is in general a very heterogeneous environment (Lastra et al., 2022).

4.3. Prevalence of infections

A mass balance approach (Eq. (1)) was applied to calculate the number of infected individuals based on the viral loads of influents, excluding outliers (Figure S1). To consider uncertainty and variability of independent variables in the used equation, we performed a Monte Carlo modeling strategy using 50,000 iterations. SARS-CoV-2 RNA gc/L of wastewater were modeled as point estimates for each date of detection and as a uniform distribution between the minimum and maximum counts observed. The results are summarized in Table S5. In the case of Dulces Nombres WWTP, the calculations determined a range of 243 to 82,690 infected individuals, which represented a prevalence of COVID-19 cases of 0.01–4.57%. For Noreste WWTP the estimated number of infections ranged from 440 to 29,659, which translated into a prevalence of 0.06–4.04%. In Norte WWTP the calculated persons infected varied between 541 and 52,311, matching a prevalence between 0.05 and 4.35%. Finally, in the Cadereyta WWTP the number of persons infected ranged between 74 and 1183, which corresponds to a prevalence of 0.11–1.74%. The aggregated data of modeled infected individuals for all four WWTPs matches in general the trend of reported cases, however, it shows a wide variation (Fig. 7).

The predicted numbers of infected individuals are in occasions considerably higher than the number of active cases in the MMA. This could be attributed to two factors. First, as mentioned above the official reports of COVID-19 cases are underestimates due to a large fraction of infected population not being tested and/or reported. According to an analysis of excess of mortality during the year 2020, the real number of deaths may be three times higher than reported figures, pointing to similar situation of real cases of infection with respect to reported cases (González-Ramírez, 2021; INEGI, 2021). Phipps et al. (2020) estimated for 15 countries of Europe and Asia that the true number of cases exceeded the reported by factors that range from 2.6 to 17.5. In addition, the official report of infection greatly oversees asymptomatic cases, while asymptomatic subjects shade virus in feces. Second, the employed model and used parameters possibly overestimated the number of infected people due to a large uncertainty in the used parameters or conceptual shortcomings in the used mass balance equation due to simplification. For example, the used mass balance approach assumes that all infected people shed the virus in their stool at a constant rate. 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.

Table 3 shows a selection of recent studies which attempted to calculate the prevalence of COVID-19 cases based on the viral loads in wastewater according to a mass balance approach. Similar to this study, the results show a wide variation in the number of infected persons. Ahmed et al. (2020a) found a prevalence of infection of 0.028% and 0.181%, based on two positive samples (19 and 120 gc/L). Gerrity et al. (2021) estimated a daily relative incidence of 0.02% and 0.03%, which was 5–8 times greater than that of confirmed COVID-19 daily relative incidence (0.004%) in Southern Nevada, USA. Chakraborty et al. (2021) reported a prevalence of 0.13% and 0.39% and concluded that these figures were in line with the COVID-19 reported cases. Hasan et al. (2021) adjusted the formula by adding the percentage of COVID-19 patients who shed virus in their stool. Their results, based on the calculation for four WWTPs, indicate that the number of infected individuals ranged from 2210 to 12,100. They don't indicate the calculated prevalence nor compare it with the COVID-19 reported cases. Pillay et al. (2021) reported that the WBE calculated infected people is considerably higher than COVID-19 cases reported in South Africa. They calculated a range of infections of 95,000 to 2.3 millions for one WWTP and 21,000 to 377,000 for another one, without indicating the size of prevalence. Saththasivam et al. (2021) calculated a number of infections from 31,181 to 542,313, considering five WWTPs studied. These values corresponded to a prevalence of 1.11% and 19.37% of the population of Qatar. This study was able to compare their results with a calculation based on an epidemiologic mathematical modeling and a nation-wide seroprevalence study and conclude that the values were according to the two models.

