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SARS-CoV-2 wastewater monitoring using a novel PCR-based method rapidly captured the Delta-to-Omicron BA.1 transition patterns in the absence of conventional surveillance evidence

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HIGHLIGHTS
• A PCR-based WBE method to detect and quantify the Omicron VOC was developed.
• Fast Delta-to-Omicron transition pattern and doubling time in wastewater was shown.
• Omicron was detected in wastewater well before its recognition in clinical samples.
• The proposed system can rapidly enable awareness and preparedness in the community.
• The methodology can be adapted for early warning of highly spreading VOCs.

ABSTRACT
Conventional SARS-CoV-2 surveillance based on genotyping of clinical samples is characterized by challenges related to the available sequencing capacity, population sampling methodologies, and is time, labor, and resource-demanding. Wastewater-based variant surveillance constitutes a valuable supplementary practice, since it does not require extensive sampling, and provides information on virus prevalence in a timely and cost-effective manner. Consequently, we developed a sensitive real-time RT-PCR-based approach that exclusively amplifies and quantifies SARS-CoV-2 genomic regions carrying the S:Δ69/70 deletion, indicative of the Omicron BA.1 variant, in wastewater. The method was incorporated in the analysis of composite daily samples taken from the main Wastewater Treatment Plant of Thessaloniki, Greece, from 1 December 2021. The applicability of the methodology is dependent on the epidemiological situation. During Omicron BA.1 global emergence, Thessaloniki was experiencing a massive epidemic wave attributed solely to the Delta variant, according to genomic surveillance data. Since Delta does not possess the S:Δ69/70, the emergence of Omicron BA.1 could be monitored via the described methodology. Omicron BA.1 was detected in sewage samples on 19 December 2021 and a rapid increase of its viral load was observed in the following 10-day period, with an estimated
early doubling time of 1.86 days. The proportion of the total SARS-CoV-2 load attributed to BA.1 reached 91.09 % on 7 January, revealing a fast Delta-to-Omicron transition pattern. The detection of Omicron BA.1 subclade in wastewater preceded the outburst of reported (presumable) Omicron cases in the city by approximately 7 days. The proposed wastewater surveillance approach based on selective PCR amplification of a genomic region carrying a deletion signature enabled rapid, real-time data acquisition on Omicron BA.1 prevalence and dynamics during the slow remission of the Delta wave. Timely provision of these results to State authorities readily influences the decision-making process for targeted public health interventions, including control measures, awareness, and preparedness.

1. Introduction

The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is accompanied by the emergence and continuous reporting of several virus variants on a global scale (CDC, 2021). Among them, Variants of Concern (VOCs) are of particular importance, as they are characterized by increased transmissibility, increased virulence, clinical disease presentation, or may hamper the performance of the available diagnostic assays, vaccines, therapeutics, and public health measures (CDC, 2021; WHO, 2021a). Currently, five SARS-CoV-2 variants are designated as VOCs. The Alpha VOC (Pango lineage B.1.1.7) became the predominant SARS-CoV-2 variant worldwide in early January 2021 (ECDC, 2021a). Concomitantly, the Beta (lineage B.1.351) and Gamma (lineage P.1) variants were also identified and reported (CoVariants, 2021). In May 2021 the Delta variant (lineage B.1.617.2) subsequently became dominant, accounting by November 2021 for nearly all infections globally (Hwang et al., 2021; Tian et al., 2021).

The Omicron variant (lineage B.1.1.529) was identified more recently in multiple countries and was designated as a VOC in late November 2021 (WHO, 2021a), as it possesses many mutations, some of which may be linked with immune escape potential and enhanced transmissibility (WHO, 2021b). Omicron is driving an unprecedented surge of infections globally and initial reported spread was among younger individuals who tend to have more mild disease (WHO, 2021c). In this regard, early warning and surveillance of Omicron emergence is challenging, especially in areas where the incidence of the Delta variant is high.

