



One-Year Surveillance of SARS-CoV-2 and Rotavirus in Water Matrices from a Hot Spring Area

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Abstract

The pandemic of Coronavirus Disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is still impacting not only on human health but also all economic activities, especially in those related to tourism. In this study, in order to characterize the presence of SARS-CoV-2 in a hot spring park in Uruguay, swimming pools water, wastewater, and surface water from this area were analyzed by quantitative PCR. Wastewater from Salto city located next to the hydrothermal spring area was also evaluated as well as the presence of Rotavirus (RV). Overall, SARS-CoV-2 was detected in 13% (13/102) of the analyzed samples. Moreover, this virus was not detected in any of the samples from the swimming pools water and was present in 18% (3/17) of wastewater samples from the hotels area showing the same trend between the titer of SARS-CoV-2 and the number of infected people in Salto city. SARS-CoV-2 was also detected in wastewater samples (32% (11/34)) from Salto city, detecting the first positive sample when 105 persons were positive for SARS-CoV-2. Rotavirus was detected only in 10% (2/24) of the wastewater samples analyzed in months when partial lockdown measures were taken, however, this virus was detected in nearly all wastewater samples analyzed when social distancing measures and partial lockdown were relaxed. Wastewater results confirmed the advantages of using the detection and quantification of viruses in this matrix in order to evaluate the presence of these viruses in the population, highlighting the usefulness of this approach to define and apply social distancing. This study suggests that waters from swimming pools are not a source of infection for SARS-CoV-2, although more studies are needed including infectivity assays in order to confirm this statement.

Keywords SARS-CoV-2 · Rotavirus · Wastewater-based epidemiology · Geothermal hot spring

Introduction

Since the first detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in Wuhan, China Zhu et al., 2019) and the posterior declaration of the pandemic by the World Health Organization (WHO) (WHO, 2020), many actions have been taken around the world to contain the virus dissemination.

Diagnosis tools as PCR were described rapidly to diagnose people who were coursing the Coronavirus Disease 2019 (COVID-19) or asymptomatic patients (Chu et al., 2020; CDC, 2020; Corman et al., 2020), in order to identify and isolate infected people and avoid viral dispersion. Other tools as immunology-based techniques were developed with the same purpose (Chau et al., 2020) such as detection of viral antigens or antibodies.

The high number of cases that rapidly started to grow and the extensive population around the world makes that

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clinical diagnoses were not enough to know the real number of people infected in a region or country. Therefore, a tool already described by WHO to evaluate environmental surveillance of poliovirus in the world (WHO, 2003) became widely used by many scientific groups. Wastewater-Based Epidemiology (WBE) has been used for poliovirus and other enteric viruses in wastewater and other water matrixes in order to evaluate the viral circulation in the population (Fumian et al., 2019; Gharbi-Khelifi et al., 2007; Pina et al., 2001). Rotavirus, one of the most important causal agents of gastroenteritis in children is an example of an enteric virus already evaluated by a WBE strategy (Kumazaki and Usuku, 2015). Unfortunately, in our country, we do not have a surveillance of Rotavirus in order to provide information of its national incidence, however, the presence of this virus in wastewater samples was already described in the region previous to COVID-19 pandemic, evidencing a wide dissemination in this kind of matrix (Victoria et al., 2014; Lizasoain et al., 2018).

WBE has many advantages, a large number of people could be evaluated with only one sample, detection and quantification of viruses could be used to take decisions about social distancing or lockdown, and viral diversity could be evaluated with the same sample (Murakami et al., 2020). The use of this tool already describes the presence of other coronavirus in water matrix samples previous to the COVID-19 pandemic (Bibby & Peccia, 2013), therefore it was rapidly used to evaluate the presence of SARS-CoV-2 in environmental matrices. Some authors even associate (by statistical estimations) the number of people infected with SARS-CoV-2 with the viral load in wastewater (Ahmed et al., 2020).

Social distancing and lockdown measures forced many shops, gyms, swimming pools, and tourist attractions to close their facilities. Swimming pools present special attention since the transmission of SARS-CoV-2 by the fecal–oral route is not ruled out (Chen et al., 2020; Xiao et al., 2020; Dergham et al., 2021). Moreover, the presence of an infected person in a swimming pool can contaminate this water through the nasal secretion with high viral titer (10^{10} copies/ml in individuals with only runny nose as symptoms [Savela et al., 2022]) which can infect a susceptible person sharing the pool.

