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Wastewater surveillance of SARS-CoV-2 variants in October–November 2022 in Italy: detection of XBB.1, BA.2.75 and rapid spread of the BQ.1 lineage

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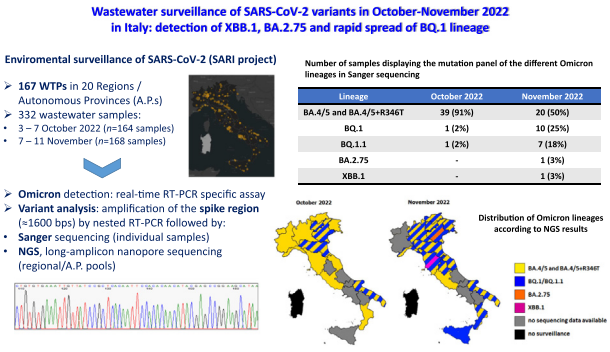
HIGHLIGHTS

- 332 sewage samples were collected throughout Italy in October–November 2022.
- Sanger and NGS sequencing of the spike protein was used for screening of SARS-CoV-2 variants.
- Prevalence of Omicron BQ.1/BQ.1.1 increased from 5 % (October) to 43 % (November).
- The detection of BA.2.75 and XBB.1 was documented in November 2022.
- Environmental surveillance tracked the spread of SARS-CoV-2 variants in the population.

GRAPHICAL ABSTRACT



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ABSTRACT

This study adds insight regarding the occurrence and spread of SARS-CoV-2 Variants of Concern (VOCs) and Variants of Interest (VOIs) in Italy in October and November 2022, by testing urban wastewater collected throughout the country. A total of 332 wastewater samples were collected from 20 Italian Regions/Autonomous Provinces (APs) within the framework of national SARS-CoV-2 environmental surveillance. Of these, 164 were collected in the first week of October and 168 in the first week of November. A ~1600 bp fragment of the spike protein was sequenced by Sanger (for individual samples) and long-read nanopore sequencing (for pooled Region/AP samples).

In October, mutations characteristic of Omicron BA.4/BA.5 were detected in the vast majority (91 %) of the samples amplified by Sanger sequencing. A fraction of these sequences (9 %) also displayed the R346T mutation. Despite the low prevalence documented in clinical cases at the time of sampling, amino acid substitutions characteristic of sublineages BQ.1 or BQ.1.1 were detected in 5 % of sequenced samples from four Regions/APs.

A significantly higher variability of sequences and variants was documented in November 2022, when the rate of sequences harbouring mutations of lineages BQ.1 and BQ1.1 increased to 43 %, and the number of Regions/APs positive for the new Omicron subvariant more than tripled ($n = 13$) compared to October. Moreover, an increase in the number of sequences with the mutation package BA.4/BA.5 + R346T (18 %), as well as the detection of variants never

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observed before in wastewater in Italy, such as BA.2.75 and XBB.1 (the latter in a Region where no clinical cases associated with this variant had ever been documented) was recorded.
The results suggest that, as predicted by the ECDC, BQ.1/BQ.1.1 is rapidly becoming dominant in late 2022. Environmental surveillance proves to be a powerful tool for tracking the spread of SARS-CoV-2 variants/subvariants in the population.

1. Introduction

The WHO recognises environmental surveillance as a powerful tool for providing additional evidence on SARS-CoV-2 circulation at population level, including presence or absence, early warning of increasing or decreasing trends, and information on variants (WHO, 2022a, b). During the COVID-19 pandemic there has been increased interest in using environmental surveillance to monitor the spread and evolution of SARS-CoV-2 and support public health responses (Ai et al., 2022; Cutrupi et al., 2022; Medema et al., 2020; Bonanno Ferraro et al., 2021; Lundy et al., 2021). Wastewater surveillance can provide sentinel surveillance for the early detection of SARS-CoV-2 Variants of Concern (VOCs) and Variants of Interest (VOIs). Indeed, several studies have reported the key role of the sequencing of SARS-CoV-2 in wastewater to help understand the variants/subvariants circulating in a community (Brunner et al., 2022; Westcott et al., 2022; Gregory et al., 2022; Wilhelm et al., 2022; Farkas et al., 2023; Smith et al., 2023; Gafurov et al., 2022).

Three VOCs are circulating in European Union/European Economic Area (EU/EEA) countries as of 8 December 2022: Omicron BA.2, BA.4, and BA.5 (ECDC, 2022a, b). The ECDC also currently recognises three Variants of Interest (VOI): Omicron BA.2.75, BQ.1, and XBB. BA.2.75, first detected in India in early May 2022, was reclassified from a Variant Under Monitoring (VUM) to a VOI by the ECDC on 14 July 2022. BQ.1, including its sub-lineage BQ.1.1, is a descendant of the Omicron BA.5 subvariant. It was designated as VOI by the ECDC on 20 October 2022. This variant and its sub-lineages will probably contribute to a further increase in cases of COVID-19 in the EU/EEA in the coming weeks and months. According to ECDC estimates, >50 % of SARS-CoV-2 infections will be caused by BQ.1/BQ.1.1 by December 2022 (ECDC, 2022a, b). XBB.1 is a recombinant lineage of BJ.1 (BA.2.10.1.1) and BM.1.1.1 (BA.2.75.3.1.1.1), with a break point in the spike region, and it was included among the VOIs by the ECDC on 8 December 2022. At least 25 EU/EEA countries have detected the circulation of the SARS-CoV-2 variant sub-lineage BQ.1 (https://cov-lineages.org/lineage.html?lineage=BQ.1). In Italy, as of late October 2022, the percentage of Omicron BQ1/BQ1.1 was low, with BQ1 and BQ1.1 representing 2.36 % and 1.92 %, respectively, of SARS-CoV-2 sequences obtained from COVID-19 cases (Istituto Superiore di Sanità, 2022a). As at 2 December there had been an increase in sequences from COVID-19 cases attributable to BQ*, with BQ.1 and BQ.1.1 accounting for 3.33 % and 13.25 %, respectively (Istituto Superiore di Sanità, 2022b).

Currently, no data is available in the literature on the sub-variants of Omicron BQ1/BQ1.1, XBB.1, and BA.2.75 in wastewater. This study aims to increase knowledge of the occurrence and prevalence of SARS-CoV-2 VOCs (BA.2, BA.4, BA.5) and VOIs (BA.2.75, BQ.1*, and XBB.1) in Italy in October–November 2022 by monitoring urban wastewater at the inlet of wastewater treatment plants (WTPs).

