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**Flash survey on SARS-CoV-2 variants in urban wastewater in Italy  
14<sup>th</sup> Report  
(Study period: October 3rd to 7th, 2022)**

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### Main findings:

- During the week of October 3rd to 7th, 2022, a total of 164 wastewater samples were collected from 18 Regions and 2 Autonomous Provinces.
- The characteristic mutations of the Omicron variant were detected in 14 Regions/Autonomous Provinces, while no sequences were obtained from the remaining regions.
- Of the sequences obtained, 81.4% showed amino acid substitutions of sublineage BA.4/5, 9.3% of BA.4/5 + R346T, 2.3% of BQ.1, and 2.3% of BQ.1.1.
- NGS results confirmed the widespread presence of sublineages BA.4/BA.5 across the majority of the regions/provinces and also confirmed the circulation of sublineages BA.4/5 + R346T, BQ.1 and BQ.1.1.

### Introduction

On March 17th, 2021, the European Union Commission issued Recommendation 2021/472, encouraging Member States to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewater by October 1st, 2021. In response to this recommendation, the Istituto Superiore di Sanità (ISS) initiated "flash surveys", which are periodic, monthly sampling campaigns conducted at various locations throughout Italy over a short period of time. The purpose of this report is to provide a summary of the findings from the 14th national Flash Survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from October 3rd to October 7th, 2022

### Methodology

The survey involved the collection of 164 sewage samples from 161 wastewater treatment plants (WTPs) located across 18 Regions and 2 Autonomous Provinces during the period from October 3rd to October 7th, 2022. Information on the WTPs participating in the SARS-CoV-2 surveillance in urban wastewater in Italy can be found on the ISS website<sup>1</sup>. The samples were processed and the viral concentration was determined by the SARI network laboratories using the protocol "Sorveglianza di SARS-CoV-2 in reflui urbani - Protocollo progetto SARI - rev.3"<sup>2</sup>. The purified RNAs were then sent to ISS for variant detection using both Sanger and NGS methods.

A real-time RT-PCR assay was used to screen for the presence of the Omicron variant<sup>3</sup>. A long nested RT-PCR assay (~1600 bps, spanning amino acid residues 58 to 573 of the spike protein) was used to detect multiple nucleotide changes distinctive of Variants of Concern (VoCs) and Variants of Interest (VoIs) in the spike protein<sup>4</sup>. Amplicons from the long nested assay were sequenced using both Sanger (single samples) and Next Generation Sequencing (NGS) (pools by Regions/AP) with the Oxford

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<sup>1</sup> Surveillance of SARS-CoV-2 in urban wastewater in Italy 1° Report (Study period: 01 October 2021 - 31 March 2022) [8e5e2edb-bae0-f1b0-ee6e-08255c76484f \(iss.it\)](https://www.iss.it/sites/default/files/8e5e2edb-bae0-f1b0-ee6e-08255c76484f_iss.it)

<sup>2</sup> DOI [10.5281/zenodo.5758724](https://doi.org/10.5281/zenodo.5758724).

<sup>3</sup> La Rosa G, Iaconelli M, Veneri C, Mancini P, Bonanno Ferraro G, Brandtner D, Lucentini L, Bonadonna L, Rossi M, Grigioni M; SARI network; Suffredini E. The rapid spread of SARS-COV-2 Omicron variant in Italy reflected early through wastewater surveillance. *Sci Total Environ.* 2022 Sep 1;837:155767. doi: 10.1016/j.scitotenv.2022.155767. Epub 2022 May 6. PMID: 35533857; PMCID: PMC9074219.

<sup>4</sup> G La Rosa, P. Mancini, G. Bonanno Ferraro, C. Veneri, M. Iaconelli, L. Lucentini, L. Bonadonna, S. Brusaferrero, D. Brandtner, A. Fasanella, L. Pace, A. Parisi, D. Galante, E. Suffredini. Rapid screening for SARS-CoV-2 variants of concern in clinical and environmental samples using nested RT-PCR assays targeting key mutations of the spike protein, *Water Research*, 2021, Volume 197, 1 June 2021, 117104. <https://doi.org/10.1016/j.watres.2021.117104>.

