NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

WASTEWATER-BASED EPIDEMIOLOGY FOR SARS-CoV-2 SURVEILLANCE IN SOUTH AFRICA

Detection, quantitation and genomic sequencing at sentinel sites in South Africa, March 2021- February 2023
WEEK 06 2023

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OVERVIEW

This report summarises and interprets findings from detection, quantification and sequencing of SARS-CoV-2 by the National Institute for Communicable Diseases (NICD) Centre for Vaccines and Immunology from influent (untreated) wastewater in 17 wastewater treatment plants (WWTPs) across five South African provinces. The results obtained and interpretations analysis of levels of SARS-CoV-2 in WWTP from 70 additional plants across South Africa were tested by SACCESS partners: the National Institute for Occupational Health (NIOH), Lumegen Laboratories, GreenHill Laboratories, SAMRC-TB Platform (until March 2022) and Praecautio to cover all provinces in South Africa. Levels of SARS-CoV-2 in wastewater correlate with population levels of SARS-CoV-2 over time and indicate the geographic distribution of disease. Variants of SARS-CoV-2 can be identified in wastewater through detection of single-nucleotide polymorphisms (SNPs) that are specific to each variant. These variants are shown to correspond to variants prevalent in clinical cases, across time and place. SARS-CoV-2 is shed from symptomatic and asymptomatic persons in stool but is not transmitted by faecal-oral route nor via wastewater. This report is based on data collected from June 2021 until 7 February 2023 (Epidemiological week 06, 2023). Results from wastewater testing should be read and interpreted together with testing and genomic reports generated by the Centre for Respiratory Diseases and Meningitis found at https://www.nicd.ac.za/diseases-a-z-index/disease-index-covid-19/surveillance-reports/

- Part 1 of this report presents methods and results of quantitative testing of wastewater.
- Part 2 of this report presents methods and results from sequencing of SARS-CoV-2 RNA fragments in wastewater.

SARS-CoV-2 levels in wastewater are generally low across the country. Omicron lineages XBB.3, BQ.1, BQ.1.1.28, BE.8, XBB.1.5, BE.1.1, CH.1.1, BQ.1.1.20, BE.7, BA.5.11 are overall, circulating in January in South Africa, followed by delta lineage AY.120.1, as of week 4, 2023. Detailed analyses are described below.

HIGHLIGHTS – sample collection dates up to 7th February 2023 (Epi week 06)

SARS-CoV-2 levels in wastewater:

- Our results for week 06, 2023 are based solely on sites tested by the NICD, using the cycle threshold (Ct) value to represent SARS-CoV-2 levels in wastewater
- SARS-CoV-2 levels are seen to be dropping in some plants across the country in Epi week 06, while level in other areas have remained at moderate levels
- Areas with low wastewater levels of SARS-CoV-2 are situated in Tshwane district (Rooiwal WWTP), eThekwini Metro (Central and Northern WWTPs) and Mangaung Municipality (Bloemspruit and Sterkwater WWTPs). Areas with moderate levels of SARS-CoV-2 are in Johannesburg (Northern WWTP) and the City of Ekurhuleni (Vlakplaats and Hartebeesfontein WWTPs)

*Note: The presence and increase/decrease of SARS-CoV-2 RNA in wastewater signify ongoing and increasing/decreasing transmission of the virus amongst populations that are serviced by particular sewer networks. The determination of a resurgence (or 'wave') of SARS-CoV-2 is made through evaluation of clinical testing data (including numbers of positive tests, percentage testing positive), hospitalisation and mortality data.

SARS-CoV-2 genomics in wastewater:

Sequencing data available up to week 4 (26th January, 2023) shows that Omicron lineages Sequencing data from week 04, 2023 show that Omicron lineages XBB.3, BQ.1, BQ.1.1.28, BE.8, XBB.1.5, BE.1.1, CH.1.1, BQ.1.1.20, BE.7, BA.5.11 are overall, circulating in January in South Africa, followed by delta lineage AY.120.1, as of week 4, 2023.

PART 1: Detection and quantification of SARS-CoV-2 in wastewater

Background

The detection and monitoring of SARS-CoV-2 through wastewater was first proposed in April 2020. Initial reports describing the feasibility and practical usefulness of this approach emerged simultaneously from several countries during August 2020. Recent evidence has shown that SARS-CoV-2 can be detected in wastewater prior to the appearance of clinical cases, and longitudinal tracking of SARS-CoV-2 viral load in wastewater correlates with the burden of clinically diagnosed cases. Furthermore, the sequencing of SARS-CoV-2 RNA fragments in wastewater has identified variants of concern as well as mutations not detected in clinical cases.

In South Africa, SARS-CoV-2 epidemiology is monitored through laboratory testing of clinical cases using reverse-transcriptase polymerase chain reaction (RT-PCR) tests and rapid antigen tests, COVID-19 hospital admissions and COVID-19 related deaths. Laboratory testing data is sent by testing laboratories to the National Institute for Communicable Diseases (NICD) via the DATCOV system. From these data sources, epidemiological indicators including incidence rates of testing and case detection, hospitalisation and death rates are made available to key stakeholders and the general public.

Clinical epidemiology based on reporting of laboratory-confirmed cases of SARS-CoV-2 has limitations. Household transmission studies in South African urban and rural settings have demonstrated that a large proportion of cases are asymptomatic or so mild as not to elicit health-seeking, and that laboratory-confirmed cases likely represent less than 10% of SARS-CoV-2 cases prevalent in a community at any given time. Secondly, there is increasing use of rapid antigen detection tests in clinical settings. Results of these tests may not be reported to surveillance networks. Consequently, laboratory diagnosis is increasingly less representative of the burden of disease.

In November 2020, a network of testing laboratories, which became known as the South African Collaborative COVID-19 Environmental Surveillance System (SACCESS) network, was established in order to support the development of a common testing methodology, identify and address challenges, and share best practices related to qualitative, quantitative and sequencing of SARS-CoV-2 in wastewater. Treatment of wastewater in South Africa is the responsibility of local government. Approximately 1050 wastewater treatment works (WWTPs) are administered by metropolitan councils and local government and treat industrial and domestic waste. SACCESS partners and the NICD have engaged with local government to support sample collection, interpretation and utilisation of the results for public health purposes.

The SACCESS network aims to detect and quantify SARS-CoV-2 in wastewater in urban settings in South Africa, to compare trends, temporal and geographic distribution of SARS-CoV-2 levels in wastewater with trends in clinical epidemiology so as to support the use of wastewater-based epidemiology for COVID-19 outbreak prevention and response activities.

Methods

Outbreak context and clinical case epidemiology

Since the first case of SARS-CoV-2 in South Africa was detected on 3rd March 2020, laboratories in the country have conducted **over 25 million RT-PCR and antigen tests**. Five distinct waves of SARS-CoV-2 infection have occurred so far, peaking in June 2020, December 2020, July 2021, December 2021 and June 2022, respectively. The current de-duplicated and geospatially allocated national line list of laboratory-confirmed cases of SARS-CoV-2 (identified by RT-PCR or antigen test) is provided by the NICD for comparison with results from SARS-CoV-2 testing of wastewater.

