

1 **Time-evolution of SARS-CoV-2 in wastewater during the first**
2 **pandemic wave of COVID-19 in the metropolitan area of Barcelona.**

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14 **Running Head:** Evolution of SARS-CoV-2 RNA in sewage in a pandemic

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19 **Keywords:** SARS-CoV-2, COVID-19, epidemiology, surveillance, early warning,
20 sewage,

21 **ABSTRACT** Two large wastewater treatment plants (WWTP), covering around 2.7 M
22 inhabitants, which represents around 85% of the metropolitan area of Barcelona, were
23 sampled before, during and after the implementation of a complete lockdown. Five one-
24 step RT-qPCR assays, targeting the polymerase (IP2 and IP4), the envelope E and the
25 nucleoprotein (N1 and N2) genome regions, were employed for SARS-CoV-2 RNA
26 detection in 24-h composite wastewater samples concentrated by polyethylene glycol
27 (PEG) precipitation.

28 SARS-CoV-2 was detected in a sewage sample collected 41 days ahead of the
29 declaration of the first COVID-19 case. The evolution of SARS-CoV-2 genome copies
30 in wastewater evidenced the validity of water-based epidemiology to anticipate COVID-
31 19 outbreaks, to evaluate the impact of control measures and even to estimate the
32 burden of shedders, including presymptomatic, asymptomatic, symptomatic and
33 undiagnosed cases. For this latter objective, a model was applied for the estimation of
34 the total number of shedders, evidencing a high proportion of asymptomatic infected
35 individuals. In this way, an infection prevalence of 2.0-6.5% was figured. On the other
36 hand, a proportion of around 0.12% and 0.09% of the total population was determined
37 to be required for positive detection in the two WWTPs.

38 At the end of the lockdown, SARS-CoV-2 RNA apparently disappeared in the
39 WWTPs but could still be detected in grab samples from four urban sewers. Sewer
40 monitoring allowed for location of specific hot spots of COVID-19, enabling the rapid
41 adoption of appropriate mitigation measures.

42 **IMPORTANCE** Water-based epidemiology (WBE) is a valuable early warning tool
43 for tracking the circulation of the virus among the population, including not only
44 symptomatic patients, but also asymptomatic, presymptomatic and misdiagnosed

45 carriers, which represent a high proportion of the infected population. In the specific
46 case of Barcelona, wastewater surveillance anticipated several weeks not only the
47 original COVID-19 pandemic wave, but also the onset of the second wave. In addition,
48 SARS-CoV-2 occurrence in wastewater evidenced the efficacy of the adopted lockdown
49 measures on the circulation of the virus.

50 Health authorities profited from WBE, to complement other inputs, and adopt rapid
51 and adequate measures to mitigate the effects of the pandemic. As an example, sentinel
52 surveillance of specific sewers helped to locate COVID-19 hot spots and to conduct
53 massive RT-PCR tests among the population.

54 Despite COVID-19 is a respiratory disease, the prolonged shedding of large amounts of
55 coronavirus genomes in the feces (1, 2), that ultimately reach wastewater (3, 4), has
56 been reported. Hence, SARS-CoV-2 surveillance in sewage is considered a sensitive
57 tool to monitor the spread of the virus among the population (5-7). There is no
58 epidemiological evidence that sewage could be a transmission route for SARS-CoV-2,
59 through contamination of bathing areas or irrigation waters, because very few studies
60 report culture of infectious virus from stool (8). As a matter of fact, Zang and coworkers
61 reported that SARS-CoV-2 released into the intestinal lumen is inactivated by human
62 colonic fluid, and hence infectious virus is seldom recovered from the stool specimens
63 of COVID-19 patients (9). In addition, the specific infectivity of SARS-CoV-2 in
64 respiratory samples has been reported to be of around one infectious unit per 10^6 or 10^7
65 genome copies (10, 11). Hence, infectious SARS-CoV-2 is unlikely to be present in
66 wastewater.

