Potential Use of Untreated Wastewater for Assessing COVID-19 Trends in Southern Italy

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Abstract: As a complement to clinical disease surveillance, the monitoring of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in wastewater can be used as an early warning system for impending epidemics. This study investigated the dynamics of SARS-CoV-2 in untreated wastewater with respect to the trend of coronavirus disease 2019 (COVID-19) prevalence in Southern Italy. A total of 210 wastewater samples were collected between May and November 2020 from 15 Apulian wastewater treatment plants (WWTP). The samples were concentrated in accordance with the standard of World Health Organization (WHO, Geneva, Switzerland) procedure for Poliovirus sewage surveillance, and molecular analysis was undertaken with real-time reverse-transcription quantitative PCR (RT-(q) PCR). Viral ribonucleic acid (RNA) was found in 12.4% (26/210) of the samples. The virus concentration in the positive samples ranged from $8.8 \times 10^{2}$ to $6.5 \times 10^{4}$ genome copies/L. The receiver operating characteristic (ROC) curve modeling showed that at least 11 cases/100,000 inhabitants would occur after a wastewater sample was found to be positive for SARS-CoV-2 (sensitivity = 80%, specificity = 80.9%). To our knowledge, this is the first study in Italy that has applied wastewater-based epidemiology to predict COVID-19 prevalence. Further studies regarding methods that include all variables (meteorological phenomena, characteristics of the WWTP, etc.) affecting this type of wastewater surveillance data would be useful to improve data interpretation.

Keywords: coronavirus; SARS-CoV-2; wastewater-based epidemiology; surveillance

1. Introduction

Several studies have investigated the presence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in wastewater [1–5] in the world. Viral shedding in stool occurs in 50% of symptomatic, asymptomatic, pre- and post-symptomatic patients with coronavirus disease 2019 (COVID-19). The shedding duration at a load of $10^{2}$ to $10^{4}$ ribonucleic acid (RNA) copies/g varies among patients, with an average of 14–21 days [6–11].
Some authors [12–14] have reported a high correlation between the detection of SARS-CoV-2 in wastewater and the number of COVID-19 cases in the catchment area served by wastewater treatment plants (WWTPs), suggesting that the monitoring of wastewater could be a useful tool for predicting trends in COVID-19 prevalence. This approach, known as wastewater-based epidemiology (WBE), could solve certain limitations in existing surveillance systems that have been highlighted during the current COVID-19 pandemic or during previous ones (e.g., asymptomatic carriers, timing of diagnosis) [15].

To date, wastewater monitoring has been implemented as a successful strategy to track other health-related chemical and biological biomarkers, for example, in relation to illicit drug consumption, pharmaceutical use/abuse, water pollution, and the occurrence of antimicrobial resistance genes [16–20].

In Italy, the first detection of SARS-CoV-2 in a sewage sample was documented in northern Italy in December 2019 [21], though the first autochthonous case of COVID-19 was recognized in February 2020. Italy has been among the most severely affected countries in the world, to the point that the Ministerial Decree issued on 11 March 2020 [22] limited the movement of people throughout the nation and implemented social, recreational, and cultural lockdown measures that were enforceable [23–25]. In Italy, after a decline in infections owing to the vaccination campaign that began in January 2021, as of 21 July 2021, the national weekly incidence increased (19/100,000 inhabitants), primarily because of the circulation of the Delta variant [26]. This trend is similar to that of other European countries. To date, although the correlation between the occurrence of SARS-CoV-2 in wastewater and the number of COVID-19 cases [4,12–14] has been demonstrated, few studies have been conducted regarding the application of WBE to predict COVID-19 prevalence due to the complexity and uncertainties associated with the process [27].

Here, we report the first detection of SARS-CoV-2 RNA in untreated wastewater samples in Southern Italy, collected from WWTPs in the Apulia region. The aims of this study were: (1) To investigate the dynamics of SARS-CoV-2 in untreated wastewater with respect to the trend of COVID-19 cases in the region; (2) To apply WBE to predict COVID-19 prevalence.

2. Materials and Methods

2.1. Study Design

Apulia is a region of Southern Italy that hosts a population of approximately 4 million inhabitants distributed in six provinces: Bari (BA), Barletta-Andria-Trani (BT), Brindisi (BR), Foggia (FG), Lecce (LE), and Taranto (TA). Apulia covers approximately 20,000 km² and extends for 834 km along the coast.

The Apulian Water Agency manages the largest European aqueduct, with an approximate 20,000 km water network and a 10,000 km sewerage pipe network. Currently, 184 WWTPs (BA = 27; BT = 12; BR = 18; FG = 69; LE = 39; TA = 22) serve the region, covering 74% of total need [28]. Of these, 15 WWTPs were selected for the investigation and are uniformly distributed throughout the region: BA (two plants, A and B), BT (three plants, A–C), BR (one plant, A), FG (four plants, A–D), LE (three plants, A–C), and TA (two plants, A and B) (Figure 1). These WWTPs serve a total of 1,857,189 inhabitants (47.0% of Apulia’s population, Table 1).
Figure 1. Locations of the included WWTPs in the Apulia region (Southern Italy).

Table 1. Population and current capacity of the included WWTPs (source: Apulian Water Agency, http://www.aqp.it, accessed on 1 August 2021 [28]).

| Province and WWTP a | Served population b | Current capacity c (m³/d) d |
|---------------------|---------------------|-----------------------------|
| BA-A                | 224,830             | 84,854                      |
| BA-B                | 380,924             | 66,634                      |
| BT-A                | 130,000             | 13,907                      |
| BT-B                | 127,728             | 15,061                      |
| BT-C                | 66,232              | 12,960                      |
| BR-A                | 116,022             | 18,000                      |
| FG-A                | 183,695             | 33,148                      |
| FG-B                | 75,895              | 10,000                      |
| FG-C                | 15,969              | 2150                        |
| FG-D                | 33,789              | 4900                        |
| LE-A                | 43,302              | 6344                        |
| LE-B                | 37,576              | 10,546                      |
| LE-C                | 147,307             | 25,753                      |
| TA-A                | 34,754              | 8757                        |
| TA-B                | 239,166             | 34,045                      |

a WWTP: Wastewater treatment plant; b Population connected to the wastewater treatment plant; c Average water flow observed during the study period; d m³/d, water flow expressed as volume per day; BA-A, Bari-plant A; BA-B, Bari-plant B; BT-A, Barletta-Andria-Trani, plant A; BT-B, Barletta-Andria-Trani, plant B; BT-C, Barletta-Andria-Trani, plant C; BR-A, Brindisi, plant A; FG-A Foggia, plant A; FG-B, Foggia, plant B; FG-C, Foggia, plant C; FG-D, Foggia, plant D; LE-A, Lecce-plant A; LE-B, Lecce-plant B; TA-A, Taranto-plant A and TA-B, Taranto-plant B.
2.2. Sample Collection

