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Monitoring the evolution of SARS-CoV-2 on a Spanish university campus through wastewater analysis: A pilot project for the reopening strategy

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HIGHLIGHTS
• Wastewater surveillance was applied at the building level of a university campus.
• WBE allowed following the evolution of a COVID-19 outbreak at the student's residence.
• SARS-CoV-2 RNA detection depended on incidence, occupants, and mobility features.
• Detected viral variants gave an indication of the evolution of the virus over time.
• WBE was integrated as part of the universities response system against the pandemic.

ABSTRACT
Wastewater surveillance is a fast and cost-effective tool that enables tracing of both symptomatic and asymptomatic transmission of SARS-CoV-2. In this paper, a pilot program carried out at the University Jaume I for monitoring the trends of the presence of SARS-CoV-2 in wastewater. To the best of our knowledge, this is the first such project conducted on a university campus in Spain. Wastewater samples (n = 838) were collected when students returned to campus, from October 2020 until August 2021, at a confluence sewer point and at the building level including different academic departments and services, the library, administration offices and the university student residence. It has been observed that the probability of SARS-CoV-2 RNA detection in wastewater depended on COVID-19 incidence on campus and visitors/occupants of the buildings i.e., high-, or low-traffic buildings with high or low frequency of potential contacts. Moreover, the third wave in Spain (after Christmas 2020) and an outbreak that occurred at the university student's residence could be carefully followed, allowing confirmation of the end of the outbreak. In addition, viral variants (i.e., mutations and linages) from selected time points were detected by sequencing and gave an indication of the evolution of the virus over time. The results illustrate the potential of wastewater-based epidemiology to provide an early warning for SARS-CoV-2 within the university, especially in buildings with low traffic and more defined populations, like the student residence. The strategy and experience gathered in this study will allow for implementation of improvements for reliable monitoring in the future.
1. Introduction

The COVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) places a significant burden on health systems worldwide, as well as disease surveillance and diagnosis systems. Faced with SARS-CoV-2's numerous shortcomings in controlling the spread of the virus, one of the major challenges is knowing the number of infected people and the evolution of the pandemic. Epidemiological surveillance is a fundamental tool, which is mainly supported on incidence studies based on the detection of new cases in the population and the investigation of contacts, either through syndromic surveillance (suspected cases due to symptoms, without testing) or laboratory-confirmed cases. The use of wastewater surveillance can contribute to public health surveillance and complement clinical testing (Medema et al., 2020; Wu et al., 2020).

At the beginning of the pandemic, primary measures to restrain the spreading of the virus included home confinement, closing of non-essential activities, and closing of schools and universities. Social distancing and the wearing of face masks allowed the return of a “normal” situation. However, monitoring the pandemic became essential to control regional and local situations, such as students re-entering university campuses in autumn 2020. Several universities embraced wastewater-based epidemiology (WBE) as a surveillance tool to monitor the trends of infection in combination with clinical testing (Betancourt et al., 2021; Gibas et al., 2021; Harris-Lovett et al., 2021). Wastewater surveillance, adapted from an established methodology in other areas (e.g., illicit drug consumption), is a fast and cost-effective tool that enables tracing of both symptomatic and asymptomatic transmission of SARS-CoV-2 (Medema et al., 2020; Wu et al., 2020).

The successful application of WBE described by different universities (Betancourt et al., 2021; Gibas et al., 2021), specifically when monitoring student residences, provided strong confidence that this approach has the potential as an early-warning tool that can avert COVID-19 outbreaks. Therefore, our university (University Jaume I (UJI), Castellón, Spain) with approximately 15,000 students and staff, initiated a pilot program, which to the best of authors' knowledge, is the first study performed in a university environment in Spain. However, unlike many universities in the United States, only a small part of the student bodies lives on the campus. The majority commonly live off-campus in the surrounding community, or commute from their parents' house to university. The nature of this being a primarily commuter campus has the potential to affect the spreading of the virus as the number of off-campus contacts increase.

In this work the main objectives were: (i) to monitor the trends of infection of SARS-CoV-2 throughout the 2020–2021 academic year, (ii) to monitor viral variants (mutations and lineages) through next-generation sequencing from selected time points and samples, and (iii) to implement wastewater surveillance within the campus, integrating it as part of the response system against the pandemic. To these aims, the UJI, created a multidisciplinary group of researchers including microbiologists and technicians specialized in PCR analysis, WBE experts, engineers, health professionals and doctors specialized in epidemiology. WBE data was compared and correlated with epidemiological data provided by the medical services of the University and the Epidemiology Department of the Public Health Center of the city of Castellón. We discuss the challenges and suggest improvements for future implementation of WBE monitoring on a university campus or similar setting.

2. Materials and methods

2.1. Wastewater sampling

The UJI, with approximately 15,000 students and staff, is a relatively young university (operating since 1991) and has a well-defined infrastructure and sewage system. In total, 12 sampling locations were selected including the main buildings: School of Technology and Experimental Sciences, Health Sciences, Humanities, Economics, Research Institutes and central UJI services, Spin-off companies, Library, Administration offices, student residence hall (giving home to only 250 students) and a confluence sewer point. The confluence point is located at the exit of the UJI, where a sample could be collected covering the complete north and south parts of the campus. Each individual building has easily accessible sewage cleanouts through a man-sized tunnel network (Fig. 1). Hence, each sampling point was the sole source of a single building (except for the confluence sewer point).