Establishment of the laboratory testing network

Commencing in 2018, the NICD had been conducting testing of wastewater for poliovirus as part of the National Department of Health's polio surveillance programme. In 2020, the NICD commenced testing of influent wastewater samples from these 18 sites, including eight in Gauteng Province, two in the City of Cape Town (Western Cape Province), two in Mangaung (Free State Province), two in eThekwini (KwaZulu- Natal Province) and four in Eastern Cape Province (two in Buffalo City Metro and two in Nelson Mandela Metro). Quantitative testing results for these sites are available from week 8 of 2021, onwards.

Additional wastewater plants across all metropolitan areas as well as sentinel site plants in smaller provinces were included from February 2021. From August 2021, quantitative testing was conducted on all specimens submitted to partner laboratories for testing. Presently, samples from 87 WWTPs are being tested for SARS-CoV-2. The supplementary Table 1 at the bottom of the page shows all the data for these plants, including their geographical location, the surrounding suburbs, water service authority, the testing laboratory, and dates testing began in these sites.

SARS-CoV-2 detection and quantitation methodology

The general approach of SARS-CoV-2 detection in wastewater used at all network laboratories is virus concentration, followed by nucleic acid extraction and molecular detection. At the identified wastewater treatment facilities grab or passive samples of influent are collected and transported at $<5^{o}C$ to the testing facility. Table 1 summarises the sample collection, processing and detection methodology used by laboratories involved in the surveillance project. The levels of SARS-CoV-2 in wastewater are reported in copies/mL of wastewater. However, in the present report the cycle threshold (Ct) value was used to represent levels of SARS-CoV-2 at sites covered by the NICD.

Table 1. Sampling and methodology used by laboratories involved in the NICD-WRC led COVID-19 wastewater surveillance project.

Name of laboratory	Sampling	Virus concentration	Nucleic acid extraction	Molecular analysis	Molecular analysis platform
National Institute for Communicable Diseases (NICD)	Grab	Ultrafiltration (Centricon® Plus-70 centrifugal ultra-filter device)	QIAamp® viral RNA mini kit	RT-qPCR[a] using the Allplex™ 2019-nCoV Assay and the EDX SARS-CoV-2 standard	7500 Real-Time PCR System (Applied Biosystems
GreenHill Laboratories / Praecautio	Grab	Ultrafiltration (Amicon® Ultra-15 Centrifugal Filter Unit)	Omega Bio-Tek Mag-Bind® Viral DNA/RNA 96 Kit	RT-qPCR using the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel	Rotor-Gene Q (Qiagen)
National Institute for Occupational Health (NIOH)	Grab	Skimmed milk flocculation	MagMAX Viral/ Pathogen Nucleic Acid Isolation Kit	RT-qPCR using the TaqPath COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher)	QuantStudio™ 5 Real-Time PCR System 96-well, 0.1 mL, desktop (Applied Biosystems)
Waterlab/University of Pretoria	Grab	Skimmed milk flocculation	QIAamp® Ultrasens® Virus kit	RT-qPCR using the Allplex™ 2019-nCoV Assay and the using the 2019_ nCoV_N positive control plasmid (Integrated DNA Technologies, Inc, Coralville, IA)	QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)
South African Medical Research Council – Tuberculosis	Grab	None – sample is centrifuged then	ZymoBiomics RNA Extraction Kit	RT-qPCR[a] using the Allplex™ 2019-nCoV	QuantStudio 5 (Applied Biosystems)

Platform (SAMRC-TB Platform)		supernatant analysed		Assay and the EDX SARS-CoV-2 standard	
Lumegen	Passive	Passive sampler and resuspension in phosphate buffered saline	MN DNA/RNA pathogen extraction Kit	RT-qPCR using the TaqPath COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher)	QuantStudio 5 (Applied Biosystems)
Council for Scientific and Industrial Research (CSIR)	Grab	Polyethylene Glycol precipitation	Omega Bio-tek ENZA total RNA Kit II	RT-qPCR using the 2019-nCoV CDC EUA Kit	Qiagen Rotor-Gene 6000 (5-plex) (Qiagen)
Durban University of Technology – Institute of Wastewater Management	Grab	Ultrafiltration (Centricon® Plus-70 centrifugal ultra-filter device)	QIAamp® viral RNA mini kit	RT-ddPCR[b] using CDC 2019-nCoV_N2 Primers, Fam Labelled, double quenched probes	QX200 AutoDG Droplet Digital PCR System (Bio-rad)

Interpretation of SARS-CoV-2 levels in wastewater

Interpretation of SARS-CoV-2 wastewater levels is evolving. We have elected to use interpretive principles outlined in Table 2 to support public health preparedness and response activities. In general, increasing or decreasing trends in levels are reported based on two or more results, as a single sample that increases or decreases compared with the result from the previous week may represent an outlier. Small changes (up to 0.5 log copies/ml) are not regarded as significant changes unless they form part of a general upward or downward trend. Comparison of results over time when quantification is done by the same laboratory using the same quantitative methodology is meaningful. The use of different methodologies by different laboratories precludes comparison of quantitative results across laboratories. The Ct values is an alternative for quantification. Changes in the Ct value of SARS-CoV-2 give an indication of whether the burden of disease is increasing or decreasing

Table 2. Principles of SARS-CoV-2 detection and quantification on influent samples from wastewater treatment plants and interpretive principles to guide application of test results to support COVID-19 public health responses, South Africa.

Testing modality	Interpretive principles to support public health responses
Detection of SARS-CoV-2	When a test result changes from • positive to negative, this signifies fewer/no cases in population • negative to positive, this indicates the need for increased population awareness and action • Qualitative results (presence or absence) are comparable between laboratories • Changes in the Cycle threshold (Ct) value of SARS-CoV-2 give an indication of whether the burden of disease is increasing or decreasing
Quantification of SARS-CoV-2	• The concentration of SARS-CoV-2 at a particular facility may be used to infer the burden of SARS-CoV-2 in the population served by the wastewater treatment facility. • Changes in the concentration of SARS-CoV-2 give an indication of whether the burden of disease is increasing or decreasing • Quantitative results between laboratories are not comparable. Quantitative results should be interpreted for a single wastewater treatment plant tested by the same laboratory using the same methodology over time.

Results

Gauteng Province

A: City of Tshwane North (sub-district 5)

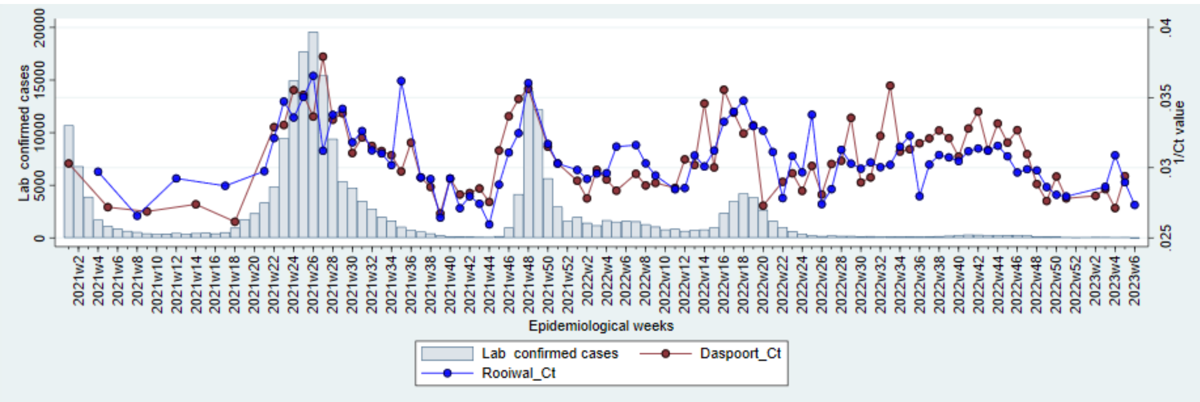


Figure 1A Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) for selected wastewater treatment plants (WWTP) and metropolitan areas in Tshwane District Municipality (Tshwane North), Gauteng Province during epidemiological weeks 1 of 2021 to week 06 of 2023.

