



Title	First detection of SARS-CoV-2 RNA in wastewater in North America : A study in Louisiana, USA
Author(s)	Sherchan, Samendra P.; Shahin, Shalina; Ward, Lauren M.; Tandukar, Sarmila; Aw, Tiong G.; Schmitz, Bradley; Ahmed, Warish; Kitajima, Masaaki
Citation	Science of the total environment, 743, 140621 <a href="https://doi.org/10.1016/j.scitotenv.2020.140621">https://doi.org/10.1016/j.scitotenv.2020.140621</a>
Issue Date	2020-11-15
Doc URL	<a href="http://hdl.handle.net/2115/86171">http://hdl.handle.net/2115/86171</a>
Rights	© <2020>. This manuscript version is made available under the CC-BY-NC-ND 4.0 license <a href="https://creativecommons.org/licenses/by-nc-nd/4.0/">https://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Rights(URL)	<a href="https://creativecommons.org/licenses/by-nc-nd/4.0/">https://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Type	article (author version)
File Information	STOTEN-D-20-13830R1.pdf



[Instructions for use](#)

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

2 Samendra P. Sherchan<sup>a</sup>, Shalina Shahin<sup>a</sup>, Lauren M. Ward<sup>a</sup>, Sarmila Tandukar<sup>b</sup>, Tiong G. Aw<sup>a</sup>, Bradley  
3 Schmitz<sup>c</sup>, Warish Ahmed<sup>d</sup>, Masaaki Kitajima<sup>e</sup>

4 <sup>a</sup>Department of Environmental Health Sciences, Tulane University, 1440 Canal Street, Suite 2100, New  
5 Orleans, LA 70112, USA

6 <sup>b</sup>Interdisciplinary Center for River Basin Environment, University of Yamanashi, 4-3-11 Takeda, Kofu,  
7 Yamanashi 400-8511, Japan

8 <sup>c</sup>Loudoun Water, 44865 Loudoun Water Way, Ashburn, VA 20147

9 <sup>d</sup>CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia

10 <sup>e</sup>Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13 West 8,  
11 Kita-ku, Sapporo, Hokkaido 060-8628, Japan