In Uruguay, the first case of COVID-19 was confirmed in March 18th, 2020, from that date until November 7th, 2020, daily new COVID-19 cases per million people were below 15. The first peak of detection was in January 15th, 2021 with 275 cases. The second and third peaks were in April 12th and May 27th, 2021 with 1134 and 1102 daily new cases per million people, respectively.

In this work, different water matrixes from a geothermal hot spring area were studied in order to evaluate the presence of SARS-CoV-2 to know the epidemiology of these viruses

in tourists that visit the hydrothermal complex and in the population that resides in the same area. Nonpharmaceutical interventions such as social distancing and lockdown likely affect not only the epidemiology of SARS-CoV-2, but also the epidemiology of other viruses. In this sense, an enteric virus like Rotavirus was also evaluated.

Materials and Methods

Sampling Sites

Grab samples were collected in a period of one year from August 2020 to July 2021, the first 6 months of samples were taken every 15 days, while in the last period (January–July 2021), samples were taken monthly (with exception of February when samples could not be collected).

Wastewater, swimming pools water (regular swimming pool with hot water from the hot spring with 1.5 ppm chlorination) from the Daymán Hot Spring Park, and surface water from a stream close to the park were evaluated. Wastewater samples were obtained in two sites from Salto city (Obras Sanitarias del Estado—OSE) and two sites of the hydrothermal spring. In Salto city one wastewater sampling site collect wastewater from the Northern region of the city, while the other site collects wastewater from the southern region. In the hydrothermal springs also two sampling sites were defined, one collects wastewater from the hydrothermal park, and the other collects wastewater from hotels located next to the hot spring park. Doña Jacinta stream receives wastewater from a neighborhood that surrounds the hot spring park. All grab samples were collected at 8:00 A.M. for Salto samples and 10:00 A.M. for Dayman samples. 34 wastewater samples were from Salto city, 34 samples from Dayman wastewater, 17 samples from swimming pools (one composite sample from two swimming pools), and 17 samples from Doña Jacinta stream. Swimming pool samples were always collected on Monday, after the weekend where the hot spring park is crowded. Sampling sites are shown in Fig. 1.

Viral Concentration

In the case of wastewater a volume of 500 ml was collected, while 10 L of swimming pool water and surface water of Doña Jacinta stream were collected. Swimming pools waters were sampled in two sites of collection and a composite sample was analyzed. In all cases, samples were refrigerated and transported to the laboratory to be analyzed immediately.

As a control of the viral concentration process, 100 μ L and 50 μ L of PP7 bacteriophage (1.0×10^6 genomic copies/ μ L) were added to 10 L of surface water and 500 ml of wastewater samples, respectively. Wastewater samples were