67 To date, Spain ranks in the ninth place in absolute number of cases worldwide, and
68 tops the list in Europe regarding the number of cases and deaths per 1 million
69 inhabitants, with Barcelona the second most affected area in the country
70 (<https://www.worldometers.info/coronavirus/>). The first case in Barcelona (actually the
71 first in continental Spain) was reported on February 25, 2020. A complete lockdown
72 was implemented in Spain in March 15, that gradually came to an end between May 25
73 and June 21. The total number of reported cases in metropolitan Barcelona at the end of
74 the lockdown in May 2020 was over 29,000 ([https://salutweb.gencat.cat/ca/inici/nota-
75 premsa/index.html?id=385948#googtrans\(ca|en\)](https://salutweb.gencat.cat/ca/inici/nota-premsa/index.html?id=385948#googtrans(ca|en))).

76 Two large wastewater treatment plants, WWTP1 (capacity 525 million liters per day
77 - MLD) and WWTP2 (capacity 420 MLD) cover around 2.7 M inhabitants, representing

78 around 85% of the densely populated metropolitan area of Barcelona. The present
79 extended study describes the evolution of the occurrence of SARS-CoV-2 RNA in these
80 large WWTPs, before, during and after the lockdown, evidencing the validity of water-
81 based epidemiology (WBE) to i) anticipate COVID-19 outbreaks, ii) evaluate the
82 impact of the control measures and iii) estimate the burden of infected patients,
83 including presymptomatic, asymptomatic, symptomatic and undiagnosed cases.

84

85 **RESULTS AND DISCUSSION**

86 **Time-evolution of SARS-CoV-2 RNA in wastewater during the pandemic.** The
87 evolution of SARS-CoV-2 genome copies in sewage from the two main WWTPs in the
88 metropolitan area of Barcelona is shown in Fig. 1. In WWTP1, maximum genome copy
89 numbers of SARS-CoV-2 were detected in the initial sample collected on April 13. A
90 progressive decrease was observed thereafter. This decrease was observed employing
91 IP2 and IP4 targets, (Fig. 1, panel A) and confirmed with E and N1 (Fig. 1, panels B
92 and C), and N2 (Fig. S1, Supplemental file) targets. On May 18, genomes disappeared,
93 although residual levels could be again detected on May 25 employing the N1 target.

94 For WWTP2, samples from December 2019 to May 2020 were available, which
95 opened the possibility to better analyze the dynamics of genome copy numbers in
96 sewage. The analysis of archival samples revealed the increasing occurrence of SARS-
97 CoV-2 genomes in samples from January 15 to March 4 employing the IP2, IP4 and E
98 targets (Fig. 1, panels D and E). Genome copy numbers peaked between March 4 and
99 May 4 independently of the used target (Fig. 1, panel D-F). Of note, SARS-CoV-2 was
100 detected in sewage 41 days (January 15) ahead of the declaration of the first COVID-19
101 case (February 25), clearly evidencing the validity of wastewater surveillance to

102 anticipate cases in the population. Again, as for WWTP1, genomes became undetectable
103 on May 18 (Fig. 1, panels D and E) except when employing the N1 target, whose signal
104 completely disappeared on May 25 (Fig. 1, panel F). The progressive decline in genome
105 copy numbers in both WWTPs paralleled the diminution in the estimated cumulative
106 number of shedders, based on the actual number of reported symptomatic cases, and
107 figured for 7-day, 14-day and 21-day excretion periods before the sampling date (Fig.
108 2). This genome copy decay evidences the effectivity of the lockdown measures on the
109 spread of the infection.

110 On May 25, phase 1 of the gradual deconfinement was implemented (Fig. 1, Table
111 S1). However, despite the apparent disappearance of SARS-CoV-2 RNA in the WWTPs
112 around May 18-25, the analysis of grab samples, collected 8-9 AM on May 18 and 25,
113 from four urban sewers revealed the occurrence of virus genomes (Fig. 3), indicating
114 that the virus was still circulating in the population. A higher dilution factor applies in
115 the WWTPs than in the sewers, which together with possible differences applying
116 between grab and composite samples, as well as bowel habits (12), could explain why
117 the WWTP samples came out to be negative for the virus, while genome copies could
118 still be detected in the sewer samples. Sewer analysis may provide most relevant
119 information for the specific localization of areas where COVID-19 cases reappear,
120 enabling immediate response to prevent spread of the outbreak. Nevertheless, it
121 represents a more laborious and costly approach than surveillance through WWTP
122 monitoring.

