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The rapid spread of SARS-COV-2 Omicron variant in Italy reflected early through wastewater surveillance

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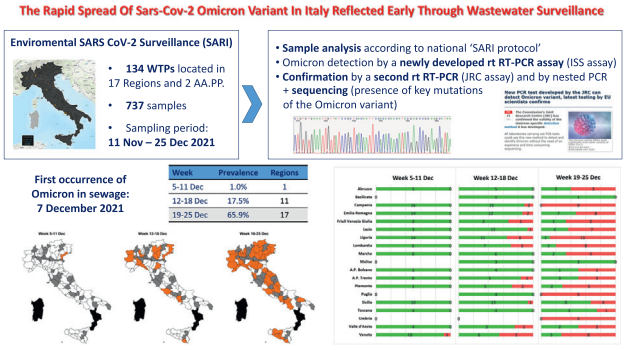
HIGHLIGHTS

- A real-time RT-PCR assay was designed for the rapid detection of the Omicron variant.
- 737 sewage samples collected throughout Italy (11 Nov – 25 Dec 2021) were tested.
- The first occurrence of Omicron was on 7 December 2021, in Veneto, North Italy.
- Omicron detection in sewage increased rapidly, raising from 1.0% to 65.9% in 3 weeks.
- In the same period, the variant spread over the country, spreading from one Region to 17.

GRAPHICAL ABSTRACT



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ABSTRACT

The SARS-CoV-2 Omicron variant emerged in South Africa in November 2021, and has later been identified worldwide, raising serious concerns.

A real-time RT-PCR assay was designed for the rapid screening of the Omicron variant, targeting characteristic mutations of the spike gene. The assay was used to test 737 sewage samples collected throughout Italy (19/21 Regions) between 11 November and 25 December 2021, with the aim of assessing the spread of the Omicron variant in the country. Positive samples were also tested with a real-time RT-PCR developed by the European Commission, Joint Research Centre (JRC), and through nested RT-PCR followed by Sanger sequencing.

Overall, 115 samples tested positive for Omicron SARS-CoV-2 variant. The first occurrence was detected on 7 December, in Veneto, North Italy. Later on, the variant spread extremely fast in three weeks, with prevalence of positive wastewater samples rising from 1.0% (1/104 samples) in the week 5–11 December, to 17.5% (25/143 samples) in the week 12–18, to 65.9% (89/135 samples) in the week 19–25, in line with the increase in cases of infection with the Omicron variant observed during December in Italy. Similarly, the number of Regions/Autonomous Provinces in which the variant was detected increased from one in the first week, to 11 in the second, and to 17 in the last one. The presence of the Omicron variant was confirmed by the JRC real-time RT-PCR in 79.1% (91/115) of the positive samples, and by Sanger sequencing in 66% (64/97) of PCR amplicons.

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In conclusion, we designed an RT-qPCR assay capable to detect the Omicron variant, which can be successfully used for the purpose of wastewater-based epidemiology. We also described the history of the introduction and diffusion of the Omicron variant in the Italian population and territory, confirming the effectiveness of sewage monitoring as a powerful surveillance tool.

1. Introduction

The SARS-COV-2 Omicron variant emerged in South Africa on 24 November 2021 and has later been identified in numerous countries worldwide. On 26 November 2021, WHO designated B.1.1.529 as a Variant of Concern, named Omicron, asking to enhance surveillance and sequencing efforts to better understand circulation of SARS-CoV-2 variants.

As of 20 January 2022, Omicron has been identified in all EU/EEA countries, and as on 30 January it was the dominant variant (accounting for >50% of sequenced viruses) in 19 of the 22 EU/EEA countries with adequate sequencing volume, with 268.835 Omicron cases reported (https://www.ecdc.europa.eu/en/covid-19/country-overviews).

In Italy, the first Omicron clinical case was described on 22 November in Milan, in an Italian man who had returned on 11 November from Mozambique (first Omicron sequence submitted in GISAID with accession ID EPI_ISL_6777160). Later on, the highly contagious Omicron variant spread quickly. As on 8 February 2022, a total of 8588 omicron sequences have been submitted to GISAID from Italy, from 16 of the 19 Italian Regions: namely Abruzzo (576), Basilicata (10), Calabria (3), Campania (1319), Emilia Romagna (664), Friuli Venezia Giulia (528), Lazio (730), Liguria (60), Lombardia (639), Piemonte (582), Puglia (280), Sicilia (2007), Toscana (58), Trentino Alto Adige (152), Umbria (425), Valle d'Aosta (16), Veneto (538), and from the two autonomous provinces (AA. PP.) of Bolzano and Trento. No sequences were available for the Regions Marche, Molise, and Sardegna.

Wastewater testing for SARS-CoV-2 has emerged as a valuable warning system for its circulation in the population and tracking the presence of novel variants in sewage is recognized as a key tool to control the spread of SARS-CoV-2 and ensure public health response. The European Commission has recommended to the EU member states to set up the monitoring of SARS-CoV-2 in wastewater by 1st October 2021 (Rec. 2021/472 of 17 March 2021), focusing on the emergence and spread of SARS-CoV-2 variants. Italy has been monitoring urban sewage since July 2020, at the beginning as a pilot study, and then, since October 2021, in the framework of the EU wastewater surveillance. The periodic detection of SARS-CoV-2 variants and the analysis of genetic diversity is part of the surveillance. In March and May 2021 Variants of Concern (VoCs) and Variants of Interest (VoI) have been identified in urban sewage in Italy (La Rosa et al., 2021a; La Rosa et al., 2021b). Since October 2021 regular national "flash surveys" - i.e. periodic sampling campaigns, held simultaneously in all regions over a short period of time - are conducted in Italy, aimed at assessing the diversity of SARS-CoV-2 in wastewater (see Acque reflue - ISS for the published monthly reports). The reports related to samples collected between 1 and 5 November (https://doi.org/10.5281/zenodo.5985196) and 28 November and 3 December 2021 (https://doi.org/10.5281/zenodo.5985276) showed, as expected, the predominance of the Delta variant associated to significant variability, and no presence of the Omicron variant by analysis of raw data of Next Generation amplicon sequencing.

As soon as the detection of the SARS-CoV-2 Omicron variant was first reported in Italy, we designed a real-time RT-PCR assay to enable rapid identification of the novel VoC, targeting characteristic mutations in the spike gene, and evaluated its specificity and sensitivity against other SARS-CoV-2 variants. In parallel, we tested an assay developed by the European Commission, Joint Research Centre (JRC) designed in silico for the detection of Omicron targeting a spike region with a unique cluster of mutations (Petrillo et al., 2021). The JRC invited control laboratories worldwide to validate it in vivo on clinical samples (https://joint-research-centre.ec.europa.eu/jrc-news/efficient-tracing-omicron-new-pcr-test-2021-12-13_en). Subsequently, both the assays were used to test sewages collected throughout Italy in the period between 11 November (date of entry in Italy of the first Omicron case in Italy) and 25 December 2021, to investigate how the Omicron variant spread over time and geographically. Moreover, Sanger sequencing was performed on positive samples, to validate results and study sequence variability.