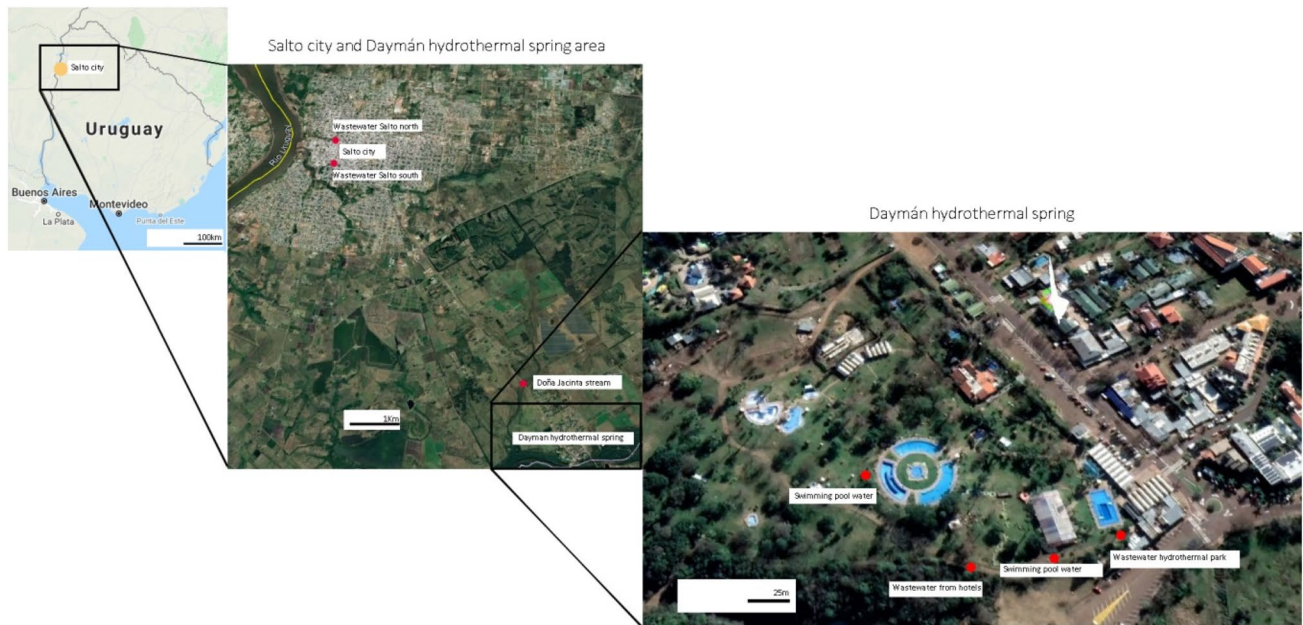


Fig. 1 Sampling sites (shown in red dots) in Salto city and Daymán hydrothermal spring area. Images from Google Earth®

concentrated with PEG precipitation method, according to the protocol described by Lewis and Metcalf (1988) with modifications (Salvo et al., 2021). Briefly, weights of centrifugation pots were registered, 500 ml of sample was distributed in two bottles (250 ml each) and centrifuged at 4750 xg for 20 min at 4 °C. Supernatant (S1) was maintained at 4 °C to be used later. Pots were weighted again and weight of each pellet was calculated. Sediment was mixed with 3 volumes respect to pellet volume with 3% Beef extract (Oxoid, Hampshire, England), 2 M NaNO₃ eluant (pH 5.5). Volumes of the same sample were mixed in one pot, pH was adjusted to 5.5 and sample was stirred for 1 h at 4 °C. Solids were then removed by centrifugation at 10000 xg for 20 min and the eluted was mixed with the first supernatant obtained (S1) and adjusted to pH 6.5–7.2. PEG 6000 (Sasol, Hamburg, Germany) was added to a final concentration of 10% (w/v) and NaCl to 2% (w/v). The resulting suspension was stirred for 6 h at 4 °C and centrifuged at 10,000 xg for 25 min. The supernatant was discarded and the pellet was resuspended in 5 ml of PBS buffer (Dako Inc., California, USA) (pH 7.2), adjusted to pH 8.0, incubated for 1 h with occasional vortex, and centrifuged at 10,000 xg for 20 min. Supernatant was stored for nucleic acid extraction.

Surface and swimming pool waters were concentrated by using the skimmed milk flocculation, according to the protocol described by Calgua et al. (2008). Briefly, a pre-flocculated 1% (w/v) skimmed milk solution (PSM) was prepared, the sample was carefully acidified to pH 3.5 by adding HCl 1 N and the PSM was added. Samples were stirred for 8 h at room temperature and flocs were allowed

to form sediment by gravity for another 8 h. The supernatant was carefully removed and a final volume of about 500 ml containing the sediment was transferred to a centrifuge tube and centrifuged at 7000 xg for 30 min at 12 °C. The supernatant was carefully removed and the pellet dissolved in 10 ml of phosphate buffer (1:2, v/v of Na₂HPO₄ 0.2 M and NaH₂PO₄ 0.2 M) at pH 7.5. The viral concentrate was stored at – 80 °C.

Viral Detection

Nucleic acid extraction was performed with QIAmp Viral RNA mini kit according to the manufacturer's instructions (QIAGEN, Germany). Detection of SARS-CoV-2 was performed using a one-step multiplex qPCR kit developed by the University of the Republic (UdelaR), ATGen and Institute Pasteur of Montevideo based on the protocol described by Chu et al. (2020). The same reaction allows the detection of SARS-CoV-2 (N gene) and Ribonuclease P (RNase P), used as an endogenous control. The SARS-CoV-2 amplicon inserted into the TOPO™ TA cloning vector provided by the kit was quantified and serially diluted in our laboratory for the construction of the standard curve in order to quantify SARS-CoV-2 in the analyzed samples. Standard curve for SARS-CoV-2 has a range of four logs (10⁰–10³ genome copies/reaction). The cycling conditions were as follows: reverse transcription for 5 min at 50 °C, initial denaturation for 20 s at 95 °C followed by 40 cycles for 15 s at 95 °C and 60 °C for 30 s.

