



Wastewater-based epidemiology for early warning of SARS-CoV-2 circulation: A pilot study conducted in Sicily, Italy

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ABSTRACT

There is increasing evidence of the use of wastewater-based epidemiology to integrate conventional monitoring assessing disease symptoms and signs of viruses in a specific territory. We present the results of SARS-CoV-2 environmental surveillance activity in wastewater samples collected between September 2020 and July 2021 in 9 wastewater treatment plants (WTPs) located in central and western Sicily, serving over 570,000 residents. The presence of SARS-CoV-2, determined in 206 wastewater samples using RT-qPCR assays, was correlated with the notified and geo-referenced cases on the areas served by the WTPs in the same study period. Overall, 51% of wastewater samples were positive. Samples were correlated with 33,807 SARS-CoV-2 cases, reported in 4 epidemic waves, with a cumulative prevalence of 5.9% among Sicilian residents. The results suggest that the daily prevalence of SARS-CoV-2 active cases was statistically significant and higher in areas with SARS-CoV-2 positive wastewater samples. According to these findings, the proposed method achieves a good sensitivity profile (78.3%) in areas with moderate or high viral circulation (≥ 133 cases/100,000 residents) and may represent a useful tool in the management of epidemics based on an environmental approach, although it is necessary to improve the accuracy of the process.

1. Introduction

The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) brought an unprecedented impact worldwide. According to the World Health Organization data, as of January 2022, about 336 million cumulative cases have been reported worldwide, including more than 5.5 million deaths ([World Health Organization dashboard at](https://www.who.int/dashboards/covid19)). In Italy, the pandemic has led to the

implementation of extraordinary public health measures ranging from the closure of schools and services/activities to local and national lockdown ([Ministero della Salute - Istituto Superiore di Sanità, 2020](https://www.governo.it/Ministero-della-Salute-Istituto-Superiore-di-Sanita-2020)). Because of these special measures, the COVID-19 outbreak has disrupted old habits, routines, and lifestyles, affecting human relationships and the productivity of the entire country ([Cerami et al., 2020](https://www.cerami.it/Cerami-et-al-2020)). In the pandemic context, real-time analyses of epidemiological data played an eminent role to address prompt interventions ([Nahla Khamis, 2020](https://www.nahla.com/Nahla-Khamis-2020)) whereas the

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SARS-CoV-2 circulation needed continuous and rapid microbiological detection to track and isolate cases, also including testing individuals who do not turn to the health system (eg. paucisymptomatic or asymptomatic). According to some authors, the frequency and infectivity of asymptotically infected persons could be the main reasons why COVID-19 has become a pandemic (Nikolai et al., 2020). The integration between epidemiological/microbiological and environmental surveillance systems may help to create an "early warning" strategy able to anticipate the viral spread in these territories and to support decision-making processes based on the risk analysis defined at the regulatory level. Surveillance on wastewater, namely wastewater-based epidemiology (WBE), is based on the principle that pathogens could be excreted by infected subjects through faeces and body fluids, for long or short periods, reaching the purification plants through the sewer system (Hamouda et al., 2021). Therefore, raw sewage entering the wastewater treatment plants (WTPs) constitute an important observation point on the circulation of pathogenic agents in the population, through the analysis of aggregate wastewater samples obtained from entire urban centres and/or differently aggregated urban areas through appropriate sampling strategies (Murakami et al., 2020). Even though SARS-CoV-2 is a respiratory pathogen, the persistence and replication of the virus in the gastrointestinal tract and shedding through faeces has been well documented (Jones et al., 2020; Guo et al., 2021; Mohan et al., 2021; Parasa et al., 2020; Wang et al., 2020a, 2020b). Some people who are infected have higher levels of virus particles in their faeces than others (about 50%). These levels are not determined by whether a person has many or few symptoms, or entirely asymptomatic (National Institute for Public Health and the Environment NIPHE – Ministry of Health, Welfare and Sport, 2021). Many factors can impact the shedding rate of viruses in the faeces, including viremia, the duration, severity and the stage of the disease, or age (Chen and Li, 2020). Moreover, SARS-CoV-2 seems to persist longer in the stool than in the respiratory tract (nearly 22 days) (Zhang et al., 2021). During the current pandemic, traces of SARS-CoV-2 genomes have been identified in wastewater in many areas of the world (Bonanno Ferraro et al., 2021), including Europe (Castiglioni et al., 2021; Hillary et al., 2021; La Rosa et al., 2020; Medema et al., 2020; Randazzo et al., 2020; Westhaus et al., 2021), USA (Gonzalez et al., 2020; Sherchan et al., 2020; Peccia et al., 2020; Wu F. et al., 2020), Australia (Ahmed et al., 2020), China (Mao et al., 2020), Japan (Haramoto et al., 2020; Hata et al., 2021), United Arab Emirates (Albastaki et al., 2021; Hasan et al., 2021) and Africa (Johnson et al., 2021; Jmii et al., 2021). In Italy, a retrospective WBE study coordinated by the Italian National Health Institute (NHI) showed that SARS-CoV-2 was already circulating in Northern Italy at the end of 2019, before the country's first confirmed cases in mid-February (La Rosa et al., 2021a). These bodies of evidence highlight the importance of sewage water as a sentinel tool to monitor the presence of epidemic viruses circulating in the general population and to identify outbreaks even before cases are reported to the healthcare system. To this end, in July 2020 the NHI launched a nationwide wastewater pilot monitoring program to investigate the spread of SARS-CoV-2 during the summer in tourist locations and then in autumn and winter seasons. Our study was conducted in the framework of the national surveillance program and reports the results of SARS-CoV-2 surveillance in wastewater samples from 9 conventional treatment plants in place in 8 Sicilian cities, during four different waves characterized by the different spread of the virus. Moreover, the relationship between the presence of the pandemic virus in wastewater and the trend of cases of infection in the population of Western Sicily was investigated to evaluate the application of this method as a possible early warning of SARS-CoV-2 circulation in the general population.