B: City of Johannesburg Metropolitan Municipality

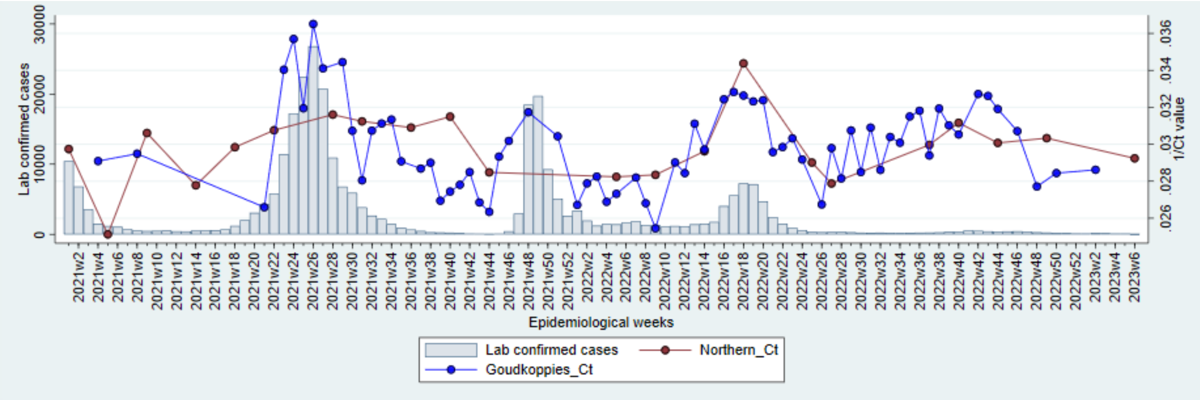
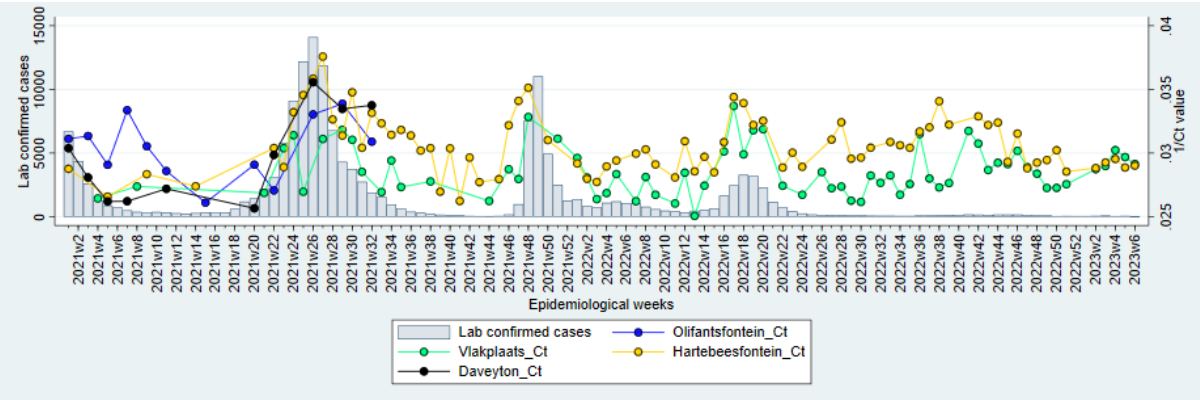


Figure 1B. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) for selected wastewater treatment plants (WWTPs) in the City of Johannesburg Metropolitan Municipality, Gauteng Province during epidemiological weeks 1 of 2021 to week 06 of 2023.

C: City of Ekurhuleni



Figures 1C. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) for selected wastewater treatment plants (WWTP) in Ekurhuleni Metropolitan Municipality, Gauteng Province during epidemiological weeks 1 of 2021 to week 06 of 2023.

In Epi week 06, the SARS-CoV-2 levels at Rooiwal WWTP in Tshwane district dropped to minimal levels. However, the moderate levels of SARS-CoV-2 remained in Vlakplaats and Hartebeesfontein in the City of Ekurhuleni. Similarly, moderate levels of SARS-CoV-2 were also observed at Johannesburg's Northern WWTP.

KwaZulu-Natal Province

2: eThekwini Metropolitan Municipality

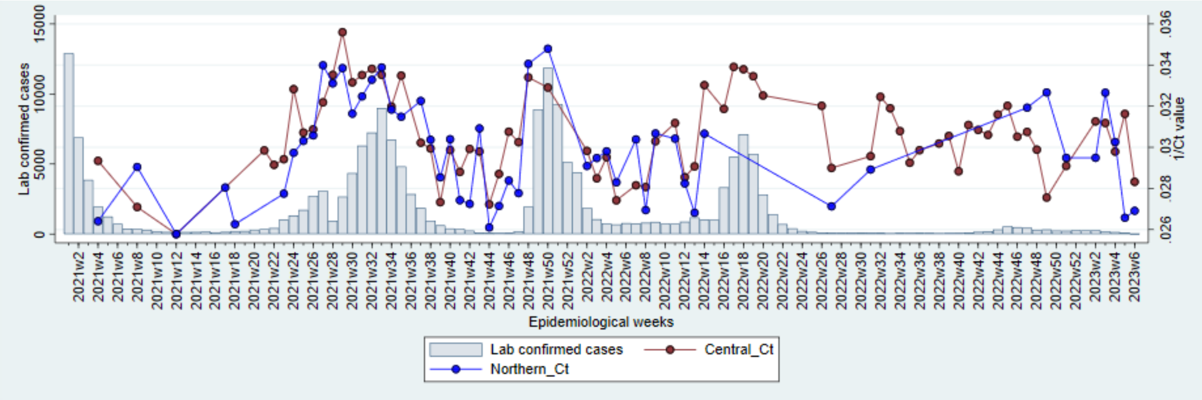
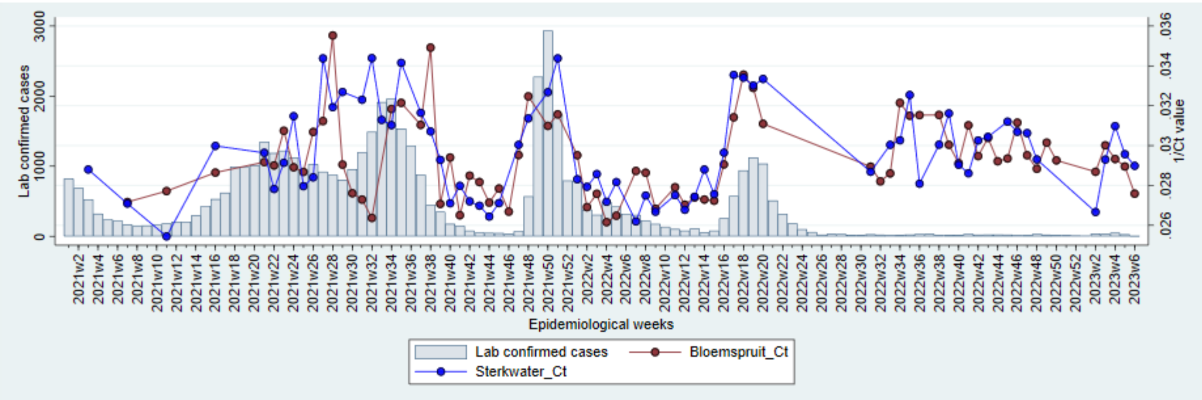


Figure 2. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) from wastewater treatment plants (WWTP) in eThekwini, Kwa-Zulu Natal Province during epidemiological weeks 1, 2021 and week 06, 2023.