For the detection of Rotavirus, a first reverse transcription reaction was performed with random primers pd(N)6 (Macrogen, South Korea) and Revert Aid reverse transcriptase (Revert Aid RT Thermo Scientific™, Carlsbad, CA, USA) according to the manufacturer's instructions. After the synthesis of the cDNA a Real-Time PCR reaction with TaqMan® technology was carried out. SensiMix™ II Probe kit (BIOLINE®, England) and Rotor Gene Q thermocycler (Qiagen®, Germany) were used following the manufacturer's instructions. A set of RVA-specific primers that amplify NSP3 gene and one probe with FAM fluorophore were used in the reaction. A standard curve was generated by using tenfold dilutions (10^7 – 10^0 genome copies reaction⁻¹) of a plasmid containing an amplicon of 86 bp (Zeng et al., 2008).

The cycling conditions were as follows: initial denaturation at 95 °C for 10 min followed by 45 cycles at 95 °C for 10 s and extension at 60 °C for 1 min.

Positive and negative controls were included in each step of the viral detection and samples that were negative for SARS-CoV-2 and RV were diluted 1/10 to evaluated possible inhibition of the enzymatic reactions.

Physicochemical Parameters Determinations

In order to compare the presence of the analyzed viruses with physicochemical parameters, temperature, dissolved oxygen (DO), redox potential (ORP), and free ammonia were measured in situ in swimming pools water and Doña Jacinta stream. Temperature and ORP were obtained using a multiparametric probe Sense ION HACH (MM150), ammonium and dissolved oxygen were determined with sensors IntelliCAL, HACH 30HQd, (IntelliCAL Nitrate ISENO3181, and LDO101, respectively). Calibration buffers were purchased from HACH.

In addition, to characterize Daymán geothermal water, a composed sample was collected at a flow rate of 70–80 m³/min of the geothermal borehole. The composed sample was obtained collecting 10 subsamples every 10 min and mixing. Hydrochemical characterization of the composed sample by majority ions analyzed provides a better awareness of the chemical baseline of this geothermal groundwater source. Temperature, Electrical Conductivity (EC), ORP, and pH were measured in situ. Sample was filtered through a 0.45 µm membrane (mixed cellulose esters-Merck Millipore), and major ions were analyzed in Ion Chromatograph DIONEX THERMO® with cation and anion standards and, respectively, solvents from THERMO®. MilliQ water was obtained from Arium Mini, SARTORIUS®.

Ion Chromatography, Alkalinity, HCO₃⁻, and Hardness were analyzed in the Water and Soils Laboratory from Water Department, Litoral North Regional Centre of the University of the Republic in Uruguay, according to the methodology established by Standard Methods (APHA, 2017).

Piper Diagram (Piper, 1944) and Schöeller-Berkaloff Diagram from EASY CHEM 5.0 (2012) software were performed to characterize the groundwater.

Results and Discussion

In this study, the contamination of different water matrices was assessed in order to determine their contamination with SARS-CoV-2 and Rotavirus comparing also the dynamic of SARS-CoV-2 in wastewater with the infected population in the same area.

PP7 was added as a control of the viral concentration process and was detected in all the analyzed samples (100% recovery success), with means of viral recovery of 26% for wastewater, 7% for swimming pools water, and 2% for Doña Jacinta stream. Differences in recovery percentage between wastewater and surface water can be explained by the fact that different methods were used for viral concentration and different volumes were analyzed causing different dilution factor for PP7.

Human RNase P amplified in 66 out of 67 wastewater samples, 15 out of 17 swimming pool water, and 5 out of 17 surface water of Doña Jacinta stream. RNase P according to the Center for Disease Control and Prevention protocol (CDC, 2019) serves as an endogenous control in clinical samples, and no results can be emitted if negative samples for SARS-CoV-2 are also negative for RNase P. In water matrices such as wastewater, RNase P have also been used as an endogenous control (Peccia et al., 2020; Ahmed et al., 2021). Our results confirm the advantages of use RNase P as an extra control, where 99% of the analyzed wastewater samples were positive for RNase P. Only one sample (1/67) was negative and corresponds to a wastewater sample from the hot spring park, in this sample the presence of chemical products could be more concentrated than the other wastewater samples evaluated and degradation of RNA/DNA molecules are more likely to occur. Moreover, this sample was positive for Rotavirus, thus this result is supported by the fact that RNase P is more vulnerable to degradation than viral genes as suggested by previous results (Ahmed et al., 2021). For swimming pools and Doña Jacinta stream, the percentage of positive results for RNase P were 88% and 29%, respectively. This is consistent with the matrix analyzed, as a mayor dilution factor is present in the stream samples, suggesting that this endogenous control could be used when wastewater matrices are analyzed for viral presence. Negative and positive viral controls presented correct results for all the analyzed samples for PP7, RNase P, Rotavirus, and SARS-CoV-2.

