SARS-CoV-2 RNA titers in wastewater anticipated COVID-19 occurrence in a low prevalence area

Walter Randazzo[1,2], Pilar Truchado[3], Enric Cuevas-Ferrando[2], Pedro Simón[4], Ana Allende[3], Gloria Sánchez[2*]

[1]Department of Microbiology and Ecology, University of Valencia, Av. Dr. Moliner, 50, Burjassot, 46100 Valencia, Spain;

[2]Department of Preservation and Food Safety Technologies, Institute of Agrochemistry and Food Technology, IATA-CSIC, Av. Agustín Escardino 7, Paterna, 46980, Valencia, Spain;

[3]Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, Campus Universitario de Espinardo, 25, 30100, Murcia, Spain;

[5]ESAMUR, Avenida Juan Carlos, s/n - Edificio Torre Jemeca, Murcia, Spain.

Running title: First detection of SARS-CoV-2 in untreated wastewater in Spain.

*Correspondence to: Gloria Sánchez, gloriasanchez@iata.csic.es.

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 200,000 reported COVID-19 cases in Spain resulting in more than 20,800 deaths as of April 21, 2020. Faecal shedding of SARS-CoV-2 RNA from COVID-19 patients has extensively been reported. Therefore, we investigated the occurrence of SARS-CoV-2 RNA in six wastewater treatments plants (WWTPs) serving the major municipalities within the Region of Murcia (Spain), a low prevalence area. Firstly, an aluminum hydroxide adsorption-precipitation concentration method was tested using a porcine coronavirus (Porcine Epidemic Diarrhea Virus, PEDV) and mengovirus (MgV). The procedure resulted in average recoveries of 10.90 ± 3.54% and 10.85 ± 2.11% in influent water and 3.29 ± 1.58% and 6.19 ± 1.00% in effluent water samples for PEDV and MgV, respectively. Then, the method was used to monitor the occurrence of SARS-CoV-2 from March 12 to April 14, 2020 in influent, secondary and tertiary effluent water samples. By using the real-time RT-PCR (RT-qPCR) Diagnostic Panel validated by US CDC that targets three regions of the virus nucleocapsid (N) gene, we estimated quantification of SARS-CoV-2 RNA titers in untreated wastewater waters of 5.29 log genomic copies/L on average. Moreover, we tested as negative all secondary and tertiary treated water samples, highlighting that current water disinfection treatments applied in the analyzed WWTP are able to remove SARS-CoV-2 RNA.

This environmental surveillance data were compared to declared COVID-19 cases at municipality level, revealing that SARS-CoV-2 was circulating among the population even before the first cases were reported by local or national authorities in many of the cities where wastewaters have been sampled. The detection of SARS-CoV-2 in wastewater in early stages of the spread of COVID-19 highlights the relevance of this strategy as an early indicator of the infection within a specific population. At this point,

45 this environmental surveillance could be implemented by municipalities right away as a
46 tool, designed to help authorities to coordinate the exit strategy to gradually lift its
47 coronavirus lockdown.

48

49 **Keywords:** environmental surveillance, influent water, effluent water, reclaimed water,
50 concentration protocol, RNA virus.

1. Introduction

Coronaviruses (CoVs) are a family of viruses pathogenic for humans and animals associated to respiratory and gastro-intestinal infections. CoVs used to be considered as minor pathogens for humans as they were responsible of common cold or mild respiratory infections in immunocompetent people. Nonetheless, the emergence of novel and highly pathogenic zoonotic diseases caused by CoVs such as Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS) and most recently SARS-CoV-2 arises questions to be addressed to guide public health response.

CoVs are mainly transmitted through respiratory droplets. However, as for SARS and MERS, SARS-CoV-2 RNA has been detected in stool samples from patients suffering COVID-19 and from asymptomatic carriers (1–5). The duration of viral shedding has been observed to vary among patients with means of 14-21 days (6, 7), as well as the magnitude of shedding varies from 10^2 up to 10^8 RNA copies per gram (1, 2, 8).

Infectious viruses deriving from fecal specimen have been cultured in Vero E6 cells and observed by electron microscopy (9). In addition, gastric, duodenal, and rectal epithelial cells are infected by SARS-CoV-2 and the release of the infectious virions to the gastrointestinal tract supports the possible fecal-oral transmission route (10). Even though the possibility of faecal-oral transmission has been hypothesized, the role of secretions in the spreading of the disease is not clarified yet (6, 7, 9, 11).

Wastewater monitoring has been a successful strategy pursued to track chemical and biological markers of human activity including illicit drugs consumption, pharmaceuticals use/abuse, water pollution, and occurrence of antimicrobial resistance genes (12–15). Viral diseases have been also surveilled by the detection of genetic material into wastewater as for enteric viruses (16–18), re-emerging zoonotic hepatitis E virus (19, 20), and poliovirus during the global eradication programme (21).

