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Optimised use of passive samplers enabled early detection of SARS-CoV-2 variants BA.4 and BA.5 in sewage water

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Wastewater-based epidemiology emerged as a valuable method to monitor the COVID-19 epidemic and the dynamic of SARS-CoV-2 variants. Because of its ease of deployment and low cost, membrane-based passive sampling is a prime alternative for deploying a monitoring network in wastewater, especially when automatic samplers cannot be used. However, the performance of these strategies for the identification of low-abundance viruses needs to be evaluated. Passive sampling using nylon membranes and grab sampling were carried out in parallel in the sewers of two French cities in April and May 2022, for the detection of norovirus GII (NoV GII) and SARS-CoV-2. SARS-CoV-2 sequencing was performed to compare the performance of passive samplers and their paired grab sampler in identifying Omicron sub-lineages. Direct lysis and elution methods from nylon membranes were equally effective for virus recovery and SARS-CoV-2 sequencing. For all sites, the virus concentrations in passive and grab samples were very similar. A near-complete genome coverage at a depth of 30 was obtained for most samples, using ARTIC multiplex PCR (V4.1) and Illumina MiSeq. There was a high proportion of low-frequency mutations for both methods and rare mutations in the S gene were detected, which could reflect the presence of cryptic lineages. Even though a large proportion of BA.2 lineage was detected in sewage, most importantly this study provides the first evidence that the use of passive sampling enables early detection of SARS-CoV-2 variants BA.4 and BA.5, that is, before they are identified in the population.

Water impact

This study demonstrates that membrane-based passive sampling is an effective, low-cost method for wastewater surveillance, enabling early detection of emerging SARS-CoV-2 variants. By providing genome-level insights into viral diversity from sewage, it advances water monitoring strategies, enhances public health preparedness, and supports early warning systems when conventional sampling is limited or impractical.

1. Introduction

The detection of SARS-CoV-2 RNA in wastewater led to the unprecedented development of wastewater-based epidemiology (WBE) during the COVID-19 pandemic.[1–5] WBE can improve estimates of infection spread by sampling all community members, including asymptomatic infected individuals. This strategy is particularly important when the population screening rate is low. Because wastewater contains a mixture of mostly partial genomes, particular attention must be paid to the sampling strategy, and to optimizing nucleic acid extraction, detection, sequencing and data analysis, notably

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when viruses are poorly abundant.[6,7] The emergence of Omicron sub-lineages in Spring 2022 France, with rather low incidence rates in some regions, provides a suitable framework for the assessment of these methods.

Previous studies have demonstrated the importance of sequencing SARS-CoV-2 in wastewater and detecting single-nucleotide variants (SNVs) which reflect the diversity of variants circulating at the population level.[8,9] As SARS-CoV-2 may be present in relatively low concentrations in sewage, an enrichment step is required to improve the sensitivity of sequencing, such as oligonucleotide-based capture[9] or multiplex tiling PCR-based targeted amplification.[10,11] Sequencing of SARS-CoV-2 is mainly based on 24 h composite water samples collected from wastewater treatment plant (WWTP) inlets and less often in the sewer network.[12,13]

The sampling procedure has a major impact when performing viral monitoring of wastewater as the virus is shed sporadically into sewage depending on the time of the day. Therefore, the main selection criteria for monitoring are (i) the acquisition of a 24 h composite sample and (ii) its suitability for different types of sites, from WWTPs to building sewers for near-source monitoring.[14] However, due to the cost of automatic samplers, they cannot be deployed on a large scale, and they are not always suitable for monitoring in sewers. Consequently, in resource limited countries, grab sampling is more common (57%) than in rich countries (24%).[15] Grab sampling is a possible alternative, although it does not reflect the viral load variations that occur over time. Various passive samplers have been proposed to detect SARS-CoV-2 including gauze, tampons, and nitrocellulose membranes; they show a sensitivity that is suitable for detection in populations with a low prevalence of COVID-19 infections.[16–19] More recently, other human pathogens have been monitored (*e.g.* norovirus, enterovirus, human adenovirus and influenza A) demonstrating a major interest in WBE, and the need for optimised methods for both enveloped and non-enveloped viruses.[16,17,19–23] As the major cause of acute gastroenteritis, norovirus monitoring is also important for describing temporal dynamics of the disease in the population and shows an inverse relationship to SARS-CoV-2 emergence.[24]

Many questions still need to be answered before using passive sampling as an early warning strategy. First, passive sampling is mainly a qualitative method. Therefore, a better understanding of the virus adsorption kinetics on membranes is required to obtain quantitative estimates of the viral load. Very little research has been carried out on this issue, and the available research has mainly been performed under laboratory exposure conditions.[22,25–27] Data from field studies could provide a basis for a semi-quantitative approach based on a comparison of the concentrations measured by composite *versus* passive sampling.[19,28,29] So far, the comparison of both sampling methods is based solely on a presence/absence analysis.[21,30–32] In addition, it is essential to check that the different sampling techniques reflect comparable degrees of viral diversity, especially when viruses are present at a very low concentration in sewage. For this purpose, evaluating viral diversity by sequencing requires optimisation of sampling procedures, viral nucleic acid extraction techniques and sequence analysis. Cha *et al.* (2023) found that SARS-CoV-2 sequencing from passive and composite samplers at the building scale was consistent with clinical data in identifying the new BA.2 variant.[33] Farkas *et al.* (2024) used tampons to identify emerging variants of concern (VOCs) from university building sewers.[23] More recently, Alamin *et al.* (2024) demonstrated that improved elution of Moore swab was more performant in identifying Omicron variants than grab samplers.[28] Robust identification of emerging variants implies the need for a high genome coverage. However, SARS-CoV-2 whole genome sequencing remains a challenging objective, due to the low genome concentration.

In the present study, we aimed to optimise virus recovery from nylon membranes, for both enveloped and non-enveloped viruses, and to analyse the representativeness of passive and grab sampling. This was done in parallel for SARS-CoV-2 and norovirus genogroup II (NoV GII) using paired passive and grab sampling collected at the neighbourhood scale. Finally, we performed SARS-CoV-2 whole genome sequencing to assess the ability of passive sampling to detect SARS-CoV-2 variants at an early stage.

2. Materials and methods

2.1 Preparation of membranes and passive sampler devices

A nylon membrane (thickness of 100 μm) (Mougel, Nantes, France) was used as a passive sampler as described previously (Vincent-Hubert *et al.*, 2022).[21] A single membrane, 8.5 cm in diameter, was put inside a 3D-printed device designed for SARS-CoV-2 detection by Hayes *et al.*, 2021.[17] The COVID-19 sewer cage (COSCa) is a hollow sphere that is 10 cm in diameter and has 26 holes, each with a diameter of 1.5 cm to foster non-restrictive flow.

