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Tracking SARS-CoV-2 variants in wastewater in San Pedro de la Paz, Chile

Andressa S. Reis[a], Christian Castro[a], Paulina Assmann[b], Katherine Salgado[a], M. Estrella Armijo[a], María José Navarrete[c], Cesar Echeverria[c], Aldo Gaggero[d], Carlos Farkas[e] and Matias I. Hepp [a,e,*]

[a] Centro de Vigilancia de Aguas Residuales, Centinela Biobío, Facultad de Medicina, Universidad Católica de la Santísima Concepción, Concepción, Chile
[b] Centro de Astrofísica y Tecnologías Afines, Departamento de Astronomía, Universidad de Concepción, Casilla 160-C, Concepción, Chile
[c] ATACAMA-OMICS, Laboratorio de Biología Molecular y Genómica, Facultad de Medicina, Universidad de Atacama, 1532502 Copiapó, Chile
[d] Laboratorio de Virología Ambiental, Programa de Virología, Instituto de Ciencias Biomédicas, Universidad de Chile, Santiago, Chile
[e] Laboratorio de Investigación en Ciencias Biomédicas, Departamento de Ciencias Básicas y Morfología, Facultad de Medicina, Universidad Católica de la Santísima Concepción, Concepción, Chile
*Corresponding author. E-mail: mhepp@ucsc.cl

MIH, 0000-0003-0993-8794

ABSTRACT

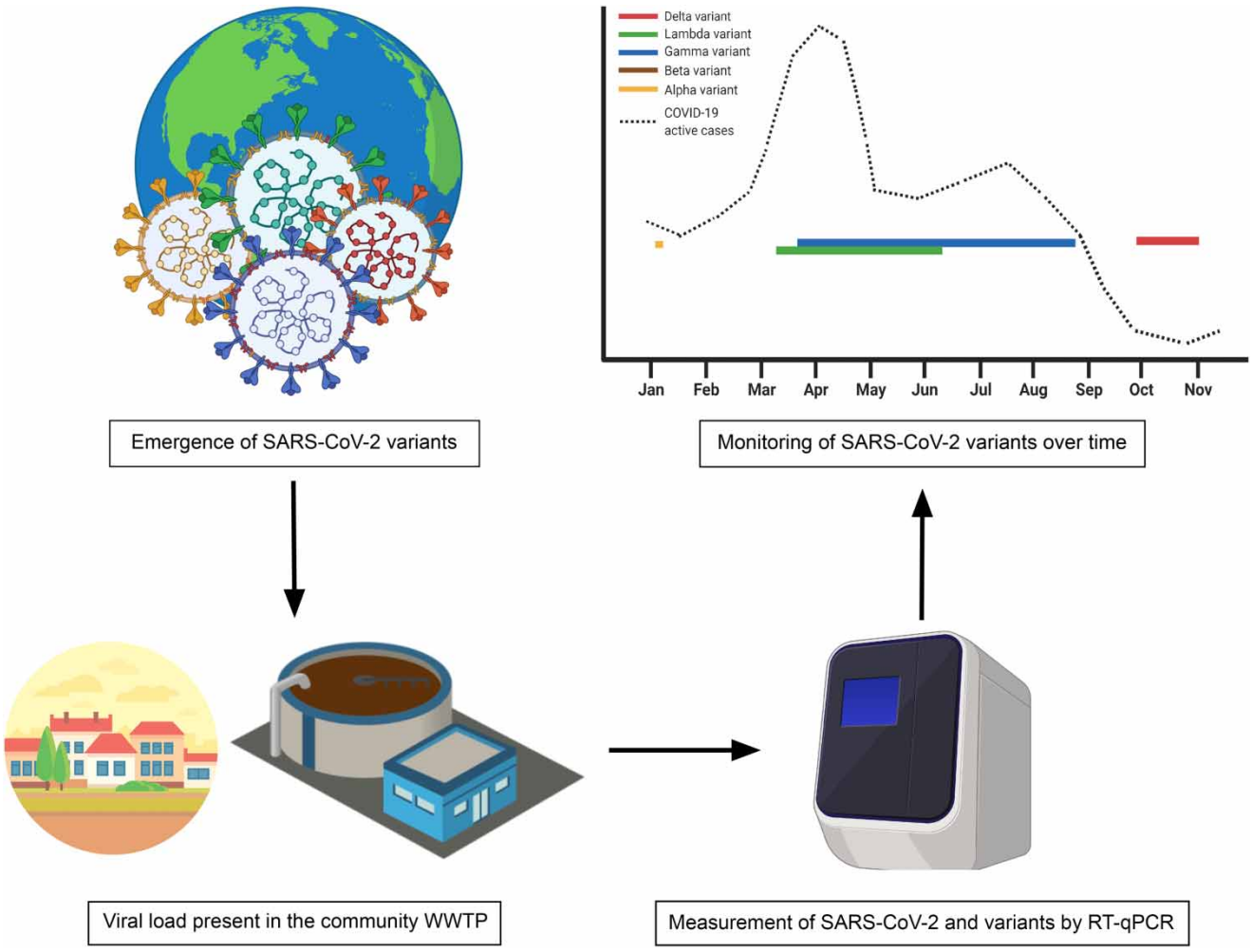
Studies have shown the presence of SARS-CoV-2 in the stool of both symptomatic and asymptomatic COVID-19 patients, enabling wastewater-based surveillance (WBS) to complement clinical monitoring. The emergence of variants can enhance viral transmissibility, highlighting the need for ongoing surveillance to detect and control infectious diseases. This study aimed to detect SARS-CoV-2 variants in wastewater from a treatment plant in San Pedro de la Paz, Chile, between January and November 2021. Wastewater samples were concentrated using the polyethylene glycol method, and RT-qPCR assays were performed to analyze SARS-CoV-2 and its variants (Alpha, Beta, Gamma, Lambda, and Delta), with results compared to Illumina amplicon sequencing. The concentration method achieved about 11% viral recovery. The detection of viruses and variants in wastewater proved sensitive and consistent with clinical data, providing additional surveillance insights. Notably, Lambda and Delta variants were the most frequently detected during the second and third infection waves, with some variants identified in wastewater before the first confirmed clinical cases. However, Illumina sequencing lacked sufficient genome coverage, suggesting the need for better sequencing methods for this matrix. This study demonstrates that WBS is a rapid, cost-effective tool for detecting SARS-CoV-2 and its mutations, particularly useful during overwhelming clinical situations or when cost is prohibitively high.