Samples of wastewater were collected from October 28, 2020 until July 29, 2021 i.e., throughout the 2020–2021 academic year. A total of 838 wastewater samples were collected and analysed for SARS-CoV-2 (Table 1). Composite samples (approx. 500 mL) were manually collected by combining grab samples of approximately 50 mL every 45 min from 8 am to 4:30 pm to cover the hours when the main activities occur within the university. Seven sampling points were collected on Tuesdays and Thursday (marked as blue dots in Fig. 1) and six sampling points on Mondays and Wednesdays (marked as red dots in Fig. 1) corresponding to the south and the north parts of the campus, respectively. Moreover, frequency of sampling of the student residence (point 4) increased on the request from the University Health Department i.e., three times a week including Fridays. Failure to collect samples occurred only few times during university holiday periods and at the administration offices (point 7), where working from home was highly encouraged. In these cases, it was not possible to take samples due to low flow at the collection sites. All grab samples were stored in small refrigerators located near the sewage cleanouts. At the end of each day the composite samples were transported to the laboratory where they were stored at 4 °C to be processed and analysed the following day (within 24 h).

2.2. Sample processing and RNA extraction

Water samples were subjected to a concentration process using a protocol based on adsorption and precipitation by aluminium (Randazzo et al., 2020). Briefly, a wastewater aliquot of 350 mL was taken from each sample and adjusted to pH 6.0. Subsequently, an Al (OH)₃ precipitate was formed by adding 1/100 v:v of a 0.9 N AlCl₃ solution (Across Organics, Fisher Scientific). After re-adjustment to pH 6.0, samples were agitated slowly for 25 min (150 rpm, room T°). Samples were then subjected to centrifugation (1700 × g) for 20 min to separate the precipitate from the supernatant. Pellets were resuspended into 20 mL of 3 % beef extract (pH 7.4, Gibco™, Fisher Scientific), and agitated for 10 min (150 rpm, room T°). After centrifugation (1900 × g, 30 min) the precipitates were resuspended in 1 mL of PBS 1x (10x, Gibco™, Fisher Scientific) and agitated for 10 min (150 rpm, room T°). After centrifugation (1900 × g, 30 min) the precipitates were resuspended in 1 mL of PBS 1x (10x, Gibco™, Fisher Scientific) and stored for RNA extraction. As a process control, samples were spiked with 10⁵ PCR units of Menvirus (MgV) vMCO (CECTI00000) according to ISO15216-1: 2017 (https://www.une.org/encuentra-tu-norma/busca-tu-norma?c=N0058882).

RNA extraction was performed using the Nucleospin RNA virus kit (Macherey-Nagel) following the recommended protocol along with an initial pre-treatment step with Plant RNA Isolation Aid (Ambion, USA) (Randazzo et al., 2020). Extracted nucleic acid was eluted in a volume of 70 μL and stored at −80 °C until analysis.

2.3. RT-qPCR for viral RNA quantification in wastewater

The presence of SARS-CoV-2 RNA was determined using the PrimeScript™ One Step RT-PCR kit (Takara Bio, Sumilab), in a StepOne Plus Applied Biosystem instrument, following the manufacturer’s instructions. To determine the presence of SARS-CoV-2 specific primers and probes for nucleocapsid N1 target regions (2019-nCoV RU0 kit) and envelope (E) target gene, the Charité Berlin procedures were used (Corman et al., 2020). A calibration curve was acquired using the 2019-nCoV N and 2019-nCoV E positive controls (IDT). Cycle threshold (Ct) values were used to calculate genomic copies (gc)/L in the original sample. For MgV, detection was carried out using primers and probe described by ISO 15216-1:2017 (Haramoto et al., 2018). Standard curves for MgV
quantifications were measured using extracted MgV genomic RNA, in triplicate and with serial 10-fold dilutions.

Ct values were used to calculate the viral load, which was reported as gc/L, in the original sample. Ct values lower than 40 were considered positive for SARS-CoV-2, as proposed previously (Wang et al., 2020). Therefore, for each sampling day on which SARS-CoV-2 was detected, the average of gc/L was calculated from the RT-qPCR duplicate wells containing undiluted RNA for each of the selected targets and using the corresponding standard curve and volume tested.

In order to determine the limits of quantification (LoQ) and limits of detection (LoD) of the control plasmids, different calibration curves were carried out and the average was used. For both control plasmids, the lower LoQ was 8 copies/μL, equating to 32,000 gc/L and lower LoD was 457 gc/L for 2019-nCoV_N_Positive Control and 92 gc/L for 2019-nCoV_E_Positive Control.

### Table 1
Overview of total number of wastewater samples tested for each sampling point, negative and positive samples for SARS-CoV-2 detection and percentage of positive cases (total and for each sampling point). Results from N1 and E target genes.

| Sampling point | 10A | 10B | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 11 | 12 | Total |
|----------------|-----|-----|----|----|----|----|----|----|----|----|----|----|-----|-------|
| No. samples tested | 78  | 78  | 58 | 57 | 63 | 105 | 70  | 63  | 62  | 63 | 59 | 36 | 36  | 838   |
| Percentage of positive samples | N1  | 43.0 | 66.6 | 15.5 | 33.3 | 15.8 | 40.9 | 20.6 | 19.3 | 14 | 35.4 | 37.7 | 2.7 | 8.3 | 31.5  |
| E              | 44.3 | 64.3 | 14.3 | 33.3 | 7.4 | 40.9 | 24.1 | 21.3 | 20.3 | 27.8 | 29.5 | 2.7 | 2.7 | 30.5  |

1. Research institutes and central UJI services 2. School of Technology and Experimental Sciences (STES). 3. Health Sciences. 4. University Residence. 5. Library. 6. Humanities. 7. Administration offices. 8. Economics. 9. Ceramic Institute. 10. Joint sewer point -North (A) / South (B). 11, 12. Spin-off companies (ESPAITEC). (*collected 3 times a week, also on Fridays).

