First detection of SARS-CoV-2 RNA in wastewater in North America: A study in Louisiana, USA

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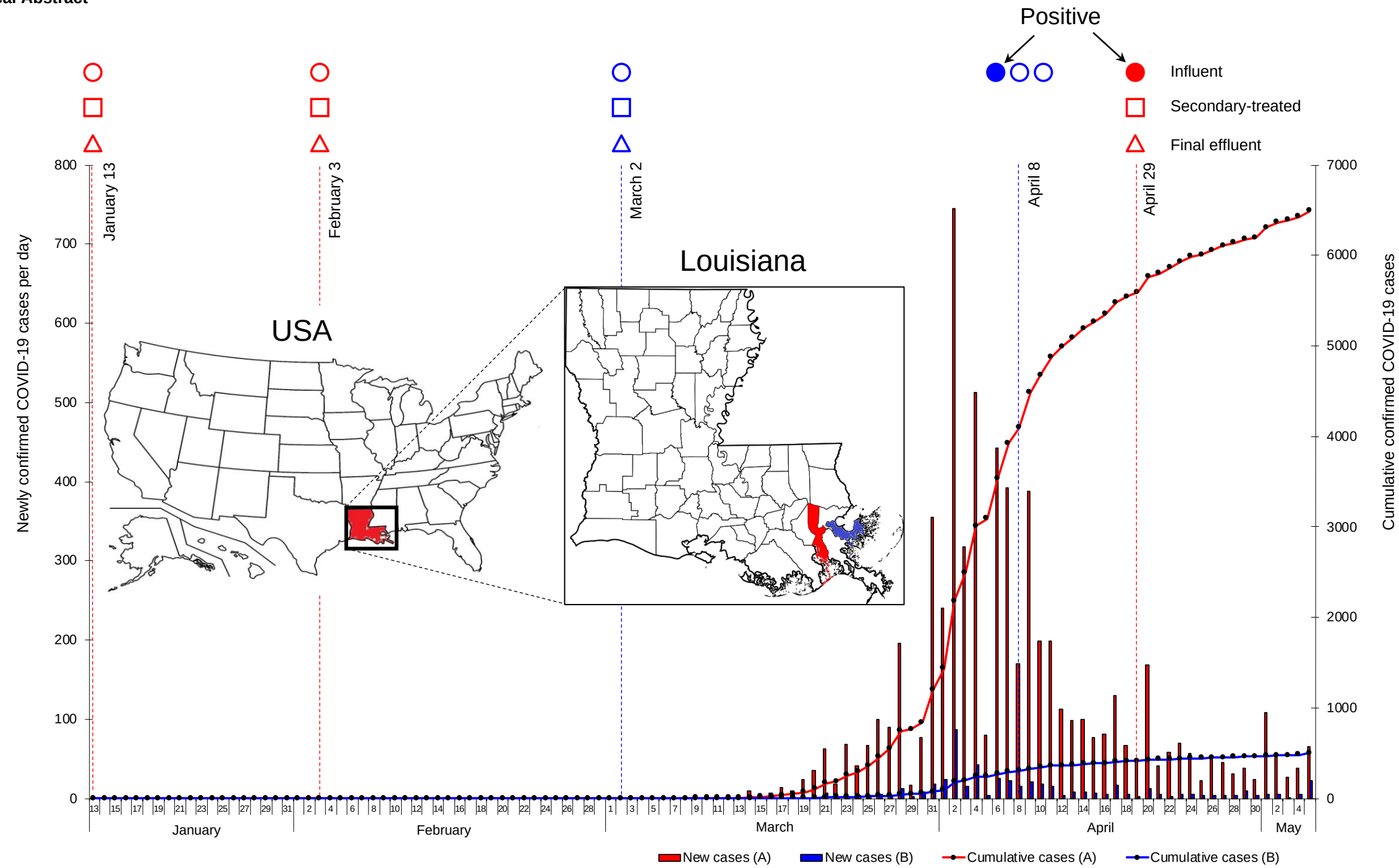
***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- First study in Louisiana, USA reporting the detection of SARS-CoV-2 RNA in wastewater using ultrafiltration.
- Two out of seven untreated wastewater samples tested positive for SARS-CoV-2 RNA.
- None of the secondary treated and final effluent samples tested positive.
- Concentration methods and RT-qPCR assays applied for SARS-CoV-2 RNA detection need further refinement.