12

13 Running head: SARS-CoV-2 RNA in Louisiana wastewater

14

15 Corresponding author:

16 Samendra Sherchan, Ph.D.,

17 Department of Global Environmental Health Sciences #8360

18 School of Public Health and Tropical Medicine

19 Tulane University of Louisiana

20 1440 Canal Street Suite 2100

21 New Orleans, LA, 70112

22 Phone: 504-988-7283

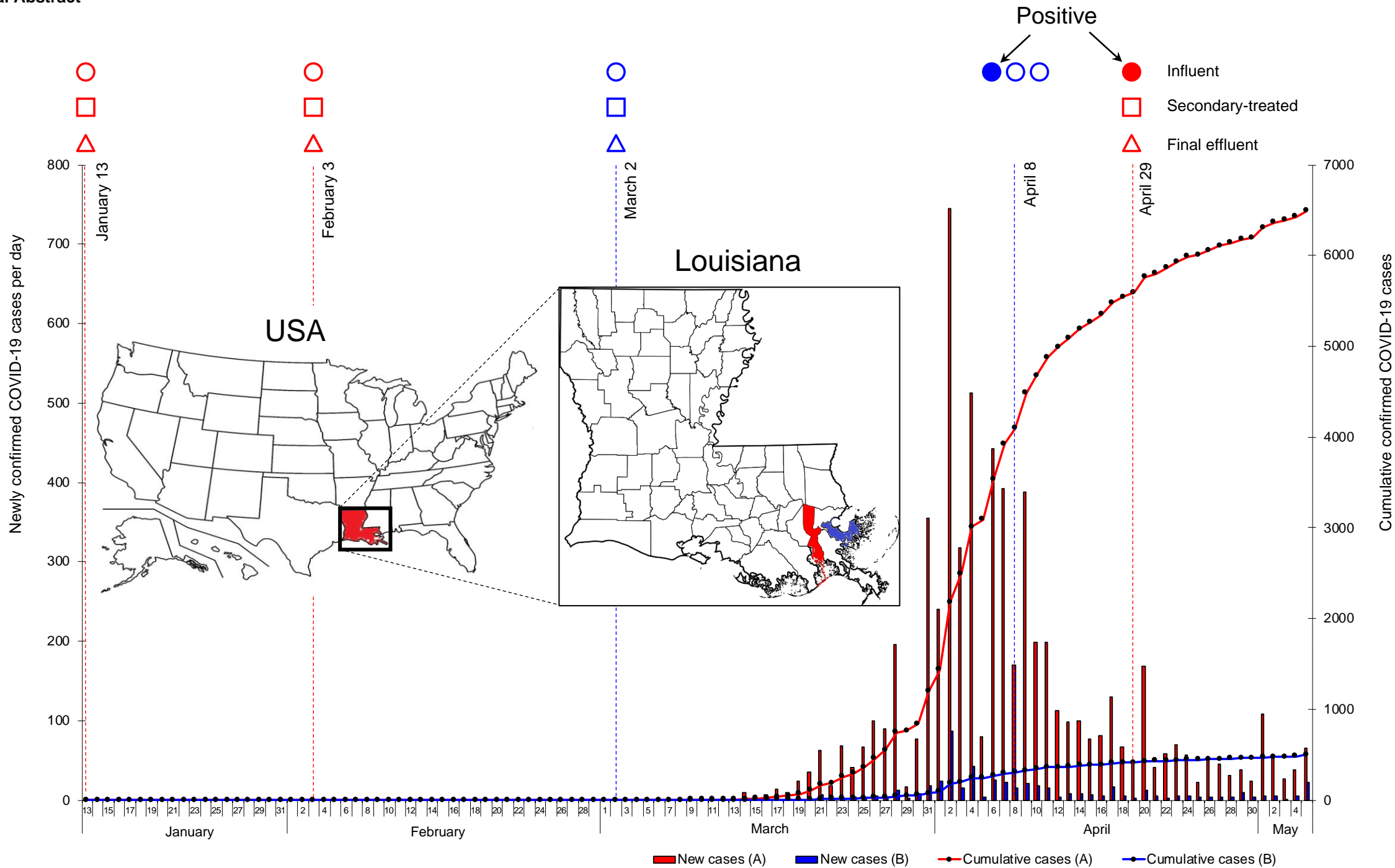
23 Email: sshercha@tulane.edu

24

25

## 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.



26 **Abstract**

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

39

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

41 **1. Introduction**

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

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

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

59 Wastewater-based epidemiology (WBE) has been used to advance our understanding of the emergence  
60 and epidemiology of pathogenic viruses such as polioviruses and noroviruses in communities around the  
61 world (Kitajima et al., 2020). Recently, the detection of SARS-CoV-2 RNA in municipal wastewater has  
62 been reported from a number of countries including Australia (Ahmed et al., 2020a), Spain (Randazzo et  
63 al., 2020), Italy (La Rosa et al., 2020), Netherlands (Medema et al., 2020), and Japan (Haramoto et al.,  
64 2020), suggesting the applicability of WBE approach to monitor COVID-19.

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

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

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

## 88 **2. Materials and Methods**

### 89 **2.1. Wastewater Sample Collection**

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

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

## 97 2.2. Concentration and nucleic acid extraction

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

106 Method B (adsorption–elution method using an electronegative membrane) was performed as  
107 described previously (Schmitz et al., 2016; Tandukar et al., 2020). Briefly, 2.5 M  $MgCl_2$  was added to all  
108 samples (100 mL influent and 750 mL secondary treated and final effluent) to obtain a final  
109 concentration of 25 mM  $MgCl_2$ . Samples were subsequently passed through an electronegative filter  
110 (90-mm diameter and 0.45- $\mu$ m pore size; Merck Millipore, Billerica, USA; Catalog no. HAWP-09000)  
111 attached to a glass filter holder (Advantec, Tokyo, Japan). Magnesium ions were then removed by the  
112 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  
113 of 1.0 mM NaOH (pH 10.8). The eluate was recovered in a tube containing 50  $\mu$ L of 100 mM  $H_2SO_4$  and  
114 100  $\mu$ L of 100 $\times$  Tris-EDTA buffer for neutralization. 10 mL was then centrifuged using a Centriprep YM-30  
115 (Merck Millipore) containing an ultrafiltration membrane with an NMWL of 30 kDa (Merck Millipore,  
116 Billerica, MA) to obtain a final volume of approximately 650  $\mu$ L.