2. Materials and methods

2.1. Study design and sample collection

This observational study was carried out in Sicily (Italy), the largest island in the Mediterranean Sea accounting for about 5 million inhabitants. Nine wastewater treatment plants (WTPs) located in 8 cities and four different provinces of central and western Sicily were selected, serving a total of 574,107 inhabitants (ranging from 7062 to 241,206; 11.2% of total residents of the island). Wastewater samples (N = 206) were collected every 15 days for approximately 12 months (between July 21, 2020, and August 16, 2021). The position and the characteristics of the WTPs are shown in Fig. 1. For each WTP, 1 L of a 24-h composite sample of raw sewage was collected by an automatic sampling device. Collected samples were transferred on ice to the laboratory, stored at +4 °C, and analyzed for the detection of SARS-CoV-2 RNA within 12 h from sampling. In four cases, in which it was not possible to carry out the transport quickly, samples were immediately stored at –20 °C and transported to the laboratory still frozen within 15 days from the date of collection.

2.2. Laboratory methods

2.2.1. Virus concentration

Before the concentration, 10 µL of a standard murine norovirus (MNV-1) suspension (supplied by NHI) was added to each sample (250 mL) as extraction control to evaluate the efficiency of the whole process. Sample concentration took place using a two-phase (PEG-dextran method) separation as detailed in the 2003 WHO Guidelines for Environmental Surveillance of Poliovirus protocol (World Health Organization, 2003a) with modifications to adapt the protocol to enveloped viruses (La Rosa et al., 2020).

2.2.2. RNA extraction

Viral RNA extraction was carried out using a semi-automated extraction method with buffer lysis and magnetic silica. In detail, the lysis phase was performed using 10 mL of lysis buffer (NucliSENS, bioMerieux, Marcy l'Etoile, France) with 5 mL of a concentrated sample, after an incubation of 20 min at room temperature, 100 µL of magnetic silica beads (bioMerieux, Marcy l'Etoile, France) were added and, after further incubation of 10 min, an automated procedure was performed by nucleic acid purification system (Auto-Pure96, All Sheng Instruments, Zhejiang, China). Before molecular tests, the extracted nucleic acids were purified from potential PCR inhibitors using the OneStep PCR Inhibitor Removal Kit (Zymo Research, CA, USA).

