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

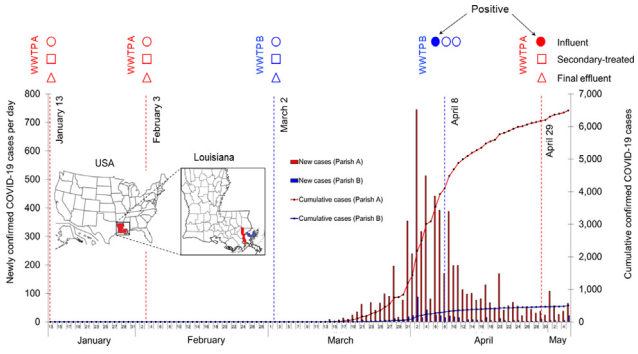
Samendra P. Sherchan [a,*], Shalina Shahin [a], Lauren M. Ward [a], Sarmila Tandukar [b], Tiong G. Aw [a], Bradley Schmitz [c], Warish Ahmed [d], Masaaki Kitajima [e]

[a] Department of Environmental Health Sciences, Tulane University, 1440 Canal Street, Suite 2100, New Orleans, LA 70112, USA
[b] Interdisciplinary Center for River Basin Environment, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan
[c] Loudoun Water, 44865 Loudoun Water Way, Ashburn, VA 20147, USA
[d] CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia
[e] Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13 West 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

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



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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 on five occasions 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 membranes (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. To our knowledge, this is the first study reporting the detection of SARS-CoV-2 RNA in wastewater in North America, including 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.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the *Coronaviridae* family, emerged in Wuhan, China in

* Corresponding author at: Department of Environmental Health Sciences #8360, School of Public Health and Tropical Medicine, Tulane University of Louisiana, 1440 Canal Street Suite 2100, New Orleans, LA 70112, USA.
E-mail address: sshercha@tulane.edu (S.P. Sherchan).

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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 USA, the total number of cases is 2,414,870 with 124,325 deaths as of June 26, 2020 (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). The 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.

A number of 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; Wölfel 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 (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 major 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., 2020b 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 (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, including the USA.

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. 24-hour composite samples were collected using an autosampler whereas grab samples were collected early in the morning (7am-11 am). 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. Samples collected on January-March were stored at −80 °C until further analysis whereas samples from April 2020 were processed within 6 h of sample collection.

2.2. Concentration and nucleic acid extraction

Two virus concentration methods were used to maximize the chance of SARS-CoV-2 RNA detection in wastewater. Method A (ultrafiltration) was performed with centrifugation of 250 mL of the sample for 30 min at 3000*g* to remove large particles and suspended solids. 70-140 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 (1500*g* for 15 min). The filter unit was inverted and centrifuged at 1000*g* for 2 min to recover the viral concentrate of approximately 350 μL, which 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) 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 non-inhibitory control. 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

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

Assay	Target gene	Primer/probe	Sequence (5′–3′)[a]	Reference
CDC N1	Nucleocapsid (N)	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	CDC (2020b)
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	
		2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	
CDC 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-IBFQ	

[a] FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1; ZEN, ZEN internal quencher; IBFQ, Iowa Black fluorescent quencher.

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 were 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), 0.1 μL of 100 μM each primer and probe, and 2.5 μL of cDNA template. CDC N1 and N2 primers and probes used in this study are shown in Table 1. The qPCR condition for SARS-CoV-2 was as follows: 95 °C for 10 min 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 standard curves. Molecular biology grade water was used as non-template controls. The amplification efficiencies (E) were calculated based on the equation: $E = 10^{(-1/\text{slope})} - 1$. Negative and positive controls were included in each qPCR run and all qPCR assays were performed in duplicate according to the MIQE guidelines (Bustin et al., 2009).

