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Science of the Total Environment 982 (2025) 179648

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Upstream SARS-CoV-2 variant surveillance at Breede Valley municipality using wastewater-based epidemiology

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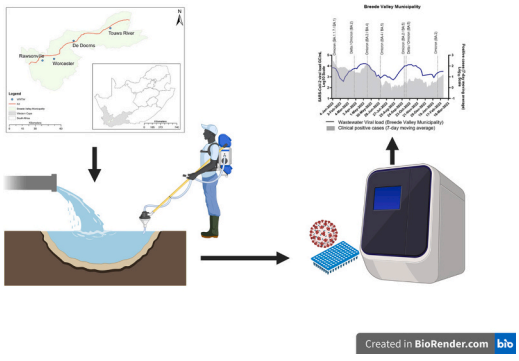
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HIGHLIGHTS

- WBE was used to track COVID-19 progression via wastewater monitoring.
- Rawsonville WWTP showed persistently emergence and re-emergence of the Deltacron variant.
- Upstream WWTP surveillance is crucial for tracking variant origins

GRAPHICAL ABSTRACT

Graphical abstract illustrating the SARS-CoV-2 wastewater surveillance workflow in the Breede Valley Municipality. The image includes a map of sampling sites and outlines the process from sample collection at wastewater treatment plants to laboratory analysis and RT-qPCR for viral detection and quantification.



ARTICLE INFO

Editor: Lingyan Zhu

ABSTRACT

The COVID-19 pandemic has strained economies and health systems worldwide, particularly in low- and middle-income countries, where limited resources were further stretched by the crisis. In response, innovative strategies

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https://doi.org/10.1016/j.scitotenv.2025.179648
Received 19 November 2024; Received in revised form 10 April 2025; Accepted 9 May 2025
Available online 15 May 2025
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Keywords:
SARS-CoV-2
COVID-19
Wastewater
Delta variant
Pandemic
South Africa
Epidemiology

like wastewater-based epidemiology (WBE) have emerged as cost-effective and efficient alternatives to traditional surveillance. In Breede Valley Municipality, Western Cape, South Africa, weekly monitoring of wastewater treatment plants (WWTPs) showed consistently high SARS-CoV-2 viral loads in Rawsonville, especially of the Delta variant (AY.32 and B.1.617.2), despite Omicron's dominance in other regions. Total RNAs of all samples were extracted using the Qiagen RNeasy PowerSoil Kit, from which both RT-qPCR and whole genome sequencing were performed to determine the amount of SARS-CoV-2 RNA and their variants, respectively. Rawsonville had the highest median viral load, measured in genome copies per millilitre (gc/mL) at 2211 gc/mL, compared to Worcester (1790 gc/mL), De Doorns (1438 gc/mL), and Touws River (1253 gc/mL). Neighbourhoods in Rawsonville (RV) upstream of the main WWTP, where manholes were sampled, indicated that the suburban manhole on Grey Street (RV_G, 2347 gc/mL) and the peri-urban manhole on Rugby Street (RV_R, 714 gc/mL) were the primary contributors to the SARS-CoV-2 viral load. In contrast, the manhole near public toilets (RV_P, 470 gc/mL) had the lowest viral load. Notably, sequencing data indicated that only Omicron (BA.5) was detected in upstream sites RV_G and RV_R when Deltacron was present in Rawsonville, suggesting complex transmission dynamics requiring further study. These findings underscore the importance of targeted upstream surveillance to trace variant emergence and inform public health interventions.

1. Introduction

In 2024, the coronavirus disease 2019 (COVID-19) pandemic has resulted in over 7 million confirmed deaths globally (World Health Organisation, 2024). The pandemic decimated many economies and health systems, particularly those in developing countries (United Nations, 2021; Chriscaden, 2020; Pak et al., 2020). Although many countries implemented measures of social distancing to curb the transmission of the virus, bioaerosols remained a concern. Research indicates that viral particles can persist in the air beyond the recommended 2-m distance, particularly in stagnant environments. This challenge highlighted the limitations of distancing alone and underscored the need for a multifaceted approach, including improved ventilation and surface hygiene, to effectively mitigate the spread of COVID-19 (Guzman, 2021).

The severity of the infection expedited the implementation of methods to monitor the spread of the disease in communities, with wastewater-based epidemiology (WBE) emerging as a novel public health tool to track SARS-CoV-2. Before the onset of the COVID-19 pandemic, WBE has been used for polio surveillance, especially in areas where vaccination coverage was low (Asghar et al., 2014). During the COVID-19 pandemic, WBE has proven to be a cost-effective strategy for wide scale community screening and tracking and, as such, has been used as a complementary SARS-CoV-2 surveillance tool in various countries (Ahmed et al., 2021; Barril et al., 2021; Medema et al., 2020; Johnson et al., 2022a). The effectiveness of this tool as a rapid, non-invasive screening method was confirmed when the European Commission recommended that all Member States implement wastewater monitoring as of 17 March 2021 (Preparedness, 2021).

