



# Wastewater-Based Epidemiology: Detection of SARS-CoV-2 RNA in Different Stages of Domestic Wastewater Treatment in Santa Fe, Argentina

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**Abstract** The COVID-19 pandemic affected human life at every level. In this study, we analyzed genetic markers (N and ORF1ab, RNA genes) of SARS-CoV-2 in domestic wastewaters (DWW) in San Justo City (Santa Fe, Argentina), using reverse transcription-quantitative real-time PCR. Out of the 30 analyzed samples, 30% were positive for SARS-CoV-2 RNA. Of the total positive samples, 77% correspond to untreated DWW, 23% to pre-chlorination, and no SARS-CoV-2 RNA was registered at the post-chlorination sampling site. The viral loads of N and ORF1ab genes decreased significantly along the treatment process, and the increase in the number of viral copies of the N gene could anticipate, by 6 days, the number of clinical cases in the

population. The concentration of chlorine recommended by the WHO ( $\geq 0.5 \text{ mg L}^{-1}$  after at least 30 min of contact time at pH 8.0) successfully removed SARS-CoV-2 RNA from DWW. The efficiency of wastewater-based epidemiology (WBE) confirms the need to control and increase DWW treatment systems on a regional and global scale. This work could contribute to building a network for WBE to monitor SARS-CoV-2 in wastewaters during the pandemic waves and the epidemic remission phase.

**Keywords** SARS-CoV-2 RNA · Wastewater-based epidemiology · Domestic wastewater treatment · Reverse transcription-quantitative real-time PCR

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## 1 Introduction

An efficient treatment of DWW has become a great challenge with the outbreak of the COVID-19 pandemic. The relevance of the treatment and management of wastewaters has augmented as a consequence of the increment in the number of biomedical residues that are thrown in cloacal stages. Frequently, these residues contain pathogens alive that could reach the water bodies, posing risks to human and environmental health (Iyer et al., 2021).

In the San Justo City, the first positive case for SARS-CoV-2, the etiological agent of the COVID-19 pandemic, was confirmed by reverse transcription-quantitative real-time PCR (RT-qPCR) on August 10, 2020 (<https://www.santafe.gob.ar/ms/covid19/datosabiertos/>).

It was reported that the SARS-CoV-2 can be found in the urine and feces of an infected person, even after the virus was not detected in the respiratory tract (Chen et al., 2020), and persist in the interior of fecal particles of domestic wastewater (DWW) (Arslan et al., 2020; Zhang et al., 2020). Another study showed that could remain viable, i.e., infectious, for up to 4.3 or 6 days in sewage or water, respectively (Giacobbo et al., 2021). It was communicated that over half of the 74 tested patients had stools that remained positive for SARS-CoV-2 RNA for an average of 11.2 days after the nasal swabs became negative (Wu et al., 2020).

This situation could be further aggravated in less developed countries, especially in areas with poor sanitation (Lodder and Roda Husman, 2020). Although important efforts have been made by the governments to limit the circulation of the virus, the transmission of SARS-CoV-2 may continue mainly due to the high infectiousness of new variants of the virus (Ullah et al., 2021). Recently, evidence has shown that contact with wastewater may lead to contamination (Giacobbo et al., 2021), but current knowledge does not confirm virus infectivity in wastewater yet, and knowledge gaps persist regarding its detection and the potential role of wastewater in its transmission (Kitajima et al., 2020).

RT-qPCR for diagnosis of the respiratory system through nasal swabs is an epidemiological tool developed and refined by environmental scientists over the last 20 years. WBE approach is now been used worldwide to understand the actual incidence of the COVID-19 pandemic, also standing out as an early-warning tool for predicting outbreaks in the future (Daughton, 2020; Sherchan et al., 2020; Chavarria-Miró et al., 2021; Xu et al., 2021a, 2021b; Ahmed et al., 2021, Randazzo et al., 2020a, 2020b; Lu et al., 2020; Orive et al., 2020; Bertels et al, 2022), besides many other ahead of print or preprints publications (Bloemen et al., 2022; Boehm et al., 2022; Hoar et al., 2022; Smith et al, 2022; Zhao et al., 2021; among others).

