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## **Epidemiological surveillance of SARS-CoV-2 by genome quantification in wastewater applied to a city in the northeast of France: Comparison of ultrafiltration- and protein precipitation-based methods**

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### ► To cite this version:

Isabelle Bertrand, Julie Challant, Helene Jeulin, Cédric Hartard, Laurence Mathieu, et al.. Epidemiological surveillance of SARS-CoV-2 by genome quantification in wastewater applied to a city in the northeast of France: Comparison of ultrafiltration- and protein precipitation-based methods. *International Journal of Hygiene and Environmental Health*, 2021, 233, pp.113692. 10.1016/j.ijheh.2021.113692 . hal-03167302

**HAL Id: hal-03167302**

**<https://hal.univ-lorraine.fr/hal-03167302>**

Submitted on 11 Mar 2021

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1 **Epidemiological surveillance of SARS-CoV-2 by genome quantification in wastewater applied to a**  
2 **city in the northeast of France: comparison of ultrafiltration- and protein precipitation-based**  
3 **methods**

4  
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29

30 **Abstract**

31 The aim of the present study was to develop a simple, sensitive, and specific approach to quantifying  
32 the SARS-CoV-2 genome in wastewater and to evaluate this approach as a means of epidemiological  
33 surveillance. Twelve wastewater samples were collected from a metropolitan area in north-eastern  
34 France during April and May 2020. In addition to the quantification of the SARS-CoV-2 genome, F-  
35 specific RNA phages of genogroup II (FRNAPH GGII), naturally present in wastewater, were used as  
36 an internal process control for the viral concentration and processing of RT-PCR inhibitors. A  
37 concentration method was required to allow the quantification of the SARS-CoV-2 genome over the  
38 longest possible period. A procedure combining ultrafiltration, phenol-chloroform-isoamyl alcohol  
39 purification, and the additional purification of the RNA extracts was chosen for the quantification of  
40 the SARS-CoV-2 genome in 100-mL wastewater samples. At the same time, the COVID-19 outbreak  
41 was evaluated through patients from the neighbouring University Hospital of Nancy, France. A  
42 regular decrease in the concentration of the SARS-CoV-2 genome from  $\sim 10^4$  gc/L to  $\sim 10^2$  gc/L of  
43 wastewater was observed over the eight weeks of the study, during which the population was  
44 placed under lockdown. The SARS-CoV-2 genome was even undetectable during one week in the  
45 second half of May and present but non-quantifiable in the last sample (28 May). A concordant  
46 circulation in the human community was highlighted by virological diagnosis using respiratory  
47 samples, which showed a decrease in the number of COVID-19 cases from 677 to 52 per week over  
48 the same period. The environmental surveillance of COVID-19 using a reliable viral quantification  
49 procedure to test wastewater is a key approach. The real-time detection of viral genomes can allow  
50 us to predict and monitor the circulation of SARS-CoV-2 in clinical settings and survey the entire  
51 urban human population.

52

53 **Keywords:** SARS-CoV-2; Wastewater; Concentration Methods; Prevalence

54 **Highlights**

- 55 • Wastewater-based epidemiology is an interesting approach for SARS-CoV-2.
- 56 • Ultrafiltration is a reliable method to concentrate SARS-CoV-2 from wastewater.
- 57 • FRNAPH naturally present in wastewater can be used as internal process control.

58

## 59 **1. Introduction**

60 In December 2019, the first outbreak of a new coronavirus disease (COVID-19) was reported in  
61 Wuhan, China. A new coronavirus linked to Severe Acute Respiratory Syndrome (SARS-CoV-2) was  
62 identified. It displayed more than 80% sequence homology with the previous SARS-CoV, identified  
63 in 2003 (Cheung et al., 2020). By March 2020, 114 countries reported outbreaks and the WHO  
64 declared a pandemic linked to SARS-CoV-2 (WHO, 2020). France was severely affected, with more  
65 than 2,550,000 confirmed COVID-19 infections and more than 62,000 deaths by the end of 2020.

66 Even though many cases of COVID-19 are asymptomatic, when symptomatic it is characterized  
67 mainly by fever and respiratory symptoms, but there are also a variety of other symptoms  
68 frequently associated with the disease. These include gastrointestinal complaints, including  
69 anorexia, diarrhoea, and vomiting/nausea at frequencies of up to 27%, 12% and 10%, respectively  
70 (Cheung et al., 2020). SARS-CoV-2 is able to productively replicate in human gut enterocytes (Lamers  
71 et al., 2020) and the SARS-CoV-2 genome has been detected in the stools of both symptomatic and  
72 asymptomatic patients (Cai et al., 2020; Gao et al., 2020; Holshue et al., 2020; Tang et al., 2020;  
73 Wölfel et al., 2020; Zhang et al., 2020). Viral RNA may be detected in stools up to 10 days after viral  
74 clearance from the respiratory tract, regardless of the severity of the disease (Cheng et al., 2020).  
75 The viral concentration can be high, potentially reaching  $10^7$  genome copies (gc)/g (Wölfel et al.,  
76 2020).