3. Results

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

Concentrated wastewater samples were seeded with *Pseudomonas* bacteriophage Φ6 as a process control to monitor RNA extraction-RT-qPCR efficiency. The geometric mean recovery efficiencies of *Pseudomonas* bacteriophage Φ6 were 56% (n=15) and 54% (n=15) for the methods A and B, respectively. The slope of the standards for Φ6, CDC N1 and N2 assays were −3.34, −3.07 and −3.01. 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 RNA, indicating that the virus was removed by wastewater treatment processes to undetectable level (Table 2). 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 a geometric mean of 7.5×10^3

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

Location	Types of samples	Sampling date	Sample type	RT-qPCR[a] Copies/L			
				Method A (ultrafiltration)		Method B (adsorption-elution)	
				CDC N1	CDC N2	CDC N1	CDC N2
WWTP A	Composite	01/13	Influent	-	-	-	-
			Secondary treated	-	-	-	-
			Final effluent	-	-	-	-
		02/03	Influent	-	-	-	-
			Secondary treated	-	-	-	-
			Final effluent	-	-	-	-
		04/29	Influent	-	3.1×10^3	-	-
			Secondary treated	-	-	-	-
			Final effluent	-	-	-	-
WWTP B	Grab	03/02	Influent	-	-	-	-
			Secondary treated	-	-	-	-
			Final effluent	-	-	-	-
		04/08	Influent	7.5×10^3	4.3×10^3	-	-
			Influent	-	-	-	-
			Influent	-	-	-	-

[a] The concentrations were calculated as geometric mean from cDNA copy numbers of the two qPCR tubes.
- : Not detected.
LOD: 1.0×10^3 copies/L for Method A and 1.7×10^2 copies/L for Method B.