SARS-CoV-2 2023 levels in Central WWTP dropped to minimal level in Epiweek 06. Similarly, the levels at Northern WWTP remained low in Epi week 06.

Free State Province - Mangaung

A: Bloemfontein sub-district



Figures 3. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) from wastewater treatment plants (WWTPs) in Mangaung, Free State Province (Bloemfontein during epidemiological weeks 1, 2021 to 06, 2023.

SARS-CoV-2 levels in Sterkwater appear to be declining further in Epi Week 06, while levals in Bloemspruit have dropped to a minimal level.

Eastern Cape Province

A: Nelson Mandela Metropolitan Municipality

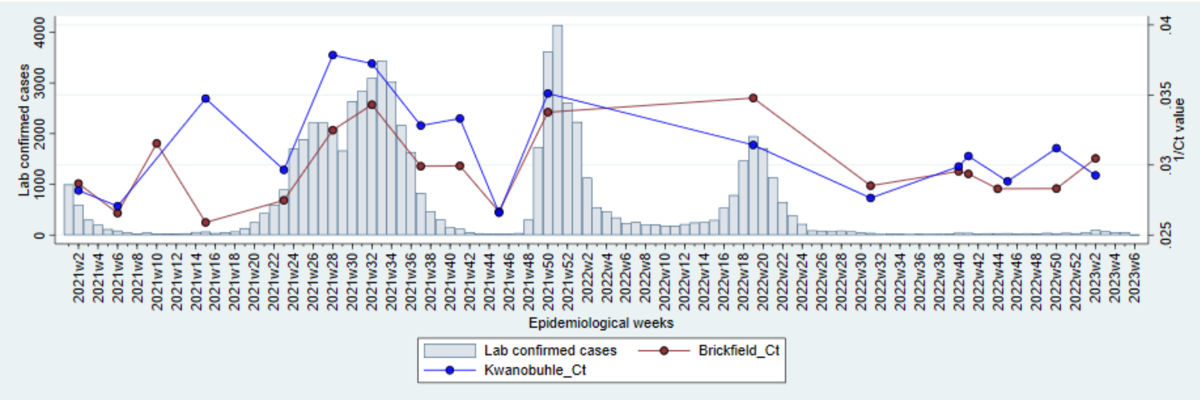


Figure 4A. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) from wastewater treatment plants (WWTPs) in Nelson Mandela Metro, Eastern Cape Province during epidemiological weeks 1, 2021 to 06, 2023.

B. **Buffalo City**

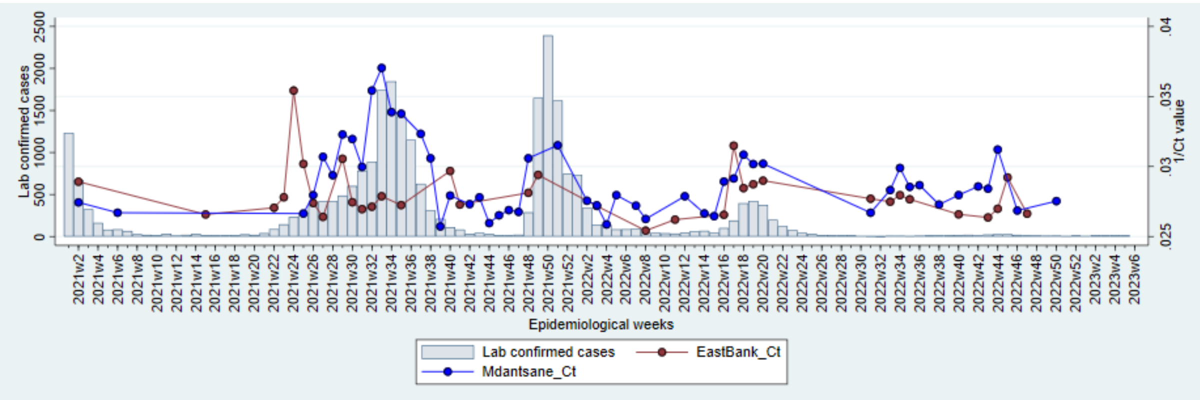


Figure 4B. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 taken as inverse of CT values (coloured lines) from wastewater treatment plants (WWTPs) in Nelson Mandela Metro, Eastern Cape Province during epidemiological weeks 1, 2021 to 06, 2023.

As of week 03, SARS-CoV-2 levels were at intermediate levels at Bricksfield and Kwanabuhle WWTPs respectively in Nelson Mandela district (4A) and Buffalo City (4B) respectively.

Readers are referred to the SAMRC wastewater dashboard for more in-depth data regarding levels of SARS-CoV-2 in wastewater plants in Nelson Mandela Metro (https://www.samrc.ac.za/wbe/).

Western Cape Province

City of Cape Town

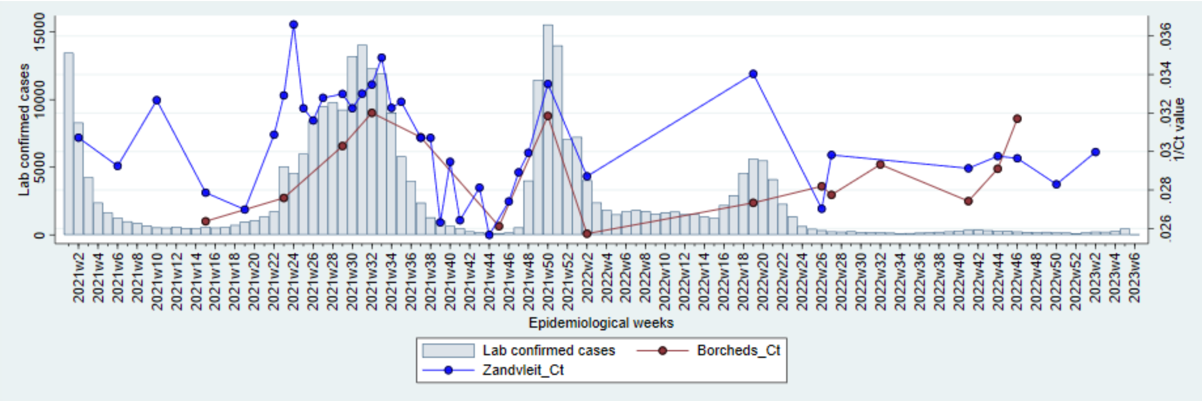


Figure 5. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in taken as inverse of CT values (coloured lines) from wastewater treatment plants (WWTPs) in the City of Cape Town, Western Cape Province during epidemiological weeks 1, 2021 to 06, 2023.

SARS-CoV-2 levels in Zandvleit WWTP in Cape Town were at intermediate levels as of week 03, 2023.