2.2.3. RT-qPCR

All RT-qPCR assays for SARS-CoV-2 were performed in the same laboratory with a QuantStudio 7K Flex Real-Time PCR System (Thermo Scientific). The reaction mixture (15 µL) consisted of 3.9 µL of Master Mix (QuantiNova Pathogen kit - Qiagen, CA, USA), 0.45 µL of 30 µM each primer, 0.3 µL of 10 µM of probe, water to the final volume of 10 µL and 5 µL of RNA template. The oligonucleotide sequences of primers and probe and thermal RT-qPCR conditions were described in La Rosa et al. (2020). All reactions were performed in quadruplicate. Molecular biology water served as non-template control. Verification of PCR inhibition was performed as a quality parameter of the determinations. To verify the inhibition, the PCR Ct (cycle threshold) value obtained from the sample added with 1 µL of a Ct 18 clinical positive control RNA was compared with the PCR Ct value of a water for molecular biology sample added with 1 µL of the same RNA according to the following formula: $\Delta Ct = Ct(\text{sample} + \text{control RNA}) - Ct(\text{water} + \text{control RNA})$. The sample was considered acceptable if ΔCt was ≤ 2 . The limit of detection (LoD) of the assay targeting ORF1ab gene was also evaluated. To assess the concentration/extraction efficiency of the method, prior to concentration, 100 µL of a process control virus solution (Murine Norovirus

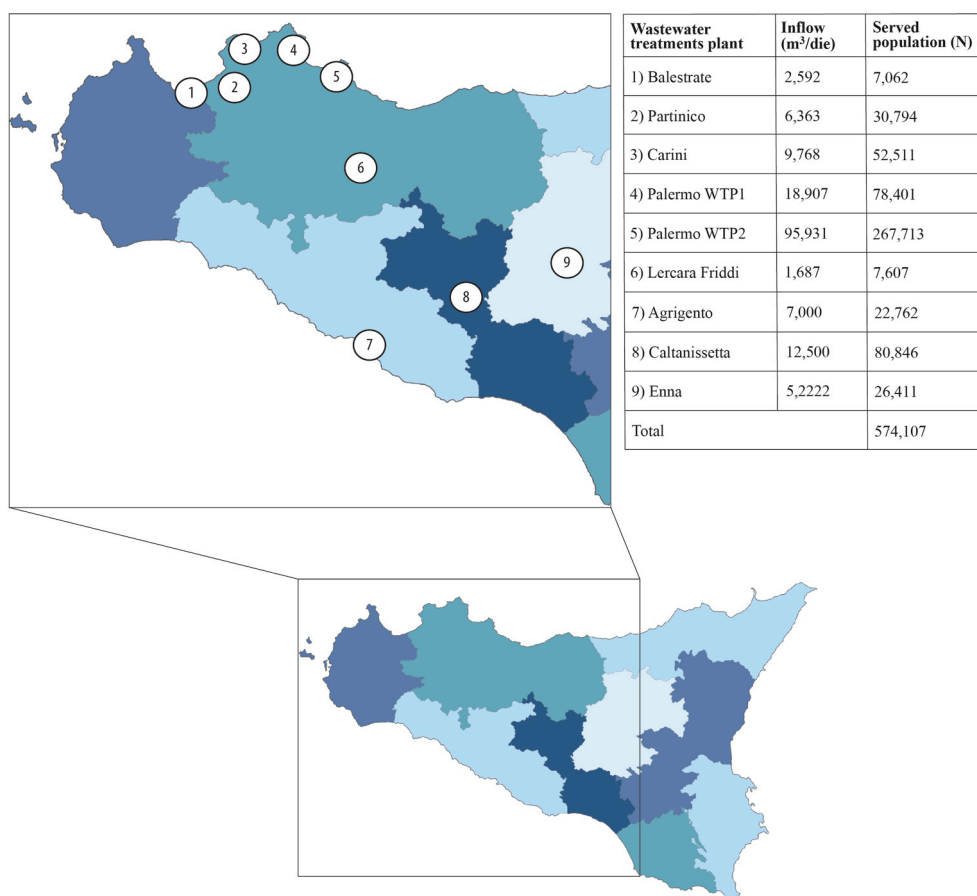


Fig. 1. Map of Sicily showing the locations of the wastewater treatment plants.

at 2.0×10^3 genomic copies/ μL) were added to 250 mL of each wastewater sample. The samples were then concentrated and extracted. For the PCR assays, serial ten-fold dilutions of a stock solution of the process control virus were used to produce standard curves. The Ct values of the reaction of samples spiked with process control virus were compared to the Ct value of the reaction containing the undiluted process control virus and the concentration/extraction efficiency (%) was calculated according to the following formula $= 10^{(\Delta\text{Ct}/m)} \times F \times 100$ ($\Delta\text{Ct} = \text{Ct sample} - \text{Ct undiluted solution of control process virus}$; $m = \text{slope of standard curve of control process virus}$; $F = \text{fraction of the initial sample processed}$). The sample was considered acceptable if the concentration/extraction efficiency was $\geq 1\%$, as suggested by the protocol of NHI.