Key words: COVID-19, public health, RT-qPCR, SARS-CoV-2 variants, sewage surveillance

HIGHLIGHTS

- Wastewater-based epidemiology (WBE) is a fast and cost-effective approach to screen specific mutations of SARS-CoV-2 through RT-qPCR.
- The first study to track SARS-CoV-2 variants in wastewater in Chile.
- SARS-CoV-2 variants were detected in wastewater before the first confirmed cases.
- The variants were responsible for driving the second and third waves.
- Integrating RT-qPCR and broad-spectrum sequencing could enhance our capacity to monitor and control disease.

GRAPHICAL ABSTRACT



INTRODUCTION

Wastewater-based surveillance (WBS) has emerged as a viable and cost-effective tool that provides objective, real-time information about the health and well-being of communities (Daughton 2020; Hart & Halden 2020). During the Coronavirus disease 2019 (COVID-19) pandemic, various WBS programs and campaigns were established to monitor Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its variants, which has been crucial for informing decision-making in several countries, including the United States and various member states of the European Union (European Commission 2021; Wu *et al.* 2021). In the case of Chile, significant studies on wastewater surveillance focusing on the detection of COVID-19 have been carried out since July 2020 in different communities. These investigations have proven to be a valuable source of new information regarding the propagation and circulation of the virus within the population (Ampuero *et al.* 2020; Gallardo-Escárate *et al.* 2021; Olivares-Pacheco *et al.* 2022). The results of these studies, along with others conducted in North and South America, Europe, and Oceania, demonstrate that WBS is an effective early warning system for identifying diseases (Medema *et al.* 2020a, 2020b; Polo *et al.* 2020; Wurtzer *et al.* 2020; Ahmed *et al.* 2021; Giraud-Billoud *et al.* 2021; Prado *et al.* 2021). This system allows for the assessment of disease incidence or presence by analyzing concentrations of SARS-CoV-2 in community wastewater.

Despite the efforts deployed to implement containment measures and carry out mass vaccination campaigns, the constant emergence of new variants of interest (VOI) and variants of concern (VOC) underscores the urgent need to establish long-term surveillance systems. These systems are essential not only for monitoring and controlling the COVID-19 pandemic but also for identifying and managing outbreaks of other emerging diseases. VOCs of SARS-CoV-2 exhibit groups of

mutations in the spike protein, which can increase the virus's transmissibility (Davies *et al.* 2021) and confer greater resistance to neutralization by antibodies generated by vaccines (Harvey *et al.* 2021; Wang *et al.* 2021). In this context, WBS can be considered a key tool for conducting real-time community-level surveillance, enabling the implementation of effective public health measures to control COVID-19 and future pandemics.

Several studies have utilized next-generation sequencing (NGS) in WBS to detect emerging variants of SARS-CoV-2 (Martin *et al.* 2020; Bar-Or *et al.* 2021; Crits-Christoph *et al.* 2021; Fontenele *et al.* 2021; Izquierdo-Lara *et al.* 2021). However, the high cost of this method renders it a less viable option in many resource-limited countries, such as those in Latin America and other developing nations, where the complexity of the sample matrix adds an additional level of difficulty. In contrast, the use of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays with TaqMan probes presents a more accessible alternative, offering rapid and reliable results, high precision, lower cost, and easier data analysis. Previous studies have developed RT-qPCR assays specifically designed for detecting variants of SARS-CoV-2 in wastewater samples (La Rosa *et al.* 2021; Lee *et al.* 2021). The widespread implementation of this technique, particularly in developing countries, could be significantly facilitated by using commercial kits produced by clinical diagnostic companies, given their good availability and rapid supply.

The aim of this study was to analyze the appearance of several SARS-CoV-2 variants (VOC and VOI) in wastewater samples collected between January and November 2021 in San Pedro de la Paz, Chile. For this, we utilized the TaqPath COVID-19 kit (Thermo Fisher Scientific, USA), commonly applied to human saliva samples, to detect mutations and/or deletions of five variants of the virus in wastewater samples. Although this method presents certain challenges due to the proprietary details of the primers and probes (Thermo Fisher Scientific, USA), which complicates more precise quantification of the SARS-CoV-2 variants, it remains possible to monitor the emergence, circulation, and transmission of these variants. This is achieved because the technique employs a workflow similar to that of RT-qPCR diagnostic assays for SARS-CoV-2. Through this method, we were able to verify the emergence and displacement of five SARS-CoV-2 variants (Alpha, Beta, Gamma, Lambda, and Delta) in San Pedro de la Paz, a city with an approximate population of 140,000 habitats and a well-connected sewage system covering the entire community, located in The Great Concepción, one of the largest and most important urban areas in Chile. This study highlights WBS as an effective, rapid, and impartial approach to tracking variants of SARS-CoV-2 in communities using RT-qPCR assays. Our proposal is especially relevant for developing countries where NGS techniques are not easily accessible or economically viable. The implementation of WBS could significantly contribute to improving public health responses to pandemics and disease outbreaks, benefiting the health of communities in a challenging global context.

MATERIAL AND METHODS

Study site and wastewater samples

The samples used in this study were obtained from the wastewater treatment plant (WWTP) located in San Pedro de la Paz, Biobío Region, Chile (Figure 1), which serves a population of 139.174 people.

Approximately 2 L of 24-h composite samples of influent water were collected by an automatic sampler (compact composite sampler (GLS) model), once a week. The sampling period began on 14th January 2021, and ended on 28th November 2021. The wastewater samples were delivered to our laboratory and processed on the same day of collection. Samples were immediately stored at 4 °C to preserve ribonucleic acid (RNA) integrity. The pH value of the wastewater collected from the WWTP was between the range of 7.2–8.2.

Virus concentration and RNA extraction methods

In total, 120 mL of each wastewater sample with neutral pH (6.0–7.0) were concentrated to a volume of 1 mL, by precipitation with polyethylene glycol 8000 (PEG). This precipitation method is commonly used to concentrate viruses from waterborne matrices (Wu *et al.* 2020). PEG and NaCl were added to the specific volume of the original sample in proportions of 10 and 2% w/v, respectively. The sample was then placed in centrifuge tubes and incubated at 20 °C for 15 min on an orbital shaker set at 150 rpm. After incubation, the tubes were centrifuged at 8,000 × *g* for 30 min at 4 °C, obtaining a pellet. Most of the supernatant was discarded and the remaining 5 mL, centrifuged at 8,000 × *g* for 5 min at 4 °C, generated a final pellet (1 mL), which was resuspended in 800 μL of TRIzol (Thermo Fisher Scientific, USA). Then, 200 μL of chloroform was added, and the sample was incubated for 2–3 min and centrifuged at 12,000 × g for 15 min at 4 °C. The upper aqueous phase was transferred to a new column, and RNA extraction was performed using the E.Z.N.A. total RNA Purification System

Figure 1 | Location of WWTP in San Pedro de la Paz, Biobío Region, Chile. This figure shows a sequence of magnifications, beginning with South America and ending with San Pedro de la Paz, Chile. The image on the left shows South America with central Chile in the red square. In the middle bottom image, the south-central region of Chile is shown, with an area of the Biobío region indicated in the red box. In the upper middle image, the sector of San Pedro de la Paz is indicated by the red box. Finally, the image on the far right shows a detailed image of San Pedro de la Paz, with the red point indicating the location of the WWTP where the samples were taken (maps obtained from http://www.geoportal.cl/visorgeoportal/, Ministerio de Bienes Nacionales de Chile).