A total of 210 wastewater samples were collected from 15 Apulian WWTPs, as well as 28 from BA, 56 from FG, 28 from LE, 42 from TA, 42 from BT, and 14 from BR. The sampling of untreated wastewater was performed by the Regional Environmental Protection Agency twice per month from May to November 2020. Composite samples over a 24 h period were collected from the WWTP influent post the inlet screens, immediately stored at −20 °C, and dispatched frozen to a regional reference laboratory for SARS-CoV-2 analysis (the Environmental and Food Hygiene Laboratory of the Department of Biomedical Science and Human Oncology, University of Bari, Aldo Moro, Italy, hereafter referred to as EnLab). The samples were stored frozen until further analysis. The samples were processed using Class II biological safety cabinets, and standard precautions were applied (hand hygiene products and personal protective equipment such as gloves, gowns, and face and eye protection).

Before virus concentration, the samples were thawed and underwent a 30 min treatment at 57 °C to inactivate the possibly present infectious viral particles to increase the safety of the analytical protocol for both the laboratory personnel and the environment [2].

2.3. Virus Concentration

Sample concentration was carried out via a two-phase separation (the polyethylene glycol–dextran method) in accordance with the World Health Organization (WHO, Geneva, Switzerland) Guidelines for the Environmental Surveillance of Poliovirus [29], with modifications as reported by La Rosa et al. [2] to adapt the protocol to enveloped viruses. In brief, a wastewater sample (250 mL) was centrifuged to pellet the solids, and the pellet was stored at 4 °C for further processing. The clarified wastewater was mixed with dextran (22%), NaCl (5 N), and polyethylene glycol 6000 (29%), and the mixture was agitated on an orbital shaker for 30 min at 4 °C. The mixture was then transferred to a separation funnel and allowed to stand overnight at 4 °C. The bottom layer and the interphase were then collected drop-wise, and this concentrate was added to the pellet from the initial centrifugation. The combined sample was treated with chloroform (1: 5 v/v) and assayed for the presence of virus.

2.4. RNA Extraction

The concentrated sample underwent viral RNA extraction using the NucliSSENS miniMAG semi-automated extraction system with magnetic silica, following the manufacturer’s instructions (bioMerieux, Marcy l’Etoile, France) with some modifications. In particular, the lysis phase was prolonged from 10 to 20 min, and a short centrifugation (2000 × g, 1 min) was used to pellet the sediment. Additionally, instead of 50 µL, 100 µL of magnetic silica beads was added to each sample. The extracted nucleic acids were further purified by polymerase chain reaction (PCR) inhibitors using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) and stored at −20 °C until molecular analysis.

After the RNA extraction, a portion of each sample was processed in the EnLab laboratory (Bari, Italy) for qualitative assessment, and a portion was transferred to the Istituto Superiore di Sanità laboratory (Rome, Italy, hereafter referred to as ISS) for quantitative assessment.

2.5. Real-Time Reverse-Transcription Quantitative PCR (Real-Time RT-(q) PCR)

Real-time RT-(q) PCR analysis was performed as described in La Rosa et al. [19]. Briefly, each 25 µL reaction contained 5 µL of RNA, 12.5 µL of 2× reaction buffer provided with the AgPath-ID™ One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA, 1 µL of 25× RT-PCR enzyme mix, 1 µL of forward primer (12.5 µM), 1 µL of reverse primer (22.5 µM), 1 mL of probe (6.25 µM), 1.83 µL of nuclease-free water (not DEPC, diethylpyrocarbonate-treated), and 1.67 µL of detection enhancer for real-time PCR (Applied Biosystems, Foster City, CA, USA). The following primer and probe sequences were used: CoV-2-F: ACA TGG CTT TGA GTT GAC ATC T (code 2297); CoV-2-R: AGC
AGT GGA AAA GCAT GTG G (code 2298); CoV-2-P: FAM-CAT AGA CAA CAG GTG CGC TC-MGBEQ (code 2299) [21]. The RT-PCR experiments were carried out in triplicate using the CFX96 Touch Deep Well Real-Time PCR System (Bio-Rad, Hercules, CA, USA) for detection and the Quant Studio 12K Flex (Applied Biosystems, Foster City, CA, USA) for quantification. Thermal cycling conditions included an initial reverse transcription step at 50 °C for 30 min, inactivation of reverse transcriptase at 95 °C for 10 min, and 45 cycles of amplification at 95 °C for 15 s and 60 °C for 45 s (30 s when using Quant Studio 12K Flex (Applied Biosystems, Foster City, CA, USA)) with the fast thermal profile). The cycle threshold (Ct) values of RT-qPCR were used as indicators of the copy number of SARS-CoV-2 RNA in the sewage samples, with lower Ct values corresponding to higher viral copy numbers. A Ct value less than 40 was interpreted as positive for SARS-CoV-2 RNA. The limit of detection (LOD50) and the limit of quantification (LOQ) for this assay were calculated in a previous study, as described in La Rosa et al., 2021 [21], and were found to be, on pure samples of target RNA, an LOD50 of 0.41 g.c./µL and an LOQ of 3.71 g.c./µL; in sewage samples, LOD50 and LOQ were 1.46 g.c./µL RNA and 7.35 g.c./µL, respectively. To construct a standard curve, the targeted region was synthetized and purified by BioFab Research (Rome, Italy) and quantified by fluorometric measurement (Qubit, Thermo Fisher Scientific, Waltham, MA, USA). Tenfold dilutions were used to construct the standard curve (range 5 × 100–5 × 104 copies/µL). In vitro-synthetized RNA containing the target region was used as an external amplification control to check for PCR inhibition.