2.2.4. Clinical data sources

Sicilian SARS-CoV-2 cases were recorded according to the protocol shared by the integrated national surveillance system established by NHI (Istituto Superiore di Sanità, 2020). SARS-CoV-2 patients were considered eligible if they met the following inclusion criteria: being a resident of Sicily (or temporarily domiciled in Sicily) and having a laboratory-confirmed SARS-CoV-2 positive result using a reverse transcriptase real-time polymerase chain reaction (Real-Time RT-PCR) of nasal, pharyngeal, or nasopharyngeal swabs between January 1st, 2020, and August 31st, 2021. For each case, the following data were extracted: demographics (birth date, sex, place of residence); collection date of the first SARS-CoV-2 PCR positive test, collection date of the first SARS-CoV-2 PCR negative test (or end of quarantine), and clinical status. Subjects included in the analyses were anonymized and geocoded by an automated information algorithm performed in accordance with the privacy law. Thus, cases were attributed to the Sicilian areas served by

the 9 wastewater treatment facilities. The number of resident populations for each area was calculated by considering the census sections included in that area as reported by the last available census report carried out by the Italian National Institute of Statistics (ISTAT) in 2011. Each SARS-CoV-2 case was considered as an active case during the window period between the collection date of the first SARS-CoV-2 PCR positive test and the collection date of the first SARS-CoV-2 PCR negative test (or end of quarantine). Otherwise, a SARS-CoV-2 case was considered as a new SARS-CoV-2 case only on the day when they got the first SARS-CoV-2 PCR positivity.

2.3. Statistical analyses

Categorical variables were summarized as percentages, whereas quantitative variables were presented as median (and interquartile range). For each area, the following occurrence measures per day of the study period were calculated:

- prevalence of active SARS-CoV-2 cases: number of active SARS-CoV-2 cases/residents*100,000
- incidence of new SARS-CoV-2 cases: number of new SARS-CoV-2 cases/residents*100,000.

Active SARS-CoV-2 incidence rates were compared by Poisson regression analysis. A logistic regression model was calculated to evaluate the association between the active SARS-CoV-2 incidence rates and the probability of positive PCR results of wastewater samples. The logistic regression model results were used to calculate the fitted predicted values for positive and negative PCR results of wastewater samples. A receiver operating characteristic (ROC) curve was used to assess the

active SARS-CoV-2 incidence rates for each PCR sample and to identify an optimal cut-off value that could predict the active SARS-CoV-2 incidence rate (ROCR Package and Optimal Cutpoints Package). Youden's index has been used for calculating the best cut-off value in the ROC curve. All the analyses were performed with the R software package and a p-value < 0.05 was considered statistically significant.

3. Results

The LoD was determined by spiking wastewater extracts with solutions of concentrations of approx. 1,000, 100, 50, 20, 10, 2 and 1.0 g.c./μL. Ten replicates of each dilution were tested. The LoD was determined as the lowest concentration to which all ten replicates were positive. The assay had a LoD of 2 g.c./μL. The recovery rate of SARS-CoV-2 from sewages may have varied according to the physico-chemical properties of wastewater samples (Mean = 11.31 ± 14.79%; Range = 1.0–61.0%; CI 95% = 3.58)". Overall, 51% (n = 105/206) of wastewater samples were positive to SARS-CoV-2 (range 23.1%–70% in the 9 different facilities). For each WTP, SARS-CoV-2 was detected as follows: 23.1% (n = 6/26) in WTP1, 58.3% (n = 14/24) in WTP2, 53.6% (n = 15/28) in WTP3, 72.7% (n = 16/22) in WTP4, 57.1% (n = 16/28) in WTP5, (n = 10/22) 45.4% in WTP6, 42.8% (n = 9/21) in WTP7, 70.0% (n = 14/20) in WTP8, and 25.0% (n = 5/20) in WTP9. SARS-CoV-2 cumulative prevalence was 5.9% considering the whole Sicilian area studied (p = NS). Prevalence of SARS-CoV-2 active cases was statistically significant higher in areas with SARS-CoV-2 positive wastewater samples than in area with negative SARS-CoV-2 wastewater samples (273.8 cases/100,000 residents vs. 46.9 cases/100,000 residents; p < 0.001) (Table 1). Differences were also observed either considering only symptomatic patients (87.4/100,000 vs. 18.9/100,000; p < 0.001) or asymptomatic active cases (170.9/100,000 vs. 25.5/100,000; p < 0.001).