*Graphical Abstract



Abstract

We investigated the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in wastewater samples in southern Louisiana, USA. Untreated and treated wastewater samples were collected over a four-month period from January to April 2020. The wastewater samples were concentrated via ultrafiltration (Method A), and an adsorption–elution method using electronegative membrane (Method B). SARS-CoV-2 RNA was detected in 2 out of 15 wastewater samples using two reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays (CDC N1 and N2). None of the secondary treated and final effluent samples tested positive for SARS-CoV-2 RNA. Our results suggest that wastewater-based epidemiology could be utilized to monitor the prevalence of COVID-19 in the community, and to predict disease circulation and upcoming waves. To our knowledge, this is the first study reporting the detection of SARS-CoV-2 RNA in wastewater in the USA. However, concentration methods and RT-qPCR assays need to be refined and validated to increase the sensitivity of SARS-CoV-2 RNA detection in wastewater.

Keywords: wastewater-based epidemiology, SARS-CoV-2, surveillance, COVID-19, RT-qPCR, wastewater

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the *Coronaviridae* family, emerged in Wuhan, China in December 2019 with a total of 9,473,214 confirmed cases and 484,249 deaths around the world (as of June 26, 2020) (World Health Organization, 2020). In the US, the total number of cases is 2,414,870 with 124,325deaths as of June 26 (CDC, 2020a). WHO announced an official name of the disease [coronavirus disease 2019 (COVID-19)] caused by SARS-CoV-2 and classified it as a global pandemic (WHO, 2020). Although SARS-CoV-2 is primarily respiratory in nature, studies have confirmed the viral RNA can be detected in the feces of infected individuals, even after respiratory

symptoms have subsided (Kitajima et al., 2020). State of Louisiana is heavily impacted by COVID-19 in the USA. The first case of COVID-19 was recorded on March 9, 2020 in Jefferson Parish and there have been 53,415 confirmed cases and 3,164 deaths as of June 26, 2020 (CDC, 2020a). A major annual festival, Mardi Gras, in February 2020 in New Orleans, LA, may have contributed to this surge.

Several studies have reported the detection of SARS-CoV-2 RNA in stool samples from infected individuals (Wang et al., 2020; Wu et al., 2020; Holshue et al., 2020; Xiao et al., 2020; Tang et al., 2020; To et al., 2020; Woelfel et al., 2020; Yeo et al., 2020; Harcourt et al., 2020; Zhang et al., 2020). This implies that SARS-CoV-2 may be excreted through feces and other bodily secretions, such as saliva and urine, from infected individuals, and subsequently transported to the wastewater treatment plants (WWTPs) (Kitajima et al., 2020; Maal-Bared et al., 2020).

Wastewater-based epidemiology (WBE) has been used to advance our understanding of the emergence and epidemiology of pathogenic viruses such as polioviruses and noroviruses in communities around the world (Kitajima et al., 2020). Recently, the detection of SARS-CoV-2 RNA in municipal wastewater has been reported from a number of countries including Australia (Ahmed et al., 2020a), Spain (Randazzo et al., 2020), Italy (La Rosa et al., 2020), Netherlands (Medema et al., 2020), and Japan (Haramoto et al., 2020), suggesting the applicability of WBE approach to monitor COVID-19.

