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The presence of SARS-CoV-2 RNA in different freshwater environments in urban settings determined by RT-qPCR: Implications for water safety

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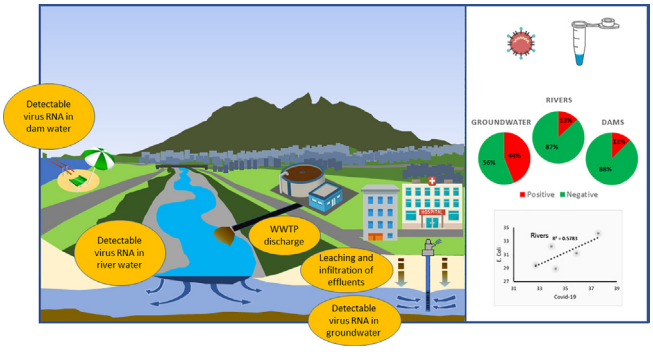
HIGHLIGHTS

- Virus RNA detected in 44, 13 and 12% of groundwater, river and dam water, respectively
- Significant correlation between SARS-CoV-2 and sucralose in groundwater
- Positive correlation between SARS-CoV-2 and *E.coli* in river water
- Ranges of freshwaters three degrees of magnitude lower than raw wastewater
- Preliminary risk assessment predicts a low risk for recreational activities.

GRAPHICAL ABSTRACT



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ABSTRACT

This study is the first focused on the presence of SARS-CoV-2 in different freshwater environments in an urban setting. Groundwater and surface water reservoirs for drinking water as well as water from receiving rivers of the Monterrey Metropolitan Area were sampled repeatedly during a SARS-CoV-2 peak phase between October 2020 and January 2021, and viral RNA was measured by quantitative reverse transcription polymerase chain reaction. Forty-four percent of the groundwater samples had detectable viral loads between 2.6 and 38.3 copies/ml. A significant correlation between viral load and sucralose concentration in groundwater reaffirmed the hypothesis of leaching and infiltrating effluent from surface and/or failing sewage pipes and emphasized the importance of water disinfection. Twelve percent of the surface water dam samples tested positive for viral RNA, with values varying between 3.3 and 3.8 copies/ml. Finally, 13% of the river samples were positive for viral RNA, with concentrations ranging from 2.5 to 7.0 copies/ml. Untreated wastewater samples taken in the same period showed viral loads of up to 3535 copies/ml, demonstrating a dilution effect and/or wastewater facilities efficiency of three orders of magnitude. Variations in the viral loads in the groundwater and surface water over time and at the submetropolitan level generally reflected the reported trends in infection cases for Monterrey. The viral loads in the freshwater environments of Monterrey represent a low risk for recreational activities according to a preliminary risk assessment model. However, this result should not be taken lightly due to uncertainty regarding data and model constraints and the possibility of situations where the infection risk may increase considerably.

1. Introduction

Since the outbreak of the COVID-19 disease, various routes of transmission of SARS-CoV-2 have been verified and others have been hypothesized. Currently, the main transmission is known to occur

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between people through respiratory droplets (diameter > 5–10 μm) produced by infected individuals when coughing or sneezing. Another presumed way of transmission is indirect contact with surfaces or objects in the immediate environment used by the infected person or on the infected person (Chan et al., 2020; Li et al., 2020; WHO, 2020).

An increasing number of studies have detected the presence of viral RNA in stool from COVID-19 patients (Wang et al., 2020; Kang et al., 2020; Xiao et al., 2020). Based on stool samples, Wu et al. (2020a) suggested that SARS-CoV-2 may replicate for 11 days in the gastrointestinal tract of patients even after samples from the respiratory tract become negative. According to another experiment, SARS-CoV-2 remained viable for 2 to 6 h in adult feces and up to 2 days in children's feces (Liu et al., 2020). This opens potential modes of fecal transmission.

Regarding the presence and persistence of SARS-CoV-2 in wastewater, there is sufficient evidence that indicates that wastewaters may contain both RNA fragments and viable particles of SARS-CoV-2 (Langone et al., 2021). Several studies have reported the new coronavirus in untreated and treated wastewater in the USA (Wu et al., 2020b; Nemudryi et al., 2020; Sherchan et al., 2020; Green et al., 2020; Peccia et al., 2020), Japan (Haramoto et al., 2020; Hata et al., 2021), France (Wurtzer et al., 2020; Trottier et al., 2020), Italy (La Rosa et al., 2020a; Rimoldi et al., 2020), Spain (Randazzo et al., 2020; Balboa et al., 2020), India (Kumar et al., 2020; Chakraborty et al., 2021), Pakistan (Sharif et al., 2020; Yaqub et al., 2020), Netherlands (Medema et al., 2020), Australia (Ahmed et al., 2020a), Turkey (Kocamemi et al., 2020), Israel (Bar-Or et al., 2020), Germany (Westhaus et al., 2021), and Czech Republic (Mlejnkova et al., 2020). Wastewater treatment plants (WWTPs) with tertiary disinfection have been found to be negative for SARS-CoV-2 (Rimoldi et al., 2020), while effluents from secondary treatments have been found to be positive (Randazzo et al., 2020; Rimoldi et al., 2020). The presence of SARS-CoV-2 RNA in sewage sludge was reported in a 10-week monitoring study in New Haven, Connecticut, USA (Peccia et al., 2020).

Although several authors have hypothesized about potential routes in water environments, to date, there exists little evidence of the presence of SARS-CoV-2 virus in freshwater (La Rosa et al., 2020b; Langone et al., 2021; Kumar et al., 2021). Rimoldi et al. (2020) detected viral RNA in three receiving rivers in the Milan area indicating the partial efficiency of the sewage system in the metropolitan area. Haramoto et al. (2020) collected three river samples between March and May 2020 in Japan and reported that no samples tested positive for SARS-CoV-2 RNA. Guerrero-Latorre et al. (2020) reported viral loads during a peak of the outbreak from three different sites of a river receiving untreated sewage from Quito, Ecuador. To our knowledge, to date, no evidence of the presence of the virus in surface water reservoirs and aquifers has been reported.

Water safety starts with the protection of water resources in catchment; therefore, it is mandatory to prevent surface and groundwater from coming into contact with fecal material. It is hypothesized that pathogen removal occurs in groundwater due to soil filtration, adsorption on sediment grains and progressive inactivation, and viruses in surface waters are exposed to several potentially inactivating stressors, including sunlight, oxidants, and predation by microorganisms (Langone et al., 2021). An ongoing research question is how persistent SARS-CoV-2 virus is in different water matrixes.

Chemical markers are indicators that may help evaluate the proper functioning of WWTPs and determine the level of human wastewater effluent in groundwater systems. The characteristics of an ideal wastewater indicator include: (i) source specificity, (ii) sustained effluent release because the indicator is not rapidly degraded by biological treatment processes, (iii) a demonstrated analytical methodology, (iv) no attenuation during transport, and (v) virtually zero background with a sufficiently large discharge to detection level ratio able to exceed receiving water dilution factors (Gasser et al., 2010; Oppenheimer et al., 2011). Several anthropogenic organic compounds with known characteristics have been used as chemical markers of pollutant loading due to their behavior as persistent aqueous organic pollutants (Benotti et al., 2009; Buerge et al., 2009). Among them, sucralose is one of the most popular artificial sweeteners and thus serves as a tracer of human wastewater, and its concentration is correlated with people connected to the sewage system (Kokotou et al., 2012; Voss et al., 2019). This organic compound is stable over a broad pH range and is heat stable, nonvolatile, highly polar and chiral. It is also strongly recalcitrant, degrading only under strongly oxidizing conditions, and is not metabolized by animals or microbes (Soh et al., 2011). These characteristics makes sucralose an excellent marker for human wastewater effluents, and it may help to confirm the presence of human pathogens such as SARS-CoV-2.

In the present study, we evaluated the presence of genetic material from SARS-CoV-2 RNA in different freshwater environments in the Monterrey Metropolitan Area (5.3 million inhabitants) in northern Mexico. The aim of the study was to perform a survey of viral dispersion and potential implications for the environment and public health during a peak phase of the epidemic. To address this goal, we collected untreated groundwater, river water and water from dams repeatedly between October 2020 and January 2021 and measured SARS-CoV-2 RNA by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For the groundwater, the concentration of the artificial sweetener sucralose was measured in parallel.

