Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
From Alpha to Omicron BA.2: New digital RT-PCR approach and challenges for SARS-CoV-2 VOC monitoring and normalization of variant dynamics in wastewater

Sebastien Wurtzer a,⁎, Morgane Levert b, Eloïse Dhenain b, Heberte Accrombessi a, Sandra Manco a, Nathalie Fagour a, Marion Goulet a, Nicolas Boudaud c, Lucie Gaillard c, Isabelle Bertrand d,1, Julie Challant d, Sophie Masnada e, Sam Azimi f, Miguel Gillon-Ritz g, Alban Robin a, Jean-Marie Mouchel b,1, Obepine Sig b, Laurent Moulin a,1

a Eau de Paris, Research & Development, 33 avenue Jean Jaures, FR-94200 Ivry sur Seine, France
b Sorbonne Universite, CNRS, EFHE, UMR 7169 Meta, e-LTER Zone Atelier Seine, F-75005 Paris, France
c ACTALIA, Food Safety Department, F-50000 Saint-Lô, France
d University of Lorraine, CNRS, LCPME, F-54000 Nancy, France
e SIAM – STV, Avenue de la courtiere, FR-77400 Saint Thibault des vignes, France
f SIAAP, Innovation Department, 82 Avenue Kléber, FR-92700 Colombes, France
g Direction de la Propreté et de l’Eau - Service Technique de l’Eau et de l’Assainissement, Rue du Commandeur, FR-75014 Paris, France

HIGHLIGHTS

• Wastewater samples were analyzed using dRT-PCR for monitoring SARS-CoV-2 mutations.
• Mutation dynamics in wastewater reflected the VOC spreading within the Paris population.
• Movements of populations have been corrected using drinking water consumption.
• Mutation frequency determined by dRT-PCR showed less variability than by sequencing in wastewater.

ABSTRACT

Throughout the COVID-19 pandemic, new variants have continuously emerged and spread in populations. Among these, variants of concern (VOC) have been the main culprits of successive epidemic waves, due to their transmissibility, pathogenicity or ability to escape the immune response. Quantification of the SARS-CoV-2 genomes in raw wastewater is a reliable approach well-described and widely deployed worldwide to monitor the spread of SARS-CoV-2 in human populations connected to sewage systems. Discrimination of VOCs in wastewater is also a major issue and can be achieved by genome sequencing or by detection of specific mutations suggesting the presence of VOCs. This

http://dx.doi.org/10.1016/j.scitotenv.2022.157740
Received 3 April 2022; Received in revised form 26 July 2022; Accepted 27 July 2022
Available online 30 July 2022
The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is marked by the continuous emergence of numerous variants (World Health Organization, 2022). Among these, some variants show clear evidences of increased transmissibility, pathogenicity or better ability to escape human immune response induced by natural invariants. The Beta (lineage B.1.351; clade 20H) and Gamma (lineage P.1; clade 20J) VOCs were also responsible for the fourth wave observed in France during the last quarter of 2021. At the same time, the Vunch VOC (Pango lineage B.1.1.7; clade 20E) became the dominant SARS-CoV-2 variant worldwide in early 2021. At the same time, the Beta (lineage B.1.351; clade 20H) and Gamma (lineage P.1; clade 20J) VOCs were also identified around the world. From May 2021, the Delta VOC (lineage B.1.617.2, clade 21A) subsequently became dominant and was mainly responsible for the fourth wave observed in France during the last quarter of 2021. At the end of 2021, the Omicron variant (lineage B.1.1.529) was identified in multiple European countries and was designated as a VOC in November 2021 (European Centre for Disease Prevention and Control, 2022). Recently, 3 sub-lineages of Omicron VOC were described based on phylogenetic analysis and were designated BA.1 (clade 21K, the former that defined the Omicron lineage), BA.2 (clade 21L) and BA.3 very minor to date (Covariants.org, 2022). Omicron VOC has a panel of mutations associated with enhanced transmissibility and high potential for vaccine-induced immune escape (Cele et al., 2022). In contrast to previous variants, Omicron VOC is thought to have a lower overall severity in vaccinated populations (Ferré et al., 2022; Maisa et al., 2022). However, Omicron VOC has been reported to induce moderate infections in younger populations, with a notable increase in multi-systemic inflammatory syndrome cases according to French health authorities (Santé Publique France, 2022a). In the Greater Paris region, the first Omicron BA.1 VOC case was identified by November 29th, 2021 as indicated by the health authorities, during a Delta wave initiative in November 2021. Omicron BA.1 VOC has led to an unprecedented global resurgence of infection (7-day rolling incidence >5000 cases/100,000 inhabitants) during the month of December 2021 in France until Delta VOC was fully replaced at the end of December (Santé Publique France, 2022b). In this regard, early warning of Omicron emergence and surveillance of its sub-lineage dynamics must be implemented.

In order to meet this high demand for clinical screening, the French individual testing strategy has significantly changed since January 2022. Thus, antigenic tests alone have become sufficient to make a diagnosis of COVID-19 infection, without confirmation by RT-PCR analysis. Although this approach has made it possible to streamline the workload of medical analysis laboratories in towns and hospitals, the possibilities of performing molecular analyses on these samples, screening by PCR for specific mutations of interest and sequencing, have been greatly affected, leading to a bias in patient recruitment and a potentially partial view of the overall viral circulation and the prevalence of each variant. Identification of the infection dynamics and involved VOCs is of great importance, but if the variants become less and less virulent, the clinical epidemiology may no longer correlate with true viral circulation in the populations.

Since the beginning of the pandemic, a complementary epidemiological approach based on fecal shedding of virus and its detection in wastewater has been proposed (Medema et al., 2020; Prasek et al., 2022). Although many methodological approaches have been described and a more standardized protocol is required (Ahmed et al., 2021; Bivins et al., 2021a, 2021b), the add-value of this approach has been widely demonstrated through the monitoring of the global viral burden of SARS-CoV-2. In this regard, the European Commission recommendation (EU) 2021/472 of 17 March 2021 called for the establishment of a systematic and harmonized surveillance of the SARS-CoV-2 genome and its variants in raw wastewaters in the European member states (Official Journal of the European Union, 2021).