SARS-CoV-2 and Rotavirus were not detected in swimming pools water and surface water of the Doña Jacinta stream. The absence of viruses in the volume analyzed of swimming pools water is in concordance with evidence

that swimming pools would not be a route of transmission for SARS-CoV-2 (CDC, 2020; Romano Spica et al., 2020; Brown et al., 2021; Sobsey, 2022).

In wastewater, Rotavirus was detected in samples collected in the hot spring (7/17), in wastewater from hotels next to the hot spring (6/17) and from Salto city (22/34). The frequency of detection and quantification for this virus in wastewater samples is shown in Fig. 2.

From August to October in 2020, the frequency of Rotavirus in wastewater was around 10%, which is a low frequency compared with the previous results on the same area, and in other region of the country during the pre-pandemic period (Victoria et al., 2014; Lizasoain et al., 2018). In this period

with a low frequency of Rotavirus, social distancing and partial lockdown measures were applied by government authorities. When these measures were relaxed (November 2020–March 2021), the frequency of Rotavirus was higher (roughly 90%). This phenomenon was already described by other authors in different regions of the world, mainly for enteric viruses (Wang et al., 2021; Chan, 2022; Shen et al. 2022).

SARS-CoV-2 in the hot spring area was detected in three wastewater samples from hotels next to hot spring corresponding to samples collected in January, May, and June of 2021. In Fig. 3, the number of active cases of SARS-CoV-2 in Salto and the concentration of SARS-CoV-2 observed in wastewater of the area of hydrothermal spring are shown.

Fig. 2 Frequency of Rotavirus (blue bars) in wastewater samples from hot spring area and Salto city. Mean (red square), maximum and minimum of Rotavirus concentrations are also shown

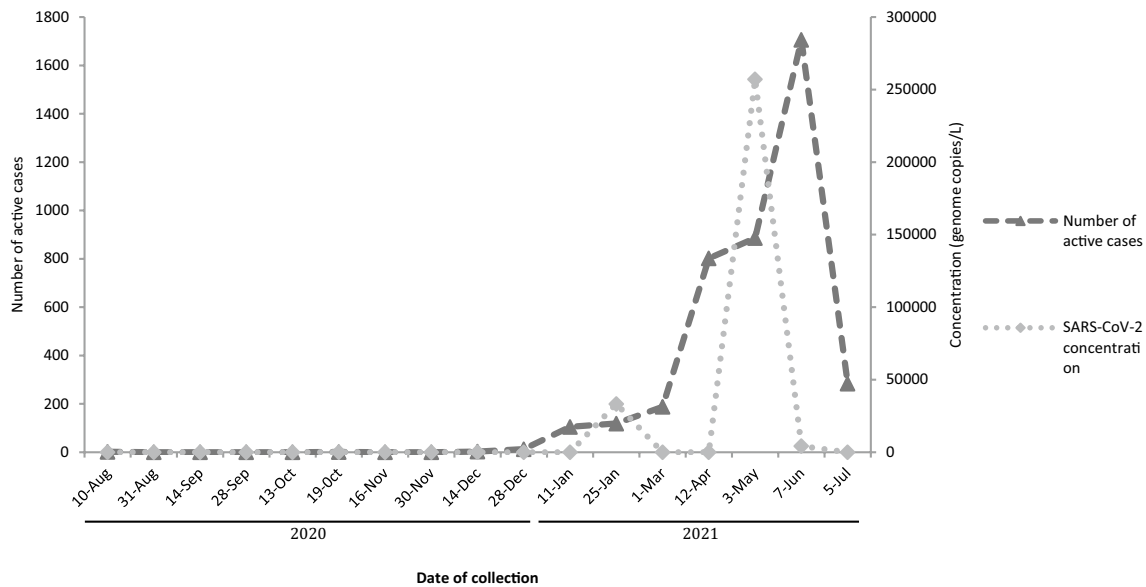
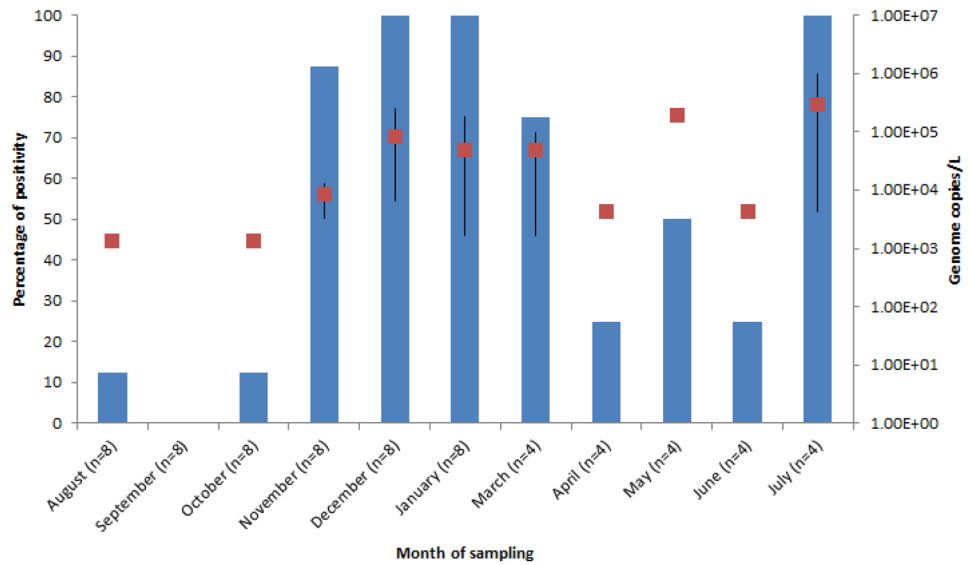