2. Material and methods

2.1. Real-time RT-PCR design and evaluation of specificity and sensitivity

A new real-time RT-PCR assay targeting the spike region of the Omicron variant was designed using the Primer3Plus software (Primer3Plus (bioinformatics.nl). The spike region of Omicron harbours a number of mutations, some of which are in common with other variants (e.g. the deletion 69/70 shared with Alpha, T95I and G142D shared with Delta, N501Y shared with Alpha, Beta and Gamma), while other are unique for Omicron, and therefore suitable for the design of specific assays. We selected the region harbouring mutations H655Y, N679K and P681H, which are each present in >98.9% of the Omicron variant sequences (Supplementary materials, Fig. S1) as on 8 January 2022. Only mutation P681H is in common with the Alpha variant which was de-escalated by the European Centre for Diseases Control on 23 September 2021 due its drastically reduced circulation in Europe.

For the optimization of the assay different reaction conditions were evaluated: primer/probe concentrations, annealing temperatures (58 °C and 60 °C), and real time RT-PCR reagents (UltraSense One-Step qRT-PCR System by Invitrogen and AgPath-ID One-Step RT-PCR Reagents by Applied Biosystems). The newly designed assay was tested on a RNA sample extracted from a nasopharyngeal swab of a patient resident in the region of Apulia, collected on 7 December 2021, and kindly provided by Dr. Maria Chironna, Department of Biomedical Sciences and Human Oncology, School of Medicine and Surgery, University of Bari Aldo Moro. This sample had been previously characterized as Omicron variant by whole genome sequencing and submitted to GISAID with ID EPI_ISL_7565149. Viral RNA was quantified using a previously described real-time RT-qPCR (La Rosa et al., 2021c; Petrillo et al., 2021) and was standardized at 6×10^2 genome copies (g.c.)/μl for the use as a positive control in PCR runs and for sensitivity assessment. For the latter, the standardized RNA was used to prepare serial dilutions which were analysed in eight replicates to calculate the limit of detection (LOD_{50}) of the assay on pure target. The same dilutions were also used to spike nucleic acids extracted from wastewater samples collected in July 2021 (i.e. before the emergence of the Omicron variant) to assess the real-time assay LOD_{50} on environmental samples. Calculation were performed according to Wilrich and Wilrich (2009), with the spreadsheet available at https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html.

The specificity of the assay was evaluated using RNAs extracted from clinical samples characterized as Alpha, Beta, Gamma, and Delta variants (sample IDs 7652, 7584, 7654 and 8019, respectively), kindly provided by Dr. Paola Stefanelli at the Department of Infectious Disease at the Italian National Institute for Health (ISS). All RNAs had been previously standardized at a concentration of 1×10^3 genome copies (g.c.)/μl. Moreover, the in-house assay was evaluated for specificity using the European Virus Archive – EVA GLOBAL (EVAg) panel, consisting of RNAs from different Alfa- and Beta- coronaviruses, namely HCoV-NL63, HCoV-229E, HCoV-OC43, MERS-CoV, SARS-CoV and the prototype strain of SARS-CoV-2 (kindly provided by the Erasmus University Medical Center (Rotterdam, The Netherlands).

Another real-time RT-qPCR assay, developed by the European Commission, Joint Research Centre (JRC) (https://doi.org/10.5281/zenodo.5747872), and found to be able to successfully detect the Omicron variant in silico, was tested to confirm results and compare the performance of the two assays as the JRC invited control laboratories worldwide to validate the assay in vivo on field samples.

Primers and probes used in the present study are shown in Table 1.

2.2. Real-time RT-PCR assay on wastewater samples

Real-Time RT-PCR was performed on 737 wastewater samples (see Supplementary Material, Table S2) collected throughout Italy from 134 wastewater treatment plants – WTPs – located in 17 Regions and 2 AA.PP. (Supplementary materials, Fig. S2). These samples were collected between 11 November - the day of the incoming flight of the first recognized case of Omicron variant in Italy - and 25 December 2021, as a part of the SARS-CoV-2 surveillance established under Rec. 2021/472 of 17 March 2021. Of these, 123 samples collected in the period 28 November – 3 December 2021, had already been analysed in the monthly "flash surveys" on SARS-CoV-2 variants by Sanger and NGS (Covid-19: flash survey sulle acque reflue - dicembre 2021). Samples were collected, registered, processed for virus concentration and subjected to RNA extraction by the members of the SARS-CoV-2 environmental surveillance network in Italy (SARI network). The same reference analytical protocol (SARI protocol rev. 3, https://doi.org/10.5281/zenodo.5758725) was adopted by all network members. Briefly, 24 h composite sewage sample was concentrated by PEG precipitation followed by centrifugation following the method described by Wu and collaborators (Wu et al., 2020) with minor modifications (sample pasteurization at 56 °C per 30 min and removal of solid debris before precipitation through centrifugation at 4500 ×*g* per 30 min). RNA was extracted by magnetic silica beads and subsequently purified by the OneStep PCR Inhibitor Removal Kit (Zymo Research, CA, USA). RT-qPCR testing for SARS-CoV-2 was conducted according to previously published protocols (La Rosa et al., 2021c; Pierri et al., 2021) and quality insurance controls (process control virus, inhibition control) were included in the process to assess viral recovery and PCR inhibition. Purified RNAs were then shipped in dry ice to the Department of Environment and Health at ISS. Detection of the Omicron variant by real-time PCR was carried out with two different protocols:

a) Newly designed assay (PCR ID_999 assay)

Following optimization of conditions, the RT-PCR mix (25 μL total volume) was prepared using the AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems, MA, USA), and 5 μL of sample RNA were added to reactions containing 1× buffer, 1 μL of RT-PCR enzyme mix, 1.67 μL of detection enhancer. Primer/probe (Table 1) concentrations were as follow: 500 nM, 900 nM, and 250 nM of primer 2356_Omicron_fw, 2357_Omicron_rev, and probe 2358_Omicron_probe, respectively. Amplification conditions included reverse transcription for 30 min at 50 °C, inactivation for 5 min at 95 °C and 45 cycles of 15 s at 95 °C and 30 s at 60 °C.

b) JRC assay (OmMet assay)

As no PCR conditions were specifically provided in the reference publication, the reaction mix was prepared as for the PCR ID_999 and the same thermal profile was adopted. Amplification with the JRC assay was performed for confirmation purpose on samples testing positive by the real-time PCR assay ID_999.