4.4. Future outlook

The present surveillance study is based on a weekly sampling program collecting 24-h composite WWTP samples using autosamplers and grab samples from hospital effluents. Compared to grab samples, composite wastewater samples collected with an autosampler are much better suited to adequately cover diurnal variations (Ahmed et al., 2020b, 2021). In a recent evaluation of WBE surveillance of over 40 US cities, Wu et al. (2021) concludes that a weekly frequency may not be enough for a robust interpretation. Feng et al. (2021) suggested that a
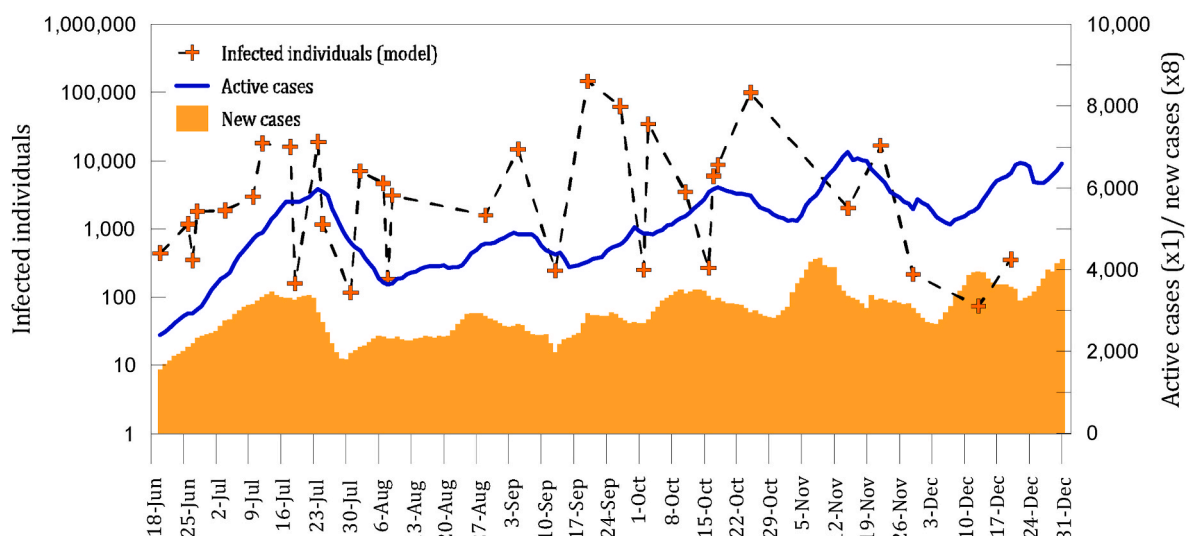

Fig. 7. Model estimates of infected individuals (aggregated) versus reported new and active clinical cases in MMA.

Table 3
Results from recent literature on estimation of infected people based on SARS-CoV-2 viral loads in wastewater.

Reference	Country	Treatment facilities and served population	Monitoring time and number of samples	Extraction and quantification methods	Range of viral loads	Range of prevalence %[a]	Infected people
Ahmed et al. (2020a)	Australia	2 WWTP and 1 pumping station 0.6 million	12 days 9 samples	Direct extraction and ultrafiltration RT-qPCR	19 and 120 gc/L	0.028 to 0.181	171 to 1090
Chakraborty et al. (2021)	India	4 WWTPs and 5 pumping stations 9.6 million	2 months 17 samples	Composite, supernatant, sediment and syringe filtration RT-qPCR	9.66×10^4 to 1.99×10^5 gc/L	0.13 and 0.39[b]	3983 and 5523
Hasan et al. (2021)	United Arab Emirates	11 WWTPs and 38 locations	2 months 81 samples	Ultrafiltration and polyethylene glycol RT-qPCR	7.5×10^2 to 3.4×10^4 gc/L	Not reported	2210 to 12,100
Pillay et al. (2021)	South Africa	4 WWTPs	4 months 56 samples	Ultrafiltration RT-ddPCR	1.55×10^5 to 7.32×10^6 gc/L	Not reported	95,000 to 2.3 million and 21,000 to 377,000
Saththasivam et al. (2021)	Qatar	5 WWTP 2.8 million	2 months 43 samples	polyethylene glycol RT-qPCR	7.8×10^3 to 5.4×10^5 gc/L	1.11 to 19.37[b]	31,181 to 542,313
Gerrity et al. (2021)	USA	2 WWTPs 1.06 million	3 months 36 samples	ultrafiltration qPCR	3.6×10^4 and 1.2×10^5 gc/L	0.02 and 0.03	20 and 200
This study	Mexico	4 WWTPs 3.8 million	6 months 581 samples	Extraction RT-qPCR	1.9×10^3 to 3.5×10^6 gc/L	0.02 to 4.6[c]	74 to 82,690

[a] NOTE: All studies used equation (1) (see 2. Methods).
[b] NOTE: Calculated by authors with the data shown in the study.
[c] NOTE: Minimum and maximum of all WWTPs.