2. Materials and methods

2.1. Study area

The Monterrey Metropolitan Area (MMA) is the second most important city in Mexico in terms of population and the economy. It comprises 12 municipalities with a total population of approximately 5.3 million inhabitants (INEGI, 2021). The climate is semiarid with a mean annual temperature and rainfall of 22.3 °C and 622 mm, respectively, with a dry season (November–April) and rainy season (May–October). The urban area is bordered to the west and south by mountain ranges varying in composition from clastic marine to carbonate sedimentary rocks reaching elevations up to 2100 m above sea level (masl) (Fig. 1).

The MMA sits in a valley at 580 masl on Quaternary alluvial deposits eroded from the surrounding mountain ranges. The valley is mostly composed of fluvial and alluvial sedimentary deposits as terraces that occurred during accumulation-erosion cycles in the early Quaternary (Martinez and Werner, 1997).

Water for the MMA is supplied from surface water (58%) and groundwater (42%) reservoirs (SADM, 2021). Surface water is extracted from the El Cuchillo dam (4.69 m^3/s), Cerro Prieto dam (2.83 m^3/s), and La Boca dam (0.45 m^3/s). Water from the El Cuchillo dam and Cerro Prieto dam is conveyed 108 km and 133 km to the MMA, respectively, while the La Boca dam connects to the Cerro Prieto aqueduct (Fig. 1**b**). Raw water from all three dams is purified before distribution throughout the city through two water-supply pipelines over 70 km in length each (Fig. 1**c**).

Groundwater is extracted from several aquifer units and wellfields and disinfected locally before being introduced into the supply network (Torres-Martínez et al., 2020) (Fig. 1**a**): The Buenos Aires (BA) well field (2.11 m^3/s) located in a side valley close to the city consists of La Huasteca horizontal filtrating gallery and 23 deep wells with water table depths between 20 and 120 m below the ground, extracting water from Early Cretaceous limestone formations; the Santiago (SA) groundwater system (1.27 m^3/s) consists of La Estanzuela spring and three horizontal filtrating galleries; the Monterrey Metropolitan Zone (ZM) aquifer (1.08 m^3/s) includes wells throughout the metropolitan area, providing water from an unconfined aquifer which consists of altered lutite, conglomerate, gravel, sand and clay, with an average depth to groundwater of 20 m; finally, the Mina well field (1.20 m^3/s) is located approximately 35 km northwest of the MMA.

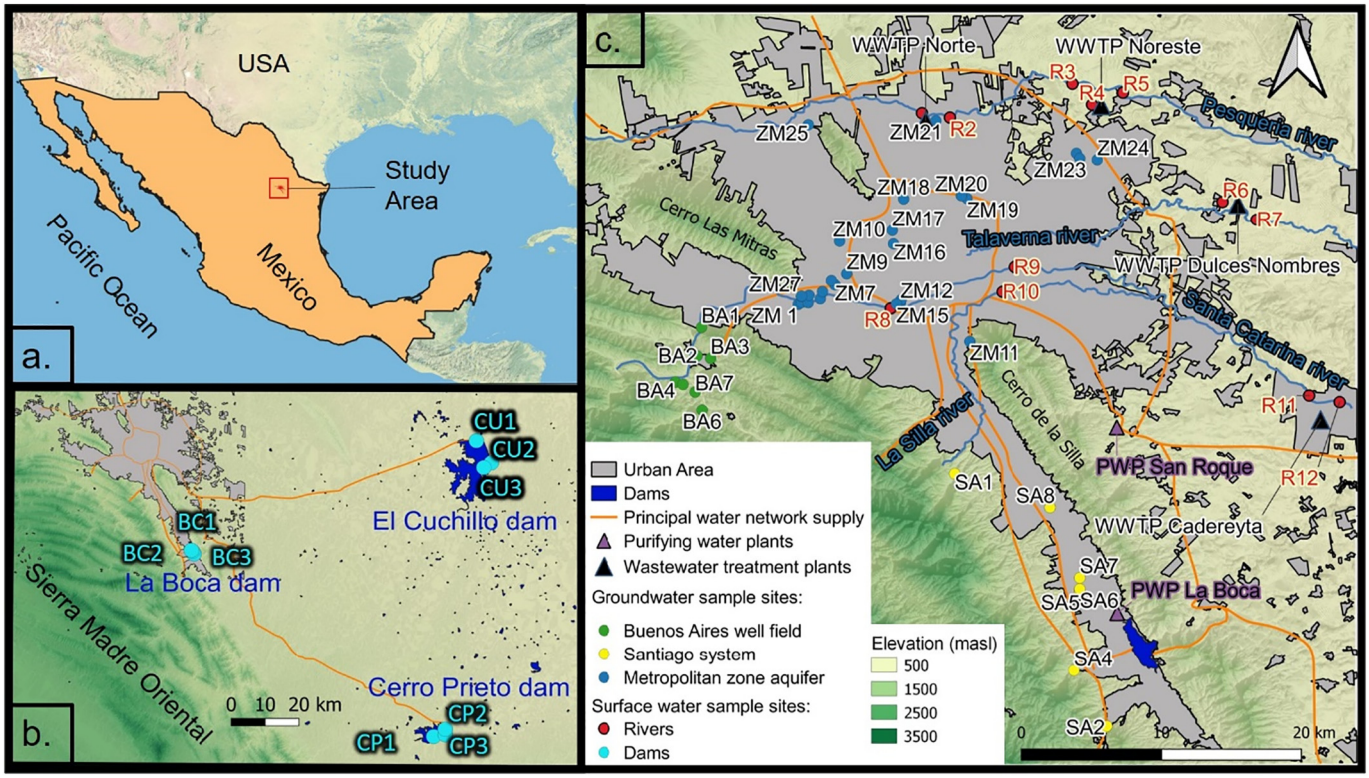

Fig. 1. (a) Location of the study area; (b) regional view showing surface-water reservoirs with sampling points El Cuchillo (CU), Cerro Prieto (CP) and La Boca (BC) and (c) the urban area with the main features and sampling points of the groundwater systems Buenos Aires (BA), Santiago (SA) and Metropolitan zone (ZM), and urban rivers (R).

Used domestic water is over 90% treated by public wastewater facilities that include primary and secondary stages in the treatment process. The most important wastewater treatment plants (WWTPs) are Dulces Nombres (7.5 m^3/s), Norte (4.0 m^3/s), Noreste (1.9 m^3/s) and Cadereyta (0.25 m^3/s) (Fig. 1**c**). All the noted WWTPs discharge the treated water directly or indirectly to the Pesquería River, except for the Cadereyta WWTP that discharges treated wastewater into the Santa Catarina River. Both rivers are tributaries of the San Juan River which in turn flows into the Rio Bravo/Rio Grande. The Pesquería and Santa Catarina Rivers had discharges that decreased from 5.5 to 4.4 m^3/s at the Pesquería hydrometric station and from 5.6 to 2.5 m^3/s at the Cadereyta hydrometric station between October 2020 and December 2020, respectively (SMN, 2020).

2.2. Field methods

Groundwater and surface water grab samples were collected at different sites and on different dates between October 2020 and January 2021. For the groundwater, 42 sites corresponding to production wells of supplying aquifer units of Monterrey (BA well field, ST system and ZM aquifer) were sampled initially between October 29 and November 3, 2020. Of these wells, 37 wells were public drinking water supply wells and five wells were used for industrial purposes. A subset of wells ($n = 10$) was resampled two more times in cycles of approximately one month to observe changes over time (Table 1).

Similarly, samples were obtained from three sites of three surface water reservoirs supplying Monterrey (El Cuchillo, Cerro Prieto and La Boca) on October 22–23, 2020, and sampling was repeated two more times. Finally, a total of 12 river water grab samples were taken along the three urban rivers Pesquería, Santa Catarina and La Silla on December 10–11, 2020, and his process was repeated on January 5–6, 2021. The river sites were selected strategically upstream and downstream of WWTP discharge into the rivers. For reference, 24-h composite samples of influent of a Dulces Nombres WWTP were taken weekly during the same period.

All samples were collected in sterile 125 ml HDPE bottles, stored at 4 °C and analyzed within 48 h. SARS-CoV-2 is highly stable at 4 °C (Chin et al., 2020). Groundwater samples were included for analysis of sucralose, using 125 ml HDPE bottles.