Fig. 2 depicts the SARS-CoV-2 incidence rate (primary y-axis) and the relative frequency of PCR positive samples on all 9 WTPs (secondary y-axis) with respect to the observation time (x-axis). Overall, the cumulative SARS-CoV-2 epidemic curve observed in all WTPs overlapped the prevalence of SARS-CoV-2 positive wastewater samples. A ROC curve was applied to identify the level of SARS-CoV-2 cases/100,000 inhabitants able to predict the wastewater sample results (Fig. 3A and B). The analysis showed a best cut-off value of 133 active cases/100,000 residents (sensitivity = 80.3%, specificity = 76.5%, accuracy = 78.3%) that should be considered equal to 9.5 new cases/day/100,000 inhabitants. Finally, a logistic regression analysis was implemented to evaluate the association between wastewater positivity and SARS-CoV-2 cases. The probability of wastewater positivity was found to increase by about 0.86% per active case/100,000 inhabitants (p < 0.001). The fitted probability of positive (and negative) wastewater samples according to SARS-CoV-2 prevalence was reported in Fig. 4. In depth, the probability of a positive sample is quite low (<24.6%) when SARS-CoV-2 active cases were below 50 active cases/100,000, whereas it was relatively

Table 1
Variables involved in determining PCR positivity in samples collected from wastewater treatment plants.

Variable	PCR positive wastewater samples	PCR negative wastewater samples	p-value
- SARS-CoV-2 active cases [median (IQR) *100,000]	273.8 (118.6–408.2)	46.9 (14.0–125.7)	<0.001
- SARS-CoV-2 active cases (only symptomatic patients) [median (IQR) *100,000]	87.4 (53.2–128.9)	18.9 (3.8–64.9)	<0.001
- SARS-CoV-2 asymptomatic active cases [median (IQR) *100,000]	170.9 (61.3–273.4)	25.5 (4.4–66.0)	<0.001

high (>91.9%) when SARS-CoV-2 active cases were above 400 active cases/100,000.

4. Discussion

To date, four epidemic waves of SARS-CoV-2 were recorded in Sicily, the first of which showed very low intensity, probably underestimated due to a low number of diagnostic tests performed, while the others presented at a significantly higher intensity. Although the research of SARS-CoV-2 in wastewater was performed occasionally during the first wave, it has been evaluated systematically since the beginning of the second wave of the pandemic in Sicily which occurred in July 2020. The aim of searching SARS-CoV-2 in WTPs of cities with a variable number of inhabitants (varying between approximately 6200 and 670,000) of central and western Sicily is to evaluate whether WBE could represent a good proxy of the early spread of the virus. The study findings have shown that SARS-CoV-2 was detected in all monitored sites, both in small and large treatment plants. Furthermore, the probability of detecting the viruses in wastewater samples changed in relation to the number of SARS-CoV-2 cases detected in the population. The analysis allowed estimation with 78.3% accuracy in the presence of more than 133 active cases/100,000 inhabitants (equal to 9.5 new cases per day per 100,000) when a positive wastewater sample was detected. According to this data, a proportional increase in the frequency of sampling could be needed when the expected SARS-CoV-2 active cases could be very low. The sensitivity reported in our research may appear to be lower than that reported in other studies (De Giglio et al., 2021; Hewitt et al., 2021). However, several factors may have contributed to these discrepancies, including the recovery rate of method, the variation in the number of asymptomatic and pre-symptomatic individuals, the COVID-19 testing rates, the incidence rates observed during the study period, the WTPs or environmental characteristics as plant daily capacity, and the rainfall events. Nevertheless, despite the global clinical surveillance for COVID-19 has been established, remain several cases of asymptomatic individuals, and those with very mild symptoms, that would not be identified, and therefore, together with contacts not traced, a large part of the real transmission could be potentially missed (Angulo et al., 2021; Havers et al., 2020; Larsen et al., 2020; Wu S.L. et al., 2020). The wastewater surveillance of SARS-CoV-2 has proven to be a powerful tool to evaluate disease incidence at the community level, but it still needs to be integrated into other public health initiatives (e.g., campaign-based and randomized testing of individuals such as the presence of pathogen or antibodies, clinical case reporting, and mobile-based contact-tracking and self-reporting systems) (Larsen et al., 2020). This represents a significant challenge considering the poor integration of the environmental and clinical science communities (Boulos and Geraghty, 2020).