(Omega, USA) according to the manufacturer's instructions, generating RNA samples of 100 μL (elution). All RNA samples were stored at −80 °C and subjected to RT-qPCR analysis on the same day of RNA extraction to avoid damage and/or degradation of the samples associated with storage, as well as freezing and thawing of RNA samples.

Virus recovery

Pseudomonas phage PP7 (Bacteriophage PP7) was used to estimate SARS-CoV-2 recovery (Fumian *et al.* 2010) for biosafety reasons (Kitajima *et al.* 2020). For this, a sample of one liter of random wastewater was inoculated with 10^4 plaque forming units (PFU)/mL of the phage PP7, previously quantified (10^{13} PFU/mL). The recovery was calculated against an aliquot of the initial inoculum, corresponding to the same sample volume (120 mL) of our concentration protocol. We performed the method based on centrifugation with and without PEG precipitation to concentrate and then detect the phage PP7 and obtain genome recovery. A previously described monoplex qPCR assay (Rajal *et al.* 2007) was used to detect the PP7 genome with a Light Cycler multiplex RNA virus master mix on a LightCycler 480 (Roche). The sensitivity of the qPCR assay was evaluated by a standard curve generated using nucleic acid purified from 10^{13} PFU/mL of phage PP7, equivalent to 82 ng/μL. The amplification efficiency for this polymerase chain reaction (PCR) was 104%. The limit of detection (LOD) was 4.1 pg/μL. The phage PP7 recovery efficiency of the method was calculated based on the copies quantified by RT-qPCR as follows:

$$\text{Recovery efficiency } \% = \frac{\text{Total viral RNA gene copies recovered} \times 100}{\text{Total viral RNA gene copies seeded}}$$

The mean and standard deviation for the concentration method was calculated.

Virus detection and quantification

The detection of viral RNA in wastewater samples by RT-qPCR targeted the SARS-CoV-2 viral gene. They were identified using a real-time fluorescent RT-qPCR kit to detect SARS-CoV-2 (SARS-2019-nCoV, BGI Genomics Co. Ltd, China) on a LightCycler 480 (Roche), according to the manufacturer's instructions. The thermal cycling variables used were as follows: 40 cycles of 95 °C for 10 s and 60 °C for 20 s. In addition, we compared different reaction volumes to obtain the best efficiency since the matrix used is very variable and can affect the PCR. Quantification of our samples was established by constructing a standard curve using a positive control sample provided with the kit, made based on RNA standards. With this material, the slope of the ORF1ab region of the SARS-CoV-2 genome standard curve and the β-actin region of the human gene were -3.417 and -3.574, respectively. The Y-intercept values were 36.84 (ORF1ab) and 35.05 (β-actin). The amplification efficiencies for these two assays were 96% (ORF1ab) and 92% (β-actin). The LOD was 0.65 copies/μL for ORF1ab and 1.3 copies/μL for β-actin. Which was obtained in a practical way by carrying out serial dilutions of different concentrations until linearity was lost. The latter allows us to define PCR inhibition factors. Each concentration and PCR was made twice.

Detection of virus variants

The detection of the specific mutations of SARS-CoV-2 RNA in the wastewater samples by RT-qPCR was targeted to the S (SPIKE) viral gene. Using a TaqMan™ SARS-CoV-2 Mutation Panel (Thermo Fisher Scientific, USA), the kit compared a reference and mutant sequence and was run and analyzed on a QuantStudio 3 (Thermo Fisher Scientific, USA). Specifically, probes targeting six individual mutations were employed, of which one probe was used for the Alpha, Beta, Gamma, and Lambda variants and two probes for the Delta variant (see Table 1). We performed the genotyping analysis through the Design and Analysis Software 2.6 program (Thermo Fisher Scientific, USA). Each PCR was made in duplicate. In addition, different dilutions were tested to analyze the detection efficiency in samples, and the PCR inhibition was established by serial dilutions.

Quality control

To minimize qPCR contamination, the RNA extraction and the RT-qPCR setup were performed in separate laboratories. A blank sample was included for each concentration method and also included during nucleic acid extraction to account for any contamination during extraction. No contamination was detected in our analyses.

Sequencing analyses

The raw Illumina amplicon-seq datasets were first subjected to trimming using Fastp (Chen *et al.* 2018), where each pair of R1 and R2 files generated trimmed outputs. The trimmed reads were then aligned to the SARS-CoV-2 reference genome (NC_045512.2) using minimap2 in short-read alignment mode (Li 2018). This alignment was immediately followed by sorting and indexing of the BAM files with SAMtools (Li *et al.* 2009). Variant calling was performed on these sorted BAM files using Freebayes (https://github.com/freebayes/freebayes), where the parameters were specifically adjusted to recognize a minimum allele frequency of 0.1 to enhance the detection accuracy of viral variants. The resultant variant calling files (VCFs) were then merged using Jacquard (https://github.com/umich-brcf-bioinf/Jacquard), which included all variant information from the input files, followed by genotype filtering to refine the data to only necessary genetic information using the vcflib library (https://github.com/vcflib/vcflib). Further processing included alignment and refinement of the VCF entries using

Table 1 | List of analyzed variants of SARS-CoV-2

Variant	Variant type	Gene S mutation
Alpha	VOC	del 69-70
Beta	VOC	del 242-244
Gamma	VOC	K417T
Delta	VOC	L452R
Delta	VOC	P681R
Lambda	VOI	L452Q

tools like vcfleftalign, which ensured correct alignment with the reference genome, and vcffixup for adjusting entries based on the reference comparison. Viral frequencies were calculated by excluding problematic sites.

Visualization of the processed data was achieved with pyGenomeTracks (Lopez-Delisle *et al.* 2021), where BAM files were converted to BigWig format using the bamCoverage package from deepTools (Ramirez *et al.* 2014). The parameters set included a bin size of 10 and normalization using counts per million, using an effective genome size of 29,903 bases of the SARS-CoV-2 genome. These tracks were then used to generate genomic heatmaps and tracks to effectively interpret and present the sequencing data.