### 2.4. SARS-CoV-2 genome sequencing and analysis

Nine wastewater samples with (Ct) values below 33 were selected for sequencing analysis. Genomic sequencing of SARS-CoV-2 was carried out by multiplex PCR amplification using the ARTIC primer scheme version 3 (https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye). Libraries were built with the Nextera Flex kit (Illumina) and sequenced on an Illumina MiSeq platform by paired-end reads (2 × 200). Obtained reads were cleaned using cutadapt software (Martin, 2011) for adaptors removal. Nucleotides with Phred score lower than 30 were also removed using the reformat.sh option from the bbmap software (www.sourceforge.net/projects/bbmap/). Clean reads were aligned to the genome of SARS-CoV-2 isolate Wuhan-Hu-1 (MN908947.3) with the Burrows-Wheeler Aligner v0.7.17-r1188 using default parameters (Li and Durbin, 2009). Alignments were indexed by samtools (Li et al., 2009).
Genomic coverage was calculated for each sample taking into account only regions with depths higher to 20×. Nucleotide variations from the reference SARS-CoV-2 genome (MN908947.3) were analysed using mpileup from samtools (Li et al., 2009) and the command variants of ivar software (Grubaugh et al., 2019). Nucleotide polymorphism was assumed in the cases where the nucleotide variation was at least 50× deep and with a quality score > 30. To avoid misidentifications of nucleotide substitutions that corresponded to incorrectly trimmed adaptors, alignments were manually curated (Nemudryi et al., 2020).

2.5. Epidemiological study

Incidence of COVID-19 cases (per 1000 population) globally observed on campus was calculated considering the number of accumulated positive reported cases within staff and students during time frames of approximately 28 days, and a total population of 13,892 people. Additionally, the reported COVID 19 cumulative incidence among the student population attending the School of Technology and Experimental Sciences, Economics, Humanities and Health Sciences was collected from the UJI web site where COVID-19 information has been publically available: (https://www.uji.es/coronavirus/docs/situacioepidemiologica). In this respect, positive cases where the nucleotide variation was at least 50× deep and with a quality score > 30. To avoid misidentifications of nucleotide substitutions that corresponded to incorrectly trimmed adaptors, alignments were manually curated (Nemudryi et al., 2020).

3. Results and discussion

3.1. Sample collection and sampling points

Collecting multiple grab samples, as performed in this study, simulates automatic time-proportional sample collection (Ort et al., 2010). The frequency of manual sampling is generally lower, which introduces a higher uncertainty (Castiglioni et al., 2013), as it increases the chance to miss viral shedding of an infected person. Nevertheless, grab sampling has shown to be effective for SARS-CoV-2 monitoring, especially during peak wastewater flow hours (Betancourt et al., 2021). Despite their costs, purchasing small sized portable autosamplers should be considered for future studies as manual sampling is physically intense and demanding. Alternatively, passive samplers should be contemplated. Passive samplers have demonstrated the ability to retain viral fragments and are cost-effective (Li et al., 2022).

3.2. Overview of wastewater surveillance results

Wastewater samples were processed and analysed within 24 h following collection. The internal mengovirus control recovery rates were ≥ 1 % in nearly all cases, which fulfilled the ISO15216-2:2017 standards (Haramoto et al., 2018) and were in agreement with studies that used this non-enveloped virus for the same purposes (Randazzo et al., 2020). In particular, the mean recovery rate of mengovirus was 6.4 ± 0.08 %.

The presence of SARS-CoV-2 in wastewater was determined and quantified by RT-qPCR using the genetic target region N1 and E gene (Table 1). A sample was considered positive for SARS-CoV-2 when both the nucleocapsid N1 target region and the E target gene were determined at Ct values lower than 40. When one of the genes failed to be detected, the sample was labelled as “suspicious”. Overall, 234 samples were found positive, from a total of 838 samples analysed, which represents a total positive detection rate of 27.9 %. Although similar frequencies of viral detection were obtained for both the N1 and E target genes, the nucleocapsid genetic region N1 seems to be a more precise and reliable indicator for the presence of SARS-CoV-2.

In the confluence sewer located at the exit of UJI (sampling points 10A (north campus) and 10B (south campus)), the frequency of viral positives was higher in the south part (66.6 %) than the north part (43 %). In addition, results of SARS-CoV-2 RNA from these two end points indicate that the virus was circulating on campus over the course of the project. Considering sampling point 10B, the maximum levels of virus genetic material were detected November 3, (6.6 × 10^6 gc/L) (Fig. 2). SARS-CoV-2 RNA continued to be detected during December, even for a few days over the Christmas period, showing moderately high virus concentration (average of 5. 55 × 10^6 gc/L). However, COVID-19 incidence on campus was still low during this period (4.9 positive cases reported per 1000 population) (Table 2). Therefore, the wastewater surveillance results suggest that the reported cases of COVID-19 on campus might be underestimated.

After the Christmas break, SARS-CoV-2 RNA concentrations increased, with the highest concentration observed at sampling point 10B on January 28 (1.19 × 10^6 gc/L). Data of this period correlated with the COVID-19 epidemiological data recorded in relation to the number of positive cases on campus (i.e., 18.4 positive cases per 1000 population in February 2021), and the 3rd wave registered in the city of Castellón.

Wastewater surveillance at the institutional level (i.e., by monitoring at the sewer confluence located at the exit of the campus) can give an indication of the general health of the community with respect to COVID-19 incidence. Although it would not lead to a direct action with respect to clinical testing, an increase in the virus concentration encouraged the need to perform a more intensive wastewater monitoring of specific buildings e.g., the student residence. Adding an additional collection day (Friday) allowed for better surveillance and eventually led to enhanced targeted clinical testing (see also Section 3.4). Considering that SARS-CoV-2 RNA can be measured in feces of nearly 66 % of infected patients (Chan et al., 2021; Lo et al.,
et al., 2021; Davó et al., 2021; Gibas et al., 2021; Harris-Lovett et al., with the results reported by (Wang et al., 2022). However, it is noteworthy of SARS-CoV-2 RNA detection in wastewater in our study, and is consistent contacts. These different characteristics seem to explain some discrepancies and the sampling methods or laboratory protocols used (Castro-Gutierrez mended. Nevertheless, there are also other aspects that may affect the de-