## 117 2.3. RT-qPCR inhibition and quality control



118 *Pseudomonas* bacteriophage  $\Phi 6$  (DSM 21518, DSMZ, Braunschweig, Germany) was used as a sample  
119 process control (SPC) to determine the efficiency of RNA extraction and RT-qPCR. Briefly, 2  $\mu\text{L}$  of  
120 *Pseudomonas* bacteriophage  $\Phi 6$  ( $2.0 \times 10^5$  copies/ $\mu\text{L}$ ) was seeded into 200  $\mu\text{L}$  of concentrated  
121 wastewater samples and molecular biology grade water was used as a control (i.e., no inhibition). The  
122 extraction-RT-qPCR efficiency (E) % was calculated as described previously (Schmitz et al., 2016;  
123 Tandukar et al., 2020).

#### 124 2.4. Viral RNA extraction and reverse transcription (RT)

125 Viral RNA was extracted from the concentrated wastewater sample seeded with *Pseudomonas*  
126 bacteriophage  $\Phi 6$  process control (202  $\mu\text{L}$  in total) using a ZR Viral RNA Kit (Zymo Research, Irvine, USA)  
127 to obtain a final volume of 100  $\mu\text{L}$  RNA according to the manufacturer's protocol. RT was performed  
128 using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) (Schmitz et  
129 al., 2016; Tandukar et al., 2020).

#### 130 2.5. RT-qPCR for SARS-CoV-2

131 RT-qPCR assays for SARS-CoV-2 was performed with a CFX96 Real-Time PCR Instrument (BioRad  
132 Laboratories, Hercules, CA). Reaction mixtures (25  $\mu\text{L}$ ) consisted of 12.5  $\mu\text{L}$  of Perfecta qPCR ToughMix  
133 (Quantabio, Beverly, MA), 100  $\mu\text{M}$  primers and probe, molecular grade water, and 2.5  $\mu\text{L}$  of cDNA  
134 template. CDC N1 and N2 primers and probes used in this study are shown in Table 1. The PCR condition  
135 for SARS-CoV-2 was as follows: 95  $^{\circ}\text{C}$  for 10 minutes and 45 cycles of 95  $^{\circ}\text{C}$  for 10 s and 55  $^{\circ}\text{C}$  for 30 s  
136 (CDC, 2020b). The PCR condition for *Pseudomonas* bacteriophage  $\Phi 6$  was 94 $^{\circ}\text{C}$  for 3 min followed by 35  
137 cycles of 94 $^{\circ}\text{C}$  for 15 s and 60 $^{\circ}\text{C}$  for 1 min with a plate reading after the elongation step (Gendron et al.,  
138 2010). Serial ten-fold dilutions of the standard plasmid of SARS-CoV-2 or gBlocks for *Pseudomonas*  
139 bacteriophage  $\Phi 6$ , obtained from IDT (Coralville, IA) were used to produce a standard curve. Molecular  
140 biology grade water was used as a non-template control. The amplification efficiencies (AE) were

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

143

### 144 **3. Results**

#### 145 3.1 Efficiency of viral nucleic acid extraction and RT-qPCR and qPCR assay performance

146 Concentrated wastewater samples were seeded with *Pseudomonas* bacteriophage  $\Phi 6$  as a process  
147 control to monitor RNA extraction-RT-qPCR efficiency. The mean recovery efficiency of *Pseudomonas*  
148 bacteriophage  $\Phi 6$  was  $92.21 \pm 19.2\%$ . The slope of the standards for  $\Phi 6$ , N1 and N2 assays were -3.34, -  
149 3.07 and -3.01 respectively. Y-intercept values were -41 ( $\Phi 6$ ), -39.17 (N1), and -38.49 (N2). The  
150 correlation coefficient ( $R^2$ ) values for these assays were 0.996% (N1), 0.991% (N2), and 0.999% ( $\Phi 6$ ),  
151 respectively.