Post-processing included the annotation of detected variants with SnpEff, configured specifically for SARS-CoV-2 on Galaxy (usegalaxy.org), to elucidate the potential impacts of mutations at the protein level. This annotated data were combined with probed-based data on Python to map mutations to specific samples. Once the data frame was built, we compared NGS-based variants versus probed-based variants, and we fitted a logistic regression to model the relationship between sequencing coverage and the probability of mutation detection. This modeling helped in establishing the efficiency of amplicon-seq in detecting SARS-CoV-2 variants from wastewater samples.

RESULTS

Recovery efficiency

After three independent concentrations with or without the PEG precipitation, the qPCR assay detected a mean of 0.018 ng of PP7 genome from a raw sewage load with 0.164 ng of PP7 (3×10^4 PFU/mL). The percentages of recovery were 10.99% with PEG precipitation and 0.78% without it (Figure 2).

Viral RNA detection in wastewater samples

The viral RNA normalized concentration range from January to November 2021 in San Pedro de la Paz is detailed in Figure 3.

The first peak of concentration of the COVID-19 virus was detected on 11 March 2021 (see Figure 3), 2 weeks before the peak of active cases (566 active cases on 26 March 2021) in the city. Following the Paso a pasö program implemented by the Chilean government to control the spread of the COVID-19 virus, communities with high infection rates were required to implement either full 7-day home lockdowns with only essential services available (Phase 1), or home lockdowns on weekends only, with limited daily occupancy of commercial establishments (Phase 2). The highest concentrations of the SARS-CoV-2 virus coincided with the most critical period of the outbreak in the city, in which the full 7-day lockdown was

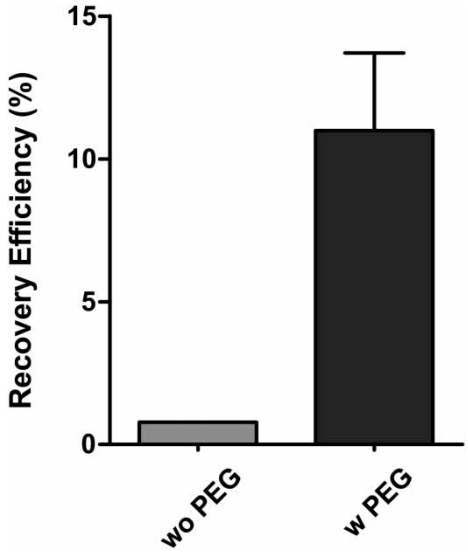


Figure 2 | Recovery efficiency percentage of viral concentration. The graph represents the efficiency percentage of the *Pseudomonas Φ7* (PP7) genome recovery in samples without PEG 8000 precipitation (wo PEG) or with PEG precipitation (w PEG). The assay was performed in triplicate.

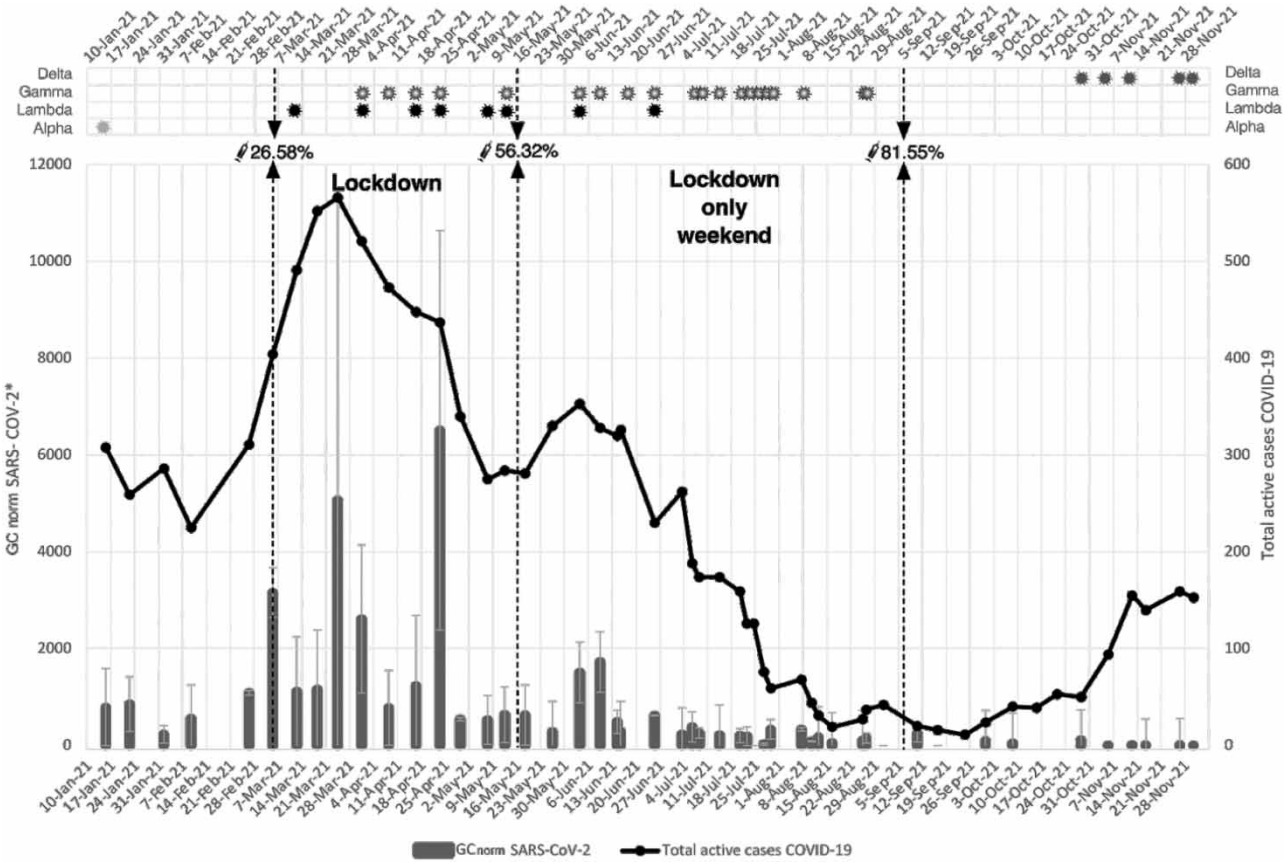


Figure 3 | Detection of SARS-CoV-2 and presence of variants in wastewater samples. Samples were taken from 14th January to 30th November in San Pedro de la Paz, Biobío Region, Chile. The graph shows the detection of SARS-CoV-2 genome copies/normalized against human charge (genome copies (GC) norm) in the wastewater, the number of total cases of COVID-19, and the detection of different variants on San Pedro de la Paz from 15 January to 29 November 2021. The gray bars represent the viral load detected for this pathogen. The values of the GC norm are plotted on the left axis. The black line shows the number of total cases for this city, with the number of positive cases plotted on the right axis. The upper part of the figure shows the variants detected in wastewater and the respective dates. The percentage of the vaccinated population (syringe image with a number) and the type of city-wide lockdown during each period are indicated.

implemented for 2 months, between 6 March and 10 May 2021 (Figure 3). At the start of the lockdown, 26.58% of city residents were vaccinated with two doses or a single dose, and at the end, this exceeded 56% of the population (Figure 3). After the end of this lockdown, between 11 May and 30 August 2021, the city protocol changed from Phase 1 to Phase 2, consisting of a lockdown only on weekends (Figure 3). At the beginning of this phase, there was an increase in the number of cases (Figure 3). During this phase, compared to the previous one, there was a decrease in both the number of active cases and the COVID-19 virus concentrations detected in the city's wastewater (Figure 3).