2.2 Sampling design and methodology

Two large cities in France (Lyon, with 1 417 000 inhabitants and Nantes, with 672 400 inhabitants) belonging to two different regions were chosen for this study. Sampling was performed in Lyon (12 April 2022 and 14 April 2022) and in Nantes (18 May 2022 and 20 May 2022). For both cities, four neighborhood sampling sites were chosen (sites I–IV). At each site, one COSCa was deployed in the morning for 24 h and grab sampling of sewage was performed when the COSCa was recovered; a second series of sampling was carried out 2 days later, for a total of 16 samples per city. For the grab sampling of sewage, 1 L of water was collected, carefully homogenised, transferred to a 1 L polyethylene bottle and transported to the laboratory at 4 °C. After retrieval, the devices were stored at 4 °C until arrival to the laboratory, where the nylon membrane was rinsed in sterile water to remove large particles. Wastewater and membrane

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samples were analysed immediately for the detection of SARS-CoV-2 and NoV GII.

2.3 Optimisation of virus recovery with elution and sonication

Optimization of virus recovery involved testing the effect of elution with Tween 20, which is usually used to elute viruses from the membrane[17] and sonication, which is known to improve virus recovery in WWTP samples.[34] Laboratory exposures were conducted with raw sewage collected in February 2022 from the Nantes WWTP inlet and scored positive for SARS-CoV-2 and NoV GII. Nylon membranes (8.5 cm diameter each) were immersed in 500 ml of freshly collected raw sewage for 24 h at room temperature (18 °C) with continuous stirring. A separate set of two membranes was subjected to either elution ± sonication or to our reference method, that is, direct lysis. Three independent experiments with two biological replicates in each experiment were conducted.

After exposure for 24 h, the membranes were rinsed in sterile water for 30 s to eliminate particles. Elution was performed with Tween 20 as described in ref. 17. Briefly, membranes were incubated 2 × 5 min with 4 ml of a 0.075% Tween 20 + 25 mM Tris-HCl-based buffer. The eluate (4 ml) was used directly for nucleic acid extraction (see section 4) or subjected to three cycles of sonication for 1 min at room temperature at maximum power in a cup-horn adaptor before extraction (Bandelin, HD 2200, Berlin, Germany), followed by 1 min on ice.[34]

To compare the performance of elution ± sonication *versus* direct lysis, the recovery rate was calculated as follows:

$$\text{Recovery rate}\ (\%) = \frac{\text{gc}\ M}{\text{gc EM}} \times 100$$

where gc M = copies of viral genome measured with qRT-PCR in the membrane nucleic acid extract and gc EM = copies of viral genome added in the exposure media (genome copies in sewage).

2.4 Nucleic acid extraction

All nucleic acids were extracted using a NucliSENS extraction kit (bioMérieux, Lyon France). The sewage samples collected by grab sampling were homogenized, 11 mL was ultracentrifuged as described previously,[3] the pellets were resuspended in 200 μL of PBS 1× and 2 ml of lysis solution was added. Mengovirus (MgV), a murine picornavirus, was used as a process control for nucleic acid extraction from liquid sewage samples, as described previously.[35] The membranes were immersed directly in 8 mL of lysis solution to which 4 mL of 1× PBS were added as described.[21] Eight milliliters of lysis buffer were added to the eluates (from section 2.3). The following steps are as described in the supplier's protocol for both matrices. Nucleic acids were treated with the one step PCR inhibitor removal kit (Zymo Research Kit, USA), and eluted with 100 μL of nuclease-free water (Qiagen, France). Nucleic acids were used immediately for RT-qPCR and then stored at −80 °C for up to 5 months until sequencing.

2.5 Detection of viral genomes by one-step quantitative RT-PCR and quantification

RT-qPCR was performed using the UltraSense one-step quantitative RT-PCR kit (Invitrogen, USA) on an MX3000 (Stratagene, Massy, France). The MgV qRT-PCR assay was carried out as described previously on 5 μL of pure and 10-fold diluted nucleic acids, to assess the presence of PCR inhibitors and to calculate the extraction efficiency.[36] For SARS-CoV-2 and NoV GII detection, all the samples were analysed in triplicate using 5 μL of pure nucleic acid extract. For SARS-CoV-2, one set of primers and probe was used: IP4, targeting the polymerase gene[37] with cycling conditions as described.[38] For NoV GII, qRT-PCR was carried out as described previously.[36] A negative amplification control (water) was included in each amplification series. For quantification, duplicate five-point standard curves were prepared with SARS-CoV-2 RNA transcript (CNR des virus respiratoires, Pasteur Institute), and with a synthetic DNA fragment containing nucleotides 4191–5863 of the GII.4 Houston virus (Genbank EU310927). Concentrations were normalized to the membrane surface or to 10 mL of sewage.

2.6 Amplicon-based sequencing using ARTIC V 4.1

All the samples with a cycle threshold (Ct) between 28 and 36 were used for SARS-CoV-2 genomic sequencing. cDNA was generated with 10 μL of nucleic acids using the SuperScript IV reverse transcriptase at 37 °C for 50 min following the manufacturer's protocol and the specific recommendation for random hexamers (Invitrogen, USA). SARS-CoV-2 genome amplification *via* multiplex PCR was performed using ARTIC version V4.1.[39] The ARTIC SARS-CoV-2 amplicon panel generates 98 amplicons of approximately 400 bp covering the entire genome. 8.5 μL of cDNA were used as a template for the PCR performed with two pools of primers, each in duplicate following the manufacturer's instructions: heat-inactivation at 98 °C for 30 s, and 40 cycles of denaturation (95 °C, 15 s), annealing and extension (63 °C, 5 min) (Integrated DNA Technologies, USA). Amplicons of the same sample were pooled, purified with SPRI select beads (Beckman Coulter, Fullerton, CA, USA) and eluted in 10 μL of nuclease-free water as described.[35] The quality was checked by electrophoresis of 1 μL DNA on a Bioanalyzer using the Agilent DNA 7500 kit (Agilent Technologies, USA). These purified amplicons (10–200 ng per sample) were used for the preparation of libraries using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, France), with an enrichment step of seven to nine PCR cycles depending on the initial concentration. Final libraries were sequenced on

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a 2 × 250 standard cartridge on the MiSeq (MiSeq Reagent Kit v2 500-cycles), loading 8pM with 15% PhiX.