Currently, various studies detected SARS-CoV-2 RNA in wastewater worldwide (22–26), and wastewater testing has been suggested as a non-invasive early-warning tool for monitoring the status and trend of COVID-19 infection and as an instrument for tuning public health response (27–29). Under current circumstance, this environmental surveillance could be implemented in wastewater treatment plants as a tool, designed to help authorities to coordinate the exit strategy to gradually lift its coronavirus lockdown. Here, we report the first detection of SARS-CoV-2 RNA in untreated wastewater samples in Spain collected from six different wastewater treatment plants (WWTPs) in Murcia, the lowest prevalence area in Iberian Peninsula. Additionally, the efficacy of the secondary and tertiary treatments implemented in the WWPTs against SARS-CoV-2 has been confirmed. The outcomes of the environmental surveillance reflect the epidemiological data in a low COVID-19 diagnosed cases setting, thus supporting the need of developing and implementing advanced models for wastewater-based epidemiology (WBE).

2. Material and methods

2.1. Sampling sites and samples collection

Influent and effluent water samples were collected from six WWTPs located in the main cities of the Region of Murcia, Spain (Figure 1). Technical data on WWTPs are provided in Table 1.

A total of 42 influent, and 18 secondary and 12 tertiary treated effluent water samples were collected from 12 March to 14 April 2020 and investigated for the occurrence of SARS-CoV-2 RNA. Collected samples were transferred on ice to the laboratory and concentrated on the same day of sampling or the day after.

2.2. Wastewater and effluent water concentration

The porcine epidemic diarrhea virus (PEDV) strain CV777 and the mengovirus (MgV) vMC0 (CECT 100000) were preliminary used to evaluate the aluminum hydroxide adsorption-precipitation method previously described for concentrating enteric viruses from wastewater and effluent water (30, 31). In brief, 200 mL of biobanked influent (n=2) and effluent water samples (n=2) were artificially inoculated with PEDV and MgV. Then pH was adjusted to 6.0 and $Al(OH)_3$ precipitate formed by adding 1 part 0.9N $AlCl_3$ (Acros organics, Geel, Belgium) solution to 100 parts of sample. The pH was readjusted to 6.0 and sample mixed using an orbital shaker at 150 rpm for 15 min at room temperature. Then, viruses were concentrated by centrifugation at 1,700 × g for 20 min. The pellet was resuspended in 10 mL of 3% beef extract pH 7.4, and samples were shaken for 10 min at 150 rpm. Concentrate was recovered by centrifugation at 1,900 × g for 30 min and pellet resuspended in 1 mL of PBS.

All wastewater and effluent water samples included in this study were processed as described and MgV (5 log PCRU) was spiked as process control.

2.3. Viral extraction, detection and quantification

Viral RNA was extracted from concentrates using the NucleoSpin RNA virus kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions with some modifications. Briefly, 150 µL of the concentrated sample was mixed with 25 µL of Plant RNA Isolation Aid (Thermo Fisher Scientific, Vilnius, Lithuania) and 600 µL of lysis buffer from the NucleoSpin virus kit and subjected to pulse-vortexing for 1 min. Then, the homogenate was centrifuged for 5 min at 10,000 × g to remove the debris. The supernatant was subsequently processed according to the manufacturer's instructions.

Viral RNA was detected by TaqMan real-time RT-PCR (RT-qPCR) on LightCycler 480 instrument (Roche Diagnostics, Germany). MgV RNA was detected by using UltraSense One-Step kit (Invitrogen, SA, US) and the RT-qPCR assay as in ISO 15216-1:2017 (32, 33). Undiluted and ten-fold diluted MgV RNA was tested to check for RT-qPCR inhibitors.

PEDV RNA was detected by using PrimeScript™ One Step RT-PCR Kit (Takara Bio, USA) and the TaqMan RT-qPCR assay described by (34).

SARS-CoV-2 RNA was detected by using PrimeScript™ One Step RT-PCR Kit and the RT-qPCR diagnostic panel assays validated by the US Centers for Disease Control and Prevention (35). The first version of the kit with three sets of oligonucleotide primers and probes was used to target three different SARS-CoV-2 regions of the nucleocapsid (N) gene. The sets of primer and probes (2019-nCoV RUO Kit) as well as the positive control (2019-nCoV_N_Positive Control) were provided by IDT (Integrated DNA Technologies, Leuven, Belgium). Each RNA was analyzed in duplicate and every RT-qPCR assay included negative (nuclease-free water) and positive controls.

Biobanked samples (n=4) collected in October 2019, before the first COVID-19 case was documented, were used as relevant negative control to exclude false positive reactions. SARS-CoV-2 RNA was quantified as genome copies (gc) by plotting the quantification cycles (Ct) to an external standard curve built with 10-fold serial dilution of a quantified plasmid control (IDT). MgV and PEDV RNA were quantified by plotting the Cts to an external standard curve generated by serial end-point dilution method.

MgV recovery rates were calculated and used as quality assurance parameters according to ISO 15216-1:2017 (33).