The establishment of such a surveillance system is of significant interest in a context of low viral circulation to alert on potential viral emergence or re-emergence. The monitoring of variants, especially VOC, can also be performed in raw wastewater but with some limitations. Sequencing, the most frequently described method (Agrawal et al., 2022a; Crits-Christoph et al., 2021; Fontenele et al., 2021; Pérez-Cataluña et al., 2021; Rios et al., 2021), is complex to perform in wastewater due to the relatively low viral load compared to clinical samples and the potentially degradation and/or fragmentation of the viral RNA in such samples (Lou et al., 2022). In addition, the presence of multiple lineages in raw wastewater does not allow the proper assembly of reads to determine the complete sequences of circulating genomes and correct lineage assignment without a complex workflow (Agrawal et al., 2022a; Jahn et al., 2021; Smyth et al., 2022). Even if the data quantity generated by sequencing is relevant, this methodology is also expensive and time-consuming. To overcome these limitations, targeted and specific RT-qPCR approaches have been proposed to detect certain mutations suggestive of variant presence or functional mutations whose evolution could reveal the spread of variants designated as VOC (Wurtz et al., 2022). Different RT-qPCR methods have been proposed based on either relative quantification of mutations to the total SARS-CoV-2 genomes (Erster et al., 2022; Wurtz et al., 2022; Yaniv et al., 2021), either on wild-type and mutated allelic discrimination (Graber et al., 2021), quantification of mutations and total genomes by digital PCR (Boogaerts et al., 2022; Caduff et al., 2022; Heijnen et al., 2021; Lou et al., 2022). These approaches have been developed and applied to the emergence of Alpha and Delta VOC. To our knowledge, few data are available regarding the emergence and the dynamics of the Omicron BA.1 and BA.2 VOC (Agrawal et al., 2022a; Ahmed et al., 2022; Chassalevris et al., 2022; Kirby et al., 2022; Smyth et al., 2022).
In this study, the specific monitoring of VOC-suggestive mutations was performed by digital RT-PCR. The objectives were to date the emergence of the Omicron BA.1 and BA.2 sub-variants that have been recently detected in France, but also to model the propagation dynamics of these specific mutations in comparison with open statistical data from the national sequencing program (EMERGEN) of clinical samples (Santé Publique France, 2022b). Population replacement kinetics of dominant variants (from Alpha to Omicron BA.2) was determined. A comparison of the mutation proportions determined by digital RT-PCR and sequencing of wastewater samples was also performed. In addition, methods of data normalization, including human fecal biomarker, were tested on the data set.

2. Material & methods

2.1. Sample collection

Eight wastewater treatment plants (WWTP) were sampled twice a week in the greater Paris area and 7 in other French regions. Sixteen sewers were weekly sampled in Paris, France (See map in Supplementary Fig. S1). Twenty-four-hours composite samples (according to NF T 90-90-523-2) were taken by automated samplers. Sampling was proportional to the flow rate, it started at 7:00 AM and finished at J + 1, 7:00 AM. A minimum of 144 sub-samples per day were taken during dry weather periods. Samples were taken by suction and collected in a refrigerated polyethylene tank at 5 °C (+ or −3 °C). The final collected volume was between 8.7 and 14 L. Then samples were carefully homogenized, distributed in a 100 mL polyethylene bottle, transported to the laboratories at 4 °C and processed in <24 h after sampling. A total of 625 samples from the greater Paris area were analyzed and 126 from other French regions.

2.2. Concentration methods

All samples from the Paris greater area, including WWTP and sewer samples, were processed as previously described (Wurtzer et al., 2020). Briefly, samples were homogenized, then 11 ml were centrifuged at 200,000 × g for 1 h at 4 °C using a XPN80 Coulter Beckman ultracentrifuge using a swing rotor (SW41Ti). Pellets were resuspended in 200 μL of Dulbecco’s Phosphate-buffered saline (DPBS) 1 × (reference 14190144, ThermoFisher Scientific) and pretreated for dissociating viruses and organic matter that was then removed from supernatant for improving RNA extraction efficiency, according to the manufacturer’s recommendations. Supernatant was then lysed, and total nucleic acids were purified using PowerFecal Pro kit (QIAGEN) on a QIAasymplym automated extractor (QIAGEN) and eluted in 50 μL of elution buffer according to manufacturer’s protocol.

Samples from WWTPs located outside the greater Paris area were processed by other laboratories according to an alternative protocol (Bertrand et al., 2021). Briefly, 5 mL of raw wastewater were used for nucleic acid extraction using 10 mL of NucliSENS® lysis buffer. A purification step using phenol/chloroform/isoamyl alcohol 25:24:1 could be performed depending on the laboratories. The nucleic acid extraction was continued using phenol/chloroform/isooamyl alcohol 25:24:1 could be performed depend- on the laboratories. The nucleic acid extraction was continued using phenol/chloroform/isooamyl alcohol 25:24:1 could be performed depend-

2.3. Molecular quantification by digital RT-PCR

A panel of oligonucleotides was designed using AlleleID software versions 7 (Premier Biosoft) in order to detect and quantify mutations reported in Table 1. Briefly, total SARS-CoV-2 genomes were quantified based on gene E amplification (Corman et al., 2020). Alpha and Omicron BA.1 VOC have been reported for sharing a deletion of amino acids H69 and V70 in the spike protein (Sdel69-70) (Wurtzer et al., 2022), but Omicron BA.1 VOC can be distinguished from Alpha VOC by an additional synonymous substitution (C/T) at the position 21762 (codon 67 of the spike protein). Delta VOC has been reported to carry the S:452R mutation in the spike protein. Bioinformatic analyses revealed that Omicron BA.2 variant carried the ORF6:D61L mutation in the ORF6. In addition, PMMoV genome was also detected using previously described oligonucleotides and reported in Table 1 (Haramoto et al., 2013).