On 17 March 2021, the European Commission recommended Member States establish systematic surveillance of SARS-CoV-2 and its variants in Europe by 1 October 2021 (Rec. 2021/472 of March 17, 2021). As a result of this Recommendation, within the wastewater surveillance system implemented in Italy, activities are regularly conducted to monitor SARS-CoV-2 and its variants nationwide through monthly monitoring.

Here we describe the first detection of Omicron subvariants BQ1/BQ1.1, XBB.1, and BA.2.75 in wastewater in Italy, and the rapid spread of BQ1/BQ1.1 throughout the country within one month.

2. Materials and methods

2.1. Sample collection

As part of the environmental surveillance of SARS-CoV-2 in Italy, regular monthly monitoring campaigns to study SARS-CoV-2 variants have been carried out since October 2021. Samples are collected from across the country, usually in the first week of each month. In the period 3–7 October 2022, 164 composite (24 h) wastewater samples were collected from 161 WTPs in 18 Regions and two Autonomous Provinces (APs) (representing the entire country with the exception of the Region of Sardinia). Additional 168 wastewater samples were collected from the same WTPs in the period 7–11 November 2022.

2.2. Viral concentration and nucleic acid extraction

Following heat inactivation at 56 °C for 30 min, 45 mL of the samples was concentrated using a polyethylene glycol (PEG)-based concentration (Wu et al., 2020). Samples were centrifuged at 4500 × *g* for 30 min to remove larger particles and debris; subsequently, 40 mL of the samples was mixed with polyethylene glycol 8000 8 % (wt/vol) and NaCl (0.3 M) (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 12000 × *g* for 2 h. The final pellet was resuspended in 2 mL of NucliSENS Lysis Buffer reagent (bioMerieux, Marcy-l'Étoile, France) for RNA extraction. Nucleic acid extraction was performed using magnetic silica beads. The eluted RNA (100 μL) was purified using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) and stored at −80 °C until molecular analysis.

2.3. Real-time RT-PCR and nested PCR

Samples were initially tested for SARS-CoV-2 using a previously published real-time RT-qPCR with primers/probe designed in the orf1b, nsp14 region of the genome (La Rosa et al., 2021a), and then screened for the Omicron variant using a real-time RT-PCR assay targeting the spike region (PCR ID 999, mutations H655Y, N679K, and P681H) (La Rosa et al., 2022). Samples showing *Cq* values <40 were regarded as positive. Subsequently, the samples underwent amplification using previously described nested RT-PCR (ID 979/980) (La Rosa et al., 2021b; La Rosa et al., 2021), able to amplify a long fragment (1592 bps) of the spike region to detect distinctive mutations of SARS-CoV-2 variants. The amplification mix was performed using the SuperScript™ IV One-Step RT-PCR System (Invitrogen) for the first-cycle PCR, and the Platinum SuperFi II Green PCR Master Mix (Invitrogen) for the second (nested) PCR. Amplifications were performed in a T100 PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). RNA from the SARS-CoV-2 Alpha variant, kindly provided by the Infectious Diseases Department of the Istituto Superiore di Sanità [National Institute of Health-ISS], and molecular grade water were included in each PCR run as positive and negative controls, respectively. The mutations detectable by the long PCR assays for VOCs and VOIs (current EDCD classification) are shown in Table 1 and in Supplementary Fig. 1. After amplification, high-sensitivity capillary electrophoresis was performed using a QIAxcel instrument supplied by Qiagen and the QIAxcel DNA Fast Analysis Kit (DNA Size Marker 50 bp – 1.5 kb). PCR products were purified using Montage PCRm96 Micro-Well Filter Plates (Millipore, Burlington, MA, USA).

Table 1
Mutations detectable by the long PCR assays (ID 980) in lineages recognised as Variants of Concern (VOC) and Variants of Interest (VOI) according to the European Centre for Disease Control as of 8 December 2022.

	Lineage	Mutations detectable by the long PCR assay (prevalence of each mutation in the lineage)
VOC	**BA.2**	**G142D** (97.4 %), **V213G** (98.4 %), **G339D** (96.6 %), **S371F** (94.3 %), **S373P** (96.3 %), **S375F** (95.9 %), **T376A** (94.6 %), **D405N** (96.4 %), **R408S** (92.2 %), **K417N** (93.5 %), **N440K** (85.7 %), **S477N** (93.6 %), **T478K** (93.8 %), **E484A** (93.7 %), **Q493R** (92.1 %), **Q498R** (91.1 %), **N501Y** (91.4 %), **Y505H** (91.4 %)
	BA.4[a]	**DEL69/70** (94.0 %), **G142D** (98.4 %), **V213G** (99.2 %), **G339D** (97.7 %), **S371F** (95.7 %), **S373P** (97.9 %), **S375F** (97.5 %), **T376A** (96.6 %), **D405N** (97.8 %), **R408S** (87.7 %), **K417N** (94.1 %), **N440K** (89.6 %), **L452R** (94.2 %), **S477N** (96.8 %), **T478K** (96.7 %), **E484A** (96.6 %), **F486V** (96.1 %), **Q498R** (95.0 %), **N501Y** (95.3 %), **Y505H** (95.3 %)
	BA.5*[a]	**DEL69/70** (96.5 %), **G142D** (98.2 %), **V213G** (98.9 %), **G339D** (97.8 %), **S371F** (96.6 %), **S373P** (97.7 %), **S375F** (97.4 %), **T376A** (96.7 %), **D405N** (97.8 %), **R408S** (92.6 %), **K417N** (94.6 %), **N440K** (91.0 %), **L452R** (94.6 %), **S477N** (96.4 %), **T478K** (96.4 %), **E484A** (96.5 %), **F486V** (95.9 %), **Q498R** (95.3 %), **N501Y** (95.6 %), **Y505H** (95.7 %)
VOI	**BA.2.75***	**G142D** (78.1 %), **K147E** (85.9 %), **W152R** (86.5 %), **F157L** (87.1 %), **I210V** (88.2 %), **V213G** (88.6 %), **G257S** (89.3 %), **G339H** (91.6 %), **S371F** (91.4 %), **S373P** (92.0 %), **S375F** (91.1 %), **T376A** (91.0 %), **D405N** (93.8 %), **R408S** (85.2 %), **K417N** (86.2 %), **N440K** (89.5 %), **G446S** (89.9 %), **N460K** (91.0 %), **S477N** (92.9 %), **T478K** (92.5 %), **E484A** (91.5 %), **Q498R** (91.5 %), **N501Y** (91.7 %), **Y505H** (91.2.7 %)
	BQ.1*[b]	**DEL69/70** (95.8 %), **G142D** (97.6 %), **V213G** (98.4 %), **G339D** (96.2 %), **S371F** (95.2 %), **S373P** (96.0 %), **S375F** (95.8 %), **T376A** (95.7 %), **D405N** (97.5 %), **R408S** (93.2 %), **K417N** (93.4 %), **N440K** (95.7 %), **K444T** (95.5 %), **L452R** (95.9 %), **N460K** (95.4 %), **S477N** (96.9 %), **T478K** (96.9 %), **E484A** (97.1 %), **F486V** (97.2 %), **Q498R** (96.9 %), **N501Y** (97.1 %), **Y505H** (97.1 %)
	XBB.1	**V83A** (95.5 %), **G142D** (96.4 %), **DEL144** (90.0 %), **H146Q** (90.3 %), **Q183E** (97.1 %), **V213E** (97.8 %), **G252V** (83.6 %), **G339H** (94.1 %), **R346T** (94.5 %), **L368I** (90.1 %), **S371F** (91.6 %), **S373P** (92.2 %), **S375F** (91.7 %), **T376A** (91.7 %), **D405N** (92.3 %), **R408S** (89.0 %), **K417N** (88.2 %), **N440K** (94.7 %), **V445P** (94.3 %), **G446S** (94.5 %), **N460K** (95.1 %), **S477N** (96.6 %), **T478K** (96.4 %), **E484A** (96.4 %), **F486S** (94.5 %), **F490S** (96.2 %), **Q498R** (96.8 %), **N501Y** (96.8 %), **Y505H** (96.9 %)