2.6. Statistical Analyses

A descriptive analysis was performed with regard to the distribution of the results of the WWTP samples analyzed from each province. To correlate the wastewater results to the number of COVID-19 cases during the May–November 2020 study period, we first correlated the number of cases to the number of patient specimen collection swabs taken per day in the area served by each WWTP. This operation effectively reduced the underestimation of COVID-19 cases. The maximum daily number of swabs carried out in Apulia during the examined period was 10,265; thus, the following formula was applied in Equation (1):

\[
A = \text{No. of COVID-19 cases on day } x \times \left(\frac{10,265}{\text{No. swabs carried out on day } x}\right)
\]

where A represents the estimated number of cases on day x in order to eliminate the uncertainty owing to the number of swabs carried out on day x.

Subsequently, to report the estimated number of COVID-19 cases (A) in the population served by each plant, the following formula was applied in Equation (2):

\[
B = \left(\frac{A}{\text{population served by plan } X}\right) \times 100,000
\]

where B represents the number of COVID-19 cases/100,000 inhabitants served by each plant. This operation allowed us to compare the number of cases that occurred in areas with different population sizes.

After the preliminary operations, R software version 4.0.5 (Brandon Greenwell, Cincinnati, Ohio) was used for the statistical analysis, and a p-value < 0.05 was considered statistically significant.

Three types of analyses were carried out with regard to the occurrence of COVID-19 during the 15 days before and after wastewater sampling:

1. Chi-squared (χ²) test with Yates’s correction and the odds ratio to compare the percentage of COVID-19 cases in relation to the results of the wastewater sample for SARS-CoV-2 in the following time periods:
   a. 15 days before: positive vs. negative wastewater samples
   b. 15 days after: positive vs. negative wastewater samples
   c. 15 days before vs. 15 days after: positive wastewater samples
d. 15 days before vs. 15 days after: negative wastewater samples

This analysis showed if and how many COVID-19 cases, detected previously and subsequently to wastewater samples positive for SARS-CoV-2, influenced the SARS-CoV-2 detection in wastewater.

2. A Poisson regression model was used to perform multivariate analysis on the viral load and the PCR Ct values of the positive wastewater samples in comparison with the following parameters:
   a. COVID-19 case trend in the 15 days before wastewater sampling
   b. COVID-19 case trend in the 15 days after wastewater sampling
   c. Population served by each plant
   d. Current average daily capacity (m$^3$/d) of each WWTP

To standardize the different units of measurement of the four independent parameters, the data were normalized using the following Equation (3) [30]:

\[
x_{\text{normalized}} = \frac{(x - x_{\text{min}})}{(x_{\text{max}} - x_{\text{min}})}
\]

where \(x\) is each of the four variables indicated above; \(x_{\text{max}}\): max value of each variable; \(x_{\text{min}}\): min value of each variable.

The final model included only variables with a \(p\)-value of <0.05 in the preliminary model of all variables.

To quantify the effects of the above parameters on the viral load and Ct values, the relative risk (RR) of each parameter was calculated [31,32].

3. A receiver operating characteristic (ROC) curve was used to assess the trend of cases 15 days after each sampling event to identify an optimal cutoff value that could predict how many cases of COVID-19 per 100,000 inhabitants (served by the plant) might occur within 15 days after a positive wastewater sample. The ROC curve shows the tradeoff between the true positive fraction (TPF) and false positive fraction (FPF), and is generated by the plot of TPF (sensitivity) versus FPF (1-specificity) across varying cut-offs. The concept of an ROC curve is based on the notion of a “separator” (or decision) variable as one change in the criterion for positivity [33]. The ROC curve corresponding to the progressively greater discriminant capacity of diagnostic tests (max values of sensibility and specificity) are located progressively closer to the upper-left-hand corner in “ROC space”. In our case, we calculated the cases that occurred 15 days after the wastewater sampling (both positive and negative for SARS-CoV-2). The ROC identified the optimal cut-off value, above which were included most of the positive wastewater samples for SARS-CoV-2 (sensitivity) and under which were included most of the negative wastewater samples (specificity).

3. Results

Overall, SARS-CoV-2 RNA was present in 12.4% (26/210) of the samples. In particular, 32.1% (9/28) of the wastewater samples from BA were positive, followed by 7.1% (4/56) from FG, 7.1% (3/42) from LE, 17.8% (5/28) from TA, and 11.9% (5/42) from BT. No positive samples were detected from BR.

The results reported by the EnLab laboratories were all confirmed by ISS, except in eight samples. The Ct and genome copies/liter (g.c./L) values of the positive samples are reported in Table 2.

The virus concentrations of the positive samples ranged from $8.8 \times 10^2$ to $6.5 \times 10^4$ g.c./L. The highest concentrations were recorded in a sample from TA-B in November 2020 ($6.5 \times 10^4$ g.c./L) and in a sample from FG-C in May 2020 ($6.2 \times 10^4$ g.c./L).

The distribution of the number of cases in the presence of positive wastewater samples in the study area is shown in Table 3.
Table 2. Positive SARS-CoV-2 detections in wastewater samples by qualitative (Ct value) and quantitative (g.c./L) real-time PCR, May–November 2020.

| Province and WWTP | Sampling Date (Year 2020) | EnLab | ISS |
|-------------------|---------------------------|-------|-----|
|                   |                           | Ct Value | g.c./L |
| BA-A              | October 6                 | 36.11  | n.d. |
|                   | October 22                | 36.93  | n.d. |
|                   | November 2                | 37.62  | 3.5 x 10^3 |
|                   | November 16               | 36.17  | 4.8 x 10^3 |
| BA-B              | July 27                   | 38.73  | 1.4 x 10^3 |
|                   | October 6                 | 37.02  | n.d. |
|                   | October 22                | 37.81  | 8.8 x 10^2 |
|                   | November 2                | 36.17  | 1.7 x 10^3 |
|                   | November 16               | 35.46  | 4.8 x 10^3 |
| BT-A              | November 10               | 35.94  | 4.1 x 10^3 |
|                   | November 23               | 35.43  | 5.1 x 10^3 |
| BT-B              | October 22                | 37.19  | 1.5 x 10^3 |
|                   | November 10               | 38.42  | 1.3 x 10^3 |
|                   | November 23               | 35.02  | 1.5 x 10^4 |
| FG-A              | October 20                | 37.57  | n.d. |
| FG-C              | May 7                     | 32.04  | 6.2 x 10^4 |
|                   | May 29                    | 34.04  | 1.2 x 10^4 |
|                   | June 17                   | 33.63  | 3.4 x 10^4 |
| LE-B              | September 16              | 37.19  | n.d. |
|                   | October 29                | 38.12  | n.d. |
|                   | November 4                | 38.15  | 1.4 x 10^3 |
| TA-A              | September 8               | 37.12  | n.d. |
|                   | November 17               | 37.09  | 2.4 x 10^3 |
| TA-B              | October 27                | 38.47  | n.d. |
|                   | November 3                | 38.24  | 6.5 x 10^4 |
|                   | November 17               | 36.52  | 1.7 x 10^3 |

g.c./L = genome copies/liter; n.d. = not detected; Ct = Cycle threshold; EnLab = Environmental and Food Hygiene Laboratory; ISS = Istituto Superiore di Sanità.