In this study, wastewater samples were collected from 11 buildings with different populations in terms of size and traffic i.e., frequency of potential contacts. These different characteristics seem to explain some discrepancies of SARS-CoV-2 RNA detection in wastewater in our study, and is consistent with the results reported by (Wang et al., 2022). However, it is noteworthy that each building has a distinct population (degree program) with different characteristics in terms of traffic, and thus the possibility for being in contact. Hence, for future studies improved characterization of the mobility of the population contributing to each sample at different times is recommended. Nevertheless, there are also other aspects that may affect the detection rate of SARS-CoV-2 in wastewater, such as disease prevalence, and the sampling methods or laboratory protocols used (Castro-Gutierrez et al., 2021; Davó et al., 2021; Gibas et al., 2021; Harris-Lovett et al., 2021; Karthikeyan et al., 2021; Scott et al., 2021; Sweetapple et al., 2022; Wang et al., 2022).

The population size represented by a single wastewater sample was very heterogeneous among the sampling points included. Population size ranged from a relatively fixed number of students (i.e., the university residence, point 4, with approximately 250 students) to buildings with some but limited movement of individuals as it mainly consist of offices and research laboratories, to high-traffic buildings with larger movements of students and university staff due to in-person theoretical and practical classes. These high-traffic buildings result in an unpredictable number of individuals passing through but the number of enrolled students in each semester still acts as a good estimate of population i.e., School of Technology and Experimental Sciences (with approx. 2819 students enrolled in 12 degrees); Health Sciences (with approx. 1162 students enrolled in 3 degrees); Humanities (with approx. 3658 students enrolled in 10 degrees) and Economics (with approx. 3441 students enrolled in 8 degrees). Moreover, the university residence has a different dynamic, where traffic and thus frequency of potential contacts is considered low and where it can be assumed that most students will use the toilet of their residence.

Table 1 indicates that the highest rate of SARS-CoV-2 positive detections in wastewater from buildings, that are known to have experienced relatively high student traffic, were observed at the Economics (35.4 % for N1 and 27.8 % for E), followed by the School of Technology and Experimental Sciences (33.3 % for both N1 and E), Humanities (19.3 % for N1 and 21.3 % for E) and Health Sciences (15.4 % for N1 and 7.4 % for E). These results also correlate with the number of COVID-19 positive cases registered on campus (data collected from the university’s web site, from the beginning of the pandemic until July 2021) for students enrolled in degrees carried out in these buildings. The highest COVID 19 incidence reported within the student population was the School of Technology and Experimental Sciences (5.7 %), followed by the Economics (5.2 %), Humanities (4.9 %) and Health Sciences (4.6 %) (Table 2). Moreover, Fig. 3 shows the temporal trends of SARS-CoV-2 (N1 data) in wastewater and a similar pattern was observed within these programs, highlighting two main periods in which viral RNA was more frequently detected. The first period was between November 12 to December 2, with the highest average values of virus concentration detected at Economics (2.5 × 10^5 gc/L) and at the School of Technology and Experimental Sciences (8.1 × 10^5 gc/L). The second period was between 12th January to 20th February, with the highest average values of virus concentration also observed at the School of Technology and Experimental Sciences (4.5 × 10^5 gc/L), and at the Health Sciences (1.8 × 10^5 gc/L). After that, there was only intermittent detection of SARS-CoV-2 RNA at those buildings, which correlates with the lowest COVID-19 incidence observed across campus (Table 2).

In addition, as we indicated for wastewater surveillance at the institution level (see Section 3.2), sampling points at buildings which include a large part of the campus population can reliably provide an understanding of viral transmission among the campus population. These points also

Table 2

| Time frame evaluated | Incidence of positive cases/1000 UJI population |
|----------------------|-----------------------------------------------|
| Dec. 1–27, 2020      | 4.9                                           |
| Dec. 28, 2020–Jan. 24, 2021 | 18.4                                         |
| Jan. 25–Feb. 21, 2021 | 5.0                                           |
| Feb. 22–Mar. 21, 2021 | 0.5                                           |
| Mar. 22–Apr. 18, 2021 | 0.6                                           |
| Apr. 18–May 30, 2021  | 0.6                                           |
| May 31–Jun. 27, 2021  | 1.0                                           |
| Jun. 28–Jul. 25, 2021 | 6.2                                           |

* Total population calculated: 13892. Data from November is not available, in that registration of monthly COVID-19 incidence on campus, that began from December 2020.

Fig. 3. Temporal trends in SARS-CoV-2 wastewater data on campus from wastewater sampling point at the main Faculties from November 2020 to July 2021.
provide the baseline of SARS-CoV-2 levels on campus as a whole, as was reported by other campus wastewater surveillance studies (Gibas et al., 2021).

SARS-CoV-2 RNA was also monitored in wastewater from low-traffic buildings with a more stable occupancy i.e., limited visiting students and occupied mainly by staff. These buildings mostly consist of offices and research laboratories (i.e., administration offices, research institutes and spin-off companies, points 1, 7, 9, 11 and 12). Overall, these locations showed the lowest rate of positives detected in wastewater, with the exception of point 9, the Research Institute for Ceramic Technology (Table 1). It is important to highlight that the majority of the staff members, normally active in these buildings, were principally working from home (i.e., from January to May 2021), which matched with the lack of viral RNA detection observed in wastewater samples at e.g., points 11 and 12, the spin-off building with global positive N1 and E rates of 2.7 % (both) and 8.3 % and 2.7 %, respectively. However, between June to July 2021, when people started working on campus again, an increase in SARS-CoV-2 genetic material was observed at those sampling points. In particular, viral RNA detection was observed only for point 11 (first week of June with 50 % positive detected) and three times for point 12 (first week of June, and second and third weeks of July, all with 50 % positive detected) (Table 3).