2.3. Laboratory methods

2.3.1. RNA and DNA extraction – QIAamp® viral RNA mini

We followed standard procedures to extract and purify nucleic acids from the water samples. Briefly, after viral thermal inactivation (95 °C; 5 min), a volume of 500 μl of the water sample was centrifuged for 10 min at 1500 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 mix was vortexed for 15 s, incubated at

Table 1
Summary of sampling campaigns.

Freshwater type	Environments	Samples (first campaign)	Samples (second campaign)
Groundwater	Buenos aires well field, Santiago system, Zona Metropolitana aquifer	40	10
Rivers	Pesquería River, Santa Catarina River, La Silla River	12	12
Surface water reservoirs	El Cuchillo dam, Cerro Prieto dam, La Boca dam	7	9

room temperature (15–25 °C) for 10 min and briefly centrifuged to remove drops from the interior surface of the lid. A volume of 560 μl of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 s.

After mixing, the tube was briefly centrifuged to remove drops from the interior surface of the lid. Then, this solution (~630 μl) was filtered through a QIAamp Mini column (Qiagen, USA) to retain the nucleic acids originally present in the sample. The retained material was repeatedly washed with different buffer solutions to elute contaminants and purify the nucleic acids. Then, 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 ×*g* (8000 rpm) for 1 min.

After centrifugation, the QIAamp Mini column was placed into a clean 2 ml collection tube, and the filtrate was discarded. In the first raising 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 centrifuged at 6000 ×*g* (8000 rpm) for 1 min. Following these two centrifugation stages, 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 ×*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 ×g; 14,000 rpm) for 3 min.

Then, 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 ×g; 8000 rpm) for 1 min.

For the DNA extraction, 500 μl of the water sample was centrifuged for 5 min at 5000 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 remainder of the extraction protocol was analogous to that previously described.

2.3.2. RNA and DNA amplification

We amplified RNA segments of SARS-CoV-2 using two sets of primers (commonly referred to as N1 and N2) in each amplification reaction. Both of these primers were directed to sequences that encode the N protein of SARS-CoV-2. These primer sets have been recommended and extensively used for the diagnosis of COVID-19 in human samples (González-González et al., 2020; Nalla et al., 2020) and wastewater (Medema et al., 2020; Wu et al., 2020b; Nemudryi et al., 2020; Randazzo et al., 2020; Haramoto et al., 2020; Sherchan et al., 2020; Peccia et al., 2020).

Similarly, we used two sets of primers to amplify the LAC and LAM regions of the genome of *Escherichia coli* in the same reaction. *E. coli* is used as a biological indicator of the presence of fecal content in water (Bej et al., 1990; Mo et al., 2002; Reza et al., 2014). The sequences of both the forward and reverse primers used are shown in **Table S1**.

Quantitative amplification was conducted in a quantitative PCR thermal cycle (Rotor gene Q 5plex, Qiagen, Germany). 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. For the amplification of DNA sequences of *E. coli*, the amplification mix (final volume of 20 μl) consisted in 10 μl of 2× QuantiNova Syber Green RT-Master Mix, 1 μl of 10× primer mix (0.5 μM final concentration), and 9.0 μl of DNA 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 (copies/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 to 100,000 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., 2021). The estimated lower limit of detection was ~1 copy of the N gene of SARS-CoV-2 per ml of water. The lowest positive value was 2.5 copies/ml.

2.3.3. Sucralose quantification

Sucralose is used as an artificial sweetener and useful tracer to demonstrate the presence of human wastewater in groundwater (Kokotou et al., 2012; Voss et al., 2019). Sucralose presence was determined using high performance liquid chromatography and mass spectrometric detection (HPLC-MS/MS) after solid-phase extraction (SOE). Isotope-labeled internal standards and an external calibration in tap water were used for quantification. Details of the analytical method are given in Table S2. The analysis was performed at DVGW-Technologiezentrum Wasser, Karlsruhe, Germany.

2.4. Monitoring of COVID-19 cases in Monterrey metropolitan area

To obtain an indication of the sensitivity of the monitoring of the urban water cycle, a proxy for the period prevalence of COVID-19 in the MMA was created using the reported number of COVID-19 cases per day (CONACyT, 2021) and the normalized cumulative number of reported COVID-19 cases per day for 2020. Normalization was performed by dividing the cumulative number of reported cases by the population size.

3. Results

3.1. Reported cases

The number of reported COVID-19 cases in each of the 12 municipalities and MMA shows that the pandemic evolved at different rates in each of the municipalities as it spread during 2020 (Fig. 2**a**). The first infection was reported on March 10, and the number of cases remained relatively low until mid-May, when another increase occurred, and starting from June 10, the infection maintained a constant increase in the MMA, with the exception of November, when the number of cases dropped. Santiago and Monterrey municipalities reported the most cases, followed by Santa Catarina, Guadalupe and San Nicolas.

However, it is worth to noting that these numbers are not directly comparable to other countries or regions because the collection methods are not necessarily standardized, and the sampling efforts are probably different from and asynchronized respect to the real infection dates (Sims and Kasprzyk-Hordern, 2020). Freshwater sampling for this report was performed during the second peak of the outbreak of the epidemic: end of October, end of November 2020, mid-December 2020 and beginning January 2021 (Fig. 2 **ab**).

3.2. Groundwater

Two field campaigns were performed for groundwater. Regarding the first campaign, the qRT–PCR concentration threshold (Ct) average values for SARS-CoV-2 ranged from 30.2 to over 40 (Table 2). Interestingly, nearly half of the samples (19 of 40) were positive, and 38% of the samples that tested positive had Ct values below the value of 33. In this study, a sample was arbitrarily defined as “positive” when a Ct

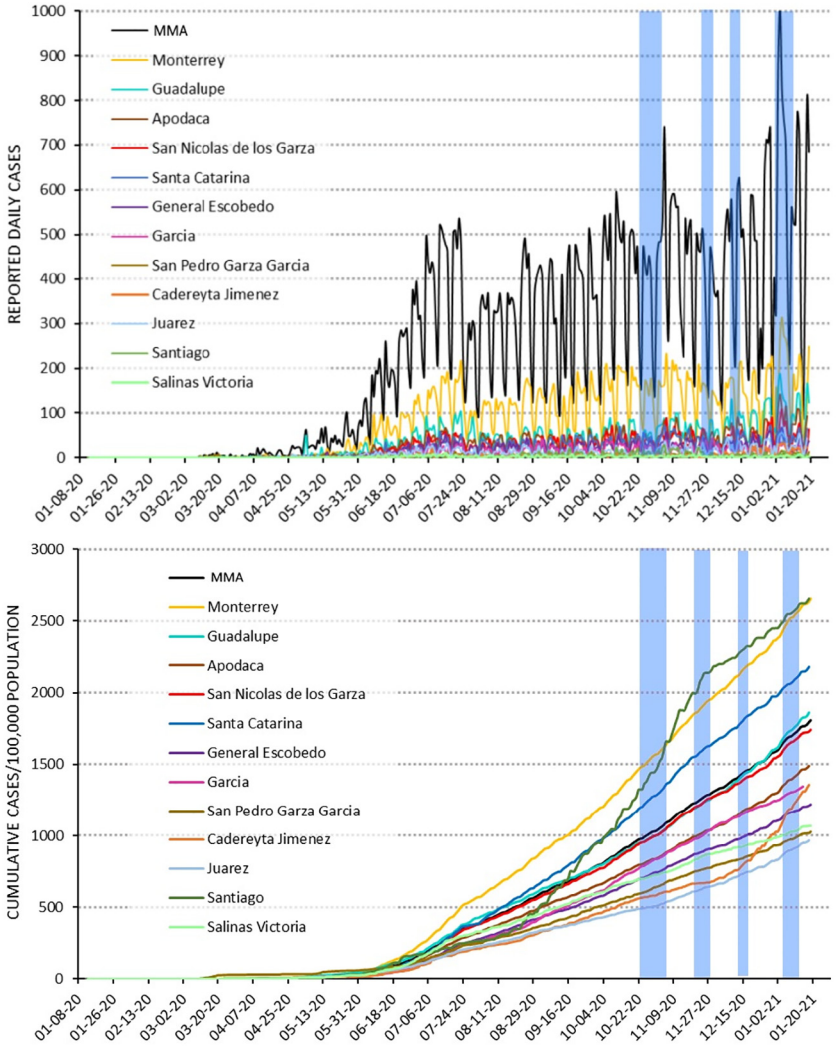

Fig. 2. Reported cases for the MMA and its 12 municipalities: (a) reported daily cases of infection; and (b) normalized cumulative cases. Note: Date retrieved from CONACyT (2021). The vertical shaded blue lines indicate the sampling periods.

value was detected in at least two of three replicates. Two of seven samples in the BA well field were detected positive, with Ct values of 30.2 and 32.4. Galeria 4 is a well at the entrance to the Huasteca highway, with a high urban development in the area prior to the entrance, while Pozo 39 is in the lower area of the Sierra Madre close to ranches and houses. Five out of eight samples in the SA system were reported to be positive, with a Ct value between 32.5 and 36.3. Estanzuela is in a woodland-rural environment, while Cola de Caballo Tunnels and San Francisco Tunnel represent horizontal galleries in piedmont shrubland. Finally, Margarita is a well located in an urban development area. Thirteen out of 26 production wells in the ZM aquifer had positive samples, with Ct values between 30.3 and 34.2. These sites are dispersed in the urbanized MMA. A trend showed a higher proportion of sites affected in the downstream area in the northeastern portion (Apodaca), and no positive samples in the southeastern portion (Contry) of the ZM aquifer.