3. Results and Discussion

3.1. Performance of the concentration method

The aluminum hydroxide adsorption-precipitation method was tested by spiking influent and effluent samples with MgV and PEDV. On average, MgV was recovered at ranges of 10.85 ± 2.11% in influent and 6.19 ± 1.00% in effluent water. PEDV was recovered at ranges of 10.90 ± 3.54% in influent and 3.29 ± 1.58% in effluent water. These results are in line with the MgV recoveries reported for enteric viruses concentration in water samples by the same aluminum-based method (19, 30) and higher than the 1% as the quality assurance parameter indicated for bottled water into ISO 15216-1:2017 (33).

Similarly, MgV was successfully used as recovery control for hepatitis E virus concentration from influent and effluent water samples (5-13%) by applying a polyethylene glycol (PEG) precipitation method (20). A similar PEG-based protocol was recently used to recover SARS-CoV-2 from wastewater, although recovery control was not included in the study (23).

Moreover, filtration through 10 kDa Centricon® Plus-70 centrifugal device successfully recovered SARS-CoV-2 in wastewater with recovery efficiencies of F-specific RNA

phages of 73% (22). However, concentration by electropositive membrane should be further evaluated given a SARS-CoV recovery from wastewater of 1% (36).

Rigorous limits of detection should be established by spiking SARS-CoV-2 cell-culture adapted strain or positive COVID-19 fecal samples in influent and effluent wastewater samples to be concentrated following the aluminum hydroxide adsorption-precipitation method. Nonetheless, the need of a BSL3 laboratory facility to handle SARS-CoV-2 represents the main limitation of this experiment.

3.2. SARS-CoV-2 titers in wastewater and effluent water

A total of 42 influent, and 18 secondary and 12 tertiary treated effluent water samples were collected from 12 March to 14 April 2020 and investigated for the occurrence of SARS-CoV-2 RNA. Samples were considered positive for Ct below 40 (as in Medema et al., 2020 and F. Wu et al., 2020) and titrated by using the quantified plasmid control for each of the RT-qPCR targets.

The 85.7% (36 positive samples out of 42) influent samples were tested positive for at least one SARS-CoV-2 RT-qPCR target. None (0 out of 30) of the secondary and tertiary effluent samples tested positive for any of the SARS-CoV-2 RT-qPCR target (Figure 2). A relevant number of influent water samples (12%) showed Ct ranging between 37 and 40, even though lower Ct of 34-37 were observed (29%). In all samples, MgV recoveries were above 1% (10.05 ± 14.10%). Detection of SARS-CoV-2 RNA in influent water has been reported worldwide (22–24, 26), and only one study tested treated wastewater that resulted positive (Paris) (25).

On average, SARS-CoV-2 RNA titers of 5.15 ± 0.25, 5.53 ± 0.24, and 5.49 ± 0.27 log gc/L were quantified in wastewater by using N1, N2 and N3 primer/probe mixes,

189 respectively. Titer of 4 and 5 to more than 6 log gc/L have been reported in Massachusetts
190 and France, respectively (23, 25).
191 We observed discrepancies among RT-qPCR N1, N2 and N3 assays for several water
192 samples in agreement to a previous report (22). This could be due to the different
193 analytical sensitivity among the assays as well as the detection of possible false positive
194 samples by RT-qPCR N3 in low concentrated samples (37, 38). The latter possibility has
195 been solved by excluding the N3 primers/probe set from the US CDC 2019-nCoV RT-
196 qPCR diagnostic panel in its last revision (March, 30) (39). In addition, a partial inhibitory
197 effect of the matrix is not to be completely excluded despite the controls included in the
198 assays. A more sensitive estimation of SARS-CoV-2 loads in wastewater should be
199 studied by digital RT-qPCR (dRT-qPCR). dRT-qPCR could be used to quantify samples
200 with low viral loads, even though it may not be the best practical and economically
201 sustainable option for environmental surveillance.
202 Even though the SARS-CoV-2 RNA detection in wastewater is functional for WBE
203 purposes, the risk for human health associated to the water cycle is still under debate as
204 infectivity of viral particles remain to be confirmed as well as its potential fecal-oral
205 transmission.
206 In spite of the high concentration of viral RNA in specimen and the evidence of
207 gastrointestinal infection (10), infectious viruses from stools have been isolated in one
208 study (9) while another attempt resulted without success (2).
209 The potential transmission of SARS-CoV-2 via wastewater has not been proven (40, 41)
210 and it seems unlikely given the poor viral stability in environmental conditions and its
211 elevated sensitivity to disinfectants (26, 42, 43).
212

213 **3.3. Environmental surveillance**

Epidemiological data on COVID-19 in the Murcia Region have been retrieved from the publically available repository of the "Servicio de epidemiologia" of the "Consejería de Salud de la Región de Murcia" (available at http://www.murciasalud.es/principal.php) (Table 3) and plotted to the SARS-CoV-2 RNA mean loads as detected by three RT-qPCR assays (Figure 2).