Briefly, total SARS-CoV-2 genomes and specific mutations were quantified by digital RT-PCR in multiplex reaction. All samples from the greater Paris area were processed using a Qiacuity instrument (QIAGEN). Multiplex reactions were performed using QIAcuitly One-Step Viral RT-PCR mastermix (QIAGEN) with 800 nM of each primer and 250 nM of each probe in 26 k Nanoplates. In order to improve the detection specificity of single nucleotide polymorphism, the thermal profile included a touchdown step. Briefly, reverse transcription was done at 50 °C for 10 min, followed by activation step at 95 °C for 2 min. Then touchdown was composed by 5 cycles including a 5-s denaturation step at 95 °C followed by a hybridization/elongation step from 63 °C with a decrease of 1 °C/cycle for 40 s. Amplification was done by 45 cycles of incubation at 95 °C for 5 s and 58 °C for 40 s. One fluorescent partition has been considered as positive result. The use of these nanoplates provided a minimum of 24 k valid partitions for each sample and an estimated detection limit of 1800 copies/L (considering the volume of nucleic acids used per reaction and the concentration factor of the sample). The amplification of PMMoV and bovin coronavirus have been done using the same cycling conditions and oligonucleotides reported in the Table 1.

Samples from WWTPs located outside the greater Paris region were processed for quantifying Sde69-70 and ORF6:D61L mutations among total genomes using QX200 instrument (Bio-Rad). The ddrt-PCR assays were performed in a 20 μL-reaction mixture containing 5 μL of extracted nucleic acids and 15 μL of One-Step RT-ddPCR™ Kit for Probes (Bio-Rad). The reaction mix contained 909 nM of each primer and 227 nM of probe. The samples were placed in the droplet generator using 70 μL of generator oil. The resulting picolitre droplet emissions (40 μL) were transferred to a T100 Thermal Cycler or CFX96 (Bio-Rad). The reverse transcription was performed with 60 min hold at 50 °C. The cDNA amplification was performed with 10 min hold at 95 °C, 40 cycles of 95 °C for 30 s then 58 °C for 60 s with a ramp rate of 2 °C per sec, followed by 10 min hold at 98 °C and 30 min hold at 4 °C, and finally the maintain of the samples at 12 °C until data analysis. After amplification, the plate was transferred to QX200 Droplet Reader (Bio-Rad) using QX Manager Edition™ Software (Bio-Rad) to measure the number of positive and negative droplets based on fluorescence amplitude. The detection limit was estimated to be 2000 copies/L.

Negative and variant positive control were included in each experiment to ensure no contamination and to set up the thresholds for considering one partition as positive. In order to prevent misassignment of polymorphism, detection specificity of each variant was assessed in each reaction using positive RNA controls kindly provided by Pr. Charlotte Charpentier (University Hospital Center Bichat-C. Bernard, Paris, France) and Pr. Evelyne Schvoerer (CHRU Nancy, France).

2.4. Sequencing of wastewater samples

Sequencing was performed based on targeted whole genome library preparation of SARS-CoV-2 using the QIAseq DIRECT SARS-CoV-2 kit (QIAGEN). Five microliters of extracted RNA from each sample were used to make this library. Amplicons were quantified using an ultra-sensitive
RT-PCR on wastewater samples as early as the mid-November 2021. The heat maps presented in Fig. 1 highlighted a decrease of the Omicron BA.2 variant, was implemented on the samples as early as December 25th, 2021. The spread dynamics of S:del69-70 in wastewater samples, started at the end of November 2021 and became a very small minority (<10 % of sequences) by mid-January 2022 (Fig. 2, panel C). How-ever, this S:L452R mutation was still detectable in wastewater by March 27th, 2021. The increase in the proportion of the S:L452R mutation was strikingly associated with the regional dynamics of vaccination (first dose) (Fig. 2, panel A) (Santé Publique France, 2022b). Nevertheless, the concentration of SARS-CoV-2 genomes in raw wastewater associated with this Delta VOC epidemic wave was greatly reduced compared to the Alpha VOC wave (Fig. 2, panel B). These mutation dynamics were compared to local open data from the national sequencing program (EMERGEN) as well (Fig. 2, panel A) (Santé Publique France, 2022b). This program was only implemented in France in mid-February 2021 explaining the shape of the curve. The Delta VOC was reported for the first time through sequencing from patient samples on May 10, 2021. The second period began in November 2021, during which time Delta VOC represented 100 % of identified viral sequences isolated from patients samples (Santé Publique France, 2022b) and the S:L452R mutation was present in about 100 % of circulating sequences in wastewater (Fig. 2, panel C). The decrease in the average S:L452R mutation frequency in wastewater, concomitant with the decrease of the Delta VOC in clinical samples, started at the end of November 2021 and became a very small minority (<10 % of sequences) by mid-January 2022 (Fig. 2, panel C). However, this S:L452R mutation was still detectable in wastewater by March 15th, 2022 suggesting the circulation of variants carrying this mutation (Delta VOC or other non-VOC variants). The S:del69-70 mutation was once more detected in a wastewater sample from November 15th, 2021 in the greater Paris area. Generalization and increase of this mutation were initiated at the end of November 2021 to become the majority on December 25th, 2021. The spread dynamics of S:del69-70 in wastewater