Lineages listed with a final asterisk (*) pool the mutation frequencies of the lineage and of all descendent sub-lineages. Mutations with prevalence >75 % in each lineage are shown. Lineage comparison based on 13,458,946 sequences from GISAID (10 December 2022), according to Gangavarapu et al., accessed on 10 December 2022.

[a] The spike gene region covered by the long PCR assays (ID 980) does not allow differentiation between BA.4 and BA.5, therefore sequencing results displaying the mutations associated to these two lineages are expressed as BA.4/BA.5 (abbreviated to BA.4/5).

[b] Omicron BQ.1.1 displays the distinctive mutation R346T (96.6 %) compared to BQ.1*.

2.4. Sanger and NGS sequencing

Positive amplicons were sequenced by Sanger sequencing using PCR forward and reverse PCR primers and internal sequencing primers (see Table 2). Mutations (deletions and amino acid substitutions) were identified using the CoVsurver tool - Mutation Analysis of hCoV-19 available at https://gisaid.org/database-features/covsurver-mutations-app/ against the GISAID reference strain hCoV-19/Wuhan/WIV04/2019.

Since Sanger sequencing on PCR amplicons may underestimate the occurrence of the less common sequences, long-read NGS was also performed using nanopore technology for a more in-depth analysis to identify the co-occurrence of mutations, as previously described (La Rosa et al., 2021b). PCR amplicons from the same Regions/APs were combined in a single pool. The entire workflow for library preparation, nanopore sequencing, and data analysis are described in La Rosa et al. (2021b). Briefly, nanopore sequencing was performed using a MinION sequencer (Oxford Nanopore Technologies, Oxford, UK). Libraries were prepared using the cDNA-PCR Sequencing kit (SQK-PCS109) following the manufacturer's instructions, with the Native barcoding kit. Pooled libraries (25 fmol) were loaded into MinION flow cells (FLO-MIN106 R9.4.1), and sequencing was carried out over 72 h using MinKNOW software version 4.5.4. The raw data were base called using the high-accuracy model (HAC) and then demultiplexed using Guppy basecalling suite version 5.1.15 on a Ubuntu 18.04 LTS machine to obtain the final FASTQ files. Subsequently, reads consistent with the PCR amplicon product length (1400–1700 bases) were selected and mapped to the SARS-CoV-2 reference sequence (NC_045512, isolate Wuhan-Hu-1) to eliminate non-specific signals. Subsequent analysis for amplicon assignation was performed by classification (supervised approach) as previously described (La Rosa et al., 2021b) querying BAM files using panels of ad hoc mutations in linkage based on mutation occurrence in VOCs/VOIs (see Table 1).

Some of the currently circulating variants have high nucleotide identity in the spike protein region; therefore, a threshold was introduced to validate the assignment of subvariants distinguished by a unique mutation (e.g., BA.4/5 from BA.4/5 + R346T, or BQ.1 from BQ.1.1, which also have the additional mutation R346T), to avoid misassignment. This threshold was based on the independent and heavily benchmarked data for the error rate of Nanopore sequencing technology (Guppy basecaller generation) (Delahaye and Nicolas, 2021). In our study, the discrimination of BA.4/5 + R346T versus BA.4/5, and of BQ1.1 versus BQ.1 was considered correct if the number of reads assigned to the sublineage (parental mutations + one mutation) was higher than 6 % of the total number of reads collectively assigned to the parental lineage plus the sublineage, which ensures that the reads are not the result of sequencing errors.

Finally, Omicron sub-variants abundance in the different Regions/APs was assessed by summing up all assigned reads per pool and calculating, for each sub-variant, the relative percentage. The barchart was realized

Table 2
Primers used in this study for real-time RT-PCR, nested RT-PCR and sequencing.