As of September 2021, after the weekend lockdown, more than 80% of the San Pedro de la Paz population had received two or a single vaccine dose, and the lowest COVID-19 virus concentrations were detected in the city's wastewater and the lowest number of active cases throughout the period analyzed (Figure 3).

Out of a total of 53 wastewater samples collected from areas with confirmed COVID-19 cases during the pandemic, 49 samples (92.5%) tested positive for the presence of the SARS-CoV-2 virus. This high detection rate demonstrates the reliability and effectiveness of our method for community-level virus monitoring.

The concentrations of SARS-CoV-2 in the positive samples ranged from 3.916 to 11887.566 copies normalized, with a mean concentration of approximately 666.3 copies normalized and a standard deviation of 278.9 copies norm. The 95% confidence interval for the mean concentration is between 387.4 and 945.2 copies/L. This variability and confidence interval reflects fluctuations in the viral load within the population over time, likely influenced by changes in infection rates and public health interventions.

Virus variants detection

All four variants analyzed were detected in the wastewater of San Pedro de la Paz, except for the Beta variant (Figure 3), which has not been detected in community cases in Chile. The first VOC to be detected in San Pedro de la Paz was Alpha (PANGO lineage B.1.1.7). This variant was only detected on 15 January, which also corresponds to the start of the second wave of the COVID-19 outbreak in San Pedro de la Paz (Figure 3). The first reported case of the Alpha variant in Chile was on 15 December 2020. In the Region of Biobío, where San Pedro de la Paz is located, the first clinical case was reported only in April 2021 (Minsal Reporter).

The next variant detected in the San Pedro de la Paz wastewater was the Lambda (PANGO lineage C.37), on 12 March 2021, more than 2 weeks before the first clinical case was reported on April 4 (Minsal Reporter). This variant was consistently present in the wastewater samples from then until 25 June 2021, which is precisely the time period with the highest rates of cases in the city (Figure 3).

The Gamma variant (PANGO lineage P.1) was detected for the first time in the sample collected on 30 March 2021, while the first known case imported into Chile was registered on 30 January 2021 (Minsal Reporter). Until 13 March, there was no reported case of this variant in the Region of Biobío. This changed on 24 March, when the first case was confirmed in the city of San Pedro de la Paz in clinical testing. The Gamma variant was present in 38% of the wastewater samples from the end of March until 26 August 2021 (Figure 3).

For 8 weeks following 26 August, none of the five analyzed variants were detected. On 27 October 2021, the Delta variant (PANGO lineage B.1617.2) was detected in the wastewater of San Pedro de la Paz for the first time and was the only variant detected in the following weeks until the end of November (Figure 3) when the study ended. The first clinical case of the Delta variant in San Pedro de la Paz was reported on 6 August 2021 (Minsal Reporter).

Evaluating SARS-CoV-2 detection by amplicon sequencing in environmental samples

We conducted a comprehensive benchmarking of SARS-CoV-2 probe detection utilizing standard amplicon sequencing across various samples from San Pedro de la Paz, Bío-Bío, Chile (see Figure 4). Panel (a) shows whole genome coverage of SARS-CoV-2, with particular emphasis on the spike protein region (positions 21,492–25,259). The coverage graph demonstrates a highly variable depth of sequencing achieved across the SARS-CoV-2 viral genome, as previously described (Jacot *et al.* 2021; Nicot *et al.* 2023; Lipponen *et al.* 2024). In panel (b), a comparative analysis of the presence or absence of key viral variants detected by both probe-based methods and NGS is presented. This comparison notably highlights the founder effects observed through variants Gly339Asp and Lys417Asn, which are expected results from geographically related samples (Farkas *et al.* 2020). Panel (c) features a Sankey diagram that traces the mutations detected by probes to their verification status in NGS, directly linking mutations to their respective samples. The efficiency of amplicon sequencing in matching probe detection was achieved in two out of seven samples. Finally, panel (d) introduces a logistic regression model that elucidates the relationship between sequencing coverage and the probability of mutation detection. This model determines the necessary coverage threshold to achieve a 50% detection probability, demonstrating a positive correlation between sequencing depth and reliable variant identification. Despite increased sequencing efforts, amplicon sequencing did not reach the 50% efficiency threshold, suggesting that alternative technologies such as shotgun metagenomic sequencing might provide better detection rates through more uniform coverage (Nicot *et al.* 2023; Kandel *et al.* 2024).

DISCUSSION

In the following paragraphs, we discuss the WBS to track the Alpha, Lambda, Gamma, and Delta variants discussed in the above section, following roughly their chronological detection in Figure 3, as compared to the appearance of these variants in Chile and the world. The Beta variant was undetected at the community-level during the entire period in Chile, appearing only in four travelers arriving from abroad (MINSAL 2021a).

Alpha variant

The first sample of the Alpha variant, PANGO lineage B.1.1.7, to be documented was in the United Kingdom in September 2020 and the first reported clinical case of the Alpha variant in Chile was on 15 December 2020 (MINSAL 2021a) in a traveler returning to Chile. In February 2021, 10 community cases of B.1.1.7 had been detected out of 133 samples sequenced (MINSAL 2021b). These cases were distributed among the regions of Coquimbo, Metropolitana, and Ñuble (MINSAL 2021b). In the Region of Biobío, where the city of San Pedro de la Paz is located, the first community case was confirmed