Nanopore Technology MinION platform. Bioinformatics analysis was carried out and variant calling was performed for recognized VoCs as previously described<sup>5</sup>.

To differentiate between the Omicron sublineages BA.4 and BA.5, which have identical spike proteins in the sequenced region, a nested PCR was carried out to detect a mutation in the M gene at position D3 (BA.5 has the D3N mutation).

## Results

In total, 158 out of 164 samples (96.3%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method adopted for environmental surveillance (Table 1). The viral concentrations ranged from 4.13 E+02 to 1.09E+06 genome copies (g.c.) per liter of sewage. Additionally, 152 out of the 164 (92.6%) samples tested positive for the Omicron variant through the RT-qPCR assay, with cycle threshold (CT) values ranging from 30.1 to 39.7.

### *Sanger Sequencing*

The real-time PCR assays, long nested PCR, and sequencing results are summarized in Table 1. A total of 45 samples from 14 Regions/Autonomous provinces were amplified by the long nested PCR assay, but long amplicons could not be obtained from samples collected in the regions of Abruzzo, Basilicata, Campania, Molise, Sicily, and Umbria. High quality Sanger sequences were obtained from 43 samples, while one sample resulted in mixed electropherograms due to the simultaneous presence of more than one strain. One sequence was unsuccessful due to a high background or noisy sequencing signal. All amplicons were characterized as the Omicron variant through Sanger sequencing.

Specifically, the Omicron BA.4/5 sublineage (which is characterized by the amino acid substitutions L452R and F486V in addition to those typical of BA.2) was detected in 35 samples (81.4%) from 14 Regions/Autonomous Provinces, such as Calabria, Emilia-Romagna, Friuli-Venezia Giulia, Lazio, Liguria, Lombardia, Marche, Piemonte, Puglia, Toscana, Valle d'Aosta, Veneto, Autonomous Provinces of Bolzano, and Trento. Four samples (9.3%) were characterized as the sublineage BA.4/5 + R346T mutation. Only one sample presented the key mutations of BQ.1 (characterized by the amino acid substitutions K444T and N460K in addition to those typical of BA.4/5) and another sample showed the characteristic mutations of BQ.1.1 (characterized by the amino acid substitutions R346T, in addition to those typical of BQ1). In two samples (4.65%), the presence of double peaks in correspondence to the spike mutation sites 1038, 1332, and 1380 (corresponding to the aminoacids R346, K444, and N460, respectively) suggested the simultaneous presence of more than one sublineage in different combinations (BA.4/5, BA.4/5 + R346T, BQ.1, or BQ.1.1) in these samples.

Using the assay targeting the M gene, 33 out of 35 samples were assigned to the Omicron BA.5 subvariant (due to the presence of the D3N mutation). Only two samples showed the presence of both subvariants BA.4 and BA.5 (indicated by a double A/G peak in correspondence to the mutation site).

### *Next Generation Sequencing*

NGS results were successfully obtained for all tested Regional pools, which showed the defining mutations of the Omicron variant (Table 1).

For ease of reading, the mutations were grouped into panels ('mutation packages') as follow:

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<sup>5</sup> La Rosa, G.; Brandtner, D.; Mancini, P.; Veneri, C.; Bonanno Ferraro, G.; Bonadonna, L.; Lucentini, L.; Suffredini, E. Key SARS-CoV-2 Mutations of Alpha, Gamma, and Eta Variants Detected in Urban Wastewaters in Italy by Long-Read Amplicon Sequencing Based on Nanopore Technology. *Water* **2021**, *13*, 2503. <https://doi.org/10.3390/w13182503>

- **Package A** (Omicron BA.4/5, long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package B** (Omicron BA.4/5 + R346T), long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package C** (Omicron BQ.1, long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **K444T**, **L452R**, **N460K**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package D** (Omicron BQ.1.1, long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **K444T**, **L452R**, **N460K**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H

