



# Trends in SARS-CoV-2 clinically confirmed cases and viral load in wastewater: A critical alignment for Padua city (NE Italy)

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## ABSTRACT

Since the beginning of the COVID-19 pandemic, wastewater-based epidemiology (WBE) has been depicted as a promising environmental surveillance tool and early warning system. Predictive models for the estimate of COVID-19 cases from wastewater viral loads also earned lot of interest and are currently under development. Hereby a pilot study that compares WBE surveillance data with confirmed cases, total hospitalizations, doses of vaccine administered and predominance of coronavirus variants.

Composite 24hrs wastewater samples were collected weekly between September 2021 and July 2022 from Padua wastewater treatment plant. Samples were processed following a previously published method. One-step RT-qPCR was performed for quantification, adapting an Orf1b-nsp14 gene assay. Variant replacement was derived from the monthly bulletins of the Italian National Health Institute. Aggregate data on vaccine doses administered and on COVID-19 prevalence and hospitalizations were retrieved from official reports.

Eighty-two samples were processed. Viral loads highlighted 3 major peaks in January, April and July 2022. Quantitation of SARS-CoV-2 in wastewater and clinical surveillance resulted temporally juxtaposable. However, variation of the two curves is not proportional. SARS-CoV-2 showed its highest peak in April, whereas maximum COVID-19 prevalence was achieved in January. Total hospitalizations followed the prevalence trend. Omicron BA.1 started to replace the Delta variant in December 2021. Subsequently, the shift towards Omicron BA.2 occurred between February and April 2022. Finally, BA.4/5 attested around June, somehow preceding the summer peak.

Emergence of Omicron BA.1 over Delta could be a possible driver of the increase in both clinical cases and wastewater viral load in January 2022. In late March 2022, Omicron BA.2 replaced BA.1: this reflected in a steep increase of wastewater viral load, but not of COVID-19 confirmed cases. When a dramatic drop in the testing capacity of clinical surveillance occurred, WBE was possibly capable of detecting a substantial increase in viral circulation.

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## 1. Introduction

Wastewater-based epidemiology (WBE) has proved a useful environmental surveillance tool, both to track the spread of COVID-19 and to support public health decisions. In addition, a value-added is concealed in the structural limit of the diagnostic testing capacity, the widespread occurrence of asymptomatic cases and a growing pandemic fatigue [1].

Nucleic acid of COVID-19 etiologic agent SARS-CoV-2 enters sewer systems via multiple sources, including stool, urine, saliva and sputum [2]. Asymptomatic and pre-symptomatic individuals and mild cases, which all may unlikely be diagnosed and tested clinically, can also contribute to the shedding [3,4].

Viral quantitative detection in wastewater can provide an indirect estimate of the prevalence of SARS-CoV-2 infection in the general population [5]. However, this is not easy to achieve, considering that only around half of COVID-19 patients shed faecal RNA in the week following diagnosis and approximately 4% of individuals eliminate faecal viral RNA up to 7 months after the infection [6]. Moreover, other factors (e.g. extensive vaccination campaigns and the rapid variant turnover over time) are thought to influence not only the pandemic dynamics but also the detection of SARS-CoV-2 in wastewater, given their possible implications on the incubation period, the initiation and the duration of viral shedding in stool, and symptoms onset. A recent paper especially addressed the potential of WBE to provide valuable information on the circulation of SARS-CoV-2 variants of concern (VOCs) in particular geographic areas and investigated differential faecal shedding among viral variants in stools of hospitalized patients. Faeces Ct values were compared with those of the diagnostic respiratory swab, notably suggesting that faecal excretion of Omicron BA.1 variant was overall lower than Delta, Omicron BA.2 and BA.5 [7].

Nevertheless, attempts to build a reliable predictive model of COVID-19 infection have been several [3–5,8,9].

The aim of the present study was to detect and quantify SARS-CoV-2 in wastewater samples and to compare analytical results with the number of confirmed cases identified either by nasal or oro-nasopharyngeal swabs, the number of doses of vaccine administered, the hospitalizations and the predominance of coronavirus variants in the considered population.

