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

Gemma Chavarria-Miró[a], Eduard Anfruns-Estrada[a], Adán Martínez-Velázquez[a], Mario Vázquez-Portero[a], Susana Guix[a], Miquel Paraira[b], Belén Galofré[b], Gloria Sánchez[c], Rosa M. Pintó[a#*], Albert Bosch[a#*]

[a] Enteric Virus laboratory, Department of Genetics, Microbiology and Statistics, Section of Microbiology, Virology and Biotechnology, School of Biology, and Institute of Nutrition and Food Safety, University of Barcelona, Barcelona, Spain.

[b] Aigües de Barcelona, Barcelona, Spain.

[c] Department of Preservation and Food Safety Technologies, Institute of Agrochemistry and Food Technology, IATA-CSIC, Paterna, Valencia, Spain.

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

#Address correspondence to Rosa Pintó; rpinto@ub.edu; Albert Bosch, abosch@ub.edu.

[*] *Co-corresponding authors.*

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

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

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

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

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

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

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

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

Between June 2-8, SARS-CoV-2 genomes reappeared in both studied WWTPs and increased thereafter. All the RT-qPCR targets but the E target revealed this gradual raise (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-
145 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

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

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

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

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

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

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

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

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

Material and Methods

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

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

Nucleic acid extraction and virus quantification. Nucleic acid extraction was performed from 1mL of the concentrate and eluted in 50 µL using the NucliSENS® miniMAG® extraction system (bioMérieux).

Five one-step RT-qPCR assays (RNA UltraSense™ One-Step Quantitative RT-PCR System, Invitrogen, Life Technologies) were employed targeting the RNA-dependent RNA polymerase (RdRp) gene, IP2 and IP4 fragments, from Institute Pasteur, Paris (Institut Pasteur, Paris. Protocol: Real-time RT-PCR assays for the detection of SARS-CoV-2. 2020 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), the envelope protein (E) gene, E fragment, from Charité, Berlin (16), and the nucleoprotein (N), N1 and N2 fragments, from CDC, Atlanta (Centers for Disease Control and Prevention A. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. 2020 https://www.fda.gov/media/134922/download).

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

Quality control and quality assurance to determine any potential contamination and/or inhibition, were ascertained using negative and positive controls, respectively. Positive controls consisted of the addition of two distilled water samples containing $5x10^3$ copies of the Twist RNA, which were run in parallel in each RT-qPCR plate. Direct and 1/10 diluted replicas were assayed to ascertain assay inhibition. All quantitative assays were performed in duplicate, hence depicted genome copy numbers correspond to the mean of four values. Negative controls comprised five distilled water samples per run: two from the beginning of the assay, to control any potential contamination during the RNA extraction, and three in the RT-PCR, to control any potential contamination during nucleic acids amplification.

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

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

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

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

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

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

ACKNOWLEDGEMENTS

This research was supported in part by the REVEAL project, funded by SUEZ Spain. We thank our colleagues from Aigües de Barcelona, Cetaqua and Labaqua, who provided insight and expertise for the research. We are indebted with the Metropolitan Area of Barcelona (AMB) for a fruitful collaboration. We are grateful to M. Jané-Checa and A. Martínez-Mateo of the Public Health Agency of Catalonia (ASPCAT) for providing epidemiological data and useful discussion. We are indepted to Drs Luis Enjuanes and Isabel Sola (National Center of Biotechnology, Cantoblanco, Madrid) for providing us with the attenuated PUR46-MAD strain of transmissible gastroenteritis virus.

306 **References**

307 1. Pan Y, Zhang D, Yang P, Poon LLM, Wang Q. 2020. Viral load of SARS-CoV-
308 2 in clinical samples. Lancet Infect. Dis. 20:411-412.

309 2. Zhang J, Wang S, Xue Y. 2020. Fecal specimen diagnosis 2019 novel
310 coronavirus–infected pneumonia. J. Med. Virol. 92:680-682.

311 3. Medema G, Heijnen L, Elsinga G, Italiaander R, Brouwer A. 2020. Presence of
312 SARS-Coronavirus-2 in sewage. medRxiv:2020.03.29.20045880.