123 Between June 2-8, SARS-CoV-2 genomes reappeared in both studied WWTPs and
124 increased thereafter. All the RT-qPCR targets but the E target revealed this gradual raise
125 (Fig. 1). Failure of the E target may be explained by the increasing circulation of viral

126 variants with a recently described recurrent mutation affecting the probe-binding site
127 (13). Throughout our study, five different RT-qPCR assays, targeting different genome
128 regions were employed for SARS-CoV-2 detection in order to increase the robustness
129 of our data. From our own experience in this and other unpublished studies on the
130 occurrence of SARS-CoV-2 in wastewater, only 10% of the samples come out to be
131 positive for the five RT-qPCR targets, indicating the need to employ more than one of
132 these. In samples positive for all the five targets, the observed differences in Cq values,
133 did not translate into major differences in genome copies in the corresponding standard
134 curves (Fig. S1 and Fig. S2, supplemental file). However, since in our hands the N2
135 target provided some inconsistent results in comparison with the rest of the employed
136 targets, for the sake of clarity, data generated with the N2 target are only shown in Fig.
137 S3 of the Supplemental file. Current RT-PCR assays employed for SARS-CoV-2 in
138 WBE studies are diverse and demand harmonization, as a step forward towards the
139 development of standardized methodologies.

140 Phases 2 and 3 of the deconfinement were eventually applied on June 8 and 21,
141 respectively (Fig. 1); phase 3 was delayed due to the SARS-CoV-2 levels detected in
142 sewage. Nevertheless, in early July, a huge outbreak was declared (over 300 cases
143 confirmed in 2 weeks and around 10,000 cases in 14 weeks
144 ([https://canalsalut.gencat.cat/ca/inici/nota-](https://canalsalut.gencat.cat/ca/inici/nota-premsa/index.html?id=387275#googtrans(ca|en))
145 [premsa/index.html?id=387275#googtrans\(ca|en\)](https://canalsalut.gencat.cat/ca/inici/nota-premsa/index.html?id=387275#googtrans(ca|en))) in a neighborhood whose sewers
146 (Sewer3 and Sewer4, Fig. 3) drain into WWTP2, where genome copy numbers had
147 started to increase around 3-4 weeks in advance (Fig. 1, panels D and F).

148 **Estimation of the total number of active shedders from SARS-CoV-2 RNA**
149 **levels in wastewater.** WBE constitutes a valuable complementary tool for the

150 surveillance of current infectious agents among the population (14, 15). In particular,
151 WBE may contribute to a comprehensive management of the spread of SARS-CoV-2
152 infection. Nevertheless, information is required to relate the detected genome copy
153 numbers in wastewater with the numbers of infected individuals in the community,
154 encompassing both symptomatic, presymptomatic, asymptomatic and undiagnosed
155 shedders.

156 A simple and intuitive model was elaborated based on SARS-CoV-2 genome copy
157 numbers per L of sewage, wastewater flow at the sampling point during the sampling
158 period (Table S2), and genome copy numbers shed in the feces of infected individuals.
159 Wölfel and colleagues (11) reported SARS-CoV-2 shedding in stool based on RT-qPCR
160 employing the E target (16) (V.M. Corman, Charité Berlin, personal communication).
161 Data generated with the E target were available from April 13 to May 11 and from
162 March 31 to May 11, in samples from WWTP1 and WWTP2, respectively. The number
163 of shedders, including symptomatic, presymptomatic, asymptomatic and undiagnosed
164 cases, could be estimated following the model (Fig. 4). On April 13, this estimation was
165 of 30,096 and 28,747 shedders, which accounted for a 2.0% and 2.4% prevalence, in
166 WWTP1 and WWTP2, respectively. Yet, on March 31, the estimation was of 77,994
167 shedders and 6.5% prevalence in WWTP2. The applied model provided a sound
168 estimation of the number of shedders in our setting. However, the simplicity of the
169 model enables further refinements related with the percent of shedders, that in our case
170 was assumed to be 100%, and/or variations in virus load in feces of symptomatic,
171 presymptomatic and asymptomatic shedders, when reliable data are available. An
172 additional adjustment to the model is related with the threshold of genome copies in
173 sewage to discern between periods of high and low stool excretion that in our case was