In South Africa, only a select number of laboratories and surveillance programs have been put in place to monitor the presence of SARS-CoV-2 in wastewater (Johnson et al., 2022a; Abdullah et al., 2022; Davies et al., 2022) (NICD dashboard, SAMRC dashboard), with most of the studies conducted within South African. At the time of this study (2022), Omicron and its sub-variant XBB.1.1.5 were in circulation, with clinical symptoms less severe than those associated with previous variants (Abdullah et al., 2022; Davies et al., 2022). A study by (Johnson et al., 2022b) examined the genomic diversity of SARS-CoV-2 in the Western Cape, South Africa from November 2021 to April 2022, and was the first to observe a peculiar circulating variant, Deltacron, in wastewater samples in the Breede Valley municipality. The study also reported that samples collected at the Rawsonville town consistently exhibited the presence of the Delta variant in wastewater throughout the Omicron wave in January 2022, suggesting that a particular region within the Rawsonville town could be the source or origin of this new variant. Consequently, an extended period of weekly monitoring for SARS-CoV-2 in wastewater was initiated at Rawsonville, with a specific focus on locations upstream of the primary wastewater treatment plant (WWTP). Thus, the aim of this study was to explore the spread of SARS-CoV-2 within the Breede Valley Municipality with a focus on the Rawsonville town's upstream wastewater treatment sites to track and identify the source of new emerging variants. The study also aimed to identify hotspots for individualised interventions that may help to mitigate the spread of SARS-CoV-2.

2. Methodology

2.1. Sample location

2.1.1. Breede valley municipality

The Breede Valley Municipality, which is located approximately 126.4 km from Cape Town, has a population of 212,682 (Census 2022), of which 87.7 % reside in formal housing. In this Municipality, flush toilets that are connected to a sewer are present in at least 94.9 % of housing units. The Municipality is also home to four sizable towns: Rawsonville, Worcester, De Doorns, and Touws River, which according to the 2011 Census, (with the latest 2022 data for these towns not readily available at the time of this manuscript), have population numbers of 3099, 97,078, 11,278 and 8126, respectively (Fig. 1). The Breede Valley Municipality is predominantly a grape farming district and is one of the largest wine-producing regions in South Africa with many tourist attraction sites. It is in the greater Cape Winelands District Municipality, which has two major wine routes, the Breedekloof Wine Route and the Worcester Wine & Olive Route (Winetourism.com, 2023). The Hex River Valley produces the largest annual quantity of table grapes in the Southern Hemisphere (Department of Agriculture and Forestry and Fisheries, 2020).

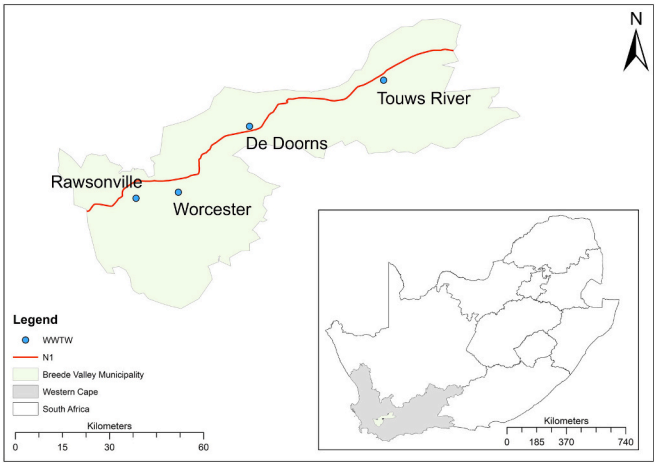


Fig. 1. Map of the Breede Valley Municipality, including the towns that were sampled: Rawsonville, Worcester, De Doorns, and Touws River. Map was produced using ArcGIS 10.6.1. Abbreviations: WWTW, wastewater treatment works, N1; National route 1.

2.1.2. Rawsonville upstream sites

Sampling points upstream of the Rawsonville WWTP included a manhole that serves public toilets central to the public transport hub (RV_P) for commuters traveling to and from Rawsonville, a manhole located at a street that serves a suburban neighbourhood (RV_G), and lastly, a manhole located in a street that serves a peri-urban neighbourhood (RV_R).

2.2. Sampling method

The wastewater sampling procedure was carried out as follows: a volume of 500 mL of untreated wastewater grab samples from the Breede Valley Municipality sites (De Doorns, Worcester, Rawsonville and Touws River) (Fig. 1) were collected between January 2022 and March 2023 at the raw inlet after removal of non-biodegradable solids. Additional wastewater grab samples (500 mL) were collected from upstream sites (sewer manholes) between May 15, 2022, and March 18, 2023. Samples were collected by trained personnel with fully kitted personal protective wear which included boots, gloves, overalls and N-95 masks to minimise the risk of contact with the samples. Appropriate sampling equipment, such as sterile polyethylene sample bottles and grab samplers was used. The samples were stored on ice for 1–2 h in designated cooler boxes equipped with temperature-monitoring thermometers to maintain sample integrity during transportation to the laboratory. Upon arrival, samples were processed immediately for RNA extraction.