313 4. Lodder W, de Roda Husman AM. 2020. SARS-CoV-2 in wastewater: potential
314 health risk, but also data source. The Lancet Gastroenterol. Hepatol. 5:533-534.

315 5. Ahmed W, Angel N, Edson J, Bibby K, Bivins A, O'Brien JW, Choi PM,
316 Kitajima M, Simpson SL, Li J, Tscharke B, Verhagen R, Smith WJM, Zaugg J,
317 Dierens L, Hugenholtz P, Thomas KV, Mueller JF. 2020. First confirmed
318 detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of
319 concept for the wastewater surveillance of COVID-19 in the community. Sci.
320 Total Environ. 728:138764.

321 6. La Rosa G, Iaconelli M, Mancini P, Bonanno Ferraro G, Veneri C, Bonadonna
322 L, Lucentini L, Suffredini E. 2020. First detection of SARS-CoV-2 in untreated
323 wastewaters in Italy. Sci. Total Environ. 736:139652.

324 7. Randazzo W, Truchado P, Cuevas-Ferrando E, Simón P, Allende A, Sánchez G.
325 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a
326 low prevalence area. Wat. Res.181:115942-115942.

327 8. Fei X, Jing S, Yonghao X, Fang L, Xiaofang H, Heying L, Jingxian Z, Jicheng
328 H, Jincun Z. 2020. Infectious SARS-CoV-2 in Feces of Patient with Severe
329 COVID-19. Emerg. Infec. Dis. 26: 1920-1922.

9. Zang R, Castro MFG, McCune BT, Zeng Q, Rothlauf PW, Sonnek NM, Liu Z, Brulois KF, Wang X, Greenberg HB, Diamond MS, Ciorba MA, Whelan SPJ, Ding S. 2020. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. Science Immunol. 5:eabc3582.

10. Bullard J, Dust K, Funk D, Strong JE, Alexander D, Garnett L, Boodman C, Bello A, Hedley A, Schiffman Z, Doan K, Bastien N, Li Y, Van Caeseele PG, Poliquin G. 2020. Predicting infectious SARS-CoV-2 from diagnostic samples. Clin. Infec. Dis. 71: 2663–2666.

11. Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Muller MA, Niemeyer D, Jones TC, Vollmar P, Rothe C, Hoelscher M, Bleicker T, Brunink S, Schneider J, Ehmann R, Zwirglmaier K, Drosten C, Wendtner C. 2020. Virological assessment of hospitalized patients with COVID-2019. Nature 581:465-469.

12. Heaton KW, Radvan J, Cripps H, Mountford RA, Braddon FE, Hughes AO. 1992. Defecation frequency and timing, and stool form in the general population: a prospective study. Gut 33:818-24.

13. Artesi M, Bontems S, Göbbels P, Franckh M, Maes P, Boreux R, Meex C, Melin P, Hayette M-P, Bours V, Durkin K. 2020. A Recurrent Mutation at Position 26340 of SARS-CoV-2 Is Associated with Failure of the E Gene Quantitative Reverse Transcription-PCR Utilized in a Commercial Dual-Target Diagnostic Assay. J. Clin. Microbiol. 58:e01598-20.

14. Sims N, Kasprzyk-Hordern B. 2020. Future perspectives of wastewater-based epidemiology: Monitoring infectious disease spread and resistance to the community level. Environ. Int. 139:105689.

15. Barras C. 2018. Going to waste. Nature Med. 24:1484-1487.

16. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T, Brunink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MP, Drosten C. 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 25:2000045.

17. Tokars JI, Olsen SJ, Reed C. 2017. Seasonal Incidence of Symptomatic Influenza in the United States. Clin. Infec. Dis. 66:1511-1518.