The global phylogeny of Omicron in early 2022 showed the presence of 3 subclades; BA.1, BA.2, and BA.3. Omicron variant strains, except for those within subclade BA.2 have an in-frame 6 nucleotide deletion at the Spike (S) protein gene, resulting in the absence of amino acids 69 and 70 (S:Δ69/70). As of 15 December 2021, BA.1 accounted for 99 % of the sequences submitted to GIAGID. Additionally, >95 % of the Omicron variant sequences reported include the S:Δ69/70 (WHO, 2021b). Amino acids 69 and 70 are located at the N-terminal domain of the S1 fragment and it has been hypothesized that deletion of these residues may allosterically change S1 conformation (Hwang et al., 2020). This genomic trait also affects the performance of COVID-19 diagnostic tests that are based on the S-gene target failure (SGTF), as it has been previously described for the Alpha variant, which also possesses the deletion.

Whole genome sequencing (WGS) via the use of the next-generation sequencing (NGS) technology constitutes the reference methodology for the detection of SARS-CoV-2 variants. However, the turn-around time of this approach is long and thus, its capability to help assist towards rapid public health responses is limited (ECDC, 2021b). Besides being time-consuming, the universal application of NGS is also currently not feasible, since it is characterized by high running costs, requires highly trained personnel and is labor-intensive. Lastly, its capacity is limited, especially in samples with low viral loads, where failures are also frequent (Huang et al., 2019). Consequently, alternative methodologies for the rapid detection of mutations and variants have been proposed by the ECDC, including the use of RT-PCR-based assays (ECDC, 2021b). Examples of such assays for SARS-CoV-2 typing in clinical and/or environmental samples can be found by reviewing the scientific literature (De Pace et al., 2022; Hughes et al., 2022; La Rosa et al., 2021; Migueles et al., 2021; Vega-Magaña et al., 2021; Zelyas et al., 2021). In addition, real-time RT-PCR-based methodologies were developed by our team to facilitate identification of critical SNPs associated with Alpha, Beta/Gamma and Delta variants, and the performance of the assays was subsequently evaluated in previously characterized human and veterinary clinical specimens (Chaintoutis et al., 2021a, 2021b).

Although variant identification in clinical samples is of importance, not all individual samples are expected to be subjected to variant typing in a resource-preservation effort, and thus, the analyzed samples may not represent the target population. In parallel with conventional surveillance, wastewater surveillance is being applied extensively for the monitoring of SARS-CoV-2 loads and mutations. It has been shown that SARS-CoV-2 RNA concentrations in sewage correlate with COVID-19 incidence in the respective areas, and for this reason, tracking variants through wastewater testing comprises an appealing and arguably a more accurate approach (Yu et al., 2021). The common approach to monitor SARS-CoV-2 in wastewater is by real-time RT-PCR, accompanied by NGS, so as to identify and quantify mutations and lineages (Pechliianakis et al., 2022; Petala et al., 2022). However, the use of sequencing-based variant identification can be affected by the nature of sewage samples, which is likely to comprise a “pool” of genetic material from multiple variants shed from different individuals, that reduces the breadth of sequence coverage (Wurzter et al., 2021). On the contrary, via the utilization of RT-PCR-based approaches, target mutations in wastewater can be identified very fast, even if the respective variants are present at very low concentrations (Lee et al., 2021). As an example, the development and use of a droplet-digital RT-PCR assay has been described to monitor abundance of mutations present in the Alpha and Delta variants in wastewater settled solids (Yu et al., 2021).