One of the biggest challenges in COVID-19 WBE studies is the efficiencies of concentration and recovery of SARS-CoV-2 and detection of its RNA in wastewater (Ahmed et al., 2020b). Little is known regarding the recovery efficiency of SARS-CoV-2 from wastewater. It has been suggested that the recovery efficiency of enveloped SARS-CoV-2 may be different than that of non-enveloped enteric viruses (Kitajima et al., 2020; La Rosa et al., 2020). Recent studies have used several virus concentration methods to recover SARS-CoV-2 from wastewater. For instance, Medema et al. (2020) used 100 kDa Centricon® Plus-70 (Millipore, Amsterdam, the Netherlands) centrifugal ultrafiltration device to recover

SARS-CoV-2 from untreated wastewater in the Netherlands. Ahmed et al. (2020a) utilized the adsorption-extraction method using electronegative membrane as well as the Centricon® Plus-70 centrifugal ultrafiltration device. La Rosa et al. (2020) used a two-phase (PEG-dextran method) separation as described in the 2003 WHO Guidelines for Environmental Surveillance of poliovirus protocol and reported that 6 out of 12 wastewater samples in Italy tested positive for SARS-CoV-2. Randazzo et al. (2020) used an aluminum hydroxide adsorption-precipitation method for the detection of SARS-CoV-2 RNA in wastewaters in Spain. However, none of these studies have reported the percent recovery of SARS-CoV-2 RNA from wastewater. A recent study evaluated seven concentration methods by seeding murine hepatitis virus (MHV) in untreated wastewater samples and the mean MHV recoveries ranged from 26.7 to 65.7% for the concentration methods used with highest recovery obtained from adsorption-extraction method pre-treated with $MgCl_2$ (Ahmed et al., 2020b).

In the present study, we investigated the presence of SARS-CoV-2 RNA in wastewaters in southern Louisiana, USA using two concentration methods followed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To our knowledge, this is the first study reporting the detection of SARS-CoV-2 RNA in wastewater in North America. Our initial data suggest that WBE could be a potential tool to monitor the prevalence of COVID-19 in the community.

2. Materials and Methods

2.1. Wastewater Sample Collection

Nine composite and six grab wastewater samples were collected monthly at two wastewater treatment plants (WWTPs) (A and B), respectively located in southern Louisiana through January to April 2020. During this period, untreated wastewater (*n* = 7), secondary treated (*n* = 4), and final effluents after chlorine disinfection (*n* = 4) were collected. The population served by the WWTPs A and B were 244,627 and 45,694 respectively. Both WWTPs used conventional activated sludge followed by chlorine

disinfection. One liter of wastewater was collected for each untreated wastewater, secondary treated, and final effluents in sterile 1 L Nalgene bottles and transported on ice to the laboratory.

2.2. Concentration and nucleic acid extraction

Two virus concentration methods were used to maximize the chance of SARS-CoV-2 detection and to compare the effectiveness of the methods for SARS-CoV-2 recovery in wastewater. Method A (ultrafiltration) was performed with centrifugation of 250 mL of the sample for 30 min at 3,000 *g* to remove large particles and suspended solids. 70 mL of the 250 mL supernatant was then concentrated using the Centricon® Plus-70 centrifugal filter with a nominal molecular weight limit (NMWL) of 100 kDa (Merck Millipore; part no UFC710008) via centrifugation (1,500 *g* for 15 min). The sample was further centrifuged at 1,000 *g* for 2 min and approximately 350 µL of viral concentrate was then collected from the sample reservoir using a pipette.