18. Pollan M, Perez-Gomez B, Pastor-Barriuso R, Oteo J, Hernan MA, Perez-Olmeda M, Sanmartin JL, Fernandez-Garcia A, Cruz I, Fernandez de Larrea N, Molina M, Rodriguez-Cabrera F, Martin M, Merino-Amador P, Leon Paniagua J, Munoz-Montalvo JF, Blanco F, Yotti R. 2020. Prevalence of SARS-CoV-2 in Spain (ENE-COVID): a nationwide, population-based seroepidemiological study. Lancet 396:535-544.

19. Havers FP, Reed C, Lim T, Montgomery JM, Klena JD, Hall AJ, Fry AM, Cannon DL, Chiang CF, Gibbons A, Krapiunaya I, Morales-Betoulle M, Roguski K, Rasheed MAU, Freeman B, Lester S, Mills L, Carroll DS, Owen SM, Johnson JA, Semenova V, Blackmore C, Blog D, Chai SJ, Dunn A, Hand J, Jain S, Lindquist S, Lynfield R, Pritchard S, Sokol T, Sosa L, Turabelidze G, Watkins SM, Wiesman J, Williams RW, Yendell S, Schiffer J, Thornburg NJ. 2020. Seroprevalence of Antibodies to SARS-CoV-2 in 10 Sites in the United States, March 23-May 12, 2020. JAMA Intern Med. 2020 Jul 21. doi: 10.1001/jamainternmed.2020.4130.

20. Hart OE, Halden RU. 2020. Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally:

Feasibility, economy, opportunities and challenges. Sci. Total Environ. 730:138875.

21. Coma E, Mora N, Prats-Uribe A, Fina F, Prieto-Alhambra D, Medina-Peralta M. 2020. Excess cases of influenza suggest an earlier start to the coronavirus epidemic in Spain than official figures tell us: an analysis of primary care electronic medical records from over 6 million people from Catalonia. medRxiv:2020.04.09.20056259.

22. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, Tan TY, Ng TY, Cui L, Said Z, Kurupatham L, Chen MI, Chan M, Vasoo S, Wang LF, Tan BH, Lin RTP, Lee VJM, Leo YS, Lye DC. 2020. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. JAMA 323:1488-1494.

23. Lauer SA, Grantz KH, Bi Q, Jones FK, Zheng Q, Meredith HR, Azman AS, Reich NG, Lessler J. 2020. The Incubation Period of Coronavirus Disease 2019 (COVID-19) From Publicly Reported Confirmed Cases: Estimation and Application. Ann. Intern. Med. 172:577-582.

24. Wang Y, Kang H, Liu X, Tong Z. 2020. Asymptomatic cases with SARS-CoV-2 infection. J. Med. Virol. 92:1401-1403.

25. Blanco A, Abid I, Al-Otaibi N, Perez-Rodriguez FJ, Fuentes C, Guix S, Pinto RM, Bosch A. 2019. Glass Wool Concentration Optimization for the Detection of Enveloped and Non-enveloped Waterborne Viruses. Food Environ. Virol. 11:184-192.

403 26. Sanchez CM, Jimenez G, Laviada MD, Correa I, Sune C, Bullido M, Gebauer F,
404 Smerdou C, Callebaut P, Escribano JM, et al. 1990. Antigenic homology among
405 coronaviruses related to transmissible gastroenteritis virus. Virology 174:410-7.
406 27. Wu Y, Guo C, Tang L, Hong Z, Zhou J, Dong X, Yin H, Xiao Q, Tang Y, Qu X,
407 Kuang L, Fang X, Mishra N, Lu J, Shan H, Jiang G, Huang X. 2020. Prolonged
408 presence of SARS-CoV-2 viral RNA in faecal samples. Lancet Gastroenterol.
409 Hepatol. 5:434-435.
410 28. Penn R, Ward BJ, Strande L, Maurer M. 2018. Review of synthetic human
411 faeces and faecal sludge for sanitation and wastewater research. Wat. Res.
412 132:222-240.
413 29. Rose C, Parker A, Jefferson B, Cartmell E. 2015. The Characterization of Feces
414 and Urine: A Review of the Literature to Inform Advanced Treatment
415 Technology. Crit. Rev. Environ. Sci. Technol. 45:1827-1879.

FIGURE LEGENDS

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

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

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

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