In general, RT-qPCR amplification signals have been detected in wastewaters when cases were diagnosed within the municipality. Positive wastewater samples have been detected with at least two out of three RT-qPCR assays in low prevalence municipalities as in Murcia (96 cases, 21.18 cases per 100,000 inhabitants), Cartagena (36 cases, 16.76) and Molina de Segura (12 cases, 16.69). Of note, positive wastewater samples were detected 12-16 days before COVID-19 cases were declared in Lorca, Cieza and Totana municipalities.

A similar study conducted in Paris (France) demonstrated the detection of viral genome before the exponential phase of the epidemic (25). However, our results indicate that SARS-CoV-2 can be detected weeks before the first confirmed case. The early detection of SARS-CoV-2 RNA in wastewater could have alerted about the imminent danger, giving a valuate time to the managers to coordinate and implement actions to slow the spread of the disease. Therefore, our outcomes support that WBE could be used as an early warning tool to monitor the status and the trend of COVID-19 infection within a community.

On the other hand, we believe that this environmental surveillance could be used as an instrument to drive the right decisions to reduce the risk of lifting restrictions too early. In fact, a very important question is to determine what needs to be implemented to have reliable data to reduce the risk of a "second wave". Massive population tests are the first choice, but in their absence, wastewater monitorization of SARS-CoV-2 RNA can give a

239 reliable picture of the current situation. Our wastewater data do not quantitatively
240 resemble the prevalence of COVID-19 confirmed cases. To this end, a quantitative model
241 that includes and corrects all the variables affecting these wastewater surveillance data
242 would be useful for a better interpretation. For instance, not all COVID-19 positive
243 patients excrete SARS-CoV-2 RNA in faeces, and when it occurs, the titers and the
244 duration of shedding vary among individuals and across time (1, 2, 5, 7). On the other
245 hand, the real number of positive cases within the Murcia Region remains unknown
246 because of the large number of mild or asymptomatic carriers that have not been included
247 in epidemiological statistics.
248 These aspects together with environmental variables (e.g., rainfall events, temperature)
249 increase the uncertainties linked to the correlation between SARS-CoV-2 RNA detection
250 in wastewater samples and the prevalence of COVID-19 that could be explored by using
251 complex models.
252 In conclusions, wastewater surveillance and WBE may represent a complementary
253 approach to estimate the presence and even the prevalence of COVID-19 in communities.
254 This represents an effective tool that needs to be further explored in order to direct public
255 health response, especially in cases of limited capacity for clinical testing.
256

257 **Funding**

258 The study was funded by the projects 20180705 of ESAMUR, 202070E101 of CSIC and
259 "VIRIDIANA" AGL2017-82909 (AEI/FEDER, UE) of MICIU. WR is supported by
260 APOSTD/2018/150 postdoctoral fellowship of Generalitat Valenciana. EC-F is recipient
261 of a predoctoral contract from the MICINN, Call 2018. PT is holder of the RYC2018-
262 025510-I Ramón y Cajal contract from the MICIU.
263

Acknowledgments

The authors acknowledge the "Entidad Regional de Saneamiento y Depuración de Aguas Residuales (ESAMUR)" for authorizing the sampling and Prof. Ana Carvajal (Faculty of Veterinary Medicine, University of Leon, Spain) for kindly providing PEDV.

References

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

2. Woelfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Mueller MA, Niemeyer D, Vollmar P, Rothe C, Hoelscher M, Bleicker T, Bruenink S, Schneider J, Ehmann R, Zwirglmaier K, Drosten C, Wendtner C. 2020. Clinical presentation and virological assessment of hospitalized cases of coronavirus disease 2019 in a travel-associated transmission cluster. medRxiv 2020.03.05.20030502.

3. Young BE, Ong SWX, Kalimuddin S, Low JG, Tan SY, Loh J, Ng O-T, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, Tan TY, Ng TY, Cui L, Said Z, Kurupatham L, Chen MI-C, Chan M, Vasoo S, Wang L-F, Tan BH, Lin RTP, Lee VJM, Leo Y-S, Lye DC, Team for the S 2019 NCOR. 2020. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. JAMA.

4. Zhang W, Du R-H, Li B, Zheng X-S, Yang X-L, Hu B, Wang Y-Y, Xiao G-F, Yan B, Shi Z-L, Zhou P. 2020. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. Emerg Microbes Infect 9:386–389.

5. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, Lau YC, Wong JY, Guan Y, Tan X, Mo X, Chen Y, Liao B, Chen W, Hu F, Zhang Q, Zhong M, Wu Y, Zhao L, Zhang F, Cowling BJ, Li F, Leung GM. 2020. Temporal dynamics in viral shedding and transmissibility of COVID-19. Nat Med.

6. Wu Y, Guo C, Tang L, Hong Z, Zhou J, Dong X, Yin H, Xiao Q, Tang Y, Qu X, Kuang L, Fang X, Mishra N, Lu J, Shan H, Jiang G, Huang X. 2020. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. Lancet Gastroenterol Hepatol 5:434–435.