Sucralose was detected in 22 out of 40 samples (55%) (Table 2), and its concentrations varied between 0.07 and 2.9 μg/l. In the BA well field, which represents desert and piedmont shrubland with a low population density, none of the samples had detectable levels. In the SA system, one site (Andares) had concentrations of sucralose close to the detection limit, and one site (Margaritas) had a sample with one of the highest concentrations. These sites represented residential areas. In the urbanized ZM aquifer, 20 out of 25 well sites (80%) had detectable concentrations of sucralose, whose values ranged between 0.1 and 2.7 μg/l. These results are generally consistent with the land use distribution, and all except one site in urbanized or industrial plots had samples with sucralose. In addition, we found a significant correlation between sucralose and Ct values ($r^2 = 0.62$, $p = 0.043$) but no correlation between Ct values and groundwater depth.

The samples that were positive in the first sampling campaign and not located close to each other were repeated for a second campaign (Table 2). In the second sampling campaign only 3 out of 10 sites tested positive for SARS-CoV-2. This suggests that groundwater was less affected on the second sampling date, and only three sites had samples that were consistently positive on both dates, namely, California 2, Lincoln 2 and Puentes 1 in Monterrey municipality. It is notable that the depth-to-water table of these sites was less than 22 m.

3.3. Surface water

Two sampling campaigns were performed in surface water reservoirs between the end of October and mid-December 2020 (Table 3). For the first period in October 2020 none of the samples were detected positive. For the second sampling period two sites had samples that tested positive, one in the La Boca dam (33.8) and another in the Cerro Prieto dam (33.6). It was not possible to analyze the correlation between the Ct values of SARS-CoV-2 and *E. coli* because only two pairs had quantitative data.

Table 2
Summary of the results of determination of SARS-CoV-2 RNA and sucralose presence in groundwater in Monterrey. Note: The Ct value represents the average of triplicate analysis for each sample (Tables S3 and S4), 'n.d.' indicates not detected and '-' indicates not measured.

Code	Site	Municipality	Geology	Land use		Campaign 1		Campaign 2
					Groundwater level	29 Oct–4 Nov 2020		26–30 Nov 2020
					(m below ground)	Ct	Sucralose	Ct
						(cycles)	(μg/l)	(cycles)
BA1	Galeria 4	Santa Catarina	Limestone	Urban Area	0.0	30.2	n.d.	n.d.
BA2	Pozo 39	Santa Catarina	Limestone	Desert Shrubland	43.0	32.3	n.d.	n.d.
BA3	Pozo 28	Santa Catarina	Limestone	Desert Shrubland	43.0	n.d.	n.d.	–
BA4	Pozo 1	Santa Catarina	Alluvial Deposits	Piedmont Shrubland	40.7	n.d.	n.d.	–
BA5	Pozo 4	Santa Catarina	Alluvial Deposits	Piedmont Shrubland	43.1	n.d.	n.d.	–
BA6	Pozo 14	Santa Catarina	Alluvial Deposits	Desert Shrubland	75.0	n.d.	n.d.	–
BA7	Pozo 2	Santa Catarina	Alluvial Deposits	Desert Shrubland	0.0	n.d.	n.d.	–
SA1	Estanzuela	Santiago	Shale	Urban Area	0.0	32.5	n.d.	–
SA2	Tunel 1 Cola de Caballo	Santiago	Limestone	Piedmont Shrubland	0.0	32.6	n.d.	–
SA3	Tunel 2 Cola de Caballo	Santiago	Shale	Mixed woodland	0.0	33.9	n.d.	–
SA4	Tunel San Francisco	Santiago	Shale	Piedmont Shrubland	0.0	32.8	n.d.	–
SA5	Andares	Santiago	Alluvial Deposits	Urban Area	11.8	n.d.	0.07	–
SA6	Condado de Asturias	Santiago	Alluvial Deposits	Urban Area	12.3	n.d.	n.d.	–
SA7	Pozo Rodriguez	Santiago	Alluvial Deposits	Urban Area	15.9	n.d.	n.d.	–
SA8	Pozo Margaritas	Santiago	Alluvial Deposits	Urban Area	11.3	36.3	2.90	–
ZM1	Auditorio San Pedro	San Pedro	Alluvial Deposits	Urban Area	21.2	30.5	0.54	n.d.
ZM2	Humberto Lobo	San Pedro	Alluvial Deposits	Urban Area	14.4	n.d.	1.80	–
ZM3	Suchiate II	San Pedro	Alluvial Deposits	Urban Area	10.9	n.d.	0.51	–
ZM4	Pozo Profundo Monterrey I	Monterrey	Alluvial Deposits	Urban Area	20.9	30.3	n.d.	–
ZM5	Pozo Profundo Monterrey II	Monterrey	Alluvial Deposits	Urban Area	20.1	31.2	n.d.	n.d.
ZM6	San Jerónimo II	Monterrey	Alluvial Deposits	Urban Area	26.1	n.d.	2.70	–
ZM7	Pozo Profundo Monterrey III	Monterrey	Alluvial Deposits	Urban Area	17.4	n.d.	n.d.	–
ZM8	Pozo Profundo Monterrey VI	Monterrey	Shale	Urban Area	9.8	n.d.	n.d.	–
ZM9	Hospital Civil Norte	Monterrey	Alluvial Deposits	Urban Area	115.0	n.d.	1.20	–
ZM10	Lincoln II	Monterrey	Alluvial Deposits	Urban Area	22.2	33.2	0.67	34.0
ZM11	Monterrey V	Guadalupe	Limestone	Urban Area	69.1	n.d.	n.d.	–
ZM12	Metro Rey Oriente	Monterrey	Alluvial Deposits	Urban Area	6.2	n.d.	0.44	–
ZM13	Metro Rey Poniente	Monterrey	Alluvial Deposits	Urban Area	7.9	n.d.	0.46	–
ZM14	Macro Plaza II	Monterrey	Alluvial Deposits	Urban Area	3.9	31.5	0.46	n.d.
ZM15	Plaza Hidalgo	Monterrey	Alluvial Deposits	Urban Area	12.3	34.2	0.46	–
ZM16	Somero California II	San Nicolás	Alluvial Deposits	Urban Area	14.8	30.8	1.00	33.1
ZM17	Estadio Beisbol	San Nicolás	Alluvial Deposits	Mixed woodland	14.2	n.d.	0.43	–
ZM18	Somero El Roble	San Nicolás	Alluvial Deposits	Urban Area	22.9	n.d.	0.49	–
ZM19	Somero Puentes Avenida	San Nicolás	Alluvial Deposits	Urban Area	44.0	n.d.	1.40	–
ZM20	Somero Puentes II	San Nicolás	Alluvial Deposits	Urban Area	11.2	31.5	1.30	33.1
ZM21	Tecno Centro I	San Nicolás	Conglomerate	Urban/Industrial	10.3	30.7	0.77	–
ZM22	Papa 02	Apodaca	Alluvial Deposits	Urban	13.3	30.9	0.20	n.d.
ZM23	Papa 03	Apodaca	Alluvial Deposits	Urban	13.5	30.7	0.16	n.d.
ZM24	Pozo PIMSA II	Apodaca	Alluvial Deposits	Urban/Industrial	7.4	n.d.	0.97	–
ZM25	Topo Chico III	Monterrey	Limestone	Urban Area	25.8	33.0	0.10	–

With respect to river water, two sampling campaigns were performed in December 2020 and in January 2021. In December, three out of twelve samples tested positive, with Ct values ranging from 32.7 to 34.2. The sites with positive values were the Pesqueria River downstream of WWTP Norte, Santa Catarina River upstream of WWTP Cadereyta, and La Silla River upstream of Tolteca Park. For the second sampling period, two out of twelve samples were positive, namely, the Pesquería River upstream WWTP Norte and La Silla River at upstream of Tolteca Park (Table 4). The result for La Silla River was notable because this river receives no treated wastewaters of domestic origin. The Ct values of SARS-CoV-2 correlated with those of *E. coli* ($r^2 = 0.75$, $p = 0.088$); however the correlation was weak due to the low number of pairs.

