Surveillance of SARS-CoV-2 in Urban Wastewater in Italy

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***Abstract* — The presence of SARS-CoV-2 RNA in wastewaters was demonstrated early into the COVID-19 pandemic. Data on the presence of SARS-CoV-2 in urban wastewater can be exploited for different aims, including: i) description of outbreaks trends, ii) early warning system for new COVID-19 outbreaks or for the spread of the virus in new territories, iii) study of SARS-CoV-2 genetic diversity and detection of its variants, and iv) estimating the prevalence of COVID-19 infections. Therefore, wastewater surveillance (known as Wastewater Based Epidemiology, WBE) can be a powerful tool to support the decision-making process on public health measures. Italy was among the first EU countries investigating the occurrence and concentration of SARS-CoV-2 RNA in urban wastewaters, virus detection being accomplished at an early phase of the epidemic, between February and May 2020 in north and central Italy. The present study reports on the methodological issues, related to sample data collection and management, encountered in establishing the systematic, wastewater-based SARS-CoV-2 surveillance, and describes the results of the first six months of surveillance.**

Keywords — wastewater analysis; environmental surveillance; epidemiology; Covid-19 epidemic; GIS database

I. Introduction

Faecal shedding of SARS-CoV-2 by infected individuals (both symptomatic and asymptomatic ones) was demonstrated early into the COVID-19 pandemic [1]. Consequently, detection of SARS-CoV-2 in urban wastewater and data on its concentration were proposed for different public health aims such as studying outbreak trends, exploring the SARS-CoV-2 genetic diversity and variants, and estimating the prevalence of infections [2]. Through these activities, wastewater surveillance (also known as Wastewater Based Epidemiology, WBE) can be a powerful tool to support the decision-making process on public health. In February 2022, the World Health Organization published the interim guidance "Environmental surveillance for SARS-CoV-2 to complement public health surveillance" [3], a document providing support for the integration of

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environmental surveillance into COVID-19 control strategies, as a complementary tool in the decision-making process. The G7 Health Ministers meeting, held in Berlin, Germany, on 19-20 May 2022, committed to explore options to support national authorities in the efforts to implement non-invasive surveillance methods, countrywide wastewater surveillance systems, which had rapidly and significantly evolved during the COVID-19 pandemic, to support the early detection and investigate the spread of new infectious agents or of emerging threats (e.g. influenza virus, enteric viruses like poliovirus, and antimicrobial resistant pathogens) [4].

The national Institute of Health (ISS) in Italy was among the first research centres to explore SARS-CoV-2 RNA occurrence in urban sewage. Virus detection, indeed, was achieved between February and May 2020 in wastewater treatment plants (WTPs) located in areas with both high and low epidemic circulation (Milan and Rome, respectively) [5]. Later, a retrospective study performed on archive samples, collected before the first Italian autochthonous case, demonstrated that SARS-CoV-2 had been circulating in northern Italy since December 2019 and that the circulation involved different geographic regions [6]. Several studies have demonstrated that detecting SARS-CoV-2 in raw wastewater can be a powerful tool to detect epidemics, study outbreak trends, assess the prevalence of infections, and to describe circulating variants by studying viral diversity [7].

Based on this, in July 2020, ISS launched a pilot project named "SARI" (Epidemiological Surveillance for SARS-CoV-2 in urban sewage) [8], and created, under his coordination, a voluntary national network (without dedicated fundings) for SARS-CoV-2 surveillance in wastewaters. Participation to the network was granted by Health Competent Authorities (C.A.) such as Regions and Autonomous Provinces (A.P.), and by other institutions as regional environmental protection agencies (ARPA), zooprophylactic institutes (IZS), universities, wastewater service providers, and research centres.

The "EU Commission Recommendation 2021/472 on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU" of the 17th of March 2021 encouraged the Member States to build national wastewater surveillance systems to obtain data on SARS-CoV-2 and its variants [6]. Following this Recommendation, the SARI project activities were progressively converted into a surveillance system, with a dedicate funding by the Italian government, and by the European Commission. The surveillance is coordinated by ISS. This paper describes the implementation of the surveillance network and results obtained during the period October - March 2022.