### Table 1

| Oligonucleotides | Sequence | Gene | VOC | Reference |
|------------------|----------|------|-----|-----------|
| Beta (Wurtzer et al., 2022) | ACAGTGACGTTAATAGTAATTAGGCT | $E$ | SARS-CoV-2 | (Gorman et al., 2020) |
| Alpha (Santé Publique France, 2022b) | ATATGACGGCAGTCGACACCA | Spike | Alpha | (Wurtzer et al., 2022) |
| S:del69-70 polymorphisms | TATCGTTGCATCGTATCIT | Spike | Omicron BA.1 | (This study) |
| PMMoV | GGTGTCGGTTGCAATGCAAGT | Spike | Delta | (This study) |
| PMMoV-Probe | PMMoV-Probe | Spike | Del69-70 | (Wurtzer et al., 2022) |

flourescent nucleic acid stain for quantitating double-stranded DNA (Quant-iT™ PicoGreen® dsDNA reagent, Thermo). Samples normalized at 4.0 nM were pooled into a single library and diluted to 10 pM concentration according to the manufacturer recommendations. Sequencing was performed with the MiSeq V2 chemistry with 2 × 150 cycles according to the manufacturer’s protocol. Data analysis was performed using the Galaxy tools. Briefly, no quality filtering was applied, all reads were considered. Reads were mapped with BWA-MEM (version 0.7.17.1) against reference genome (NC_45512). Duplicates were cleaned from the BAM dataset using Mark Duplicates (version 2.18.2.2). Variant calling was performed using Lofreq (version 2.1.5) and annotation of SARS-CoV-2 variants using SnpEff eff version 4.5 covid19.

### 2.5. Mathematical and statistical methods

Viral dynamics (using either gene concentrations or signature mutation frequency) were modeled based on daily average concentration (when multiple locations were sampled on the same day) using a LOWESS smoothing method on 10 adjacent points (GraphPad Prism v9.0), allowing to limit outlier effects. Correlations between different datasets were estimated using Spearman test (GraphPad Prism v9.0). First derivative of dynamics curves was calculated using dedicated function of GraphPad Prism software. Normalized data were calculated by dividing raw mutation concentrations by PMMoV concentrations or by daily drinking water consumption.

### 3. Results

#### 3.1. Dating of the emergence of Omicron variants in France

The S:del69-70 mutation of the Omicron BA.1 VOC spike protein and the S:1452R mutation carried by the Delta VOC were quantified by digital RT-PCR on wastewater samples as early as the first week of November 2021. Quantification of the ORF6:D61L mutation in the ORF6, suggestive of the Omicron BA.2 variant, was implemented on the samples as early as mid-November 2021. The heat maps presented in Fig. 1 highlighted a detection of S:del69-70 mutation in samples from the greater Paris area as early as January 3rd, 2022, (later in other French regions) heralding the emergence and rapid spread of the Omicron BA.2 variant. The density of samples taken in the greater Paris area, both in wastewater treatment plants and sewage collectors of the city of Paris, allowed to observe at first punctual detections of these mutations, then the generalization of the positivity of the sampling sites was a precursor of the large spread of the BA.1 and BA.2 lineages of the Omicron VOC in the population and to the acceleration of their propagation.

#### 3.2. Dynamics of mutation in wastewater and of variant based on patient sequencing

Two periods were considered, and 625 samples were processed. The first period concerned the first half of 2021 during which the S:del69-70 and S:1452R mutations, carried by Alpha VOC and Delta VOC respectively, were quantified. The proportions of each mutation were estimated in comparison to the total SARS-CoV-2 genome concentrations based on gene E quantification. This period was marked by a strong predominance of S:del69-70 mutation suggesting Alpha VOC before decreasing after April 2021. It should be noted that the S:1452R mutation was detected as early as January 2021 and could be found in nearly 15 % of the circulating genomes in the wastewater. Its increasing frequency from mid-April 2021 was accompanied by a decrease in the proportion of S:del69-70. In wastewater, the S:1452R mutation became the main mutation detected by May 27th, 2021. The increase in the proportion of the S:1452R mutation was strikingly associated with the regional dynamics of vaccination (first dose) (Fig. 2, panel A) (Santé Publique France, 2022b). Nevertheless, the concentration of SARS-CoV-2 genomes in raw wastewater associated with this Delta VOC epidemic wave was greatly reduced compared to the Alpha VOC wave (Fig. 2, panel B). These mutation dynamics were compared to local open data from the national sequencing program (EMERGEN) as well (Fig. 2, panel A) (Santé Publique France, 2022b). This program was only implemented in France in mid-February 2021 explaining the shape of the curve. The Delta VOC was reported for the first time through sequencing from patient samples on May 10, 2021. The Delta variant became predominant in clinical samples on June 21th, 2021 (Santé Publique France, 2022b).