2.7 SARS-CoV-2 sequence analysis

The ASPICov pipeline (version 1.1.7; https://gitlab.com/vtilloy/aspicov), an automated pipeline designed for clinical investigation for the identification of SARS-CoV-2 nucleotide variants, was used to analyse the reads obtained from MiSeq sequencing.[40] This multistep Nextflow pipeline can process raw-read sequences allowing alignment, genome coverage, variant calling, and S gene mutation analysis. The genome coverage percentage was analysed at a depth of 30. Adapters were trimmed, and the reads were aligned to the SARS-CoV-2 reference (NM908947.2). Only mutations with a frequency >1% were considered when analysing S gene mutations.

To analyse the diversity of sub-lineages in these mixed samples, and following the generation of BAM files by ASPICov, VaRaPS (variants ratios from pooled sequencing) was employed for subsequent analysis and compare it with Freya.[41,42] VaRaPS performed variant calling and mutation extraction from individual reads within the BAM files. Mutations were considered when present at a frequency of at least 1% and in at least 3 reads. Subsequently, variant proportions were computed by using a co-occurrence-based method. This approach, adapted from VirPool,[43] leverages the co-occurrence patterns of mutations within individual reads to estimate the relative abundance of different SARS-CoV-2 lineages.

The variant/mutation profile matrix necessary for this deconvolution was constructed using 118 228 high-quality SARS-CoV-2 sequences from Europe, collected between 28 January 2020 and 7 January 2024, and obtained from the global initiative on sharing avian influenza data (GISAID) database. Sequences were selected based on stringent criteria: completeness (>29 000 nucleotides), low ambiguity (<1% undefined bases), and high quality (<0.05% unique amino acid mutations). Nextclade was employed for clade assignment and mutation calling against the Wuhan reference sequence. The resulting matrix comprised 32 lineages and 438 mutations.

2.8 Statistical analysis

Student's t test was performed to compare the recovery methods (elution ± sonication compared with direct lysis). The Spearman correlation test was performed to analyse the relationship between $\log_{10}$ gc per membrane and $\log_{10}$ gc per grab. Analysis of variance (ANOVA) was carried out to determine whether the genome coverage depends on the Ct, and whether the water sampling method has an impact on the Ct and the diversity of sub-lineages. RStudio version 4.2.2 and GraphPad Prism version 10 were used for graphical representations and statistical analysis.

3. Results

3.1 Virus recovery: evaluating the elution and direct lysis methods

Because a fraction of the viral material is adsorbed on suspended particles, the first step of this study was to assess whether an elution step, with or without sonication, could enhance virus recovery as suggested.[44] These procedures were compared with our previously published protocol.[21] As shown in Table 1, the recovery rates for both viruses were significantly lower when using the elution-based methods (about two times less for SARS-CoV-2 and three times less for norovirus) ($p < 0.05$). The recovery rate was about two times lower for SARS-CoV-2 and three times lower for norovirus ($p < 0.05$). The direct lysis method yielded the highest recovery rate for norovirus (17 ± 4%) and for SARS-CoV-2 (3.27 ± 2%) compared to the other two methods. Moreover, the recovery rate of norovirus was significantly higher than for SARS-CoV-2 ($p < 0.05$). The sequencing data showed that the three methods yielded nearly complete genome coverage at a depth of 30 of SARS-CoV-2, with no significant differences between the methods (Table 1). The sequencing depth per amplicon was high (between 3 and 4 $\log_{10}$), with much higher heterogeneity for the second method (Tween 20 elution) compared with the two others (Fig. 1). Based on these assays, we retained the direct lysis procedure for this study.

3.2 SARS-CoV-2 and NoV GII detection in city sewers: comparison of passive and grab sampling

We quantified SARS-CoV-2 and NoV GII from paired samples (passive and grab samples) collected in the sewers of Lyon and Nantes (Table S1), and after confirming that extraction efficiency and inhibition were within the criteria established in previous reports, we observed mean RT-qPCR inhibition levels of 35% and 15% for Lyon and Nantes samples,

Table 1 Optimization of virus recovery and SARS-CoV-2 sequencing metrics

	NoV GII	SARS-CoV-2				
Protocol	Recovery rate (%)	Recovery rate (%)	Total reads (10^6)	Mapped reads (%)	Median depth ($\log_{10}$)	Genome coverage_30 (%)
Direct lysis	17 ± 4	3.27 ± 2.8	0.3	90 ± 11	3.4 ± 3	89 ± 0.2
Elution	6 ± 4	1.9 ± 0.7	0.35	71 ± 30	3.2 ± 2.7	89 ± 4.5
Elution + sonication	6.3 ± 4.5	1.4 ± 0.8	0.33	79 ± 17	3.3 ± 2.6	91 ± 2.4

The data are presented as the mean ± standard deviation (n = 3) for the recovery rate (n = 6) and metrics values (n = 3). Two viral elution protocols were tested: direct lysis (NucliSens) and viral elution with Tween 20 ± sonication followed by NucliSens. The libraries were prepared as described in the Materials and methods section and then, sequenced on Illumina MiSeq™. The data were analysed with the ASPICov pipeline.