WBE was theorized in 2001 (Daughton and Ternes, 2001), then implemented to trace illicit drug and pharmaceutical consumption in communities (Lopardo et al., 2018; Maida et al., 2017; Nikolaos et al., 2017; van Nuijs et al., 2011; Zuccato et al., 2005) and human pathogens during the global polio eradication program (Hovi et al., 2012; Ndiaye et al., 2014; Roberts, 2013). Today, we know that human pathogens in wastewater can represent a good proxy of the concentrations excreted by the population afferent to the treatment plant if they persist long enough (2–4 days) to be detected (Brouwer et al., 2018; Carducci et al., 2020; Kitajima et al., 2020). Therefore, monitoring temporal changes in viral concentrations and diversity in community wastewater samples can be used not only to determine the true extent of the infection in the population but also the emergence of new viral strains and the early detection of new viral outbreaks (Ahmed et al., 2020; Daughton, 2012; Johnson et al., 2021; Hart and Halden, 2020; La Rosa et al., 2021b; Monteiro et al., 2022). While retrospective studies have already demonstrated the feasibility as an alert system, WBE for real-time early warning cannot be realized without frequent sampling, rapid sample delivery, analytical turnaround, and reporting. For this reason, based on

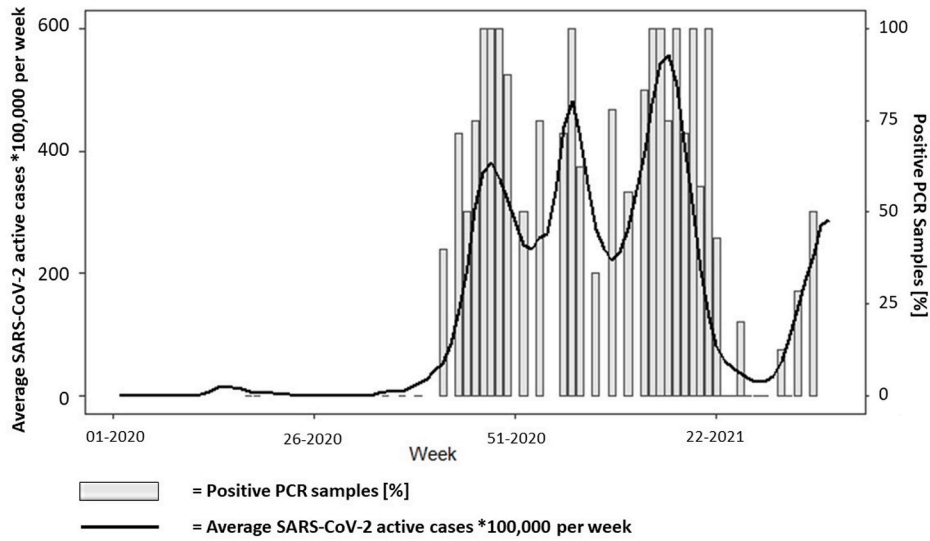


Fig. 2. PCR results and average active cases observed on a weekly basis in the areas connected with the WTPs.