7. Xu Y, Li X, Zhu B, Liang H, Fang C, Gong Y, Guo Q, Sun X, Zhao D, Shen J, Zhang H, Liu H, Xia H, Tang J, Zhang K, Gong S. 2020. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. Nat Med 26:502–505.

8. Lescure F-X, Bouadma L, Nguyen D, Parisey M, Wicky P-H, Behillil S, Gaymard A, Bouscambert-Duchamp M, Donati F, Le Hingrat Q, Enouf V, Houhou-Fidouh N, Valette M, Mailles A, Lucet J-C, Mentre F, Duval X, Descamps D, Malvy D, Timsit J-F, Lina B, van-der-Werf S, Yazdanpanah Y. 2020. Clinical and virological data of the first cases of COVID-19 in Europe: a case series. Lancet Infect Dis.

9. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. 2020. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA.

10. Xiao F, Tang M, Zheng X, Li C, He J, Hong Z, Huang S, Zhang Z, Lin X, Fang Z, Lai R, Chen S, Liu J, Huang J, Xia J, Li Z, Jiang G, Liu Y, Li X, Shan H. 2020. Evidence for gastrointestinal infection of SARS-CoV-2. medRxiv 2020.02.17.20023721.

11. Yeo C, Kaushal S, Yeo D. 2020. Enteric involvement of coronaviruses: is faecal–oral transmission of SARS-CoV-2 possible? Lancet Gastroenterol Hepatol 5:335–337.

12. Choi PM, Tscharke BJ, Donner E, O'Brien JW, Grant SC, Kaserzon SL, Mackie R, O'Malley E, Crosbie ND, Thomas K V, Mueller JF. 2018. Wastewater-based epidemiology biomarkers: Past, present and future. TrAC - Trends Anal Chem.

13. Lorenzo M, Picó Y. 2019. Wastewater-based epidemiology: current status and future prospects. Curr Opin Environ Sci Heal.

14. Mercan S, Kuloglu M, Asicioglu F. 2019. Monitoring of illicit drug consumption via wastewater: development, challenges, and future aspects. Curr Opin Environ Sci Heal.

15. de Oliveira M, Frihling BEF, Velasques J, Filho FJCM, Cavalheri PS, Migliolo L. 2020. Pharmaceuticals residues and xenobiotics contaminants: Occurrence, analytical techniques and sustainable alternatives for wastewater treatment. Sci Total Environ.

16. Prevost B, Lucas FS, Goncalves A, Richard F, Moulin L, Wurtzer S. 2015. Large scale survey of enteric viruses in river and waste water underlines the health status of the local population. Environ Int 79:42–50.

17. Santiso-Bellón C, Randazzo W, Pérez-Cataluña A, Vila-Vicent S, Gozalbo-Rovira R, Muñoz C, Buesa J, Sanchez G, Díaz JR. 2020. Epidemiological surveillance of norovirus and rotavirus in sewage (2016–2017) in Valencia (Spain). Microorganisms.

18. Hellmer M, Paxeus N, Magnius L, Enache L, Arnholm B, Johansson A, Bergstrom T, Norder H. 2014. Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks. Appl Environ Microbiol 80:6771–6781.

19. Cuevas-Ferrando E, Randazzo W, Pérez-Cataluña A, Sánchez G. 2020. HEV Occurrence in Waste and Drinking Water Treatment Plants . Front Microbiol .

20. Miura T, Lhomme S, Le Saux J-C, Le Mehaute P, Guillois Y, Couturier E, Izopet J, Abranavel F, Le Guyader FS. 2016. Detection of Hepatitis E Virus in Sewage After an Outbreak on a French Island. Food Environ Virol 8:194–9.

21. Asghar H, Diop OM, Weldegebriel G, Malik F, Shetty S, El Bassioni L, Akande AO, Al Maamoun E, Zaidi S, Adeniji AJ, Burns CC, Deshpande J, Oberste MS, Lowther SA. 2014. Environmental surveillance for polioviruses in the Global Polio Eradication Initiative. J Infect Dis 210 Suppl:S294-303.

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

23. Wu F, Xiao A, Zhang J, Gu X, Lee WL, Kauffman K, Hanage W, Matus M, Ghaeli N, Endo N, Duvallet C, Moniz K, Erickson T, Chai P, Thompson J, Alm E. 2020. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. medRxiv 2020.04.05.20051540.

24. Ahmed W, Angel N, Edson J, Bibby K, Bivins A, O'Brien JW, Choi PM, Kitajima M, Simpson SL, Li J, Tscharke B, Verhagen R, Smith WJM, Zaugg J,

Dierens L, Hugenholtz P, Thomas K V, Mueller JF. 2020. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. Sci Total Environ 138764.

25. Wurtzer S, Marechal V, Mouchel J-M, Moulin L. 2020. Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlates with COVID-19 confirmed cases. medRxiv 2020.04.12.20062679.

26. Lodder W, de Roda Husman AM. 2020. SARS-CoV-2 in wastewater: potential health risk, but also data source. Lancet Gastroenterol Hepatol.