Already from the beginning of the pandemic, the wastewater-based epidemiology group of the Aristotle University of Thessaloniki in Greece has been engaged in analyzing the viral load in daily wastewater samples taken from the Wastewater Treatment Plant (WWTP) of Thessaloniki that serves an estimated population of about 700,000 people (Aristotle University of Thessaloniki Research Committee, 2021). The group has published several papers that go beyond the conventional monitoring of viral load and contribute to the reliability of determinations. Hence, a methodology has been introduced which rationalizes virus shedding rate data in wastewater with regards physicochemical parameters of wastewater to cope with virus loss by adsorption to sewage suspended particles (Petala et al., 2021). The presence of chemical and biological species in wastewater that compete with virus particles in occupying adsorption sites on suspended solids along with topological complications of large-scale sewage networks have been considered in a separate publication to assess the spatial distribution of virus shedding rate along the wastewater piping system of a city (Kostoglou et al., 2021). In addition, contrary to earlier simplistic statistical approaches, a rigorous model has been proposed to estimate the number of infected people from the viral load in wastewater based on detailed population balances of infected people dynamics and shedding rate variability during disease days (Petala et al., 2022).
December, Omicron had been identified in 3 clinical samples. This evidence became publicly available on 30 December 2021, by the National Genomic Surveillance Network for SARS-CoV-2 mutations (National Public Health Organization-Greece (EODY), 2021a). By the end of 2021, the city experienced an unprecedented outburst of daily reported cases. Specifically, 4920 cases were reported on 31 December 2021, which was the highest number of cases recorded since the beginning of the pandemic (National Public Health Organization-Greece (EODY), 2021b). Thus, the credibility and usefulness of the developed methodology was assessed in a real-world scenario, as surveillance for BA.1 was incorporated in the daily viral load monitoring in the wastewater of Thessaloniki.

2. Methods

2.1. Oligonucleotides

A primer pair was designed to amplify a 145 bp fragment of the SARS-CoV-2 S gene. The selected downstream primer (OmDo2: 5′-AGTAGTACC AAAATGCGGCTCT-3′, Tm 65.6 °C) was designed to be specific to all 5 designated VOCs, including the Omicron. The upstream primer (OmUp: 5′-TCCA ATGTTAATCTTGTCCCATGTATCTC-3′, Tm 65.2 °C) targets the region that contains the 6-nucleotide deletion associated with S:Δ69/70 (nucleotide sequence TACATG; positions 21765–21770 on GenBank acc. no. NC_045512, isolate “Wuhan-Hu-1”). More specifically, the OmUp primer can hybridize to the target sequences as well, despite the T/C mismatch position (bold) was included in this particular probe to provide the ability to discriminate variants based on the detection of the S:T95I amino-acid substitution (C/T nucleotide mutation) which is present in Omicron strains (CoVariants, 2021). The effects of LNA modifications on mismatch discrimination were taken into consideration during probe design (Owczarzy et al., 2011; You et al., 2006). The length of the probe was also kept short (16 nucleotides) to improve mismatch discrimination. The philosophy behind the design of LNA TaqMan probes for SARS-CoV-2 mutation typing via RT-PCR-based SNP identification has been presented in detail in our previous works (Chaintoutis et al., 2021a, 2021b). Via this approach, discrimination between the Omicron and the Alpha is feasible on the bases of the obtained fluorescence.

2.2. Real-time RT-PCR assay

The reactions (20-μl) were comprised by: EnzyQuest’s One-step RT-qPCR kit (Product No.: RN010; EnzyQuest, Heraklion, Greece), 4 mM Mg2+, the primers OmUp and OmDo2 at concentrations of 0.2 μM each; the OmProbe at concentration of 0.15 μM, and 4 μl of sample RNA extract. Optimization of reactions took place on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The following thermal cycling conditions were applied: 55 °C for 15 min (reverse transcription), 94 °C for 15 min (inactivation of reverse transcriptase and activation of Taq polymerase), and 48 cycles in 2 steps: i) 94 °C for 10 s (denaturation), and ii) 58 °C for 35 s (combined annealing/extension), followed by FAM fluorescence measurement. Fluorescence data acquisition and analysis were performed using the CFX Maestro Software (v4.1; Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Analytical sensitivity, specificity, and diagnostic performance

For the evaluation of the analytical sensitivity parameters, a patient-derived SARS-CoV-2-positive RNA extract was used and the presence of Omicron variant genome in it was verified by NGS analysis. The RNA extract was subsequently subjected to real-time RT-PCR-based SARS-CoV-2 quantification, utilizing the N2 protocol proposed by the CDC for the diagnosis of COVID-19 in humans, with the use of the synthetic single-
stranded RNA standard “EURM-019” (Joint Research Centre, European Commission) as a calibrator. Based on the quantification results, the sample RNA was serially diluted (10-fold) and dilutions representing from 15 × 10^6 down to 15 viral RNA copies/reaction were prepared. Each prepared dilution was tested in triplicate, to determine the amplification efficiency and linear range of the developed assay. The RNA extract was further diluted at 25, 20, 15, 10, 5 and 2.5 copies/reaction, in a background of Delta variant genome-containing RNA extract from sewage. These dilutions were tested in 20 technical replicates each. The LOD was subsequently determined with 95 % probability of detection, by applying probit regression analysis using the MedCalc V20 (MedCalc Software, Mariakerke, Belgium).