77 As expected, very high concentrations of the SARS-CoV-2 genome have also been detected in  
78 wastewater (Ahmed et al., 2020; Hindson, 2020; Medema et al., 2020; Nemudryi et al., 2020;  
79 Randazzo et al., 2020; Wu et al., 2020a; Wurtzer et al., 2020). Concentrations may reach close to  
80  $10^6$  gc/L (Wu et al., 2020a; Wurtzer et al., 2020). The presence of SARS-CoV-2 in wastewater  
81 indicates a potential health risk, but also a data source that can be used for epidemiological  
82 purposes (Lodder and de Roda Husman, 2020; Thompson et al., 2020). Wastewater-based  
83 epidemiology is not a new idea. It has been used to detect chemicals (Choi et al., 2020) and other

84 viruses, such as norovirus, hepatitis A virus, poliovirus, and Aichivirus (Asghar et al., 2014; Hellmer  
85 et al., 2014; Lodder et al., 2012, 2013). Many countries (including The Netherlands, Spain, France,  
86 Australia, and Israel) now support the idea of using this type of epidemiological approach in the  
87 surveillance of human populations and to possibly launch an early warning system to predict future  
88 outbreaks (Ahmed et al., 2020; Bar Or et al., 2020; Medema et al., 2020; Wu et al., 2020; Wurtzer  
89 et al., 2020).

90 Whatever its purpose — whether epidemiological or to assess public health risk — the first step is  
91 to define a method of quantifying SARS-CoV-2 in wastewater. The methodologies that have been  
92 used to date are highly diverse. The concentration methods were based on various principles,  
93 including ultrafiltration (Ahmed et al., 2020; Medema et al., 2020; Nemudryi et al., 2020; Trottier et  
94 al., 2020); protein precipitation (Kumar et al., 2020; La Rosa et al., 2020b; Wu et al., 2020a);  
95 electronegative membranes (Ahmed et al., 2020) and ultracentrifugation (Wurtzer et al., 2020).  
96 Recovery rates are currently unknown or extrapolated from those of similar viruses such as porcine  
97 epidemic diarrhoea virus (PEDV) and mengovirus (MgV) (Randazzo et al., 2020).

98 The aim of this study was to define the dynamics of viral concentration in wastewater during the  
99 first lockdown of the French population. This objective required a simple, specific, and sensitive  
100 approach to quantifying the SARS-CoV-2 genome in wastewater. In the present study, we took  
101 advantage of the wide circulation of SARS-CoV-2 in north-eastern France to compare two methods  
102 of virus concentration. We assessed the recovery rate of SARS-CoV-2 by virus quantification directly  
103 in four unconcentrated wastewater samples. We also included an internal process control, using F-  
104 specific RNA phages of genogroup II (FRNAPH GGII). We applied the most reliable method to  
105 investigate the temporal variations of SARS-CoV-2 genome concentrations in wastewater over 12  
106 weeks in an area of France with 250,000 inhabitants, which has been significantly impacted by  
107 COVID-19.

108

## 109 **2. Material and methods**

### 110 **2.1. Sewage samples**

111 Samples were collected between 2 April and 28 May 2020 in the French Grand Est region. In that  
112 region, the population was under lockdown from 17 March to 10 May 2020. A total of 12 water  
113 samples (400 mL) were collected from a wastewater treatment plant (WWTP) weekly or twice  
114 weekly. The average volume of influent treated in this WWTP was  $\sim 118,000$  m<sup>3</sup>/day ( $\sim 250,000$   
115 inhabitants). The wastewater samples were captured at regular intervals, just after decantation, but  
116 before activated sludge treatment. The samples were stored at  $-20^{\circ}\text{C}$  until analysis.

### 117 **2.2. Clinical samples**

118 At the same time, 19,850 samples were examined by the laboratory of the local University Hospital  
119 to ascertain the presence of the SARS-CoV-2 genome in nasopharyngeal swabs. These samples were  
120 collected between week 10 and week 22 from suspected COVID-19 patients hospitalized in the local  
121 university hospital (60%) and from retirement home residents (40%). More than 95% of the people  
122 sampled during this period were resident in the urban area of the WWTP. The procedure used for  
123 the detection of the SARS-CoV-2 genome was based on primers and probes designed to target two  
124 RdRp (RNA-dependent RNA polymerase) gene segments (RdRp\_IP2 and RdRp\_IP4), in accordance  
125 with a procedure developed by the French National Reference Centre for Respiratory Viruses  
126 (Institut Pasteur, Paris, France).