All real-time reactions were run on a QuantStudio 12 K Flex (Applied Biosystems). Samples with *Ct* values <40 were considered positive; samples with *Ct* values >40 were considered uncertain but were retained for nested RT-PCR testing.

A summary of the characteristics of the samples testing positive by the two real-time PCRs is shown in Table 2.

2.3. Nested RT-PCR and sequencing

A newly designed nested RT-PCR assay targeting the spike protein and designed PCR ID_995/996 (first cycle/nested reaction), was used for further confirmation and to study viral genetic diversity though sequencing. The Omicron's Spike protein has several amino acid mutations (substitutions and deletions) in the selected region that are distinct compared to other VoCs (Kannan et al., 2022). Specifically, the nested assay amplifies a region of 577 bps, covering the amino acids 220 to 412 of the spike protein.

First-strand cDNA was synthesized using Super Script IV Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions, using reverse primer 2347. First PCR reaction was performed with 4 μL of cDNA and 1 μL of each primer (10 μM) in a final volume of 25 μL (Kit Platinum SuperFi Green PCR Master Mix, Thermo). The PCR conditions were as follows: 98 °C for 2 min, followed by 35 cycles at 98 °C for 10 s, 62 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 5 min. Nested PCR was performed using 2 μL of the first PCR product under the same conditions, with the exception of the annealing temperature raised at 63 °C. To avoid false-positive results, standard laboratory precautions were taken.

The PCR products were visualised by gel electrophoresis purified using a Montage PCRm96 Microwell Filter Plate (Millipore, Billerica, MA, USA), and were then sequenced on both strands (BioFab Research, Rome, Italy). Mutations were detected using the CoVsurver mutation Analysis of hCoV-19 implemented in GISAID (GISAID - CoVsurver mutations App).

Table 1
Primer and probes used in the present study, targeting the spike region.

PCR ID	Primer/probe name and orientation	Nucleotide sequence	Position in the SARS-CoV-2 Omicron genome[a]	Amplicon size (bps)	Targeted mutations
999	2356_Omicron_fw	5′-GGCTGTTTAATAGGGGCTGAATA-3′	23,429–23,451	141	H655Y, N679K, P681H
	2357_Omicron_rev	5′-GGCAATGATGGATTGACTAGC-3′	23,569–23,549		
	2358_Omicron_probe	5′-FAM-TCAGACTCAGACTAAGTCTCATCGG-BHQ1-3′	23,509–23,533		
OmMet[b]	Omt-F	5′-AACAAACCTTGTAATGGTGTTGC-3′	22,916–22,938	138	S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H
	Omt-R	5′-TGCTGGTGCATGTAGAAGTTC-3′	23,053–23,033		
	Omt-P	5′-FAM-GATCATATAGTTTCCGACCCACTTATGGTG TTGGTC-BHQ1-3′	22,965–23,000		
995 (1st cycle)	2351_fw	5′-CACGCCTATTATAGTGCGTGA-3′	22,102–22,122	693	G339D, S371L, S373P, S375F[c]
	2347_rev	5′-CAAGCTATAACGCAGCCTGT-3′	22,794–22,775		
996 (nested)	2352_fw	5′-TTCGGCTTTAGAACCATTGGTAG-3′	22,147–22,169	577	
	2349_rev	5′-TGGAGCGATTTGTCTGACTTC-3′	22,723–22,703		

[a] Position related to GISAID sequence accession ID: EPI_ISL_6913995.
[b] Petrillo et al., 2021 (https://doi.org/10.5281/zenodo.5747872); probe quencher QSY was replaced with BHQ1.
[c] List restricted to mutations with a prevalence in the lineage of at least 75%, data from https://outbreak.info/compare-lineages?pango=Omicron&gene=S&threshold=75&nthresh=1&sub=false&dark=true

Table 2
Summary of the positive samples, including geographic region and sampling date, WTPs data, SARS-CoV-2 concentrations, results of the two real-time assays and availability of sequencing data.