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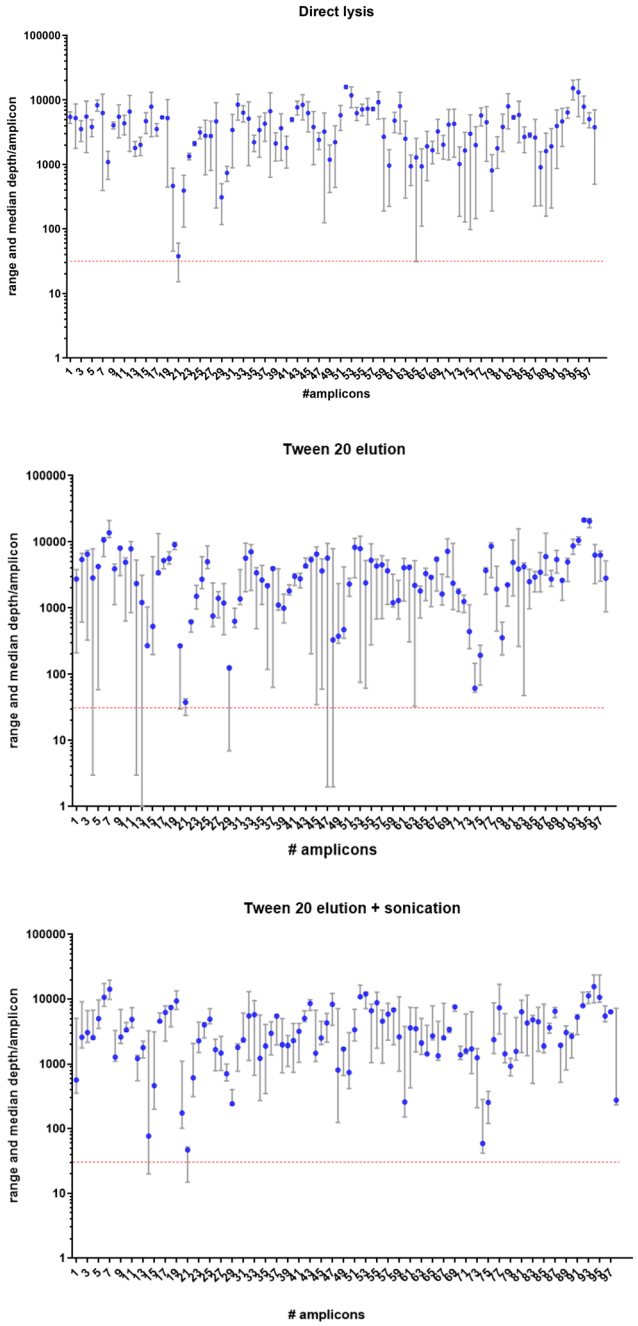

Fig. 1 Comparison of the depth per amplicon according to the virus recovery protocol. Coverage analysis of the SARS-CoV-2 genome using the ARTIC sequencing protocol. The range (grey floating bars) and median depth (blue dot) for each of the 98 amplicons of the ARTIC multiplex PCR are shown. Three virus recovery methods were compared: direct lysis, Tween 20 elution and Tween 20 elution + sonication.

respectively, while the mean extraction efficiency was 1.9% for Lyon and 14% for Nantes.[36,45,46] Overall, we detected higher SARS-CoV-2 concentrations on membranes compared with grab samples ($p < 0.001$) in Lyon, whereas there was no significant difference between the two sampling series.

We compared the quantities of virus collected on the membranes with the wastewater concentrations measured in grab samples (Fig. 2). Two trends emerged: (i) for SARS-CoV-2 in Lyon only, there was a positive correlation between the quantities accumulated on the membranes and in the corresponding grab samples (Spearman rank correlation analysis, $p < 0.05$), and (ii) for NoV GII in both cities and SARS-CoV-2 in Nantes, the membrane concentrations were similar to their paired grab samples or lower, suggesting that the membrane did not accumulate viruses over time in a concentration-dependent manner.

3.3 SARS-CoV-2 sequencing using grab and passive sampling

Despite the low concentration of SARS-CoV-2 in our samples, the sequencing success rate was high (15/16 for each method) (Table S1). Due to ineffective cDNA synthesis, one sample could not be sequenced. As expected, the coverage at a depth of 30 was related to Ct values (ANOVA, $p < 0.05$), while the sampling method had no significant impact (Fig. 3). For the samples with lower viral loads, ($34 < Ct < 39$), the genome coverage at a depth of 30 varied from 75% to 95%, except for one grab sample. As the SARS-CoV-2 genome coverage was not significantly different at depth 10 and 30, we chose a depth of 30× to increase our confidence in identifying cryptic mutations and low-frequency variants (data not shown). The total number of reads was the same for both sampling methods (with a median at 0.3×10^6 reads). The percentage of reads mapped on the SARS-CoV-2 reference sequence was not statistically different between the membrane and the grab samples (with a median at 84% and 90% respectively), as there was relatively high heterogeneity (52.8% to 96% for grab samples and 56% to 94% for the membrane samples) (Table S1). The median sequencing depth was high and did not differ according to the sampling method (1.9×10^3 and 2.2×10^3 reads per base, respectively for the membrane and grab samples). The genome coverage at a depth of 30 did not vary significantly between membrane and grab samples, with median of 92% and 90%, respectively. There was near-complete genome coverage at a depth of 30 (>90% coverage) for 73% of the membrane samples and 47% of the grab samples. For the majority of the grab samples (93%), the coverage was >83%.

The multiplex ARTIC PCR generates 98 overlapping amplicons allowing for complete amplification of the SARS-CoV-2 genome, but the efficiency may vary depending on the amplicon.[47] We analysed the sequencing depth per amplicon to identify amplicons that are poorly sequenced, that is, below a threshold depth of 30 (Fig. 4). We removed one grab sample (Lyon, site IV, membrane) from the dataset as its amplification efficiency was very poor for many amplicons. Overall, the median depth/amplicon was high and varied throughout the genome for both sampling methods (3–4 log). Of note, there was a lower depth of sequencing in some regions of the genome for both methods and notably for amplicon 21 (depth <30 in the majority of our samples), suggesting a lower efficiency of cDNA synthesis, possibly due to fragmented RNA, rather than a lower efficiency of the ARTIC PCR (Fig. 4).

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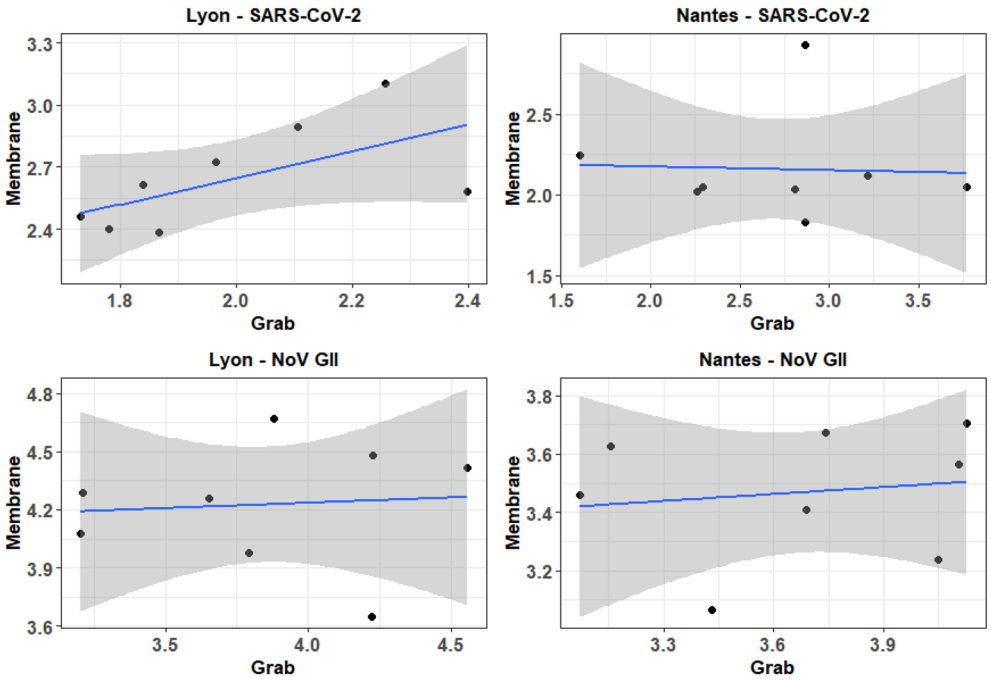