#### 152 3.2 Detection of SARS-CoV-2 RNA in wastewater samples

153

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

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

#### 165 **4. Discussion**

166 Several studies have been conducted for the quantification of SARS-CoV-2 in untreated wastewater  
167 during the ongoing COVID-19 pandemic (Table 3). However, further studies are needed to assess the  
168 existing methods for concentration and recovery of viruses from wastewater with varying characteristics  
169 for the accurate detection and quantification of SARS-CoV-2 RNA in wastewater. For the assessment of  
170 RNA extraction-RT-qPCR efficiency, we used *Pseudomonas* bacteriophage  $\Phi 6$ . The mean recovery  
171 efficiency was quite high, indicating that there was no considerable inhibition or loss occurred during  
172 the RNA extraction and RT-qPCR. In Ye et al. (2016), the recovery of *Pseudomonas* bacteriophage  $\Phi 6$   
173 was  $18.2 \pm 9.5\%$  using an optimized ultrafiltration method. Ye et al., (2016) spiked  $\Phi 6$  into wastewater  
174 before concentration and quantified using plaque assay, evaluating the concentration efficiency.  
175 Medema et al. (2020) also used an ultrafiltration method (100 kDa Centricon® Plus-70 centrifugal  
176 device) and determined the recovery of F-specific RNA phages by the purification and concentration  
177 steps using plaque assay, which yielded a mean recovery efficiency of 73%. A recent study conducted by  
178 Ahmed et al. (2020b) evaluated seven different concentration methods using a surrogate coronavirus  
179 (CoV), i.e., murine hepatitis virus (MHV). The recovery efficiencies of MHV using Amicon Ultra -15 and  
180 Centricon Plus-70 ultrafiltration centrifugal devices were  $56.0 \pm 32.3\%$  and  $28.0 \pm 9.10\%$ , respectively.  
181 According to Ahmed et al. (2020b), an adsorption-extraction method with  $MgCl_2$  pre-treatment was the  
182 most efficient method to concentrate MHV. However, since the present study was initiated before the  
183 results presented in Ahmed et al. (2020b) was obtained, we were unable to include the adsorption-  
184 extraction method with  $MgCl_2$  pre-treatment when we designed the present study.

185 In the present study, we used two virus concentration methods, namely, ultrafiltration and adsorption-  
186 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<sub>10</sub> 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<sub>10</sub> copies/L), but lower than  
210 those reported by Randazzo et al. (2020) in Spain (5.1–5.5 log<sub>10</sub> copies/L) (Table 3). This could be due to

211 differences in abundance of SARS-CoV-2 in wastewater and methodologies for viral RNA detection  
212 including virus concentration methods, viral RNA extraction strategies, and RT-qPCR assays. Randazzo et  
213 al. (2020) used an aluminum hydroxide adsorption-precipitation method. Ahmed et al. (2020a) used the  
214 membrane adsorption-direct RNA extraction method using the electronegative membranes followed by  
215 N\_Sarbeco and NIID\_2019-nCoV\_N RT-qPCR assays.

216 Epidemiological data on COVID-19 confirmed cases in the State of Louisiana have been retrieved from  
217 the USA facts (<https://usafacts.org/visualizations/coronavirus-covid-19-spread-map/>). The first  
218 confirmed case of COVID-19 in Louisiana was reported on March 9, 2020 (CDC, 2020a). On April 8 and  
219 29, when the samples tested positive for SARS-CoV-2 RNA in influent, the total confirmed number of  
220 COVID-19 cases were 6,173 and 308 in locations served by WWTPs A and B, respectively. Even though  
221 we tested samples from January, we were not able to detect the viral RNA in wastewater until April  
222 2020. This result suggests that concentrations of the viral RNA in wastewater were not detectable in  
223 wastewater until the cases started increasing in the studied area. We found no evidence for the  
224 presence of SARS-CoV-2 RNA in wastewater from Louisiana before the first COVID-19 case was reported  
225 in the community on March 9. There are some limitations of this study, which may have attributed the  
226 detection of SARS-CoV-2 RNA in wastewater in the early stage of the pandemic. For example, a small  
227 number of samples were tested from two WWTPs, and only two virus concentration methods were  
228 used. Also, some of the samples were grab samples collected at a time point when the viral RNA levels  
229 could have been low in the wastewater streams. Therefore, it seems prudent to test more wastewater  
230 samples and evaluate the performance of several other concentration methods including the  
231 adsorption-direct RNA extraction method (Ahmed et al., 2020b) and molecular assays including droplet  
232 digital PCR.