Method B (adsorption–elution method using an electronegative membrane) was performed as described previously (Schmitz et al., 2016; Tandukar et al., 2020). Briefly, 2.5 M $MgCl_2$ was added to all samples (100 mL influent and 750 mL secondary treated and final effluent) to obtain a final concentration of 25 mM $MgCl_2$. Samples were subsequently passed through an electronegative filter (90-mm diameter and 0.45-µm pore size; Merck Millipore, Billerica, USA; Catalog no. HAWP-09000) attached to a glass filter holder (Advantec, Tokyo, Japan). Magnesium ions were then removed by the passage of 200 mL of 0.5 mM H_2SO_4 (pH 3.0) through the filter, and the viruses were eluted with 10 mL of 1.0 mM NaOH (pH 10.8). The eluate was recovered in a tube containing 50 µL of 100 mM H_2SO_4 and 100 µL of 100× Tris-EDTA buffer for neutralization. 10 mL was then centrifuged using a Centriprep YM-30 (Merck Millipore) containing an ultrafiltration membrane with an NMWL of 30 kDa (Merck Millipore, Billerica, MA) to obtain a final volume of approximately 650 µL.

2.3. RT-qPCR inhibition and quality control

Pseudomonas bacteriophage Φ6 (DSM 21518, DSMZ, Braunschweig, Germany) was used as a sample process control (SPC) to determine the efficiency of RNA extraction and RT-qPCR. Briefly, 2 µL of *Pseudomonas* bacteriophage Φ6 (2.0×10^5 copies/µL) was seeded into 200 µL of concentrated wastewater samples and molecular biology grade water was used as a control (i.e., no inhibition). The extraction−RT-qPCR efficiency (E) % was calculated as described previously (Schmitz et al., 2016; Tandukar et al., 2020).

2.4. Viral RNA extraction and reverse transcription (RT)

Viral RNA was extracted from the concentrated wastewater sample seeded with *Pseudomonas* bacteriophage Φ6 process control (202 µL in total) using a ZR Viral RNA Kit (Zymo Research, Irvine, USA) to obtain a final volume of 100 µL RNA according to the manufacturer's protocol. RT was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) (Schmitz et al., 2016; Tandukar et al., 2020).

2.5. RT-qPCR for SARS-CoV-2

RT-qPCR assays for SARS-CoV-2 was performed with a CFX96 Real-Time PCR Instrument (BioRad Laboratories, Hercules, CA). Reaction mixtures (25 µL) consisted of 12.5 µL of PerfecTa qPCR ToughMix (Quantabio, Beverly, MA), 100 µM primers and probe, molecular grade water, and 2.5 µL of cDNA template. CDC N1 and N2 primers and probes used in this study are shown in Table 1. The PCR condition for SARS-CoV-2 was as follows: 95 °C for 10 minutes and 45 cycles of 95 °C for 10 s and 55 °C for 30 s (CDC, 2020b). The PCR condition for *Pseudomonas* bacteriophage Φ6 was 94◦C for 3 min followed by 35 cycles of 94◦C for 15 s and 60◦C for 1 min with a plate reading after the elongation step (Gendron et al., 2010). Serial ten-fold dilutions of the standard plasmid of SARS-CoV-2 or gBlocks for *Pseudomonas* bacteriophage Φ6, obtained from IDT (Coralville, IA) were used to produce a standard curve. Molecular biology grade water was used as a non-template control. The amplification efficiencies (AE) were

calculated based on the equation: AE = $10^{(-1/slope)} - 1$. All qPCR assays were performed in duplicate while following the MIQE guidelines (Bustin et al. 2009).

3. Results

3.1 Efficiency of viral nucleic acid extraction and RT-qPCR and qPCR assay performance

Concentrated wastewater samples were seeded with *Pseudomonas* bacteriophage Φ6 as a process control to monitor RNA extraction-RT-qPCR efficiency. The mean recovery efficiency of *Pseudomonas* bacteriophage Φ6 was 92.21 ± 19.2%. The slope of the standards for Φ6, N1 and N2 assays were -3.34, -3.07 and -3.01 respectively. Y-intercept values were −41 (Φ6), -39.17 (N1), and −38.49 (N2). The correlation coefficient (R^2) values for these assays were 0.996% (N1), 0.991% (N2), and 0.999% (Φ6), respectively.