Tables 4 and 5 present the COVID-19 test swab results in relation to SARS-CoV-2 detection in wastewater from the Apulia region during the study period in the areas served by the investigated WWTPs. Table 4 shows the results from the 15 days before wastewater sampling, and Table 5 shows the results from the 15 days after sampling.

The $\chi^2$ results with Yates’s correction and odds ratios are reported in Table 6.

Compared with wastewater samples that are negative for SARS-CoV-2, when wastewater samples are positive, there is a 45.8-fold greater risk of cases in the 15 days prior to sampling and a 32.5-fold greater risk in the 15 days after sampling.

Compared with the results 15 days before wastewater sampling, when samples are positive for SARS-CoV-2, there is a two-fold greater risk of swabs also being positive for SARS-CoV-2 (and therefore a two-fold risk of identifying COVID-19 cases) in the 15 days after sampling.
| Province WWTP | 30 April 2020 | 31 May 2020 | 30 June 2020 | 31 July 2020 | 31 August 2020 | 30 September 2020 | 31 October 2020 | 30 November 2020 |
|---------------|--------------|-------------|--------------|--------------|----------------|-------------------|-----------------|-----------------|
| BA            |              |             |              |              |                |                   |                 |                 |
|               | 1313         | 1483        | 1491         | 1504         | 1890           | 3034              | 7668            | 20,839          |
|               | 10.5         | 11.8        | 11.9         | 12.0         | 15.1           | 24.2              | 61.4            | 166.4           |
|               | A+B          |             |              |              |                |                   |                 |                 |
|               | 19           | 0.3         | 27           | 0.4          | 28             | 0.4               | 68              | 1.1             |
|               | 10.5         | 11.8        | 11.9         | 12.0         | 15.1           | 24.2              | 61.4            | 166.4           |
|               |              |             |              |              |                |                   |                 |                 |
| BT            |              |             |              |              |                |                   |                 |                 |
|               | 373          | 9.6         | 380          | 9.7          | 382            | 9.8               | 694             | 17.8            |
|               |              |             |              |              |                |                   |                 |                 |
|               | A+B          |             |              |              |                |                   |                 |                 |
|               | 2            | 0.1         | 2            | 0.1          | 2              | 0.1               | 2               | 0.1             |
|               |              |             |              |              |                |                   |                 |                 |
| FG            |              |             |              |              |                |                   |                 |                 |
|               | 1044         | 16.8        | 1155         | 18.2         | 1170           | 18.8              | 1186            | 19.1            |
|               |              |             |              |              |                |                   |                 |                 |
|               | A+B          |             |              |              |                |                   |                 |                 |
|               | 2            | 0.1         | 2            | 0.1          | 2              | 0.1               | 2               | 0.1             |
|               |              |             |              |              |                |                   |                 |                 |
| LE            |              |             |              |              |                |                   |                 |                 |
|               | 487          | 6.1         | 515          | 6.5          | 521            | 6.6               | 557             | 7.0             |
|               |              |             |              |              |                |                   |                 |                 |
|               | A+B          |             |              |              |                |                   |                 |                 |
|               | 2            | 0.1         | 2            | 0.1          | 2              | 0.1               | 2               | 0.1             |
|               |              |             |              |              |                |                   |                 |                 |
| TA            |              |             |              |              |                |                   |                 |                 |
|               | 258          | 4.5         | 281          | 4.9          | 281            | 4.9               | 313             | 5.4             |
|               |              |             |              |              |                |                   |                 |                 |
|               | A+B          |             |              |              |                |                   |                 |                 |
|               | 16           | 0.5         | 16           | 0.5          | 17             | 0.5               | 17              | 0.5             |

Table 3. Epidemiological data summary of COVID-19 cases in the study area and their relation to positive wastewater samples.

*Data from “Epidemia COVID-19—Bollettino Epidemiologico Regione Puglia” ([http://www.regione.puglia.it/web/speciale-coronavirus/elenco-notizie](http://www.regione.puglia.it/web/speciale-coronavirus/elenco-notizie), accessed on 1 August 2021) [34]; * Cumulative Incidence: the percentage of diagnosed cases per 10,000 inhabitants; Bold = cases concomitant with positive wastewater samples.
Table 4. Swab results in relation to SARS-CoV-2 detection in wastewater in the 15 days preceding wastewater sampling.

| Outcome of Wastewater Samples for SARS-CoV-2 | No. (%) of Negative Swabs for SARS-CoV-2 | No. (%) of Positive Swabs for SARS-CoV-2 |
|---------------------------------------------|------------------------------------------|------------------------------------------|
| Negative                                    | 453,911 (99.9)                           | 529 (0.1)                                |
| Positive                                    | 77,049 (95.0)                            | 4101 (5.0)                               |
| Total                                       | 530,960 (99.1)                           | 4630 (0.9)                               |

Table 5. Swab results in relation to SARS-CoV-2 detection in wastewater in the 15 days following wastewater sampling.

| Outcome of Wastewater Samples for SARS-CoV-2 | No. (%) of Negative Swabs for SARS-CoV-2 | No. (%) of Positive Swabs for SARS-CoV-2 |
|---------------------------------------------|------------------------------------------|------------------------------------------|
| Negative                                    | 453,163 (99.7)                           | 1277 (0.3)                               |
| Positive                                    | 74,350 (91.6)                            | 6800 (8.4)                               |
| Total                                       | 527,513 (98.5)                           | 8077 (1.5)                               |

Table 6. The percentage of COVID-19 cases in relation to the wastewater sample outcomes for SARS-CoV-2 detection in the 15 days before and after sampling.