Table 3
Results of determination of SARS-CoV-2 RNA and *E. coli* presence in surface water reservoirs. Note: The Ct value represents the average of triplicate analysis for each sample (**Table S5**), 'n.d.' indicates not detected, and '-' indicates not measured.

ID	Site	Campaign 1		Campaign 2	
		22–23 Oct 2020		14–15 Dec 2020	
		Ct (SARS-CoV-2)	Ct (*E. coli*)	Ct (SARS-CoV-2)	Ct (*E. coli*)
		(cycles)	(cycles)	(cycles)	(cycles)
BO1	La Boca 1	n.d.	31.3	n.d.	34.5
BO2	La Boca 2	–	–	n.d.	33.9
BO3	La Boca 3	–	–	33.8	32.4
CP1	Cerro Prieto 1	n.d.	29.7	n.d.	32.5
CP2	Cerro Prieto 2	n.d.	30.6	n.d.	32.2
CP3	Cerro Prieto 3	n.d.	30.7	33.6	33.2
CU1	El Cuchillo 1	n.d.	31.5	n.d.	31.5
CU2	El Cuchillo 2	n.d.	30.8	n.d.	31.4
CU3	El Cuchillo 3	n.d.	30.8	n.d.	33.0

3.4. Wastewater

For reference, untreated wastewater from the influent of the Dulces Nombres WWTP was measured for SARS-CoV-2. Between October 25, 2020, and December 13, 2020, 3 out of 8 samples (38%) were positive. The Ct value of positive samples ranged from 23.5 to 31.2 (**Table S7**).

4. Discussion

4.1. Contextualization of the findings in freshwater environments

This is the first study that quantifies the presence of SARS-CoV-2 in different freshwater environments of an urban setting. Previous studies

Table 4
Results of the determination of SARS-CoV-2 RNA presence in rivers in the MMA. Note: The Ct value represents the average of triplicate analysis for each sample (**Table S6**) and 'n.d.' means not detected.

ID	Site	Campaign 1		Campaign 2	
		10–11 Dec 2020		5–6 Jan 2021	
		Ct SARS-CoV-2 (cycles)	Ct *E. coli* (cycles)	Ct SARS-CoV-2 (cycles)	Ct *E. coli* (cycles)
R1	Pesquería River upstream WWTP Norte	n.d.	31.7	35.9	33.8
R2	Pesquería River downstream WWTP Norte	34.2	28.9	n.d.	31.5
R3	Channel upstream WWTP Noreste	n.d.	31.0	n.d.	31.0
R4	Pesquería River upstream WWTP Noreste	n.d.	30.9	n.d.	30.9
R5	Pesquería River downstream WWTP Noreste	n.d.	31.4	n.d.	31.4
R6	Channel upstream WWTP Dulces Nombres	n.d.	29.5	n.d.	29.5
R7	Channel downstream WWTP Dulces Nombres	n.d.	29.1	n.d.	29.1
R8	Santa Catarina River downtown	n.d.	32.5	n.d.	32.5
R9	Santa Catarina River after downtown	n.d.	33.1	n.d.	33.1
R10	La Silla River	33.9	32.2	37.5	34.5
R11	Santa Catarina River upstream WWTP Cadereyta	32.7	29.4	n.d.	31.1
R12	Santa Catarina River downstream WWTP Cadereyta	n.d.	30.4	n.d.	30.4

that aimed to detect the virus in freshwater focused on receiving rivers (Table 5). For example, Rimoldi et al. (2020) collected grab samples at three sites of receptor rivers in the Milan area on April 14 and 22, 2020. In the first sampling round, all three samples were positive, while in the second round only one out of three samples was positive. A quantitative analysis was not performed. Similarly, Haramoto et al. (2020) collected grab water samples in a river in Yamanashi Prefecture, Japan, on three different occasions between April 22 and May 7, 2020; they reported that no sample tested positive for SARS-CoV-2 RNA.

Guerrero-Latorre et al. (2020) reported viral loads during a peak of the outbreak (June 5, 2020) from three different sites of a river receiving untreated sewage from Quito city. The authors used RT-qPCR for these determinations and two different primer sets, namely N1 and N2. All samples were found to be positive, and the values ranged from 284 to 3190 GC/ml and from 207 to 2230 GC/ml in assays using the N1 and N2 target regions, respectively. These values could be related clearly to COVID-19 cases reported in the contributing areas.

4.2. Explanation of viral loads in receiving waters

In the present study, 13% of all river water samples (3 out of 24) were positive regarding viral RNA, and the viral RNA amounts in the positive samples varied between 2.5 and 7.0 GC/ml (Fig. 3ab). Importantly, during this period no significant rainfall was recorded in the Monterrey area that could have had an impact on virus concentration in the water (**Tables S8** and **S9**). These loads are two to three orders of magnitude lower than those reported by Guerrero-Latorre et al. (2020) for Quito's river. This could be because Monterrey treats more than 95% of its municipal wastewater, while the urban rivers of Quito are impacted by the direct discharge of sewage water from the city (3 million inhabitants). Similarly, the negative results derived from the analysis of river water samples from Yamanashi Prefecture, Japan (Haramoto et al., 2020) and Milan, Italy (Rimoldi et al., 2020) could be attributed to the fact that both studies collected water from rivers receiving treated wastewater.

It is expected that wastewater from WWTPs that is completely treated would test negative. Thus, the occurrence of SARS-CoV-2 RNA in a few samples in La Silla and Pesquería River water could stem from different wastewater sources coexisting in the same basin. For example, aliquots of untreated sewage can be present because of illegal discharges, local malfunctions of sewerage systems, and their increased relative contribution during dry periods (Mosley, 2015). The lack of separation of urban runoff water from the domestic effluents, which causes combined sewer overflows (CSOs), could also be a reason for this occurrence of viral loads (Rimoldi et al., 2020). CSOs occur usually during high rainfall events. However, the accumulated rainfall between December 2020 and January 2021 in Monterrey was only 3 mm.

Another reason for the high aliquots of untreated sewage in river water could be the organization of local football derbies, whose high loads in short time periods may overburden the capacity of WWTPs to release untreated wastewater to the Pesquería river (SADM, 2020). The case of the La Silla River is notable because it receives no relevant treated municipal wastewater due to sanitary drainage to the other two rivers; therefore illegal discharges or a local sewage system malfunction is a plausible explanation for the presence of SARS-CoV-2 genetic material in this water course.

Regarding dam water, only 12% of the samples (2 out of 16) tested positive for SARS-CoV-2 RNA (Fig. 3a), with no positive result in the first campaign (22–23 October 2020). The positive samples (which contained 3.3 and 3.8 viral copies/ml) occurred during the second campaign (14–15 December 2020) and only at one site in the La Boca and at one site in the Cerro Prieto dam, respectively. In both cases, a village is located nearby, which suggests that the presence of the virus might be due to failure of the local sewage system. The observed values were comparable to the range of values in the urban rivers in Monterrey. The lack of viral loads in the first campaign and the presence of viral loads at two of the nine sites in the second campaign may reflect the increasing trend in reported cases of infection in the corresponding municipalities during the same period (Fig. 1**a**).