2.3. Sample concentration and RNA extraction

RNA was extracted from wastewater grab samples using a modified method described previously (Peccia et al., 2020). Samples were concentrated (pelleted) by centrifuging 100 mL of the raw wastewater at 2500*g* for 20 min at room temperature (22–23 °C), and the resulting pellet was resuspended in 5–10 mL of the supernatant. Total RNA was extracted using the Qiagen RNeasy® Powersoil® Total RNA Kit (Qiagen, Hilden, Germany) following a modified protocol. Briefly, the resuspended pellet from each sample was added to a 15 mL Powerbead® tube containing lysis buffer and equal volumes, 1:1 ratio, of phenol:chloroform solution. Thereafter, samples were vortexed at maximum speed (2400 rpm) for 15 min, followed by centrifugation at 2500*g* for 15 min to obtain phase separation. The resultant upper aqueous phase containing nucleic acid was carefully transferred to a new 15 mL tube for further clean-up steps. The nucleic acid isolate was then transferred to the RNeasy® JetStar Mini Column for binding and eluting using SR6. Following elution, the extracted RNA was incubated for 10 min at −20 °C and centrifuged at 13000*g* for 15 min. The resultant supernatant was discarded, and the RNA was air dried and resuspended with 70 μL of nuclease-free water. The quantity and quality of the extracted RNA was assessed using the Nanodrop ND-1000 instrument (Nanodrop Technologies, Wilmington, NC, USA), by measuring absorbance at 260 nm and 280 nm. The purity of the RNA was evaluated using the 260/280 ratio, with values close to ~2.0 indicating high-quality RNA suitable for downstream applications. The instrument was calibrated using a blank measurement with nuclease-free water to establish a baseline and account for background absorbance before sample analysis.

2.4. Reverse transcription–quantitative polymerase chain reaction

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed following the method described by Johnson et al. (2021) (Johnson et al., 2021). Primer/probe sets targeting two different regions (N1 and N2) of the SARS-CoV-2 nucleocapsid gene were used for the detection of SARS-CoV-2 RNA in wastewater samples. To quantify SARS-CoV-2 viral RNA, a one-step RT-qPCR reaction was performed using iTaq™ Universal Probes One-Step Reaction Mix in a final reaction volume of 10 μL as per the manufacturer's instruction (Bio-Rad Laboratories, Richmond, CA). For the RT-qPCR reaction, 200 ng of total RNA was used, and the assays were performed using an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System (ABI Technologies). The viral RNA copy number was quantified using the standard curve method with the 2019-nCoV-N- Positive plasmid, which was supplied at 200000 copies/μL, as standard in 1:10 serial dilutions (Qauntabio, Beverley, MA). For quality control, all reactions were done in duplicate with positive and negative controls for RNA extraction and RT-qPCR. The positive and negative controls for RNA extraction consisted of a previously positive sample and nuclease-free water, respectively. The PCR control comprised a 200 copies/μL sample that was included as an unknown for the positive control and a non-template control that contained nuclease-free water instead of RNA as the negative control.

2.5. Whole genome sequencing

Sequencing and mutation detection were carried out using Variant Caller v5.16.0.5 with default settings, following a method similar to that described by Johnson et al. (2022b), using the Thermo Fisher Ion Torrent Research Panel. RNA samples were sent to the Central Analytical Sequencing Facility in Stellenbosch, South Africa, for next-generation sequencing (NGS) analysis. Library preparation was performed using the Ion AmpliSeq SARS-CoV-2 research panel (Life Technologies, Carlsbad, USA), which targets the full SARS-CoV-2 genome through highly multiplexed PCR amplification. The preparation process included reverse transcription of RNA into complementary DNA (cDNA), followed by targeted amplification using primers specific to the SARS-CoV-2 genome. After amplification, libraries were quantified, purified, and subjected to adapter ligation before sequencing. Sequencing was conducted on the Ion Torrent platform, generating high-depth sequencing reads. Data quality control, read alignment, and variant calling were performed using the Torrent Suite Software, ensuring high-confidence mutation detection and variant identification in the analyzed samples.

2.6. Statistical analysis

Data analysis and statistical tests were carried out using GraphPad Prism v8.0.1. The SARS-CoV-2 viral loads of wastewater were reported using summary statistics. Normality was tested using the Shapiro-Wilk test. Outliers were identified using Grubb's test and the wastewater sample matrices were correlated using Spearman's rank correlation.

3. Results and discussion

This study investigated the temporal and geographic trends of SARS-CoV-2 in wastewater, exploring its correlation with clinical case data and the persistence of specific variants to trace the source of new variant introductions. Spatiotemporal analysis revealed a positive correlation between SARS-CoV-2 viral load detected in wastewater and clinical case data in the Breede Valley Municipality (Fig. 2). Similar findings on the correlation between wastewater data and clinical case data have been observed in research conducted in Houston (Stadler et al., 2020), Utah (Weidhaas et al., 2021), Milwaukee (Feng et al., 2021), and San Diego County (Karthikeyan et al., 2021). Following this, various studies also reported on the circulating variants of concern (VOC), using either PCR-based detection or Whole genome sequencing (WGS), further affirming the value of WBE (Karthikeyan et al., 2021; Lang et al., n.d.). From our previous study, Johnson et al. (2022b) reported on the onset of the Omicron-driven fourth wave in the Breede Valley and City of Cape Town Municipalities, after which Omicron rapidly becoming the predominant sublineage detected in most of the WWTPs under investigation. Conversely, although Omicron was dominant in >90 % of the WWTPs in January 2022, Omicron persisted in the Breede Valley, but with periodic co-circulation with the Delta variant. Hence, this study set out to better