II. Materials and Methods

Network organization, sample collection and processing

After more than one year of pilot project, the official surveillance system started in October 2021 and as of 31 March 2022, 19 of the 21 Italian Regions/A.P. (95% coverage) are officially involved in the environmental surveillance program. Each Region/A.P. participating to the surveillance has selected and appointed the laboratories for the analytical activities, consisting in the detection and quantification of SARS-CoV-2 RNA in wastewater samples. The laboratories include different institutions: environmental protection agencies, zooprophylactic institutes, universities, and other research institutions, as well as wastewater service laboratories. Overall, 43 laboratories are involved all over the country, as shown in Figure 1.

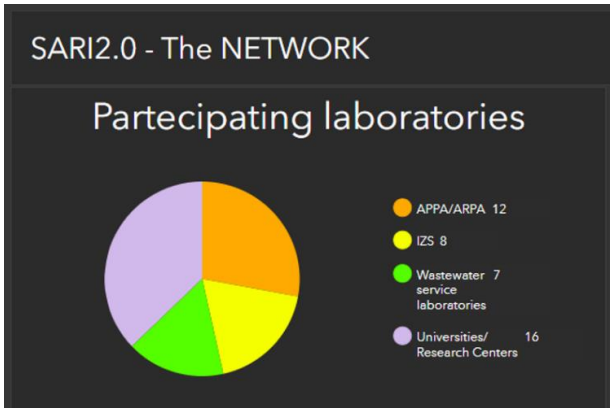

Fig. 1. Laboratories participating to the SARI network

In agreement with EU Commission Recommendation 2021/472, the monitoring network includes WTPs located in large centers with more than 150.000 inhabitants. Urban centers with a population between 50.000 and 150.000 inhabitants were also included in the program, to increase population and geographic coverage. Overall, a total of 167 WTPs are monitored within the environmental surveillance network (see Figure 2), serving a total of approximately 32 million population equivalents [1]. Plants serving large urban centers (>150.000 inhabitants) are sampled twice per week as recommended by Rec. 2021/472, while small WTPs are monitored once per week. Overall, this sampling plan collects about 200 wastewater samples per week throughout the country.

Analytical protocols for SARS-CoV-2 detection and quantification in sewage were elaborated by ISS during the pilot project and shared with the participating laboratories. Moreover, reference materials required for the implementation of the analytical procedures were shipped to all the laboratories of the network [9]. Methods were modified over time, with revisions of the national protocol, including improvements accordingly to knowledge updates. Briefly, wastewater composite samples (24-hour composite, resulting from a combination of a series of equal-volume aliquots collected via an automatic sampler or manually over the period), are collected at the inlet of WTPs, before any treatment, and are transported under refrigerated

[1] The concept of "population equivalent" (PE) is referred to the quantity of oxygen needed to treat the biodegradable organic load in the raw sewage. A common assumption is given by one unit equaling 54 grams of BOD (biochemical oxygen demand) per 24 hours

condition to laboratories for further processing. The first phase of the analytical process is the concentration of viral particles from the wastewater samples, which is performed by polyethylene glycol (PEG) precipitation followed by centrifugation, as previously described [9]. Afterward, viral RNA is extracted using the "boom technology" with magnetic silica beads. Finally, extracted and purified RNA is subjected to a quantitative real time PCR to determine SARS-CoV-2 concentrations, a method which combines amplification and detection into a single step. The results are finally expressed as genome copies/L of wastewater. A normalization step is then needed, to account for variations of viral concentrations due to rain or dilution with other water sources. This is performed by normalizing quantitative data for the WTP daily flowrate. Moreover, normalization for the population equivalent of the WTP is needed to allow comparison of viral concentrations in different urban areas, Finally, data are reported as number of genome copies/day*inhabitant.