Readers are referred to the SAMRC website, which provides data from additional wastewater treatment plants in the City of Cape Town and other Western Cape districts (https://www.samrc.ac.za/wbe/) to contextualise the results.

Limitations

It is not possible to estimate population burden of disease using wastewater testing of SARS-CoV-2 as sources of variability are multiple, including variation in length and concentration of SARS-CoV-2 excretion by infected persons, variation in degradation rate of viral RNA in wastewater and sampling error. Interpretation of results from the levels of SARS-CoV-2 in wastewater is enhanced when the population served by the wastewater treatment plants is well characterised in terms of SARS-CoV-2 testing rates, health seeking behaviour, hospital admissions and deaths due to SARS-CoV-2, as well as other general indicators of health. Further exploration of the relationship between levels of SARS-CoV-2, local trends in clinical case burden, environmental factors, and test methodology will support the interpretation of observed fluctuations in RNA levels. Quality assessment and inter-laboratory comparisons are underway to ensure participating laboratories are providing consistent and comparable results.

PART 2: Results from sequencing of SARS-CoV-2 RNA fragments in wastewater

Background

SARS-CoV-2 has been classified into different variants, that are continually emerging as a result of viral evolution. These variants acquire or lose mutations coding for various epitopes found on key viral proteins which lead to changes in transmissibility dynamics, response to treatment or ability to evade neutralisation by antibodies. WHO classified SARS-CoV-2 variants into variants of concerns (VOCs) and variants of interest (VOIs). VOCs have included Alpha, Beta, Delta, and Gamma, and Omicron. Of these, Beta and Omicron were first reported in South Africa. VOIs include Lambda and Mu (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/).

The Network for Genomics Surveillance of South Africa (NGS-SA) monitors the epidemiology of SARS-CoV-2 variants in PCR-confirmed cases in South Africa. In clinical cases, variant detection is performed using whole genome sequencing and other methods such as real-time PCR. During the first wave (June to August 2020), the Wuhan SARS-CoV-2 strain dominated amongst clinical cases while in the second wave (November 2020 to February 2021), the Beta variant was discovered and was predominant. The third wave (May to September 2021) was characterized by the dominance of the Delta variant and the fourth wave (November 2021 to January 2022) by the Omicron variant.

Several groups have sequenced SARS-CoV-2 from wastewater including groups in the Netherlands which generated near whole genome sequence from wastewater (Lara *et al*., 2020). In the United States, wastewater sequencing provided comparable results to clinical testing and contained sequences with previously undescribed mutations before they appeared in clinical samples (Crits-Christoph *et al*., 2021).

Here, we report on SARS-CoV-2 sequences and variants of concern present in wastewater samples collected at sentinel wastewater treatment plants in South African urban metros from week 14 in 2021 to week 4 of 2023.

Methods

Wastewater sites

In 2020, the National Institute for Communicable Diseases commenced with sequencing of influent wastewater samples for SARS-CoV-2 RNA from 15 wastewater treatment plants in metropolitan areas, including five in Gauteng Province, four in Eastern Cape province, two in the City of Cape Town (Western Cape Province), two in Mangaung (Free State Province), two in eThekwini (KwaZulu- Natal Province) (Table 1).

Table 1. Characteristics of wastewater treatment facilities and of samples submitted for SARS-CoV-2 sequencing from these sites, 2021-2023

Province	Metro or District	Plant name	Population size served by the facility	Genomic testing			
				Epidemio-logical week when sequencing started in 2021	Number of samples submitted for sequencing	Number of samples with coverage > 50	% of samples with useable quality sequences
Eastern Cape	Buffalo City Metro	East Bank	141000	15	33	11	33,33
		Mdantsane	112900	25	47	20	42,55
	Nelson Mandela Metro	Brickfield	40000	15	16	12	75,00
		KwaNobuhle	100320	15	18	12	66,67
Free State	Mangaung	Sterkwater	200000	16	58	33	56,90
		Bloemspruit	350000	16	62	44	70,97
Gauteng	Ekurhuleni Metro	Daveyton	100000	20	5	0	0,00
		Hartebeesfontain	100000	14	65	39	60,00
		Vlakplaats	200000	21	56	36	64,29
	Johannesburg Metro	Northern	1200000	14	16	10	62,50
		Goudkoppies	500000	21	56	29	51,79
	Tshwane Metro	Rooiwal	unknown	17	72	37	51,39
		Daspoort	unknown	14	69	38	55,07
KwaZulu-Natal	eThekwini Metro	Northern	316425	17	38	18	47,37
		Central	350000	17	58	35	60,34
Western Cape	City of Cape Town Metro	Borcherd's Quarry	380000	15	13	9	69,23
		Zandvliet	460000	15	32	17	53,13
Total					714	400	

Sample collection, RNA extraction, amplification and sequencing

One litre of grab sewage samples were collected and transported at 4°C. Viruses were concentrated from the sample by ultrafiltration (Ikner, Soto-Beltran and Bright, 2011), and RNA was extracted using the QIAamp Viral RNA kit (Qiagen, GmbH, Germany). SARS-CoV-2 was detected by RT-PCR using Allplex^{TM} 2019- nCoV Assay from Seegene kit (Seoul, Korea). RNA was re-extracted from SARS-CoV-2 positive concentrates and subjected to amplicon-based whole genome sequencing using the Sinai protocol with some modifications (Gonzalez-Reiche *et al.*, 2020). Libraries were prepared using the COVIDSeq Kit (Illumina Inc, USA), and sequencing was performed using Illumina COVIDSeq kits as described in (Bhoyar *et al.*, 2021) at the Sequencing Core Facility at the NICD.

Sequence analysis

The ARTIC protocol for sequence analysis (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html) was used in the Galaxy pipeline for sequence analysis (RC, 2005). Reads were trimmed and filtered according to published criteria (Khailany, Safdar and Ozaslan, 2020). At least 10 reads required at each nucleotide position for downstream analysis. Mutations present at 10% or less were removed from the analysis. Reads were mapped against the reference genome (Wuhan strain/ NC_045512.2) and amino acid variation was analysed. Table 2 illustrates an example of amino acids variation file (https://usegalaxy.eu/).

Table 2: Illustration of amino acids variations. A shows sample ID. B is QC filter, which is quality indicator. C is the number of reads produced for each sample. D is the effect of the mutation detected in the gene. E is the name of the gene where mutation occurred. F is the mutation detected. G is the frequency of the reads in the mutation.