3.2 Detection of SARS-CoV-2 RNA in wastewater samples

Two of the fifteen (13%) wastewater samples tested positive with RT-qPCR assays, as shown in Table 2, and these were both untreated wastewater samples. Secondary-treated wastewater and final effluent samples tested negative for SARS-CoV-2, indicating that the virus was removed by wastewater treatment processes (Figure 1). On April 29, 2020, an untreated wastewater from WWTP A tested positive using the CDC N2 assay. Untreated wastewater samples collected on April 8, 2020 tested positive with both CDC N1 and N2 assays. Positive samples were found at mean concentrations of 3.2 $\log_{10}$ copies/L from the N1 assay and 2.5 and 3.0 $\log_{10}$ copies/L from the N2 assay. Wastewater samples processed through Method A yielded positive results, while all samples tested negative using the Method B. When the samples tested positive for SARS-CoV-2 RNA in influent, the total confirmed

number of COVID-19 cases were 6,173 and 308 in locations served by WWTPs A and B, respectively (Figure 1).

4. Discussion

Several studies have been conducted for the quantification of SARS-CoV-2 in untreated wastewater during the ongoing COVID-19 pandemic (Table 3). However, further studies are needed to assess the existing methods for concentration and recovery of viruses from wastewater with varying characteristics for the accurate detection and quantification of SARS-CoV-2 RNA in wastewater. For the assessment of RNA extraction-RT-qPCR efficiency, we used *Pseudomonas* bacteriophage Φ6. The mean recovery efficiency was quite high, indicating that there was no considerable inhibition or loss occurred during the RNA extraction and RT-qPCR. In Ye et al. (2016), the recovery of *Pseudomonas* bacteriophage Φ6 was 18.2 ± 9.5% using an optimized ultrafiltration method. Ye et al., (2016) spiked Φ6 into wastewater before concentration and quantified using plaque assay, evaluating the concentration efficiency. Medema et al. (2020) also used an ultrafiltration method (100 kDa Centricon® Plus-70 centrifugal device) and determined the recovery of F-specific RNA phages by the purification and concentration steps using plaque assay, which yielded a mean recovery efficiency of 73%. A recent study conducted by Ahmed et al. (2020b) evaluated seven different concentration methods using a surrogate coronavirus (CoV), i.e., murine hepatitis virus (MHV). The recovery efficiencies of MHV using Amicon Ultra -15 and Centricon Plus-70 ultrafiltration centrifugal devices were 56.0 ± 32.3 % and 28.0 ± 9.10 %, respectively. According to Ahmed et al. (2020b), an adsorption-extraction method with $MgCl_2$ pre-treatment was the most efficient method to concentrate MHV. However, since the present study was initiated before the results presented in Ahmed et al. (2020b) was obtained, we were unable to include the adsorption-extraction method with $MgCl_2$ pre-treatment when we designed the present study.

In the present study, we used two virus concentration methods, namely, ultrafiltration and adsorption-elution, for the detection of SARS-CoV-2 in wastewaters. Of the two methods tested, method A

187 (ultrafiltration) successfully yielded detection of SARS-CoV-2 RNA in two untreated wastewater samples,
188 while none of the secondary treated and final effluent samples tested positive for SARS-CoV-2 RNA. Our
189 findings suggest the removal of SARS-CoV-2 during wastewater treatment processes. However,
190 Randazzo et al. (2020) used an aluminum hydroxide adsorption-precipitation method and found 11% (2
191 out of 18 samples) positive in secondary treated water with at least one SARS-CoV-2 RT-qPCR assay
192 (Table 3). Another study by Haramoto et al. (2020) in Japan detected SARS-CoV-2 RNA in 20% (1/5) of
193 secondary-treated wastewater samples using N_Sarbeco RT-qPCR assay (Table 3).