Fig. 2 Comparison of the log 10 (gc per membrane) and log 10 (gc per grab) values for paired samples. For each site, both passive sampling and grab sampling were conducted, and SARS-CoV-2 was detected in each sample type. The resulting paired data are presented in this figure to evaluate the correlation between the two methods. The number of genome copies (log gc per grab or membrane) is indicated. Two trends were observed: (i) saturation of membranes in Lyon (NoVG II), Nantes (NoVG II) and Nantes (SARS-CoV-2) with no correlation for these three, and (ii) accumulation of SARS-CoV-2 on the membrane (Lyon), (Spearman correlation analysis, rho = 0.61, $p < 0.05$).

3.4 Passive samplers led to the early detection of SARS-CoV-2 variants

We wondered whether grab or passive sampling could capture enough viral diversity to identify low-frequency SARS-CoV-2 variants. We analysed allelic mutations of the SARS-CoV-2 genome with the following criteria to exclude coincidental factors such as sequencing errors: nucleotide variations occurring with a frequency of >1% at depth of >30 were defined as a mutation based on a global analysis of Omicron SNPs.[48] The mean number of mutations in genes that are frequently mutated (corresponding to N, S and ORF1ab genes for Omicron BA.2) and the total mutation number are presented in Fig. 5. In total, we found 268 and 312 mutations, respectively, in the membrane and grab samples. Half of them were low-frequency mutations (<10%), and 71 and 61 mutations, respectively, for membrane and grab samples, were high-frequency mutations (>50%). The mutations occurred preferentially in the S gene and the ORF1ab regions, which are known as mutation hot spots of SARS-CoV-2 (ref. 49 and 50) (Fig. S1 and 5). In the S gene, there were more low-frequency mutations in the grab samples (mean: 31 ± 26) compared with the membrane samples (mean: 14 ± 15), but the difference was not significant (Fig. 5). Overall, the total number of low-frequency mutations (<10%) was not impacted by the sampling method (ANOVA, $p < 0.05$).

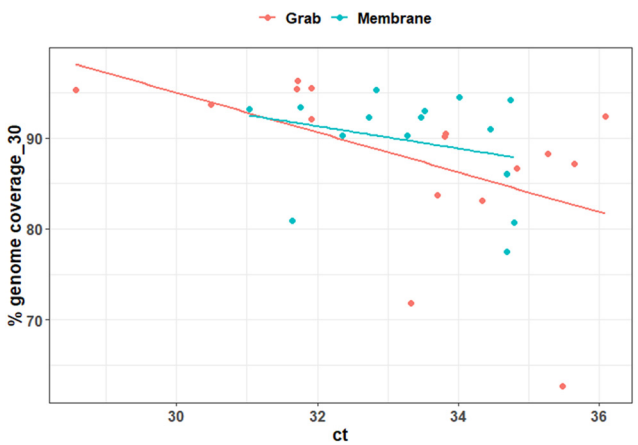

Fig. 3 Effect of the SARS-CoV-2 concentration (Ct value) on depth 30 genome coverage. Two water sampling methods were examined: grab sampling (sewage) and passive sampling (membrane). ANOVA was used to test the effect of the sampling method on the genome coverage at depth 30, and the relation between Ct and coverage ($p < 0.05$). The water sampling method did not have a significant effect.

We used the ASPICov pipeline to analyse the amino acid mutations in the S gene. We detected 30 amino acid changes, most of them specific to the Omicron sub-lineage BA.2 (27/30) associated with a few signatures detected at lower and variable frequencies, such as D405N, N764K and D796T (Fig. S2). We also detected the L452R mutation, a signature of the BA.4 and BA.5 variants, at low frequencies in a few grab samples from three sites in Nantes only (Fig.

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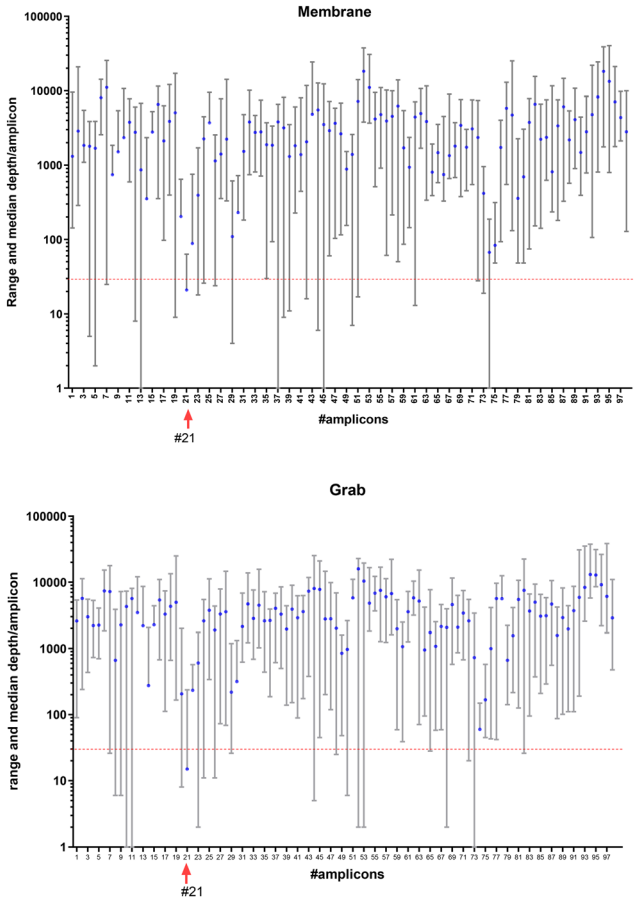

Fig. 4 Depth per amplicon for membrane and grab samples. Coverage analysis of the SARS-CoV-2 genome using the ARTIC sequencing protocol. The range (grey floating bars) and median depth (blue dot) for each of the 98 amplicons of the ARTIC multiplex PCR are shown. Fifteen samples were pooled for each water sampling method, namely passive sampling (membrane) and grab sampling (grab).