A	B	C	D	E	F	G
Sample	**QC filtre**	**Number of reads**	**Mutation effect**	**Gene**	**Mutation**	**Frequency of mutations**
ENV-COV-21-285_S337_001.fastq	PASS	12	NON_SYNONYMOUS_CODING	ORF1ab	K790Q	0.833333
ENV-COV-21-285_S337_001.fastq	PASS	644	NON_SYNONYMOUS_CODING	ORF1ab	K798N	0.057453
ENV-COV-21-285_S337_001.fastq	PASS	14	NON_SYNONYMOUS_CODING	ORF1ab	F800L	0.857143
ENV-COV-21-285_S337_001.fastq	PASS	44	SYNONYMOUS_CODING	ORF1ab	G45	0.863636
ENV-COV-21-285_S337_001.fastq	min_af_0.05Xmin_dp_1Xmin_dp_alt_10	44	FRAME_SHIFT	ORF1ab	Y46L?	0.045455
ENV-COV-21-285_S337_001.fastq	PASS	1347	NON_SYNONYMOUS_CODING	ORF1ab	T54P	0.123979
ENV-COV-21-285_S337_001.fastq	PASS	153	SYNONYMOUS_CODING	ORF1ab	T54	0.078431

SARS-CoV-2 in the sewage system is fragmented and the genome originated from multiple different individuals, therefore, the generation of a consensus sequence for each sample is not meaningful. Therefore, to identify variants at each geographic location, we analysed amino acid variation in each individual sample. For each VOC or VOI, unique single nucleotide polymorphisms were identified by comparing the new lineage with the Wuhan strain in a public database (https://outbreak.info/). Using the amino acid variation data file, we used STATA software (v 17.1) (https://www.stata.com/) to collate spike-gene mutations in a matrix such that the columns represented the amino acid positions of the spike protein and each row recorded all mutations detected in each sample at every locus across the spike gene, for each epidemiological week. The matrix was used to create a heatmap using conditional formatting on Excel and both low and high read frequency mutations were included. The matrix was also used to plot a mutational profile by filtering out positions were mutations were not present in that respective week and the list of signature mutations present for each VOC and VOI in the spike protein region, listed by WHO (Table 3) were used to deduced the variant or lineage circulating in each week. To further capture evolution and spread of the virus, Freyja, a tool used to estimate the relative abundance of virus lineages present in wastewater. Freyja uses a “barcode” library of lineage defining mutations to uniquely define all known SARS-CoV-2 lineages and solves for lineage abundance using a depth-weighted, least absolute deviation regression approach. Freyja is free to use and available at (https://github.com/andersen-lab/Freyja).

Table 3: Signature mutations and lineages of concern or under monitoring listed and identified by The World Health Organization (WHO) (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/).

Pango lineage	One or more of these mutations in the spike protein	Relationship to circulating VOC lineages
BA.5	S:R346X. S:K444X. S:V445X . S:N450D or S:N460X	BA.5 sublineages (e.g. BF.7. BF.14. BQ.1)
BA.2.75	**BA.2.75:** S:K147E. S:W152R. S:F157L. S:I210V. S:G257S. S:D339H. S:G446S. S:N460K. S:Q493R **BA.2.75.2:** S:R346T. S:F486S. S:D1199N	BA.2 sublineages
BA.4.6	S:R346T. S:N658S	BA.4 sublineage
BJ.1	S:V83A. S:Y144-, S:H146Q. S:Q183E. S:V213E, S:G339H. S:R346T. S:L368I. S:V445P. S:G446S. S:V483A. S:F490V. S:G798D. S:S1003I	BA.2 sublineage (B.1.1.529.2.10.1.1)
XAY	S:R21G. S:W152L. S:F186L. S:T95I. S:F486P. S:P621S. S:A706V. S:T111I	Recombinant (Omicron and delta)
XBA	S:R21G. S:W152L. S:F186L. S:T95I. S:F486P. S:P621S. S:A706V. S:T111I	Recombinant (Omicron and delta)
XBB.1.5	S:V83A, S:Y144-, S:H146Q, S:Q183E, S:V213E, S:R346T, S:L368I, S: F486P, S:F490V, S:M1233V	Recombinant (BA.2 sub-lineages)

Results and discussion

Detection of SARS-CoV-2 variants and lineages from wastewater samples using Freyja

Up to the **26th January, 2023**, a total of **714** wastewater samples from sites listed in Table 1 underwent RNA extraction, amplification and sequencing. Of these **714** samples, **400 (56.02%)** yielded SARS-CoV-2 RNA sequences that had a coverage >50%, which were considered for the variants and lineages analysis. Overall, the distribution of SARS-CoV-2 variants in South Africa from wastewater has progressed from the predominance of Beta variant in January 2021, to Delta variant (June 2021) to Omicron in early 2022, which continues to circulate to date (Figure 1). Furthermore, Omicron lineages including; XBB.3 (light pink), BQ.1 (pale blue), BQ.1.1.28 (tan), BE.8 (purple), XBB.1.5 (red), BE.1.1 (orange) and CH.1.1 (green), BQ.1.1.20 (olive green), BE.7 (dark tan), BA.5.11 (dark blue), circulating in January in South Africa, followed by delta lineage AY.120.2 (neon blue), as of week 4, 2023 (Figure 2).

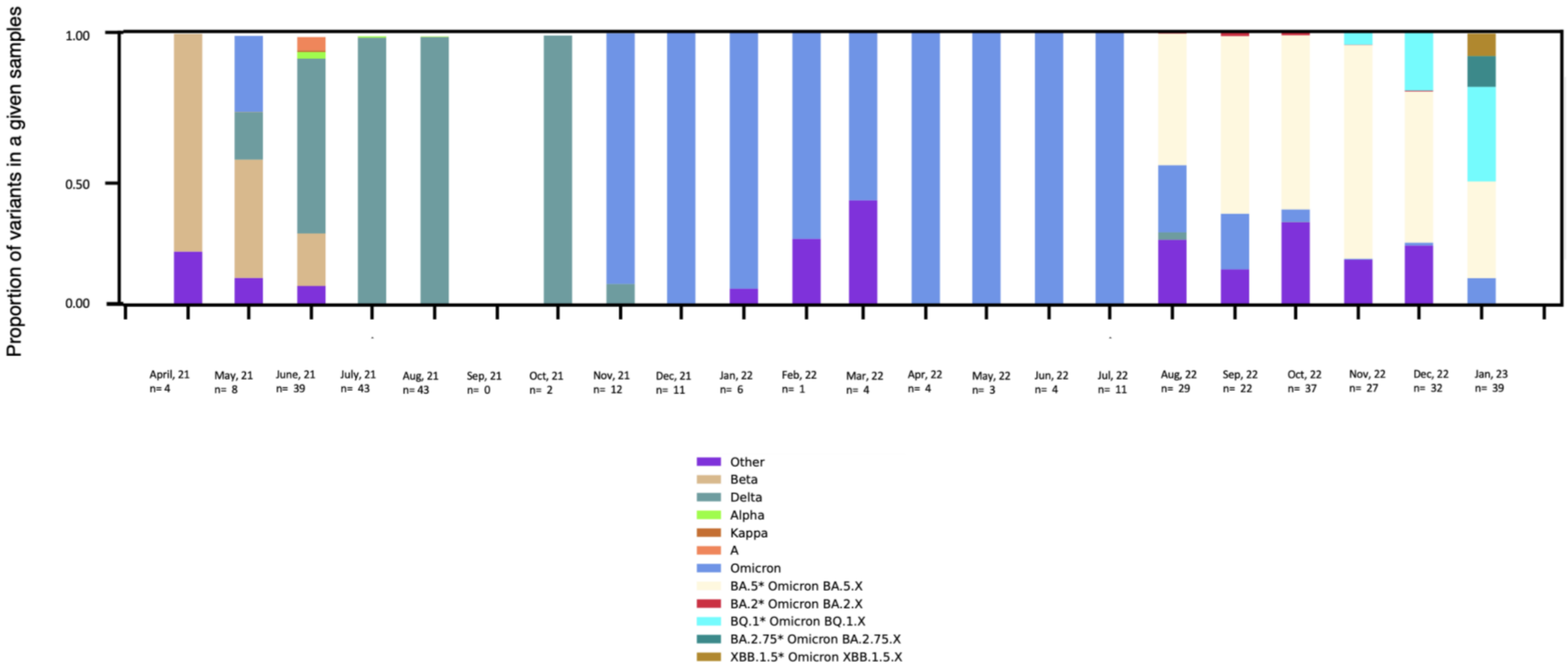


Figure . The proportion of SARS-CoV-2 variants in the environmental samples sorted by month and year (April 2021-January, 2023) from all South African provinces. The number of samples processed each month, with a coverage >50% are indicated as n.