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

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

238

### 239 **Acknowledgments**

240 This research was partially supported by the Board of Regents grant number LEQSF (2018-21)-rd-a-21 to  
241 Dr. Samendra Sherchan and the Japan Society for the Promotion of Science (JSPS) through the  
242 Promotion of Joint International Research (Fostering Joint International Research (B)) Grant Number  
243 JP18KK0270 to Dr. Masaaki Kitajima. The authors would like to thank the plant managers from two  
244 anonymous wastewater treatment plants in southern Louisiana for their collaboration.

245

246

247

248

### 249 **References**

250 Ahmed, W., Angel, N., Edson, J., et al., 2020. First confirmed detection of SARS-CoV-2 in untreated  
251 wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the  
252 community. *Sci. Total Environ.* <https://doi.org/10.1016/j.scitotenv.2020.138764>.

253 Ahmed, W., P. Bertsch, A. Bivins, et al., (2020b) Comparison of virus concentration methods for the RT-  
254 qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater,  
255 *Sci. Total Environ.* <https://doi.org/10.1016/j.scitotenv.2020.139960>

256 Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl,  
257 M. W.; Shipley, G. L.; et al. The MIQE guidelines: Minimum Information for publication of quantitative  
258 real-time PCR experiments. *Clin. Chem.* 2009, 55 (4), 611–622.

259 CDC (2020a) <https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/cases-in-us.html>

260 CDC. (2020b) 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Primer and Probe Information.  
 261 <https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html>. Published 2020.  
 262 Accessed May 5, 2020.

263 de Roda Husman, A. M., W. J. Lodder, S. A. Rutjes, J. F. Schijven, and P. F. Teunis. 2009. Long-term  
 264 inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell  
 265 culture. *Appl. Environ. Microbiol.* 75:1050-1057.

266 Gendron, L., Verreault, D., Veillette, M., Moineau S., Duchaine, C., (2010) Evaluation of Filters for the  
 267 Sampling and Quantification of RNA Phage Aerosols, *Aerosol Science and Technology*, 44:10, 893-901,  
 268 DOI: 10.1080/02786826.2010.501351

269 Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence  
 270 of SARS-CoV-2 RNA in wastewater and river water in Japan. *Science of the Total*  
 271 *Environment*. **737**:140405. [DOI: 10.1016/j.scitotenv.2020.140405](https://doi.org/10.1016/j.scitotenv.2020.140405)

272 Harcourt J, Tamin A, Lu X, Kamili S, Sakthivel SK, Murray J, Queen K, Tao Y, Paden CR, Zhang J, Li Y,  
 273 Uehara A, Wang H, Goldsmith C, Bullock HA, Wang L, Whitaker B, Lynch B, Gautam R, Schindewolf C,  
 274 Lokugamage KG, Scharton D, Plante JA, Mirchandani D, Widen SG, Narayanan K, Makino S, Ksiazek TG,  
 275 Plante KS, Weaver SC, Lindstrom S, Tong S, Menachery VD, Thornburg NJ. Severe Acute Respiratory  
 276 Syndrome Coronavirus 2 from Patient with 2019 Novel Coronavirus Disease, United States. *Emerg Infect*  
 277 *Dis*. 2020. doi:10.3201/eid2606.200516.