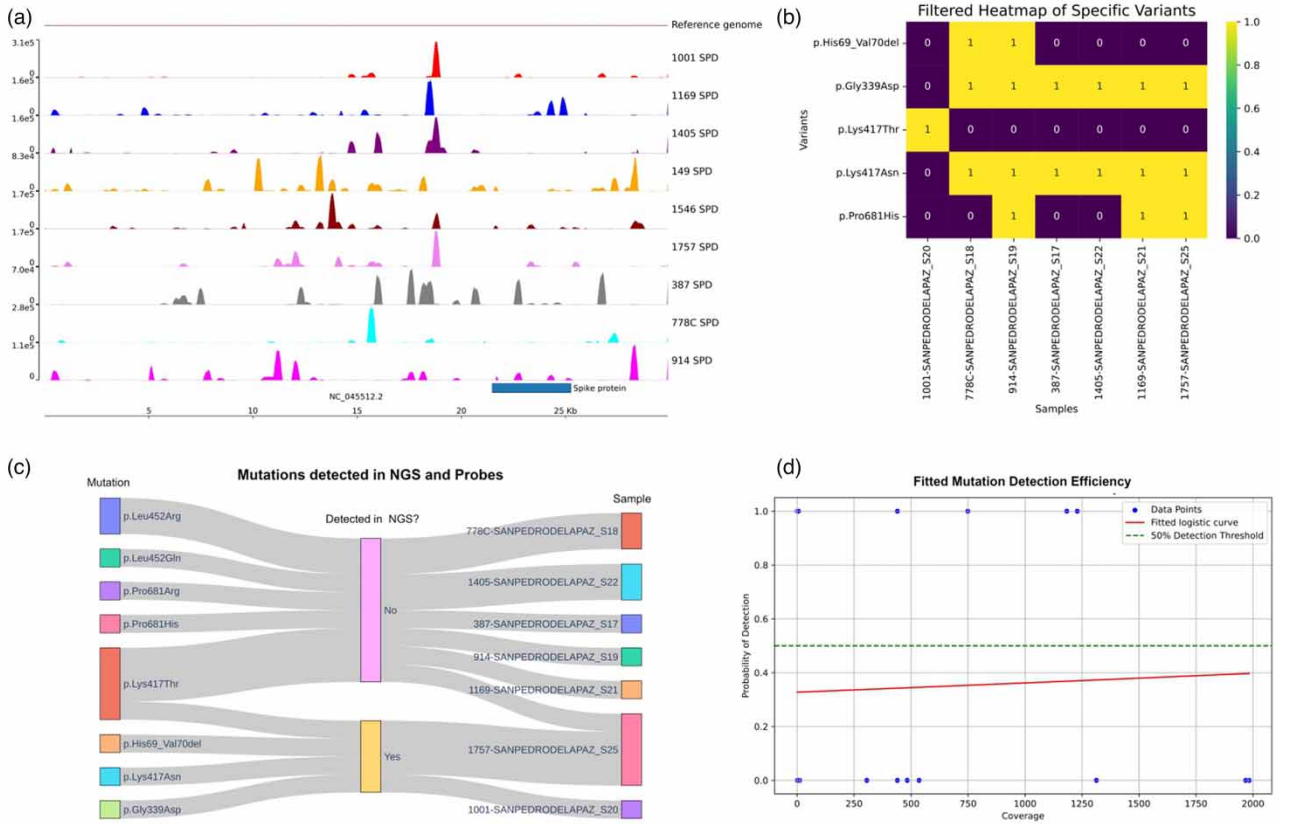


Figure 4 | Benchmarking of SARS-CoV-2 probe detection by amplicon sequencing. (a) Genome coverage for each sample was calculated using deepTools (counts per million (CPM) normalization). pyGenomeTracks was utilized to display the sequencing depth across the viral genome with a specific emphasis on the spike protein region (positions 21,492 to 25,259). (b) The presence or absence of key viral variants as detected by both probe-based methods and NGS offers a comparative view of the methodologies. Notably, the founder effect across samples can be observed by inspecting variants Gly339Asp and Lys417Asn, respectively. (c) Sankey diagram depicting the mutations detected by probes and their confirmation status in NGS, directly linking to the associated samples, thus providing a visual representation of detection efficiencies. (d) A logistic regression model is plotted to demonstrate the relationship between sequencing coverage and the probability of mutation detection. The model elucidates the coverage threshold necessary to achieve a 50% probability of detecting mutations, emphasizing adequate sequencing depth in reliable viral variant identification. Despite increased coverage, amplicon sequencing failed to reach 50% efficiency, suggesting that other technologies like shotgun metagenomic sequencing may surpass this threshold.

only in early April 2021 (MINSAL 2021b). However, our analysis indicates that the Alpha variant was already present in San Pedro de la Paz since January 2021, when it was detected with the del69-70 probe for the first and only time in the wastewater collected on the 15th of that month. This difference between the date of the first reported case (in April) in the region and the detection of this variant in wastewater in the region's city (in January) may be a result of the low number of new COVID-19 cases sequenced in the Region of Biobío, between December 2020 to March 2021 (25 of 58,855 new cases, see MINSAL 2021c). This highlights the valuable contribution of wastewater monitoring to health surveillance at both the local and national levels to anticipate and understand the emergence and circulation of SARS-CoV-2 variants in the community, as evidenced here for the Alpha variant.

According to the Global Initiative on Sharing All Influenza Data (GISAID), compared to all other continents and the United Kingdom, South America had the lowest percentage of VOC Alpha GRY (B.1.1.7 + Q.*), with a peak of 8.6% of total COVID-19 cases sequenced ($n = 2{,}189$) from 15 countries from 29 March to 4 April 2021. In Chile, the Alpha variant had a peak of 13% of cases sequenced in April ($n = 820$) (MINSAL 2021a) and in November corresponded to 0.01% of the sequenced cases ($n = 15{,}427$) (MINSAL 2021c). In the Region of Biobío, from January to November 2021, the Alpha variant corresponded to 0.3% ($n = 22$) of all cases analyzed by sequencing ($n = 1{,}152$) and by RT-PCR ($n = 5{,}162$) (MINSAL 2021c). However, from August to November 2021, the Alpha variant was not detected in any of the cases analyzed ($n = 5{,}636$), so the

22 cases reported in the region correspond to 5.7% of the total cases analyzed until July 2021 ($n = 381$) (MINSAL 2021c). In the wastewater sampled in San Pedro de la Paz, Region of Biobío, from the second half of January to the end of November 2021, the Alpha variant was no longer detected, which also coincides with the low incidence of COVID-19 cases with this variant in the city.