However, in South America, few studies have been reported (Fongaro et al. 2020; Michael-Kordatou et al., 2020; De Souza et al., 2020), and to the best of our knowledge, until now, five studies have been conducted to detect SARS-CoV-2 in Argentinian wastewaters (Iglesias et al. 2020; Barril et al. 2021; Giraud-Billoud et al., 2021; Barrios et al., 2021; Masachessi

et al., 2022). Although the setup for the Argentinian treatment plants is different from the ones at San Justo City, none of them has studied the presence of SARS-CoV-2 RNA at different stages of DWW treatment.

In the present work, we sampled DWW at three stages of the treatment process: (1) untreated DWW; (2) treated DWW in secondary facultative lagoons; and (3) post-chlorinated DWW, to detect the presence/absence of viral RNA, to monitor physicochemical variables, and to check the effectiveness of the chlorination process.

We applied viral concentration methods and RNA isolation methods to each sample at different stages of the DWW treatment process to detect and quantify genetic markers of SARS-CoV-2 RNA by RT-qPCR and to confirm the effectiveness of chlorination as a pre-treatment step before sewage discharge into the river. In addition, we compared the temporal changes in the number of SARS-CoV-2 RNA genetic copies in DWW with the COVID-19 daily active cases reported in San Justo City. We expect that this approach could contribute to gaining knowledge on SARS-CoV-2 surveillance in DWW.

## 2 Materials and Methods

### 2.1 Study Area and Sample Collection

Santa Fe province is located in the center east of Argentina. In San Justo City, the city's Public, Social, Social Assistance, and Housing Services Cooperative constructed the wastewater treatment plant (WWTP) and maintain the sewage network since 1998.

The samples were taken between July and August 2021 at the WWTP (30° 78' 93" S, 60° 62' 09" W). The WWTP processes a flow of 135 m<sup>3</sup> h<sup>-1</sup> of DWW from approximately 25,000 inhabitants. In addition, the WWTP receives the DWW brought by atmospheric trucks and from septic tanks located in areas of the city without a sewer network. The DWWs are treated by a stabilization and chlorination lagoon system before being discharged into the receiving aquatic environment, the Salado River (Fig. 1A). The treatment plant consists of (i) two anaerobic lagoons; (ii) two primary facultative lagoons; (iii) two secondary facultative lagoons; and (iv) a chlorination chamber.

To analyze SARS-CoV-2 RNA, ten 50-mL samples per sampling site (30 samples in total) at different

sampling times between July and August 2021 were taken at three sampling sites, corresponding to the stages of the DWW treatment: site 1 (S1), untreated DWW, site 2 (S2), junction of two secondary facultative lagoons (prechlorination), and site 3 (S3), post-chlorination (Fig. 1B). Physicochemical and bacteriological parameters were also analyzed at each sampling site. No rainfall or storm events occurred on the sampling days or before them.

## 2.2 Physicochemical and Bacteriological Parameters of DWW

Some physicochemical and bacteriological parameters were recorded during the sampling period. At sites S1 and S2, the following parameters were determined: pH, chemical oxygen demand (COD, mg O<sub>2</sub> L<sup>-1</sup>), biological oxygen demand (BOD, mg O<sub>2</sub> L<sup>-1</sup>), sulfur (mg L<sup>-1</sup>), total suspended solids (TSS, mg L<sup>-1</sup>), nitrates (mg NO<sub>3</sub><sup>-</sup> L<sup>-1</sup>), nitrites (mg NO<sub>2</sub><sup>-</sup> L<sup>-1</sup>), ammonia (mg NH<sub>4</sub><sup>+</sup> L<sup>-1</sup>), phosphate (mg PO<sub>4</sub><sup>3-</sup> L<sup>-1</sup>), total coliform, and fecal bacteria (MPN 100 mL<sup>-1</sup>). At S3, all the mentioned parameters and residual chlorine (mg Cl<sub>2</sub> L<sup>-1</sup>) were recorded. All analyses were made according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 2017).

## 2.3 Pretreatment of Samples for SARS-CoV-2 RNA Detection

The DWW samples were stored in polypropylene containers and kept refrigerated (4 °C) until centrifugation and filtration (Róka et al. 2021; Kumar et al., 2020). The samples were centrifuged for 30 min at 4500×g and filtered twice. For the first filtering, glass fiber filters (Sartorius MGC, 1.2-µm nominal pore size) and for the second filtering, Sartorius filters (0.2-µm nominal pore size) were used. Between filtering the samples, all the elements used were disinfected with a 10% sodium hypochlorite solution. After this process, all samples were stored at - 80 °C for 10 days until the analysis of SARS-CoV-2 RNA.