27. Daughton C. 2020. The international imperative to rapidly and inexpensively monitor community-wide Covid-19 infection status and trends. Sci Total Environ 138149.

28. Mallapaty S. 2020. How sewage could reveal true scale of coronavirus outbreak. Nature.

29. Naddeo V, Liu H. 2020. Editorial Perspectives: 2019 novel coronavirus (SARS-CoV-2): what is its fate in urban water cycle and how can the water research community respond? Environ Sci Water Res Technol.

30. Randazzo W, Piqueras J, Evtoski Z, Sastre G, Sancho R, Gonzalez C, Sanchez G. 2019. Interlaboratory Comparative Study to Detect Potentially Infectious Human Enteric Viruses in Influent and Effluent Waters. Food Environ Virol.

31. AAVV. 2011. Standard Methods For the Examination of Water and Wastewater (9510)Detection of enteric viruses. American Public Health Association.

32. Costafreda MI, Bosch A, Pintó RM. 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl Environ Microbiol 72:3846–3855.

33. ISO 15216-1. 2017. Microbiology of the Food Chain – Horizontal Method for Determination of Hepatitis A Virus and Norovirus Using Real-Time RT-PCR – Part 1: Method for Quantification. ISO 15216-1:2017.

34. Zhou X, Zhang T, Song D, Huang T, Peng Q, Chen Y, Li A, Zhang F, Wu Q, Ye Y, Tang Y. 2017. Comparison and evaluation of conventional RT-PCR, SYBR green I and TaqMan real-time RT-PCR assays for the detection of porcine epidemic diarrhea virus. Mol Cell Probes 33:36–41.

35. CDC. 2020. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel.

36. Wang X-W, Li J-S, Jin M, Zhen B, Kong Q-X, Song N, Xiao W-J, Yin J, Wei W, Wang G-J, Si B-Y, Guo B-Z, Liu C, Ou G-R, Wang M-N, Fang T-Y, Chao F-H, Li J-W. 2005. Study on the resistance of severe acute respiratory syndrome-associated coronavirus. J Virol Methods 126:171–177.

37. Jung YJ, Park G-S, Moon JH, Ku K, Beak S-H, Kim S, Park EC, Park D, Lee J-H, Byeon CW, Lee JJ, Maeng J-S, Kim SJ, Kim S Il, Kim B-T, Lee MJ, Kim HG. 2020. Comparative analysis of primer-probe sets for the laboratory confirmation of SARS-CoV-2. bioRxiv 2020.02.25.964775.

38. Vogels CBF, Brito AF, Wyllie AL, Fauver JR, Ott IM, Kalinich CC, Petrone ME, Casanovas-Massana A, Muenker MC, Moore AJ, Klein J, Lu P, Lu-Culligan A, Jiang X, Kim DJ, Kudo E, Mao T, Moriyama M, Oh JE, Park A, Silva J, Song E, Takehashi T, Taura M, Tokuyama M, Venkataraman A, Weizman O-E, Wong P, Yang Y, Cheemarla NR, White E, Lapidus S, Earnest R, Geng B, Vijayakumar P, Odio C, Fournier J, Bermejo S, Farhadian S, Dela Cruz C, Iwasaki A, Ko AI, Landry M-L, Foxman EF, Grubaugh ND. 2020. Analytical sensitivity and efficiency comparisons of SARS-COV-2 qRT-PCR assays. medRxiv 2020.03.30.20048108.