The key mutations of the Omicron sublineage BA.4/5 (Package A) were found in 14 Regions/AP in Italy: Calabria, Emilia Romagna, Friuli-Venezia Giulia, Lazio, Liguria, Lombardia, Marche, Piemonte, Puglia, Toscana, Valle d'Aosta, Veneto, and the A.P. of Bolzano and Trento. The key mutations of the Omicron sublineage BA.4/5 + R346T (Package B) were detected in seven Regions/AP: Emilia Romagna, Friuli-Venezia Giulia, Liguria, Puglia, Veneto, Trento, and Bolzano. The amino-acid substitutions of the sublineage BQ.1 (Package C) were found in Emilia Romagna, Puglia and Veneto. The sublineage BQ.1.1 (Package D) was detected in Emilia Romagna, Puglia, Veneto, and A.P Trento.

**Table 1. PCR and sequencing results**

Sample ID	Region/A.P.	City	WTP	RT-qPCR (c.g./L)	RT-qPCR Omicron-ID 999 (CT value)	Mutations found by Sanger sequencing (long PCR ID_980)	SARS-CoV-2 variant (Sanger sequencing)	Sequencing results (NGS)	SARS-CoV-2 variant (NGS)
1	14701	Abruzzo	Pescara	Villa Carmine	1,83E+03	35.69			
2	14702		Teramo	Villa Pavone	7,76E+02	34.35			
3	14703		Pescara	Via Raiale	6,02E+02	36.61			
4	14704		Chieti	S. Martino	5,56E+02	39.08			
5	14705		L'Aquila	Pile	1,46E+03	36.05			
6	14665	Basilicata	Potenza	Tiera di Vaglio	6,00E+02	N/A			
7	14666		Matera	Pantano	1,45E+03	37.27			
8	14616	Calabria	Cosenza	Cosenza - Code di volpe	5,81E+04	35.26		• PACKAGE A	• Omicron BA.4/5
9	14617		Cosenza	Cosenza - Sant'Angelo	7,18E+04	37.69			
10	14618		Crotone	Crotone - località Papaniciaro	3,91E+04	39.36			
11	14619		Catanzaro	Catanzaro Lido - Loc. Verghello	6,79E+04	36.58			
12	14620		Reggio Calabria	Ravagnese - località Aeroporto	5,13E+04	36.39	PACKAGE A	Omicron BA.4/5 <sup>a</sup>	
13	14621		Catanzaro	Catanzaro - Zona industriale	5,23E+04	36.07			
14	14648		Campania	Salerno	Salerno	2,07E+03	39.72		
15	14649	Salerno		Nocera Sup	5,72E+03	39.36			
16	14650	Napoli		Napoli OVEST - Ingresso Principale	7,03E+03	38.12			
17	14651	Napoli		Napoli OVEST - ex ingresso Camaldoli	7,84E+03	36.79			
18	14652	Napoli		Area Nolana	4,41E+03	36.64			
19	14653	Napoli	Napoli EST	1,24E+03	N/A				