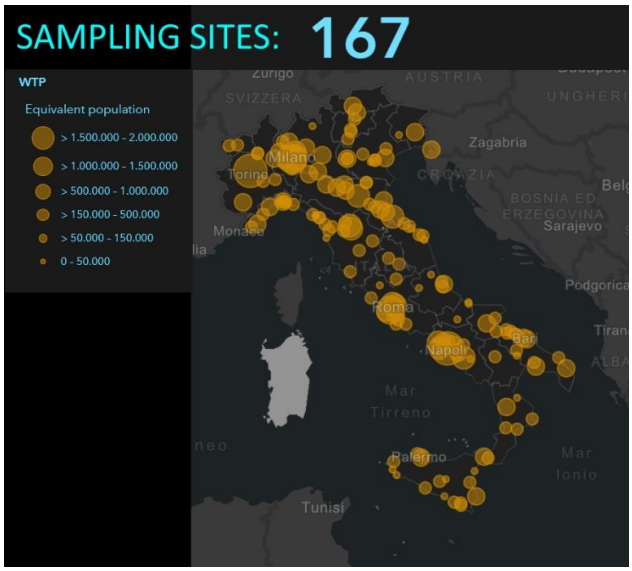

Fig.2. WTPs sampling points in Italy. Each dot's size is scaled based on the population equivalent of the associated WTP

The study of SARS-CoV-2 diversity and geographic distribution of viral variants is another main aim of the environmental surveillance of SARS-CoV-2, as per EU Recommendation 2021/472. To investigate SARS-CoV-2 variants in sewage Italy, "*flash surveys*" are regularly organized to sequence samples collected throughout the country within a short time window (5 days). The first pilot flash survey was conducted in July 2021. Since October 2021 the surveys are performed with a monthly frequency. For tracking SARS-CoV-2 variants, samples are amplified using a long-nested RT-PCR assay of about ~1600 bp, targeting the region coding for the spike protein. This long fragment includes several significant nucleotide changes differentiating the major known SARS-CoV-2 variants [14, 15]. Both Sanger sequencing and Next Generation Sequencing (NGS) using MinION platform (Oxford Nanopore Technology) are performed on these samples. All *flash survey* results are regularly published in the ISS website [16].

Data and users' management

Participation in the project of the Local Structures ("*strutture territoriali*", ST) is subject to confirmation of the availability of each structure to conduct the experimental activities and is arranged through assignation to one or more of the following four different levels, on the basis of specific availability, expertise and resources:

- ST-1 level: sampling, storage of raw waste sample and dispatch to the upper tier facility.
- ST-2 [2] level: virus concentration from sample, storage, and dispatch of concentrated sample to ST3.
- ST-3 level: extraction of nucleic acids, molecular screening, and data transmission to ST3R.
- ST-3R level: regional reference structure that coordinates operations and sees the results of the Region.

A specific Web App for entering and editing the information related to the wastewater samples has been developed by the engineers involved in the project to simplify the workflow of the laboratory personnel enrolled in the surveillance network. The Web App consists of a digital form that the user should fill in by entering information on the collected wastewater samples:

1) WTP name/location, sampling date, WTP flow rate, etc.)

2) methods for the analysis: sampling, concentration, extraction of nucleic acids, molecular methods for qualitative (presence/absence) and quantitative (viral loads, etc.) determinations. The compilation of the digital form is partially automatized, to reduce manual intervention to a minimum.

The registration of the sample in the database is assigned to the structure of level ST1. When the user accesses the App with his credentials, the homepage is shown (Fig. 3), where he can add a new sample by pressing the button "Collect". The "Collect" function will open a brief questionnaire (Fig. 4). When the record is sent, the central database also records the information associated with the WTP (location, municipality, province, region, population equivalent) and generates a unique code, which the user will receive in the Inbox section (Fig. 3c) so that it can be physically placed on the test sample.

Information about the sample entered by ST1 is transferred to the "Inbox" section of the ST2. From the Inbox, where the ST2 will find the samples compiled by all the ST1s submitting samples to the structure, the ST2 can complete the analytical information related to sample preparation (sample volume, sample type, storage temperature, volume of the concentrated sample, final volume of the concentrated sample) (Fig. 4) and submit the filled record to ST3.

[2] Dismissed after the release of the modified protocol (DOI:10.5281/zenodo.5758725) which foresees a unique ST performing the entire protocol, from viral concentration to SARS-CoV-2 viral loads determination.