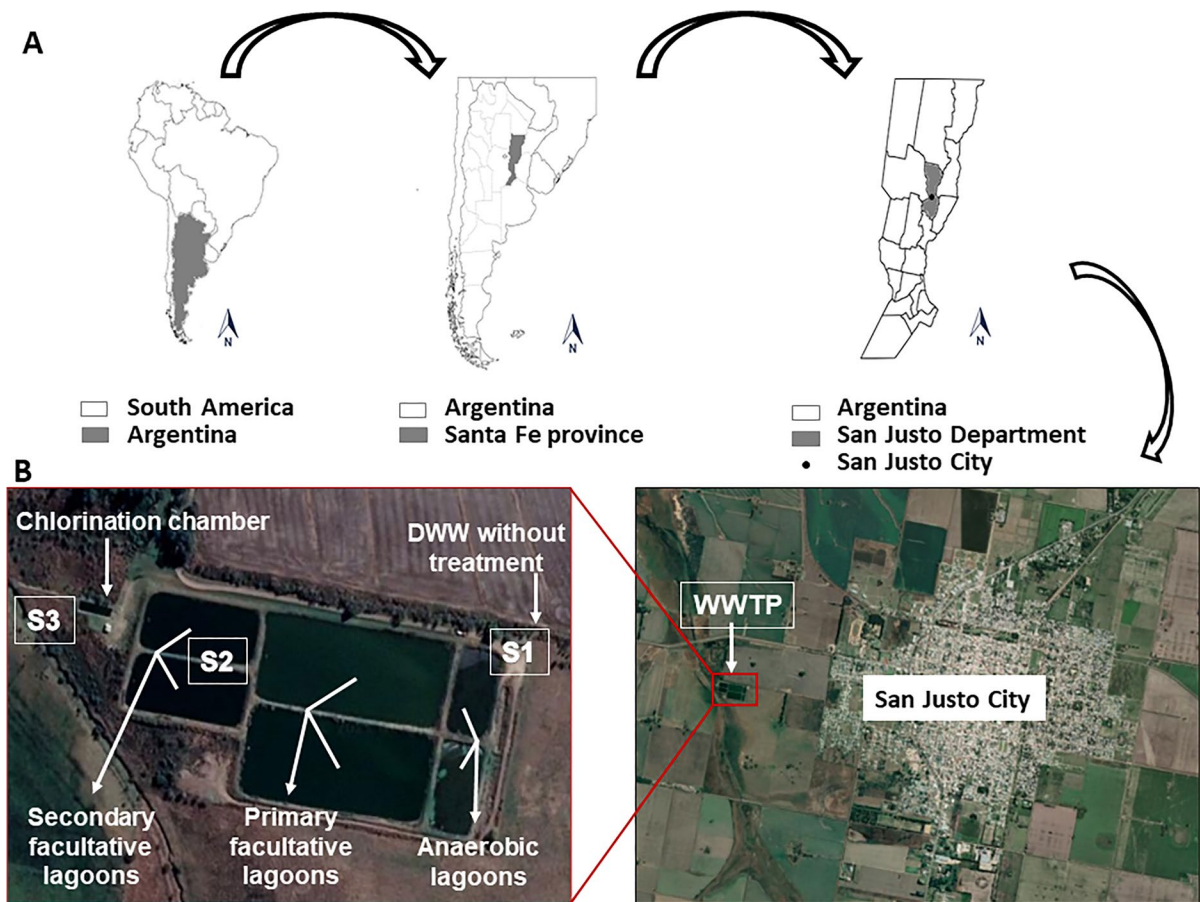
## 2.4 RNA Isolation and Extraction, SARS-CoV-2 Genetic Marker Detection and Quantification

RNA was extracted from the samples using a Thermo Scientific™ KingFisher automated system, with Invitrogen™ MagMAX™ kit for nucleic acid purification. Following Ahmed et al. (2020), sample volumes

of 200 µL were extracted and incubated for 5 min at 65 °C with lysis buffer (265 µL of MagMAX™), proteinase K (5 µL), and magnetic beads (10 µL). After digestion, washes were performed with wash buffer 1 (500 µL) and 80% ethanol (500 µL) to obtain an RNA/DNA elution of 50 µL.