20	14654		Caserta	Villa Literno	1,38E+03	N/A			
21	14655		Caserta	Area Casertana	9,73E+02	N/A			
161	14633	Emilia Romagna	Reggio Emilia	Mancasale	1,37E+04	37.27		• PACKAGE A	• Omicron BA.4/5
163	14630		Piacenza	Borgoforte	1,25E+03	36.44		• PACKAGE B	• Omicron BA.4/5 + R346T
164	14631		Parma	Parma Ovest	1,90E+04	34.29		• PACKAGE C	• Omicron BQ.1
22	14545		Modena	Carpi	<LOD	N/A		• PACKAGE D	• Omicron BQ.1.1
23	14679		Bologna	IDAR	2,25E+04	32.28	PACKAGE A		Omicron BA.4/5 <sup>b</sup>
24	14681		Bologna	Imola	3,38E+03	35.42			
25	14683		Ravenna	Faenza	1,75E+03	37.31			
26	14684		Ravenna - Forlì-Cesena	Ravenna	1,05E+04	N/A	PACKAGE A+ Y144DEL+ G181R		Omicron BA.4/5 <sup>b</sup>
27	14685		Forlì-Cesena	Cesena	4,90E+04	31.77			
28	14688		Forlì-Cesena	Forlì	8,00E+03	34.18	PACKAGE B		Omicron BA.4/5 + R346T
29	14690		Rimini - Forlì-Cesena	S. Giustina	4,80E+03	33.72	PACKAGE A		Omicron BA.4/5 <sup>b</sup>
30	14691	Modena	Naviglio	4,75E+03	33.97	PACKAGE A		Omicron BA.4/5 <sup>b</sup>	
31	14397	Friuli-Venezia Giulia	Trieste	Servola	4,90E+03	33.99	PACKAGE A + S247I		• Omicron BA.4/BA.5
32	14717		Udine	Udine	<LOD	34.02	Sequence failure <sup>c</sup>	• PACKAGE A	• Omicron BA.4/5 + R346T
33	14718		Pordenone	Cordenons	<LOD	34.36		• PACKAGE B	
34	14523	Lazio	Roma	Civitavecchia Fiumaretta	<LOD	N/A		• PACKAGE A	• Omicron BA.4/5
36	14572		Latina	Latina Loc Latina Est	3,35E+03	37.20			
37	14573		Latina	Aprilia (Via del Campo)	1,41E+03	37.00			
38	14574		Roma	Anzio - Colle Cocchino	1,84E+03	37.51			
39	14575		Roma	Guidonia - Ponte Lucano	4,58E+02	N/A			
40	14576		Roma	Velletri (LA CHIUSA-SORBO)	1,30E+03	36.43	PACKAGE A		Omicron BA.4/5 <sup>b</sup>
41	14577		Roma	Pomezia - Via Cincinnato	4,13E+02	N/A			

42	14692		Roma	Roma Est (linea 1 + linea 2)	3,49E+04	32.49			
43	14693		Roma	Roma Nord	6,53E+04	32.90			
44	14694		Roma	Roma Sud	<LOD	34.31			
45	14695		Roma	Ostia	2,28E+04	34.04			
46	14696		Roma	Fregene	2,40E+04	34.16			
47	14590		Savona	Savona	1,09E+06	31.45	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	<ul style="list-style-type: none"> <li>• PACKAGE A</li> <li>• PACKAGE B</li> </ul>
48	14591		Savona	Borghetto Santo Spirito	2,20E+05	33.25	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
49	14592		Genova	Pegli	1,47E+05	33.58			
50	14593		Genova	Voltri	3,37E+05	32.24	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
51	14594		Genova	Quinto	5,82E+04	34.44			
52	14595		Genova	Rapallo	2,51E+05	31.98	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
53	14596		Genova	Sestri P	2,33E+05	32.69			
54	14600		Genova	Sturla	2,37E+04	36.57	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
55	14602	Liguria	Imperia	Imperia	3,14E+05	31.58	PACKAGE B	Omicron BA.4/5 + R346T	
56	14604		Imperia	Sanremo - località Capo Verde	1,81E+05	32.99			
57	14606		La Spezia	Camisano	2,46E+05	32.20	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
58	14607		La Spezia	Silea	8,22E+05	30.13	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
59	14608		La Spezia	La Spezia	4,98E+05	30.79	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
60	14609		Genova	Darsena	2,05E+05	33.16	PACKAGE B	BA.4/5 + R346T	
61	14610		Genova	Punta Vagno Genova	4,30E+05	31.46	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
62	14611		Genova	Valpolcevera	1,66E+05	32.53	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
162	14627		Genova	Punta Vagno genova	1,34E+04	38.13			
63	14534		Milano	Bresso	2,50E+04	N/A			<ul style="list-style-type: none"> <li>• PACKAGE A</li> <li>• Omicron BA.4/5</li> </ul>
64	14535	Lombardia	Milano - Monza e della Brianza	Peschiera Borromeo	3,99E+04	32.60			