Evaluation of the assay’s specificity also involved testing of the currently predominant Delta variant in sewage RNA extracts. This involved testing diluted RNA extracts from clinical samples containing 5 × 10^7 Delta variant genomic copies/reaction in 5 replicates each. These concentrations were chosen to reflect highest viral RNA concentrations in concentrated sewage RNA extracts. Higher concentrations were also tested. In addition, the specificity in sewage samples was evaluated by testing sewage samples (N = 22) obtained from Thessaloniki city over a 20-day period, between 9 and 30 November 2021, spanning the peak of the epidemic wave in Thessaloniki due to the Delta variant. The input SARS-CoV-2 RNA copy number ranged between 484 and 1662 copies/assay. Quantification of the aforementioned samples was performed during the routine monitoring of SARS-CoV-2 viral load in the city’s wastewater (Petala et al., 2022). Specificity testing also involved testing 4 patient RNA samples containing Alpha variant genome. Amplification curves and the relative fluorescence unit values at the plateau phase were compared to Delta variant RNA samples also tested by the assay.

2.4. Omicron BA.1 subclade variant surveillance results in wastewater samples from Thessaloniki city

After its evaluation, the assay was incorporated in the routine monitoring of SARS-CoV-2 viral load in wastewater samples of Thessaloniki city, which is being performed daily, by collecting 24-h composite samples (100 ml collected per hour). Routine analysis of sewage samples of a given day is performed in 3 replicates, as described previously in detail (Petala et al., 2022). Briefly, after particle removal, three 40-ml aliquots of each wastewater sample supernatant are being filtered through 0.45 µm MCE membrane filters (HAWP04700; Merck Millipore Ltd., Tullagreen, Ireland). Membranes undergo a phenol/chloroform-based RNA extraction procedure coupled with magnetic bead binding, followed by a PCR inhibitor removal step (OneStep PCR Inhibitor Removal Kit; Zymo Research, Irvine, CA, USA). RNA quantification is being performed in triplicates using the EOG SARS-CoV-2 RT-qPCR kit (Product No. RN01; EnzyQuest, Heraklion, Greece) targeting two genomic regions that encode the N and E proteins, respectively. A quantification precision threshold at 35 % CV for each individual sewage sample is chosen, assessed from the measurements of the 3 electronegative membranes. The recovery of virus concentration procedure is 63.3 % (CV = 12.1 %) and it was estimated by spiking heat-inactivated SARS-CoV-2 from a human clinical sample (10^6 viral particles) in three 40-ml replicates of different UV irradiated sewage samples (N = 4). The recovery of the RNA extraction procedure is 31.4 % (CV = 3.9 %) and it was estimated by spiking SARS-CoV-2 quantified RNA (10^6 genomic copies) onto membrane filters (N = 8) with UV irradiated sewage concentrates.

Quantitative measurements of SARS-CoV-2 RNA in the tested samples undergo rationalization with respect to the adsorption of virus onto suspended solids and the role of wastewater organic load on it by means of a physicochemical model (Petala et al., 2021). The results of rationalization are expressed in the form of a relative shedding rate, that is, the ratio of the shedding rate at any day versus a reference shedding rate value. With- out loss of generality, as reference value was selected the average shedding rate - a low value but above the LOQ - during the epidemiologically calm period in Thessaloniki at the first week of October 2020. Since 10 December 2021, RNA extracts (3 per day) that were subjected to routine total SARS-CoV-2 load determination, also underwent specific analysis for the quantification of genomes carrying the S:Δ69/70 deletion, by testing each extract in 10 replicates, i.e., 30 reactions/day. Samples obtained between 1 and 9 December were also analyzed retrospectively via the developed assay. In positive reactions, the Omicron-specific standard curve was used for quantification. The resultant values underwent rationalization (Petala et al., 2021) and viral loads were expressed as relative shedding rates. Relative shedding rates of variants without the targeted deletion were also calculated for these extracts, by subtracting Omicron BA.1 relative shedding rates from the total SARS-CoV-2 respective values.