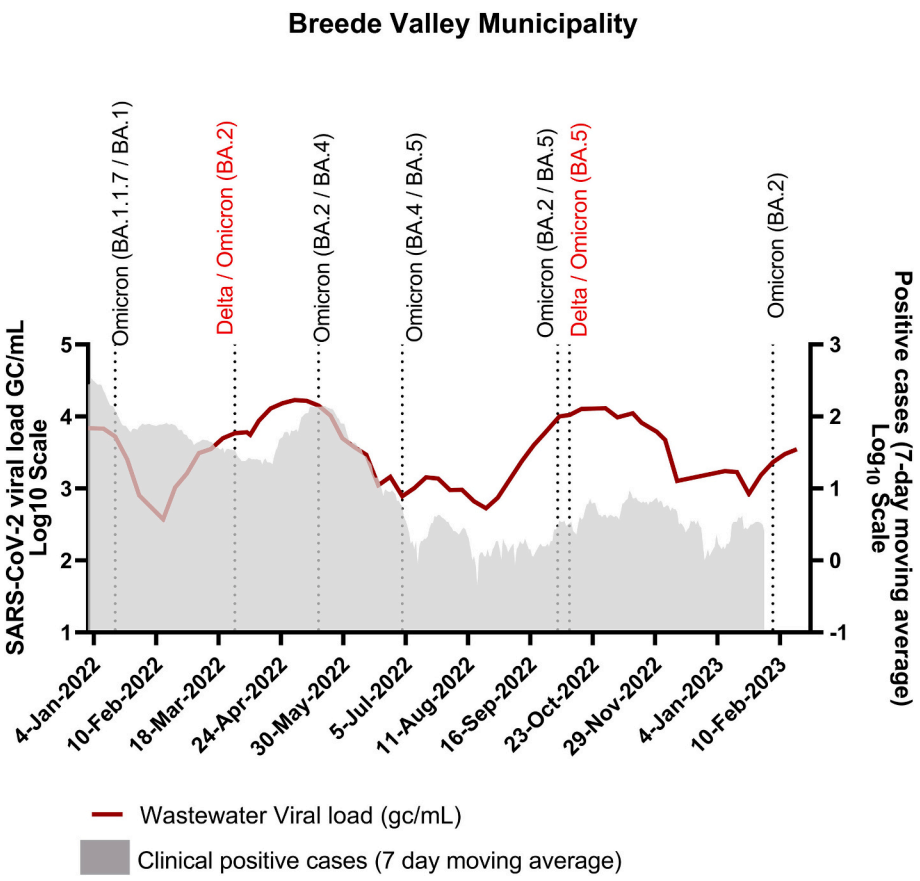


Fig. 2. Analysis of circulating variants within the Breede Valley Municipality. Graphs show wastewater SARS-CoV-2 RNA viral load measured with RT-qPCR using N1 and N2 primer/probe sets (solid line) overlayed Cape Winelands positive case data (grey shade) and circulating variants. Key amino acid changes (sublineages) of the Omicron variant were classified by Pangolin (https://pangolin.cog-uk.io/ version 4.0.5, lineages version, 30 March 2023). The solid line represents the median log10 SARS-CoV-2 load based on the mean N1 and N2 concentration for the four Breede Valley Municipality WWTPs (De Doorns, Touws River, Worcester and Rawsonville). Clinical case data were obtained from the Department of Health's COVID-19 Response dashboard, which is accessible to the public on the Western Cape Government of South Africa website (http://coronavirus.westerncape.gov.za/COVID-19-dashboard). Abbreviations: gc, genome copies; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WWTPs, wastewater treatment plant

understand the factors that may have enabled the persistent re-emergence of the Delta variant from January 2022 to March 2023, while other towns remained in the Omicron-driven wave.

WGS analysis confirmed that while the Omicron variant and its sub-variants were in circulation in the City of Cape Town, the Delta variant continued dominance in the Breede Valley Municipality towards the end of March 2022 and re-emerged in October 2022 (Fig. 2). Interestingly, between September 22, 2022, and October 23, 2022, the viral load of both the Delta and Omicron variants in wastewater samples did not show statistically significant differences. This lack of significance suggests that during this period, the transmission dynamics of both variants were similar in the sampled regions, despite their distinct biological characteristics. It is important to note that although Omicron was already the dominant variant in many regions globally, local factors such as population immunity, public health interventions, and environmental conditions may have influenced viral shedding in wastewater, leading to comparable levels of both variants in the wastewater samples. This could imply that both variants were circulating concurrently at similar intensities, or that the methods used for viral load quantification were not sensitive enough to detect subtle differences between the two variants in the given timeframe. Further, the lack of statistical significance might also be attributed to the complex nature of viral load dynamics, where factors like sampling frequency, sample volume, and the presence of other confounding variables can impact the results. These findings warranted further investigation to identify a possible entry point within the Breede Valley Municipality where the Delta variant was likely introduced. The Breede Valley Municipality is a prominent grape farming region known for housing one of the largest pre-cooling facilities in the Southern Hemisphere. It also boasts the longest harvest season of any wine-producing area in the world (Winetourism.com, 2023). As a result, the municipality experiences a significant influx of seasonal farm workers during harvest time. This influx may have contributed to and introduced variants of SARS-CoV-2 from other areas that were not previously in circulation. Additionally, the municipality boasts various tourist attractions, which, according to studies on virus transmission patterns, have been shown to increase the likelihood of introducing and spreading different SARS-CoV-2 variants. Tourists traveling from other regions and countries have been identified as potential vectors for new variants, contributing to regional viral diversity (Winetourism.com, 2023; Lang et al., n.d.; Maharaj et al., 2021).