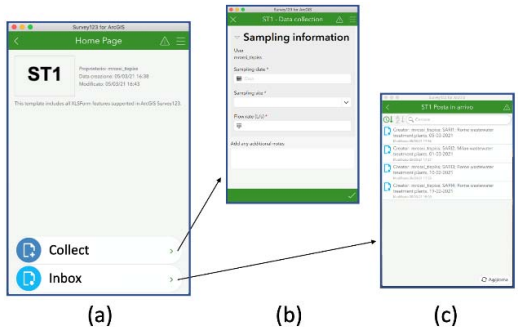

Fig. 3 Web App for the ST1: user interface and illustration of operation.

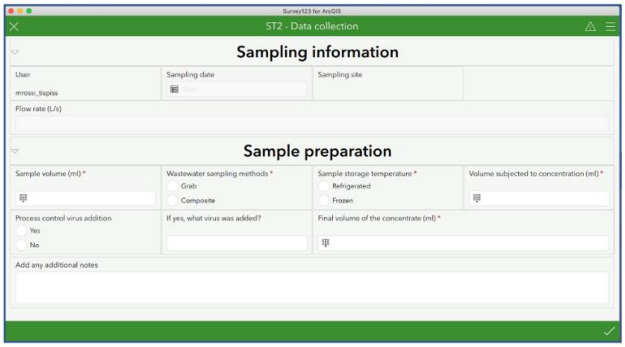

Fig. 4. ST2 Web App: user interface.

The ST3 App is analogous to those of ST2 and ST1 (Fig. 5) and in the Inbox ST3 will find the records of the samples created by ST1/ST2, which can be updated with the information related to the extraction of nucleic acids and the analysis results of the molecular method (real-time RT-PCR). The samples remain stored in the inbox until the answer to the question "Input finished", set by default to the value "no", passes to the value "yes".

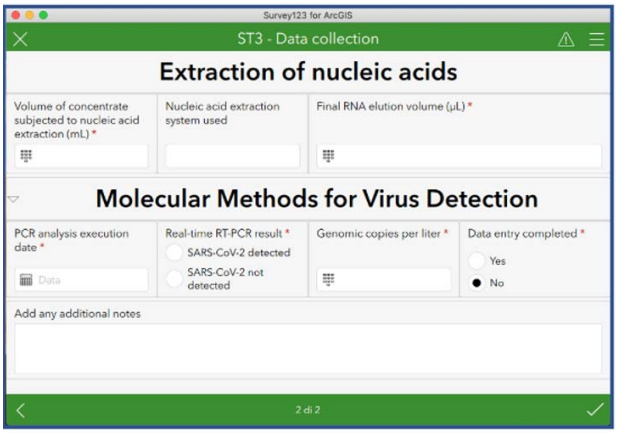

Fig. 5 – ST3 Web App: user interface.

Finally, the data entered is collected in a national database and Health C.A. can view – on an interactive and updated in real time dashboard – the results of the samples collected from all the WTPs of their territory of competence (i.e. regional C.A. can access their own data, while national C.A. can visualize the whole dataset). Username and password are applied to access the system, for data entry and for data viewing.

Data collection and visualization: GIS database SARI 2.0

A system based on GIS technology has been developed by the by the engineers involved in the project at the ISS National Centre for Innovative Technologies for Public Health. The database (current version SARI 2.0) includes the following information:

- laboratories enrolled in the surveillance network, with their role (sample collection and oranalysis)
- coordinators for laboratories and Region/A.P. (name, role, telephone and email contact);
- Data describing WTPs sampling points (latitude, longitude, location, catchment area, population equivalent, sampling frequency, etc.);
- Sample (meta)data, including associated sampling point and date, analytical data (procedure, laboratory performing the analyses, starting and closing date of testing, etc.), SARS-CoV-2 detection and quantification (raw viral loads and viral concentrations normalized for WTP flow rate and population equivalent)
- other accessory information.

A "User manual for data entry in the SARI.2.0 database" was elaborated and shared with the network to detail methods for sampling, data entry and data management [17]. Effort are ongoing in order to interface the SARI 2.0 database with the European DEEP platform (Digital European Exchange Platform; https://wastewater-observatory.jrc.ec.europa.eu/), which is under implementation at the date of writing, with the aim of ensuring that the results of the wastewater surveillance are quickly shared by electronic means and conjointly used for the assessment of SARS-CoV-2 trends in the EU countries.