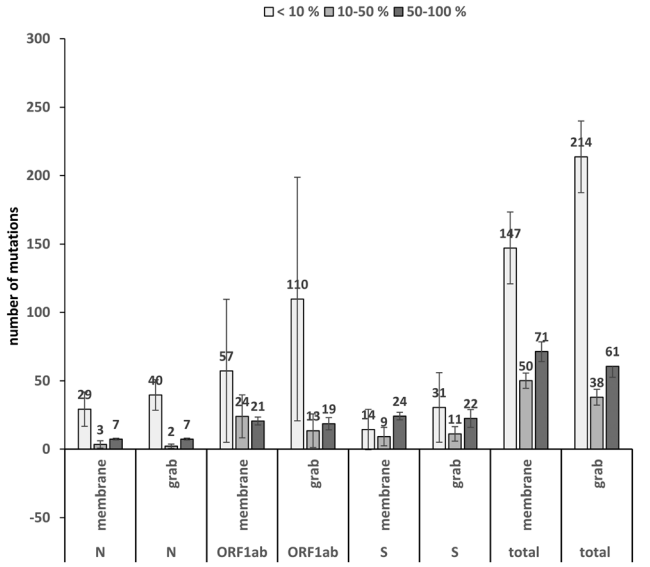

Fig. 5 The number of mutations per gene preferentially mutated in the SARS-CoV-2 genome. The data are presented as the mean number of mutations ± standard deviation. All types of mutations were plotted, that is, those with a high frequency (>50%), a medium frequency (10–50%) and a low frequency (<10%). Three genes were preferentially mutated in the SARS-CoV-2 genome: N, ORF1ab and S. Two water sampling methods were evaluated: passive sampler (membrane) and grab sampling (grab sampler). The total indicates the sum of mutations occurring in the whole SARS-SoV-2 genome.

S2). There were also five potentially new mutations: T887T (1%), Y489Y (3–7%), G700C (2–45%), A694S (27–83%) and I410I (26–100%).

Regarding the sampling methods, the majority of mutations (26 out of 30) were detected at all sites by both methods or by one method or the other (*e.g.* G142D, V213G, K417, N440L and Q954H) (Fig. S2). Only one signature mutation, G339D, was not detected at all sites. G339D, covered by amplicon #75, has a very low depth only at the mutation site (nt 22 578) and is not covered by the primer.

The monitoring of incidence rate (Fig. 6A) showed that our sampling dates were after the peak of Omicron BA.2 at Lyon (nationwide frequency 98.8%) and before the Omicron BA.5 wave at Nantes with potential mixed viral variant population. The incidence rate was 822/100 000 inhabitants in April in Lyon (8×10^6 inhabitants) and 264/100 000 inhabitants in May in Nantes (3.8×10^6 inhabitants).[51] We recently developed the VaRaPS package to evaluate the SARS-CoV-2 variant ratios.[41] This package was compared with Freyja[42] and other well-known variant deconvolution pipelines and showed equal or better performances in lineage proportion estimation.[52] As shown in Fig. 6B (left), Freyja did not identify any minor lineage, it only identified BA.2. Using VarAps we identified 32 distinct Omicron sub-lineages including a large proportion of BA.2 lineages (membrane = 83% and grab = 86%), 2 minority lineages (BA.4 [6%] and BA.5 [membrane = 4.7% and grab = 6.7%]) and others that were poorly represented (BA.1 [<1%], BQ.1 [membrane = 0.4% and grab = 0.002%] and BN.1 [1%]) (Fig. 6B, right). The estimated proportions of the remaining variants were close to zero ($<10^{-4}$) (referred to "others" in Fig. 6B).

Depending on the sampling site and city, the proportion of BA.2 sub-lineages varied from 61% to 94% but did not differ by more than 20% according to the sampling method, except for site I in Lyon (A-I). BA.4 and BA.5 were detected with very similar proportions, whatever the sampling method, except at sites A-I for BA.4, A-IV for BA.5, and B-IV for BA.5 (Fig. 6C). BN.1 proportion was low (0.7 to 2.2%) corresponding to the emerging status of this sub-lineage at the time of the study.

To compare this diversity of sewage sequencing with that of clinical sequencing, we recovered from GISAID SARS-CoV-2 sequences corresponding to the two administrative regions of these cities. The first identification of Omicron BA.5 in the population was in mid-April 2022 (Lyon) and 1 May 2022 (Nantes), with the peak in July in these two cities, while the sewage frequencies were already >4.7%. Moreover, the first identification of BA.4 in the population was in mid-April (Lyon) and mid-June (Nantes), showing that sequencing in wastewater by passive and grab sampling enabled early