Fig. 3 Number of active cases of SARS-CoV-2 in Salto city and concentration of SARS-CoV-2 in wastewater of the area of the hot spring

The first positive sample for SARS-CoV-2 in wastewater from the hot spring area was observed when the number of active cases exceed 100 which is in accordance with the moment when social distancing and partial lockdown measures started to be relaxed. Moreover, the peak of detection of SARS-CoV-2 in wastewater was observed previous to the peak of active cases in the Salto population which is consistent with the previous studies, suggesting the use of wastewater as an early warning of the number of SARS-CoV-2 infections in the population (Robotto et al., 2022; Prado et al., 2021).

In Salto city, SARS-CoV-2 was detected in 11 out of 34 wastewater samples analyzed, 5 corresponding to the Northern sampling site and 6 to the Southern sampling site (Fig. 4).

In these samples, the association between the number of active cases and genome copies/L detected is more evident. Concentration of SARS-CoV-2 in the Northern and Southern pump stations of the city was clearly accompanied by the number of active cases in the city. These results confirm that, as it was already described by various authors around the world, the use of WBE is a powerful tool to describe the viral dissemination in the population based on the analysis

of wastewater samples not only for enteric viruses but also for SARS-CoV-2 (Randazzo et al., 2020; Peccia et al., 2020; Xagorarakis and O'Brien, 2019). The first detection of SARS-CoV-2 in wastewater from Salto city was observed when 105 active cases were reported. Taking in consideration the difference between populations, concentration methods, environmental, and other factors, the number of infected people and the presence of a positive wastewater sample for SARS-CoV-2 are consistent with the results from Ahmed et al. (2020) where they detect SARS-CoV-2 in wastewater samples when the number of active cases was higher than 171 active cases.

Differences observed in the dynamic of SARS-CoV-2 in wastewater between both sites (hot spring park and Salto city) respect to SARS-CoV-2 active cases in Salto could be explained since that hot spring receives tourists coming from different places of the country.

The absence of virus in swimming pools and Doña Jacinta stream water did not allow a possible association between the physicochemical parameters evaluated in these matrices and the presence of virus. In any case, the results obtained for these parameters are shown in Fig. 5.