Results

Over the first six months of surveillance, between October and March 2022, 3.865 wastewater samples were collected throughout Italy. Of these, 85% tested positive for SARS-CoV-2 RNA. Figure 6 shows the results of SARS-CoV-2 detection (presence/absence data) by week of sampling. Week 40 (2021) represent the period 04.10.2021 - 10.10.2021. Week 1 (2022) correspond to 03.01.2022 - 09.01.2022. Red and green and colours represent positive and negative samples, respectively. Samples represented in blue are yet to be analyzed at the time of data extraction.

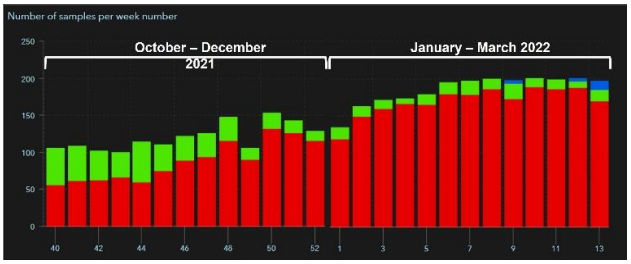

Fig. 6. Positive/negative results by week of sampling. Red=positive sample; green=negative sample; blue=under testing. 2021: Week 40= 04.10.2021 - 10.10.2021; week 52= 27.12.2021 - 02.01.2022. 2022: week 1= 03.01.2022 - 09.01.2022; week 13= 28.03.2022 - 03.04.2022

As shown in the graph, there was a considerable increment in the number of collected samples, which doubled in the six-month period (from 420 in October 2021 to 850 in March 2022). This is the result of the progressive activation of new Regions or further WTP sampling points in the surveillance plan. During 2022 (period January-March), there was also an increase in the proportion of positive (red) samples compared to October-December 2021. Indeed, the first quarter of 2022 corresponds to the period of the introduction and spread of the Omicron variant in the country.

Figure 7 shows the dashboard representing quantitative data obtained in the six months of surveillance at national level (genome copies of SARS-CoV-2 per inhabitant/24 hours) while Figure 8 shows data obtained in one of the 19 Regions/A.P.

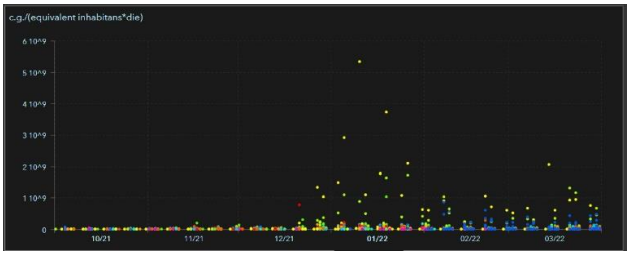

Fig. 7. SARS-CoV-2 quantitative data expressed as g.c/(equivalent inhabitant*die). Different colours represent different Regions/A.P.

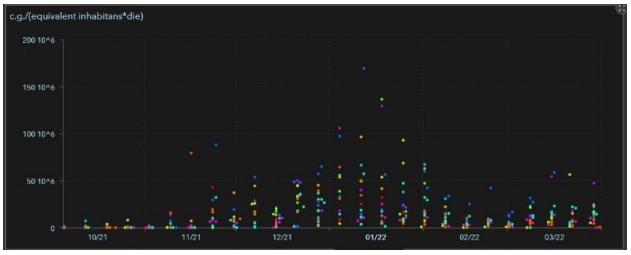

Fig. 8. SARS-CoV-2 quantitative data expressed as g.c/(equivalent inhabitant*die) in one of the 19 Italian Region/A.P. operating in the network. Different colours represent different WTPs.

The highest viral loads were detected in January 2022, during the Omicron variant introduction/spread, with concentrations increasing since December 2021 and then decreasing in February 2022. Afterwards, a second increase of SARS-CoV-2 loads in wastewaters was recorded in February-March 2022, smaller than the previous one.