65	14536		Milano - Varese	Canegrate	1,15E+05	N/A			
66	14537		Varese	Varese	5,79E+04	31.61			
67	14538		Milano - Varese	Lonate Pozzolo	6,20E+04	32.18			
68	14622		Bergamo	Bergamo	1,91E+04	32.12			
69	14623		Cremona	Citta di Cremona	5,45E+03	34.34			
70	14625		Brescia	Verziano	1,34E+04	32.90			
71	14647		Sondrio	Sondrio	3,06E+05	32.04			
152	14546		Milano	Milano Nosedo	1,12E+05	36.02			
153	14547		Milano	Milano San Rocco	1,15E+05	36.57			
154	14548		Como	Como	1,20E+05	35.53			
155	14549		Pavia	Pavia	3,73E+04	39.03			
156	14550		Como - Lecco - Milano - Monza e della Brianza	Monza	4,73E+04	37.13	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	
157	14551		Pavia	Vigevano	3,68E+04	38.07			
72	14659	Marche	Pesaro-Urbino	Borgheria	2,32E+04	33.58	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	• PACKAGE A • Omicron BA.4/5
73	14660		Pesaro-Urbino	Ponte Metauro	8,98E+03	36.02			
74	14661		Pesaro-Urbino	Ponte Sasso	8,93E+03	36.55	PACKAGE A + K147N	Omicron BA.4/5 <sup>b</sup>	
75	14662		Ancona	Zipa	3,37E+04	34.26			
76	14663		Ancona	Falconara	2,04E+04	33.38			
77	14664		Ancona	Camerano	2,05E+04	34.42			
78	14706	Molise	Campobasso	Campobasso - San Pietro	<LOD	37.14			
79	14707		Campobasso	Termoli - località Porto	5,54E+02	35.92			
80	14708		Campobasso	Termoli - località Pantano Basso	1,17E+03	35.13			
81	14565	P.A. Bolzano	Bolzano	IDA Bolzano	2,15E+05	30.62	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	• PACKAGE A • Omicron BA.4/5
82	14566		Bolzano	IDA Merano	9,34E+04	30.79	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	• PACKAGE B



83	14567		Bolzano	IDA Termeno	1,06E+05	31.59	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		• Omicron BA.4/5 + R346T
84	14513	P.A. Trento	Trento	Trento nord	4,99E+05	31.81	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	• PACKAGE A • PACKAGE B • PACKAGE D	• Omicron BA.4/5
85	14514		Trento	Trento sud	2,83E+05	34.22	PACKAGE A	Omicron BA.4/5 <sup>a</sup>		• Omicron BA.4/5 + R346T
86	14515		Trento	Rovereto	5,21E+05	30.53	Double peaks in specific positions <sup>d</sup>	Omicron BA.4/5 + R346T-BQ.1.1-BQ.1		• Omicron BQ.1.1
87	14482	Piemonte	Torino	Castiglione Torinese	2,58E+04	34.15			• PACKAGE A	• Omicron BA.4/5
88	14483		Biella	Biella Nord	5,35E+03	38.54				
89	14484		Novara	Novara	1,94E+04	37.63				
90	14542		Alessandria	Alessandria	4,93E+03	36.63				
91	14543		Asti	Asti	1,63E+04	32.63	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		
92	14544		Cuneo	Cuneo	6,33E+04	31.10				
93	14459	Puglia	Bari	Bari Est	1,04E+04	32.93	Mixed electropherograms <sup>e</sup>		• PACKAGE A • PACKAGE B • PACKAGE C • PACKAGE D	• Omicron BA.4/5
94	14460		Bari	Bari Ovest	9,07E+03	33.47	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		• Omicron BA.4/5 + R346T
95	14461		Bari	Altamura	6,35E+03	32.93	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		• Omicron BQ.1
96	14489		Brindisi	Brindisi Fiume Grande	9,87E+03	34.04	PACKAGE D	Omicron BQ.1.1		• Omicron BQ.1.1
97	14495		Lecce	Lecce	6,88E+03	33.57				
98	14496		Taranto	Taranto Bellavista	2,79E+03	34.63				
99	14497		Taranto	Taranto Gennarini	2,75E+03	33.74	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		
100	14516		Barletta-Andria-Trani	Andria	5,79E+03	33.06				
101	14517		Barletta-Andria-Trani	Barletta	4,07E+03	33.80				
102	14524		Foggia	Cerignola	1,00E+04	33.34	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		
103	14525		Foggia	Foggia	1,49E+03	33.58				
104	14526		Foggia	Manfredonia	6,14E+02	33.90				
105	14540		Bari	Bitonto	1,69E+03	33.12				
106	14552	Bari	Molfetta	8,29E+02	32.39					
107	14553	Barletta-Andria-Trani	Trani	2,39E+03	32.59					