127

### 128 **2.3. Concentration procedures**

129 Two concentration procedures, based on ultrafiltration and on PEG 6000 precipitation, respectively,  
130 were compared in the present study.

131 For the ultrafiltration procedure, a Centricon<sup>®</sup> Plus-70 centrifugal ultrafilter with a cut-off of 100 kD  
132 (Merck Millipore) was used. Before processing the samples, the ultrafilter was washed with 60 mL  
133 deionised water by centrifugation at  $1,500 \times g$  for 15 min to remove the trace amounts of glycerine,



134 in accordance with the manufacturer's recommendations. Two volumes of 50 mL wastewater were  
135 both filtered by centrifugation at  $1,500 \times g$  for 15 min. After each centrifugation step, the  
136 concentrate was recovered by inverting the system and applying centrifugation ( $1,000 \times g$  for 2 min).  
137 The resulting concentrate's volume was around 1.5 mL. The ultrafilter was then washed with 3.5 mL  
138 of deionised water. The washing solution was added to the 1.5 mL concentrate to produce the final  
139 concentrate sample (5 mL). In order to recover the maximum amount of virus genome from the  
140 ultrafilter, two further washing steps were undertaken, each using 5 mL NucliSENS® lysis buffer  
141 (bioMérieux) for an incubation time of 5 min. The entire volume (15 mL) was then used for nucleic  
142 acid extraction. Twelve water samples were subjected to this procedure.

143 PEG 6000 precipitation was performed in a 250-mL centrifuge bottle containing 3 g beef extract  
144 powder, 3 g NaCl, and 0.37 g glycine for 100 mL of wastewater. After the dissolution of the beef  
145 extract powder, 20 g of PEG 6000 were added. The sample was gently stirred at 4°C for 2 h and then  
146 maintained at 4°C overnight. The pellet obtained after centrifugation at  $4,500 \times g$  and 4°C for 45  
147 min was resuspended in deionised water to obtain a concentrated 5 mL sample. Ten millilitres of  
148 NucliSENS® lysis buffer were then added to the concentrate. After incubation for 10 min at room  
149 temperature, the entire volume (15 mL) was used for nucleic acid extraction. This concentration  
150 method was tested on the first four water samples of our study.

151 In tandem with each concentration procedure, 5 mL samples of unconcentrated water were used  
152 for viral genome extraction. They were submitted to the same procedure as the concentrated  
153 samples. Ten millilitres of NucliSENS® lysis buffer was added to the water, which was incubated at  
154 room temperature for 10 min, prior to the nucleic acid extraction.

#### 155 **2.4. Nucleic acid extraction**

156 Both concentrated and unconcentrated wastewater samples reached the same volume of 15 mL  
157 after the lysis step. Phenol-chloroform-isoamyl alcohol purification was then undertaken with both  
158 concentrated and unconcentrated samples. This was realized in a 50 mL conical tube, containing 4

159 g of a mixture of high-vacuum silicon grease (Dow Corning®) and silicon dioxide (Sigma) (90:10 w/w),  
160 which was labelled the separation tube. The sample in lysis buffer (15 mL) was transferred to the  
161 separation tube and 15 mL phenol-chloroform-isoamyl alcohol (25:24:1 pH 7.8-8.2, Acros  
162 Organics™) were added. After 15 s of vigorous stirring by hand and centrifugation (3,500 × g for 5  
163 min), the hydrophile supernatant (15 mL) was recovered. The nucleic acid extraction was continued  
164 using 70 µL of magnetic silica beads and the NucliSENS® easyMAG™ platform (bioMérieux). The  
165 extracted nucleic acids were eluted in 100 µL of elution buffer.

166 A complementary step was applied to some of the samples to remove residual environmental  
167 inhibitors using OneStep PCR Inhibitor Removal kit (ZymoResearch). The RNA samples were stored  
168 at -80°C until viral genome quantification. A negative control made of sterile phosphate buffered  
169 saline was included at the beginning of each nucleic acid extraction procedure.

## 170 **2.5. Viral genome quantification**

171 Viral RNA quantification was performed using real-time RT-PCR and RT-digital droplet PCR (RT-  
172 ddPCR).

173 For the FRNAPH GGII genome, the VTB4-Fph GII set published by Wolf et al. (2010) was used. For  
174 the SARS-CoV-2 genome, two primer sets were selected: the “RdRp\_IP4” set developed by the  
175 Pasteur Institute (Paris, France: [https://www.who.int/docs/default-source/coronaviruse/real-time-  
176 rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6\\_2](https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2)) for the  
177 RdRp gene and the “E” set developed by Corman et al. (2020) for the envelope protein (E) gene. The  
178 specificity of these sets for other respiratory viruses, including human coronaviruses, has been  
179 previously described (Corman et al., 2020; Etievent et al., 2020). The SARS-CoV-2 and FRNAPH GGII  
180 genomes were quantified in the same RNA extracts. For the RdRp\_IP4, E, and VTB4-Fph GGII sets,  
181 quantification was performed using an RNA UltraSens™ One-Step Quantitative RT-PCR system  
182 (Applied Biosystems™). The RdRp\_IP4 and E sets were applied to 5 µL of RNA in a 25-µL reaction  
183 volume with final concentrations of 0.4 µM for each primer and 0.2 µM for the probe. The VTB4-