## 2. Methods

Composite 500 mL 24hrs wastewater samples were collected weekly between September 2021 and July 2022 from the inlet (i.e. untreated influent) of the main wastewater treatment plant in Padua (NE Italy), i.e. a 197 000 population equivalent activated-sludge plant. No rainfall occurred at least in the 12 h preceding the sampling. Wastewater samples were transported under refrigerated conditions at the Laboratory of Hygiene and Applied Microbiology of the Padua University and processed within 2 h.

Samples were concentrated by PEG precipitation, following the method originally proposed by Wu and colleagues [10], with minor modifications. Briefly, for viral precipitation 40 mL of each sample were processed, undergoing a first centrifugation step (4500×g for 30 min) to remove solid debris. Viral concentration was then carried out adding polyethylene glycol (PEG) 8000 (8% [wt/vol]; Millipore Sigma) and NaCl (0.3 M; Millipore Sigma) to the supernatant. Samples were vortexed at room temperature until full dissolution of chemicals and then centrifuged at 12 000×g for 2 h. Viral pellet was then resuspended in 140 µL of InhibitEX Buffer (Qiagen, Hilden, Germany) to remove PCR inhibitors. RNA was extracted with commercial kit QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, with a final elution volume of 100 µL.

One step RT-qPCR testing for SARS-CoV-2 was carried out according to a previously published assay targeting the ORF1b-nsp14 genomic region [11,12]. Performance characteristics of the nsp14 assay are: sensitivity 100%; specificity 98.7%; accuracy 99.6%. Limit of detection LOD = 0.41 gc/µL and limit of quantification LOQ = 3.71 gc/µL are also presented in the original publications. Reliability of the assay design was also double-check in terms of entropy of the targeted genome region (i.e. ORF1b-nsp14; 3'-to-5' exonuclease; positions 18 600–18699 of GenBank accession number NC\_045512), to ascertain the target was not positioned in a high-mutation rate region. Entropy check was performed with Next Strain Diversity tool (<https://nextstrain.org/ncov/open/global>) and showed an extremely low and circumscribed entropy value of 0.039, at positions 18 646–18648, supporting that the validity of the assay is not affected by the various Omicron variants.

Quality insurance controls were included in the process to assess viral recovery efficiency (RE) (i.e. spiking of raw wastewater samples with process control virus mengovirus) and PCR inhibition (monitored with an external synthetic RNA as amplification control) [13]. Percentage RE was used to normalize quantitation data.