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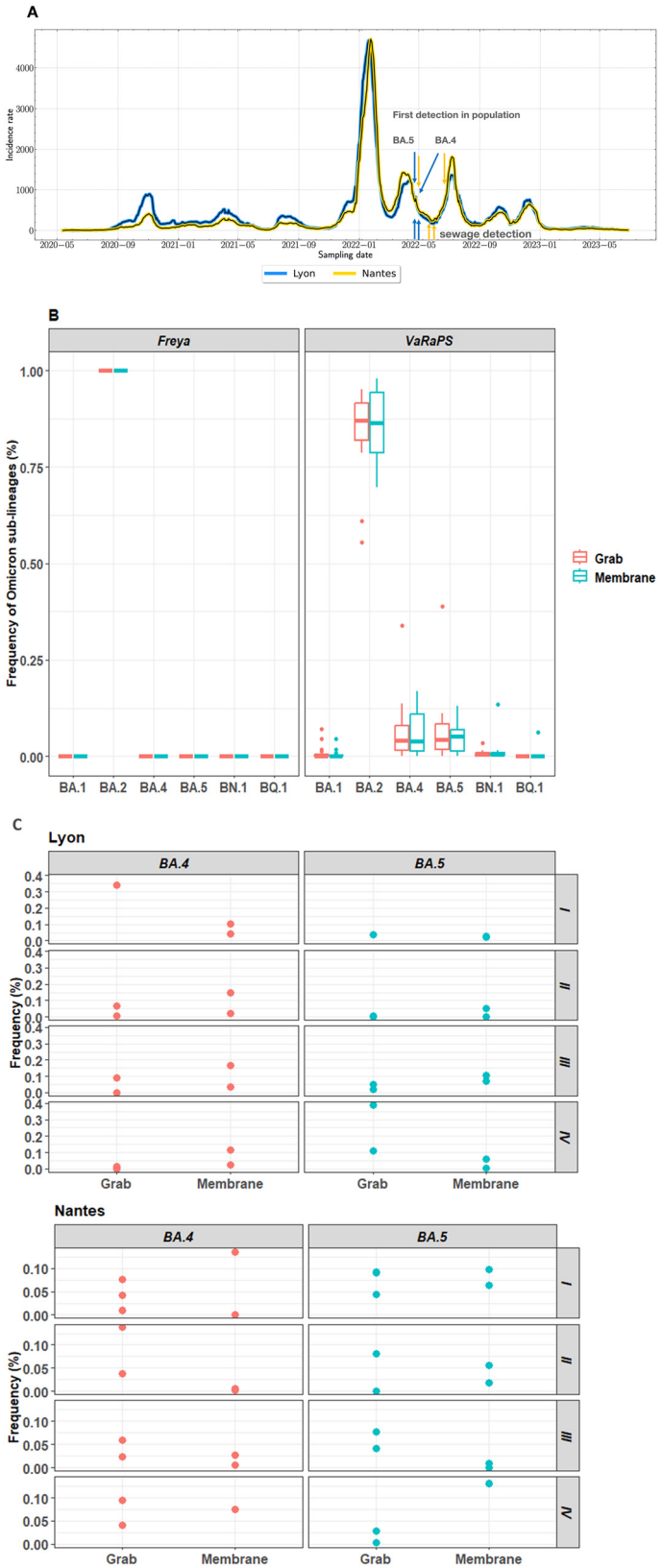

Fig. 6 Frequencies of Omicron sub-lineages in sewage in Lyon and Nantes and first detection in population. VaRaPSs was used to analyse the diversity of sub-lineages in these wastewater samples after generating the BAM files with ASPICOv. A: Incidence rate of COVID-19 in the population of Lyon and Nantes. The sewage sampling dates with membrane and grab sampling are indicated for Lyon (blue arrow) and Nantes (yellow arrow). The first detection of BA.4 and BA.5 in the population of Lyon (blue arrow) and Nantes (yellow arrow) is indicated. B: Frequencies of the major Omicron sub-lineages detected in the membrane and sewage samples with the Freyja pipeline (left) and with the VaRaPS-VirPool pipeline (right). C: Frequencies of BA.4 and BA.5 sub-lineages per site (I–IV) for the membrane and grab samples from Lyon and Nantes.

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detection of BA.4, specifically 3 weeks ahead of detection in the general population.

4. Discussion

Passive sampling is a valuable tool for SARS-CoV-2 wastewater surveillance at different scales and notably for the building and neighbourhood scales and when incidence rates are low. Nevertheless, to track the emergence of new variants, optimised virus recovery methods and whole genome sequencing are still a challenge, even with recent investigations involving Moore swabs and tampon-based passive samplers.[23,28,33] In this study, we have shown that passive and grab sampling offer comparable performance for NoV GII and SARS-CoV-2 detection and SARS-CoV-2 sequencing, even when sewage contains low concentrations of SARS-CoV-2 RNA. In particular, we have demonstrated that both techniques are effective during the early phases of the epidemic (BA.5) or before the variant is detected in the population (BA.4).

4.1 Performance of passive sampling for the detection of NoV GII and SARS-CoV-2

We chose nylon membranes based on previous studies showing that (i) the norovirus and SARS-CoV-2 detection frequencies were higher or similar compared with the zetapor membrane in seawater and sewage, (ii) nylon is not saturated as quickly as gauze as it absorbs less particulate organic matter and, as a consequence, (iii) nucleic acids extracted from nylon contain fewer PCR inhibitors than gauze.[21,25,53]

Two different methods are used to recover viruses from samplers: elution is recommended for gauze, tampon, and cheesecloth using various buffers, while direct lysis is more suitable for cellulose membranes and electronegative filters.[18,54] Although researchers have optimised recovery methods for the above mentioned passive samplers, prior to this study, there has been no optimization for sample recovery from nylon. We found that elution, with or without sonication, does not improve virus recovery compared with direct lysis on the membrane as described for cellulose-based samplers.[55] We also confirmed that the recovery rates of both viruses were in the same range as our previous findings for SARS-CoV-2 and NoV GII (Vincent-Hubert *et al.*, 2022).[21] As it has been described that sonication allows the detachment of viruses from complex matrices such as sewage and biofilm,[44,56] and given that SARS-CoV-2 is known to be mainly adsorbed on suspended solids,[27,57] these findings suggest that the concentration of suspended solids absorbed on nylon is low or that direct lysis treatment is effective enough in recovering viruses.

4.2 Passive and grab sampling: comparative analysis of quantification

Very few field studies have examined whether passive sampling can actually produce a time-integrated sample, which can be done by comparing passive and composite sampler concentrations as first proposed by Bivins *et al.*[19] We found a correlation between the SARS-CoV-2 RNA concentrations from passive and composite samples, as well as between passive and grab samples, indicating that with further optimisation these processes could be used for semi-quantification approaches.[19,28,29]

It can be critical to compare concentrations measured by passive and grab sampling, since one is supposed to reflect the concentration over several hours, while the other reflects the concentration at a given moment. However, with this comparison, we can at least expect the concentrations on the membranes to be similar, if not higher, than the concentrations in the grab samples. We showed that both sampling methods performed equally to detect SARS-CoV-2 and NoV GII, but the SARS-CoV-2 and NoV GII RNA concentrations from passive and grab samplers were not correlated, possibly due to the relatively small sample size. However, we observed two clear trends: one suggesting saturation of the membrane (NoV in both cities and SARS-CoV-2 at Nantes), and the other suggesting that the accumulation phase was still underway (SARS-CoV-2 at Lyon). These observations are based on laboratory kinetics, which demonstrated that NoV GII and SARS-CoV-2 concentrations increased with time and remained stable after 24 h.[21,25] For other samplers such as Moore swabs, tampons, and electronegative filters, it has also been demonstrated that the affinity of viruses for membranes may vary according to the type of virus, the total suspended solids, and the water.[22,27,53,58] The equilibrium, and, consequently, the optimal duration of the deployment of the membrane depends on the virus and the sample.[22,27] Therefore, additional studies are still required to make these methods semi-quantitative.