| Period Analyzed with Respect to Wastewater Sampling | Outcome of Wastewater Sample for SARS-CoV-2 | Percentage of Cases with Respect to Wastewater Sample Outcome for SARS-CoV-2 | \(\chi^2\) with Yates’s Correction | Odds Ratio (95% CI) |
|---------------------------------------------------|---------------------------------------------|--------------------------------------------------------------------------------|-----------------------------------|---------------------|
| 15 days before                                    | Positive vs. negative                       | 5.0% vs. 0.1%                                                                  | \(\chi^2 = 19.579\) p-value < 0.0001 | 45.8 (41.7–50.0)    |
| 15 days after                                     | Positive vs. negative                       | 8.4% vs. 0.3%                                                                  | \(\chi^2 = 30.398\) p-value < 0.0001 | 32.5 (30.6–34.5)    |
| 15 days before vs. 15 days after                  | Positive vs. positive                       | 5.0% vs. 8.4%                                                                  | \(\chi^2 = 715.84\) p-value < 0.0001 | 1.7 (1.6–1.8)       |
| 15 days before vs. 15 days after                  | Negative vs. negative                       | 0.1% vs. 0.3%                                                                  | \(\chi^2 = 309.59\) p-value < 0.0001 | 2.4 (2.2–2.7)       |

95% CI = 95% confidence interval.

Table 7 presents the Poisson regression modeling results to evaluate whether independent parameters affected the dependent variable “SARS-CoV-2 viral load” in wastewater samples (mean load = 1825.90 g.c./L, first and third interquartile = 0, median load = 0, range = 0–65,000).

All independent parameters had a significant influence on the wastewater SARS-CoV-2 detection results. In particular, the average daily capacity of the WWTPs was inversely proportional to the SARS-CoV-2 load of the wastewater (the lower the daily average capacity, the greater the probability of detecting the virus). By contrast, the other three parameters were directly proportional to the viral load. When the Ct value was used as a dependent variable in the Poisson regression, none of the four independent parameters listed in Table 7 had a statistically significant impact. To predict the number of COVID-19 cases/100,000 inhabitants served by the WWTPs in the 15 days after sampling, an ROC curve model was applied using the wastewater samples that were positive for SARS-CoV-2 (Figure 2). The analysis showed a cut off value for which at least 11 cases/100,000 inhabitants would occur after a wastewater sample was found to be positive for SARS-CoV-2 (sensitivity = 80%; specificity = 80.9%).
Table 7. Poisson regression model of SARS-CoV-2 load in wastewater samples: final model.

|                          | \( \beta \) | \((e^\beta - 1) = RR(\%)\) | \(p\)-Value |
|--------------------------|------------|--------------------------|-------------|
| Intercept                | 8.6647436  | <0.0001 *                |             |
| COVID-19 case trend in the 15 days after sampling | 0.0067969  | 0.68                     | <0.0001 *   |
| COVID-19 case trend in the 15 days before sampling | 0.0640235  | 6.61                     | <0.0001 *   |
| Daily average capacity (m\(^3\)/d\(^\hat{\,}\)) of each WWTP | -0.3440558 | -29.11                   | <0.0001 *   |
| Population served by each plant | 0.1569391  | 16.99                    | <0.0001 *   |

\( \beta = \) coefficient of the regression model for each independent variable; RR = Relative risk; * statistically significant; \( \hat{\,}\) = day.

Figure 2. ROC curve to predict the number of COVID-19 cases/100,000 inhabitants 15 days after SARS-CoV-2 detection in wastewater (11 cases/100,000 inhabitants).

4. Discussion

The EU Commission recommendation of 17 March 2021 strongly encourages member states to establish national wastewater surveillance systems to detect SARS-CoV-2 and its variants in wastewater [35]. These systems should be implemented as soon as possible and no later than 1 October 2021.

Our study found that SARS-CoV-2 was detected in wastewater during the period in which cases were being diagnosed within the municipalities served by the investigated WWTPs.

The virus circulation generally slowed during the summer months, as evidenced by the low incidence of cases during this time. The presence of SARS-CoV-2 in wastewater in July and September 2020 from the Bari (A+B) and Lecce (B) plants, respectively, which served municipalities without newly confirmed cases, could be related to undiagnosed asymptomatic cases and to previous cases, as the virus is excreted in the stool for some time after infection (approximately 30 days) [12]. After a small number of detections during the summer months, the presence of SARS-CoV-2 was again detected in all of the WWTPs, except for those in Brindisi. During this time, there was a concomitant increase in cases.

The positive samples were qualitatively and quantitatively confirmed by EnLab and ISS, respectively, and appeared to be related to a high level of the virus circulating in
the population. The current detection methods are not sensitive enough to detect low quantities of viral RNA in wastewater because of the complex nature of this media [36].

The non-detection of virus by the ISS in some wastewater samples may be because of viral RNA degradation, owing to transportation conditions [13,37]. A recent study found that SARS-CoV-2 RNA is partially stable at 4 °C for at least 14 days [38]; however, one review stated that freezing and thawing the sample from −20 °C or −80 °C could lead to the degradation of the SARS-CoV-2 genetic material [39].

In line with previous studies [3,13], we found a direct correlation between the SARS-CoV-2 RNA concentration in wastewater and the number of COVID-19 cases during the 15 days before and after a positive detection in wastewater. The average daily capacity of the WWTPs was inversely proportional to the SARS-CoV-2 load in the wastewater samples, probably owing to dilution (e.g., precipitation, average daily water usage).

To ensure that differences in viral concentration could not be attributed to changes in population, some authors have inserted an important step in the application of WBE, namely population normalization. For this purpose, human biomarkers as ammonium excreted in urine can be used to estimate the serviced population in an area via statistical modeling [15].

As has been demonstrated in previous examples such as the 2013–2014 silent polio epidemic in Israel [40], environmental surveillance can be used as a tool to decide when to enact restrictions, with the aim of an early introduction and avoiding premature repeal [41,42]. This surveillance approach could also be used to inform vaccination distribution [43] and to investigate emerging genomic variants circulating in the population [44–46].

The presence of SARS-CoV-2 in wastewater can be used to predict COVID-19 cases, supporting the potential of wastewater-based epidemiology (WBE) [1,8,21,47]. This approach represents a non-invasive early-warning tool for monitoring the status and trends of COVID-19 infection [48]. Here, we predicted that at least 11 cases/100,000 inhabitants would occur in the 15 days after detecting a positive wastewater sample. To our knowledge, this is the first study in Italy to use WBE to predict the COVID-19 prevalence.