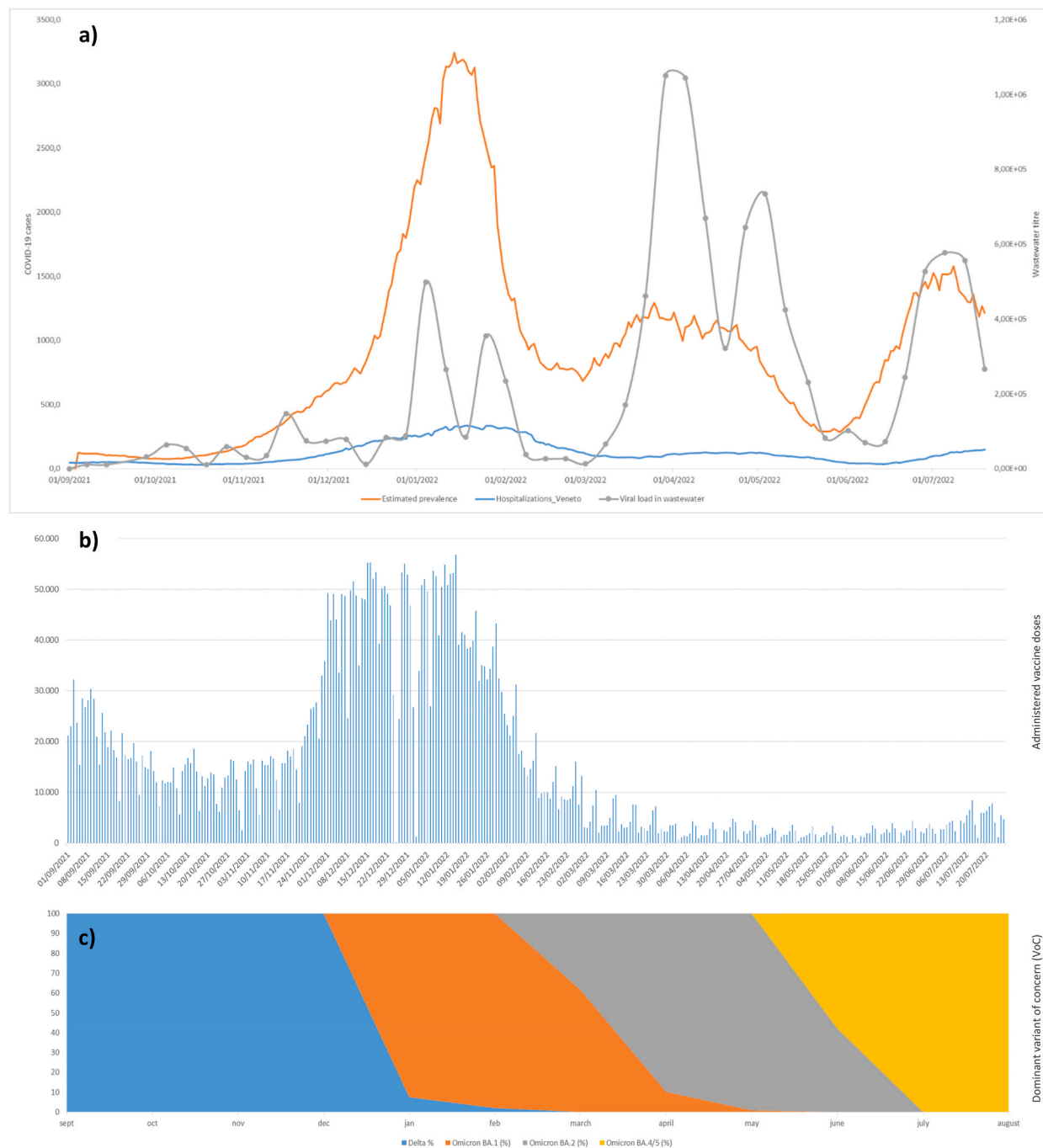
The Laboratory of Hygiene and Applied Microbiology is part of the SARS-CoV-2 environmental surveillance network in Italy (SARI network), coordinated by the Italian National Health Institute (ISS) [14]. The laboratory successfully passed two mandatory proficiency tests, held in October 2021 and March 2022 (Laboratory ID: #17) to attest correct performance of all of the above described methods. The detailed analytical protocol (SARI protocol Rev.3 25.07.2021), encompassing sampling, concentration, extraction and detection methods, can also be retrieved at <https://doi.org/10.5281/zenodo.5758725>.

Moreover, RNA extracts of samples collected during the first week of each month in the considered period were shipped in dry ice to ISS laboratory for genotyping of SARS-CoV-2 variants. Employed NGS and Sanger sequencing methods have been described elsewhere [14,15].

Aggregate data on vaccine doses administered and on COVID-19 incidence and hospitalizations in the province of Padua (i.e. a catchment area of approximately 938 000 inhabitants) were publicly available ([mappe.protezionecivile.gov.it/it/mappe-e-dashboards-emergenze/dashboards-coronavirus](https://mappe.protezionecivile.gov.it/it/mappe-e-dashboards-emergenze/dashboards-coronavirus)). In the absence of an aggregated and officially established number of prevalent cases, daily new positive cases (incident cases) were used to estimate the prevalence of infection. To this purpose, viral excretion was assumed to last between 3 days before and 15 days after a positive test result, as suggested by previous studies [16,17]. SARS-CoV-2 load in wastewater was graphically compared with the estimated prevalence of infection. Similarly, the proportion of circulating

SARS-CoV-2 variants of concern (VoC) and the number of administered doses of vaccine were juxtaposed in a separate graph to allow for visual evaluations.

Given the anonymous nature of data, neither evaluation by an ethics committee nor informed consent was required, according with



**Fig. 1.** SARS-CoV-2 in Padua Province (NE Italy): surveillance data; administered vaccine doses and dominant Variant of Concern. a) Viral RNA titres found in wastewater samples are reported as genome copies per litre (gc/L). Although single data-points were collected, a straight line follows the general trend for better readability. Estimated prevalence of COVID-19 (confirmed cases) is provided by the number of daily confirmed cases detected by the clinical surveillance network in the considered period. Daily number of total hospitalizations is also plotted. b) Number of vaccine doses administered. The increase around December 2021 in the daily number of doses is mainly due to boosters and the start of paediatric vaccination campaign. c) Turnover of the dominant variant of concern (VoC). Progressive substitution is represented as percentage. Table 1 also reports the genomic surveillance data point depicted by this plot.

the National legislation.