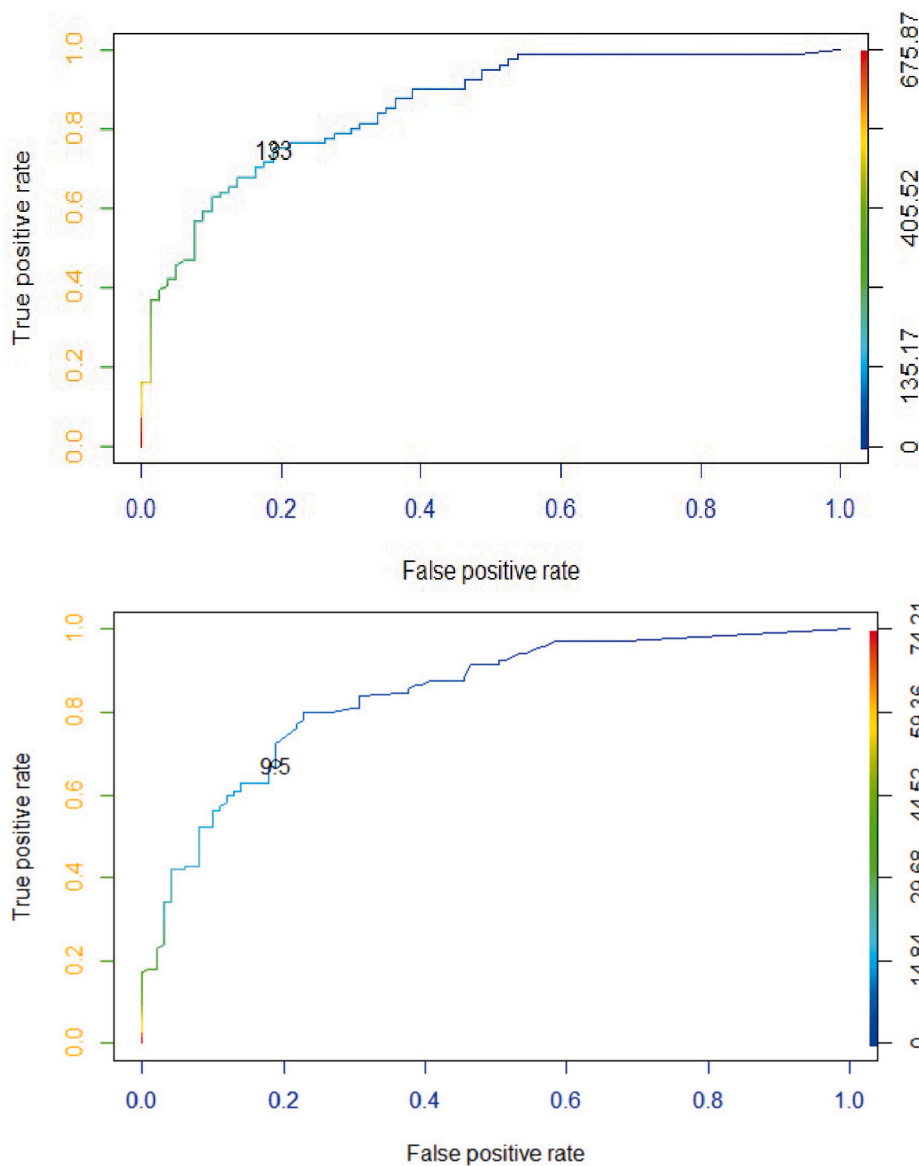


Fig. 3. ROC curve to predict the number of SARS-CoV-2 active cases/100,000 inhabitants (A) and SARS-CoV-2 new cases/100,000 inhabitants (B).A: SARS-CoV-2 active cases/100,000 inhabitants: best cut-off point 133/100,000 residents; sensitivity = 80.3% (69.2%–88.2%); specificity = 76.5% (65.8%–84.7%); AUC = 78.3% (70.9%–84.2%).B: SARS-CoV-2 new cases/100,000 inhabitants: best cut-off point 9.5/100,000 residents; sensitivity = 78.5% (69.3%–85.6%); specificity = 78.8% (69.2%–86.1%); AUC = 78.6% (72.3%–83.9%).