4.3 SARS-CoV-2 sequencing: performance of MiSeq and ARTIC PCR

Successful sequencing of SARS-CoV-2 genomes from environmental samples relies on both the depth and breadth of genome coverage and therefore on the efficiency of multiplex PCR.[59,60] With Illumina sequencing, a low viral load is often associated with low depth and coverage after either amplification or capture.[9,10] Here, we found a significant relationship between the Ct value and the breadth of coverage at a depth of 30, with no impact from the sampling method. Indeed, we obtained near-complete genome coverage (>90%) for the majority of our samples, despite challenging conditions ($28 < Ct < 36$ and using RNA that had been frozen for 6 months). We obtained the same scores as those previously described in similar studies using composite sample sequencing with the ARTIC V3 and V4 protocol, and frozen sewage samples.[35,61–64] Only three previous studies have reported SARS-CoV-2 whole genome sequencing from passive sampling; the authors of those studies used similar or less stringent quality control criteria

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compared with ours, and they did not provide information concerning the depth per amplicon.[23,28,33]

Even though we observed a high depth per amplicon, there was heterogeneity for some regions of the genome, independent of the sampling method. Amplicon dropouts have been observed with the ARTIC protocol and are generally associated with new mutations occurring as the virus evolves and develops new variants (ARTIC_Network, 2021). Here, only amplicon 21 had the lowest coverage in 19 of our 30 sequenced samples, without any mutations in the primer binding site, whereas the amplicons described as having a dropout have the correct depth (ARTIC V4 or V4.1 panel [#75,76,88]).[65,66] Reduced amplification of amplicon 21 has been reported once and correlated with a low number of mapped reads,[65] which we also observed in some of our samples. Therefore, this lower depth might be due to a poor quality and/or a low quantity of extracted RNA, probably due to the impact of freezing and thawing, which are known to impact sequencing.[6,35]

4.4 Early detection of Omicron BA.4 and BA.5 by passive and grab sampling

Wastewater-based genomic surveillance could be limited by low-quality sequence data as well as the inability to estimate relative lineage abundance in mixed samples. Despite low RNA concentrations and the use of samples that had been frozen for several months, we were able to analyse the obtained sequences and to identify variants that were poorly represented. Application of the VaRaPS package – with its co-occurrence-based method – proved to be essential for deconvoluting the complex mixture of SARS-CoV-2 lineages in our wastewater samples. This approach enabled a robust estimation of the frequency levels of 32 distinct Omicron sub-lineages across all the samples. Our analysis revealed the predominance of the BA.2 lineage (83–86%) in both membrane and grab samples, with much lower proportions of BA.4 (6%) and BA.5 (4.7–6.7%). Detection of the L452R mutation, a signature of BA.4 and BA.5, at low frequencies in grab samples from Nantes corroborated the emergence of these sub-lineages as indicated by the deconvolution results. This concordance between specific mutation detection and lineage composition analysis demonstrates the sensitivity and reliability of passive sampling in capturing the evolving viral landscape within the studied populations.

Our approach involving deep sequencing and the threshold for mutation frequencies revealed many more mutations than described recently.[48] Notably, we detected mutations in the S gene that could be related to cryptic lineages, such as G700C, A694S and I410I. Given that these mutations have never been reported, our findings highlight the ability of wastewater-based surveillance to uncover cryptic lineages.[42,67–70]

Passive sampling using swab samplers has proved to be effective in localising VOCs at the city scale in urban sewers[28] and at the building scale.[23] SARS-CoV-2 sequencing from passive samplers has demonstrated its added value in identifying a shift in viral variants (from BA1 to BA.2) from Moore swabs confirmed by individual saliva testing.[33] However, the ability of passive samplers to detect the emergence of new variants at an early stage – that is, before they are detected in the population by mass screening – remains to be established.

In France, Omicron BA.5, classified as a VOC in May 2022, was predominant in the population. According to GISAID, it was initially detected at the end of April 2022 (a 9% frequency nationwide) and peaked in August 2022 (a 92% frequency nationwide) (GISAID). BA.4 was detected at the same time, but remained as a very small minority. Based on our data, we identified the emergence of BA.5 in Lyon and Nantes (4.7–6.7%) at the same time as the first human case reported in GISAID. BA.4 was first identified in the population in Nantes in mid-June, thus showing that sewage sequencing identified BA.4 up to 3 weeks before detection through genomic surveillance in the population in Nantes. Access constraints to the sewers prevented us from taking samples over a longer period, which would have enabled us to accurately monitor the emergence of BA.5 in the two cities. Early detection of these variants in wastewater collected by traditional sampling has been reported in Germany, Belgium, and Tunisia. In Germany, BA.5 was detected in wastewater in mid-May 2022 (10% prevalence) and was dominant in July (with a 90% incidence).[71–73]

Even though many reports have shown that the presence of SARS-CoV-2 in WWTP influents correlates with genomic surveillance of the population, only a few of them have reported on measurements and sequencing upstream of WWTPs, that is, throughout the sewer network or at the building scale. Some reports found that WBE could identify local outbreaks that may be overlooked at the city-scale when the COVID-19 incidence rate is low.[74] To our knowledge, only one study compared data from wastewater sequencing using a passive sampler and clinical data in neighbourhoods.[23]

5. Conclusion

Taken together, our data demonstrate that sequencing from passive sampling with nylon membranes is as effective as grab sampling for monitoring the emergence of SARS-CoV-2 variants, therefore providing an early warning system at the community level. Given that both techniques exhibit similar performance, they could be especially useful for implementing large monitoring networks in regions far from analysis settings for a reasonable cost.

Conflicts of interest

All authors do not have any conflict of interest.

Data availability

The sequence data are available at: https://www.ebi.ac.uk/ena/browser/view/PRJEB85436. The code for ASPICov can be

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found at https://gitlab.com/vtilloy/aspicov, DOI: https://doi.org/10.1371/journal.pone.0262953. The version of the code employed for this study is version 1.1.7. The mutation variant matrix used in VaRaPS is available at: [https://github.com/hacen-ai/Varaps-data/blob/main/Variant-mutation-profile-matrix.csv]. VaRaPS is open-sourced and hosted publicly on GitHub [https://github.com/hacen-ai/varaps] and is also accessible as a package *via* PyPI [https://pypi.org/project/VaRaPS/]. The version of the VaRaPS employed for this study is version 0.7.8. Supplementary information is available. See DOI: https://doi.org/10.1039/d5ew00482a.

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