To estimate the doubling time of SARS-CoV-2 Omicron BA.1 concentration in sewage, we computed the exponential fit curve using daily relative shedding rate sewage measurements for BA.1 for the first 10 days since its detection. Doubling time (D) was calculated as follows: \( D = \ln(2)/\alpha \), where \( \alpha \) was the slope of the curve.

2.5. Ethics statement

The human samples used as controls for the determination of the performance of the developed method were collected at the emergency ward of the General Hospital of Larissa from patients, as part of the routine diagnostic procedure. The use of clinical samples for the real-time RT-PCR method evaluation was approved by the scientific committee of the hospital, in accordance with national legal and ethical standards (Decision No. 102/19-03-2021).

3. Results

The in-silico prediction of the melting profile of the OmUp primer-Delta variant genome hybridization complex indicated that in the reaction’s annealing conditions, non-specific hybridization between the upstream primer and Delta targets is expected at a percentage of approx. 60 %. However, amplification of Delta (and Beta/Gamma) templates is unexpected, due to the presence of 4 nucleotide mismatches at the 3′-end of the hybridization region of the upstream primer. Alternatively, the upstream primers may be able to hybridize at their 3′-end, but other than that the hybridization is largely unstable to promote elongation during PCR amplification (Fig. 1). Thus, through the design of the OmUp primer, amplification of Delta and Beta/Gamma targets is largely not favored.

The amplification efficiency of the assay was determined to be 100 %, with a linear range of over 5 log10 Omicron RNA copies (Fig. 2). The corresponding standard curve was also characterized by a \( R^2 \) of 0.998 and a y-intercept of 42.507. The LOD for the detection of Omicron in RNA from wastewater samples was determined at 21 copies/reaction.

Results from testing four SARS-CoV-2-positive RNA extracts containing Alpha variant strains revealed the detection of fluorescence. However, the respective amplification curves exhibited suppressed fluorescence signals compared to Omicron-specific curves (Fig. 2). In detail, fluorescence signal for Alpha variant strains at plateau phase was considerably reduced by over 80 % RFU (relative fluorescence units) compared to the respective signal for Omicron.

The in-silico prediction results about the absence of the Delta variant detection were confirmed by the experimental evaluation of the specificity of the developed assay, where extracts with viral loads equivalent to 5 × 10^3 Delta variant genomic copies/reaction were tested, and fluorescence was not detectable. Delta variant input RNA from clinical samples with very high virus concentrations could be detected, although not efficiently, since a shift by approx. 13 cycles was observed in the Ct value obtained by the assay developed herein, compared to the Ct value obtained when the same extract was tested with the CDC’s N2 assay (data not shown). Testing of sewage RNA extracts obtained from the WWTP of Thessaloniki city between 9 and 30 November 2021 gave negative results, indicating that the genome of the Delta variant that was highly abundant in sewage of that time was not detected.

After the incorporation of the assay in the sewage routine analysis of Thessaloniki wastewater (i.e., as of 10 December, along with the retrospective analysis of samples obtained between 1 and 9 December), the BA.1 subclade of Omicron was detected for the first time in the sample collected on 19 December 2021. This positive result was obtained during the next
day (20 December) and reported on 21 December 2021. Subsequently, a sharp increase in viral RNA concentrations was observed, as within a 10-day period, the levels of BA.1 in Thessaloniki wastewater increased exponentially, from 6 RNA copies/μl (19 December) to 223 RNA copies/μl (29 December). Sewage viral load attributed to the BA.1 subclade continued to increase at a lower rate, peaking at 675 RNA copies/μl (7 January 2022). The detection of the Omicron BA.1 in sewage coincided with a change in the overall trend of SARS-CoV-2 RNA concentrations. Specifically, after peaking on 25 November, the SARS-CoV-2 load in sewage of Thessaloniki kept decreasing until December 18, i.e., a day before the detection of BA.1. The evolution of the viral load (expressed as relative shedding rates) corresponding to SARS-CoV-2 genomes carrying S:Δ69/70 over time, indicative of the Omicron BA.1 subclade, is presented in Fig. 3, along with the respective viral loads that correspond to other variants without the deletion and the total measured SARS-CoV-2 load. The doubling time of BA.1 load in sewage, based on measured relative shedding rate values, was estimated at 1.86 days, for a 10-day period (19-to-29 December).