184 Fph GGII set was used on 2  $\mu$ L of RNA in a 20- $\mu$ L reaction volume with final concentrations of 1  $\mu$ M  
185 for each primer and 0.3  $\mu$ M for the probe. A StepOnePlus Real-Time PCR System (Applied  
186 Biosystems™) was used for these three real-time RT-PCR assays. For the RdRp\_IP4 and E sets, the  
187 RT step was performed at 50 °C for 20 min and PCR amplification was performed at 95°C for 2 min,  
188 followed by 50 cycles of 15 s at 95°C and 30 s at 58 °C. For the VTB4-Fph GGII set the RT step was  
189 performed at 50 °C for 30 min and PCR amplification was performed at 95°C for 2 min, followed by  
190 45 cycles of 15 s at 95°C and 40 s at 58 °C. Negative and positive controls were included in each  
191 experiment. Quantification was carried out using standard curve ranges. RNA extracted from  
192 patients who had tested positive for SARS-CoV-2 was quantified using ddRT-PCR with the E set, as  
193 described below. Quantified RNA was then used to obtain the standard curve for both RdRp\_IP4  
194 and E genes. The nCoV-ALL-Control plasmid (Eurofins genomic) was also used for the standard curve  
195 of the E gene. This plasmid containing ampicillin resistance gene was maintained in TOP10  
196 chemically competent *E. coli* (Invitrogen) and quantified using Qubit 4 fluorometer (Invitrogen). At  
197 last, RNA extracted from GA phage suspension was used to obtain the standard curve for FRNAPH  
198 GGII. RNA extracted from GA phage suspension was quantified by ddRT-PCR using VTB4-Fph the  
199 GGII set as described below. The standard curves ranged from  $1 \times 10^{-1}$  to  $1 \times 10^4$  gc/reaction for the  
200 RdRp\_IP4 and E genes of SARS-CoV-2 and from  $2.8 \times 10^{-1}$  to  $2.8 \times 10^4$  gc/reaction for the GA phage.  
201 The limit of detection (LoD) was 1 gc/RT-qPCR reaction for the RdRp and E genes. The limit of  
202 quantification (LoQ) ranged from 1 to 10 gc/RT-qPCR reaction for the RdRp gene and reached 1  
203 gc/RT-qPCR reaction for the E gene. When we take the analytical volumes of the wastewater sample  
204 concentrations subjected to RT-qPCR analysis into consideration, the LoQ ranged from  $2 \times 10^2$  to  
205  $2 \times 10^3$  gc/L of wastewater for the RdRp gene and reached  $2 \times 10^2$  gc/L of wastewater for the E gene.  
206 The ddRT-PCR assays were performed using the E set of SARS-CoV-2 and the VTB4-Fph GGII set for  
207 GA phage. Amplifications were carried out in a 20- $\mu$ L reaction mixture containing 5  $\mu$ L of RNA and  
208 15  $\mu$ L of One-Step RT-ddPCR™ Kit for Probes (Bio-Rad). The reaction mix contained 0.9  $\mu$ M of each

209 primer and 0.3  $\mu$ M of the probe. The samples were placed in the droplet generator using 70  $\mu$ L of  
210 generator oil to generate up to 20,000 droplets per sample. The resulting picolitre droplet emulsions  
211 (40  $\mu$ L) were transferred to a Veriti 96-Well Thermal Cycler (Applied Biosystems). After  
212 amplification, the plate was transferred to the QX100™ Droplet Reader (Bio-Rad) and QuantaSoft™  
213 Software (Bio-Rad) was used to measure the number of positive droplets per well. Droplets were  
214 designated positive or negative based on their fluorescence amplitude, using thresholding. The  
215 starting concentration of each target RNA molecule was then calculated, by modelling a Poisson  
216 distribution.

217 For each sample and each targeted virus (SARS-CoV-2 and FRNAPH GGII) the recovery rate was  
218 calculated as follows: recovery rate = genome copies (gc) in 5 mL unconcentrated sample  
219  $\times 20 \times 100 /$  gc in 100 mL of the concentrate. The high concentration of the FRNAPGH GGII genome  
220 allowed for the determination of the recovery rate in all wastewater samples. It was also possible  
221 to determine the recovery rate for SARS-CoV-2 in 5 of the 12 samples.