108	14554		Barletta- Andria-Trani	Bisceglie	8,01E+02	33.16		
109	14969		Trapani	Mazara del Vallo	8,43E+03	35.40		
110	14970		Trapani	Trapani	5,37E+03	38.77		
111	15010		Ragusa	Modica	7,10E+03	35.57		
112	15014		Ragusa	Vittoria	4,58E+03	36.92		
113	15015		Ragusa	Ragusa	2,65E+03	37.47		
114	15017		Messina	Messina	1,26E+04	34.36		
115	15018		Messina	Messina	1,07E+04	34.43		
116	15098		Agrigento	Agrigento	1,23E+04	34.61		
117	15099		Enna	Enna alta - Enna bassa	4,65E+03	36.23		
	15100	Sicilia	Palermo	Bagheria	5,06E+03	38.74		
118	15103		Caltanissetta	Caltanissetta - San Cataldo	9,80E+03	35.64		
119	15104		Palermo	Palermo - Villabate - Ficarazzi - Misilmeri (fraz. Portella di Mare)	3,63E+04	33.62		
120	15105		Palermo	Palermo	2,74E+04	34.06		
158	14640		Catania	Pantano d'Arci	1,15E+03	38.20		
159	14643		Siracusa	Siracusa	6,50E+02	37.73		
160	14642		Catania	Giarre	7,33E+03	38.81		
121	14635		Arezzo	Casolino - San Leo	7,73E+03	35.41		• PACKAGE A • Omicron BA.4/5
122	14667		Pisa	Pisa Nord - S. Jacopo	8,97E+04	33.20		
123	14668		Firenze	Empoli Pagnana	2,19E+04	36.95		
124	14669		Massa	Lavello 2	1,52E+05	32.22	PACKAGE A	Omicron BA.4/5 <sup>b</sup>
125	14670	Toscana	Lucca	Viareggio	1,31E+05	33.34		
126	14671		Massa	Lavello 1	3,12E+04	33.41		
127	14672		Lucca	Pontetetto	1,51E+05	33.43		
128	14673		Livorno	Rivellino	5,51E+04	33.81		

129	14674		Livorno	Rivellino	8,47E+04	33.61				
130	14709		Firenze	San Colombano	6,10E+03	37.60				
131	14710		Firenze	San Colombano	3,83E+03	35.84				
132	14711		Prato	Baciacavallo	3,60E+03	37.61				
133	14712		Prato	Baciacavallo	3,28E+03	35.72				
134	14713		Grosseto	San Giovanni - Pianetto	2,18E+04	33.74				
135	14714		Pistoia	Centrale Pistoia	3,00E+04	33.21				
136	14715		Siena	Ponte a Tressa	2,83E+04	34.64				
137	14452	Umbria	Perugia	Perugia - Pian della Genna	2,01E+04	35.49				
138	14558		Perugia	Foligno Casone	3,90E+04	34.60				
139	14559		Terni	Terni	7,55E+04	32.19				
140	14614	Valle d'Aosta	Aosta	La Salle	1,46E+04	35.94	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	• PACKAGE A	• Omicron BA.4/5
141	14615		Aosta	Brissogne	3,35E+03	38.90				
142	14509	Veneto	Padova	Padova Ca' Nordio - centro storico	1,55E+05	35.37	PACKAGE B	BA.4/5 + R346T	• PACKAGE A	• Omicron BA.4/5
143	14510		Padova	Padova Ca' Nordio - zip	2,20E+05	33.45	Double peaks in specific positions <sup>f</sup>	Omicron BA.4/5 - BQ.1	• PACKAGE B	• Omicron BA.4/5 + R346T
144	14511		Padova	Padova Guizza	1,18E+05	34.14			• PACKAGE C	• Omicron BQ.1
145	14512		Padova	Abano Terme	1,80E+05	34.10	PACKAGE A	Omicron BA.4/5 <sup>b</sup>	• PACKAGE D	• Omicron BQ.1.1
146	14529		Vicenza	Vicenza Casale	2,57E+04	33.46	PACKAGE C	Omicron BQ.1		
147	14530		Treviso	Treviso	3,16E+04	36.22				
148	14531		Venezia	Venezia Fusina	1,07E+04	34.06				
149	14587		Verona	Verona_collettore 1M	3,29E+04	N/A				
150	14588		Verona	Verona_collettore 3M	1,05E+04	33.73	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		
151	14589		Verona	Verona_collettore 8M	1,65E+04	32.49	PACKAGE A	Omicron BA.4/5 <sup>b</sup>		