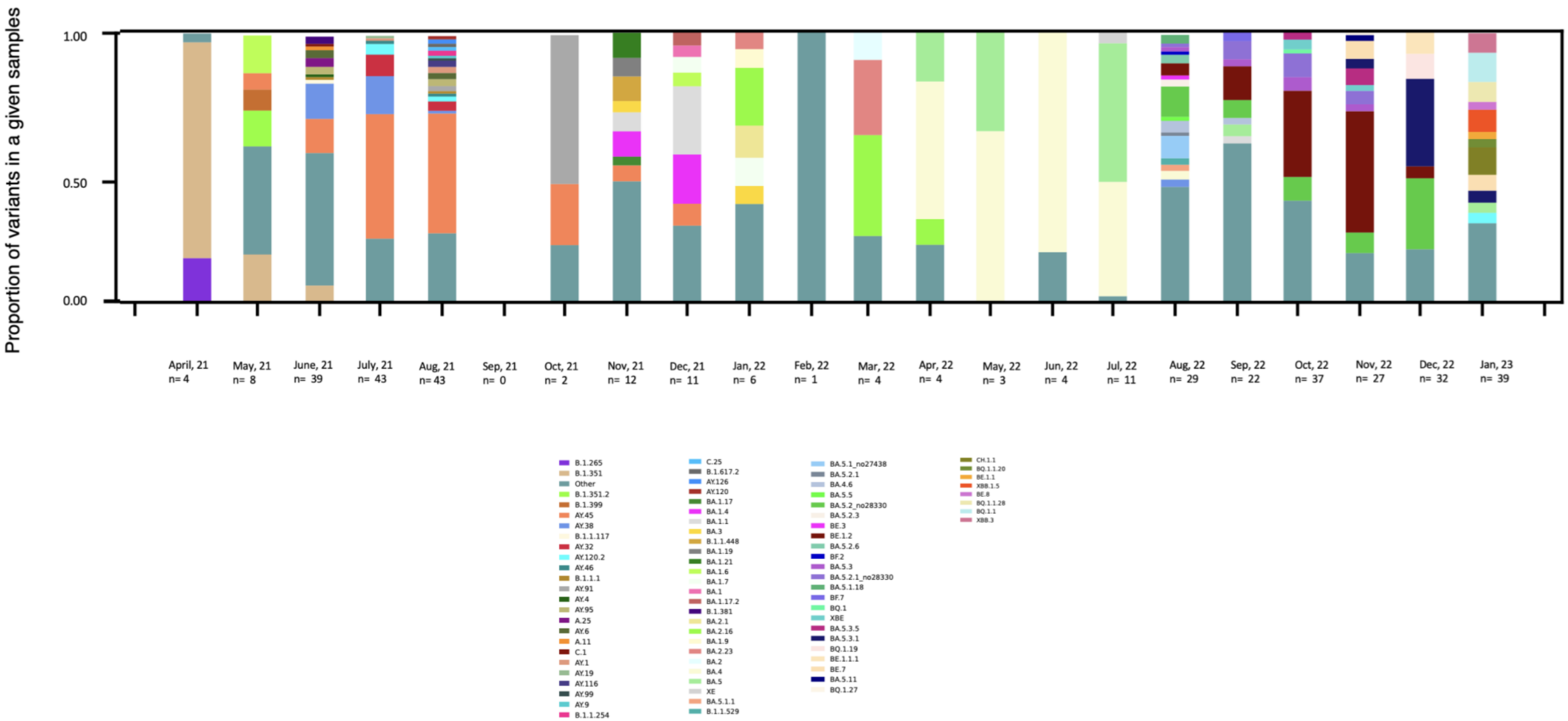


Figure . The proportion of SARS-CoV-2 lineages in the environmental samples sorted by month and year (January 2021-January 2023) from all South African provinces. The number of samples processed each month, with a coverage >50% are indicated as n.

Gauteng province

In the Gauteng province**, 189** samples yielded sequencing results displayed in Figure 1-6, which illustrates how Beta variant was present in all the sites in the Gauteng province in week 21-22, 2021 but was replaced by delta shortly after. During the interwave period (weeks 34-44, 2021) most samples submitted for sequencing failed to yield good quality sequence data, most likely due to low or absent SARS-CoV-2 RNA fragments which yielded low coverage . Omicron lineage BA.1 was first detected in week 46, 2021 in wastewater and by week 47, 2021, was found to be present in almost all sites across the province. Lineage BA.2 was then detected from week 5, 2022 in Vlakplaats, followed by the other sites. BA.3 was only detected in Goudkoppies, in week 15, 2022, however at a low read frequency. The low or absence of BA.3 in all other sites was due to either no sampling or low sequence coverage during that period. Gaps in the graph are due to either low coverage or samples were not received during that week. Due to the nature of the wastewater matrix, the genome of certain enveloped RNA viruses like SARS-CoV-2 degrade faster than nonenveloped enteric viruses and therefore have very low coverage. Omicron lineage BA.4 was detected from week 10, 2022 in the Daspoort site and shortly thereafter Omicron BA.5 emerged, causing a resurgence in hospital cases from week 15, 2022. Omicron lineages BE.2, BE.6, BE.7, BE.8, and BE.9 have now since been circulating from week 40, 2022, with BE.1 and BQ.1 dominating in proportion. BQ.1 has also since been detected in clinical case samples, along with lineage XAY (a recombinant lineage between Omicron and Delta, first detected in South Africa) dominating in proportion from week 31, 2022. In wastewater samples however, recombinant XAY was not detected by Freyja in the Gauteng province. In the recent week (week 2, 2023), Freyja has detected BE.1.1, BE.7, BE.8 and BE.9, BQ.1 and 2, BA.5 and XBB.1.5. Lineage XBB.1.5 is a recombinant between BA.2 sub-lineages and was first detected in October, 2022 in the United States of America. Recent sequences from clinical case data in South Africa have also detected XBB.1.5 in 15 patients across the Western Cape, Free State, Gauteng and Kwa-Zulu Natal. XBB.1.5 was detected in wastewater at the Goudkoppies site in week 2. In week 4, omicron lineages BQ.1 and BE.1.1 are consistently emerging with recombinant XBB.1.5 showing up in Rooiwal and XBB.3/6 in Hartbeesfontein, Rooiwal and Vlakplaats.