4.3. Viral load in groundwater reaffirms human sewage impact

The number of groundwater samples containing detectable SARS-CoV-2 RNA was surprisingly high. Twenty-two out of 50 samples (44%) had viral loads between 2.9 and 38.3 GC/ml (Fig. 3**a**). This finding suggests that a fraction of untreated sewage entered the groundwater system. The origin of the untreated sewage may have been from the surface or from a leaky sewage system. Torres-Martínez et al. (2020) used isotopic and chemical evidence to determine that nitrate pollution in groundwater from Monterrey was mainly derived from sewage leaks in urban areas. It is evident that organic and viral loads could have entered the groundwater system using the same pathway. The significant correlation between SARS-CoV-2 concentrations and sucralose at the 0.05 level is another remarkable confirmation of the contribution of raw wastewater to the groundwater and reaffirms possible leaching and infiltration of effluents from health care facilities, sewage, solid landfills, and drainage water as well as failing sewage pipes in the MMA (Fig. 3**c**).

From the three aquifer units used for water supply, the SA system (63%) was most affected, followed by the ZM aquifer (54%), and the BA well field (22%). Nevertheless, the viral loads observed in the wells

Table 5
Selected studies on municipal wastewater/sludge and receiving river waters. Note: 'NA' means not applied and 'WW' indicates wastewater.

Study region	Period/sampling rounds	Study object	Sample size and type	Sample storage/treatment before analysis	Genetic traces/genes analyzed	Results	Concentrations (GC/ml)	Reference
Paris (France)	5 March - 23 April 2020 / 7 rounds	raw WW from 3 WWTPs	27 grab samples (?)	4 °C; <24 h	RdRp	All samples positive between 5th March and 23rd April	50 (5th March) - 3000 (23rd April)	Wurtzer et al., 2020
Milan and Rome (Italy)	2 February - 2 April 2020 / 8 rounds	raw WW from 3 WWTPs	12 24-h composite samples	−20 °C; <24 h / thermal treated (30 min@56 °C)	ORF1ab, S	6 out of 12 samples positive in raw WW	NA	La Rosa et al., 2020a
Netherlands (different places)	5 February - 25 March 2020 / 4 rounds	WWTPs of 5 cities and 1 airport	30 24-h composite samples	Melting ice; <24 h	N1, N2, N3, E	No sample positive on 5–7 February; 3 out of 7 WW samples positive on March 4/5; 9 out 9 WW samples positive in the middle of March	5-7th February: n.d.; 4/5th March: 2.6–30 GC/ml; 14-16th March: 8–2200 GC/ml; 25th March: 26–1800 GC/ml	Medema et al., 2020
Milan and Monza (Italy)	14 and 22 April 2020	raw and treated WW from 3 WWTPs and receiving rivers	18 grab samples (8 raw WW, 4 treated WW, 6 river water)	No information	ORF1ab, N, E	Raw WW: First sampling with 3 out of 4 samples positive; second sampling with 1 out of 4 positive Treated WW: first sampling with 2 out of 2 negative; second sampling with 2 out of 2 negative River W: first sampling with 3 out of 3 positive; second sampling with 1 out of 3 positive	NA	Rimoldi et al., 2020
Southeastern Queensland (Australia)	20 March - 1 April 2020	raw WW from pumping station and 2 WWTP	9 composite samples	−80 °C; <24 h	N and confirmation via Sanger and MiSeq Illumina sequencing	2 out of 9 samples positive in raw WW	ND, 0.019 and 0.12	Ahmed et al., 2020a
Massachusetts (USA)	18–25 March 2020	raw WW from 1 WWTP	12 24-h composite samples	4 °C / 30 min@90 °C	N1, N2, N3,	10 out of 10 raw WW samples positive	57 to 303 / 21 to 506 after normalization of variations	Wu et al., 2020a, b
Israel (different cities and facilities)	10 March - 21 April 2020	raw WW from different WWTPs	32 24-h composite samples (6 Tel Aviv, 26 different cities)	−80 °C or − 20 °C	E	3 out of 6 WW samples in Tel Aviv positive; 2 out of 15 positive in different cities in March 2020; 8 out of 11 positive in different cities in April 2020; several cities demonstrate a correlation of the Ct values with dynamic of outbreak	NA	Bar-Or et al., 2020
Bozeman, Montana (USA)	30 March - 12 June 2020	raw WW from 1 WWTP	17 24-h composite samples	No information	N1, N2	13 out of 17 positive (1 out of 1 positive in March; 7 out of 7 positive in April; 0 out of 4 positive in May; 5 out of 5 positive in June)	ND to 5600	Nemudryi et al., 2020
Istanbul (Turkey)	8 April 2020 (?)	raw WW from 7 WWTPs and 2 manholes near hospitals	9 samples	No information	RdRp	9 out of 9 sludge samples positive	ND to 18 in WWTPs; 45 and 93 in manholes	Kocamemi et al., 2020
Murcia region (Spain)	12 March - 14 April 2020	Raw and treated WW from 6 WWTPs	72 samples (42 raw, 18 secondary and 12 tertiary treated WW samples)	4 °C; <24 h	N1, N2, N3	35 out of 42 influent samples positive; 2 out of 18 secondary samples positive; None of 12 tertiary treated samples positive	ND to 5000 in raw WW	Randazzo et al., 2020
Yamanashi Prefecture (Japan)	17 March - 7 May 2020	Raw and treated WW from 1 WWTP, river water	13 samples (5 raw and 5 treated samples from 5 rounds, 3 river samples from 3 rounds)	ice; <6 h	N1, N2	None of 5 raw samples positive; 1 out of 5 secondary treated samples positive; none out of 3 river water samples positive	240 (1 treated WW sample)	Haramoto et al., 2020
Ahmedabad, Gujara (India)	8–27 May 2020; 2 rounds	Raw and treated WW from 1 WWTP	4 composite samples (2 raw and 2 treated WW)	4 °C; 19 days for first sampling campaign, < 24 h	ORF1ab, N and S	2 out of 2 influent samples positive; 2 out 2 effluent samples negative	<0.35	Kumar et al., 2020

				for second campaign				
Louisiana (USA)	13 January - 29 April 2020	Raw WW, secondary treated WW and chlorine disinfected WW from 2 WWTP	15 samples (9 composite samples and 6 grab samples: 7 influent, 4 secondary treated effluent, 4 chlorine-disinfected effluent)	−80 °C; <4 months	N1 and N2	2 out of 15 raw wastewater samples positive; all effluent samples negative	ND; 3.1 and 4.3 (raw)	Sherchan et al., 2020
Quito (Ecuador)	5 June 2020	River water	3 samples from 3 locations	4 °C; <6 h	N1 and N2	3 out of 3 river water samples positive	284 to 3190 (N1)	Guerrero-Latorre et al., 2020
North-Rhine Westphalia (Germany)	8 April 2020	Raw and treated WW	13 samples (9 inflow, 2 secondary treated efluents, 2 desinfected effluent)	No information	N, M, E, RdRp	All samples positive	3.0 and 20 (untreated sewage); 2.7 to 37 (treated sewage)	Westhaus et al., 2021
Montpellier (France)	7 May - 20 June 2020 / 7 rounds	Raw WW from 1 WWTP	7 24-h composite samples	4 °C; immediately	N1, N3, Ebo Std	All samples positive	200–4000 (aprox.)	Trottier et al. 2020
Czech Repulic (different places)	April to June 2020	Raw WW from 33 WWTPs	112 24-h composite samples	5 ± 3 °C; <48 h		13 out of 112 positive (12%)	NA	Mlejnkova et al., 2020
Onondaga County, NY (USA)	6–13 May 2020 / 2 rounds	11 access points of WW facilities	22 24-h composite samples	4 °C; <24 h		18 out of 22 positive; 13 out of 22 in quantifiable range	<112	Green et al., 2020
Pakistan (different places)	20 March - 28 April 2020	Raw WW from 38 open drains and pumping stations	78 grab samples	< 48 h	ORF1ab, N, E	21 out of 78 samples (27%) positive	NA	Sharif et al., 2020
Ourense (Spain)	6–21 April 2020	Raw and treated WW from 1 WWTP	39 24-h composite samples (15 WW and 24 sludge)	NA	RdRP, N, E	Influent systematically positive; none of treated WW positive; primary and secondary sludge mainly positive	Influent: 7.5–15; Primary sludge: 10–40; biological sludge 7.5–10	Balboa et al., 2020
Prefectures of Ishikawa and Toyama (Japan)	5 March - 29 May 2020	Raw WW from 5 WWTPs	45 grab samples	−80 °C (initial samples) & ice / <72 h	N2, N3, NIID	7 out of 45 positive in ≥2 assays	120–350	Hata et al., 2021
New Haven, Connecticut (USA)	19 March – 1 June 2020	Primary sewage sludge from 1 WWTP	73 samples	−80 °C	N1, N2	All samples positive	Primary sludge: 17000–460,000	Peccia et al., 2020
Lahore (Pakistan)	13–25 July 2020	Untreated WW from 2 sites	28 samples	Ice cooled / 4 °C < 24 h	ORF1ab, N	22 out of 28 positive	0.267–36	Yaqub et al., 2020
Chennai (India)	10 August-14 September 2020	Composite samples from 4 WWTP, 5 sewage pumping stations and hospital	17 WWTP (influent, primary sludge, effluent) and WW pumping station samples, 12 hospital WW	Ice cooled and immediate processing	N1, N2	12/12 hospital WW samples positive	Hospital WW: 425–1620	Chakraborty et al., 2021
Student dormitory at University of Arizona Campus, Tucson (USA)	24 August – 20 November 2020	Sewer manholes	319 daily and twice-per-week samples from 13 dorms	Ice cooled and immediate processing	N1, N2	99 positive samples	ND – 993	Betancourt et al., 2021
Monterrey (Mexico)	29 October - 6 January 2021	Groundwater from 3 aquifer units, 3 rivers and 3 dams, raw WW from 1 WWTP	90 freshwater samples (50 groundwater, 24 river water, 16 dam waters) and 8 24-h raw WW samples	4 °C / <48 h (5 min@95 °C)	N1, N2	22 out of 50 groundwater samples positive; 3 out of 24 river water samples positive, 2 out of 16 dam water samples positive, 3 out of 8 raw WW samples positive	Groundwater <38.3; river water <7.0; dam water <3.8; raw WW < 3600	This study