194 We collected wastewater samples in four consecutive months (January 13, February 3, March 2, and
195 April 8 and 29). However, we were able to detect SARS-CoV-2 only during the month of April from both
196 WWTPs by Method A, suggesting that the performance of Method A for SARS-CoV-2 recovery in
197 wastewater is superior to that of Method B. The influent sample from WWTP A was positive using the
198 CDC N2 qPCR assay, whereas, the influent samples from WWTP B tested positive using both N1 and N2
199 assays. Medema et al. (2020) used all three CDC N1, N2, and N3 assays for the detection of SARS-CoV-2
200 RNA in wastewater samples in the Netherlands and obtained inconsistent results among the three qPCR
201 assays. A similar study in Spain observed discrepancies among the CDC assays for quantification of SARS-
202 CoV-2 RNA in untreated wastewater (Randazzo et al., 2020). This inconsistency among qPCR assay
203 results could be due to several factors including the sequences of the primers and probes, assay
204 specificity and reactivity, and low levels of SARS-CoV-2 RNA in wastewater (Ahmed et al., 2020; Li et al.,
205 2020; Randazzo et al., 2020). Several other factors may also affect the occurrence of viral pathogens in
206 wastewater, such as rainfall, temperature, hydraulic retention time, and PCR inhibitors (de Roda
207 Husman et al., 2009).

208 The concentrations of SARS-CoV-2 (2.5–3.2 $\log_{10}$ copies/L) in wastewater samples in this study was
209 higher than that reported by Ahmed et al. (2020a) in Australia (1.28–2.08 $\log_{10}$ copies/L), but lower than
210 those reported by Randazzo et al. (2020) in Spain (5.1–5.5 $\log_{10}$ copies/L) (Table 3). This could be due to

differences in abundance of SARS-CoV-2 in wastewater and methodologies for viral RNA detection including virus concentration methods, viral RNA extraction strategies, and RT-qPCR assays. Randazzo et al. (2020) used an aluminum hydroxide adsorption-precipitation method. Ahmed et al. (2020a) used the membrane adsorption-direct RNA extraction method using the electronegative membranes followed by N_Sarbeco and NIID_2019-nCOV_N RT-qPCR assays.

Epidemiological data on COVID-19 confirmed cases in the State of Louisiana have been retrieved from the USA facts (https://usafacts.org/visualizations/coronavirus-covid-19-spread-map/). The first confirmed case of COVID-19 in Louisiana was reported on March 9, 2020 (CDC, 2020a). On April 8 and 29, when the samples tested positive for SARS-CoV-2 RNA in influent, the total confirmed number of COVID-19 cases were 6,173 and 308 in locations served by WWTPs A and B, respectively. Even though we tested samples from January, we were not able to detect the viral RNA in wastewater until April 2020. This result suggests that concentrations of the viral RNA in wastewater were not detectable in wastewater until the cases started increasing in the studied area. We found no evidence for the presence of SARS-CoV-2 RNA in wastewater from Louisiana before the first COVID-19 case was reported in the community on March 9. There are some limitations of this study, which may have attributed the detection of SARS-CoV-2 RNA in wastewater in the early stage of the pandemic. For example, a small number of samples were tested from two WWTPs, and only two virus concentration methods were used. Also, some of the samples were grab samples collected at a time point when the viral RNA levels could have been low in the wastewater streams. Therefore, it seems prudent to test more wastewater samples and evaluate the performance of several other concentration methods including the adsorption-direct RNA extraction method (Ahmed et al., 2020b) and molecular assays including droplet digital PCR.

In summary, this is the first study that reports the detection of SARS-CoV-2 RNA in untreated wastewater samples in southern Louisiana, USA using ultrafiltration method. Further studies are needed

to improve the concentration methods and molecular assays for more sensitive detection of SARS-CoV-2 RNA in wastewater toward application of wastewater-based epidemiology approach for the sentinel surveillance of COVID-19 at the community level.

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Table

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Table 1: Oligonucleotide sequences of primers and probes used in this study.