The research institutes located at point 1 and the administration office at point 7 showed global positive N1 and E rates of 15.5 % and 14 %, and 14 % and 20.3 % respectively (Table 1). In addition, the highest positive rate observed (100 %) for sampling point 1 was between 8 and 11 of February 2021 and for sampling point 7, during 18–21 of January (Table 3). SARS-CoV-2 RNA detection then decreased at both sampling points, being even negative during April and May at point 1 and during May at point 7. Overall, we observed that the decrease in COVID-19 incidence on campus was associated with the decreased viral concentration in the wastewater over this period. In fact, between June–July, a peak of COVID-19 incidence on campus was observed (1.0 and 5.2 positive cases per 1000 population, respectively), which also correlates with an increase in virus RNA detection at both points. In particular, the research institute (point 1) showed a higher positive rate (50 %) during the first week of June and the last week of July, which could be linked to a higher occupancy at the building with highly transient footfall and thus, a higher probability of cases (Table 2).

Point 9, the Research Institute for Ceramic Technology along with the student residence, registered one of the highest average percentage of positive viral RNA detection among all sampling points monitored in this study with a value of 37.7 % (N1) and 29.5 % (E) (Table 1). Moreover, the highest monthly positive percentages (N1 data) were detected during November (first week with 50 % and the rest with 100 %) and the first two weeks of December (100 % and 50 %, respectively), 2020. At this particular sampling point, detection of SARS-CoV-2 occurred during the entire timeframe of the project, except for April and July 2021 (Table 3). A clear explanation

| Week | Number of positive wastewater samples/total (% positive detected) |
|------|---------------------------------------------------------------|
| Point 1 | Point 5 | Point 7 | Point 9 | Point 11 | Point 12 |
| Nov. 2-6th | ND | ND | 0/2 (0) | ND | ND | ND |
| Nov. 9-12th | ND | ND | 0/2 (0) | 1/2 (50) | ND | ND |
| Nov. 16-19th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 2/2 (100) | ND | ND |
| Nov. 23-25th | 1/2 (50) | 0/2 (0) | 0/2 (0) | 2/2 (100) | ND | ND |
| Nov. 30-Dec 3rd | 0/2 (0) | 0/2 (0) | 0/2 (0) | 2/2 (100) | ND | ND |
| Dec. 7-10th | 0/1 (0) ** | 0/1 (0) ** | 2/1 (100) ** | 2/1 (100) ** | ND | ND |
| Dec. 14-17th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 1/2 (50) | ND | ND |
| Dec. 21-25th | ND ** | 0/4 (0) | ND ** | ND ** | ND | ND |
| Dec. 28-30th | ND ** | 0/3 (0) | ND ** | ND ** | ND | ND |
| Jan. 4-8th | ND ** | 2/4 (50) | ND ** | ND ** | ND | ND |
| Jan. 11-14th | 0/2 (0) | 1/2 (50) | 1/2 (50) | 2/2 (100) | ND | ND |
| Jan. 18-21st | 0/2 (0) | 1/2 (100) | 1/2 (100) | 1/2 (50) | ND | ND |
| Jan. 25-28th | 0/2 (0) | 2/2 (100) | 1/2 (50) | 0/2 (0) | ND | ND |
| Feb. 1-3rd | ND ** | 0/1 (0) | 1/1 (50) | 1/2 (50) | ND | ND |
| Feb. 8-11th | 1/2 (100) | 1/1 (50) | 0/2 (0) | 2/2 (100) | ND | ND |
| Feb. 15-18th | 1/2 (50) | 0/2 (0) | 1/2 (50) | 1/2 (50) | ND | ND |
| Feb. 22-25th | 1/2 (50) | 0/2 (0) | 0/2 (0) | 0/2 (0) | ND | ND |
| Mar. 1-4th | 0/2 (0) | 0/2 (0) | 1/2 (50) | 1/2 (50) | 0/2 (0) | 0/2 (0) ** |
| Mar. 8-11th | 1/2 (50) | 0/2 (0) | 0/1 (50) ** | 0/1 (50) ** | 0/2 (0) | 0/2 (0) |
| Mar. 15-18th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| Mar. 22-25th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| Mar. 29-Apr. 1st | 0/2 (0) ** | 0/1 (0) ** | 0/2 (0) | 0/2 (0) | 1/1 (100) ** | 0/1 (0) ** |
| Apr. 5-8th | ND ** | ND ** | ND ** | ND ** | ND ** | ND ** |
| Apr. 12-15th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| Apr. 19-22nd | 0/2 (0) | 0/2 (0) | 1/2 (50) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| Apr. 26-29th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| May 3-6th | 0/1 (0) ** | 0/1 (0) ** | 0/2 (0) | 0/2 (0) | 0/1 (0) ** | 0/1 (0) ** |
| May 10-13th | ND ** | NO ** | ND ** | 0/1 (0) ** | NO ** | NO ** |
| May 17-20th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| May 24-27th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| May 31-Jun. 3rd | 0/2 (0) | 0/2 (0) | 0/2 (0) | 1/2 (50) | 0/2 (0) | 0/2 (0) |
| Jun. 7-10th | 1/2 (50) | 1/2 (100) | 1/5 (50) | 2/2 (100) | 1/2 (50) | 1/2 (50) |
| Jun. 14-17th | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| Jun. 21-23rd | 1/2 (50) | 0/1 (0) ** | 0/2 (0) | 1/2 (50) | 0/1 (0) ** | 0/1 (0) ** |
| Jun. 28-Jul. 1st | 0/1 (0) ** | 0/1 (0) ** | 0/2 (0) | 0/2 (0) | 0/1 (0) ** | 0/1 (0) ** |
| Jul. 5-8th | 0/2 (0) | 0/1 (0) ** | 1/2 (50) | 0/2 (0) | 0/2 (0) | 0/2 (0) |
| Jul. 12-15th | 0/2 (0) | 0/1 (0) ** | 0/2 (0) | 0/2 (0) | 0/2 (0) | 1/2 (50) |
| Jul. 19-22nd | 0/2 (0) | 0/2 (100) | 0/1 (0) ** | 0/2 (0) | 0/2 (0) | 1/2 (50) |
| Jul. 26-29th | 1/2 (50) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) | 0/2 (0) |