The second period began in November 2021, during which time Delta VOC represented 100 % of identified viral sequences isolated from patients samples (Santé Publique France, 2022b) and the S:1452R mutation was present in about 100 % of circulating sequences in wastewater (Fig. 2, panel C). The decrease in the average S:1452R mutation frequency in wastewater, concomitant with the decrease of the Delta VOC in clinical samples, started at the end of November 2021 and became a very small minority (<10 % of sequences) by mid-January 2022 (Fig. 2, panel C). However, this S:1452R mutation was still detectable in wastewater by March 15th, 2022 suggesting the circulation of variants carrying this mutation (Delta VOC or other non-VOC variants). The S:del69-70 mutation was once more detected in a wastewater sample from November 15th, 2021 in the greater Paris area. Generalization and increase of this mutation were initiated at the end of November 2021 to become the majority on December 25th, 2021. The spread dynamics of S:del69-70 in wastewater
Fig. 1. Detection of VOC-assigned mutation in the greater Paris area and other French regions from mid-November 2021 to mid-March 2022. Heat-maps showed the concentration of total SARS-CoV-2 genomes and those carrying specific mutations (S:L452R mutation for Delta VOC, S:del69-70 for Omicron BA.1 VOC, and ORF6:D61L for Omicron BA.2 VOC). White box meant not analyzed, grey box meant not detected.
Fig. 2. Dynamics of specific mutations in wastewater in the greater Paris area from January 2021 to March 2022. Each analyzed sample was represented by a dot, the trend curve was obtained using a LOWESS smoothing. Panel A: Temporal variation in the frequency of S:del69-70 (red curve and red dots) and S:L452R (blue curve and blue dots) mutations in wastewater from January 2021 to July 2021. The red area indicated the variation of Alpha VOC by sequencing of clinical samples, the blue area for the variation in Delta VOC. The dotted curve indicated the variation in vaccination (percentage of people receiving the first dose). Panel B: Temporal variation in the concentration of S:del69-70 (red curve and red dots) and S:L452R (blue curve and blue dots) mutations, and of the total SARS-CoV-2 genomes (grey curve and grey dots) in wastewater from January 2021 to July 2021. The dotted curve indicated the variation in vaccination (percentage of people receiving the first dose). Panel C: Temporal variation in the frequency of S:L452R (blue curve and blue dots), S:del69-70 (orange curve and orange dots) and ORF6:D61L (green curve and green dots) mutations in wastewater from November 2021 to March 2022. The blue area indicated the variation in Delta VOC by sequencing of clinical samples, the orange area for the variation in Omicron VOC (distinction of BA.1 and BA.2 lineages was not available). The dotted curve indicated the variation in vaccination (percentage of people receiving the first dose). Panel D: Temporal variation in the concentration of S:L452R (blue curve and blue dots), S:del69-70 (orange curve and orange dots) and ORF6: D61L (green curve and green dots) mutations, and of the total SARS-CoV-2 genomes (grey curve) in wastewater from November 2021 to March 2022. The dotted curve indicated the variation in the regional incidence.
was very similar to that of the Omicron VOC estimated in patient by sequencing (Santé Publique France, 2022b). In contrast, the S:del69-70 mutation began to decline by mid-January 2022 in parallel with the increase of the ORF6:D61L mutation, detected in samples as early as January 3rd, 2022. Genomes carrying the ORF6:D61L mutation became predominant by mid-February 2022 to present almost all circulating genomes by March 15th, 2022 in the greater Paris wastewater. Sequencing results from patients did not report a distinction between Omicron BA.0.1 and BA.2 sublineages to date (Fig. 2, panel C) (Santé Publique France, 2022b). The association of the S:del69-70 mutation with the Omicron BA.1 VOC was based on a polymorphism in the forward primer and the absence of residual detection of this specific mutation in the summer of 2021 (data not shown). During this second period, the concentration of SARS-CoV-2 genomes was also compared to the regional incidence. The dynamics of the viral genome increase in wastewater that preceded the dynamics of incidence was of about 13 days (Fig. 2, panel D). The concentration of viral genomes in wastewater resulting from the Omicron BA.1 VOC wave was much higher than those related to Delta VOC. To confirm the relevance of the digital RT-PCR method, the same samples were analyzed by RT-qPCR based on gene E quantification using the previously described method (Wurtzer et al., 2022). Both quantification methods showed comparable results on nearly 400 comparative analyses (Pearson correlation test: \( r = 0.809; \ p < 0.0001 \) (supplementary Fig. S2).

### 3.3. Temporal dynamics of viral concentration: interest of raw concentration normalization

Viral concentration in wastewater can be influenced both by changes in the assisted population (e.g., mobility, holidays) and by dilution (stormwater, industrial wastewater, water infiltration, sewer cleaning). To assess the impact of human population movements on the temporal dynamics of the different VOC mutations, comparison of raw mutation concentrations and normalized concentrations was performed using two indicators. The first one was related to a human biomarker, the Pepper Mild Mottle Virus genome (PMMoV), in wastewater. The second one was related to the consumption of drinking water by the local population living in Paris, France. PMMoV
genome concentration and drinking water consumption were plotted in Fig. 3, panels A and B. Mean PMMoV genome concentration ranged between $10^6$ and $10^8$ copies/L with a median value of about $10^7$ copies/L, suggesting large fluctuations. On the contrary, the volume of drinking water consumed was more stable. An evolution of both parameters showed that a decrease in drinking water consumption was observed during holidays (shaded period

![Graph A](image)

![Graph B](image)

![Graph C](image)

Fig. 4. Temporal variation in the concentration of S:L452R (blue curve), S:del69-70 (orange curve) and ORF6:D61L (green curve) mutation concentrations, and the total SARS-CoV-2 genome concentration (grey curve) in wastewater in the greater Paris area from November 2021 to March 2022. The dotted curve indicated the variation in the regional incidence. Panel A: trends based on raw concentrations. Panel B: trends based on concentrations normalized using drinking water consumption. Panel C: trends based on concentrations normalized using PMMoV concentrations measured in wastewater.
on the graph) suggesting a decrease in the population connected to the watershed. Such variations were not observed with the PMMoV genome concentration.

Both datasets underwent the same LOWESS smoothing setup over the same period. The trend dynamics of each mutation were very comparable, but some disagreements can be observed (Fig. 4, panels A, B, C). Since the values were relatively stable (except during holidays), normalization by drinking water consumption only significantly altered the dynamics, mostly over the period of population movement (Fig. 4, panels A and B). Normalization with PMMoV noticeably altered viral dynamics in wastewater. The temporal advance of viral dynamics in wastewater, compared to incidence, was also reduced. This approach highlighted an epidemic rebound during February 2022 before an increase in March 2022 related to Omicron BA.2 (Fig. 4, panels A and C).

The viral dynamics in raw wastewater having the best correlation with incidence data was determined by a Spearman correlation test. Taking into account the anticipated variation in raw viral concentrations or concentrations normalized using the drinking water consumption, these two modeling showed a very good correlation with the incidence curve ($r = 0.956; p < 0.0001$ and $r = 0.968; p < 0.0001$) respectively, while the correlation of the normalized curve using the PMMoV genome concentration seemed to be less relevant ($r = 0.761; p < 0.0001$).

Normalization of wastewater data neither changed the mutation frequencies nor their dynamics but slightly modified the reported concentration (Fig. 4, panels A, B and C).