PCR ID	Primer name	Nucleotide sequence (5′ – 3′)	Orientation	Primer position[a]	Usage	Amplicon size (bp)	Reference
979	2319	ACCCTGACAAAGTTTTCAGATCCT	+	21,675–21,698	First PCR	1728	La Rosa et al., 2021a; La Rosa et al., 2021b
	2324	CCTGATAAAGAACAGCAACCTG	−	23,402-23,381			
980	2321	TTCAACTCAGGACTTGTTCTTACC	+	21,709–21,732	Nested PCR	1583	
	2326	GTGGATCACGGACAGCATC	−	23,300–23,282			
980int	2337	TTGGCAAATCTACCAATGGTTC	−	22,253–22,232	Internal sequencing (PCR 980)		This study
	2338	CGCCCTAAATTAATAGGCGTGT	−	22,203–22,182			
	2339	CTCAGCCTTTTCTTATGGACCTT	+	22,077–22,099			
	2340	AGGACAGAATAATCAGCAACACAG	−	22,665–22,642			
	2342	CAACACAGTTGCTGATTCTCTTC	−	22,649–22,627			
	2343	GAAGTCAGCCAAATCGCTCCA	+	22,778–22,798			
	2347	CAAGCTATAACGCAGCCTGT	−	22,869–22,850			
999	2356	GGCTGTTTAATAGGGGCTGAATA	+	23,504–23,526	Real-time PCR	141	La Rosa et al., 2021c
	2357	GGCAATGATGGATTGACTAGC	−	23,644–23,624			
	2358	5′-FAM-TCAGACTCAGACTAAGTCTCATCGG-BHQ1–3′	+	23,584–23,608			

[a] Reference genome MN996528 (Severe Acute Respiratory Syndrome Coronavirus 2, isolate WIV04).

and plotted with ggplot2 R package version 3.3.6 (Wickham, 2016) in RStudio 2022.12.0 + 353.

3. Results

3.1. Real-time RT-PCR assays

SARS-CoV-2 loads in tested samples ranged from 1.3×10^3 to 1.1×10^6 (median value: 6.1×10^4) genome copies/L of wastewater (Supplementary Table 1), and the results of the screening for the Omicron variant are summarized in Supplementary Table 2. In detail, in October 2022, 156 of the 164 (95.1 %) wastewater samples tested positive with the real-time RT-PCR assay designed to detect the Omicron variant (*Cq* <40). *Cq* values ranged from 30.13 to 39.72. In November, 145/168 (86.3 %) samples tested positive for the Omicron variant, with *Cq* values ranging from 27.34 to 39.55. The variant was detected in all the Italian Regions/APs participating in the survey.

3.2. Sanger and NGS sequencing of long-nested PCR (Spike gene)

In October 2022, the long-nested PCR assay successfully amplified 43 samples collected in 14 of the 20 Italian Regions/APs (Table 3). Sanger sequencing detected the following mutation panels:

i) BA.4/BA.5 (the two Omicron variants are not distinguishable in the spike region, see Table 2) in 39 samples (91 %) distributed over all 14 Regions/APs; of these BA.4/BA.5 positive samples, four (9 %) also displayed the additional mutation R346T, which is present in BF.7 subvariant (classified as a VUM by the ECDC), and 3 samples included other additional mutations (Y144DEL + G181R, S247I, or K147N);
ii) BQ.1 (mutations of BA.4/BA.5 + K444T + N460K) in one sample (2 %);
iii) BQ.1.1 (mutations of BQ.1 + R346T) in one sample (2 %).

Moreover, two samples showed mixed electropherograms with double peaks in genome positions 22,599, 22,893, and 22,942 (NC_045512, isolate Wuhan-Hu-1), suggesting the presence of both parental/mutated amino acids R346/R346T, K444/K444T and N460/N460K, possibly due to the co-presence of BQ.1/BQ.1.1 with BA.4/BA.5 and/or BA.4/BA.5 + R346T.

Nanopore NGS (Table 3 and Fig. 1) revealed the presence of sublineages that were undetected or unresolved (mixed electropherograms) by Sanger sequencing. Overall, the mutation package BA.4/BA.5 + R346T was detected in seven Regions/APs, and the BQ.1 and BQ.1.1 lineages were detected in four Regions/APs (Veneto, Emilia Romagna, Puglia, and Trento). In three Regions (Veneto, Emilia Romagna and Puglia), the concurrent circulation of up to four lineages was observed by nanopore sequencing: Omicron BA.4/BA.5, BA.4/BA.5 + R346T, BQ.1 and BQ.1.1.

Table 3
Variants detected by Sanger and NGS sequencing in October 2022.

Oct-2022 survey ID	Sample ID	Region/AP	City	Omicron sub-variant (Individual samples, Sanger sequencing)	Omicron sub-variant (Regionally pooled samples, NGS)
12	14620	Calabria	Reggio Calabria	BA.4/5	BA.4/5
23	14679	Emilia Romagna	Bologna	BA.4/5	BA.4/5 BA.4/5 + R346T BQ.1 BQ.1.1
26	14684		Ravenna - Forlì-Cesena	BA.4/5 + Y144DEL + G181R	
28	14688		Forlì-Cesena	BA.4/5 + R346T	
29	14690		Rimini - Forlì-Cesena	BA.4/5	
30	14691		Modena	BA.4/5	
31	14397	Friuli Venezia Giulia	Trieste	BA.4/5 + S247I	BA.4/5 BA.4/5 + R346T
40	14576	Lazio	Roma	BA.4/5	BA.4/5
47	14590	Liguria	Savona	BA.4/5	BA.4/5 BA.4/5 + R346T
48	14591		Savona	BA.4/5	
50	14593		Genova	BA.4/5	
52	14595		Genova	BA.4/5	
54	14600		Genova	BA.4/5	
55	14602		Imperia	BA.4/5 + R346T	
57	14606		La Spezia	BA.4/5	
58	14607		La Spezia	BA.4/5	
59	14608		La Spezia	BA.4/5	
60	14609		Genova	BA.4/5 + R346T	
61	14610		Genova	BA.4/5	
62	14611		Genova	BA.4/5	
156	14550	Lombardia	Como - Lecco - Milano - Monza e Brianza	BA.4/5	BA.4/5
72	14659	Marche	Pesaro-Urbino	BA.4/5	BA.4/5
74	14661		Pesaro-Urbino	BA.4/5 + K147N	
81	14565	AP Bolzano	Bolzano	BA.4/5	BA.4/5 BA.4/5 + R346T
82	14566		Bolzano	BA.4/5	
83	14567		Bolzano	BA.4/5	
84	14513	AP Trento	Trento	BA.4/5	BA.4/5
85	14514		Trento	BA.4/5	BA.4/5 + R346T
86	14515		Trento	Mixed electropherograms [a]	BQ.1.1
91	14543	Piemonte	Asti	BA.4/5	BA.4/5
94	14460	Puglia	Bari	BA.4/5	BA.4/5 BA.4/5 + R346T BQ.1 BQ.1.1
95	14461		Bari	BA.4/5	
96	14489		Brindisi	BQ.1.1	
99	14497		Taranto	BA.4/5	
102	14524		Foggia	BA.4/5	
124	14669	Toscana	Massa	BA.4/5	BA.4/5
140	14614	Valle d'Aosta	Aosta	BA.4/5	BA.4/5
142	14509	Veneto	Padova	BA.4/5 + R346T	
143	14510		Padova	Mixed electropherograms [b]	BA.4/5
145	14512		Padova	BA.4/5	BA.4/5 + R346T
146	14529		Vicenza	BQ.1	BQ.1
150	14588		Verona	BA.4/5	BQ.1.1
151	14589		Verona	BA.4/5	