ID SARI	Region	Province	Sampling Date	WTP	Equivalent inhabitants	SARS-CoV-2 c. g./L	rt RT-PCR ISS (Ct)	rt RT-PCR JRC (Ct)	Sequencing data
6440		AQ	21/12/21	L'Aquila - Pile	48,000	<LOD	37,29	38,70	No
6437	Abruzzo	PE	21/12/21	Montesilvano - Villa Carmine	140,000	<LOD	>40	39,64	No
6439		TE	21/12/21	Teramo - Villa Pavone	41,824	<LOD	35,82	37,08	No
6407		AV	21/12/21	Manocalzati	140,000	4,4E + 03	37,63	–	Yes[a]
6351		CS	16/12/21	Villa Literno	631,714	<LOD	38,68	38,98	Yes
6402		CS	21/12/21	Area Casertana	370,769	2,0E + 03	38,55	39,57	Yes
6409		CS	21/12/21	Villa Literno	631,714	1,2E + 04	35,30	37,86	Yes[a]
6348		NA	16/12/21	Napoli OVEST - ex ingr. Camaldoli	250,000	6,8E + 03	36,13	39,46	Yes
6401	Campania	NA	21/12/21	Area Nolana	400,000	1,3E + 04	34,14	37,72	No
6404		NA	21/12/21	Napoli EST	1,750,000	1,3E + 04	34,78	–	Yes[a]
6406		NA	21/12/21	Napoli OVEST - ex ingr. Camaldoli	250,000	2,4E + 04	34,78	36,19	Yes
6405		NA	21/12/21	Napoli OVEST - ingr. Principale	950,000	2,4E + 04	33,88	36,42	Yes[a]
6403		SA	21/12/21	Nocera Superiore	299,121	2,2E + 04	35,91	36,93	Yes
6408		SA	21/12/21	Salerno	700,000	8,3E + 03	35,89	37,59	Yes[a]
6187	Emilia-Romagna	BO	13/12/21	IDAR Bologna	800,000	2,5E + 05	38,01	38,51	Yes
6244		BO	15/12/21	IDAR Bologna	800,000	1,1E + 05	37,24	38,95	Yes
6372		BO	20/12/21	IDAR Bologna	800,000	2,3E + 05	34,95	37,04	Yes[a]
6453		BO	22/12/21	IDAR Bologna	800,000	1,4E + 04	32,99	34,89	Yes[a]
6375		FC	21/12/21	Forlì	250,000	1,2E + 05	37,31	–	No
6373		MO	20/12/21	Naviglio	500,000	4,1E + 04	38,02	39,84	Yes
6454		MO	22/12/21	Naviglio	500,000	4,0E + 04	36,48	36,62	Yes[a]
6383		PR	22/12/21	Parma Ovest	168,000	9,0E + 03	36,29	–	Yes[a]
6382		RE	22/12/21	Reggio Emilia Mancasale	280,000	1,1E + 03	36,21	38,64	No
6456		RN-FC	22/12/21	S. Giustina	560,000	8,2E + 04	34,72	36,76	Yes[a]
6449	Friuli-Venezia	PN	21/12/21	Cordenons	15,000	2,3E + 05	35,81	37,75	Yes[a]
6312	Giulia	UD	14/12/21	Udine	200,000	8,4E + 04	>40	–	Yes
6450		UD	21/12/21	Udine	200,000	4,9E + 04	37,43	–	No
6259	Lazio	RM	14/12/21	Roma Est (linea 1 + linea 2)	900,000	4,0E + 04	37,91	39,31	Yes
6280		RM	20/12/21	Anzio Colle Cocchino	75,000	9,4E + 03	37,50	39,57	Yes
6283		RM	20/12/21	Pomezia Capoluogo	60,000	4,4E + 03	37,56	37,97	Yes
6285		RM	20/12/21	Ponte Lucano di Guidonia	50,000	9,3E + 03	35,86	39,78	Yes[a]
6284		RM	20/12/21	Velletri La Chiusa	36,700	1,1E + 04	34,24	37,37	Yes[a]
6732		RM	22/12/21	Fregene	76,000	1,5E + 05	37,57	–	Yes
6731		RM	22/12/21	Roma Ostia	350,000	1,3E + 06	33,26	34,99	Yes[a]
6730		RM	22/12/21	Roma Sud	1,100,000	1,1E + 05	35,06	36,94	Yes
6410	Liguria	GE	22/12/21	Darsena	160,000	1,2E + 05	35,59	36,01	No
6234		GE	14/12/21	Genova	60,000	4,9E + 04	34,41	38,57	Yes[a]
6387		GE	21/12/21	Pegli	40,000	2,2E + 05	32,58	35,88	Yes[a]
6386		GE	21/12/21	Punta Vagno	250,000	1,0E + 05	36,00	37,85	Yes
6400		GE	22/12/21	Punta Vagno	250,000	1,1E + 05	34,04	37,61	No
6389		GE	21/12/21	Quinto	60,000	2,5E + 05	31,87	34,69	Yes[a]
6390		GE	21/12/21	Rapallo	90,000	1,8E + 05	33,26	36,27	Yes[a]
6239		GE	14/12/21	Sestri Ponente	130,000	4,1E + 04	36,10	39,49	No
6391		GE	21/12/21	Sestri Ponente	130,000	1,1E + 05	35,61	37,84	Yes
6240		GE	14/12/21	Sturla	60,000	1,1E + 05	38,04	38,77	Yes
6392		GE	21/12/21	Sturla	60,000	2,6E + 05	33,60	35,19	Yes[a]
6385		GE	16/12/21	Valpolcevera	160,000	5,7E + 04	38,11	39,17	No
6397		GE	22/12/21	Valpolcevera	160,000	2,0E + 05	33,39	35,82	No
6388		GE	21/12/21	Voltri	62,000	9,4E + 04	34,19	38,89	Yes[a]
6396		IM	21/12/21	Camisano	40,000	1,1E + 05	36,83	37,86	Yes[a]
6393		IM	21/12/21	Sanremo - Capo Verde	75,000	2,5E + 05	36,47	38,56	Yes[a]
6242		SP	14/12/21	Camisano	40,000	1,5E + 04	36,76	–	No
6243		SP	14/12/21	Silea	17,500	3,1E + 04	34,76	38,29	Yes
6398		SP	21/12/21	Silea	17,500	1,5E + 05	32,82	34,29	Yes[a]
6399		SP	21/12/21	Stagnoni	82,000	1,9E + 05	34,44	36,18	Yes[a]
6394		SV	20/12/21	Savona	280,000	3,0E + 05	32,19	34,98	Yes[a]
6430	Lombardia	BS	21/12/21	Verziano	296,000	9,5E + 02	36,15	–	Yes
6431		BS	22/12/21	Verziano	296,000	3,5E + 02	37,37	39,52	No
6327		CO-LC-MI-MB	20/12/21	Monza San Rocco	600,000	5,3E + 04	34,48	38,94	Yes
6371		CO-LC-MI-MB	22/12/21	Monza San Rocco	600,000	3,5E + 03	35,15	–	Yes
6659		MI	12/12/21	Milano Nosedo	1,250,000	5,1E + 04	37,04	–	Yes[a]
6664		MI	14/12/21	Milano San Rocco	1,036,000	5,7E + 04	37,43	37,07	Yes[a]
6661		MI	19/12/21	Milano Nosedo	1,250,000	4,5E + 04	35,69	37,18	Yes[a]
6665		MI	19/12/21	Milano San Rocco	1,036,000	2,2E + 04	37,28	38,60	No
6251		MI-VA	14/12/21	Canegrate	137,950	6,9E + 03	36,36	37,58	Yes[a]
6445		MI-VA	21/12/21	Canegrate	137,950	3,3E + 04	31,54	34,67	Yes[a]
6446		VA	21/12/21	Varese	74,402	2,0E + 04	33,43	36,91	Yes[a]
6358	Marche	AN	21/12/21	Camerano	33,000	6,4E + 03	38,81	39,54	Yes
6356		AN	21/12/21	Zipa	100,000	8,3E + 03	37,49	–	Yes[a]
6354		PU	21/12/21	Ponte Metauro	60,000	1,1E + 04	37,61	39,64	No

Table 2 (*continued*)