Assay	Target gene	Primer/Probe	Sequence (5'–3')[a]	Reference
N1	Nucleocapsid (N)	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	CDC (2020b)
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	
		2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	
N2	Nucleocapsid (N)	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	CDC (2020b)
		2019-nCoV_N2-R	GCGCGACATTCCGAAGAA	
		2019-nCoV_N2-P	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1	
phi6 (Φ6)	phi-6S 1	phi6- F	TGGCGGCGGTCAAGAGC	Gendron et al., (2010)
		phi6- R	GGATGATTCTCCAGAAGCTGCTG	
		phi6- P	FAM/CGGTCGTCG/ZEN/CAGGTCTGACACTCGC/3IABkFQ/	

[a] FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1

Table 2: Detection of SARS-CoV-2 RNA in wastewater samples in southern Louisiana

Location	Sampling date	Sample type	RT-qPCR results (log_{10} copies/L, mean ± standard deviation)		
			Method A		Method B
			N1	N2	N1/N2
WWTP A					
	01/13/2020	Influent	-	-	-
		Secondary treated	-	-	-
		Final effluent	-	-	-
	02/03/2020	Influent	-	-	-
		Secondary treated	-	-	-
		Final effluent	-	-	-
	04/29/2020	Influent	-	2.5 ± 0.1	-
		Secondary treated	-	-	-
		Final effluent	-	-	-
WWTP B					
	03/02/2020	Influent	-	-	-
		Secondary treated	-	-	-
		Final effluent	-	-	-
	04/08/2020	Influent	3.2 ± 0.4	3.0 ± 0.3	-
		Influent	-	-	-
		Influent	-	-	-

- :Not detected.

Table 3: Currently available peer-reviewed reports on the detection of SARS-CoV-2 RNA in municipal wastewater

Sample	Sample Type	Virus concentration method	Samples positive	Concentration range for positive samples (log_{10} gene copies/L)	PCR assays	Country	References
Untreated wastewater	Composite and grab	Adsorption-direct RNA extraction Ultrafiltration	2/9	1.23-2.08	qPCR (N_Sarbeco, NIID_2019-nCOV_N)	Australia	Ahmed et al., (2020a)
Untreated wastewater	Composite	Ultrafiltration	14/24	3.4-6.3	qPCR (CDC N1, N2, N3, E_Sarbeco)	The Netherlands	Medema et al., (2020)
Untreated wastewater	Grab	Aluminum hydroxide adsorption-precipitation	35/42	5.15-5.53	qPCR (CDC N1, N2, N3)	Spain	Randazzo et al., (2020)
Secondary Treated			2/18				
Tertiary Treated			0/12				
Untreated wastewater	Composite	PEG/dextran precipitation	6/12	ND	qPCR (RdRp), nested PCR (ORF1ab and S assays)	Italy	La Rosa et al., (2020)
Untreated wastewater	Grab	Electronegative membrane-vortex (EMV) adsorption-direct RNA extraction	0/5		qPCR (N_Sarbeco, NIID_2019-nCOV_N, CDC N1, N2), nested PCR (ORF1a and S	Japan	Haramoto et al., (2020)
Secondary Treated effluent			1/5	3.38			
River water			0/3				

					assays)		
Untreated wastewater	Composite and grab	Ultrafiltration Adsorption-elution using electronegative membrane	2/7	2.5-3.2	qPCR (CDC N1, N2)	USA	This study
Secondary Treated			0/4				
Final effluent			0/4				