## 222 **2.6. Estimation of RT-qPCR inhibition**

223 The estimation of RT-qPCR inhibition in the wastewater samples was based on the concentrations  
224 of FRNAPH GGII detected. Logarithmic dilutions (1/10 and 1/100) were performed in PCR-grade  
225 water following viral RNA extraction. The RT-qPCR assay was then carried out on both undiluted and  
226 diluted RNA extracts. The percentage of inhibition was estimated for undiluted and 1/10 samples  
227 by taking the concentration obtained from the 1/100 samples as a reference, due to the high dilution  
228 of potential inhibitors.

## 229 **2.7. Statistical analysis**

230 The statistical analyses were performed using XLSTAT 2020.3.1.2 software. As normal distribution  
231 of the data and the homogeneity of variances could not be met, two non-parametric tests were  
232 used. A Kruskal-Wallis test was performed on k-independent samples ( $k > 3$ ) to compare the viral  
233 genome copy values obtained using the different concentration and purification methods. A

234 Wilcoxon signed-rank test was performed on paired samples to compare the recovery rates  
235 following the two methods and the genome concentrations of the two targeted genes. *P* values <  
236 0.05 were considered statistically significant.

237

### 238 **3. Results and discussion**

#### 239 **3.1. Concentration method for SARS-CoV-2 quantification in wastewater**

240 The epidemiological surveillance of SARS-CoV-2 by quantifying its genome in wastewater requires  
241 reliable methods of concentration and detection. Beginning our study in a region that was highly  
242 impacted by COVID-19 allowed us to estimate recovery rates for SARS-CoV-2.

243 FRNAPH are usually present in wastewater at concentrations that are relatively stable over time and  
244 around the world (Lucena et al., 2003). We obtained just under  $2.5 \times 10^7$  gc/L which was high enough  
245 to allow us to evaluate both recovery rates and PCR inhibition problems. Using these  
246 bacteriophages, the presence of PCR inhibitors was detected at even a low volume (5 mL) of  
247 unconcentrated wastewater. Indeed, the PCR inhibition varied between 88% and 100% in undiluted  
248 RNA extracts (n=5) and between 14% and 42% in 1/10 diluted RNA extracts (n=5). Following phenol-  
249 chloroform purification, the PCR inhibitors had been completely removed from 5 mL of wastewater.  
250 In these five wastewater samples, the level of contamination by SARS-CoV-2 also enabled the  
251 quantification of the virus in 5 mL unconcentrated samples and showed that, following phenol-  
252 chloroform purification, the concentrations of SARS-CoV-2 genome detected in the undiluted  
253 samples had multiplied by between a factor of 2 and a factor of 10. The positive impact of this  
254 method on viral RNA extraction from environmental samples such as wastewater, sediments and  
255 animal stool samples has already been shown by previous studies (Miura et al., 2011; Hartard et al.,  
256 2015). The phenol-chloroform purification method was systematically applied in the following  
257 experiments. A complementary method for the removal of PCR inhibitors in RNA extracts was tested  
258 during the comparison of the concentration methods (as described below).

259 Two concentration procedures, based on protein precipitation using PEG 6 000 and on ultrafiltration  
260 using the Centricon® 70-Plus 100 kD device, respectively, were compared. Recovery rates for both  
261 FRNAPH GGII and SARS-CoV-2 could be determined (Table 1) for both methods in four distinct  
262 wastewater samples collected in April 2020, since these samples tested positive for the SARS-CoV-  
263 2 genome even when only 5 mL of unconcentrated wastewater was analysed. The mean recovery  
264 rate of FRNAPH GGII reached  $47.7 \pm 18.5\%$  and  $39.2 \pm 23.9\%$  using ultrafiltration and protein  
265 precipitation, respectively. Because some of the results obtained for FRNAPH GGII showed residual  
266 PCR inhibition, the RNA extracts previously obtained by both methods were then purified using a  
267 PCR inhibitor removal kit. This treatment led to mean recovery rates of  $47.1 \pm 10.4\%$  and  
268  $43.3 \pm 20.3\%$  for ultrafiltration and protein precipitation, respectively. This supplementary  
269 treatment therefore led to a slight increase in the mean recovery rate of protein precipitation. It  
270 also led to a decrease in the standard deviation in both concentration methods. The concentration  
271 values obtained for FRNAPH GGII using the two concentration methods, with or without the use of  
272 the PCR inhibitor removal kit, were not significantly different ( $p$  value = 0.098, Kruskal-Wallis test).  
273 For SARS-CoV-2, the mean recovery rates of the RdRp\_IP4 gene reached  $64.1 \pm 50.2\%$  and  
274  $32.4\% \pm 20.2\%$  using ultrafiltration and protein precipitation, respectively. Additional purification of  
275 the RNA extracts led to mean recovery rates of  $55.8 \pm 46.9\%$  and  $23.5 \pm 15.0\%$  for ultrafiltration and  
276 protein precipitation, respectively. Thus, the mean recovery rate obtained for SARS-CoV-2  
277 (RdRp\_IP4 gene) using ultrafiltration were twice as high as those obtained using protein  
278 precipitation. Moreover, the highest recovery rate was obtained using ultrafiltration in the four  
279 wastewater samples. As observed in the case of the phage, additional purification reduced the  
280 standard deviation values. The genome concentration values were not significantly different  
281 between the two concentration methods ( $p$  value = 0.088, Kruskal-Wallis test). Amplification of the  
282 E gene was then performed on the purified RNA extracts, resulting in mean recovery rates of  
283  $64.0 \pm 41.6\%$  and  $45.0 \pm 44.6\%$  for ultrafiltration and protein precipitation, respectively (Table 1).