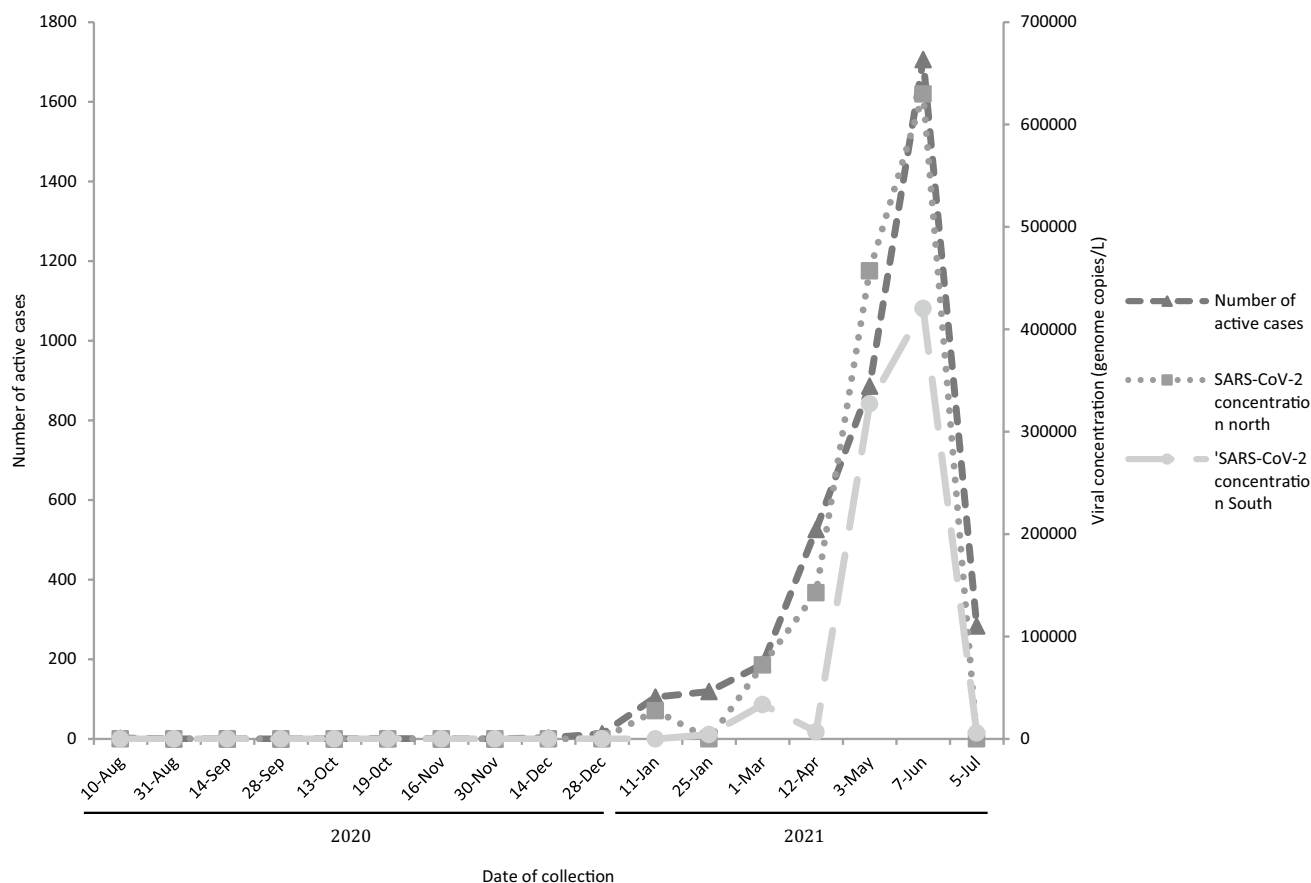


Fig. 4 Number of active cases of SARS-CoV-2 infections in Salto city and concentration of SARS-CoV-2 in wastewater of the two pump stations evaluated