However, the usage of WBE for estimating COVID-19 prevalence remains limited, owing to the complexity and uncertainties associated with the process [37]. Several studies [28,38,49] have discussed the uncertainties in using WBE to assess SARS-CoV-2 prevalence. For viral shedding, variations in the magnitude, probability, and duration were commonly observed across different studies [27]. Physiological factors such as gender, age, and pathological conditions impact the probability of virus shedding among patients. For most viruses, the water matrix plays an important role in their inactivation and decay because, without active human cells as hosts in wastewater, the infectivity of SARS-CoV-2 was reported to be reduced [50]. From currently available reports, even for the best recovery method, a considerable loss of virus RNA is commonly observed [38]. The flow inside the sewers has relatively large uncertainties due to seasonal or diurnal variations in water usage patterns among the population and any rainfall event [51]. To date, the exploration for sampling techniques in the detection of SARS-CoV-2 RNA is limited, even if the use of the composite vs. grab sampling technique is preferable due to the inherent variability in virus shedding and diurnal sewer flows [37].

One limitation of our study is that it does not take into account several parameters that influence the result, such as the precipitation, catchment size, variation of the viral load in stool, virus degradation and dilution in the WWTP, the impact of the wastewater matrix components, and the underestimation of cases owing to asymptomatic patients [7,37,41]. Moreover, the duration and distribution of SARS-CoV-2 RNA shedding in feces varies among individuals and across time may be also affected by variants and vaccination [7,10,14,26].

The EU Commission recommendation [35] states that “wastewater surveillance is a tool to observe trends and not an absolute means to draw conclusions about the prevalence of COVID-19 in the population.” Therefore, further studies of the complex methods that
include all variables that affect this type of wastewater surveillance data would be useful to improve data interpretation [28,37]. Moreover, a future development of our research (with a larger number of wastewater samples to make the analysis more robust) could foresee a validation of the statistical model used by comparing these results with those derived from innovative approaches such as an artificial neural network (e.g., machine learning) [27].

5. Conclusions
Wastewater surveillance is less resource-intensive than large-scale clinical testing, making it an optimal tool for long-term virus monitoring and for the early identification of viral circulation in a population. The early detection of SARS-CoV-2 RNA in wastewater could signal imminent danger, providing authorities with valuable time in which to coordinate and implement actions to slow disease spread.

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References
1. Ahmed, W.; Angel, N.; Edson, J.; Bibby, K.; Bivins, A.; O’Brien, J.W.; Choi, P.M.; Kitajima, M.; Simpson, S.L.; Li, J.; et al. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveil-lance of COVID-19 in the community. Sci. Total Environ. 2020, 728, 138764. [CrossRef]
2. La Rosa, G.; Iaconelli, M.; Mancini, P.; Bonanno Ferraro, G.; Veneri, C.; Bonadonna, L.; Lucentini, L.; Suffredini, E. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci. Total Environ. 2020, 736, 139652. [CrossRef] [PubMed]
3. Medema, G.; Heijnen, L.; Elsinga, G.; Italiaander, R.; Brouwer, A. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the Early Stage of the Epidemic in The Netherlands. Environ. Sci. Technol. Lett. 2020, 7, 511–516. [CrossRef]
4. Hata, A.; Hara-Yamamura, H.; Meuchi, Y.; Imai, S.; Honda, R. Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak. Sci. Total Environ. 2021, 758, 143578. [CrossRef]
5. Westhaus, S.; Weber, F.A.; Schiwy, S.; Linnemann, V.; Brinkmann, M.; Widera, M.; Greve, C.; Janke, A.; Hollert, H.; Wintgens, T. Detection of SARS-CoV-2 RNA in raw and treated wastewater in Germany-suitability for COVID-19 surveillance and potential transmission risks. Sci. Total Environ. 2021, 751, 141750. [CrossRef] [PubMed]
6. Ouali, S.E.; Achkar, J.P.; Lashner, B.; Regueiro, M. Gastrointestinal manifestations of COVID-19. Cleve. Clin. J. Med. 2021. [CrossRef]
7. Pan, Y.; Zhang, D.; Yang, P.; Poon, L.L.M.; Wang, Q. Viral load of SARS-CoV-2 in clinical samples. Lancet Infect. Dis. 2020, 20, 411–412. [CrossRef]
8. Wu, Y.; Guo, C.; Tang, L.; Hong, Z.; Zhou, J.; Dong, X.; Yin, H.; Xiao, Q.; Tang, Y.; Qu, X.; et al. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. Lancet Gastro-Enterol. Hepatol. 2020, 5, e434–e435. [CrossRef]
9. Xu, X.; Zheng, X.; Li, S.; Lam, N.S.; Wang, Y.; Chu, D.K.W.; Poon, L.L.M.; Tun, H.M.; Peiris, M.; Deng, Y.; et al. The first case study of wastewater-based epidemiology of COVID-19 in Hong Kong. Sci. Total Environ. 2021, 790, 148000. [CrossRef]
10. He, X.; Lau, E.H.Y.; Wu, P.; Deng, X.; Wang, J.; Hao, X.; Lau, Y.C.; Wong, J.Y.; Guan, Y.; Tan, X.; et al. Temporal dy-namics in viral shedding and transmissibility of COVID-19. Nat. Med. 2020, 26, 672–675. [CrossRef]
11. 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.; et al. Molecu-lar and serological investigation of 2019-nCoV infected patients: Implication of multiple shedding routes. Emerg. Microb. Infect. 2020, 9, e386–e389. [CrossRef]
12. Wu, F.; Zhang, J.; Xiao, A.; Gu, X.; Lee, W.L.; Armas, F.; Kauffman, K.; Hanage, W.; Matus, M.; Ghaeli, N.; et al. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. mSystems 2020, 5, e00614–e00620. [CrossRef]
13. Wu, F.; Xiao, A.; Zhang, J.; Moniz, K.; Endo, N.; Armas, F.; Bushman, M.; Chai, P.R.; Duvallet, C.; Erickson, T.B.; et al. Wastewater surveillance of SARS-CoV-2 across 40 U.S. states from February to June 2020. *Water Res.* 2021, 202, 117400. [CrossRef] [PubMed]