For the detection and quantification of viral RNA, PerkinElmer® SARS-CoV-2 real-time RT-PCR assay was used following the manufacturer's instructions. The assay targets specific SARS-CoV-2 genomic regions: nucleocapsid gene (N) and open reading frame gene 1ab (ORF1ab). The assay is based on the TaqMan enzyme to perform in vitro viral RNA transcription, DNA amplification, and fluorescence detection. The two amplicons were labeled with FAM (N), HEX (ORF1ab), and internal control (Cy5) fluorescent dyes, respectively, to generate a target-specific signal.

The following mix was used for the PCR: CoV-2 reagent A-1 µL/test×n (samples); CoV-2 enzyme mix-5 µL/test×n (samples); and total-6 µL/test×n (samples). For a final volume of 20 µL, 14 µL of the nucleic acid extract to be studied was added to each mix. The COBAS z480 analyzer real-time thermal cycler (Roche®) was used. The cycling conditions were temperature (25 to 60 °C); time (2 min to 30 s); and 1 to 45 cycles. Nuclease-free water provided in the PerkinElmer® SARS-CoV-2 RT-qPCR reagent kit was used as a negative control. The negative control was added during extraction to each assay plate to control contamination during this step. Negative control PCR was performed using the same PCR mix used for the DWW samples. On the other hand, positive control was included in each extraction plate to ensure reagents and instruments perform optimally. The positive control was a plasmid containing target sequences of SARS-CoV-2: the ORF1ab and N gene of SARS-CoV-2. Both targets had Ct values of ≤35 and ≤32, respectively. The reaction mixture including a primer/probe set that detects a human RNA target was used as an internal control. The internal control is Cy5 fluorescent dye-labeled RNase P (RPP30) and is used to monitor processes from nucleic acid extraction to fluorescence detection. RNase P (internal control) should not be detected in either the positive control or the negative control. Positive and negative controls, performed with PerkinElmer® SARS-CoV-2 RT-qPCR reagent kit assay, are described in Table 1 (supplementary material).



**Fig. 1** Study area. **A** Location of the WWTP. **B** Sampling sites at the different stages of the treatment (S1, S2, and S3)

The cycle threshold values ( $C_t$ ) for two viral genes (N and ORF1ab) were considered positive if SARS-CoV-2 is detected when at least one target has  $C_t \leq 40$ , following the instructions of the kit—PerkinElmer® SARS-CoV-2 real-time RT-PCR assay—and previous research (Giraud-Billoud et al. (2021) and Xu et al., (2021a, 2021b)). The results were expressed as the number of genetic copies per 100 mL of DWW, and the conversion of  $C_t$  values to several copies per  $100 \text{ mL}^{-1}$  of the sample was carried out from emergency use authorization (USA) summary PerkinElmer SARS-CoV-2 RT-qPCR.

The evaluated linear dynamic range was between 60 and 3000 genetic copies of each marker per 100 mL. The standard curves had  $R^2=0.891$  (slope =  $-309.97$ ; y-intersect =  $12.136$ ) for the N marker and  $R^2=0.763$  (slope =  $-406.01$ ; y-intersect =  $14.286$ ) for ORF1ab marker. The results are expressed as the number of genetic copies per 100 mL DWW. Results of viral loads

obtained from the dilutions performed for the genetic markers N and ORF1ab are shown in Table 2 (supplementary material).

## 2.5 Epidemiological Data

Data from daily clinically active cases were accessed through the COVID-19 Statistics Office of the local hospital, San Justo, Santa Fe. In addition, the number of accumulated cases was obtained from the Santa Fe Health Ministry (<https://www.santafe.gob.ar/ms/covid19/datosabiertos/>).

## 2.6 Data Analysis

To verify possible statistically significant differences between sampling sites (S1-S3) in the number of genetic copies of the two genetic markers used in this

work, Kruskal–Wallis nonparametric tests were performed. Spearman's correlation analyses were carried out to test correlations between COVID-19 daily clinically active cases and the number of genetic copies per 100 mL of DWW samples at the different sampling times and sampling sites (significance level at  $p < 0.05$ ) by the GraphPad Prism 5.0 program (GraphPad Software Inc. n.d., San Diego, CA, USA).