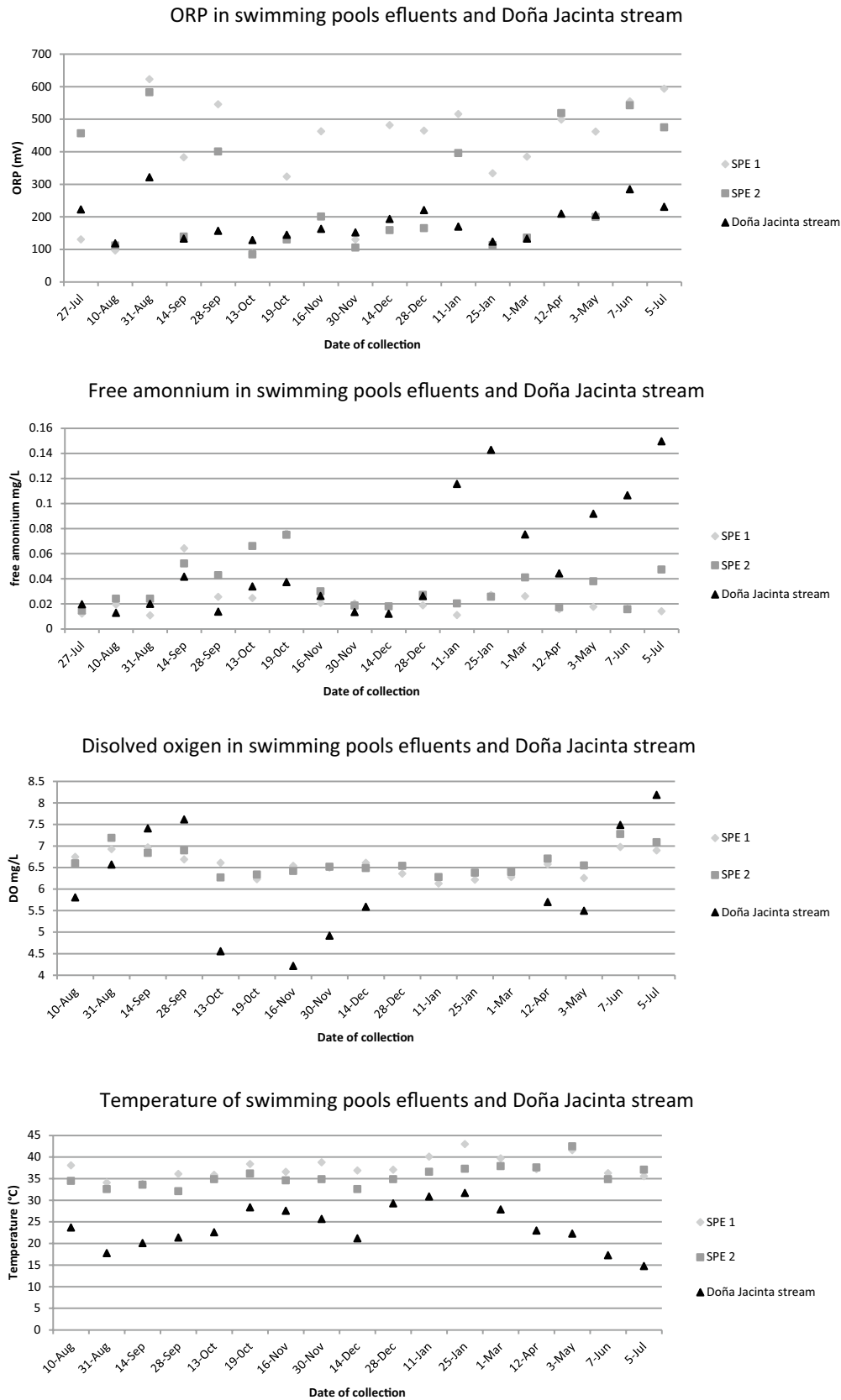


Fig. 5 Evaluation of physicochemical parameters (DO, temperature, free ammonia, and redox potential) in swimming pools effluents and Doña Jacinta stream

Regarding the hydrochemical characterization of the Daymán groundwater, this is a Hyperthermal groundwater, in situ physicochemical parameters were as follows: Temperature 43.2 °C, pH 7.32, ORP 190.3 mV, EC 754 $\mu\text{S}/\text{cm}$, and alkalinity 330.0 $\text{mgCaCO}_3/\text{ml}$ (see Table A from supplementary materials). Results from ion chromatography of majority ions analyzed by Piper Diagram indicated that this geothermal source is a Sodium bicarbonate type (See Figure A and EasyChem files from supplementary materials).

Daymán water showed no detected values of NH_4^+ . However, free ammonia was detected in swimming pools water (Fig. 5). This could be related to the possible presence of the decomposition of human urine products.

Conclusions

The absence of SARS-CoV-2 in swimming pools water in this one-year surveillance suggests that waters from swimming pools would not be a source of transmission for this virus although infectivity assays are needed in order to confirm this statement. The concentration of SARS-CoV-2 in wastewater follows the tendency of the number of infected people in the area of this study, supporting the advantage of this approach in order to determine the viral dispersion in the population and also serves as an early warning. Changes in frequencies of Rotavirus in wastewater samples evidence that the effects of social distancing and partial lockdown measures applied to the population in order to avoid SARS-CoV-2 transmission dispersion also affect the dissemination and transmission for other pathogens like enteric viruses.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12560-022-09537-w>.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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