<sup>a</sup> the presence of a double A/G peak in the triplet encoding the amino acid in position 3 of the M gene (D3/N3) is suggestive of the simultaneous presence of BA.4 and BA.5

<sup>b</sup> the presence of mutation D3N in the M gene is suggestive of the presence of BA.5

<sup>c</sup> High Background/Noisy Sequencing Signal

<sup>d</sup> Double peaks in three positions: R346 + K444 + N460

<sup>e</sup> Sequence data begins to show multiple overlapping traces after a point in the sequence

<sup>f</sup> Double peaks in two positions: K444 + N460

- **Package A** (Omicron BA.4/5, long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package B** (Omicron BA.4/5 + R346T), long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package C** (Omicron BQ.1, long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **K444T**, **L452R**, **N460K**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package D** (Omicron BQ.1.1, long fragment from PCR ID\_980) = DEL69/70, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **K444T**, **L452R**, **N460K**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H

**Table 2. Sanger sequencing results**

**CHARACTERISTIC MUTATIONS**

ID SAMPLES	DEL69/70	G142D	G142D	V213G	G339D	R346T	S371F	S373P	S375F	T376A	D405N	R408S	K417N	N440K	K444T	L452R	N460K	S477N	T478K	E484A	F486V	Q493R	Q498R	N501Y	Y505H	VARIANTS
12-23-26-29-30-31-40-47-48-50-52-54-57-58-59-61-62-72-74-81-82-83-84-85-91-94-95-99-102-124-140-145-150-151-156																										<b>Package A*</b> (Omicron BA.4/5)
28-55-60-142																										<b>Package B</b> (Omicron BA.4/5 + R346T)
146																										<b>Package C</b> (Omicron BQ.1)
96																										<b>Package D</b> (Omicron BQ.1.1)

\* Upon testing for the assay targeting the M gene, of the 35 BA.4/BA.5 sequences, 33 were assigned to Omicron BA.5, and 2 showed the presence of both sublineages.

## Limitations of the study

The geographical and population coverage of this flash survey was incomplete, as it covered 20 out of 21 of the Italian regions/Autonomous Provinces.

The molecular analytical methods used for complex environmental matrices, such as wastewater, can be hindered by low viral concentrations, poor analyte recovery, and/or PCR amplification inhibition. As a result, both detection/quantification and PCR amplification for sequencing may produce false negatives, making it difficult to achieve molecular characterization and variant detection for all samples.

Partial sequencing of the Spike region does not provide conclusive results for sublineage assignment. However, the detection of defined mutation panels that are characteristic of certain lineages/sublineages, through Sanger or NGS sequencing, should be considered as a presumptive detection.

## Conclusions and final considerations

This report is part of a monthly series on SARS-CoV-2 and its variants in wastewaters in Italy, as established by the EU Commission Recommendation 2021/472. The aim is to provide additional information on SARS-CoV-2 variants in the population, supplementing information gathered through clinical surveillance. The results indicate that the Omicron variant is the sole presence of SARS-CoV-2 in Italy, with the sublineage BA.5 being the most prevalent. However, mutations characteristic of sublineages Omicron BA.4/5 + R346T, BQ.1, and BQ.1.1 were also detected.

The sequencing of SARS-CoV-2 in wastewater provides additional information to the sequencing of clinical cases, allowing for a more accurate description of the circulating variants in the country.

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