3.4. Dynamics of variant proportions in wastewater: from Alpha to Omicron BA.2

The temporal variation in each mutation suggestive of VOC was studied over the 2 time periods. As PMMoV concentrations were not available for the first period and normalization by drinking water consumption did not significantly modify the modeling of viral dynamics, first derivatives of the raw mutation concentration trend curves were calculated to determine variant replacement rates based on the mutations studied (Table 2). These values corresponded to the kinetics of replacement of one variant by another. Thus, the proportion of the S:del69-70 mutation of Alpha VOC increased by a maximum of 2.8 % per day, compared with 5.2 % for the S:L452R mutation of Delta VOC. The S:del69-70 mutation of Omicron BA.1 VOC increased by 6.0 % per day in wastewater, while the ORF6: D61L mutation replaced it more rapidly (maximum 17.2 %/day) as soon as the wave started (March 2022). The rate of decay of the S:del69-70 mutation of Omicron BA.0.1 VOC was similar to those of S:L452R mutation.

The sum of the concentrations of the mutations of interest carried by the different genomes was compared with the total concentration of circulating genomes over the two periods studied. During the first period, the correlation between both datasets was estimated using a Spearman test ($r = 0.789; p < 0.0001$) and a shift with the SARS-CoV-2 genome curve was observed suggesting the circulation of other variants than Alpha and Delta VOC circulating in the wastewater (Fig. 5, panel A). In the second period, the sum of variants carrying the S:L452R, S:del69-70 and ORF6:D61L mutations appeared to be more consistent with the total concentration of circulating genomes, independently of the viral concentration in wastewater ($r = 0.966; p < 0.0001$) suggesting less « other variants » circulating in wastewater than these three VOCs (Fig. 5, panel B).

Table 2

Maximal replacement rate (RR) of variant in wastewater. RR + indicated the replacement rate during the increase of mutation frequency, whereas RR − provided information on the decay rate of the frequency. Values were indicated in increase or decrease of frequencies per day.

| VOC            | Alpha | Delta | Omicron BA.1 | Omicron BA.2 |
|----------------|-------|-------|---------------|--------------|
| Mutation       | S:del69-70 | S:L452R | S:del69-70 | ORF6:D61L |
| RR +           | 0.028 | 0.052 | 0.060         | 0.172        |
| RR −           | −0.036 | −0.076 | −0.072       | NA           |
were estimated by sequencing on 23 wastewater samples collected in the greater Paris area between December 15th, 2021 and February 28th, 2022. These mutation frequencies were compared to those estimated on the same samples by digital RT-PCR (Fig. 6, panel A). The SARS-CoV-2 concentrations ranged from $3.3 \times 10^4$ copies/L to $1.1 \times 10^6$ copies/L corresponding to an input for library preparation ranging from 36 to 1206 copies. A Spearman’s correlation showed a significant moderate positive correlation of mutation frequencies ($r = 0.38$, $p = 0.002$). The Sdel60-70 mutation was not found by sequencing in eight of 23 samples despite including samples where Omicron BA.1 VOC was the predominant variant and SARS-CoV-2 concentrations the highest, thus suggesting a lower sensitivity of this sequencing-based mutation monitoring. Results also showed important disparities in viral population dynamics over this period resulting from these datasets (Fig. 6, panels B, C and D). The fluctuations observed in the mutation frequencies by sequencing have made curve smoothing difficult and the dynamics of change in most prevalent populations were not evident to discern. Mutation frequencies for each sample and sequencing depth for each mutation position are shown in supplementary Table S3 and Fig. S4.

3.5. Comparison of mutation frequencies by digital RT-PCR and sequencing

The frequencies of Sdel69-70, S:L452R, and ORF6:D61L mutations were estimated by sequencing on 23 wastewater samples collected in the greater Paris area between December 15th, 2021 and February 28th, 2022. These mutation frequencies were compared to those estimated on the same samples by digital RT-PCR (Fig. 6, panel A). The SARS-CoV-2 concentrations ranged from $3.3 \times 10^4$ copies/L to $1.1 \times 10^6$ copies/L corresponding to an input for library preparation ranging from 36 to 1206 copies. A Spearman’s correlation showed a significant moderate positive correlation of mutation frequencies ($r = 0.38$, $p = 0.002$). The Sdel60-70 mutation was not found by sequencing in eight of 23 samples despite including samples where Omicron BA.1 VOC was the predominant variant and SARS-CoV-2 concentrations the highest, thus suggesting a lower sensitivity of this sequencing-based mutation monitoring. Results also showed important disparities in viral population dynamics over this period resulting from these datasets (Fig. 6, panels B, C and D). The fluctuations observed in the mutation frequencies by sequencing have made curve smoothing difficult and the dynamics of change in most prevalent populations were not evident to discern. Mutation frequencies for each sample and sequencing depth for each mutation position are shown in supplementary Table S3 and Fig. S4.

4. Discussion

During the course of this pandemic, new variants have continuously emerged. Some of them, at the origin of the wave of contaminations worldwide, have been identified based on certain signature mutations. The S:del69-70 mutation was shared by Alpha and Omicron BA.1 VOCs. Since Alpha VOC has been replaced by Delta VOC, the S:del69-70 mutation was no longer detected during the summer of 2021 in wastewater in the greater Paris region in accordance with the WHO that no longer identifies Alpha VOC as a circulating VOC (World Health Organization, 2022). The Omicron BA.1 VOC additionally carries the synonymous mutation C21762T, the presence of which allows discrimination between the S:del69-70 mutation carried by the Alpha VOC and that carried by the Omicron BA.1 VOC. The detection of the S:del69-70 mutation detected in the winter of 2021 was therefore representative of Omicron BA.1 VOC. To our knowledge, this study is the first to date the emergence of the Omicron VOC BA.1 and BA.2 subvariants in raw wastewater in France. Omicron subvariants were detected earlier in the greater Paris region compared to other French regions. The Paris region is a hub for entry into the national territory and for the movement of people between the different French regions. Monitoring of specific mutations in wastewater has led to very early identification of the Omicron BA.1 VOC (Ferré et al., 2022) as well as the BA.2 sublineage from January 2022.