278 Holshue, M.L., DeBolt, C., Lindquist, S., Lofy, K.H., Wiesman, J., Bruce, H., Spitters, C., Ericson, K.,  
 279 Wilkerson, S., Tural, A., Diaz, G., Cohn, A., Fox, L., Patel, A., Gerber, S.I., Kim, L., Tong, S., Lu, X.,  
 280 Lindstrom, S., Pallansch, M.A., Weldon, W.C., Biggs, H.M., Uyeki, T.M., Pillai, S.K., 2020. First case of  
 281 2019 novel coronavirus in the United States. *N. Engl. J. Med.* 382 (10), 929–936.  
 282 <https://doi.org/10.1056/NEJMoa2001191> (Epub2020 Jan 31).

283 Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B.,  
 284 2020. SARS-CoV-2 in wastewater: state of the knowledge and research needs. *Sci. Total Environ.*  
 285 <https://doi.org/10.1016/j.scitotenv.2020.139076>.

286 La Rosa, G., Iaconelli, M., Mancini, A., Bonanno, F., Veneri, Bonadonn, L., Lucentini, Suffredini, E., 2020.  
 287 First detection of SARS-CoV-2 in untreated wastewaters in Italy. *Sci. Total Environ.* 736, 139652.  
 288 <https://doi.org/10.1016/j.scitotenv.2020.139652>.

289 Li D, Zhang J, Li J. Primer design for quantitative real-time PCR for the emerging Coronavirus SARS-CoV-  
 290 2. *Theranostics* 2020; 10(16):7150-7162. doi:10.7150/thno.47649.

291 Maal-Bared, R.; Bastian, R.; Bibby, K.; Brisolara, K.; Gary, L.; Gerba, C.; Olabode, L.; et al. (2020) The  
 292 Water Professional’s Guide to COVID-19. *Water Environ. Federation*, [https://www.wef.org/news-](https://www.wef.org/news-hub/wef-news/the-water-professionals-guide-to-the-2019-novel-coronavirus/)  
 293 [hub/wef-news/the-water-professionals-guide-to-the-2019-novel-coronavirus/](https://www.wef.org/news-hub/wef-news/the-water-professionals-guide-to-the-2019-novel-coronavirus/)

294 Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., 2020. Presence of SARS-Coronavirus- 2 in sewage.  
 295 *EST Letters*, <https://doi.org/10.1021/acs.estlett.0c00357>

296 Ong, S. et al. (2020). Air, Surface Environmental, and Personal Protective Equipment Contamination by  
 297 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) From a Symptomatic Patient. *JAMA*.  
 298 323(16):1610-1612. doi:10.1001/jama.2020.3227

299 Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2  
300 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. *Water Res.*  
301 <https://doi.org/10.1016/j.watres.2020.115942>.

302 Schmitz BW, Kitajima M, Campillo ME, Gerba CP, Pepper IL. Virus Reduction during Advanced Bardenpho  
303 and Conventional Wastewater Treatment Processes. *Environ Sci Technol.* 2016;50(17):9524-9532.  
304 doi:10.1021/acs.est.6b01384

305 Tandukar, S., Sherchan, S.P. and Haramoto, H., 2020. Applicability of crAssphage, pepper mild mottle  
306 virus, and tobacco mosaic virus as indicators of reduction of enteric viruses during wastewater  
307 treatment. *Sci. Rep.* 10, 3616. <https://doi.org/10.1038/s41598-020-60547-9>.

308 Tang, A., Tong, Z. D., Wang, H. L., Dai, Y. X., Li, K. F., Liu, J. N., ... & Yan, J. B. (2020b). Detection of Novel  
309 Coronavirus by RT-PCR in Stool Specimen from Asymptomatic Child, China. *Emerging infectious  
310 diseases*, 26(6).

311 To, K. K. W., Tsang, O. T. Y., Chik-Yan Yip, C., Chan, K. H., Wu, T. C., Chan, J., ... & Lung, D. C. (2020).  
312 Consistent detection of 2019 novel coronavirus in saliva. *Clinical Infectious Diseases*, ciaa149, doi:  
313 10.1093/cid/ciaa149.