Lambda variant

The Lambda variant GR/452Q.V1 (C.37 + C.37.1), first detected in August 2020 in Peru, had the highest percentage of 0.6% on 7 June 2021, of the total sequenced cases ($n = 60,820$) in 140 countries (GISAID). South America (15 countries included) had the highest percentage of Lambda variant cases (346 of 3,446, or 9.8%) on 14 June 2021, which was 24.5 times higher than the second-highest percentage of 0.4% of the total cases sequenced ($n = 15,990$) in North America (18 countries included) on 21 June 2021 (GISAID). In Chile, the first four cases of the Lambda variant (PANGO lineage C.37) were identified on 20 January 2021 (MINSAL 2021d). The circulation of this variant increased in March and continued to rise until April, with 32.2% of the 903 sequenced cases (MINSAL 2021c). In July 2021, the Lambda variant was the second most frequent variant with 16.8% of the 1,843 sample cases analyzed in the country and the most frequent with 19.4% in the Region of Biobío in community cases (MINSAL 2021a). In San Pedro de la Paz, Region of Biobío, the first case of the Lambda variant was reported on 4 April 2021. However, we detected this variant, with L452Q, in the wastewater sampled on 11 March 2021, a week before the peak in active cases in the city, which corresponded to the second wave of COVID-19 infections (see Figure 3). This result indicates that this variant has already been present since March in San Pedro de la Paz, and this variant may be associated with the increase in community transmission rates, as also seen in other countries (World Health Organization 2021). In addition, the Lambda variant showed an increase in prevalence over a 299-long period of time, together with a higher incidence of COVID-19 cases in Chile (MINSAL 2021d). This can be verified both in the data reported at the national level (MINSAL 2021d) and at the local level, where in Figure 3 we can see that this variant was detected from 11 March to 25 June 2021, coinciding with the highest incidence of COVID-19 cases in San Pedro de la Paz during the time period of the present study.

Gamma variant

The Gamma variant GR/501Y.V3 (P.1 + P.1.*) was more frequent in South America (15 countries included) than in other continents, reaching a peak of 76.9% ($n = 3,672$) of sequenced cases ($n = 4,685$) between 28 June and 4 July 2021 (GISAID). This percentage was 9.5 times higher than the peak of 8.1% ($n = 5,765$) of total cases sequenced ($n = 60,820$) in countries between 7 and 13 June 2021 (GISAID). In Chile, the peak frequency of this variant was 75.36% ($n = 1,058$) of sequenced cases ($n = 1,004$) in April 2021 (MINSAL 2021c). The Gamma variant (PANGO lineage P.1) was documented in November 2020 in Brazil, and the first case in Chile was on 23 January 2021, based on a sample taken at the airport aimed at travelers from Brazil, and sequenced by the Public Health Institute of Chile (MINSAL 2021c). Two months later, in the report on 26 March, there were 45 cases of the P.1 variant reported out of 931 cases sequenced from December 2020 to March 2021. Of these 45 cases, 34 correspond to travelers, eight to community cases, and three to secondary cases, distributed among the regions of Antofagasta, Coquimbo, Valparaíso, Metropolitana de Santiago, Maule, Biobío, Los Rios, and Los Lagos (MINSAL 2021e). In the Region of Biobío, the peak frequency of the Gamma variant was 78.8% ($n = 41$) of sequenced cases ($n = 52$) (MINSAL 2021f) in early August (between 1 and 7 August). However, the peak of new cases of COVID-19 in the Biobío region was on 26 March, 2021, reaching 65 cases per 100 thousand inhabitants ($n = 1,014$, https://www.gob.cl/pasoapaso/cifrasoficiales/). The peak of new cases of COVID-19 was 4 months before the peak of sequenced cases of the Gamma variant in the region. This time difference in the frequency peaks of the Gamma variant and new COVID-19 cases suggests that this variant was not the cause of the peak in infections, and/or the number of sequenced case samples was scarce during the period from December 2020 to March 2021 ($n = 25$ in Region of Biobío, MINSAL 2021f), to the point that it is not possible to identify which variant(s) triggered and caused the peak in the second wave of COVID-19 in the Region of Biobío.

The second wave of COVID-19 in San Pedro de la Paz began in December 2020, when the documented infection rate reached 123 new cases per week per 100 thousand inhabitants. It ended in July 2021 with fewer than 121 new weekly cases per 100 thousand inhabitants, with a peak of 389 new weekly cases per 100 thousand inhabitants between 14 and

20 March 2021 (https://www.gob.cl/pasoapaso/cifrasoficiales/). On 24 March 2021, the first case of the Gamma variant was confirmed in the city. Six days later, on 30 March, we detected this variant for the first time with K417T in the city wastewater sample. The Gamma variant was detected more frequently when compared to the other four analyzed variants (Alpha, Beta, Lambda, and Delta); it was present in 38% of the wastewater sampled ($n = 50$) in San Pedro de la Paz from January to November 2021 (Figure 3). From March to August 2021, the Gamma variant was constantly detected in the wastewater of San Pedro de la Paz, having been detected for the last time on 26 August 2021 (Figure 3). The same occurred with the Lambda variant, which was constantly detected between 11 March and 25 June in the city's wastewater samples (Figure 3). Thus, according to our data from the wastewater monitoring in San Pedro de la Paz, Region of Biobío, the second wave of COVID-19 in this city was driven, initially, by the original virus and/or another lineage, indicated by the detected reference gene of the SARS-CoV-2, and by the Alpha variant, detected in early January 2021. Until then, between 10 December and 5 March, the city schools were on summer vacation, with home lockdown only on weekends, and the percentage of complete vaccinations (two doses or single dose) was lower than 20%. These are reasons that might have facilitated the higher incidence of transmission of the virus in the community. The successive increase in new cases in San Pedro de la Paz was at the end of February 2021, with more than 400 new cases per week, reaching a peak in March 2021 with 573 new cases per week (https://www.gob.cl/pasoapaso/cifrasoficiales/). This increase must have been caused by the appearance of the Lambda variant, detected on 11 March in the city's wastewater samples, and soon after with the Gamma variant, detected on 30th March, even though the city had been in a full 7-day home lockdown since 5 March and with a complete vaccination percentage greater than 26% (Figure 3). However, there was a significant decline in cases from April to 10 May 2021, in which the city was still on full lockdown and the complete vaccination percentage reached over 50% (Figure 3). As of 11 May 2021, the lockdown in the city was implemented only on weekends, and there was a prompt rise of new cases 361 of COVID-19 in the following weeks (more than 300 new cases weekly, https://www.gob.cl/pasoapaso/cifrasoficiales/). This fact demonstrates the contagious potential of the Gamma variant, as it is 1.7–2.4-fold more transmissible (Faria *et al.* 2021), which in conjunction with the long-term presence of the Lambda variant (World Health Organization 2021), contributed to the increase in the incidence of COVID-19 cases in San Pedro de la Paz, shortly after the reduction of distancing measures. However, despite the continued detection of the Gamma variant in the city's wastewater samples, exclusively from 5 July to 26 August 2021, COVID-19 cases decreased from July 2021 with fewer than 64 new cases weekly (https://www.gob.cl/pasoapaso/cifrasoficiales/). This decrease in cases may have been a result of a lower incidence of cases of the Lambda variant, which was no longer detected in wastewater samples as of July 2021, as well as the gradual increase in the application of vaccines between May and June 2021, reaching more than 80% of the population in August 2021.