The proportion of sewage RNA corresponding to BA.1 also increased rapidly from 3.52 % on 19 December 2021 to 89.78 % on 3 January 2022, indicating a fast transition from Delta-to-Omicron within the community of Thessaloniki city (Fig. 4). The following period (4–9 January 2022) the above proportion remained stable with mild fluctuations indicating a degree of persistence of the Delta at relatively low levels. Mean concentrations, standard deviations, and the coefficients of variation of BA.1 and total RNA concentrations (in genome copies/l) for each day are presented in Supplementary Material (Table S1).

The time series of SARS-CoV-2 load in wastewater against daily confirmed COVID-19 cases in the regional unit of Thessaloniki, as reported by the National Public Health Organization is presented in Fig. 5. It is evident that, shortly after the identification of Omicron BA.1 in sewage, an outburst of reported cases occurred. It can be also observed that the increases in relative viral shedding occur earlier than the increase in reported cases. While the exponential growth of cases becomes clear in the period between 27 and 31 December, the increasing trend of SARS-CoV-2 loads in sewage was observed as of 19 December, i.e., over a week earlier.

4. Discussion

Although Delta was the predominant SARS-CoV-2 variant during the fourth COVID-19 wave in many countries worldwide, the novel Omicron variant was rapidly reported and designated as a VOC. This is since it harbors a high number of mutations in the S gene, and preliminary data from South Africa indicated that this variant is characterized by immune evasion capabilities, rapid transmissibility, increased disease severity and high re-infection rates (Pulliam et al., 2021; WHO, 2021d). The rapid spread in South Africa, particularly among younger age groups, placed WHO and global health systems on high alert (Petersen et al., 2022). It is also of concern that Omicron started to rapidly spread against a backdrop of ongoing Delta variant...
transmission and high levels of natural immunity against the latter variant (Karim and Karim, 2021). In addition, since the expansion of this epidemic wave is initially driven among young individuals, most of them being asymptomatic, the initial expansion of the virus in the community cannot be effectively captured with the classical surveillance of recorded cases.

The magnitude of Omicron expansion necessitates reinforcement in the implementation of surveillance utilizing molecular diagnostics techniques. Due to the unprecedented spread of Omicron in the community, the time needed for sewage analysis with NGS constitutes a severe bottleneck that renders it incapable to be used as an early warning tool. Taking also into consideration these restrictions of the routine use of NGS, an alternative approach was urgently needed to directly detect and quantify low levels of Omicron in sewage samples, rapidly and efficiently. Wastewater-based epidemiology comprises an alternative approach for monitoring the dynamics SARS-CoV-2 outbreaks with several benefits. Importantly, SARS-CoV-2 variant monitoring in sewage can allow a fine description of the variant spreading in the monitored population, as well as the dynamics of newly introduced variants (Wurtzer et al., 2022). Besides the analysis of samples obtained from city WWTPs, wastewater analysis from incoming flights could also be utilized to monitor introductions of the variant (European Centre for Disease Prevention and Control (ECDC), 2021). The first detection of Omicron in aircraft wastewater using an SGTF assay and

![Fig. 3. Fluctuations of wastewater SARS-CoV-2 viral load levels from samples obtained from the WWTP of Thessaloniki. The relative shedding rates of total SARS-CoV-2, of viral genomes carrying the S:Δ69/70 deletion, indicative of Omicron BA.1 and of other variants without the deletion, are represented by the blue, brown, and green line, respectively. The relative shedding rate of the other variants without the deletion was calculated by subtracting the relative shedding rates of SARS-CoV-2 genomes carrying S:Δ69/70 from the total SARS-CoV-2 respective values.](image)