ID SARI	Region	Province	Sampling Date	WTP	Equivalent inhabitants	SARS-CoV-2 c. g./L	rt RT-PCR ISS (Ct)	rt RT-PCR JRC (Ct)	Sequencing data
6355		PU	21/12/21	Ponte Sasso	18,000	4,9E + 03	35,91	–	Yes
6325	P.A. Bolzano	BZ	20/12/21	IDA Merano	356,520	6,5E + 03	37,62	–	Yes
6117	P.A. Trento	TN	13/12/21	Rovereto	95,000	2,1E + 05	36,58	–	Yes[a]
6314		TN	20/12/21	Trento sud	100,000	2,2E + 05	36,22	39,94	Yes[a]
6316		TN	22/12/21	Trento nord	120,000	1,5E + 05	34,86	36,95	Yes[a]
6317		TN	22/12/21	Trento sud	100,000	1,6E + 05	34,95	36,65	Yes[a]
6148	Piemonte	AL	15/12/21	Alessandria	110,000	1,3E + 02	38,53	–	No
6334		AL	24/12/21	Alessandria	110,000	8,5E + 03	33,46	36,99	Yes[a]
6289		BI	20/12/21	Biella Sud	53,000	2,8E + 03	37,20	–	Yes
6333		CN	23/12/21	Cuneo	185,000	6,3E + 03	35,93	36,54	Yes[a]
6287		NO	20/12/21	Novara	184,000	1,2E + 03	37,52	38,61	Yes[a]
6144		TO	15/12/21	Castiglione Torinese	1,934,099	2,9E + 02	>40	–	Yes[a]
6332		TO	22/12/21	Castiglione Torinese	1,934,099	9,1E + 03	34,98	36,70	Yes[a]
6424	Puglia	BA	20/12/21	Bari Est	461,394	3,8E + 03	37,25	–	Yes
6425		BA	20/12/21	Bari Ovest	360,000	2,4E + 03	36,50	39,46	Yes
6426		BA	20/12/21	Bitonto	79,332	3,8E + 03	37,22	39,78	Yes
6427		FG	21/12/21	Cerignola	56,355	4,7E + 04	37,36	38,64	Yes[a]
6428		FG	21/12/21	Foggia	208,000	7,9E + 03	>40	39,88	Yes[a]
6306	Sicilia	AG	20/12/21	Agrigento	55,000	4,1E + 04	36,52	–	Yes
6328		CL	21/12/21	Caltanissetta	76,700	1,1E + 05	38,02	>40	Yes[a]
6308		PA	21/12/21	Acqua dei Corsari	314,973	3,5E + 04	38,16	37,07	Yes[a]
6310		PA	21/12/21	Bagheria	75,000	3,7E + 04	34,90	37,74	Yes
6112		RG	14/12/21	Vittoria C.da Mendolilli	55,000	2,9E + 03	39,95	–	Yes[a]
6364	Toscana	PI	20/12/21	Pisa Nord (San Jacopo)	52,000	2,8E + 04	37,73	39,50	Yes[a]
6290	Umbria	PG	20/12/21	Pian della Genna	90,000	4,0E + 04	34,20	36,00	Yes[a]
6330		PG	22/12/21	Foligno Casone	90,000	1,3E + 04	34,92	37,10	Yes[a]
6329		PG	22/12/21	Pian della Genna	90,000	6,8E + 04	32,78	34,84	Yes[a]
6331		TR	22/12/21	Terni	150,000	2,5E + 04	33,41	35,33	Yes
6113	Valle d'Aosta	AO	12/12/21	La Salle	60,000	8,6E + 03	37,73	39,60	No
6319		AO	19/12/21	La Salle	60,000	8,1E + 03	35,89	37,11	Yes[a]
6413		AO	21/12/21	La Salle	60,000	1,1E + 04	35,99	39,21	Yes[a]
6302	Veneto	PD	21/12/21	Padova Ca' Nordio - zip	98,500	4,1E + 04	37,62	–	Yes[a]
6339		TV	21/12/21	Treviso	70,000	3,0E + 04	34,24	39,53	Yes[a]
6007		VE	07/12/21	Venezia Fusina	400,000	1,1E + 04	38,70	–	Yes
6146		VE	14/12/21	Venezia Fusina	400,000	2,3E + 04	39,01	37,60	Yes[a]
6340		VE	21/12/21	Venezia Fusina	400,000	2,6E + 04	34,08	36,21	Yes[a]
6432		VE	23/12/21	Venezia Fusina	400,000	1,5E + 04	33,43	35,70	Yes[a]
6235		VR	16/12/21	Verona-collettore 1 M	82,000	4,7E + 04	32,04	35,89	Yes[a]
6236		VR	16/12/21	Verona-collettore 3 M	102,000	2,4E + 04	34,41	37,03	Yes[a]
6238		VR	16/12/21	Verona-collettore 8 M	226,000	1,5E + 04	34,82	37,94	Yes[a]
6433		VR	23/12/21	Verona-collettore 1 M	82,000	2,0E + 04	31,95	34,47	Yes[a]
6434		VR	23/12/21	Verona-collettore 3 M	102,000	2,4E + 04	31,84	34,82	Yes[a]
6435		VR	23/12/21	Verona-collettore 8 M	226,000	1,7E + 04	33,63	37,70	Yes[a]
6147		VI	15/12/21	Vicenza Casale	92,000	1,6E + 04	37,01	–	Yes
6341		VI	21/12/21	Vicenza Casale	92,000	7,9E + 03	34,12	37,30	Yes[a]

[a] Presence of mutations characteristic of the Omicron variant.

Sequences were submitted to GenBank under the accession numbers ON196927-ON197019.

3. Results

3.1. Real-time RT-PCR design and evaluation of specificity and sensitivity

The optimization of PCR conditions showed that the RT-PCR reagents used had a significant impact on the performance of both real-time PCR assays, with Δ*Ct* values between the results provided by the different amplification systems equal or above 5 cycles (see Supplementary Material, Fig. S3). Following optimization, the standardized Omicron RNA (6×10^2 g.c./μl) was successfully amplified by both the newly designed PCR ID_999 (ISS assay) and the OmMet PCR (JRC assay), although higher *Ct* values were obtained for the latter (30.15 ± 0.40 vs. 27.38 ± 0.66, Supplementary Material, Table S1). The ISS real-time RT-PCR provided a LOD_{50} of 0.28 g.c./μl of tested nucleic acids (1.4 g.c./reaction) on pure Omicron RNA, and of 2.67 g.c./μl (13.3 g.c./reaction) on nucleic acids extracted from sewage samples and spiked with the Omicron variant. As for the specificity of the assays, no amplification was obtained for the six Coronavirus RNAs included in the EVAg Coronavirus panel, but a partial cross-reactivity was observed with Alpha and Gamma variants which were however amplified at a lower efficiency (Δ*Ct* of 5.02 and 3.78, respectively, compared to Omicron; Supplementary Material, Fig. S4).