[a] Presence of overlapping peaks in the three positions R346, K444, N460 suggesting the simultaneous presence of a combination of BA.4/5 and BQ.1 lineages/sublineages.
[b] Presence of overlapping peaks in the two positions K444 and N460 suggesting the simultaneous presence of a combination of BA.4/5 and BQ.1 lineages/sublineages.

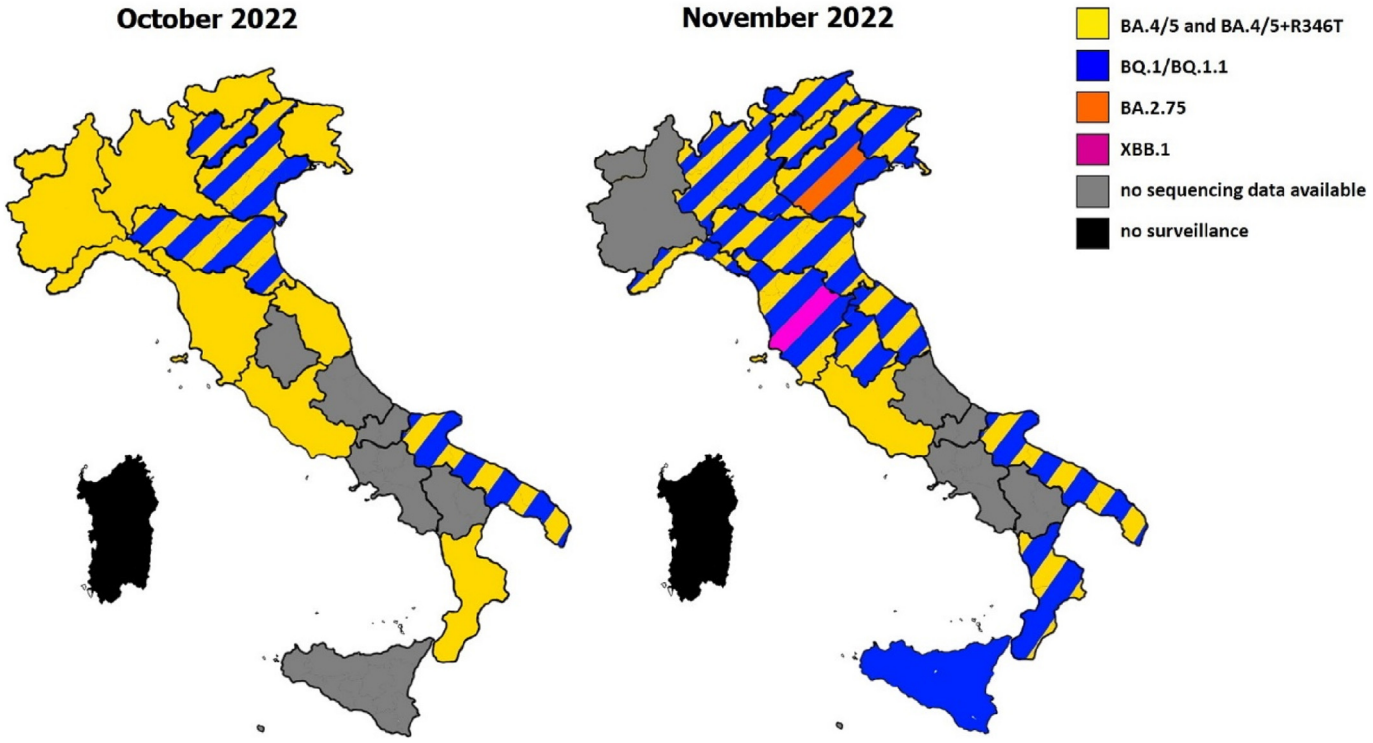

Fig. 1. Omicron lineages/sublineages detected by NGS in October and November 2022.

In November, the long-nested PCR assay successfully amplified 40 samples collected from 14 Regions/APs (Table 4) and the following mutation panels were detected by Sanger sequencing:

i) BA.4/BA.5 in 20 samples (50 %), seven (18 %) of which including the additional mutation R346T;
ii) BQ.1 in 10 samples (25 %);
iii) BQ.1.1 in seven samples (18 %);
iv) BA.2.75 (mutations G142D, K147E, W152R, F157L, I210V, V213G, G257S, G339D, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, N460K, S477N, T478K, E484A, F490S, Q498R, N501Y, Y505H) in one sample (3 %);
v) XBB.1 (mutations V83A, G142D, Y144del, H146Q, Q183E, V213E, G252V, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K, S477N, T478K, E484A, F486S, F490S, Q498R, N501Y, Y505H) in one sample (3 %).

Finally, one sample showed mixed electropherograms with double peaks in genome positions 22,599, 22,893, and 22,942.

Nanopore sequencing (Table 4 and Figs. 1 and 2) confirmed the results obtained by Sanger sequencing with regards to detection of the BA.2.75 lineage in the Region of Veneto and of XBB.1 in Tuscany. In addition, it detected BQ.1/BQ.1.1 sequences in two additional Regions, bringing the total number of Regions/APs in which circulation of this variant/subvariant was detected to 13 (Calabria, Emilia Romagna, Friuli Venezia Giulia, Liguria, Lombardy, Marche, APs Trento and Bolzano, Puglia, Sicily, Tuscany, Umbria and Veneto). Interestingly, five Regions showed simultaneous circulation of both BQ.1 and BQ.1.1, and two Regions (Tuscany and Veneto), the co-circulation of up to five lineages. Finally, despite the spread of BQ.1/BQ1.1, the widespread diffusion of lineages BA.4/BA.5 and BA.4/BA.5 + R346T (each detected in 11 Regions/APs) was also confirmed by nanopore sequencing.