Clinical data related to the study period were extracted from the Coronavirus maps of the Civil Protection Department of the Italian Government (https://mappe.protezionecivile.gov.it/en/emergencies-maps/coronavirus/coronavirus-situation-desktop) for comparison with environmental data, and are reported in Figure 9.

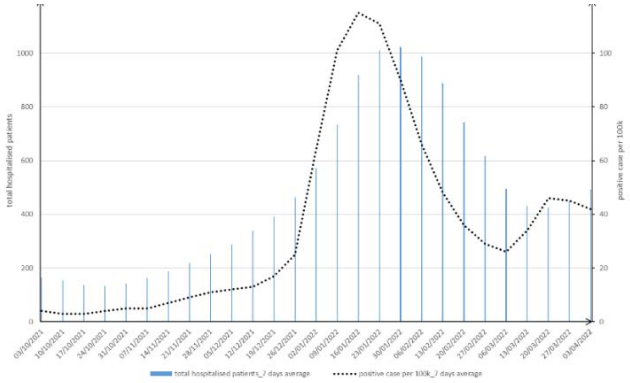

Fig. 9. Positive cases per 100.000 inhabitants and total hospitalized patients (7 days average)

The figure shows COVID-19 cases/100.000 inhabitants (7-day average) and total hospitalized patients (7-day average). The same trends observed in wastewater samples (two waves observed in the six–month period: a larger one with a peak in January and a smaller one with a peak in March) were observed in the COVID-19 cases and hospitalizations, suggesting that environmental data mirrored clinical data.

Sampling period	Main findings
04-12 Jul 2021	Predominance of the Delta variant; the Alpha and Beta variants were also detected
04-08 Oct 2021	Only the Delta variant was detected including aa substitution characteristic of sublineage AY.4.2
01-05 Nov 2021	Predominance and significant variability of the Delta variant. No other VoCs or VOIs were detected
30 Nov-03 Dec 2021	Predominance and significant variability of the Delta variant. No other VoCs or VOIs were detected
10-14 Jan 2022	Predominance and significant variability of the Omicron variant (Lineage BA.1 predominant). Mutations of the Delta variant detected in only three Regions/A.P.
07-11 Feb 2022	Predominance of sublineage Omicron BA.1; sublineage BA.2 detected in four Regions. Mutations of the Delta variant detected in four Regions.
07-11 Mar 2022	Only the Omicron variant was detected. Aminoacid substitutions of sublineage BA.2 were detected in 64% of the sequences while mutations of sublineages BA.1 and BA.1.1 were detected in 4% and 32% of the samples, respectively.

Fig. 10. Main finding observed in the seven *flash surveys* performed since July 2021

As for viral diversity and variants detection, the results of the six months monitoring are summarized in Figure 10. Predominant viral variants found in wastewaters changed over the monitoring period, according with changes in the COVID-19 epidemiological situation with the predominance of the Delta variant in July 2021 and exclusive presence of the Omicron variant in March 2022. Indeed, the Omicron variant was introduced in Italy in the first week of December 2021 and then spread quickly countrywide within 21 days, as demonstrated in a previous work [18]. In three weeks, the prevalence of Omicron-positive wastewater samples increased from 1.0% to 65.9%; similarly, the number of Regions/A.P. in which Omicron variant was detected raised from one to 17.

Currently, dashboards for presentation of the results of the *flash surveys* on SARS-CoV-2 variants are under development. Preliminary examples, under implementations, are shown in Figures 11 and 12, representing October and December, respectively.