3.2. Omicron detection by real-time RT-PCR in wastewater samples

None of the 355 samples collected between 11 November and 4 December tested positive for the Omicron variant. Of the 382 samples collected from 5 to 25 December, 111 tested positive (*Ct* values <40) for Omicron with the ISS assay (PCR ID_999) and 4 provided weak amplification signals (*Ct* values >40). An example of the results obtained is reported in Supplementary Materials, Fig. S5.Of the 115 positive samples, 91 (79.1%) were confirmed positive for the Omicron variant using the JRC assay (OmMet). Positive samples displayed an average Ct value (±SD) of 35.97 ± 2.17 (range 31.54–42.76) and 37.59 ± 1.58 (range 34.29–40.26) in the ISS and JRC assay, respectively, with a statistically significant difference of the two values (paired *t*-test, $p = 0.043$; Supplementary Materials, Fig. S6). Most of the discrepant results were associated to samples with high *Ct* values and, presumably, a lower viral concentration.

The first occurrence of the Omicron variant in wastewater in Italy was detected in a sample collected in Venice (Region of Veneto) on 07

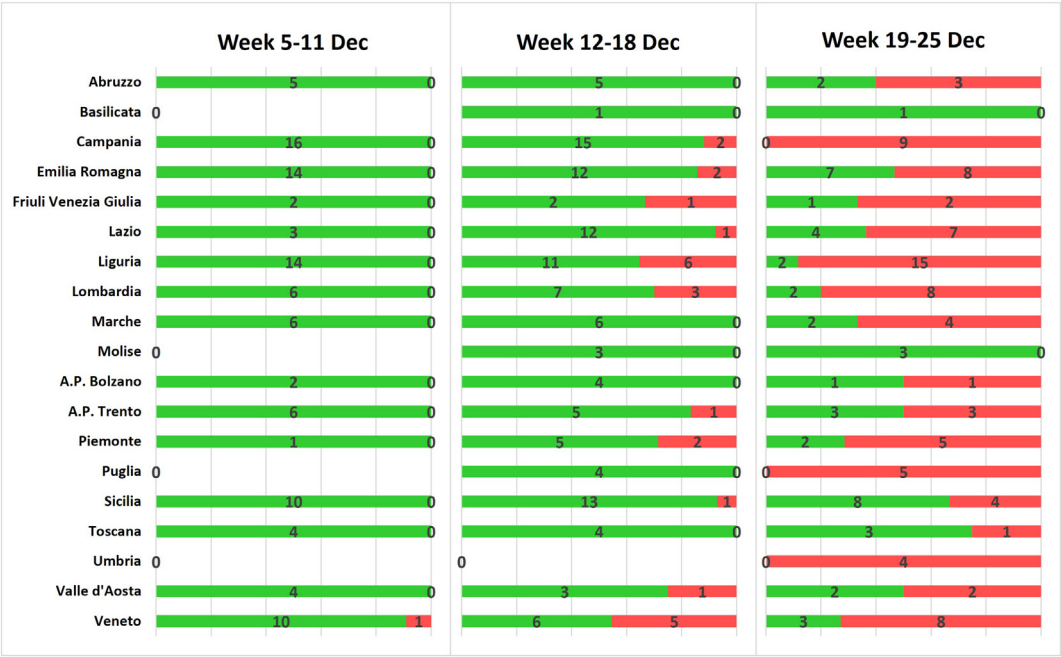

Fig. 1. Number of Omicron positive samples detected in the three weeks of December (05.12.2021–25.12.2021) divided by Region/A.P. Samples negative for Omicron variant are reported in green; positive samples are represented in red.

December 2021. Later on, a rapid spread of the variant was observed (Fig. 1) as follow: 1/104 samples (1.0%) and one Region in the week 5–11 December; 25/143 samples (17.5%) and 11 Regions/AA.PP. in the week 12–18 December; and 89/135 samples (65.9%) and 17 Regions/AA.PP. in the week 19–25 December (Supplementary Material, Fig. S7). In the last week, the Omicron variant was detected in 100% of the samples collected in the Regions of Campania, Puglia, Umbria (Fig. 2) and in the majority of the samples in other Regions (e.g. Lazio, Liguria, Lombardia, Piemonte, Veneto).

3.3. Characterization of Omicron-positive samples by Sanger sequencing

Overall, 97 of the 115 Omicron-positive wastewater samples (84.3%) were successfully amplified with the nested RT-PCR assay targeting the S protein, and subsequently sequenced by Sanger sequencing. Of these, 64 samples showed mutations associated with the Omicron variant, three samples showed amino acid substitutions not discriminatory for variant assignment, and 4 were unreadable due to mixed electropherograms (Table 3). Moreover, 26 sequences showed no mutations.

Sequence analysis showed a high degree of sequence variability within the Omicron variant sequences. In total, 10 amino acid substitutions were detected in the 577-bps fragment, with 12 different combinations (Table 3). The most frequent combination of mutations included the panel "G339D, S371L, S373P, S375F" (29 samples) followed by the panels "G339D, S371F, S373P, S375F" and "G339D, R346K, S371L, S373P, S375F" (11 samples each). This last combination of mutation is associated with sublineage BA1.1.

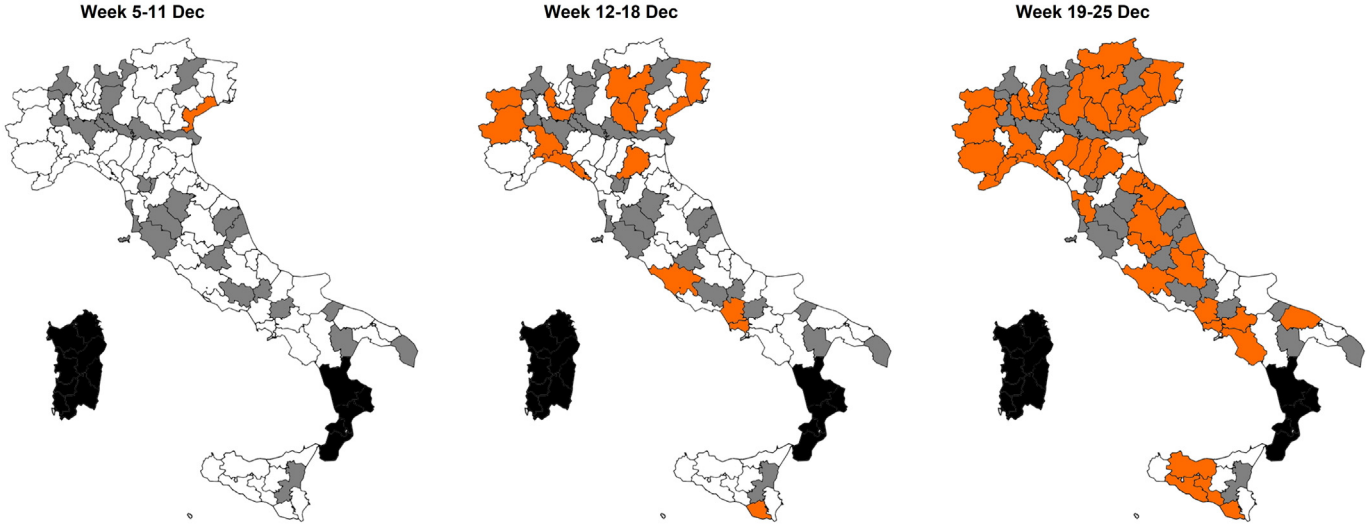

Fig. 2. Geographic spread of the Omicron positive samples during the three weeks of December (05.12.2021–25.12.2021) by province Regions in which wastewater surveillance was not yet activated at the time of the study are reported in black. Provinces for which no samples were tested in the study are reported in grey. Detection of the Omicron variant is reported in orange.