Consequently, towns within the Breede Valley Municipality were surveyed to determine the origin and factors that could possibly contribute to the reintroduction of the Delta variant. Four towns, (De Doorns, Touws River, Worcester and Rawsonville) were subjected to a 15-month-long surveillance and variant monitoring program for SARS-CoV-2. Spatiotemporal analysis revealed a median SARS-CoV-2 viral load measured in genome copies per millilitre (gc/mL) of 8799 gc/mL across the Breede Valley Municipality, with total viral loads ranging from 399 gc/mL (August 2022) to 101,290 gc/mL (October 2022).

There was a weak to moderate correlation ($r = 0.3721$, $p = 0.0023$) between clinical case data and wastewater viral load. Interestingly, WGS confirmed that the Omicron variant was present in Touws River

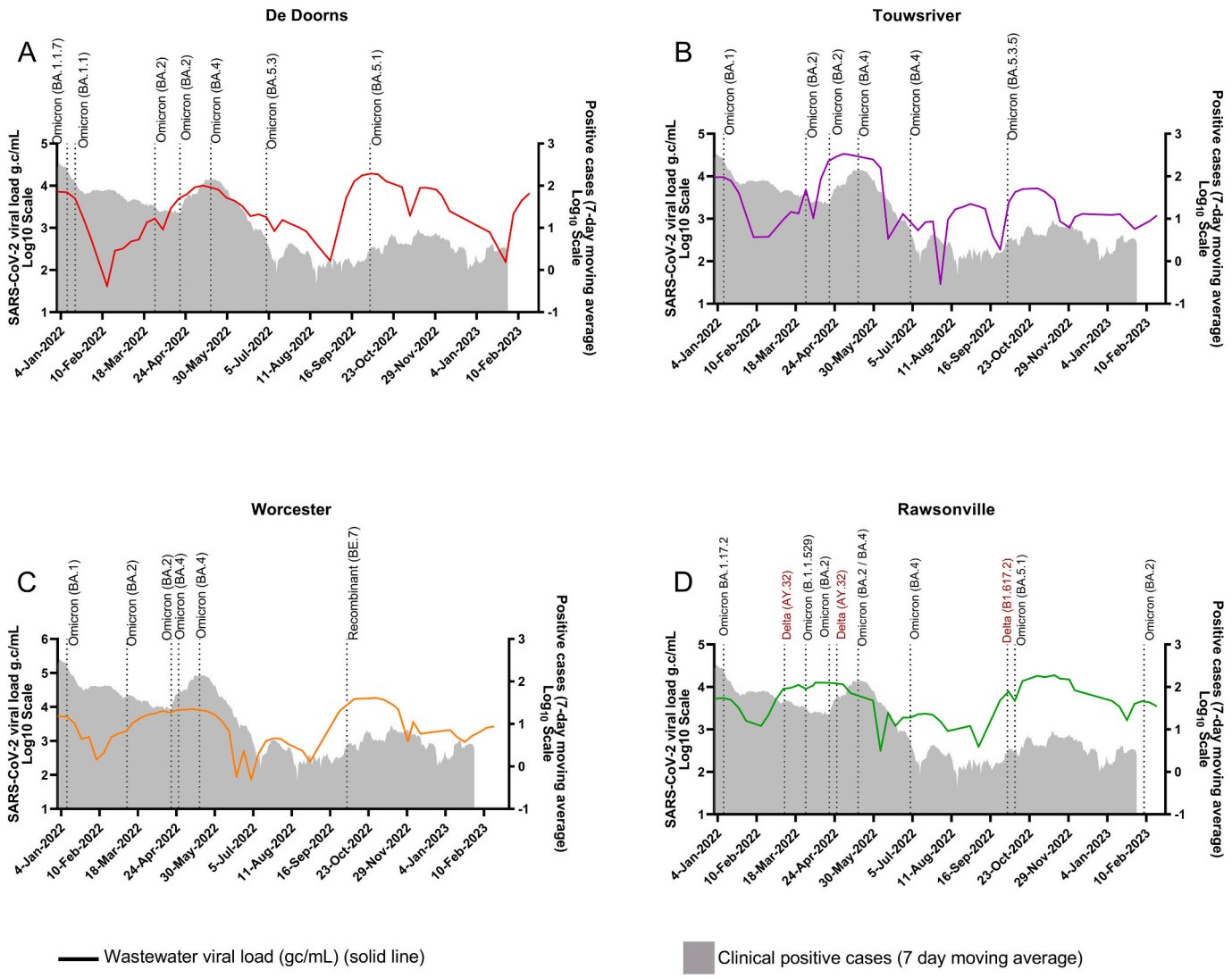


Fig. 3. Analysis of circulating variants within the Breede Valley Municipality towns. A: De Doorns, B: Touws River, C: Worcester, and D: Rawsonville Graphs show wastewater SARS-CoV-2 RNA viral load measured with RT-qPCR using N1 and N2 primer/probe sets (solid line) overlayed with Cape Winelands positive case data (grey shade) and circulating variants. Key amino acid changes (sublineages) of the Omicron variant were classified by Pangolin (https://pangolin.cog-uk.io/ version 4.0.5, lineages version, 30 March 2023). The solid line represents median of the four Breede Valley Municipality WWTPs (De Doorns, Touws River, Worcester and Rawsonville) plotted using the mean of N1 and N2. Clinical case data was obtained from the Department of Health's COVID-19 Response dashboard, which is accessible to the public on the Western Cape Government of South Africa (http://coronavirus.westerncape.gov.za/COVID-19-dashboard). Abbreviations: gc, genome copies; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WWTPs, wastewater treatment plants.