Retrospective studies have also demonstrated the presence of minor viral populations carrying the S:L452R mutation, suggestive of Delta VOC, as early as January 2021, in parallel with the strong expansion of Alpha VOC. The high transmissibility of Alpha VOC was at the origin of the third wave of COVID-19 encountered in France (Wurtzer et al., 2022). The introduction of vaccination coupled with acquired collective immunity to this VOC has probably allowed the expansion of an already circulating and better adapted variant such as Delta VOC whose S:L452R mutation allows a better escape to the immune response (Motozono et al., 2021). The high transmissibility of Alpha VOC was at the origin of the third wave of COVID-19 encountered in France (Wurtzer et al., 2022). The Omicron BA.1 VOC infection without preventing viral circulation in the populations. The dynamics of mutations assigned to a VOC in wastewater is therefore representative of Omicron BA.1 VOC. To our knowledge, this study is the first to date the emergence of the Omicron VOC BA.1 and BA.2 subvariants in raw wastewater in France. Omicron subvariants were detected earlier in the greater Paris region compared to other French regions. The Paris region is a hub for entry into the national territory and for the movement of people between the different French regions. Monitoring of specific mutations in wastewater has led to very early identification of the Omicron BA.1 VOC (Ferré et al., 2022) as well as the BA.2 sublineage from January 2022.

Retrospective studies have also demonstrated the presence of minor viral populations carrying the S:L452R mutation, suggestive of Delta VOC, as early as January 2021, in parallel with the strong expansion of Alpha VOC. The high transmissibility of Alpha VOC was at the origin of the third wave of COVID-19 encountered in France (Wurtzer et al., 2022). The introduction of vaccination coupled with acquired collective immunity to this VOC has probably allowed the expansion of an already circulating and better adapted variant such as Delta VOC whose S:L452R mutation allows a better escape to the immune response (Motozono et al., 2021). The results presented here suggested that vaccination and previous infections, could have contributed to the selection of the Delta VOC (against Alpha VOC) but also strongly reduced viral circulation with respect to the concentrations measured in the wastewater when the Delta VOC was prevalent. The introduction of vaccination coupled with acquired collective immunity to this VOC has probably allowed the expansion of an already circulating and better adapted variant such as Delta VOC whose S:L452R mutation allows a better escape to the immune response (Motozono et al., 2021). The results presented here suggested that vaccination and previous infections, could have contributed to the selection of the Delta VOC (against Alpha VOC) but also strongly reduced viral circulation with respect to the concentrations measured in the wastewater when the Delta VOC was prevalent. The large increase in viral genome concentration in wastewater during the Omicron BA.1 wave, compared with the Delta wave, was also consistent with the reduced efficacy of vaccines to prevent Omicron VOC infection (Cele et al., 2022). Similar results in wastewater were observed in other countries (Cutrupi et al., 2022). These results were consistent with a vaccine-induced reduction in severe forms of COVID-19 resulting from Omicron VOC infection without preventing viral circulation in the populations.

To date, VOCs are effectively monitored by conventional epidemiological indicators and intensive sequencing of samples collected from hospitalized patients. The dynamics of mutations assigned to a VOC in wastewater were consistent with VOC dynamics in patients, particularly with respect to
the early detections, wave dynamics and changes in dominant populations. While variants may emerge simultaneously (e.g. Alpha and Delta VOCs), there were no periods in which different SARS-CoV-2 VOCs were codominant. Over the periods studied, the co-spreading of different variants in a population was finally only very transient and each variant was bound to be replaced by another, more fitted one in the population. This constant variant replacement demonstrated the SARS-CoV-2 evolution spreading within a population that has been largely immunized collectively either by one or more infections or by vaccination. Thus, maximum VOC replacement rates were calculated for variants that have circulated extensively in the early detections, wave dynamics and changes in dominant populations. The SARS-CoV-2 concentrations in wastewater can be affected by method variability (Bivins et al., 2021a, 2021b), by fecal shedding potentially different from a variant to another (Prasek et al., 2022), by heavy rainfalls that dilute raw wastewater and population movement. Mathematical algorithms have been proposed to correct the estimated raw concentrations using the flow of wastewater inlet (Cluzel et al., 2022; Courbariaux et al., 2022). Other normalization factors of viral concentrations in wastewater should be investigated (Mazumder et al., 2022). The greater Paris area is a very touristy region and a business hub. As a very dense populated area, during the school vacations such as Christmas period (from December 20th, 2021 to January 1st, 2022) and winter season (from February 19th to March 7th, 2022), many people leave the region. PMMoV genome, a human biomarker, was measured by digital RT-PCR at the same time as drinking water consumption. The normalized viral dynamics in wastewater showed a temporal advance on the concentration in raw wastewater showed an important variability that did not re-
during the Omicron wave than during previous waves. Similar advance can be observed on SARS-CoV-2 wastewater monitoring data in the U.S (Biobot.io, 2022). This finding could be explained by a higher proportion of asymptomatic or pauci-symptomatic infections that would be detected later. Normalization based on PMMoV genome concentration suggested a viral rebound in February 2022 that was not observed on the incidence curve. This observation was probably the reason for the lower correlation. If other human biomarkers could be evaluated such as CRAssphages or specific F-RNA phages, a previous study also concluded that normalization of SARS-CoV-2 signal using fecal indicator did not improve the correlation with the incidence (Ai et al., 2021). Less severe cases of infection, resulting from acquired collective immunity or attenuation of viral pathogenicity, but also less human testing could lead to a misunderstanding of the viral circulation provided by the clinical incidence. Viral monitoring in wastewater will allow an unbiased approach of viral circulation in human population.

This study also demonstrated the interest of monitoring specific mutations evocative of VOC because of the simplicity of setting up such a measurement, its low cost and the result quickness, which are indispensable for informing health authorities. This study also highlighted the limitations of sequencing in wastewater with respect to analytical sensitivity that may result from the low concentration of SARS-CoV-2 genomes, the very low concentration of mutations in minority variant populations, insufficient sequencing depth, and non-homogeneous coverage along the entire viral genome. Similar limitations have been already reported (Caduff et al., 2022). The accumulation of mutations identified by sequencing is also challenged by the reconstruction of the genomes from which these mutations originate. Mathematical algorithms for deconvolution of isolated mutations or long fragment sequencing approaches to identify different polymorphisms in the same reads may improve the predictivity of sequencing. To date, sequencing must be interpreted with caution for quantitative assessments of mutation frequency in wastewater.