Sequences obtained in the present study were submitted to GeneBank under accession numbers OQ300138 - OQ300211.

4. Discussion

The evolution of SARS-CoV-2 variants has added an additional level of complexity to the ongoing pandemic. As new variants continue to emerge, it is essential to continuously monitor and assess their impact on the spread of the virus. The appearance of variants with increased transmission potential or resistance to current vaccines highlights the need for effective measures to be taken to slow the spread of the virus. This may include increasing testing and contact tracing efforts, as well as accelerating the rollout of vaccines to at-risk populations. Tracking the appearance and spread of new SARS-CoV-2 variants/subvariants is crucially important for controlling the COVID-19 pandemic.

In early 2021, we designed a long-nested PCR (~1600 bps) targeting the spike region to detect SARS-CoV-2 VOCs and VOIs (La Rosa et al., 2021; La Rosa et al., 2021b). Although the virus has accumulated numerous mutations in the spike region, the primers still exhibit 100 % identity with all the sequences of SARS-CoV-2 that have circulated so far, and the assay is, therefore, suitable for the amplification of all possible viral variants/subvariants. Following the original Omicron BA.1 variant, several subvariants of Omicron have emerged. As of 8 December 2021, BA.2, BA.4, and BA.5, together with associated sublineages, are described as VOCs by the ECDC (i.e. clear evidence indicates a significant impact on transmissibility, severity and/or immunity that is likely to influence the epidemiological situation in the EU/EEA) (ECDC, 2022a). Omicron BA.2.75, BQ.1 and XBB.1 are classified as VOIs (i.e. evidence regarding genomic properties, epidemiological evidence, or in-vitro evidence is available that could imply a significant impact on transmissibility, severity and/or immunity; however, the evidence is associated with major uncertainty or is still preliminary). BQ.1 is a sub-lineage of BA.5, carrying additional changes K444T and N460K in the spike receptor-binding domain (RBD) compared to BA.5, while BQ.1.1 also carries the additional mutation R346T. BQ.1 and BQ.1.1 are spreading quickly in the United States, and the U.S. Centers for Disease Control and Prevention estimated that, as at 10 December 2022, Omicron subvariants BQ.1 and BQ.1.1 account for 31.1 % and 36.8 %, respectively, of the cases of COVID-19 in the country (https://covid.cdc.gov/covid-data-tracker/#variant-proportions).

Table 4
Variants detected by Sanger and NGS sequencing in November 2022.

Nov-2022 survey ID	Sample ID	Region/AP	City	Omicron sub-variant (Individual samples, Sanger sequencing)	Omicron sub-variant (Regionally pooled samples, NGS)
11	15760	Calabria	Reggio Calabria	BA.4/5 + R346T	BA.4/5
12	15761		Catanzaro	BA.4/5	BA.4/5 + R346T
					BQ.1
28	15713	Emilia Romagna	Bologna	BQ.1	BA.4/5
					BA.4/5 + R346T
					BQ.1
36	15735	Friuli Venezia Giulia	Pordenone	BQ.1	BA.4/5
38	15737		Trieste	BA.4/5	BA.4/5 + R346T
					BQ.1
					BQ.1.1
44	15558	Lazio	Viterbo	BA.4/5	BA.4/5
52	15834	Liguria	La Spezia	BQ.1	BA.4/5
54	15836		Genova	BQ.1.1	BA.4/5 + R346T
55	15837		Genova	BA.4/5	BQ.1
57	15839		Genova	BA.4/5	BQ.1.1
58	15846		Genova	BA.4/5	
61	15849		Savona	Mixed electropherograms [a]	
63	15851		Imperia	BQ.1	
66	15854		Genova	BQ.1	
67	15663		Genova	BA.4/5	
74	15638	Lombardia	Milano	BQ.1	BA.4/5 + R346T
					BQ.1
83	15705	Marche	Pesaro-Urbino	BQ.1	BA.4/5
87	15710		Ancona	BA.4/5 + R346T	BA.4/5 + R346T
					BQ.1
91	15638	AP Bolzano	Bolzano	BQ.1	BA.4/5
92	15663		Bolzano	BA.4/5 + R346T	BA.4/5 + R346T
93	15692		Bolzano	BA.4/5 + R346T	BQ.1
94	15552	AP Trento	Trento	BQ.1.1	BA.4/5
					BQ.1.1
108	15510	Puglia	Taranto	BA.4/5	BA.4/5
109	15547		Foggia	BQ.1	BA.4/5 + R346T
112	15590		Bari	BA.4/5	BQ.1
113	15591		Barletta-Andria-Trani	BA.4/5	BQ.1.1
114	15592		Barletta-Andria-Trani	BQ.1	
115	15593		Barletta-Andria-Trani	BQ.1.1	
117	15595		Barletta-Andria-Trani	BQ.1.1	
135	15680	Sicilia	Messina	BQ.1.1	BQ.1.1
141	15696	Toscana	Grosseto	BA.4/5 + R346T	BA.4/5
144	15724		Pisa	XBB.1	BA.4/5 + R346T
					BQ.1
					BQ.1.1
					XBB.1
152	15487	Umbria	Perugia	BA.4/5 + R346T	BA.4/5 + R346T
154	15607		Terni	BQ.1.1	BQ.1.1
157	15534	Veneto	Padova	BA.4/5	BA.4/5
158	15535		Padova	BA.4/5 + R346T	BA.4/5 + R346T
159	15536		Padova	BA.4/5	BQ.1
160	15537		Padova	BA.4/5	BQ.1.1
162	15605		Verona	BQ.1.1	BA.2.75
163	15606		Verona	BA.2.75	

[a] Presence of overlapping peaks in the three positions R346, K444, N460 suggesting the simultaneous presence of a combination of BA.4/5 and BQ.1 lineages/sublineages.

According to ECDC predictions, >80 % of SARS-CoV-2 cases in the EU/EEA are expected to be due to BQ.1/BQ.1.1 by the beginning of 2023 (ECDC, 2022b).

This study reports the results of the environmental flash surveys performed by Istituto Superiore di Sanità (ISS) with the collaboration of the Regions and APs in the period 3–7 October, and 7–11 November 2022. In these weeks the number of COVID-19 cases/day in Italy ranged from 466.949 to 525.955 (mean 500.596) and from 414.942 to 418.986 (mean 417.004), respectively (https://opendatadpc.maps.arcgis.com/apps/dashboards/b0c68bce2cce478eaac82fe38d4138b1).