314 Wang, W., Xu, Y., Gao, R., Lu, R., Han, K., Wu, G., & Tan, W. (2020). Detection of SARS-CoV-2 in Different  
315 Types of Clinical Specimens. *JAMA*. <https://jamanetwork.com/journals/jama/fullarticle/2762997>

316 WHO (2020) [https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200626-  
317 covid-19-sitrep-158.pdf?sfvrsn=1d1aae8a\\_2](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200626-covid-19-sitrep-158.pdf?sfvrsn=1d1aae8a_2)

318 Wu, Y., Guo, C., Tang, L., Hong, Z., et al., 2020. Prolonged presence of SARS-CoV-2 viral RNA in faecal  
319 samples. *Lancet Gastroenterol. Hepatol.* 5, 434–435. [https://doi.org/10.1016/S2468-1253\(20\)30083-2](https://doi.org/10.1016/S2468-1253(20)30083-2).

320 Wölfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M.A., Niemeyer, D., Jones,  
321 T.C., Vollmar, P., Rothe, C., Hoelscher, M., Bleicker, T., Brünink, S., Schneider, J., Ehmann, R.,  
322 Zwirgmaier, K., Drosten, C., Wendtner, C. 2020. Virological assessment of hospitalized patients with  
323 COVID-2019. *Nature*, 1–10. <https://doi.org/10.1038/s41586-020-2196x>.

324 Xiao, F., et al. (2020). Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology March  
325 2020*. DOI: <https://doi.org/10.1053/j.gastro.2020.02.055>

326 Ye, Y., R.M. Ellenberg, K.E. Graham, K.R Wigginton, 2016. Survivability, partitioning, and recovery of  
327 enveloped viruses in untreated municipal wastewater, *Environ. Sci. Technol.*, 50 (10):5077-5085.

328 Yeo, C., Kaushal, S., & Yeo, D. (2020). Enteric involvement of coronaviruses: is faecal–oral transmission  
329 of SARS-CoV-2 possible?. *The Lancet Gastroenterology & Hepatology*, 5(4), 335-337.  
330 [https://www.thelancet.com/journals/langas/article/PIIS2468-1253\(20\)30048-0/fulltext](https://www.thelancet.com/journals/langas/article/PIIS2468-1253(20)30048-0/fulltext)

331 Zhang, Y.; Chen, C.; Zhu, S.; Shu, C.; Wang, D.; Song, J.; Song, Y.; et al. (2020) Isolation of 2019-nCoV from  
332 a Stool Specimen of a Laboratory-Confirmed Case of the Coronavirus Disease 2019. *China CDC Wkly.*, 2  
333 (8), 123–124.

334

335



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

Assay	Target gene	Primer/Probe	Sequence (5'-3') <sup>a</sup>	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 phi6- R phi6- P	TGGCGGCGGTCAAGAGC GGATGATTCTCCAGAAGCTGCTG FAM/CGGTCGTCG/ZEN/CAGGTCTGA CACTCGC/3IABkFQ/	Gendron et al., (2010)

<sup>a</sup> 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 <sub>10</sub> 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 <sub>10</sub> 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 Secondary Treated Tertiary Treated	Grab	Aluminum hydroxide adsorption-precipitation	35/42 2/18 0/12	5.15-5.53	qPCR (CDC N1, N2, N3)	Spain	Randazzo et al., (2020)
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 Secondary Treated effluent River water	Grab	Electronegative membrane-vortex (EMV) adsorption-direct RNA extraction	0/5 1/5 0/3	3.38	qPCR (N_Sarbeco, NIID_2019-nCOV_N, CDC N1, N2), nested PCR (ORF1a and S	Japan	Haramoto et al., (2020)