- ND= Not determined

Figure

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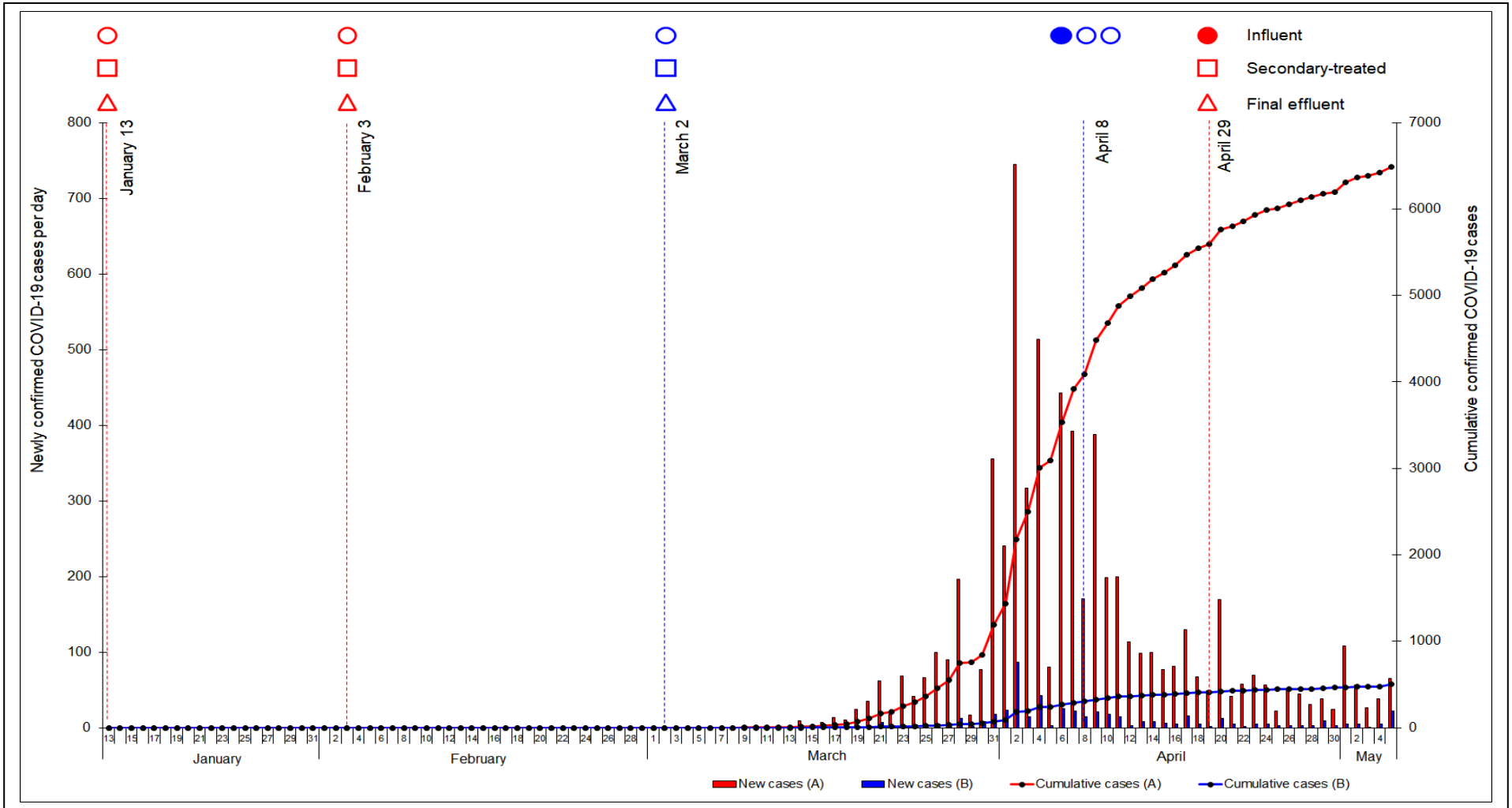

Figure 1: SARS-CoV-2 RNA detection in wastewater and confirmed COVID-19 cases in Southern Louisiana, USA. Circles, squares, and triangles represent sampling dates and sampling type (influent, secondary-treated and final effluent) respectively. Red and blue closed circle denote positive SARS-CoV-2 RNA detection from WWTPs A and B, red and blue bars denote new COVID-19 cases and red and blue line plots denote cumulative COVID-19 cases in locations served by WWTPs A and B

Conflict of Interests

The authors declare no conflict of interest.

Credit Author Statement

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