(Fig. 3B), which recorded a peak SARS-CoV-2 viral load of 70,537 gc/mL in May 2022, and in De Doorns (Fig. 3 A), which had the highest viral load of 68,956 gc/mL in October 2022. Worcester (Fig. 3C) and Rawsonville (Fig. 3D) both recorded the highest viral loads in November 2022 at 47,358 and 58,822 gc/mL, respectively. This was preceded by the resurgence of the Delta variant (B1.617.2) in Rawsonville, and the appearance of the recombinant variant (BE.7) in Worcester between September and October 2022. Another emergence of the Delta (AY.32) variant within Rawsonville coincides with the increase in SARS-CoV-2 viral load detected in wastewater in March (9950 gc/mL) and April (1247 gc/mL) of 2022. The Delta variant has been reported to have an enhanced transmission and increasing virulence owed to mutations in the S-protein (Dhawan et al., 2022; Zhan et al., 2022), hence the observed increase in SARS-CoV-2 viral load detected in wastewater. These mutations arise from ongoing infections in the host's immune system, potentially resulting in highly divergent variants that could trigger new community outbreaks.

To more precisely determine the origin of the Delta variant detected in Rawsonville, three manholes upstream the main Rawsonville WWTP were subsequently sampled over 15 months. Samples were collected weekly from public toilets, RV_P and two on-site manholes RV_G and RV_R. From this, the data indicates that RV_R contributed largely to the high viral load (6172 gc/mL) in Rawsonville during May 2022 with a further resurgence in August (38,457 gc/mL) through September (6486 gc/mL) 2022 (Fig. 4C). This peri-urban neighbourhood (RV_R) has two places of interest that draw large crowds: a church and a primary school. Since the SARS-CoV-2 viral load was observed to be higher during these periods, which correspond with the times when schools were open (5 April – 24 June and 19 July – 30 September 2022), it can be argued that the high viral load was likely caused by people moving in and out of the area during these times. Furthermore, WGS identified the circulating variant in the area during the peak in May as Omicron (BA.4) (Fig. 4C), which differed from the Delta variant detected at the Rawsonville WWTP during the same period. This discrepancy suggests that RV_R was not the source of the variant's introduction. Subsequent sequencing of the RV_R sample collected in October, when the Delta variant re-