![Fig. 4. Proportion (%) of the total wastewater viral load corresponding to Omicron BA.1 from 19 December 2021 to 9 January 2022. Red line represents a polynomial fit curve. Proportions were estimated by dividing the mean Omicron BA.1 concentration with the mean total wastewater SARS-CoV-2 RNA concentration for each day.](image)
sequencing has been reported recently in Australia (Ahmed et al., 2022). So far, variant monitoring in wastewater is applicable mostly via the use of the NGS methodology. However, a relatively high quantity of input target RNA is required for this technique and considerable time is needed for sample analysis (Pechlivanis et al., 2022). As introduction of rapidly expanding Omicron took place in areas where the Delta was predominant, its early detection could have been missed or significantly delayed.

In this context, a highly sensitive real-time RT-PCR assay was developed for immediate and targeted detection and quantification of Omicron BA.1 subclade variant in sewage samples. The assay exclusively amplifies sequences with the S:Δ69/70 deletion that was a trait of the majority of the Omicron variant strains reported worldwide during the investigation period. Since the deletion was exploited for the design of the upstream primer, amplification of the genome of variants that do not possess the deletion (e.g., of Delta) is not favored, thus addressing the issue of competition among amplicons corresponding to different variants. This ensures the applicability of the assay in use for the analysis of wastewater, as it comprises a composite sample containing genomes from various SARS-CoV-2 strains. Commercially available SGTF assays that are being used for diagnostics are different from the developed assay in this regard, as it has been shown that the S:Δ69/70 deletion is contained in the middle of the amplicon, meaning that all SARS-CoV-2 genomes are amplified, irrespective of the variant, and the SGTF relies on the inability of the probe to hybridize in deletion-containing amplicons (Public Health England, 2021). Consequently, despite being useful for testing human specimens to diagnose COVID-19, those commercial assays are inappropriate for testing composite wastewater samples, as a novel variant can either be missed or incorrectly quantified when a different variant dominates. Another approach developed for the quantitative detection of Omicron in wastewater is based on an allele-specific RT-qPCR assay that simultaneously targets mutations Q493R, G496S and Q498R (Lin Lee et al., 2021).

Evaluation of our assay in sewage samples indicated its robust performance in detecting low-copy numbers of the BA.1 subclade, a feature that is of importance to detect the introduction of Omicron in a monitored area as early as possible. The presence of Delta which was ubiquitous in the monitored wastewater samples in December 2021 did not affect the ability of the assay to detect and quantify the targeted variant. Additionally, fluorescence signals were not obtained even when much higher Delta variant RNA copy numbers were tested, compared to those that were expected to be found in wastewater samples, e.g., at the concentrations detected in sewage samples from Thessaloniki during the peak observed in November 2021. Fluorescence corresponding to the Alpha variant could be detected, as this variant also harbors S:Δ69/70. However, the fluorescence plateau values detected were considerably lower compared to those of Omicron. In addition, since Alpha has been completely displaced by Delta, the presence of the former variant in the monitored wastewater is unexpected. It should be also highlighted that the data obtained from the National Genomic Surveillance Network regarding the SARS-CoV-2 variants that circulated during each period were critical for the interpretation of the RT-PCR findings. This is based on the fact that the developed assay aims at a specific viral genomic target. In our case, up to 16 December 2021, the vast majority of the genomic sequences that were characterized from Thessaloniki belonged to the Delta variant (92.8 %, i.e., 1059 out of 1141 clinical samples that underwent typing) (National Public Health Organization-Greece (EODY), 2021c) and this assisted in drawing safe conclusions about the performance of our wastewater monitoring methodology.