174 established to be of $10^{2.5}$ gc/L. This value may vary depending on the WWTP type, the
175 million liters per day capacity or on technical factors inherent to the SARS-CoV-2
176 detection pipeline, i.e., virus concentration, RNA extraction and RT-qPCR efficiencies,
177 and the reference material used in the standard curve, which all contribute to a certain
178 degree of uncertainty. Additional sources of uncertainty are the limited number of
179 assayed replicas, in our case genome copies were determined in duplicate, while only a
180 single value of the daily wastewater flow was available. Nevertheless, for influenza, a
181 well-characterized respiratory infection with similar transmissibility and for which
182 natural and/or vaccine-induced immunity exists, a 2018 CDC study determined that the
183 percentage of the U.S. population sickened each season by flu was about 8%, with a
184 range of between 3% and 11%. When asymptomatic cases were also considered, the
185 estimate raise from 5 to 20% (17), which is not far from our estimate of COVID-19 in
186 metropolitan Barcelona.

187 Our data fall within the range of seroprevalence reported in the literature, taking into
188 account the uncertainty of the seroprevalence assays, associated with the time of sample
189 collection in the convalescence phase, the immunostatus and/or age of the patients and
190 the employed determination kit. A study conducted in Spain based on the detection of
191 antibodies directed to the S protein revealed an overall 5% seroprevalence (18), with
192 substantial geographic variability, e.g., over 10% and 7% in the Madrid and Barcelona
193 areas, respectively. Similarly, adjusted estimates of the persons seroreactive to SARS-
194 CoV-2 spike protein antibodies in the San Francisco and New York City areas were of
195 1% and 7%, respectively (19).

196 Hart and Halden (20) reported through computational analysis that, in worst-case
197 conditions, a 0.88% prevalence is required for successful detection of SARS-CoV-2 in

198 sewage, while Ahmed and coworkers reduced this requirement to a prevalence of only
199 0.025% (5). In the present study, the last positive RNA signal with the E target was
200 observed on March 11 in both WWTP (Fig. 1). Applying our model, a proportion of
201 around 0.12% and 0.09% of the total population (1,732 and 1,109 infected individuals)
202 is required for positive detection in WWTP1 and WWTP2, respectively; Fig. 4).

203 Our SARS-CoV-2 early detection in sewage supports the idea that cases may have
204 been present in the population before the first imported case was reported. COVID-19
205 cases may have been misclassified as influenza diagnoses in primary care, boosting
206 community transmission before public health measures were taken (21). Most COVID-
207 19 cases show mild influenza-like symptoms (22) and it has been suggested that some
208 uncharacterized influenza cases may have masked some COVID-19 cases in the 2019-
209 2020 season (21).

210 Our data reveal the significant proportion of presymptomatic and asymptomatic
211 carriers that nevertheless shed SARS-CoV-2 and contribute to the spread of the virus
212 (23, 24). The enormous burden in morbidity and mortality of COVID-19 calls for
213 sentinel surveillance of SARS-CoV-2 in wastewater to enable rapid mitigation measures
214 in pandemic waves and to evaluate the usefulness of lockdown and deconfinement
215 measures. Presently, surveillance networks comprising 56 WWTP in Catalonia
216 (Catalonian Health Authority, Catalonian Water Agency and Catalonian Institute of
217 Water Research, <https://sarsaigua.icra.cat/>), and 30 WWTP in Spain (VATar Project,
218 Ministry of Health and Ministry of the Environment,
219 [https://www.miteco.gob.es/es/agua/temas/concesiones-y-autorizaciones/vertidos-de-](https://www.miteco.gob.es/es/agua/temas/concesiones-y-autorizaciones/vertidos-de-aguas-residuales/alerta-temprana-covid19/default.aspx)
220 [aguas-residuales/alerta-temprana-covid19/default.aspx](https://www.miteco.gob.es/es/agua/temas/concesiones-y-autorizaciones/vertidos-de-aguas-residuales/alerta-temprana-covid19/default.aspx)) are implemented.