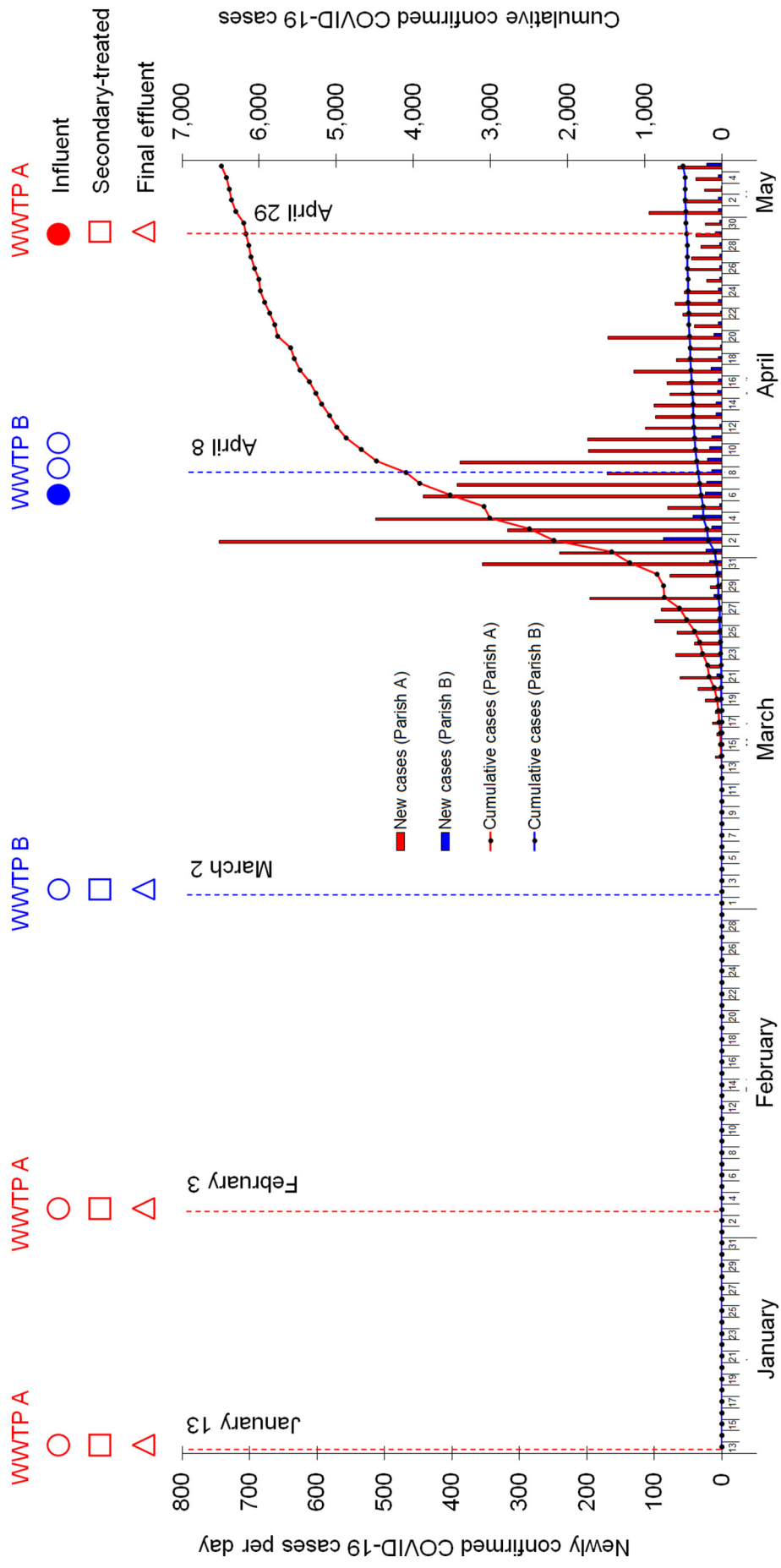

Fig. 1. SARS-CoV-2 RNA detection in wastewater and confirmed COVID-19 cases in southern Louisiana, USA. Circles, squares, and triangles represent sample types, i.e.,influent, secondary-treated, and final effluent, respectively. Red and blue symbols represent samples collected from WWTPs A and B, respectively. Closed and open symbols denote positive and negative SARS-CoV-2 RNA detections, respectively. Bars and line plots denote new and cumulative COVID-19 cases, respectively, in parishes A (red) and B (blue) where respective WWTPs (A and B) are located. Epidemiological data on confirmed COVID-19 cases in each parish in the State of Louisiana were retrieved from the USA facts (https://usafacts.org/ visualizations/coronavirus-covid-19-spread-map/).

copies/L from the N1 assay and 3.1×10^3 and 4.3×10^3 copies/L from the N2 assay. Wastewater samples processed through Method A yielded positive results, while all samples tested negative using the Method B. Epidemiological data on confirmed COVID-19 cases in each parish in the State of Louisiana were retrieved from the USA facts (https://usafacts.org/ visualizations/coronavirus-covid-19-spread-map/). When the samples tested positive for SARS-CoV-2 RNA in influent (April 8 for WWTP B and April 29 for WWTP A), the cumulative confirmed COVID-19 cases were 6173 (on April 29) in parish A where WWTP A is located and 308 (on April 8) in parish B where WWTP B is located (Fig. 1).

4. Discussion

Several studies have been conducted for the quantification of SARS-CoV-2 RNA in untreated wastewater during the ongoing COVID-19 pandemic (Table 3). However, further studies are needed to assess the recovery efficiency of existing virus concentration methods for the accurate detection and quantification of SARS-CoV-2 RNA in wastewater. For the assessment of RNA extraction and 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 analysis. Ye et al. (2016) recovered 18.2 ± 9.5% *Pseudomonas* bacteriophage Φ6 using an optimized ultrafiltration method. 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 from wastewater. However, since the present study was initiated before the results presented in Ahmed et al., 2020b, we were unable to include the adsorption-extraction method with $MgCl_2$ pre-treatment.

In this study, we used two virus concentration methods, namely, ultrafiltration and adsorption-elution, for the detection of SARS-CoV-2 RNA in wastewaters. Of the two methods tested, method A (ultrafiltration) successfully recovered SARS-CoV-2 RNA from two untreated wastewater samples. None of the secondary treated and final effluent samples tested positive for SARS-CoV-2 RNA indicating the removal of SARS-CoV-2 RNA during wastewater treatment processes to undetectable level. However, Randazzo et al. (2020) used an aluminum hydroxide adsorption-precipitation method and found 11% (2 out of 18 samples) positive in secondary treated water with at least one SARS-CoV-2 RT-qPCR assay (Table 3). Another study by Haramoto et al. (2020) in Japan detected SARS-CoV-2 RNA in 20% (1/5) of secondary-treated wastewater samples using N_Sarbeco RT-qPCR assay (Table 3).