aThere was no water in the manhole; b samples were not taken due to holidays; c frequency of sampling increased as a request from the University of health department.
for the high and relative constant detection rate cannot be given, in that only two positive COVID-19 cases were registered in November and four in January. This building had a restrictive policy during the study period, such as checking temperature to everyone passing through the building, tracking positive cases and positive contacts. However, our findings suggest that pre-symptomatic and asymptomatic cases that were not detected by the building health program, were contributing to the positive signal detected in wastewater at this sampling site.

Finally, point 5, corresponding to the Library, a public building with a broad timetable opening from 8:30 am to 9 pm and an expected higher circulation of individuals visiting and leaving the building, showed an average positive percentage of 20.6 % (N1) and 24.1 % (E) (Table 1). The highest levels of positive detection (N1 data) were observed during the first week of December (100 %), January (50 % during the first two weeks and 100 % during the last two weeks), when the second wave started after Christmas break, and the majority of the students attended their exams on campus.

Subsequently, detection of SARS-CoV-2 RNA decreased in February (12.5 %) and ended up negative from March to May. Yet, a second peak of virus load was detected in the first week of June (100 %) and July (50 % and 100 % during the first and third week, respectively) (Table 3). Once again, these results correlate well with the data of COVID-19 incidence on campus, where the number of positive cases decreased in February–March and increased in July, probably coinciding with the relaxation of restrictive measures (Table 2).

3.4. Wastewater surveillance of the student residence

Fig. 4 and Table 4 show the results of SARS-CoV-2 in wastewater collected at the student residence. A very similar temporal trend of SARS-CoV-2 in wastewater was observed for N1 and E. The highest viral load values recorded were $4.2 \times 10^7$ gc/L (N1) and $12.3 \times 10^7$ gc/L (E), corresponding to the first wastewater sample taken from the residence on November 3, and coinciding with the outbreak reported between October 27 and November 6 (the epidemiological aspects of this outbreak are discussed below in more detail). This outbreak helped us to review the sample collection frequency of the student residence, which was subsequently increased from two to three times per week i.e., Friday in addition to Tuesday and Thursday. In general, the highest detection rate of virus RNA was also observed in in every week of November, with near 100 % positive samples (for both N1 and E). In December, with almost an empty residence for nearly 2 weeks, due to the Christmas holidays, the detection frequency for N1 decreased to 66.6 % (in the first and third week) and 0 % (in the second and fourth week). For gene E, the detection frequency was 22.3 % (in the first, second and fourth weeks), reaching values of 66.6 % during the

### Table 4
Weekly detection of SARS-CoV-2 RNA in wastewater samples collected at the student residence between fall 2020 and spring 2021. Coloring was used to highlight high (red), medium-high (orange), medium-low (yellow) and low (green) values of frequency of detection of SARS-CoV-2 RNA in wastewater samples.

| Weeks          | Number of positive wastewater samples / Total number samples (Frequency of detection, %) |
|----------------|----------------------------------------------------------------------------------------|
|                |                                             | N1   | E                       |
| Nov. 2-6th     | 4/4 (100%)                                | 4/4 (100%)   |
| Nov. 9-13th    | 5/5 (100%)                                | 5/5 (100%)   |
| Nov. 16-20th   | 3/3 (100%)                                | 3/3 (100%)   |
| Nov. 23-27th   | 2/3 (66.6%)                               | 2/3 (66.6%)   |
| Nov. 30-Dec. 4th | 3/3 (100%)                           | 3/3 (100%)   |
| Dec. 7-11th    | 2/3 (66.6%)                               | 1/1 (22.2%)   |
| Dec. 14-18th   | 0/3 (0)                                   | 1/1 (22.2%)   |
| Dec. 21-23th   | 2/3 (66.6%)                               | 2/3 (66.6%)   |
| Dec. 28-30th   | 0/3 (0)                                   | 1/1 (22.2%)   |
| Jan. 4-8th     | 1/4 (25%)                                 | 1/4 (25%)   |
| Jan. 11-15th   | 1/4 (25%)                                 | 1/4 (25%)   |
| Jan. 18-22nd   | 3/3 (100%)                                | 3/3 (100%)   |
| Jan. 25-29th   | 3/3 (100%)                                | 3/3 (100%)   |
| Feb. 1-5th     | 3/3 (100%)                                | 3/3 (100%)   |
| Feb. 8-12th    | 1/1 (22.2%)                               | 1/1 (22.2%)   |
| Feb. 15-19th   | 1/1 (22.2%)                               | 2/2 (66.6%)   |
| Feb. 22-26th   | 1/1 (22.2%)                               | 1/1 (22.2%)   |
| Mar. 1-5th     | 1/1 (22.2%)                               | 1/1 (22.2%)   |
| Mar. 8-12th    | 0/3 (0)                                   | 0/3 (0)   |
| Mar. 15-19th   | 0/2 (0%) *                                | 0/2 (0%)   |
| Mar. 22-26th   | 1/1 (22.2%)                               | 1/1 (22.2%)   |
| Mar. 29-Apr. 2nd | 0/1 (0%) *                              | 0/1 (0%)   |
| Apr. 5-9th     | 0/3 (0)                                   | 0/3 (0)   |
| Apr. 12-16th   | 0/3 (0)                                   | 0/3 (0)   |
| Apr. 19-23rd   | 0/3 (0)                                   | 0/3 (0)   |
| Apr. 26-30th   | 0/3 (0)                                   | 0/3 (0)   |
| May 3-7th      | 0/3 (0) *                                 | 0/3 (0)   |
| May 10-14th    | ND *                                     | ND *   |
| May 17-21st    | 0/3 (0)                                   | 0/3 (0)   |
| May 24-28th    | 1/1 (22.2%)                               | 1/1 (22.2%)   |
| May 31-Jun. 4th | 1/1 (22.2%)                           | 1/1 (22.2%)   |
| Jun. 7-11th    | 2/3 (66.6%)                               | 0/3 (0)   |
| Jun. 14-18th   | 0/3 (0)                                   | 0/3 (0)   |
| Jun. 21-25th   | 0/2 (0%) *                                | 0/2 (0%)   |
| Jun. 28-Jul. 2nd | 0/1 (0%) *                              | 0/1 (0%)   |
| Jul. 5-9th     | 0/2 (0%) **                               | 0/2 (0%)   |
| Jul. 12-16th   | 0/2 (0%) *                                | 0/2 (0%)   |
| Jul. 19-23rd   | 0/2 (0%) *                                | 0/2 (0%)   |
| Jul. 26-30th   | 0/2 (0%) *                                | 0/2 (0%)   |