39. CDC. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel.

40. WHO WHO. 2020. Water, sanitation, hygiene and waste management for COVID-19.

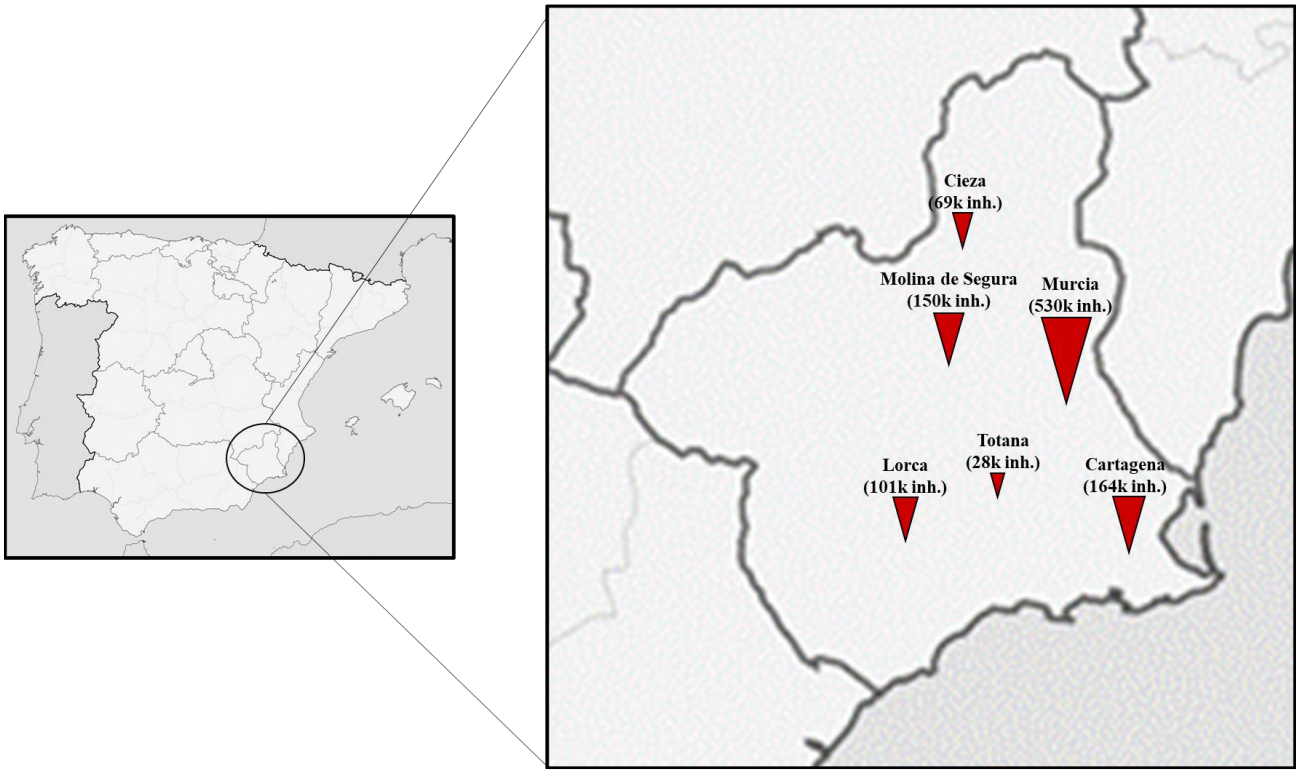
41. CDC. Water Transmission and COVID-19: Questions and Answers.

42. Chin AWH, Chu JTS, Perera MRA, Hui KPY, Yen H-L, Chan MCW, Peiris M, Poon LLM. 2020. Stability of SARS-CoV-2 in different environmental conditions. The Lancet Microbe.

43. Haas C. 2020. Coronavirus and Risk Analysis. Risk Anal 40:660–661.

417 **Figure 1.** Maps of the sampling location. Symbols represents WWTPs and are sized

418 according to the number of equivalent inhabitants.



419

420 **Table 1.** Operating characteristics of WWTPs.

	Population		**Capacity (m^3/a)**		**Reclamation processes**	**Reuse**
	Connected	Equivalent	Designed	Current		
Murcia	370,893	530,499	36,500,000	36,952,999	Activated sludge (A2O process), Disinfection, NaClO	Public domain
Cartagena	175,870	163,969	12,775,000	8,625,103	Activated sludge, Disinfection	Irrigation
Molina de Segura	67,455	150,545	9,125,000	5,699,930	Activated sludge, Decantation, Coagulation, Flocculation, Sand filtration, Disinfection, UV, NaClO	Irrigation
Lorca	73,057	101,161	7,300,000	3,366,919	Activated sludge, Coagulation, Flocculation, Sand filtration, Disinfection, UV, NaClO	Irrigation
Cieza	33,744	69,502	3,650,000	2,338,673	Activated sludge (Extended aeration), Disinfection, Coagulation, Flocculation, Sand filtration, Disinfection, UV	Irrigation
Totana	29,113	28,289	2,190,000	1,440,463	Activated sludge (Extended aeration), Disinfection, UV	Irrigation

421

Figure 2. Mean amplification cycles of SARS-CoV-2 RNA in influent, secondary and tertiary effluent waters in monitored WWTPs within Murcia Region (Spain). Results are reported for each of the three regions of the virus nucleocapsid (N) gene according to the first version of the Real-Time RT-PCR Diagnostic Panel by US CDC. Abbreviations: -, negative; white boxes, not tested.

Sampling site	Water sample	Molecular Target	12 March	16 March	18 March	26 March	02 April	07 April	14 April
Murcia	Influent	N1	-		-				
	Influent	N2	-		-		-		-
	Influent	N3	-	-			-		
	Secundary treated	N1, N2, N3					-	-	-
	Tertiary treated	N1, N2, N3					-	-	-
Cartagena	Influent	N1	-	-	-			-	
	Influent	N2	-	-	-			-	
	Influent	N3	-			-			
	Secundary treated	N1, N2, N3					-	-	-
Molina de Segura	Influent	N1	-	-	-	-			
	Influent	N2			-	-			
	Influent	N3		-					
	Secundary treated	N1, N2, N3					-	-	-
	Tertiary treated	N1, N2, N3					-	-	-
Lorca	Influent	N1	-	-		-			
	Influent	N2		-	-			-	
	Influent	N3		-		-			
	Secundary treated	N1, N2, N3					-	-	-
	Tertiary treated	N1, N2, N3					-	-	-
Cieza	Influent	N1	-			-	-	-	-
	Influent	N2	-						
	Influent	N3	-				-		-
	Secundary treated	N1, N2, N3					-	-	-
	Tertiary treated	N1, N2, N3					-	-	-
Totana	Influent	N1	-		-				-
	Influent	N2	-		-	-		-	-
	Influent	N3	-		-			-	-
	Secundary treated	N1, N2, N3					-	-	-

Ct scale: 34 | 35 | 36 | 37 | 38 | 39 | 40

Table 2. Epidemiological data[1] summary of COVID-19 cases in the area of study.