The vast majority of sequences were characterised as BA.4/BA.5. The Omicron variants BA.4 and BA.5 are not distinguishable in the spike region, therefore the sequencing results displaying the mutations associated with these two lineages are expressed as BA.4/BA.5. Within the environmental surveillance of SARS-CoV-2 in Italy we also use a short nested RT-PCR targeting the M gene to distinguish between the two variants (M: D3N mutation in BA.5). In this study, almost all of the sequences were characterised as BA.5 (data not shown).

In October 2022, for the first time, key mutations of BQ.1 and BQ.1.1 were detected by Sanger sequencingi in two wastewater samples collected in the Regions of Puglia (Southern Italy) and Veneto (Northern Italy). Additional BQ.1/BQ.1.1 sequences were detected in the Region of Emilia Romagna and the AP of Trento by nanopore deep sequencing, confirming that studies based on conventional sequencing technologies may underestimate the existence of less prevalent strains (Iaconelli et al., 2017; Suffredini et al., 2018). Similarly, the key mutations of Omicron BA.4/BA.5 plus the mutation R346T were detected in seven Regions/APs, suggesting a non-negligible circulation in the population. The same mutation package BA.4/BA.5 + R346T is indeed present in lineage BF.7, which is currently classified as a VUM by the ECDC (i.e. there is some indication that it could have properties similar to those of a VOC, however the evidence is weak or has not yet been assessed by the ECDC; ECDC, 2022a). Since the

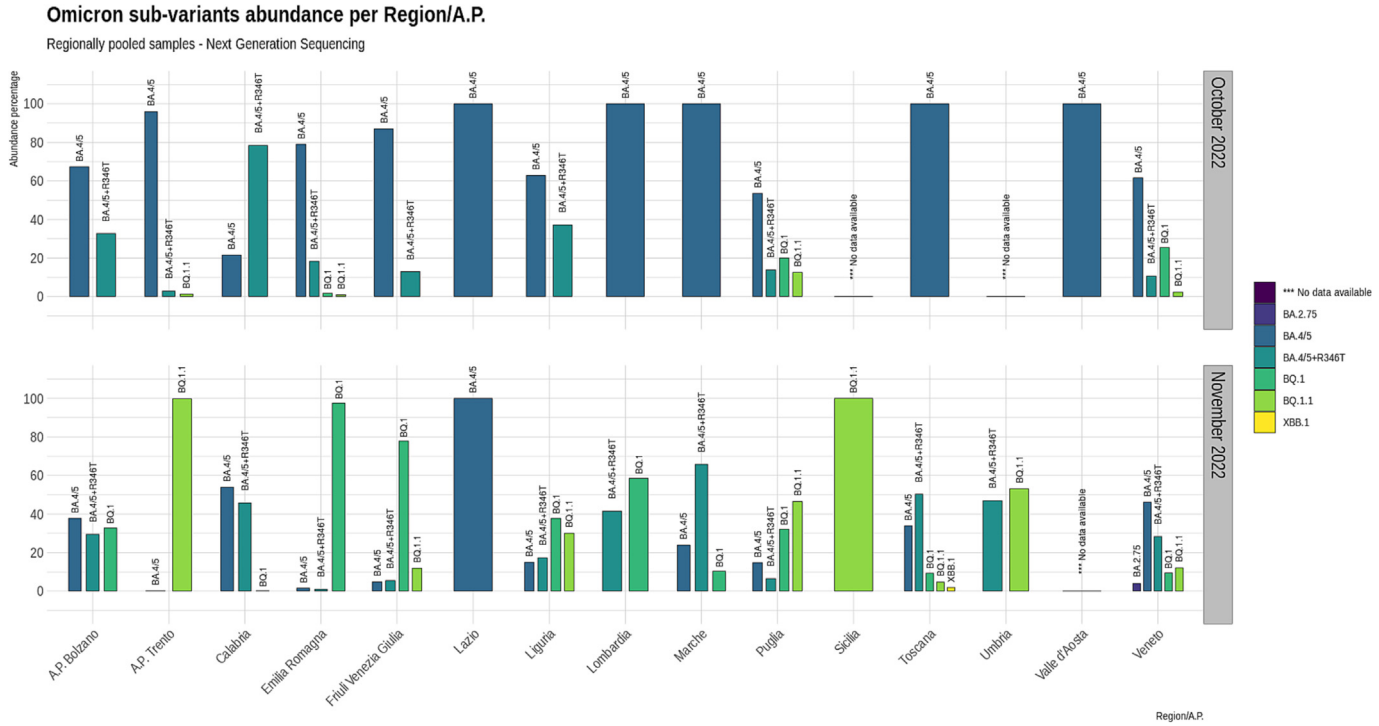

Fig. 2. Frequency of Omicron lineages/sublineages detected by NGS in October and November 2022

same mutation panel is also present in BA.4.6, these two lineages cannot be distinguished in the amplified region (BA.4.6 contains the additional mutation N658S, which is however outside the amplified region) and the results are expressed by reference to the mutation package. In October, no lineages beside BA.4/BA.5 and BQ.1/BQ.1.1 were detected.