### 3 Results

#### 3.1 Physicochemical and Bacteriological Parameters in DWW

Table 1 shows the environmental parameters of each sampling site in the WWTP of San Justo City. All physicochemical parameters at S3 were within the mandatory limits for the disposal of sewage waste according to local regulations of the province of Santa Fe (ENRESS, 2011).

#### 3.2 SARS-CoV-2 RNA Detection in DWW Samples

Out of the 30 analyzed samples, 30% were positive for SARS-CoV-2 RNA. From the total of positive

**Table 1** Physicochemical and bacteriological parameters of each sampling site in the WWTP of San Justo City, Santa Fe Province, Argentina

Physicochemical and bacteriological parameters	S1	S2	S3
pH	7.86	7.67	7.74
COD (mg O <sub>2</sub> L <sup>-1</sup> )	341.3	150.7	188.3
BOD (mg O <sub>2</sub> L <sup>-1</sup> )	154.5	41.2	41.9
Sulfur (mg L <sup>-1</sup> )	0.7	<0.2	<0.2
TSS (mg L <sup>-1</sup> )	54	38	47
Nitrates (mg NO <sub>3</sub> <sup>-</sup> L <sup>-1</sup> )	10.8	19.6	-
Nitrites (mg NO <sub>2</sub> <sup>-</sup> L <sup>-1</sup> )	<0.010	0.074	-
Ammonium (mg NH <sub>4</sub> <sup>+</sup> L <sup>-1</sup> )	61.2	36.4	39.1
Phosphorus (mg PO <sub>4</sub> <sup>3-</sup> L <sup>-1</sup> )	11.72	5.23	-
Chlorine residual (mg Cl <sub>2</sub> L <sup>-1</sup> )	-	-	0.16
Total coliforms (MPN 100 mL <sup>-1</sup> )	1.10 × 10 <sup>7</sup>	2.40 × 10 <sup>5</sup>	200
Fecal coliforms (MPN 100 mL <sup>-1</sup> )	2.10 × 10 <sup>6</sup>	9.30 × 10 <sup>4</sup>	40

Site 1 (S1), untreated DWW; site 2 (S2) junction of secondary facultative lagoons (pre-chlorination); and site 3 (S3) post-chlorination.

samples (9 samples), 77% (7 samples) corresponded to S1 (untreated DWW), 23% (2 samples) to S2 (pre-chlorination), and no positivity for SARS-CoV-2 RNA was recorded at S3 (post-chlorination). Significant differences were observed in the number of positive samples between S1 and S2 ( $p < 0.05$ ). The viral loads' average number of genetic N per 100 mL<sup>-1</sup> was as follows: at S1, 139,581 (min.=92,659, max.=179,850); at S2, 57,256 (min.=4717, max.=109,796), and at S3, the gen N dropped to 0. The ORF1ab gene viral load in S1 was average=80,890, max.=114,751, and min.=31,925, but was not detected in S2 and S3 (Fig. 2, Table 2).

Results are reported for each of the two specific SARS-CoV-2 genomic regions: nucleocapsid gene (N) and open reading frame gene 1ab (ORF1ab). —, negative results.

On the other hand, in San Justo City, 179 clinical cases were reported during the period of the study. No correlation ( $p < 0.05$ ) was found between the number of clinically active daily cases and the number of genetic copies of N per 100 mL<sup>-1</sup> at S1, which is the sampling point with the highest positivity of cases.

In Fig. 2, it can be observed that the increase in the number of viral copies of the N gene could anticipate the number of clinical cases in the population. On August 7, an increase in clinical cases was detected (71 cases), and between 7 and 8 days before, on July 29 and 30, there was an increment in the number of SARS-CoV-2 RNA viral copies of the N gene 100 mL<sup>-1</sup>, augmenting from 129,474 N gene viral copies 100 mL<sup>-1</sup> on July 28 to 179,850 and 175,200 N gene viral copies 100 mL<sup>-1</sup> on July 29 and 30, respectively. At S2, the N gene was quantified only in 20% of the samples analyzed, and at S3 (post-chlorination), no viral RNA was detected.