Table 3
Mutations detected by nested RT-PCR amplification followed by Sanger sequencing.

ID SARI	Mutations	Suggested variant
6236	G339D	
6383	S375F	
6339, 6408	G339D, S371P	
6333, 6404	S371L, S375F	
6330	T323I, G339D	
6393	G257V, G339D, S371L, S373P, S375F	
6234, 6235, 6372, 6387, 6388, 6394, 6407, 6409, 6433, 6435, 6449	G339D, R346K, S371L, S373P, S375F (BA.1.1)	
6117, 6144, 6238, 6251, 6284, 6285, 6314, 6316, 6317, 6340, 6356,	G339D, S371F, S373P, S375F	Omicron
6146, 6287, 6290, 6308, 6319, 6328, 6329, 6334, 6341, 6364, 6389, 6390, 6392, 6396, 6398, 6399,6405, 6413,6427, 6432, 6434, 6445, 6446, 6453, 6454, 6456,6659, 6661, 6664	G339D, S371L, S373P, S375F	
6428	G339D, S371F, S375F	
6112, 6302, 6731	G339D, S371P, S375F	
6332	G339D, S373P, S375F	
6007, 6147, 6240, 6243, 6244, 6259, 6280, 6283, 6289, 6306, 6310, 6312, 6325, 6348, 6351, 6358, 6373, 6386, 6391, 6403, 6406, 6425, 6426, 6430, 6730, 6732	No mutations	
6187, 6327, 6331, 6371	Mixed electropherogram	Unassigned
6355, 6424	P251L	
6402	P251L, S371P	

4. Discussion

The SARS-CoV-2 Omicron variant has rapidly replaced SARS-CoV-2 Delta variant in most European Union/European Economic Area (EU/EEA) countries (European Centre for Disease Prevention and Control, 2022). Immunity acquired through previous infection seems to be less effective against Omicron than against other variants, but the risk of severe COVID-19 remains low (Mallapaty 2022).

Omicron belongs to the Pango lineage B.1.1.529 which, compared to the Wuhan strain, is characterized by several amino acid changes in the spike protein, including A67V, DEL69/70, T95I, G142D, DEL143/145, N 211I, DEL212, G339D, S371L, S373P, S375F, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, and L981F (list restricted to mutations with a prevalence in the lineage of at least 75%; data from https://outbreak.info/compare-lineages?pango=Omicron&gene=S&threshold=75&nthresh=1&sub=false&dark=true). This lineage has recently been partitioned into four sub-lineages BA.1 (B.1.1.529.1), BA.1.1 (B.1.1.529.1.1), BA.2 (B.1.1.529.2) and BA.3 (B.1.1.529.3).

The present study describes the development of a real time RT-PCR to differentiate the Omicron variant from other SARS-CoV-2 VoCs in environmental samples and its successful use for the purpose of Wastewater-Based Epidemiology (WBE). The spike protein was selected for assay design since Omicron harbours more than 32 mutations in the Spike protein alone, 15 of these mutations reside in the receptor-binding domain (Shah et al., 2022). Another assay, also targeting the S-region and developed at JRC, was tested for comparison and for results confirmation. Since this assay has been only tested in silico, JRC invited control laboratories worldwide to validate it in vivo on clinical samples (https://joint-research-centre.ec.europa.eu/jrc-news/efficient-tracing-omicron-new-pcr-test-2021-12-13_en). The validation of the ISS assay on clinical samples confirmed the ability of the test to detect the Omicron variant, though we found that it may have cross-reaction with the Alpha and Gamma variants to a minor extent. However, since the circulation of both Alpha and Gamma has drastically reduced in the EU/EEA (https://www.ecdc.europa.eu/en/covid-19/situation-updates/variants-dashboard) and the amplification efficiency is significantly lower than Omicron, this should not currently present any major problem for the use of the assay on wastewater samples.

The newly designed PCR ID_999 assay targets amino acid substitutions H655Y, N679K, and P681H of the S-region. As on 8 February 2022, a total 1,081,970 Omicron sequences have been deposited in GISAID, 1,072,798 of which (99.1%) show the combination of amino acid substitutions Spike_H655Y, Spike_N679K, Spike_P681H. These mutations have not been detected in non-Omicron variants worldwide and are reported in 8284/8588 (96.5%) of the Omicron sequences submitted to GISAID from Italy, thus far (as on 8 February 2022). Moreover, the three mutations are present in all Omicron sublineages, therefore the assay is potentially able to detect BA.1, BA.1.1, BA.2, and BA.3, which may be useful in light of the rapid evolution of the variant's epidemiology. The Omicron subvariant BA.2, indeed, is more transmissible than BA.1. Researchers in Denmark have found that BA.2 is about 1.5 times more transmissible than BA.1, currently the dominant version of omicron worldwide, and will likely become more common in the near future; moreover, it is more adept at infecting people who are vaccinated and even boosted (Lyngse et al., 2022). Early studies suggest that the BA.2 lineage might prolong the Omicron wave, but won't necessarily cause a fresh surge of COVID-19 infections (Callaway 2022). However, until more research is performed to reveal the epidemiological characteristics of this VoC, we should remain cautious regarding Omicron BA.2 (Huang et al., 2022).