In November 2022, the picture changed considerably compared to October 2022. Indeed, the number of single samples (tested by Sanger sequencing) harbouring mutations of lineage/sublineage BQ.1/BQ.1.1 showed an eight-fold increase (from 2/43, 5 % to 17/40, 43 %). Accordingly, the number of Regions/APs in which BQ.1/BQ.1.1 sequences were detected (nanopore sequencing) increased from 4 to 13. These results suggest that these subvariants will be predominant in Italy in the very near future. Although the ECDC Country Overview Report for week 472,022, showed that BQ.1 had become the dominant variant in seven EU/EEA countries, the high prevalence of BQ.1 does not appear to be associated with a deterioration in the epidemiological situation (https://covid19-country-overviews.ecdc.europa.eu/). According to the SARS-CoV-2 Mutation Situation Report available at https://outbreak.info/situation-reports, as of 07 December 2022, 71,954 GISAID sequences with the BQ.1* [Omicron (BQ.1.X)] combined lineage had been detected worldwide (Gangavarapu et al., 2022a), with a cumulative prevalence (ratio of the sequences containing BQ.1* to all sequences collected since the first identification of this sublineage) of 1 % (period: 15.10.2020–30.11.2022). Of these, 1185 were detected in Italy, with a cumulative prevalence of 2 % (period: 11.01.2022–28.11.2022). Moreover, BQ.1* GISAID sequences have been submitted from 10 Regions, prevalence ranging from 11 % (Piedmont) to 33 % (Lazio) (Gangavarapu et al., 2022a). In this study, the mutation panels of BQ.1 and BQ.1.1 were detected between October and early November 2022 in 13 Regions/APs in which these subvariants were not yet reported, confirming the early warning potential of environmental surveillance for monitoring geographical spread in communities. Environmental data showed the first detection of BQ.1/BQ.1.1 in wastewater in early October 2022, despite the low prevalence documented in clinical cases at the time of sampling. Data on SARS-CoV-2 variants obtained from COVID-19 cases are regularly reported on the website of the National Institute of Health (ISS) (https://www.iss.it/en/cov19-varianti-del-virus). The clinical *flash survey* conducted on 4 October 2022 (during the week of wastewater sampling) found no evidence of sub-lineages BQ.1 or BQ.1.1 in Italy (Istituto Superiore di Sanità, 2022a); moreover, as of 7 October 2022, only 20 BQ.1 and 10 BQ.1.1 sequences had been submitted to GISAID from Italy. The clinical *flash survey*, conducted on 8 November 2022, showed that BQ.1 was increasing significantly, with a prevalence of 30.7 % (Istituto Superiore di Sanità, 2022b). Environmental surveillance thus detected the circulation of this subvariant before clinical surveillance and later described its rapid spread within one month, which was also documented by clinical surveillance.

November 2022 also saw an increase in the percentage of BA.4/BA.5 + R346T, the fraction of positive samples doubling in one month (from 9 % to 18 %). More interestingly, in November, we obtained the first evidence in wastewater of BA.2.75 and XBB.1 subvariants. BA.2.75, also known as NextStrain Lineage 22D, first emerged in India in May 2022, and is classified as a VOI by the ECDC, due its increased impact on immunity, although there is no evidence of an impact on severity. According to outbreak.info reports, as of 07 December 2022, 40,881 GISAID sequences with the BA.2.75* [Omicron (BA.2.75.X)] combined lineage had been detected worldwide, accounting for a 1 % cumulative prevalence (period: 31.12.2021–01.12.2022) (Gangavarapu et al., 2022b). Of these, only 166 had been detected in Italy (<0.5 % cumulative prevalence, period: 11.03.2022–23.11.2022). BA.2.75 sequences had been submitted from only 10 Italian Regions, with prevalence ranging from 1 % (Umbria) to 7 % (Liguria). In Veneto, the Region where one wastewater sample was found positive for BA.2.75, only 3 % of sequences submitted to GISAID (20/642) belonged to this subvariant. Currently, there appears to be no evidence of any increase in the prevalence of this variant in Italy.

Finally, the XBB.1 subvariant was first detected in Italy on 21 September 2022 in the Region of Lazio. As of 7 December 2022, 5995 sequences in the XBB.1 lineage had been detected worldwide, with a cumulative prevalence of <0.5 % (period: 29.01.2022–29.11.2022) (Gangavarapu et al., 2022c). Of these, only 71 sequences had been detected in Italy (cumulative prevalence of 1 %, period: 21.09.2022–28.11.2022), submitted from 9 Italian Regions, with prevalence ranging from 1 % (Umbria) to 7 % (Sicily). Interestingly, no XBB.1 sequences had been detected in the Region of Tuscany, where one wastewater sample was instead found positive for XBB.1. Therefore, environmental surveillance demonstrated the first

introduction of this novel subvariant in the Region, in the absence of documented clinical cases.

5. Limitations

In this study, long-read nanopore sequencing proved to be a powerful method for variant detection, compared to other NGS technologies generating short-read sequencing, since it made it possible to read panels of up to 30 linked mutations. However, we must also stress the limitation of this technology, which is due to the low sensitivity of the long-nested PCR. As a matter of fact, obtaining DNA fragments of around 1600 bases is difficult in complex matrices such as urban sewage, where viral concentrations are low, nucleic acids are fragmented and many inhibitors are present. We were therefore unable to obtain sequencing data from all Regions/APs, despite most of the sample being positive for the Omicron variant by real-time RT-PCR. As a consequence, while long-sequences analysis provided significant information on the diffusion of SARS-CoV-2 variants, issues remains in relation to providing a uniform geographical coverage to the surveillance.

6. Conclusions

The WHO urges countries to continue to be vigilant, to monitor and report on the different Omicron sublineages. In this study, key mutations of Omicron sublineages BQ.1 and BQ.1.1 were detected in wastewater in Italy in early October 2022, before the variant was reported in clinical cases. As at early November 2022, BQ.1/BQ.1.1 looked set to replace previously circulating variants. Whether this could affect the level of COVID-19 circulating in the community in the coming weeks/months is unknown. The results of this study also show the occurrence of rare subvariants, such as XBB.1 and BA.2.75, in wastewater in Italy. Interestingly, positivity for BQ.1/BQ.1.1 and for XBB.1 was also detected in Regions/APs where no sequences were available, according to GISAID data.

With the global spread of COVID-19, genomic surveillance of SARS-CoV-2 is of great importance for early identification of the emergence of possible new variants with an effect on disease severity or immunity escape, as well as for monitoring their circulation in the countries. Results of this study confirm that environmental surveillance has the potential of documenting the introduction of novel variants as well as their temporal and geographic spread. In light of this evidence, optimizing sequencing strategies in wastewater and integrating environmental with clinical surveillance will help the early discover of less abundant SARS-CoV-2 lineages and complement public health surveillance.

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Conceptualization	Ideas; formulation or evolution of overarching research goals and aims	GLR, ES
Methodology	Development or design of methodology; creation of models	GLR, ES
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components	DB, MI
Validation	Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs	GLR, ES
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data	GLR, ES, DB
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection	GLR, MI, CV, PM, GBF, ES, CDG, LO, the SARI Network
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools	GLR, ES, the SARI Network
Data Curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse	GLR, ES, MR, MG, CDG, LO, the SARI Network
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Writing - Review & Editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages	GLR, ES, LL, the SARI Network
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Funding acquisition	Acquisition of the financial support for the project leading to this publication	GLR, ES, LL

Data availability

Data will be made available on request.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162339.

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