221

222 **Material and Methods**

223 **Wastewater samples.** Composite raw sewage samples corresponding to 24 hours,
224 were weekly collected from two large wastewater treatment plants (WWTP1 and
225 WWTP2) in the metropolitan area of Barcelona from April 13, in the peak of the
226 COVID-19 first wave, to July 7. In addition, for WWTP2, frozen archival samples
227 monthly collected from January to March 2020 were also assayed. Furthermore, grab
228 samples were collected from sewer maintenance holes on May 18 and 25, at 8-9 AM.
229 Time of grab sample collection was selected according the bowel habits of the
230 population (12)

231 **Wastewater concentration.** Eight hundred-milliliter samples of wastewater were
232 concentrated through precipitation with 20% polyethylene-glycol 6000 and resuspended
233 in 3 mL of PBS, pH 7.4 (25). In our hands, this procedure provides a mean recovery
234 efficiency of $2.53\% \pm 0.17\%$ of the attenuated porcine coronavirus PUR46-MAD strain
235 of transmissible gastroenteritis virus (kindly provided by L. Enjuanes and I. Sola,
236 National Center of Biotechnology, Cantoblanco, Madrid; 26).

237 **Nucleic acid extraction and virus quantification.** Nucleic acid extraction was
238 performed from 1mL of the concentrate and eluted in 50 μ L using the NucliSENS®
239 miniMAG® extraction system (bioMérieux).

240 Five one-step RT-qPCR assays (RNA UltraSense™ One-Step Quantitative RT-PCR
241 System, Invitrogen, Life Technologies) were employed targeting the RNA-dependent
242 RNA polymerase (RdRp) gene, IP2 and IP4 fragments, from Institut Pasteur, Paris
243 (Institut Pasteur, Paris. Protocol: Real-time RT-PCR assays for the detection of SARS-
244 CoV-2. 2020 <https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr->

245 [assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2](https://www.pasteur.fr/medias/Default.aspx?sfvrsn=3662fcb6_2)),
246 the envelope protein (E) gene, E fragment, from Charité, Berlin (16), and the
247 nucleoprotein (N), N1 and N2 fragments, from CDC, Atlanta (Centers for Disease
248 Control and Prevention A. CDC 2019–Novel Coronavirus (2019-nCoV) Real-Time RT-
249 PCR Diagnostic Panel. 2020 <https://www.fda.gov/media/134922/download>).

250 Standard curves were constructed using the Twist Synthetic SARS-CoV-2 RNA
251 Control 2 (MN908947.3, Twist Bioscience). Figure S1 of the Supplemental file shows
252 the average standard curves for each of the targets used.

253 Quality control and quality assurance to determine any potential contamination
254 and/or inhibition, were ascertained using negative and positive controls, respectively.
255 Positive controls consisted of the addition of two distilled water samples containing
256 5×10^3 copies of the Twist RNA, which were run in parallel in each RT-qPCR plate.
257 Direct and 1/10 diluted replicas were assayed to ascertain assay inhibition. All
258 quantitative assays were performed in duplicate, hence depicted genome copy numbers
259 correspond to the mean of four values. Negative controls comprised five distilled water
260 samples per run: two from the beginning of the assay, to control any potential
261 contamination during the RNA extraction, and three in the RT-PCR, to control any
262 potential contamination during nucleic acids amplification.