*a samples were not taken due to holidays; ** Sampling frequency was reduced due to staff availability; sampling frequency increased due to the outbreak declaration.

b It begins with the usual sampling frequency of 3 samples a week.

Fig. 4. Temporal trends in SARS-CoV-2 wastewater data on campus from wastewater sampling point at the student residence from November 2020 to July 2021.
third week. Interestingly, right after the holidays and when students returned for their exams, the number of positive detections in wastewater increased, which resulted in January being the second-highest month with a detection rate of near 100 % positive samples for both target genes (Table 4). This increase indicated the presence for SARS-CoV-2 in the wastewater samples that were representative of the students living in the residence as has been observed in similar studies (Betancourt et al., 2021). Despite the relative high number of positive cases of COVID-19 in January on campus (6.2 positive cases per 1000 population) (Table 2), no outbreak was declared based on clinical testing of individuals. However, the possibility of a new outbreak, perhaps from a population of pre-symptomatic and asymptomatic students (Buitrago-Garcia et al., 2020), was of concern.

In February to the end of the project, a steady decrease in SARS-CoV-2 RNA detection was observed in wastewater. In particular, a detection frequency of 22.3 % (N1) and between 22.3 % and 66.6 % (E) was observed in February, and 22.3 % (both N1 and E) in only the third week of March. By April 2021, SARS-CoV-2 RNA was not detected in wastewater and only few samples showed positive results in May and June (Table 4). These results also matched with the reduction of COVID-19 clinical incidence observed on campus during these months, which could be explained by the fact that a great proportion of student and staff were fully vaccinated by springtime. This is consistent with the results reported by (Bivins and Bibby, 2021), in that mass vaccination was coincident with decreased shedding of SARS-CoV-2 RNA into wastewater.

Regarding the epidemiological aspects of this outbreak, immediately after the student residence outbreak was declared (October/November 2020), targeted clinical testing (nasopharyngeal RT-qPCR) was conducted among all students living in the residence (250 students). As a result, 44 cases tested positive and all affected students were required to stay in quarantine in their rooms for 14 days (both positive tested, but also those with negative PCR results that were in close contact with positive cases in the previous 72 h). SARS-CoV-2 RNA detection in wastewater from the student residence began a week after the outbreak was declared and this allowed us to monitor the contribution of viral excretion from the COVID-19 isolated students to the campus-wide viral load. Determination of the effectiveness of these interventions (i.e., quarantines) is a very important aspect for epidemiologists as previously explained. SARS-CoV-2 RNA levels (target N1) in wastewater on sampling days are plotted against the 14-day cumulative number of newly reported COVID-19 cases (Fig. 5). We observed a high and constant positive detection while students were in quarantine at their room. In addition, we observed how the viral load decreased over time, as the number of cases reported in the last 14 days also decreased. The first sample without detection was observed when there were no longer any PCR declared cases in the last 14 days. Positive detection during this period would correspond to the quarantine period of those affected. As long as there were cases in quarantine, there was also viral detection in wastewater samples. Reports have demonstrated that viral shedding in feces can continue for over two weeks after symptoms have ceased (Chen et al., 2020; Walsh et al., 2020; Zheng et al., 2020) or even before the infection is identified by clinical testing (Jones et al., 2020). Although wastewater surveillance was not used in this case as an early-warning tool for the outbreak declared at the student residence, it was able to provide strong evidence, based on a sustained negative wastewater signal over time that the outbreak was successfully contained.

3.5. SARS-CoV-2 genome sequencing

A total of nine wastewater samples positive for SARS-CoV-2 by RT-qPCR (Ct < 33) were sequenced during this study. Samples corresponded to sampling points 4 (University residence, n = 5; named as S4 + “Nov”, “Dec”, “Jan”, “Feb” and “Mar”), 10A (confluence point North, n = 3; named as S10A + “Nov”, “Jan”, “Feb”), and 10B (confluence point South, n = 1; named as S10B + “Oct”). These samples showed Ct values of N1 SARS-CoV-2 target ranging from 23.65 to 34.12. Eight of the nine sequenced samples (88.8 %) showed percentages of 20× coverage values higher than 80 % (Fig. 6), with mean values of genome coverage of 85.8 ± 19.3 %. Mean depth values ranged from 166.9 in sample S4-Jan to 1436.4 in sample S4-Nov.