### 3. Results

Eighty-two wastewater samples were collected in the considered period. SARS-CoV-2 loads in wastewater and prevalence of COVID-19 confirmed cases are shown in Fig. 1a. Among wastewater samples, a unique negative result was found on 1 September 2022. The lowest viral RNA titre was of  $1.07 \times 10^4$  gc/L (19 October 2021). Peak loads were of  $4.99 \times 10^5$ ,  $1.05 \times 10^6$ ,  $5.77 \times 10^5$  gc/L on 4 January, 29 March and 05 July 2022, respectively. Clinical surveillance also showed three main peaks of prevalence, detecting 3245 cases on 14 January 2022; 1244 on 26 March 2022 and 1579 on 8 July 2022. Occurrence of peak periods in wastewater and prevalence is temporally juxtaposable. However, variation of the two curves is not proportional. SARS-CoV-2 load in wastewater exhibited the first peak in January 2022 and was then characterised by a considerably higher peak in April 2022. Both events are characterized by a bigemini pattern, with sub-peaks separated by 21 and 35 days, respectively. COVID-19 confirmed cases also showed the highest peak in January, yet it was followed by a substantial lower prevalence in March–May 2022. On the contrary, in the last part of the study period, (i.e. from mid-June to the end of July 2022), the two curves share a similar trend. Total hospitalizations followed the same trend of the prevalence curve, although with lower numbers.

The number of administered vaccine doses and dominant viral VoC are reported in Fig. 1b and c respectively. In December there was a significant increase in the daily number of vaccine doses administered. This was mainly due to booster doses and, for a less consistent amount, to the start of the primary vaccination course in the age 5–12.

Omicron BA.1 (from 0% to 92.5%) started to replace the SARS-CoV-2 Delta VoC (B.1.617.2) in December 2021. Subsequently, the shift towards Omicron BA.2 occurred between February and April 2022 (from 0% to 89.8%). Finally, BA.4/5 attested around June, somehow preceding both the summer peak of wastewater load and prevalence. Relative abundance and replacement of variants in the considered period is summarized by Table 1.

### 4. Discussion

Theoretical applications of WBE (e.g. early warning, estimate of real COVID-19 cases, ...) are now under empirical assessment, with the returning of solid multi-seasonal data from the National surveillance networks empowered in various countries [e.g. 12]. The present study mostly focused on how, at local level, WBE may provide a complementary perspective on the ongoing pandemics, if jointly compared with clinical surveillance data (i.e. prevalence and hospitalizations). The three curves presented three coeval peaks (Fig. 1a), although the proportion diverged in occasion of the first two main peaks. Interestingly, the curves again showed a similar trend in the last month of the observation period.

Obtained results suggest that the emergence of Omicron BA.1 over the Delta variant could be a possible driver of the increase in both clinical cases and, consequently, wastewater viral load in January 2022. Later on, around March 2022, Omicron BA.2 almost replaced BA.1 and this may have reflected in a steep increase of wastewater viral load, while – interestingly – this did not happen to the SARS-CoV-2 number of diagnoses in the population. While it is not possible to exclude a dramatic drop in the testing capacity and the subsequent lower identification of positive cases, WBE seemed able to detect a substantial increase in the viral circulation. As a matter of fact, wastewater data would indirectly depict a plausible scenario of heavy defection of SARS-CoV-2 testing by citizens (i.e. the manifest social limit of clinical surveillance), possibly interlaced with a change in VoC predominance, in favour of a milder variant such as Omicron BA.2, although with an increased transmission potential [18]. Of course, differential faecal shedding among VOCs should also be considered. As interestingly observed by Rector and colleagues, whilst viral load peaks corresponded to waves of COVID-19 cases for the original Wuhan strain and subsequent VOCs, during the later Omicron BA.1 wave, wastewater viral load increased to lesser levels. A plausible interpretation resides in a lower level of fecal excretion of BA.1, with the preliminary support of data from screening of hospitalized patients, also indicating that viral loads of BA.1 in stools were lower than other VOCs [7].