263 **Estimation of SARS-CoV-2 shedders.** The number of symptomatic SARS-CoV-2
264 shedders was figured from the actual number of reported cases in the metropolitan
265 Barcelona area ([https://salutweb.gencat.cat/ca/inici/nota-](https://salutweb.gencat.cat/ca/inici/nota-premsa/index.html?id=385948#googtrans(calen))
266 [premsa/index.html?id=385948#googtrans\(calen\)](https://salutweb.gencat.cat/ca/inici/nota-premsa/index.html?id=385948#googtrans(calen))). Since SARS-CoV-2 excretion in stool
267 has been reported to be variable and long-lasting (11, 27), we calculated the cumulative
268 number of symptomatic shedders at each given date considering all cases reported on

269 this date and in each of the previous seven (1-7) days, fourteen (1-14) days, and twenty-
270 one (1-21) days.

271 The total number of SARS-CoV-2 shedders (S), including asymptomatic,
272 presymptomatic and undiagnosed virus carriers as well, was figured applying a model
273 integrating the genome copy numbers per L of sewage (gc/L), the actual 24-h flow in L
274 corresponding to each assayed composite sample (F), and the mean genome copy
275 numbers per gram (gc/g) shed per infected patient.

$$276 \quad S = \text{gc/L} * F / (\text{gc/g stool} * \text{g stool}),$$

277 Genome copy numbers in sewage were determined using the same E-targeted
278 RT-qPCR assay developed at Charité, Berlin, employed for the quantification of the
279 genomes present in stool (11, 16). The number of genomes excreted per patient, was
280 figured by the product of the mean genome copy numbers excreted per gram of stool,
281 and the average daily wet weight (w/w) of feces. This latter amount was figured to be
282 380 g, based on an excretion of 30 g (w/w) per 5.5 Kg of body weight
283 (<https://www.emedicinehealth.com/>) assuming an average weight of the Spanish
284 population of 70 Kg (<https://www.mscbs.gob.es/estadEstudios/sanidadDatos/>), which
285 falls within the previously reported range (28, 29). Genome copies shed by patients has
286 been reported to range from less than 10^3 gc/g, to over 10^7 gc/g, depending on the time-
287 course of the infection, with higher titers during the first 10 days post symptom onset
288 (11). Based on these data, we assumed a fecal excretion of $10^{5.3}$ gc/g (average for the
289 first 10 days) or $10^{4.9}$ gc/g (average for the rest of the excretion period up to 21 days),
290 depending on whether the number of genomes detected in sewage was higher or lower
291 than $10^{2.5}$ gc/L (threshold established to discern between periods of high and low
292 excretion), respectively.

293 The authors certify that they will comply with ASM's Data Policy: Data will be made
294 publicly available upon publication and upon request for peer review.

295

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416 **FIGURE LEGENDS**

417 **FIG 1** Evolution of SARS-CoV-2 genomes in two large Barcelona wastewater
418 treatment plants (WWTP). Panels A and D: detection of the RNA-dependent RNA
419 polymerase gene (IP2 and IP4 primers). Panels B and E: detection of the envelope
420 protein gene (E primers). Panels C and F: detection of the nucleoprotein gene (N1
421 primers). Absence of values at a given date is due to the unavailability of aliquots to
422 assay. Dashed lines depict limits of detection. Red, orange and green arrows indicate
423 Phase 1, Phase 2 and Phase 3 of the deconfinement, respectively.

424

425 **FIG 2** Cumulated SARS-CoV-2 shedders associated to WWTP1 and WWTP2, figured
426 estimating fecal excretion periods of 7, 14 and 21 days, based on the actual number of
427 reported symptomatic cases.

428

429 **FIG 3** SARS-CoV-2 genome copy levels in grab samples from four urban sewers,
430 detected with targets IP2, IP4, E, N1 and N2. Sewer1 drains into WWTP1, while
431 sewer2, sewer3 and sewer4 drain into WWTP2.

432

433 **FIG 4** Estimation of the total number of SARS-CoV-2 infected shedders, including
434 symptomatic, presymptomatic, asymptomatic and undiagnosed cases. A model was
435 developed based on the genome copies at the wastewater treatment plants detected
436 during the first wave of the pandemic using the E target, the reported genome copies
437 excreted in feces figured also employing the E target (11), and considering the actual
438 daily wastewater flow.