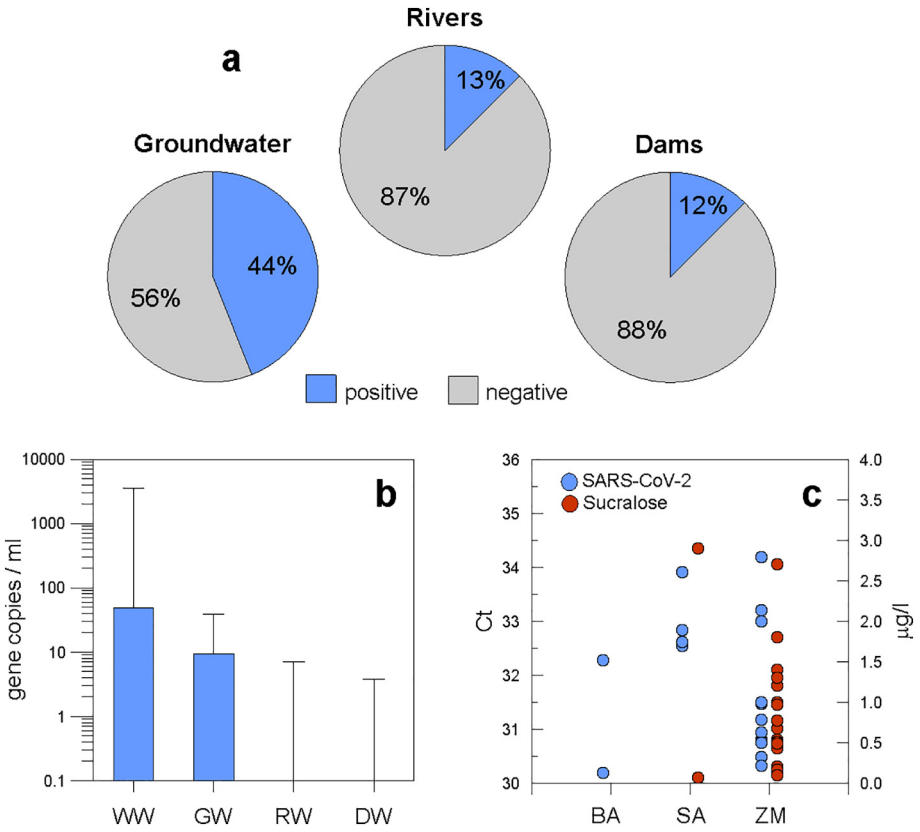

Fig. 3. (a) Share of positive and negative samples in the different freshwater environments; (b) Boxplot of viral loads of different water/wastewater types (WW = wastewater, GW = groundwater, RW = river water; DW = dam water); (c) SARS-CoV2 and sucralose scatter graph (BA = Buenos Aires, SA = Santiago; ZM = Zona Metropolitana).

of the first sampling campaign (29 October 2020–4 November 2020) were only partly reproduced one month later (26–30 November 2020), indicating a decrease in the viral load. This result demonstrates how dynamic the groundwater system is in relation to the presence of the coronavirus; the decline of the viral load in groundwater appeared to follow the decreasing trend in reported cases of infection during the month of November 2020 (Fig. 2**a**).

From the sampled municipalities in the MMA during the first campaign, Apodaca had the most positive samples at with 63% of the samples, followed by Monterrey (50%), and San Nicolas (50%). Coincidently, these are the most affected municipalities considering the officially reported daily cases of infection in Fig. 2a. Guadalupe was also among the most affected municipalities; however, it was represented by only one sampled well. Santiago, the southernmost municipality was the exception as it had a relatively lower number of cases of infection, but a high incidence of positive cases (63%). This scenario could indicate a different dynamic. Generally, the high number of positive samples in municipalities with highest number of COVID infections suggests that groundwater samples approximately mirror the infection situation at the municipality level.

4.4. Implications for public health

This study provides the first evidence that SARS-CoV-2 may enter groundwater through possible leaching events and infiltration of effluent from health care facilities, sewage, solid landfills and drainage water, as well as leakages from sewage pipes. Groundwater in the MMA is currently disinfected by gas chlorination removing pathogenic viruses and bacteria before entering the water supply system. Since coronaviruses are sensitive to oxidants such as chlorine (La Rosa et al., 2020b), it is important to continue strengthening and advancing the treatment processes of groundwater, especially in wells located in shallow aquifers and in places where sewage effluent from health care facilities, sewage, solid landfills and drainage water is not treated or treated inefficiently (Guerrero-Latorre et al., 2020) and is expected to infiltrate, or where sewage pipes could be leaky (Torres-Martínez et al., 2020).

The concentrations of SARS-CoV-2 RNA in untreated wastewater from selected studies worldwide were in the range of not detected to 5600 GC/ml (Table 5). In our study, monitoring of the influent at the Dulces Nombres WWTP showed that between October 25, 2020, and December 13, 2020, 3 out of 8 samples (38%) were positive for SARS-CoV-2, and that the maximum load was 3535 GC/ml (**Table S7;** Fig. 3**b**). This number is quite comparable to other studies of raw wastewater during outbreaks (Nemudryi et al., 2020; Randazzo et al., 2020; Trottier et al., 2020; Wurtzer et al., 2020; Medema et al., 2020; Table 5). This result shows that the concentration of SARS-CoV-2 in the surface water (<5.6 GC/ml) and groundwater (<38.3 GC/ml) in the MMA is approximately two to three orders of magnitude lower than that in raw wastewater. This means that the viral load could not be eradicated completely, as observed in Haramoto et al. (2020); however the result in this study is similar to that in Rimoldi et al. (2020).

The presence of SARS-CoV-2 genetic material in natural waters receiving treated or untreated wastewater effluents raises the important question of whether there is a risk of infection. Since urban water courses and dams are very popular places for recreation, there is concern about the risks of infection. The transmission potential of SARS-CoV-2 by ingestion is still controversial but potentially occurs (Amirian, 2020). Kumar et al. (2021) suggested a quantitative microbial risk assessment framework to estimate the potential risk from SARS-CoV-2 in natural water bodies through various water activities, based on the framework for SARS-CoV developed by Watanabe et al. (2010). The support for this approach is that there is no risk assessment model available for ingestion of water with SARS-CoV-2 and that both

SARS-CoV and SARS-CoV-2 species have similar genetics and infection mechanisms. According to this approach, the chances of infection by a virus are calculated by a dose–response model, which describes relations of viral exposure dose and the probability of infection and can be calculated by an exponential model with the following equation:

$$p\,(r/d) = 1 - \exp\left(-\frac{d}{k}\right) \quad (1)$$

where $p\,(r/d)$ is the chance of infection at the viral dose of d, d is dose of the virus (PFU, plaque-forming unit), and k is 4.2×10^2 (PFU). The expected dose of the virus is estimated from the volume of water ingested and the viral concentration in the water. The median volume of water ingested per event is reported to be 6.0 ml when swimming and 2.0 ml when fishing (Dorevitch et al., 2011). Considering a viral load of 7.0 copies/ml in the rivers of MMA (Fig. 3**b**), the estimated chance of infection per event was derived from Eq. (1) as 1.0×10^{-7} for swimming and 3.4×10^{-8} for fishing.