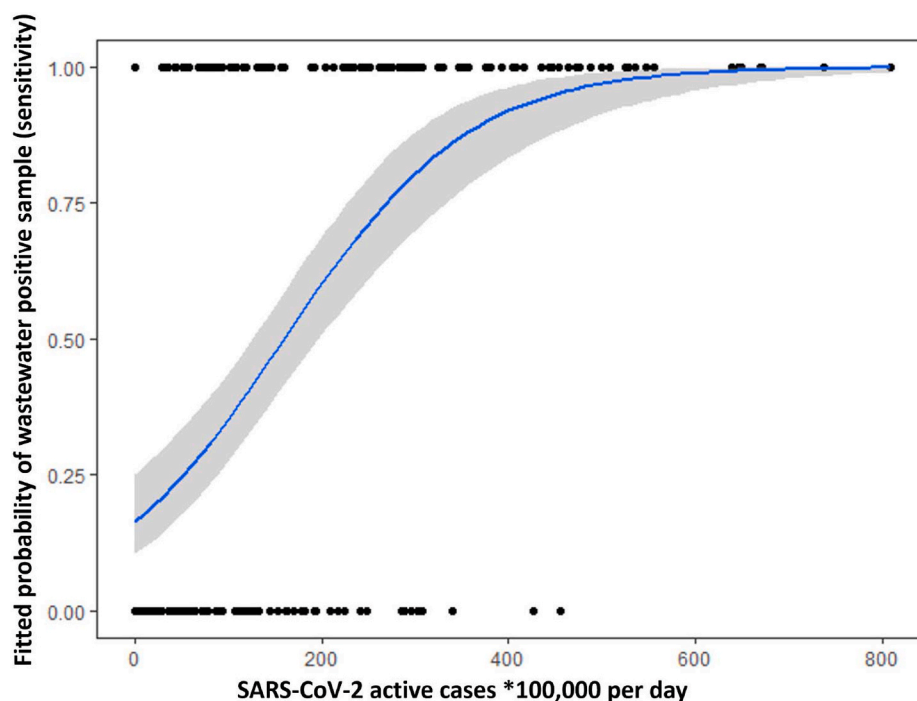


Fig. 4. Predicted probability of positive wastewater according to the active SARS-CoV-2 cases.

the EU Commission recommendation n. 2021/472 (European Commission, 2021), the EU Member States are strongly encouraged to implement as soon as possible, and no later than October 1st, 2021, a national wastewater surveillance system targeted at data collection of SARS-CoV-2 and its variants with the minimum sampling frequency on two samples per week for large cities with over 150,000 inhabitants. In Italy, cities with more than 50,000 inhabitants will also be monitored due to the low presence of cities with more than 150,000 inhabitants by a collection of one sample per week. Nevertheless, the findings presented in this study have some limitations to be further investigated to clarify their potential impact on sensitivity of the method starting from its recovery rate that seems lower than other described. As discussed by McMahan et al. (2021), the following factors are currently being studied in the new wastewater-based surveillance approach: the quantification of viral RNA to better evaluate the presence of cases in the population; the impact of physical-chemical characteristics of wastewater on viral recovery; the sewage flow rate; the presence of COVID hospitals; the size of the population residing in the area served by WTP; differences between urban and suburban centres. A further limitation could be given to the accuracy in the geo-localization due to errors in addresses input or in the exclusion of tourists who were temporary present in Sicily at the time of SARS-CoV-2 positivity, and to the unavailability of information on the location address. The effectiveness of the WBE early-warning approach, as proved by the outcome of this research, is prompting the implementation of the Italian nationwide wastewater monitoring system to monitor for the SARS-CoV-2, by effectively integrating the conventional clinical surveillance.

5. Conclusions

The detection of SARS-CoV-2 RNA in wastewater may provide a good picture of the trend of infections in a community, providing valuable real-time data on the viral spread within a spatial unit, hence, putatively requiring fewer resources than individual diagnostic testing.

To our knowledge, this is one of the first studies aimed to correlate the number of geo-referenced COVID-19 cases, within the area served by a wastewater treatment plant, with SARS-CoV-2 detection in wastewaters adducted to that plant to estimate the specificity of the method

applied. Although it is necessary to improve both the sensitivity and the accuracy of the environmental analysis, according to our findings, the current method already seems to achieve a good sensitivity profile in areas with moderate or high viral circulation and may represent a useful tool for integration in the management of epidemics. The integration between clinical and environmental surveillance systems may constitute an appropriate decision-support tool to put in place public health intervention to prevent the spreading of the epidemic virus in the general population and guide public health decisions.

CRedit authorship contribution statement

Carmelo Massimo Maida: Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Microbiological characterization. **Emanuele Amodio:** Formal analysis, Writing – review & editing. **Walter Mazzucco:** Supervision, Writing – original draft, Writing – review & editing. **Giuseppina La Rosa:** Conceptualization, Writing – review & editing. **Luca Lucentini:** Conceptualization, Writing – review & editing. **Elisabetta Suffredini:** Conceptualization, Writing – review & editing. **Mario Palermo:** Writing – review & editing. **Gina Andolina:** Microbiological characterization. **Francesca Rita Iaia:** Microbiological characterization. **Fabrizio Merlo:** Samples collection, Writing – review & editing. **Massimo Giuseppe Chiarelli:** Samples collection, Writing – review & editing. **Angelo Siragusa:** Samples collection, Writing – review & editing. **Francesco Vitale:** Supervision, Writing – review & editing. **Fabio Tramuto:** Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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