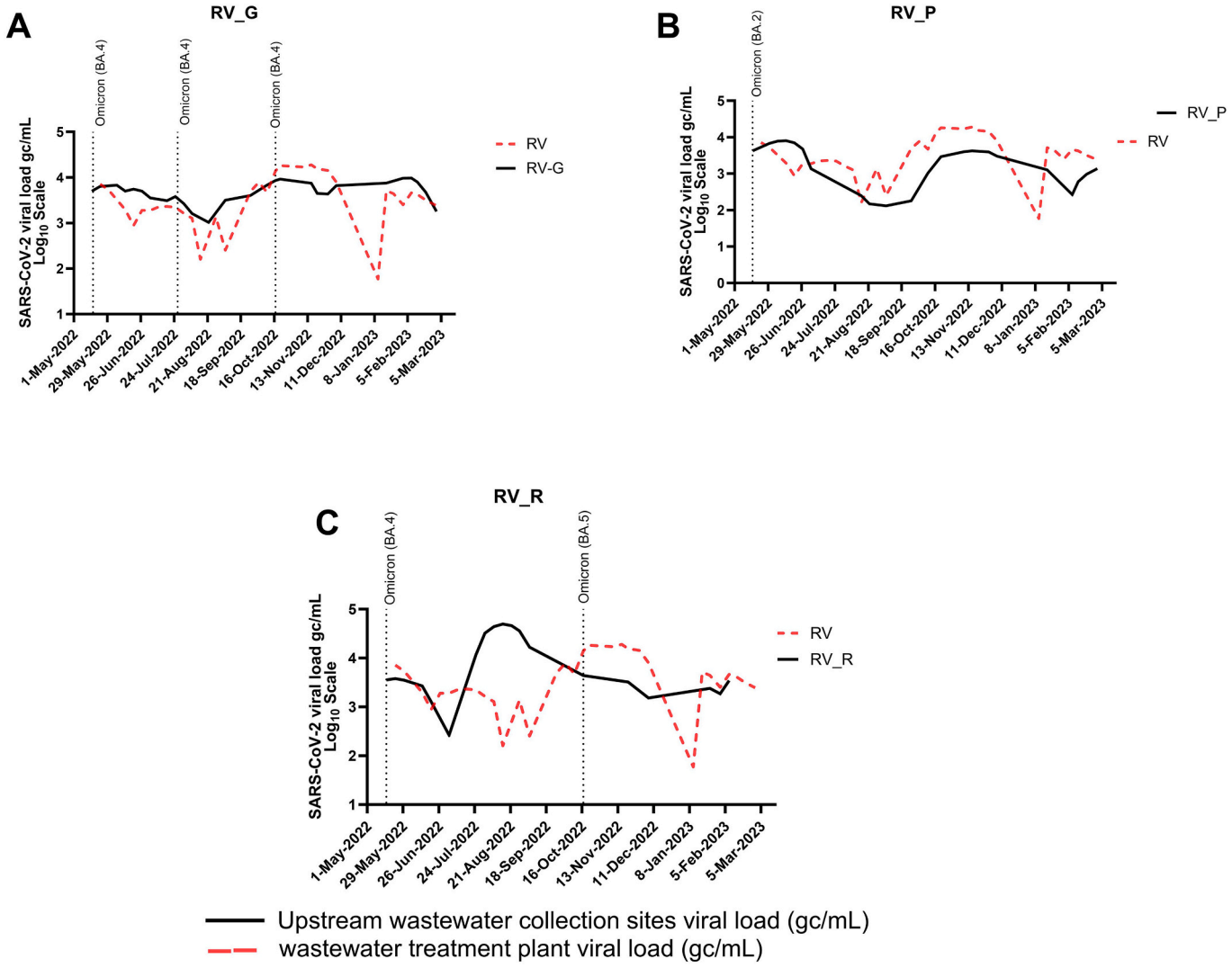


Fig. 4. Breede Valley Municipality severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) wastewater RNA viral load of the Rawsonville upstream sites. A: RV_G, B: RV_P and C: RV_R. Graphs show wastewater SARS-CoV-2 RNA viral load measured with RT-qPCR using N1 and N2 primer/probe sets (solid line) for each town, A: RV_G, B: RV_P and C:RV_R, overlayed with Rawsonville WWTP viral copy number (dotted line). Abbreviations: gc, genome copies; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WWTPs, wastewater treatment plants.

emerged in Rawsonville, once again identified Omicron (BA.5) as the dominant circulating variant, further excluding RV_R as the source of the Delta variant in both instances.

The suburban neighbourhood, RV_G, largely contributed to the high viral load in July 2022 (3815 gc/mL) with a resurgence in October 2022 (5515 gc/mL) through to November 2022 (6371 gc/mL) and into March 2023 (43,819 gc/mL) (Fig. 4 A). This is an area that separates residential and commercial areas and is surrounded by an arboriculture and fruit production farm), a church, at least 3 schools, an old age home, a medical clinic, rugby club and at least 3 lodges and restaurants. The high viral load detected in this neighbourhood may be attributed to several factors, including significant seasonal labour migration during fruit-pruning (late June to early August) and harvesting (March to May and September to January) seasons, which likely affects community health dynamics and viral spread. Additionally, the town's economy increasingly relies on tourism and events like rugby matches, both of which likely contribute to heightened viral transmission during peak seasons, as the influx of foreign and domestic tourist impacts the viral load in the area (South Africa Online SA (Pty) Ltd, 2024).

To further evaluate the possibility that the Delta variant emerged from RV_G, it's important to note that the Delta variant observed in Fig. 3D consistently preceded a decline in viral load just before Omicron became dominant. The Delta variant (B1.617.2) detected in September 2022 (Fig. 3D) may have been introduced from RV_G, as Fig. 4 A shows a similar peak in viral load just before the onset of the Omicron wave. However, WGS detected Omicron (BA.4) in May, July, and October—periods that coincided with the emergence and re-emergence of the Delta variant in Rawsonville—indicating that Omicron was the dominant circulating variant during these times.

The public toilets (RV_P, Fig. 4B) experienced a spike in viral load in June 2022, which coincided with the school holidays in the Western Cape. Located centrally near a public transport hub, these toilets pose a heightened risk for COVID-19 transmission. This risk is amplified by their proximity to eateries, hair salons, convenience stores, and, most importantly, public transportation. Moreover, with schools closed during this period (expatcapetown.com), it's likely that more people used this route for travel, either as visitors to and from surrounding regions or as tourists entering the towns for shopping and other activities. As a result, these activities may have contributed to the increased viral load observed in RV_P. Unfortunately, WGS was unable to detect the Delta variant in this area, likely due to the low SARS-CoV-2 viral load in the wastewater during the periods that coincided with the Delta variant's

emergence and re-emergence at the Rawsonville main WWTP. Consequently, the samples were insufficient for WGS analysis.