The developed assay was successfully used to detect the Omicron variant in sewage samples, demonstrating the first occurrence in Italy in the first week of December 2021 and its rapid countrywide diffusion within three weeks. In only 21 days, the prevalence of Omicron-positive wastewater samples increased from 1.0% to 65.9%, and the Regions/AA.PP. detecting its presence from one to 17. This trend reflected the progressive diffusion of the variant in the population. Indeed, as on 20 December 2021, the clinical "flash survey" conducted in Italy showed that the Delta variant was still predominant, and the prevalence of the Omicron variant was 21.0% (Stima della prevalenza delle varianti VOC - Indagine 20.12.2021). On 3 January 2022 the prevalence of the Omicron variant in nasopharyngeal swabs increased at 80.8% (Stima della prevalenza delle varianti VOC - Indagine 03.01.2022), and on 17 January to 95.8% (Stima della prevalenza delle varianti VOC - Indagine 17.01.2022). Due to the nature of wastewater surveillance, which monitors clusters of the population and not individuals, the sharp increase of the Omicron variant and its path towards the replacement of the Delta variant, were captured earlier in this study compared to the clinical data. Indeed, the regular flash surveys on wastewater performed in January (Covid-19: flash survey sulle acque reflue - gennaio 2022) and February (Covid-19: flash survey sulle acque reflue - febbraio 2022), confirm the predominance of the Omicron variant throughout Italy, with only a residual presence of the Delta variant. The Delta variant has completely disappeared in the flash survey performed in March (Covid-19: flash survey sulle acque reflue - marzo 2022) confirming that, since December 2021, Omicron has taken over in the whole country.

Positive samples by the newly designed ISS real-time RT-PCR were also tested with another assay (JRC OmMet) to further confirm the results, and compare the performances of the two assays. Overall, 79.1% of positive samples were also confirmed by the second assay, discrepancies being mostly associated with samples with *Ct* values higher than the average and, presumably, lower concentration of the target. Indeed, considering the results of the optimization tests and of the analysis on wastewater samples, the JRC assay seems to provide higher amplification stringency (no cross-reactivity with other variants), but lower sensitivity (*Ct* values on average higher than the ISS assay). Since a protocol for the OmMet assay was not given in the published article (Petrillo et al., 2021), the OmMet assay was run at the same condition of the newly designed assay, as specific optimization of the assay was outside the scope of the work. Increase of the sensitivity of the assay could be incremented by further optimize reaction conditions.

It should be noted that only qualitative real-time RT-PCR assays were used in this study, aiming at evaluating presence/absence of the target; we therefore did not exactly measure the Omicron template by comparison of the Ct values to a standard curve. Indeed the aim of the present study was not comparing Omicron SARS-CoV-2 concentrations, but describing the

history of the introduction and diffusion of the Omicron variant in the Italian population. However, by comparing absolute Ct values, we were able to show that they (and consequently the variant concentration) varied considerably. It is known that many factors impact the absolute value of Ct besides the concentration of the target, however a comparison can be done for data obtained in experiments using the same reaction conditions (instruments, reagents and assays). Therefore, the observation that the Ct value from one sample is higher than that of the other, could be valuable in concluding that the amount of template is lower in the first sample. Ct values ranged from 31,5 up to >40, that is more than 2.5 log difference, showing significant differences in sample concentrations. Lower Ct values (and therefore higher concentrations) were detected in North Italy in Veneto (where the first occurrence was also documented), Liguria and Emilia Romagna, while higher Ct values (lower concentrations) were documented in South Italy (Sicilia, Campania, and Puglia).

Further confirmation of the results obtained by the ISS assay was obtained by sequencing of positive samples. Overall, 55.6% of the positive samples (64 of 115) showed mutations characteristic of the Omicron variant. Interestingly, a considerable variability was observed within the Omicron sequences with 10 different amino acid substitutions detected in 12 combinations over a relatively short fragment (192 aa), the majority of positive samples harbouring the panel "G339D, S371L, S373P, S375F". The amino acid substitutions found by Sanger sequencing suggest the presence of the sublineage BA.1, with a subset of samples having a panel of mutation associated to BA1.1 (G339D, R346K, S371L, S373P, and S375F). Characteristic mutations of BA.2 were not detected. Indeed, the first Omicron BA.2 Italian sequence deposited in GISAID (EPI_ISL_11191121) was obtained from a nasopharyngeal swab collected on 2021-03-06 in the Region of Emilia Romagna.

Twenty-four sequences showed no mutations in the amplified fragment, which could be consistent with the presence of the Delta variant, which was still the prevalent one at the beginning of December (Covid-19: flash survey sulle acque reflue - dicembre 2021). Indeed, in the portion of the spike protein spanning amino acids 220 to 412, the Delta variant harbours few mutations with low or very low prevalence in the lineage: S221L (0.3%), A222V (10%), T250I (0.5%), P251L (0.9%), S255F (0.2%), W258L (0.2%), A262S (0.2%), V289I (0.9%), T299I (0.3%) (https://outbreak.info/compare-lineages?pango=Delta&gene=S&threshold=0.2). We can therefore assume that these samples contained both the Omicron and the Delta variants, the latter being quantitatively prevalent and therefore amplified more efficiently by the nested PCR.

A few studies have successfully detected the Omicron variant in sewage since its introduction in the population. Ahmed and co-workers published on the first detection of SARS-CoV-2 Omicron VOC in an aircraft wastewater sample from a flight arriving to Australia from South Africa on the 25th of November 2021 by RT-qPCR assays followed by sequencing (Ahmed et al., 2022). Agravall and collaborators found all characteristic mutations of Omicron in wastewater originating from Frankfurt Airport before the first confirmed clinical case (Agravall et al., 2022). In the United States, early evidence of the SARS-CoV-2 Omicron variant was documented in the community in November–December 2021 by detecting key mutations associated with the variant in wastewater (Kirby et al., 2022). Finally, SARS-CoV-2 Omicron Variant mutations were detected in wastewater samples from Denmark using Nanopore Sequencing (Rasmussen et al., 2022).

This study described the history of the introduction and diffusion of the Omicron variant in the Italian population and territory. The rapid spread of the variant was captured, confirming the effectiveness of sewage monitoring as a powerful surveillance tool. From a public health point of view, when a new variant arises policymakers need a rapid assessment of whether the new variant impacts the transmissibility of the virus, severity, and/or immunity that are likely to have an impact on the epidemiological situation. WBE can be used to study the spread of the variants of SARS-CoV-2 in the general population, and it can therefore be considered as a valid and efficient alternative method to test campaigns for surveillance purposes.

CRediT authorship contribution statement

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, MR, MG
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, MR, MG
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection	GLR, MI, CV, PM, GBF, ES, 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, the SARI Network
Writing - Original Draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation)	GLR, ES
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, LB, LL, the SARI Network
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/ data presentation	GLR, ES, CV, PM, GBF
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team	GLR, ES
Project administration	Management and coordination responsibility for the research activity planning and execution	GLR, ES
Funding acquisition	Acquisition of the financial support for the project leading to this publication	GLR, ES, LB, LL

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. The SARI network ("Sorveglianza Ambientale di SARS-CoV-2 attraverso i Reflui urbani in Italia")

Abruzzo: Giuseppe Bucciarelli, Paolo Torlontano (Regione Abruzzo); Giuseppe Aprea (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale");

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Appendix B. Supplementary data

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