14. Xu, Y.; Li, X.; Zhu, B.; Liang, H.; Fang, C.; Gong, Y.; Guo, Q.; Sun, X.; Zhao, D.; Shen, J.; et al. Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. *Nat. Med.* 2020, 26, e502–e505. [CrossRef] [PubMed]

15. Polo, D.; Quintela-Baluja, M.; Corbishley, A.; Jones, D.L.; Singer, A.C.; Graham, D.W.; Romalde, J.L. Making waves: Wastewater-based epidemiology for COVID-19 approaches and challenges for surveillance and prediction. *Water Res.* 2020, 186, 116404. [CrossRef] [PubMed]

16. Mercan, S.; Kuloglu, M.; Asioglu, F. Monitoring of illicit drug consumption via wastewater: Development, challenges, and future aspects. *Curr. Opin. Environ. Sci.* 2019, 9, 64–72. [CrossRef]

17. de Oliveira, M.; Frihling, B.E.F.; Velasques, J.; Filho, F.J.C.M.; Cavalheri, P.S.; Miglioli, L. Pharmaceuticals residues and xenobiotics contaminants: Occurrence, analytical techniques and sustainable alternatives for wastewater treatment. *Sci. Total Environ.* 2020, 705, 135568. [CrossRef]

18. Montagna, M.T.; De Giglio, O.; Calia, C.; Pousis, C.; Triggiano, F.; Murgolo, S.; De Ceglie, C.; Bagordo, F.; Apollonio, F.; Diella, G.; et al. Microbiological and Chemical Assessment of Wastewater Discharged by Infiltration Trenches in Fractured and Karstified Limestone (SCA.Re.S. Project 2019–2020). *Pathogens* 2020, 9, 1010. [CrossRef]

19. Triggiano, F.; Calia, C.; Diella, G.; Montagna, M.T.; De Giglio, O.; Caggiano, G. The Role of Urban Wastewater in the Environmental Transmission of Antimicrobial Resistance: The Current Situation in Italy (2010–2019). *Microorganisms* 2020, 8, 1567. [CrossRef]

20. Gracia-Lor, E.; Castiglioni, S.; Bade, R.; Been, F.; Castrignanò, S.; Iaconelli, M.; Bonadonna, L.; Lucentini, L.; Suffredini, E. Impact of lockdown on the microbiological status of the hospital water network during COVID-19 pandemic. *Sci. Total Environ.* 2021, 750, 141711. [CrossRef]

21. Istituto Poligrafico e Zecca dello Stato. Decreto del Presidente del Consiglio dei Ministri 11 Marzo 2020. Ulteriori Disposizioni Attuative del Decreto-Legge 23 Febbraio 2020, n. 6, Recante Misure Urgenti in Materia di Contenimento e Gestione Dell’emergenza Epidemiologica da COVID-19, Applicibili Sull’intero Territorio nazionale (20A01605); Gazzetta Ufficiale della Repubblica Italiana n. 64 del 11-03-2020; Istituto Poligrafico e Zecca dello Stato: Rome, Italy, 2020.

22. De Giglio, O.; Diella, G.; Lopuzzo, M.; Triggiano, F.; Calia, C.; Pousis, C.; Fasano, F.; Caggiano, G.; Calabrese, G.; Rafaschieri, A.; et al. Legionella and legionellosis in tourist-recreational facilities: Influence of climate factors and geostatistical analysis in Southern Italy (2001–2017). *Environ. Sci. Total Environ.* 2018, 627–635. [CrossRef] [PubMed]

23. De Giglio, O.; Diella, G.; Lopuzzo, M.; Napoli, C.; Apollonio, F.; Brigida, S.; Calia, C.; Campanale, C.; Mar-Zella, A.; et al. Legionella and legionellosis in tourist-tic-recreational facilities: Influence of climate factors and geostatistical analysis in Southern Italy (2001–2017). *Environ. Res.* 2019, 178, 108721. [CrossRef]

24. Gallè, F.; Sabella, E.A.; Ferracuti, S.; De Giglio, O.; Caggiano, G.; Protano, C.; Valeriani, F.; Parisi, E.A.; Valerio, G.; Liguori, G.; et al. Sedentary Behaviors and Physical Activity of Italian Undergraduate Students during Lockdown at the Time of COVID-19 Pandemic. *Int. J. Environ. Res. Public Health* 2020, 17, 6171. [CrossRef] [PubMed]

25. Gallè, F.; Sabella, E.A.; Da Molin, G.; De Giglio, O.; Caggiano, G.; Di Otofrio, V.; Ferracuti, S.; Montagna, M.T.; Liguori, G.; Orsi, G.B.; et al. Understanding Knowledge and Behaviors Related to COVID-19 Epidemic in Italian Under-graduate Students: The EPICO Study. *Int. J. Environ. Res. Public Health* 2020, 17, 3481. [CrossRef] [PubMed]

26. Ministero Della Salute. Nuovo Coronavirus. COVID-19 Weekly Monitoring, Report 5–11 July 2021. Available online: https://apps.who.int/iris/handle/10665/67854 (accessed on 8 August 2021).

27. Li, X.; Kulandaivelu, J.; Zhang, S.; Shi, J.; Sivakumar, M.; Mueller, J.; Luby, S.; Ahmed, W.; Coin, L.; Jiang, G. Data-driven estimation of COVID-19 community prevalence through wastewater-based epidemiology. *Sci. Total Environ.* 2021, 789, 147947. [CrossRef] [PubMed]

28. Apulian Water Agency. Available online: https://www.aqp.it (accessed on 13 August 2021).

29. World Health Organization. Guidelines for Environmental Surveillance of Poliovirus Circulation. 2003. Available online: https://apps.who.int/iris/handle/10665/67854 (accessed on 8 August 2021).