These findings suggest a very low risk of transmission of SARS-CoV-2 during recreation in waters receiving treated wastewater from the MMA. However, the presence of detectable amounts of genetic material from SARS-CoV-2 in fresh water should not be ignored. There exist situations where the infection risk may increase considerably. For example, the occurrence of CSO events during COVID-19 outbreaks may cause a substantial increase in the infection risk of SARS-CoV-2 by exposure to receiving water bodies (Kumar et al., 2021). Another situation is that residual chlorine may not be maintained in sufficient concentration to control the virus. Consider a fictional case where raw wastewater from the Dulces Nombres WWTP with 3535 copies/ml is discharged into the riverbed without dilution as may occur during a drought period, then the chances of infection increase to 5.2×10^{-5} and 1.7×10^{-5} for swimming and fishing, respectively.

Under normal operating conditions, the infection risk in groundwater is minimal if the pumping wells are on a well seal, which protects it from surface contamination, and the disinfection system is working properly. However, in a leaky pumping well, the infiltration of human effluent spills combined with a failure in the disinfection system may considerably increase the infection risk. Assuming a sludge concentration of 10^5 copies/ml infiltrates and is diluted 5 times in groundwater, then the immediate chance of infection from drinking a glass of untreated water is 0.15%. Therefore, an annual, preventative water-well maintenance inspection is important to avoid any risks of a COVID-19 infection through groundwater.

It is important to note that these values could be underestimated and have large uncertainty associated with them, because SARS-CoV-2 is potentially more infectious than SARS-CoV from which the model is derived (Kitajima et al., 2020). On the other hand, the proportion of viable RNA copies in the measured viral load was not known. SARS-CoV-2 RNA was found to be significantly more persistent than infectious SARS-CoV-2, indicating that the environmental detection of RNA alone does not substantiate the risk of infection (Bivins et al., 2020). Thus, this risk assessment model should be considered a preliminary estimation or base line of the associated health risks for SARS-CoV-2 in aquatic environments.

4.5. Limitations and future directions

This study shows the importance of monitoring programs to determine the fate of SARS-CoV-2 in the urban water cycle. To date, there is no evidence related to the fate of SARS-CoV-2 in the urban water cycle, and few datasets exist to confirm whether water or wastewater containing SARS-CoV-2 could be potentially infectious. Some studies have predicted a low risk of SARS-CoV-2 transmission via wastewater (Chin et al., 2020; Rimoldi et al., 2020), but this topic still deserves attention and further detailed examinations (Buonerba et al., 2021). It is necessary to monitor natural waters, especially in countries or areas that have limited capacities of wastewater treatment.

Future research should be oriented towards the development of a proper SARS-CoV-2 infection risk assessment model, considering the virus in its different variants. This model could be based on dose-response approaches developed for other pathogens (Watanabe et al., 2010; de Man et al., 2014) and use SARS-CoV-2 data sets yet to be developed from experiments.

Another area of opportunity is to study the SARS-CoV-2 removal efficiency of wastewater treatment processes including disinfection. One limitation of this study is a lack of understanding of how the removal efficiency of a WWTP contributes to the dilution of the viral load in the receiving river water. In general, there is still minimal knowledge about the removal of enveloped viruses in wastewater (Kumar et al., 2021).

The use of chemical and microbial markers for human wastewater could assist in not only evaluating the removal efficiency of wastewater treatment facilities but also understanding the routes and fates of SARS-CoV-2 in natural water systems. For biosafety purposes, surrogate viruses such as the murine hepatitis virus and phages were employed successfully due to their structural and morphological similarity to SARS-CoV-2 (e.g. Ahmed et al., 2020b). The combined use of selected markers could provide additional information about the dilution, decay, and inhibition factors of the new coronavirus in aquatic environments.

Studies performed to date show that there is a lack of standardized protocols for sampling, detecting and quantifying of SARS-CoV-2 in water and wastewater (Table 5). For example, in some studies grab samples were obtained, while in others 24-h composite samples were collected. In this study we used a sample size (125 ml) and recognize that larger samples would be a more appropriate choice and that would have derived in a more representative finding. Also, the sampling duration was relatively short.

There were significant differences in not only sample collection but also sample storage and treatment and the use (or not) of genetic or chemical traces (i.e., chemical agents indicating human activity or viral tracers used for normalization purposes). This may lead to discrepancies in the results. Currently, RT-qPCR has been employed widely for detection of SARS-CoV-2 in water samples; however it is imperative to develop a standard sampling procedure for accurate extraction, isolation, detection and quantification of the virus. The N gene (N1&N2) is the most abundant transcript of SARS-CoV-2 and is therefore a good target for the detection of the virus in samples (Babiker et al., 2020; Chakraborty et al., 2021). Inter- and intralaboratory comparisons such as those employed by Chik et al. (2021) may lead to global standardization.

5. Conclusions

This study evaluated the presence of SARS-CoV-2 RNA in different freshwater areas of a metropolitan area and the implications for the environment and public health. As such, this study represents a contribution to the ongoing discussion on the potential routes and fate of SARS-CoV-2 in freshwater environments receiving wastewater and water safety concerns.

This is the first study that detected and quantified SARS-CoV-2 RNA in groundwater. Nearly half of the samples showed detectable genetic material. This result suggests that in a pump well, sewage from the surface or from a leaky sewage system entered the groundwater system. Moreover, the temporal and submetropolitan variations in the viral loads in groundwater mimic the reported trend in cases of infection in ZMM.

The share of detectable SARS-CoV-2 RNA in urban rivers (13%) and dams (12%) was lower than that in groundwater. The quantitative results show that the viral loads in these waters were three orders of magnitude lower than the maximum value measured in raw wastewater during the same time period. It is assumed that aliquots of nontreated

sewage due to illegal discharges, local malfunctions of the sewage system and their increased relative contribution during the dry period may have been the factors. Again, there was a correlation between the temporal variation in the viral loads in the surface waters and the trend in the reported cases of infections. A preliminary risk assessment model suggests that, considering the viral loads found during this study in the receiving waters of Monterrey, the potential of infection was low for recreational activities (swimming, fishing, etc.). However, this situation should not be taken lightly because the occurrence of combined sewer overflow events and/or temporal failures of disinfection systems may cause substantial increases in infection risks.

This study shows that knowledge about the routes and fates of SARS-CoV-2 in the environment is still in the early stage and that datasets for water are scarce. In the short term, it is important to monitor especially natural water systems that receive untreated or poorly treated wastewaters. In the medium and long term, the COVID-19 pandemic represents an opportunity for the international community to accelerate the UN Sustainability Development Goal 6 (clean water and sanitation for all) by fostering financial and technical support to programs that increase the capacity of preventative water-well maintenance inspections and wastewater treatment, especially in less developed countries.

Future research and innovation efforts in this regard should be oriented towards: (i) the development of a proper SARS-CoV-2 infection risk assessment model for water and wastewater; (ii) an assessment of the removal efficiency of SARS-CoV-2 in wastewater treatment processes including disinfection; (iii) the combined use of chemical and microbiological markers for tracing the routes, decay and inhibition factors of SARS-CoV-2 in water; and (iv) the development of standardized protocols for sampling, detecting and quantifying SARS-CoV-2 in the environment.

CRediT authorship contribution statement

Herewith we state that all authors participated in the development of the manuscript. In the following an accurate and detailed description of their diverse contributions to the work:

Jürgen Mahlknecht: Conceptualization, Writing-Original draft preparation, Supervision, Funding acquisition, Project administration, Reviewing and editing.

Diego Padilla: Field work, Data curation, Investigation, Data curation, Methodology,

Edrick Ramos: Field work, Data analysis, Visualization, Study area description, Investigation.

Luisa Ramos: Laboratory analysis, Validation, Quality control.

Mario Moises Álvarez: Conceptualization, Laboratory methods, Funding acquisition, Reviewing and editing, Language editing.

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.scitotenv.2021.147183.

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