### 4 Discussion

Since the emergence of the COVID-19 pandemic, many studies confirm that WBE is a useful tool that allows monitoring the progress of the pandemic and predicting increases in clinical cases, thus acting as an early alarm system for COVID-19 outbreak and as a complementary approach for public health monitoring at the population level (Tran et al. 2021; Gonçalves et al., 2021).

In our study, the DWW treatment under the stabilization lagoon system followed by chlorination

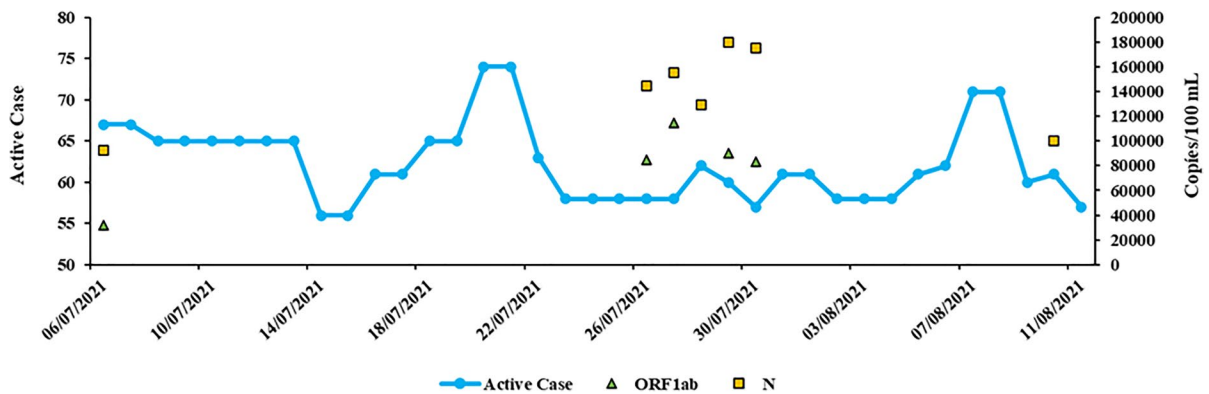
**Table 2** Amplification cycles of SARS-CoV-2 RNA in DWW without treatment, secondary facultative lagoons, and post-chlorination DWW

Sampling points in WWTP	Molecular target	6 July	26 July	27 July	28 July	29 July	30 July	4 August	5 August	10 August	11 August
DWW without treatment	N	35.5	34.5	34.1	34.9	33.3	33.5	—	—	35.8	—
	ORFI ab	34.4	33.1	32.3	—	32.9	33.1	—	—	—	—
Secondary facultative lagoons	N	39.0	—	—	—	35.6	—	—	—	—	—
	ORFI ab	—	—	—	—	—	—	—	—	—	—
Post-chlorination	N	—	—	—	—	—	—	—	—	—	—
	ORFI ab	—	—	—	—	—	—	—	—	—	—

(mean pH=7.8 and 0.16 mg L<sup>-1</sup> chlorine) was highly efficient in reducing and eliminating SARS-CoV-2 RNA, total coliforms from 2.40×10<sup>5</sup> to 200 MPN 100 mL<sup>-1</sup>, fecal coliforms from 9.30×10<sup>4</sup> to 40 MPN 100 mL<sup>-1</sup>, total coliforms diminished 45.8-folds and fecal coliforms 22.58-folds. In addition, we confirmed that the concentration of chlorine recommended by the WHO (≥0.5 mg L<sup>-1</sup> after at least 30 min of contact time at pH 8.0) successfully removed SARS-CoV-2 RNA from DWW. In a similar work, the pathogenic microorganism concentrations in effluent achieved <2 MPN L<sup>-1</sup>. The main disinfection was achieved at a chlorine dose of 2 mg L<sup>-1</sup> (Zhang et al., 2020). The effect of chlorination was considered by several studies, typically in the context of biosafety of WWTP effluents (Bertels et al., 2022; Wigginton & Kohn, 2012; Wigginton et al., 2012). Regarding the mechanism involved, it has been reported that chlorine inactivates viruses through the cleavage of the capsid protein backbone of viruses, therefore inhibiting viral genome injection into host cells (Page et al., 2010). In another study, 19% out of 100 untreated wastewater samples taken from the primary influent contained SARS-CoV-2 RNA, while secondary and tertiary effluent samples were negative for SARS-CoV-2 RNA, indicating that the secondary and tertiary treatment processes were successful in removing SARS-CoV-2 RNA (Sherchan et al. 2020), as was also found in our survey.