Taken together, our results indicate that while the Delta variant did re-emerge at times, the Omicron dominance as a SARS-CoV-2 variant is primarily due to its significantly higher transmissibility and ability to evade immunity compared to Delta (Sari et al., 2022; Ghebreyesus, 2020). For example, a study by Tian et al. (2022) (Tian et al., 2022), demonstrated that the Omicron BA.1 and BA.2 variants significantly evade neutralization in vitro using sera from vaccinated individuals. This finding aligns with another study by Bolze et al. (2022), (Bolze et al., 2022), which revealed that Omicron's high infectivity and immune escape are due to its numerous spike protein mutations—twice as many as those found in the Delta variant. Nonetheless, the Delta variant's increased transmissibility has been linked to key mutations in the S-protein, including D614G, L452R, P681R, and T478K. These mutations are thought to enhance the S-protein's affinity for the ACE2 receptor, which may contribute to the observed reduction in vaccine efficacy. Evidence suggests that the Delta variant not only spreads more easily but also shows a lower vaccine effectiveness compared to other variants of concern, such as Alpha and Beta. This may explain why the Delta variant continues to persist in the Breede Valley Municipality, even during periods of high Omicron transmission. This could be further explained by the influx of people from the surrounding areas. We hypothesize that the Delta variant could have been re-introduced by individuals such as fruit pickers, and holidaymakers that come into the town who have had less recent exposure to vaccines or boosters that are more effective against Omicron than the Delta variant. As a result, we might see a period of overlap where both variants are present, especially if Delta is still circulating at low levels in the community and are masked by the dominant Omicron sub lineages. As such, high viral loads of Omicron might not completely suppress Delta, leading to its occasional reappearance. This was also further confirmed in a study by Bolze et al. (2022) (Bolze et al., 2022), which reported that the Omicron and Delta variants co-circulated in the U.S. between November 2021 and February 2022. Conversely, it could also be that in the Breede Valley area, Delta might have specific genetic traits that allow them to persist in certain environments or populations, especially if there is a lag in the population's immunity or changes in virus behaviour due to mutations. This is referred to as an immune escape which allows for the persistent reappearance of a variant. For example, in a study by (Willett et al., 2022), it was reported that Delta could remain present in certain regions or populations despite Omicron's dominance, reflecting the complex dynamics of variant prevalence and competition.

On the other hand, the continuous re-emergence of SARS-CoV-2 variants may also be exacerbated by animal hosts. While no documented cases exist of SARS-CoV-2 transmission from infected pets to their owners, other pets, or humans, studies have shown that companion animals like dogs and cats are susceptible to experimental SARS-CoV-2 infection (Hobbs and Reid, 2021). Additionally, research indicates that SARS-CoV-2 likely originated from animal reservoirs, with animal-to-human transmission occurring on fur production farms. These environments have facilitated the emergence of new mutations from these potential amplifying hosts. This highlights the importance of monitoring animal populations as potential sources of new SARS-CoV-2 variants, which could pose additional challenges in controlling the virus's spread and evolution.

4. Conclusion

In conclusion, the underlying factors that contribute to the emergence and persistence of variants such as Delta AY.32 or B1.617.2 are critical to understanding the future trajectory of the pandemic or any viral outbreak. The ability of these variants to evade vaccines, increase virulence, and affect public health measures underscores the importance of ongoing surveillance, research, and the adaptation of medical and public health strategies to mitigate their impact. Sampling upstream sites of main wastewater treatment plants not only offers a clearer perspective on the areas contributing to the high viral load in a region but also enhances understanding of how and where new variants are introduced into the population. This approach helps pinpoint infection trends and hotspots within a specific community before the wastewater reaches the main WWTP, enabling more precise public health interventions and/or mitigation strategies needed to contain the localized spread of SARS-CoV-2. The present study suggests that the variety of activities in Rawsonville may have led to an influx of people, providing opportunities for the highly transmissible SARS-CoV-2 Delta variant to emerge and re-emerge on multiple occasions.

This study reinforces the importance of employing wastewater surveillance to pinpoint infection sources, enabling targeted mitigation strategies. These strategies may include public health campaigns, awareness initiatives, and the implementation of personal protective equipment (PPE), such as face masks, in specific hotspots. In rural areas this approach is crucial both during and before peak seasons, to prevent and contain the transmission of COVID-19 virus or other infectious pathogens.

CRediT authorship contribution statement

Kholofelo Malemela: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Noluxabiso Mangwana:** Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Stephanie Dias:** Writing – review & editing. **Jyoti Rajan Sharma:** Writing – review & editing. **Pritika Ramharack:** Writing – review & editing. **Anri Kotze:** Data curation, Writing – review & editing. **Rianita van Onselen:** Data curation, Writing – review & editing. **Sizwe Nkambule:** Writing – review & editing. **Johan Louw:** Writing – review & editing. **Mongezi Mdhluli:** Writing – review & editing. **Glenda Gray:** Writing – review & editing. **Christo Muller:** Writing – review & editing. **Angela Mathee:** Writing – review & editing. **Renée Street:** Funding acquisition, Writing – review & editing. **Rabia Johnson:** Funding acquisition, Writing – review & editing.

Funding

This research was funded in part by The South African Medical Research Council (Grant Number: 40118), The Michael & Susan Dell Foundation (Grant Number: 96829), The National Research Foundation of South Africa (Grant number: 138011) and The Solidarity Fund. The funders had no involvement in the study's design, data collection, analysis, report writing, or the decision to submit the article for publication.

Declaration of competing interest

The authors declare no competing financial or non-financial interests that could have influenced the work reported in this manuscript.

No funding organizations had a role in the study design, data collection, analysis, interpretation, or decision to submit the manuscript for publication.

All authors have approved the final manuscript and affirm that this submission does not contain any conflicts of interest that could compromise the integrity of the research or its publication in Science of The Total Environment.

Acknowledgements

We thank the South African Medical Research Council (SAMRC) for funding and support. The SAMRC acknowledges the Breede Valley Municipality for granting access to the wastewater treatment plants. We also extend our thanks to the Central Analytical Sequencing Facility (Stellenbosch, South Africa) for their support, and to the Western Cape

Government for providing the daily COVID-19 clinical case data.

Data availability

Data will be made available on request.

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