3.5.1. Overview of detected nucleotide substitutions and deletions

After sequence analysis, a total of 197 nucleotide substitution and 4 deletions were found in coding regions in relation to the reference genome MN908947.3. Among the nucleotide substitutions, 118 of them (55.9 %) corresponded to non-synonymous substitutions (Table 5, Table S1). Most of these substitutions (52.7 %) were present in the ORF1ab region. Alternative nucleotide frequencies are shown in Fig. 7. Different frequencies were found between sampling sites, showing different lineage mixtures. In this sense, several mutations with frequencies below 100 % were found in samples obtained at the confinement point North, while for the other two samples the number of mutations was lower. The highest frequencies were found in ORF10 region for all the samples, in the nucleocapsid protein gene in samples from the University residence, and in the nucleocapsid gene, ORF8 and ORF10 in samples from the confinement point S10A (Figs. 6 and 7). No nucleotide substitutions were found in coding regions for the envelope protein, the ORF6 protein or the ORF7a protein (Fig. 7). Among the detected deletions, three of them were located in ORF1ab (A3675–3676 and Δ1064 in sample S4-May, and Δ82–86 in sample 10A-Jan), while the remaining deletion corresponded to three nucleotides of ORF8 (Δ58 in sample 10B-Oct). Its position in relation to the coding regions.
of the SARS-CoV-2 genome is presented in Fig. 6. Deletion Δ3675–3676 and Δ82–86 have previously been described in Spain, deletion Δ1064 has not been described in Spanish sequenced genomes and deletion Δ58 has been detected worldwide but in low frequencies (<0.5, outbreak.info). Detection of low frequency or not described deletions is evidence for the usefulness of massive sequencing techniques for the study of new nucleotide variations.

3.5.2. Detection of Variants of Concern (VOCs) and Variants of Interest (VOIs)

Since the appearance of SARS-CoV-2, different lineages have emerged, of which certain variants have aroused clinical interest. Variants of Concern (VOCs) are variants that have been associated with higher transmissibility, higher virulence, and/or changes in response to health measures (diagnosis, vaccines, treatments) and Variants of Interest (VOIs) are variants that harbor mutations with phenotypic implications and cause community transmission or have been detected in multiple countries (https://www.who.int/publications/m/item/covid-19-weekly-epidemiological-update). Specific mutations of VOCs alpha, beta, gamma, and delta, and VOI lambda were searched for among the sequences obtained from the different samples. Samples corresponded to different sampling dates, allowing the analysis of the appearance of these mutations over time. In sample S4-May, four characteristic mutations of Lambda were found, corresponding to mutations ORF1ab:F2387V, S:G75V, S:L452Q, and S:F490S (Fig. 6). Additionally, other mutations present in lambda VOI but also in other lineages were found (i.e., ORF1ab:T1246I and N:P13L). All these mutations were found with 100% frequency. Except for sample S4-Jan, these mutations were not found in earlier samples, despite that coding regions that harbor these mutations were sequenced with high coverages (Fig. 6). The presence of these mutations in the S4-May sample and their absence in the chronologically earlier samples could indicate the introduction of this VOC between February and May 2021 (Fig. 6). Samples 10A-Jan and 10A-Feb showed the characteristic mutations of delta VOC ORF1ab:T3750I and M:I82T; however, sample 10A-Nov did not show these mutations (Fig. 6). Interestingly, in sample 10A-Jan these two mutations showed mean frequency values of 82.9 ± 2.2%, while in sample 10A-Feb these mutations reached frequencies of 100%. These results showed the introduction of the delta variant between November 2020 and January 2021 and its incremental increase in frequency in samples from January to February 2021 (Table S1). As shown in previous studies, massive sequencing allows for the detection of the introduction of new lineages and variants, as well as

Fig. 6. Genome coverage of the SARS-CoV-2 reference genome MN908947.3 (only nucleotides with depth higher to 20×) in logarithmic scale (max 4.5 log) for each sample. Red dots, non-synonymous nucleotide substitutions; Yellow triangles, deletions; blue diamond, characteristic mutations of VOI Lambda; green diamond, characteristic mutations of Delta VOC.
to students living on campus and the staff working at the student residence, the university made clear recommendations to the community, but mainly cation, surveys and taking temperature of its inhabitants daily. In addition, Hence, the surveillance of the residence was intensi-

tance size at classes.
e.g., at Economics between November 12 to December 2) and limiting at-

tendance size at classes.

## 4. Conclusions

In this paper, the strategy followed by the University Jaume I of Castellón (Spain), to monitor the evolution of SARS-CoV-2 within its campus through wastewater analysis is described and discussed. The
establishment of the wastewater surveillance program allowed reliable detection of virus RNA in different university areas (faculties, research institutes, spin-off companies, administration offices, student residence), even when COVID-19 incidence was very low. The probability of detecting SARS-CoV-2 RNA in wastewater depended on the COVID-19 incidence on campus and the number of visitors/occupants in the buildings. Wastewater analysis at the student residence illustrated its usefulness as an early warning tool in determining a potential surge in SARS-CoV-2 transmission among pre-symptomatic and asymptomatic students, even when clinical information could not confirm it. Subsequently, it also proved to be a reliable tool to track the evolution of an outbreak. Sequencing analyses showed the ability to detect new nucleotide variations that could be related with different phenotypic characteristics of the virus. The use of massive sequencing allows the detection of VOC introduction and the behaviour of these VOCs along the time.

This study was coordinated by a multidisciplinary team of professionals from different fields and disciplines (chemical and biological scientists and epidemiologists) along with professionals of the UJI’s health department and academic authorities, which was essential to integrate wastewater-based data as part of the universities response system against the pandemic. Overall, this work has shown that WBE can be used as early warning tool, but also as a complementary approach for detecting COVID-19 prevalence and transmission on a university campus without the need of individual testing.

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Availability of data

All relevant data generated or analysed during this study are included in the manuscript.

CRediT authorship contribution statement

Rosa de Llanos: Conceptualization, Project administration, Visualization, Supervision, Funding acquisition, Writing – original draft. Rocío Cejudo-Marín: Investigation, Methodology, Formal analysis, Writing – review & editing. Manuela Barneo: Investigation, Methodology,
Declaration of competing interest

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

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