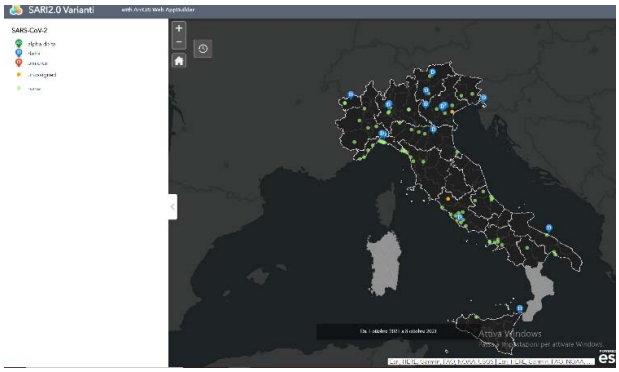

Figure 11. SARS-CoV-2 variants: October 2021

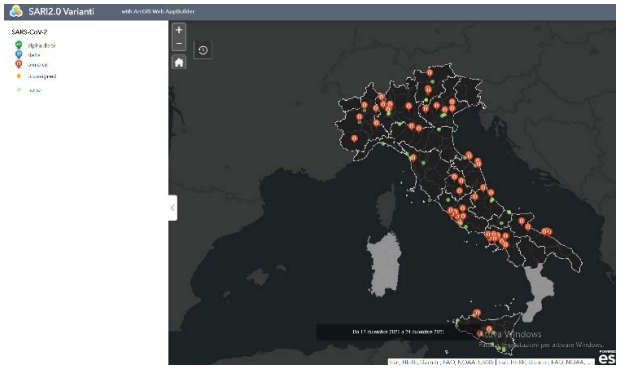

Figure 12. SARS-CoV-2 variants: December 2021

In conclusion, the official environmental surveillance of SARS-CoV-2 started in Italy on October 2021 as per EU Recommendation 2021/472. During October 2021-March 2022, an important effort has been made to build the environmental network and make it fully operational. During the semester, nationwide environmental data followed increasing and decreasing trends throughout the country, which mirrored trends observed in clinical cases in most of the Regions/A.P., confirming that environmental surveillance can effectively describe changes in viral spread in the population. We also demonstrated that WBE can successfully contribute to track SARS-CoV-2 variants at population level, providing significant information for effectively monitoring the pandemic.

REFERENCES

[1] Zhang Y, et al.. China CDC Wkly. 2020;2(8):123–4. https://doi.org/10.46234/ccdcw2020.033.

[2] Bonanno Ferraro G et al, Food Environ Virol. 2021. doi: 10.1007/s12560-021-09498-6.

[3] Environmental surveillance for SARS-COV-2 to complement public health surveillance – Interim Guidance. https://www.who.int/publications/i/item/WHO-HEP-ECH-WSH-2022.1

[4] G7 Health Ministers' Communiqué 20 May 2022, Berlin. 2022-05-20-g7-health-ministers-communique-data.pdf (g7germany.de)

[5] La Rosa G et al., Sci Total Environ. 2020. Doi: 10.1016/j.scitotenv.2020.139652.

[6] La Rosa G et al, Sci Total Environ. 2021. Doi: 10.1016/j.scitotenv.2020.141711.

[7] Bonanno Ferraro G et al,, Food Environ Virol. 2021. Doi: 10.1007/s12560-021-09498-6.

[8] "ISS, al via la rete 'sentinella' di sorveglianza epidemiologica del coronavirus nelle acque reflue" https://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero&id=4953

[9] Commission Recommendation (EU) 2021/472 on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU. (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32021H0472&qid=1628798981209)

[10] https://stats.oecd.org/glossary/detail.asp?ID=208

[11] Protocollo della Sorveglianza di SARS-CoV-2 in reflui urbani (SARI) - rev. 3 [Technical protocol (including analytical methods) of the national SARI project (Surveillance of SARS-CoV-2 in wastewaters in Italy)]. Doi: 10.5281/zenodo.5758725

[12] Wu F, et al, mSystems. 2020. Doi: 10.1128/mSystems.00614-20

[13] Pierri B et al, J Virol Methods. 2022. Doi: 10.1016/j.jviromet.2021.114420.

[14] La Rosa G et al, Sci Total Environ 2022. Doi: 10.1016/j.scitotenv.2022.155767.

[15] La Rosa, G et al, *Water* **2021.** https://doi.org/10.3390/w13182503

[16] https://www.iss.it/cov19-acque-reflue

[17] Technical protocol for data entry and data management of the national SARI project (Surveillance of SARS-CoV-2 in wastewaters in Italy). Doi: 10.5281/zenodo.5950261.

[18] La Rosa, G et al, Doi: 10.5281/zenodo.5985319.