To the authors’ knowledge, the present research reports for the first-time an early warning system based on wastewater-based surveillance that was able to specifically detect and rapidly estimate the viral load doubling time and frequency of the BA.1 subclade of Omicron in a large Greek city, prior to its recognition through clinical testing, therefore rapidly enabling awareness and preparedness among the community. Upon the detection of BA.1 in sewage of Thessaloniki on 19 December, the corresponding wastewater RNA levels were characterized by a sharp exponential growth. Omicron BA.1 influenza levels showed a > 60-fold increase within the first 10 days and > 100-fold increase within the first 15 days, with a short early doubling time. This observation is in agreement with estimates deriving from surveillance data of reported cases, from countries where Omicron prevailed. Early investigations from Gauteng Province of South Africa had estimated the early doubling time in the first 3 days after crossing the wave threshold of ten cases per 100,000 population at 1.2 days and at 1.5 days in the first week (Karim and Karim, 2021). Since then, data on the Omicron transmission dynamics rapidly accumulated, unanimously indicating significantly higher transmission rates compared to Delta. The World Health Organization has summarized recent evidence (until 17 December 2021) on the transmission of Omicron suggesting a doubling time between 1.5 and 3 days, based on various studies and reports mainly from United Kingdom and South Africa (WHO, 2021b). Data from the UK Health Security Agency on 24 December 2021 also highlight the short doubling time that is <2.5 days for various regions of England (UK Health Security Agency, 2021a).

Our results indicate that the proposed method provided evidence on the increase of the prevalence of SARS-CoV-2 genomes carrying S:Δ69/70
significantly earlier than the identification of Omicron via routine surveillance based on clinical samples. The detection of Omicron BA.1 in influent of the WWTP of Thessaloniki signified major changes in the total sewage SARS-CoV-2 viral load, immediately reversing the prevailing declining trends. This pattern change was later reflected in the epidemic curve. Reported SARS-CoV-2 cases in the regional unit of Thessaloniki started increasing exponentially approximately a week after its detection in sewage (sampling on 19 December, result obtained on 20 December), reaching high levels as of 31 December 2021. Thus, the present report provides a clear early warning example of wastewater-based epidemiology. The first detection of the Omicron BA.1 in sewage of Thessaloniki was reported on 21 December 2021, i.e., over a week before the official announcement (30 December 2021) of the National Genomic Surveillance Network for SARS-CoV-2 mutations regarding the emergence of Omicron in humans in the city. It is interesting though, that this early warning potential was not clear in data before the emergence of Omicron. This can be attributed to the fact that Omicron is more prevalent in younger individuals who are more likely to escape diagnosis. Recent observations from the United Kingdom verify that the distribution of Omicron by age currently differs markedly from Delta, as people aged 18–29-year-old have significantly higher infection rates with Omicron relative to Delta (Imperial College COVID-19 Response Team, 2021).

We also observed a pattern of extremely fast Delta-to-Omicron transition in sewage SARS-CoV-2 load, as the proportion of SARS-CoV-2 genomes carrying the Δ69/70 reached 60.2 % within 10 days, and 91.09 % within 19 days. The interpretation of clinical surveillance data reveals similar transition trends. According to UK Health Security Agency, the percentage of cases with SGTF in England increased from 2.47 % (5 December 2021) to 67.3 % (15 December 2021) in a 10-day period (UK Health Security Agency, 2021b). Moreover, the estimated proportions of sewage viral load attributed to Omicron are in line with results from the analysis of clinical samples from Thessaloniki, where the percentage of Omicron cases based on SGTF screening ranged from 67.5 % to 85.0 %, between 3 and 8 January 2022 (National Public Health Organization, personal communication). SARS-CoV-2 variant analysis in positive clinical samples from Thessaloniki city, analyzed retrospectively via the NGS technology, revealed a similar Omicron proportion increase trend. Specifically, by considering human samples obtained between 12 and 18 December a 4.17 % percentage of the Omicron variant was shown. Out of those Omicron-positive samples, the first was obtained on 15 December. This proportion increased to 27.89 % (samples obtained between 19 and 25 December), 57.53 % (26 December to 1 January 2022), 75.00 % (2 January to 8 January) and 80.95 % (9 to 11 January). Thus, through the applied wastewater-based surveillance methodology, a similar pattern of expansion in SARS-CoV-2 variants is prevalent, and together with outbreaks of seasonal influenza can indicate that the incubation period of Omicron is shorter (Jansen et al., 2021), applying overwhelming pressure to the healthcare systems. Finally, the proposed surveillance approach that targets deletions to selectively amplify SARS-CoV-2 genomic regions with deletion signatures can serve as a paradigm for monitoring the community spread of new variants that may emerge in the future.

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CRediT authorship contribution statement

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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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