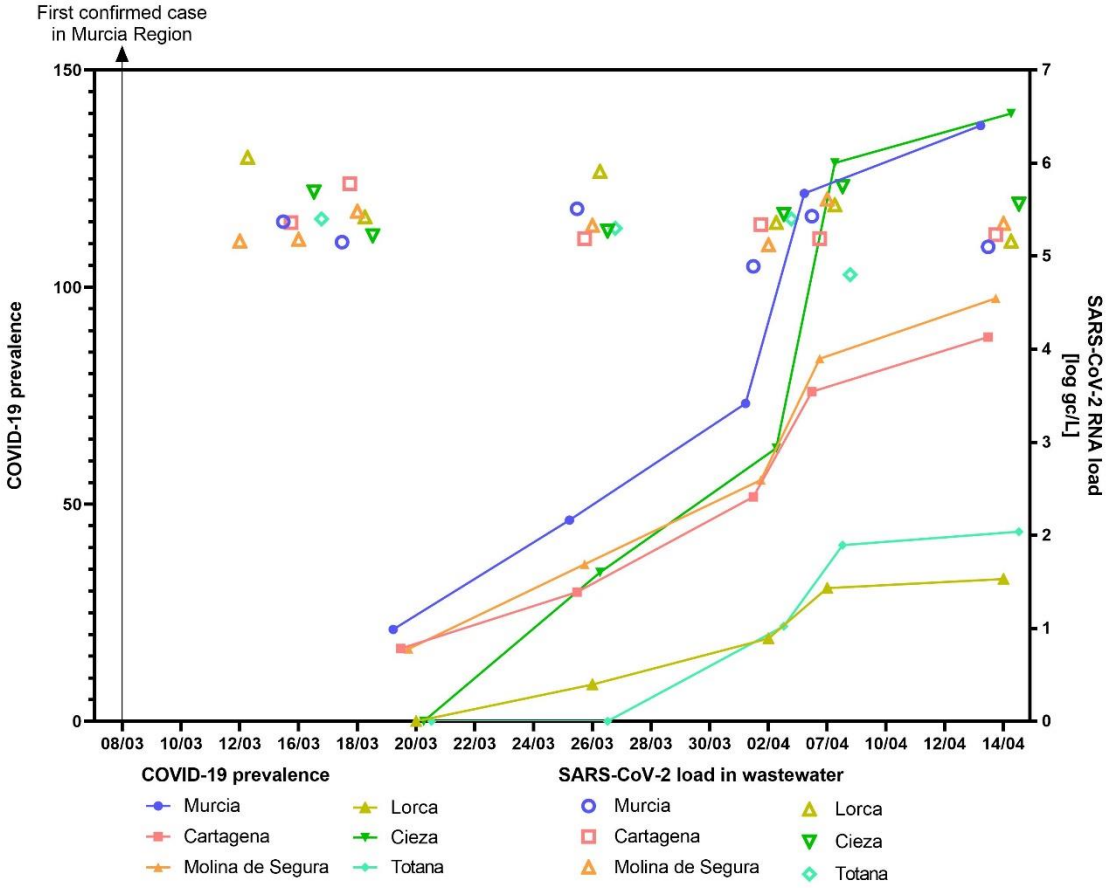
	20/03/2020		25/03/2020		30/03/2020		08/04/2020		15/04/2020	
	Cases	**Prevalence[2]**	**Cases**	**Prevalence**	**Cases**	**Prevalence**	**Cases**	**Prevalence**	**Cases**	**Prevalence**
Murcia	96	21.18	210	46.33	332	73.2	551	121.6	622	137.2
Cartagena	36	16.76	64	29.79	111	51.7	163	75.9	190	88.5
Molina de Segura	12	16.69	26	36.17	40	55.6	60	83.5	70	97.4
Lorca	-	-	8	8.47	18	19.1	29	30.7	31	32.8
Cieza	-	-	12	34.30	22	62.9	45	128.6	49	140.0
Totana	-	-	-	-	7	21.9	13	40.6	14	43.7

[1,] Data retrieved from the public repository of the "Servicio de epidemiologia" of the "Consejería de Salud de la Región de Murcia" (available at http://www.murciasalud.es/principal.php).

[2,] Prevalence, percentage of diagnosed cases per 100.000 inhabitants.

433 **Figure 3.** COVID-19 prevalence and SARS-CoV-2 RNA loads in wastewater per

434 sampling date and municipalities.



435

436