minimum of two samples collected per week were needed to maintain accuracy in trend analysis. In our case, the weekly sampling regimes may introduce bias through the frequency of sampling. A more unbiased sampling strategy including appropriate time intervals and exclusion of unusual wastewater spills would improve the present estimates of the viral transmission in the population. Lastra et al. (2022) found that a weekly sampling strategy offered adequate quantification with fixed sampling hours to reduce the effect of daily variations. However, they emphasized that laboratory results must be validated with physico-chemical parameters such as BOD to detect unusual compositions (outliers) from heavy industrial discharges or rainwater dilution. Several scholars favor the use of biomarkers such as pepper mild mottle virus for normalization of SARS-CoV-2 signal (e.g. D'Aoust et al., 2021; Haramoto et al., 2020; Kitamura et al., 2021). Further, there is a difference in the number of samples collected by month, therefore, the proportion of positive samples may have been biased. For example, June and December have fewer sampling days, while during June and July the sampling was performed three days a week to test the variability.

Similarly, the detection of SARS-CoV-2 was only performed in the liquid phase of the wastewater samples. Thus, there may have been underestimates of the viral load by not analyzing the solid phase in the same sample. Primary sludge provides a high-solids-content, mixed sample that has been shown to contain a broad diversity of human viruses, including commonly circulating coronavirus strains (Peccia et al., 2020; D'Aoust et al., 2021; Kitamura et al., 2021). Parallel detection on solid and liquid fractions could be more effective and improve the accuracy of analysis.

All the clinical data recorded remain restricted by sampling procedures to monitoring the progress of the disease in the population and consequently, the number of cases reported by the authorities. The collection method used by federal and state health authorities were not standardized and access to tests limited, and therefore the sampling efforts are different from and asynchronized respect to the real infection dates (Mahlknecht et al., 2021; Sims and Kasprzyk-Hordern, 2020).

While WBE has proven to be a valuable tool for health monitoring at population-level, one of the main challenges to implement this method for COVID-19 surveillance, is the establishment of standardized methods and procedures for a successful extraction of viral material from wastewater, as well as its analysis and concentration in the laboratory

(Saththasivam et al., 2021). This standardization will also improve comparability between studies. In addition, the emergence of new SARS-CoV-2 variants in the early 2021 represents an opportunity to improve COVID-19 surveillance. One of the burning questions is whether the measurement of variants and its frequency through sequencing will provide a tool to check the precision of environmental surveillance of COVID-19.

5. Conclusions

Based on comprehensive statistical and epidemiological analyses of data obtained from 581 wastewater samples of four WWTP and four hospitals in the Monterrey Metropolitan Area over six months (between June and December 2020), we conclude our findings as follows:

- In three out of four WWTPs, wastewater viral titers (10^3 and 10^6 gc/L) were consistent with and precede clinical COVID-19 surveillance indicators. Our findings substantiate the fact that WBE is capable of providing 2–7 days of early warning of clinical cases, which may be a critical advantage during the surge (wave), especially in developing countries.
- Influent may be a better sample to perform WBE surveillance than the effluent as indicated by our results where temporal trends of viral loads in effluents from hospitals showed a weak association with hospital admissions of COVID patients.
- Wastewater surveillance has a high probability (80%) of detection if the daily incidence exceeds 8 reported cases per 100,000 people. However, there may exist an overrepresentation of positive tested samples in the case of wastewater treatment facilities with larger sewershed.
- For all days with new clinical cases, only 34% were detected in wastewater. This is probably due to the sampling strategy applied to the case. However, the median wastewater detection rate of SARS-CoV-2 is one out of 14,300 new reported cases.
- Simultaneous measurements of viral concentrations in influents and treated wastewater in WWTPs were found similar, probably owing to a low effectivity of treatment processes or differences in the sampling procedure from authorities. In any case, the viral loads in receiving rivers were lower by three orders of magnitude.

- The COVID-19 point prevalence according to WBE ranged from 0.02 to 4.6%. Model estimates of infected individuals are highly capricious and significantly higher than active cases reported by authorities due to combination of shortcomings in clinical testing and large uncertainties in the model parameter estimation.
- Although the WBE is highly time- and cost-effective for COVID-19 surveillance of communities in comparison to clinical testing, there is still a lack of reproducibility and repeatability across studies. In other words, there is still room for improving the understanding and standardization of best practices including tracking of variants.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.112967.

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