284 The statistical analysis of the data obtained for both the RdRp\_IP4 and E genes showed significantly  
285 higher recovery rates for ultrafiltration than for protein precipitation ( $p$  value = 0.009, Wilcoxon  
286 signed-rank test).

287 Recovery rates and the inhibition of molecular detection methods applied for SARS-CoV-2 in  
288 wastewater have been poorly described. Nevertheless, our SARS-CoV-2 recovery rate was higher  
289 than those described in a recent review paper (La Rosa et al., 2020a) reporting five studies that  
290 described recovery rates for coronavirus in water matrices. But these recovery rates were all defined  
291 using artificial spiking with different types of coronaviruses (bovine enteric coronavirus,  
292 transmissible gastroenteritis virus [TGEV], murine hepatitis virus, and SARS-CoV). The recovery rate  
293 did not exceed 28% for bovine enteric coronavirus seeded in wastewater (Collomb et al., 1986) and  
294 it varied from 0 to 21.4% for SARS-CoV seeded in wastewater ( $n=4$ ) (Wang et al., 2005). Our higher  
295 recovery rates for the SARS-CoV-2 naturally present in the wastewater samples could be primarily  
296 explained by the choice of concentration method. Increasing PEG concentration might lead to a  
297 decrease in the standard deviation values as suggested by the recovery rate of  $41.9 \pm 6.5\%$  for TGEV  
298 seeded in concentrated surface water (Blanco et al., 2019). Two of the studies reviewed by La Rosa  
299 and colleagues (2020a) used adsorption/elution on glass wool with the objective of analysing large  
300 volumes (5–50 L) of surface water (Blanco et al., 2019) and dechlorinated tap water (Abd-Elmaksoud  
301 et al., 2014). Such methods are commonly used as primary concentration methods and are followed  
302 by secondary concentration methods — PEG precipitation in the case of these two studies.  
303 However, the combination of concentration methods used on large volumes of water commonly  
304 leads to a decrease in recovery rates. By working on municipal wastewater spiked with different  
305 enveloped and non-enveloped viruses, Ye et al. (2016) have shown that ultrafiltration is a more  
306 suitable method for wastewater than ultracentrifugation or PEG precipitation because the recovery  
307 rate of ultrafiltration is 25% compared with 5% by ultracentrifugation or PEG precipitation. Since Ye  
308 and colleagues (2016) used culture as their detection method, the low recovery rates may be

309 explained by the possible inactivation of coronavirus by the greater ultracentrifugal forces and the  
310 lower precipitation capacity of enveloped viruses compared with non-enveloped viruses in PEG  
311 precipitation. Wurtzer and colleagues (2020) showed the ability of ultracentrifugation to  
312 concentrate SARS-CoV-2 from wastewater, but the recovery rate was not given, and detection was  
313 done using RT-qPCR instead of culture methods. It is important to note that the ultrafiltration  
314 method could be used for the detection of infectious SARS-CoV-2 in wastewater, as shown with  
315 FRNAPH by Medema et al. (2020). The environmental conditions may have a negative impact on the  
316 integrity of the SARS-CoV-2 particles, with implications for detection methods and viral risk  
317 assessment; this requires further research.

318 The procedure combining ultrafiltration, phenol-chloroform purification, and additional purification  
319 of the RNA extracts was chosen to monitor the concentrations of the SARS-CoV-2 genome in  
320 wastewater during longitudinal wastewater sampling. Our approach based on ultrafiltration may be  
321 performed with different devices such as Centricon® (Ahmed et al., 2020; Medema et al., 2020),  
322 Corning® Spin-X® UF (Nemudryi et al., 2020) or Vivaspin® (Trottier et al., 2020) concentrators.