Chlorine inactivation efficiency can be reduced because potential alternative hosts can store SARS-CoV-2 and function as reservoirs in wastewaters. It was reported that the SARS-CoV-2 can persist in the interior of fecal particles of domestic wastewater (DWW) (Arslan et al., 2020; Zhang et al., 2020). Alternative reservoirs of SARS-CoV-2 in WW are organic and suspended solids that can protect viruses that adsorb to these particles (Gundy et al., 2008). Conversely, many factors can modify the integrity of viruses in WW, e.g., pH temperature, solid phase/liquid phase, population number, dilution by rain, presence, and characteristics of organic matter and humic acids and the influence of microorganisms and biofilms, among other factors (Amoah et al., 2020; Bertels et al., 2022).

Some authors alert about the relevance of fecal shedding factors among other sewer system-dependent factors that are not fully understood (Bertels et al., 2022). In another study, Fores et al. (2021)



**Fig. 2** Relationship between daily active cases of COVID-19 and the number of genetic copies of SARS-CoV-2 100 mL<sup>-1</sup> of DWW (principal Y-axis) and positivity of DWW samples for both genetic markers, N (yellow squares), and ORF1ab (green triangles)

observed that on average, 23% of SARS-CoV-2 N1 genes remain in the solid phase of wastewater after ultrafiltration. In this regard, Zhao et al. (2021) found that the supernatant fraction had high PCR threshold cycle (Ct) values, and most data were unquantifiable owing to low concentrations of SARS-CoV-2 RNA in the wastewater. In this work, we do not consider the fecal shedding factor. Future research should include SARS-CoV-2 loads in the solid fraction of raw influent, not only of the supernatant.

The minimum number of positive cases that there must be in a community for the virus to be detected in DWW is not yet exactly known, and a minimum number of infected people should be registered when trying to find correlations between concentrations of viral RNA and positive cases within a given population (Hong et al., 2021). The lack of correlation between the number of clinically active cases and the number of viral RNA copies of the gene N/100 mL<sup>-1</sup> could be due to changes in the temperature of the DWW (Hart & Halden, 2020), the hydraulic retention time in the system, sampling times, chemical or biological compounds that may occur at specific times (Amoah et al., 2020), the extension of the sanitation network, variations in virus excretion (van Doorn et al., 2020), and the degradation of genetic material or the number of suspended solids (Mandal et al., 2020), or simply because the number of diagnosed cases was not high enough.

Hoar et al. (2022) recently published the results of an ongoing wastewater monitoring program that tracked trends in sewershed-level SARS-CoV-2 loads.

An estimated minimum detectable case rate between 2 and 8 cases/day/100,000 people was associated with the method limit of detection in wastewater. In this sense, WBE is an attractive option for many municipalities, to confirm findings from clinical testing about population-level COVID-19 dynamics and to monitor for new outbreaks in instances when testing is inadequate.

Regarding the genetic markers selected to detect SARS-CoV-2-RNA in this work, -N1, N3, and ORF1ab genes-, these are also the most commonly found in the literature. The virus nucleocapsid (N) gene is widely used for WBE (Ahmed et al., 2021; Giraud-Billoud et al., 2021; Medema et al., 2020; Wu et al., 2020). In the detection of the SARS-CoV-2 RNA N gene, the Ct values found in this work were between 34.14 and 35.71. Similar Ct results for the N gene were reported by Medema et al. (2020), Pérez-Cataluña et al. (2021), and Giraud-Billoud et al. (2021) for DWW samples.

In general, OFR1ab genes were not as widely used as N genes to detect SARS-CoV-2 RNA. The results of our study show that the OFR1ab gene could be considered as a marker in WBE for COVID-19, but to confirm the use of this marker in WBE for COVID-19, more studies are needed. In another work, WBE surveillance through genetic material of SARS-CoV-2 viruses was also successfully applied. ORF1ab, N, and S genes of SARS-CoV-2 were found in the influent, with no genes being detected in the effluent (Kumar et al., 2020).

Considering the effectiveness of SARS-CoV-2 RNA quantification using RT-qPCR to anticipate the course of

the pandemic, in our work, the rise in the SARS-CoV-2 genetic marker N occurred 6 days earlier than the rise in clinically active COVID-19 cases, even though more research has to be done to corroborate this result.