At the beginning of the study period, most of the population was fully vaccinated (78.5% of the population above 12 years had received two doses), so that we did not consider the population vaccine coverage. It should be noted that most of the considered

**Table 1**  
Relative abundance (RA%) and chronological replacement of SARS-CoV-2 variants in Padua WWTP samples.

Sampling date	Delta (RA%)	Omicron BA.1 (RA%)	Omicron BA.2 (RA%)	Omicron BA.4/5 (RA%)
07/09/2022	100	0	0	0
04/10/2021	100	0	0	0
02/11/2021	100	0	0	0
30/11/2021	100	0	0	0
11/01/2022	7,5	92,5	0	0
08/02/2022	2	98	0	0
08/03/2022	0	61,5	38,5	0
05/04/2022	0	10,2	89,8	0
03/05/2022	0	0,86	99,1	0
07/06/2022	0	0	42,1	57,9
05/07/2022	0	0	0	100
02/08/2022	0	0	0	100

administrations during winter months were booster doses and, for a relatively smaller quota, they consisted in primary cycle in children aged 5–12 (in detail: 8% were 1st doses; 10% 2nd doses; 82% 3rd doses).

We rather preferred to consider the number of doses administered, also in the light of the fact that a 20% drop on average in vaccine efficacy and effectiveness towards infection has been demonstrated after six months from the completion of the full course and the impact of a recently administered dose may be higher on protection from infection [19]. On the whole, estimated prevalence is deemed somehow lower in the two waves that occurred starting from March 2022, if compared to the first Omicron wave that arose in December 2021, although all subsequent variants were similarly or even more contagious than BA.1 [20]. In the light of this, it is interesting to note that the peak in number of doses administered between December 2021 and February 2022 seemed to flatten the curve of positive cases, and especially hospitalizations, that occurred starting from the spring stage of the considered timeline. On the contrary, it had no effect on viral loads in wastewater, that during spring and summer months resulted actually higher than in the period December 2021–February 2022, though disjoining from the COVID-19 estimated prevalence and hospitalization trend. However, as mentioned, this finding could also be interpreted considering a reduced Omicron BA.1 faecal shedding. On the whole, beside the enhancement of vaccination coverage, additional cofactors could explain how COVID-19 estimated prevalence progressively reduced over time. For instance, the role of hybrid immunity (immunological protection developed through a combination of SARS-CoV-2 natural infection and vaccination) could be taken into account. Consistently, a recent systematic review supported how individuals with hybrid immunity obtained the highest degree and durability of protection, both against short-term reinfection and hospital admission or severe disease [21].

Although our study did not aim to further understand the faecal spread rates of SARS-CoV-2 in terms of modelling or predicting the epidemic trend, it was able to demonstrate how WBE can be fundamental, whenever revealing a mismatch between clinical surveillance and wastewater data.

Possibly the major limitation of this population-based study consist in the actual reference denominator being the people residing in Padua's province. It amounts to approximately 938 000 inhabitants and was assumed to be constant during the whole survey period. Furthermore, as a WBE approach on a provincial basis was not possible for logistical reasons, samples from the wastewater treatment plant located in the main city were assumed to be adequately representative of the entire reference population.

Discrepancy between environmental and clinical surveillance curves could suggest specific targets for the implementation or recalibration of public health interventions. Moreover, at the local level WBE can effectively provide a different perspective of the ongoing pandemic. In the near future, it would be desirable to achieve further confidence in interpreting SARS-CoV-2 RNA load in wastewaters, so to determine a more precise burden of disease for COVID-19 within a community. As future perspective, we strongly believe that the lesson we are learning from WBE investigations on SARS-CoV-2, could also lead to the development of an expertise that can be adapted to virtually any other emerging pathogen.

## Data availability statement

Data will be made available on request.

## CRediT authorship contribution statement

**Tatjana Baldovin:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Irene Amoruso:** Conceptualization, Investigation, Methodology, Writing – original draft. **Marco Fonzo:** Data curation, Formal analysis, Writing – original draft. **Chiara Bertoncetto:** Data curation, Writing – review & editing. **Vanessa Groppi:** Project administration, Validation. **Gisella Pitter:** Supervision, Validation. **Francesca Russo:** Resources, Supervision, Validation. **Vincenzo Baldo:** Funding acquisition, Supervision.

## 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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