323

### 324 **3.2. Quantification of the SARS-CoV-2 genome in wastewater**

325 Our longitudinal study was performed in a French geographical area with one of the highest  
326 prevalence rates of COVID-19. The population was placed under lockdown from 17 March until 11  
327 May 2020, but the number of cases detected at the University Hospital increased continuously until  
328 the end of March, when the wastewater sampling period started. Twelve wastewater samples,  
329 collected between 2 April and 28 May 2020, were analysed in one replicate using both the  
330 ultrafiltration procedure on 100 mL and the direct analysis of 5 mL to quantify the SARS-CoV-2 and  
331 FRNAPH GGII genomes.

332 The FRNAPH GGII genome could be quantified in both 5 mL and 100 mL of wastewater in all the  
333 samples (Figure 1). The mean concentrations reached  $2.1 \times 10^7 \pm 1.1 \times 10^7$  gc/L and



334  $1.6 \times 10^7 \pm 1.4 \times 10^7$  gc/L in unconcentrated and concentrated samples, respectively. From these  
335 concentration values, the recovery rates of FRNAPH GGII ranged from 14.1% to 133.8%.

336 We propose a recovery rate of over 10% as a quality control, to validate the results. This goes beyond  
337 the current ISO standards for the molecular detection of viruses in water and food, which require  
338 recovery efficiencies of over 1% (International Organization for Standardization - ISO 2019; Lowther  
339 et al. 2019). Quantifying FRNAPH at the same time as SARS-CoV-2 also allowed for the normalisation  
340 of faecal material content, which may vary daily, depending on rainfall or variations in faecal  
341 content. In our samples, FRNAPH genomes ranged from  $1.1 \times 10^7$  to  $6.7 \times 10^7$  gc/L, showing that  
342 the faecal pollution was relatively stable during the period in question. No significant rainfall events  
343 were reported during the sampling period. To compare the variations in SARS-CoV-2 genome  
344 concentrations in different wastewater samples may require the normalisation of genome content.

345 Other variables, such as temperature, pH, turbidity, total suspended solids, 5-day biological oxygen  
346 demand (BOD5), antecedent dry days, and conductivity may explain the variability of faecal  
347 indicator bacteria (FIB) (Mohammed et al., 2018; Paule-Mercado et al., 2016) and probably of other  
348 faecal pathogens.

349 The SARS-CoV-2 genome could be quantified in 5 mL of wastewater, without using any  
350 concentration procedures, between 2 April and 30 April (Figure 1). By analysing 100 mL of  
351 wastewater, a decrease in the concentration of the SARS-CoV-2 genome was observed over the  
352 course of 8 weeks (Figure 1). The SARS-CoV-2 genome was even undetectable during one week (22  
353 and 25 May) and present but below the LoQ in the last sample (28 May). The concentrations of RdRp  
354 and E genes in the twelve samples appeared to be similar ( $p$  value = 0.496, Wilcoxon signed-rank  
355 test). Moreover, the concentration values obtained using real-time RT-qPCR and ddRT-PCR for the  
356 E gene were similar ( $p$  value = 0.734, Wilcoxon signed-rank test). This suggests that RT-PCR inhibitors  
357 were effectively removed by our protocol. Indeed, ddRT-PCR is far less influenced by inhibitors than  
358 RT-qPCR (Sun et al., 2019).

359 The decrease in the genome concentrations detected in wastewater may be related to the decrease  
360 in the number of COVID-19 cases observed at the Nancy University Hospital (Figure 1). Between 2  
361 April and 23 April, the number of cases decreased from 677/week to 286/week and the  
362 concentration values of the SARS-CoV-2 genome detected in 100 mL of wastewater decreased from  
363  $1.2 \times 10^4$  gc/L to  $3.0 \times 10^3$  gc/L. During this period, the virus genome could be detected in only 5 mL  
364 of wastewater and decreased only from  $1 \times 10^4$  gc/L to  $7 \times 10^3$  gc/L. The analysis of only 5 mL of  
365 wastewater could be less representative of the variation in viral genome concentration. After a  
366 single data point showing an increase in genome concentration in wastewater in both 5 mL and 100  
367 mL (30 April), the SARS-CoV-2 genome was no longer detected in 5 mL of wastewater and the  
368 concentrations dropped below  $10^3$  gc/L in the 100 mL analysis. For three of the samples during this  
369 period, either no genes were detected or only one gene was detected, and the number of cases  
370 varied between 27/week and 52/week. Nevertheless, SARS-CoV-2 was still detected in wastewater  
371 in the last sample (28 May) collected 17 days after the end of the lockdown. We observed a parallel  
372 decrease in cases in patients and genome concentration in wastewater, confirming the link between  
373 the circulation of the virus in the human population and its presence in wastewater. This clearly  
374 confirms the findings of other studies showing such a relationship (Wurtzer et al., 2020). One of the  
375 first studies on the detection of the SARS-CoV-2 genome in wastewater (Medema et al., 2020) even  
376 showed that it was possible to detect the virus in wastewater during the early stages of the COVID-  
377 19 epidemic.