Delta variant

The Delta variant GK (B.1.617.2 + AY.*), different from the other four variants analyzed in this study (Alpha, Beta, Lambda, and Gamma), had high frequencies (more than 90%) in all continents in the sequenced cases (GISAID). According to GISAID, the arrival of the peak frequency of 95.8% (3,065 of 3,143 sequenced cases) of this variant was later in South America (15 countries), occurring between 29 November and 5 December 2021, when compared to equivalent frequencies from other continents and the United Kingdom (UK): UK on 19–25 July 2021, Oceania on 9–15 August 2021, Europe-NoUK and North America on 6–12 September 2021, Africa on 13–19 September 2021, and Asia on 20–26 September 2021. In Chile, the first case of the Delta variant, PANGO lineage B.1617.2, was identified on 13 June 2021, and in that month, five sequenced cases were identified out of 434 cases; all of these cases were travelers returning to Chile (MINSAL 2021g). In July 2021, detection of the Delta variant rapidly increased, corresponding to 71 cases in travelers, three cases related to travelers, and nine community cases (MINSAL 2021a). In the Region of Biobío, between the 14th and 24th of July, two cases of the Delta variant were reported, these being travelers abroad returning to the region (MINSAL 2021a). The first community case reported in the Region of Biobío was in August 2021, and as of 10 October 2021, the frequency of cases sequenced 392 for this variant was more than 90% in the region (MINSAL 2021c) and followed the same trend in November 2021. The city of San Pedro de la Paz, Region of Biobío, had its first case sequenced with the Delta variant on 6 August 2021. However, new COVID-19 cases from August to 23 October 2021, were lower than 40 new cases weekly per 100 thousand inhabitants, reaching a minimum value of five new cases weekly per 100 thousand inhabitants between 12 and 18 September 2021 (https://www.gob.cl/pasoapaso/cifrasoficiales/). From 26 August to 26 October 2021, no variant analyzed in this study, except the SARS-CoV-2 reference genes, was detected in the wastewater samples of San Pedro de la Paz. Then, on 27 October

2021, we detected the Delta variant with the P681R and L452R mutations for the first time in the wastewater samples of this city, and 3 days later, the number of new accumulated cases of COVID-19 in the city was almost twice as high ($n = 104$) as the previous week ($n = 59$) between 17 and 23 October 2021 (see Figure 3). From the first detection in the wastewater of San Pedro de la Paz, the Delta variant was detected in all subsequent samples until the end of November 2021, except for the sample on 15 November 2021 (Figure 3). This indicates that this variant may be responsible for driving the start of a new COVID-19 wave in the city of San Pedro de la Paz, which later culminated in the third wave of COVID-19 at the beginning of the year 2022.

Benchmarking between probe-based and NGS detection of SARS-CoV-2 from wastewater nucleic acids, in the assessment of SARS-CoV-2 variant detection methodologies, our findings underscore a crucial limitation of standard amplicon sequencing on wastewater samples: its failure to detect all circulating variants can be attributed to the specificity of primers and the variability in sequencing coverage (Johnson *et al.* 2022; Nicot *et al.* 2023; Kandel *et al.* 2024; Lipponen *et al.* 2024). This specificity often leads to biases in which certain variants may be preferentially amplified or missed, particularly when primer sites undergo mutations. This methodological constraint suggests that while amplicon sequencing provides valuable insights, it may not comprehensively capture the genomic diversity of SARS-CoV-2 present in complex samples such as wastewater.

To address these limitations, whole genome metagenomic sequencing presents a robust alternative. Unlike targeted amplicon sequencing, metagenomic approaches do not rely on specific primer binding and therefore offer a broader spectrum of detection, capturing a more comprehensive array of genetic variations within viral populations. This method can be particularly effective in tracking the evolution of the virus and swiftly identifying emerging variants, which is critical for timely public health responses.

Nevertheless, the application of probe detection remains a reliable and cost-effective method for monitoring SARS-CoV-2 in wastewater samples. Its efficacy and economic viability make it a pragmatic choice for routine surveillance, especially in resource-limited settings. Probe-based methods, when optimally designed, can accurately identify known variants and contribute significantly to the epidemiological tracking of the virus. Integrating both targeted and broad-spectrum sequencing strategies could, therefore, enhance our overall capacity to monitor and control the spread of COVID-19.

CONCLUSIONS

In conclusion, our study, while focused on a single WWTP, presents results that are applicable to various WWTPs. The methodology employed, which includes RNA concentration through PEG and viral detection via RT-qPCR, has proven to be efficient and reliable for measuring viral loads in wastewater samples. This suggests that other WWTPs can adopt these protocols to monitor SARS-CoV-2 RNA and its variants, providing valuable data on viral transmission within communities.

It is important to highlight that traditional amplicon sequencing may not detect certain variants in complex samples. Therefore, probe-based detection methods are a robust solution, offering greater sensitivity and specificity that enhances the accuracy of surveillance, ensuring the timely identification of emerging variants.

The methodology used is scalable and adaptable, which is fundamental for the surveillance of SARS-CoV-2 and its variants, allowing for more informed decisions and effective public health responses. Additionally, wastewater surveillance is a cost-effective and rapid method for tracking mutations, complementing clinical sequencing, especially in contexts with economic limitations. Although variants must be previously identified, a high percentage of those detected by qPCR are confirmed, suggesting that this tool may be effective for anticipating sequencing data.

In summary, while NGS is key for molecular surveillance, its widespread implementation is complicated. Alternative methodologies, such as real-time RT-qPCR kits, allow for rapid and accurate detections, supporting the network of surveillance and diagnosis of SARS-CoV-2, emerging variants, and other viruses.

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AUTHOR CONTRIBUTIONS

A. S. R. conceptualized and visualized the process, developed the methodology, rendered support in formal analysis, wrote the original draft, wrote and review and edited the article. C. C. conceptualized the process, developed the methodology, rendered support in formal analysis, wrote the original draft. P. A. conceptualized the process and rendered support in funding acquisition. K. S. developed the methodology, wrote the original draft, wrote and review and edited the article. M. E. A. visualized the work, developed the methodology, wrote and review and edited the article. M. J. N. developed the methodology and rendered support in formal analysis. C. E. developed the methodology and rendered support in formal analysis. A. G. wrote the original draft, wrote and review and edited the article. C. F. developed the methodology, rendered support in formal analysis, wrote the original draft, wrote and review and edited the article. M. H. conceptualized the process developed the methodology, wrote the original draft, wrote and review and edited the article, rendered support in project administration and funding acquisition.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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