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

The concentrations of SARS-CoV-2 RNA (3.1×10^3 - 7.5×10^3copies/L) in wastewater samples in this study was higher than that reported by Ahmed et al., 2020a in Australia (1.9×10^1- 1.2×10^2copies/L), but two orders of magnitude lower than those reported by Randazzo et al. (2020) in Spain (1.4×10^5- 3.4×10^5 copies/L) (Table 3). This could be due to differences in abundance of SARS-CoV-2 in wastewater due to pandemic level in the community and methodologies for viral RNA detection including virus concentration, RNA extraction, and RT-qPCR assays.

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 6173 and 308 in parishes 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 or below the assay limit of detection in wastewater until the cases were high in the study area. We found no evidence for the presence of SARS-CoV-2 RNA in wastewater from southern Louisiana before the first COVID-19 case was reported in the community on March 9. Only a small number of samples were tested

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

Types of samples	Virus concentration method	Sample	Samples positive	Concentration range for positive samples (gene copies/L)	PCR assays	Country	References
Composite and grab	Adsorption-direct RNA extraction and Ultrafiltration	Untreated wastewater	2/9	1.9×10^1 - 1.2×10^2	RT-qPCR (N_Sarbeco, NIID_2019-nCOV_N)	Australia	Ahmed et al., (2020a)
Composite	Ultrafiltration	Untreated wastewater	14/24	2.6×10^3 - 2.2×10^6	RT-qPCR (CDC N1, N2, N3, E_Sarbeco)	The Netherlands	Medema et al., (2020)
Grab	Aluminum hydroxide adsorption-precipitation	Untreated wastewater	35/42	1.4×10^5 - 3.4×10^5	RT-qPCR (CDC N1, N2, N3)	Spain	Randazzo et al., (2020)
		Secondary Treated	2/18	<LOQ- 2.5×10^5			
		Tertiary Treated	0/12	NA			
Composite	PEG/dextran precipitation	Untreated wastewater	6/12	ND	RT-qPCR (RdRp), nested PCR (ORF1ab and S assays)	Italy	La Rosa et al., (2020)
Grab	Electronegative membrane-vortex (EMV) and Adsorption-direct RNA extraction	Untreated wastewater	0/5	NA	RT-qPCR (N_Sarbeco, NIID_2019-nCOV_N, CDC N1, N2), nested PCR (ORF1a and S assays)	Japan	Haramoto et al., (2020)
		Secondary Treated	1/5	2.4×10^3			
		River water	0/3	NA			
Composite and grab	Ultrafiltration and Adsorption-elution using electronegative membrane	Untreated wastewater	2/7	3.1×10^3 - 7.5×10^3	RT-qPCR (CDC N1, N2)	USA	This study
		Secondary Treated	0/4	NA			
		Final effluent	0/4	NA			

ND: Not determined; NA: Not Available; LOQ: limit of quantification.

from two WWTPs, and only two virus concentration methods were used in this study. 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-extraction method (Ahmed et al., 2020b) and molecular assays using droplet digital PCR.

In summary, we detected SARS-CoV-2 RNA in untreated wastewater samples in southern Louisiana, USA using ultrafiltration method. This is the first proof of concept study that reports the detection of SARS-CoV-2 RNA in wastewater in North America, including the USA. 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.

CRediT authorship contribution statement

Samendra P. Sherchan: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Shalina Shahin:** Methodology, Validation, Formal analysis, Writing - original draft. **Lauren M. Ward:** Methodology, Writing - original draft. **Sarmila Tandukar:** Validation, Writing - original draft. **Tiong G. Aw:** Writing - review & editing. **Bradley Schmitz:** Writing - review & editing. **Warish Ahmed:** Writing - review & editing. **Masaaki Kitajima:** Validation, Visualization, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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