---

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

---

- ND= Not determined

**Figure**  
[Click here to download Figure: Figure.docx](#)

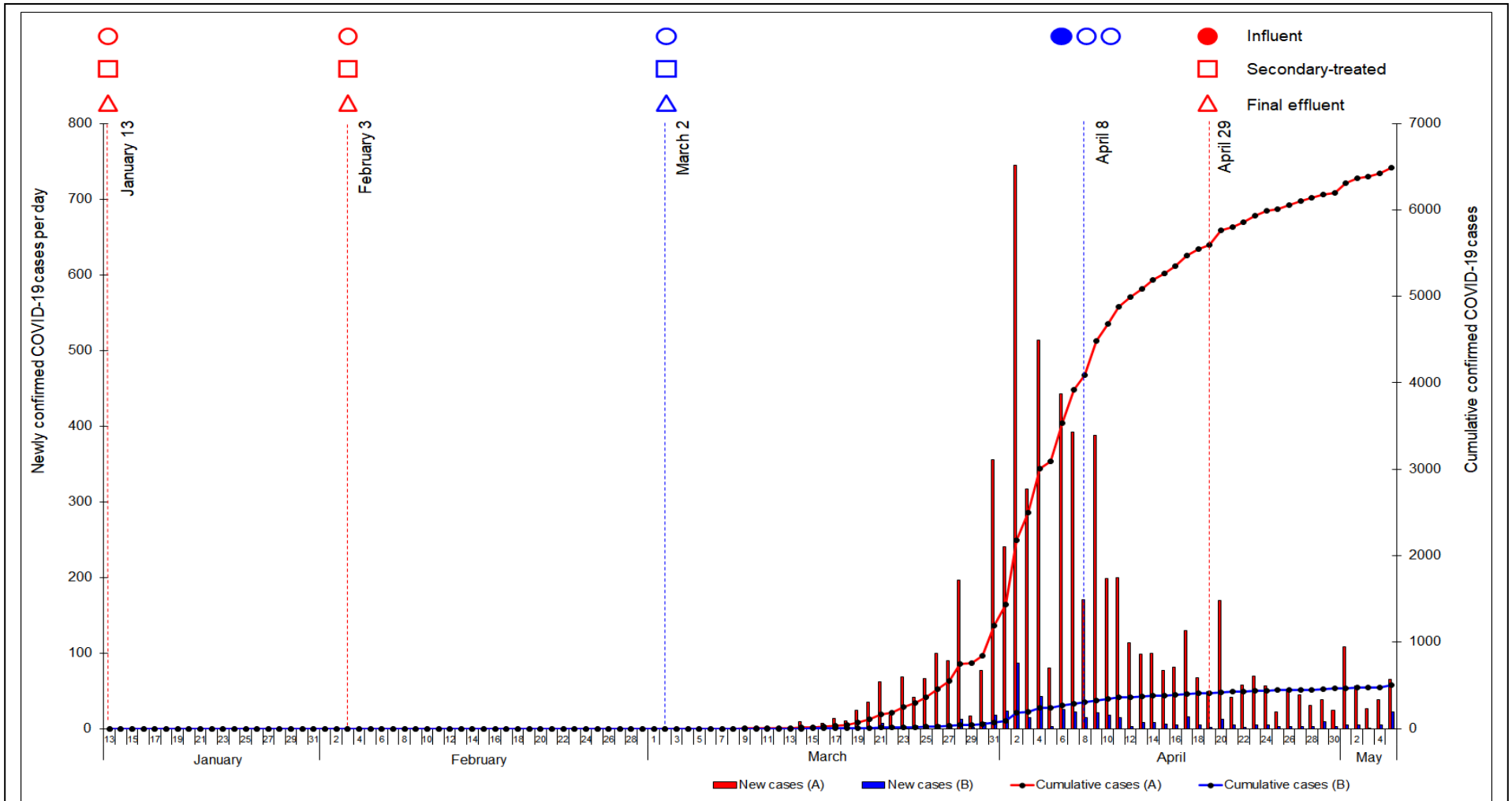


Figure 1: SARS-CoV-2 RNA detection in wastewater and confirmed COVID-19 cases in Southern Louisiana, USA. Circles, squares, and triangles represent sampling dates and sampling type (influent, secondary-treated and final effluent) respectively. Red and blue closed circle denote positive SARS-CoV-2 RNA 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.

Author Contributions: Conceptualization, S.P.S.; methodology, S.S., L.W. and S.P.S.; validation, S.S., S.T., B.S., S.P.S., and M.K.; formal analysis, S.S., S.P.S.; investigation- S.S., S.P.S.; writing—original draft preparation, S.P.S., S.T., L.W., S.S.; writing—review and editing, S.T., S.P.S., T.A., B.S., W.A., and M.K.; supervision, S.P.S.; funding acquisition, S.P.S. All authors have read and agreed to the published version of the manuscript.