378

#### 379 **4. Conclusion**

380 We developed a method of concentrating SARS-CoV-2 from wastewater with one of the highest  
381 recovery rates described in the literature. The quantification of the SARS-CoV-2 genome was carried  
382 out in both concentrated and unconcentrated wastewater which was naturally contaminated; this  
383 gives our results a high degree of reliability. Moreover, this study provides additional data to validate

384 proof-of-concept for a link between the outbreak in the human community and the concentrations  
385 of faecally excreted viruses in wastewater. This method can contribute to the monitoring of the  
386 epidemic and improve the management of potential viral recirculation.

387

### 388 **Declaration of interest**

389 None

390

### 391 **Funding**

392 This work has benefited from the financial support of the French Government's Ministry of Higher  
393 Education and Research and Innovation (Ministère de l'enseignement supérieur, de la recherche et  
394 de l'innovation [MESRI]). This study was supported by the French scientific interest group Obépine  
395 for the surveillance of SARS-CoV-2 in wastewater. All the authors' salaries were funded by either  
396 Lorraine University (France), the University Hospital of Nancy (France) or SUEZ (France).

397

### 398 **Acknowledgements**

399 The authors would like to thank Sibel Berger for her contribution to the acquisition of the RT-qPCR  
400 assays for SARS-CoV-2 at LCPME. The authors would also like to thank Christophe Merlin and Hélène  
401 Guilloteau for their contributions to the plasmid replication.

402

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562

563 **Table 1: Recovery rates (%) of SARS-CoV-2 and FRNAPH GGII genomes in wastewater samples using two concentration methods (protein**  
 564 **precipitation and ultrafiltration) both alone and combined with the use of a PCR inhibitor removal kit.**

565

<b>Virus / Primers set</b>	<b>SARS-CoV-2 / RdRp_IP4</b>		<b>SARS-CoV-2 / E</b>		<b>FRNAPH GGII / VTB4-Fph GGII</b>	
<b>Concentration method</b>	<b>PP</b>	<b>UF</b>	<b>PP</b>	<b>UF</b>	<b>PP</b>	<b>UF</b>
<b>Without PCR Inhibitor removal kit</b>						
<b>Sample 1</b>	56.0	131.0	ND	ND	58.2	49.1
<b>Sample 2</b>	12.0	56.0	ND	ND	4.5	26.0
<b>Sample 3</b>	41.8	60.2	ND	ND	43.9	71.1
<b>Sample 4</b>	19.8	9.2	ND	ND	50.0	44.4
<b>Mean ± SD</b>	<b>32.4 ± 20.2</b>	<b>64.1 ± 50.2</b>	ND	ND	<b>39.2 ± 23.9</b>	<b>47.7 ± 18.5</b>
<b>With PCR Inhibitor removal kit</b>						
<b>Sample 1</b>	40.5	119.0	108.0	119.0	68.2	52.7
<b>Sample 2</b>	27.0	61.0	23.0	45.0	19.1	37.5
<b>Sample 3</b>	22.4	32.6	43.0	70.0	37.2	58.9
<b>Sample 4</b>	4.3	10.6	6.0	22.0	50.0	39.4
<b>Mean ± SD</b>	<b>23.5 ± 15.0</b>	<b>55.8 ± 46.9</b>	<b>45.0 ± 44.6</b>	<b>64.0 ± 41.6</b>	<b>43.3 ± 20.3</b>	<b>47.1 ± 10.4</b>

566

567 PP: protein precipitation, UF: ultrafiltration, ND: not done

568 **Figure 1. Quantitative time-course monitoring of SARS-CoV-2 and FRNAPH-GGII genomes in**  
569 **wastewater samples in the Nancy metropolitan area and evolution of the number of cases per**  
570 **week at the local University Hospital.** Quantification in 100 mL of wastewater (ultrafiltration) of the  
571 SARS-CoV-2 genome targeting RdRp (full triangles) and E (full squares) genes and of the FRNAPH  
572 GGII genome (full diamonds). Quantification in 5 mL of wastewater: open forms. Negative samples  
573 of the SARS-CoV-2 genome are marked with an asterisk. The sample positive for the RdRp gene but  
574 below the LoQ is marked with a plus sign. The number of cases of COVID-19 detected at the local  
575 University Hospital is shown per week (bars). The limit of detection (LoD) for RdRp and E genes  
576 ( $2 \times 10^2$  gc/L) is represented by a white arrow on the left y-axis. The lines given for genome  
577 concentration values are only designed to provide a visual guide.  
578