30. Fasano, F.; Addante, A.S.; Valenzano, B.; Scannicchio, G. Variables Influencing per Capita Production, Separate Collect-ion, and Costs of Municipal Solid Waste in the Apulia Region (Italy): An Experience of Deep Learning. *Int. J. Environ. Res. Public Health* 2021, 18, 752. [CrossRef] [PubMed]

31. Conza, L.; Casati, S.; Limoni, C.; Gaia, V. Meteorological factors and risk of community-acquired Legionnaires’ disease in Switzerland: An epidemiological study. *BMJ Open* 2013, 3, e002428. [CrossRef]

32. De Giglio, O.; Fasano, F.; Diella, G.; Lopuzzo, M.; Napoli, C.; Apollonio, F.; Brigida, S.; Calia, C.; Campanale, C.; Mar-Zella, A.; et al. Legionella and legionellosis in tourist-tic-recreational facilities: Influence of climate factors and geostatistical analysis in Southern Italy (2001–2017). *Environ. Res.* 2019, 178, 108721. [CrossRef]
34. Epidemia COVID-19-Bollettino Epidemiologico Regione Puglia. Available online: https://www.regione.puglia.it/documents/65725/216593/Bollettino+Covid_25102020.pdf/5c41e524-836e-44e2-5859-c56a0954a1e7?Expires=1603637526522 (accessed on 13 August 2021).

35. European Commission. Recommendation (EU) 2021/472 of 17 March 2021 on a Common Approach to Establish a Systematic Surveillance of SARS-CoV-2 and Its Variants in Wastewaters in the EU; OJ L 98; European Commission: Brussels, Belgium, 2021; pp. 3–8.

36. Pulicharla, R.; Kaur, G.; Brar, S.K. A year into the COVID-19 pandemic: Rethinking of wastewater monitoring as a preemptive approach. J. Environ. Chem. Eng. 2021, 9, 106063. [CrossRef]

37. Li, X.; Zhang, S.; Shi, J.; Luby, S.P.; Jiang, G. Uncertainties in estimating SARS-CoV-2 prevalence by wastewater-based epidemiology. Chem. Eng. J. 2021, 415, 129039. [CrossRef] [PubMed]

38. Chin, A.W.H.; Chu, J.T.S.; Perera, M.R.A.; Hui, K.P.Y.; Yen, H.L.; Chan, M.C.W.; Peiris, M.; Poon, L.L.M. Stability of SARS-CoV-2 in different environmental conditions. Lancet Microbe 2020, 1, e10. [CrossRef]

39. Alygizakis, N.; Markou, A.N.; Rousis, N.I.; Galani, A.; Avgeris, M.; Adamopoulos, P.G.; Scorilas, A.; Lianidou, E.S.; Par-askevis, D.; Tsiodras, S.; et al. Analytical methodologies for the detection of SARS-CoV-2 in wastewater: Protocols and future perspectives. Trends Anal. Chem. 2021, 134, 116125. [CrossRef] [PubMed]

40. Brouwer, A.F.; Eisenberg, J.N.S.; Pomeroy Connor, D.; Shulman, L.M.; Hindiyeh, M.; Manor, Y.; Grooto, I.; Koopman, J.S.; Eisenberga, M.C. Epidemiology of the silent polio outbreak in Rahat, Israel, based on modeling of environmental surveillance data. Proc. Natl Acad. Sci. USA 2018, 115, E10625–E10633. [CrossRef] [PubMed]

41. Randazzo, W.; Truchado, P.; Cuevas-Ferrando, E.; Simón, P.; Allende, A.; Sánchez, G. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res. 2020, 181, 115942. [CrossRef]

42. Montagna, M.T.; De Giglio, O.; Calia, C.; Pousis, C.; Apollonio, F.; Campanale, C.; Diella, G.; Lopuzzo, M.; Marzella, A.; Triggiano, F.; et al. First Detection of Severe Acute Respiratory Syndrome Coronavirus 2 on the Surfaces of Tourist-Recreational Facilities in Italy. Int. J. Environ. Res. Public Health 2021, 18, 3252. [CrossRef]

43. Smith, T.; Cassell, G.; Bhatnagar, A. Wastewater surveillance can have a second act in COVID-19 vaccine distribution. JAMA Health Forum 2021, 2, e201616. [CrossRef]

44. Crits-Christoph, A.; Kantor, R.S.; Olm, M.R.; Whitney, O.N.; Al-Shayeb, B.; Lou, Y.C.; Flamholz, A.; Kennedy, L.C.; Greenwald, H.; Hinkle, A.; et al. Genome sequencing of sewage detects regionally prevalent SARS-CoV-2 variants. mBio 2021, 12, e02703–e02720. [CrossRef]

45. Izquierdo-Lara, R.; Elsinga, G.; Heijnen, L.; Munnink, B.B.O.; Schapendonk, C.M.E.; Nieuwenhuijse, D.; Kon, M.; Lu, L.; Aarestrup, F.M.; Lynch, S.; et al. Monitoring SARS-CoV-2 circulation and diversity through community wastewater sequencing, the Netherlands and Belgium. Emerg. Infect. Dis. J. 2021, 27, 1405–1415. [CrossRef]

46. La Rosa, G.; Mancini, P.; Bonanno Ferraro, G.; Veneri, C.; Iaconelli, M.; Lucentini, L.; Marzella, L.; Brusaferro, S.; Brandtner, D.; Fanelli, A.; et al. Rapid screening for SARS-CoV-2 variants of concern in clinical and environmental samples using nested RT-PCR assays targeting key mutations of the spike protein. Water Res. 2021, 197, 117104. [CrossRef]

47. Saawarn, B.; Hait, S. Occurrence, fate and removal of SARS-CoV-2 in wastewater: Current knowledge and future perspectives. J. Environ. Chem. Eng. 2021, 9, 104870. [CrossRef] [PubMed]

48. Daughton, C. The international imperative to rapidly and inexpensively monitor community-wide COVID-19 infection status and trends. Sci. Total Environ. 2020, 726, 138149. [CrossRef] [PubMed]

49. Bhattacharya, P.; Kumar, M.; Islam, M.T.; Haque, R.; Chakraborty, S.; Ahmad, A.; Niaz, N.K.; Cetecioglu, Z.; Nilsson, D.; Ijumulana, J.; et al. Prevalence of SARS-CoV-2 in Communities Through Wastewater Surveillance—A Potential Approach for Estimation of Disease Burden. Curr. Pollut. Rep. 2021, 7, 160–166. [CrossRef] [PubMed]

50. Bivins, A.; Greaves, J.; Fischer, R.; Yinda, K.C.; Ahmed, W.; Kitajima, M.; Munster, V.J.; Bibby, K. Persistence of SARS-CoV-2 in water and wastewater. Environ. Sci. Technol. Lett. 2020, 7, 937–942. [CrossRef]

51. Sharma, K.; Gangue, R.; Yuan, Z. pH dynamics in sewers and its modeling. Water Res. 2013, 47, 6086–6096. [CrossRef]