5. Conclusion

The quantification of mutations assigned to VOCs circulating in wastewater has allowed a depiction of the VOC propagation dynamics in the greater Paris area population. This approach also allowed early detection of the Omicron VOC BA.1 and BA.2 lineages emergence in this area. The observed dynamics confirmed the impact of vaccination on the selection of the Delta variant, which escapes acquired immunity better than the Alpha variant, but also highlighted the relative effectiveness of vaccination in reducing the intensity of viral circulation of the Delta variant. The observed dynamics confirmed that the vaccination strategy, as well as exposition of previous variants, probably contributed to the selection of Delta VOC to the detriment of Alpha VOC, but also to the strong decrease of the viral circulation in the human population during the same period, confirming the effectiveness of the vaccine response against these VOCs. Moreover, the normalization of concentrations based on the drinking water consumption in Paris seemed to be a relevant method to follow the population movements in this region. This normalized approach provided a viral dynamic in close relationship with the regional incidence curve. Spreading of variants leading to less severe infections could also lead to a detection delay through patient-centered syndromic surveillance tools. Thus, raw wastewater testing will still allow this tracking, as well as the VOC dynamics monitoring during the spread of SARS-CoV-2 in populations.

Sophie Masnada: Methodology (sampling); Sam Azimi: Methodology (sampling); Miguel Guillen-Ritz: Methodology (sampling); Alban Robin: Funding acquisition; Jean-Marie Mouchel: Methodology (transportation); Obepine Sig: Funding acquisition; Laurent Moulin: Conceptualization, Data curation, Visualization, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank all the people who have made it possible to collect all these wastewater samples since the beginning of the pandemic, especially the team of sewage workers of Paris, France, who set up a specific monitoring. Technical teams of WWTP (SIAM, SIAAP and other utilities) should also be thanked. We also thank all the people who participated in the exchange of ideas and who cannot be listed as co-authors of the study. We also warmly thank Mickael Boni for his critical reading of the manuscript.

Funding

Sample collection and nucleic acid extraction were carried on the OBEpine Research grant (French ministry of Research and French ministry of Health). Development of tools for the detection of variants by digital PCR and sequencing was funded both by Eau de Paris and a grant from the Emerenuea project. A part of the analyses was also financially supported by the “Syndicat Intercommunal du Bassin d’Arcachon”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157740.

References

Agrawal, S., Orschler, I., Schubert, S., Zachmann, K., Heinjen, L., Tavazzi, S., Gawkli, B.M., de Graaf, M., Medema, G., Lackner, S., 2022a. Prevalence and circulation patterns of SARS-CoV-2 variants in European sewage mirror clinical data of 54 European cities. Water Res. 214, 118162. https://doi.org/10.1016/j.watres.2022.118162.
Agrawal, S., Orschler, I., Tavazzi, S., Greither, R., Gawkli, B.M., Lackner, S., 2022b. Genome sequencing of wastewater confirms the arrival of the SARS-CoV-2 omicron variant at Frankfurt airport but limited spread in the city of Frankfurt, Germany, in November 2021. Microbiol. Resour. Announc. 11, e01229.21. https://doi.org/10.1128/MRA.01229-21.
Ahmed, W., Simpson, S., Bertsch, P., Bibby, K., Bivins, A., Blackall, L., Boffill-Mas, S., Bosch, A., Brandoz, J., Choi, P., Ciriakiski, M., Donner, E., D’Souza, N., Fernleiten, A., Gerrity, D., Gonzalez, R., Griffith, J., Gyawali, P., Haas, C., Hamilton, K., Hapushanachchi, C., Harwood, V., Haque, R., Jackson, G., Khan, S., Khan, W., Kitajima, M., Krajacic, A., Rosa, G.L., Layton, B., Lipp, E., McEllan, S., McMinn, B., Medema, G., Metcalfe, S., Meijer, W., Mueller, J., Murphy, H., Naughton, C., Noble, R., Payyappatt, S., Petterson, S., Pitkanen, T., Rajal, V., Reineke, B., Roman, F., Rose, J., Ruiniol, M., Sadowsky, M., Salo-Comorera, T., Setoh, Y.X., Sherchan, S., Sirikanancha, K., Smith, W., Steele, J., Sabburg, R., Simmonds, E., Thai, P., Thomas, K., Tynan, Y., Trow, S., Thompson, J., Whiteley, A., Wong, J., Sano, D., Wuetz, S., Xagoraraki, I., Zhang, Q., Zimmer-Faust, A., Shanks, O., 2021. Minimizing Errors in RT-PCR Detection and Quantification of SARS-CoV-2 RNA for Wastewater Surveillance. https://doi.org/10.20944/preprints202104.0811.v1.
Ahmed, W., Bibvis, A., Smith, W.J.M., Metcalfe, S., Stephens, M., Jennison, A.V., Moore, F.A.J., Bourke, J., Schlebusch, S., McMahon, J., Hewitson, G., Nguyen, S., Barelzon, J., Jackson, G., Mueller, J.F., Ehret, H., Hoseoglu, I., Tian, W., Wang, H., Yang, L., Bertsch, P.M., Tynan, J., Thomas, K.V., Bibby, K., Gruber, T.E., Zieles, R., Simpson, S.L., 2022. Detection of the Omicron (B.1.1.529) variant of SARS-CoV-2 in aircraft wastewater. Sci. Total Environ. 820, 153171. https://doi.org/10.1016/j.scitotenv.2022.153171.
Ai, Y., Davis, A., Jones, D., Lemeshow, S., Tu, H., He, F., Ru, P., Pan, X., Bohrerova, Z., Lee, J., 2021. Wastewater SARS-CoV-2 monitoring as a community-level COVID-19 trend tracker and variants in Ohio, United States. Sci. Total Environ. 801, 149757. https://doi.org/10.1016/j.scitotenv.2021.149757.