Similar findings revealed that the genetic markers N1 and N2 of SARS-CoV-2 increased between 3 and 6 days earlier than the confirmed cases of COVID-19 (Giraud-Billoud et al., 2021). In another study, the increase in SARS-CoV-2 RNA concentrations in primary sewage sludge was observed between 1 to 4 days before the admission of patients to local hospitals and 6 to 8 days before positive test results for SARS-CoV-2 (Peccia et al., 2020). Other studies reported that wastewater surveillance anticipated, by several weeks, not only the original COVID-19 pandemic wave but also the onset of the second wave (Chavarria-Miró et al., 2021), while Róka et al. (2021) detected the first positive signal in sewage 2 weeks before the rise in case of numbers could predict the second wave of the outbreak.

Another aspect to be considered is the observed variability in virus counting reported in the literature. In this line, a source of unexplained variability in SARS-CoV-2 surveys in WWTPs is presented by Bloemen et al. (2022). Disconnected sewer systems (e.g., septic tanks) reduce the number of persons connected to the sewer network. This is especially important for WWTPs in rural areas. In San Justo City, this variability was minimized because the WWTP receives the DWW brought by atmospheric trucks and from septic tanks located in areas of the city without a sewer network. No rainfall events occurred during the sampling period, thus excluding another source of potential variability.

The presence of SARS-CoV-2 virion-free RNA in urban wastewater from the city of San Justo was determined from two specific molecular markers. However, these results do not demonstrate the transmission capacity of SARS-CoV-2 from urban wastewater. Different authors have discussed the possibility of SARS-CoV-2 transmission to humans through sewage or through contact with surface water that receives untreated or inefficiently treated sewage. Ahmed et al., 2021, among many others). In this sense, Anand et al. (2022) reported that, depending on the fate and decomposition in wastewater, SARS-CoV-2 has a low risk of transmission to humans, but his review paper also shows that the knowledge of SARS-CoV-2 transmission routes and environmental fates still needs to be improved and that the effective surveillance and deactivation are critical. Although

virion-free RNA was reported in this study, its deactivation allows us to hypothesize that it would not pose a risk to the environmental and human health of plant operators and users of the receiving water body (Salado River).

In synthesis, since the outbreak of the COVID pandemic, literature has accumulated considering WBE as a real-time low costing, noninvasive, and individual-independent methodology to track and prevent, to some extent, coronavirus events (Bertels et al., 2022). Several countries have successfully implemented WBE as a complementary system to clinical testing and acquired relevant qualitative and (semi-)quantitative data on community infection levels (Hasan et al., 2021).

Recent research considers that wastewater surveillance has become essential for monitoring the spread of SARS-CoV-2 (Pipes et al., 2022). Moreover, Smith et al. (2022) emphasize that publicly available health department incidence rates vastly underestimate true community incidence. Considering the relevance of rapid detection of the continuous appearance of new variants of the virus, it was documented that regional replacement of BA.1 with BA.2 is in agreement with, and ahead of, clinical sequencing data (Boehm et al., 2022).

Considering that wastewater represents a biological composite sample from the contributing community, continued population-level monitoring from wastewater data could become increasingly useful in areas where clinical testing rates decline or resources for clinical testing are limited (Hoar et al., 2022). In this line, this work could contribute to building a network for WBE to monitor SARS-CoV-2 RNA in wastewaters during the pandemic waves and the post-pandemic.

## 5 Conclusions

- The presence of SARS-CoV-2 virion-free RNA in urban wastewater was determined from two specific molecular markers.
- The viral loads of N and OFR1ab genes decreased significantly between S1 and S2, and the number of positive samples decreased as follows: S1>S2>S3, finally eliminating SARS-CoV-2 RNA.
- Results presented here can assist local authorities in taking action on public health, enabling the



cooperation between research and management institutions.

- Assessment of wastewater matrix effects should be considered for future research aiming to characterize uncertainty in WBE data. It is recommended that solid-phase analyses should complement aqueous phase samples.

- The results of this work contribute to the WBE for COVID-19, confirming that it is an early warning system, which allows detecting if the virus is in a community, based on its presence, concentrations, or absence, in the different stages of the DWW treatment.

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**Declarations**

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