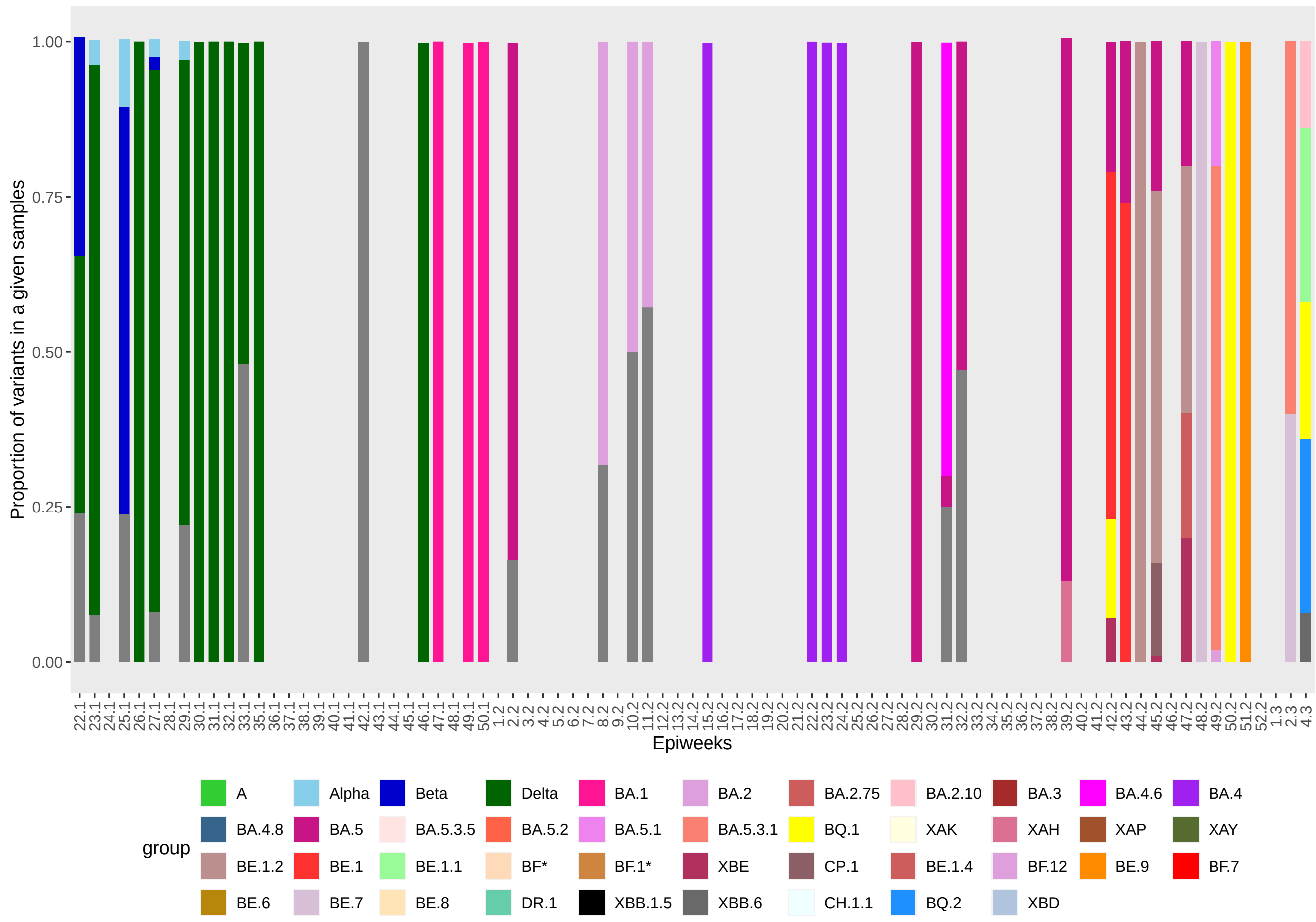


Figure 1: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Hartbeesfontein, in the Ekhurukeni region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

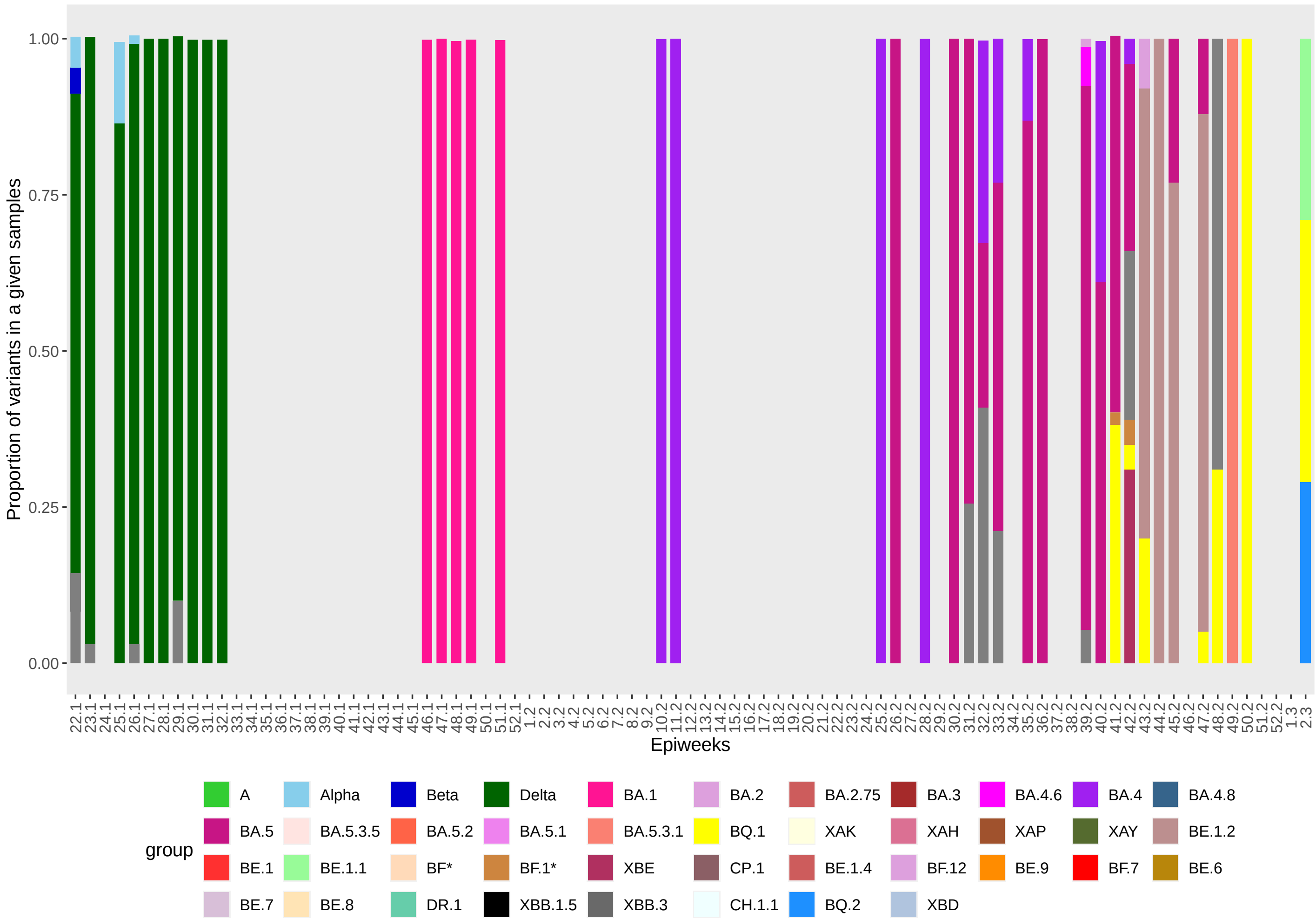


Figure 2: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Daspoort, in the Tshwane region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

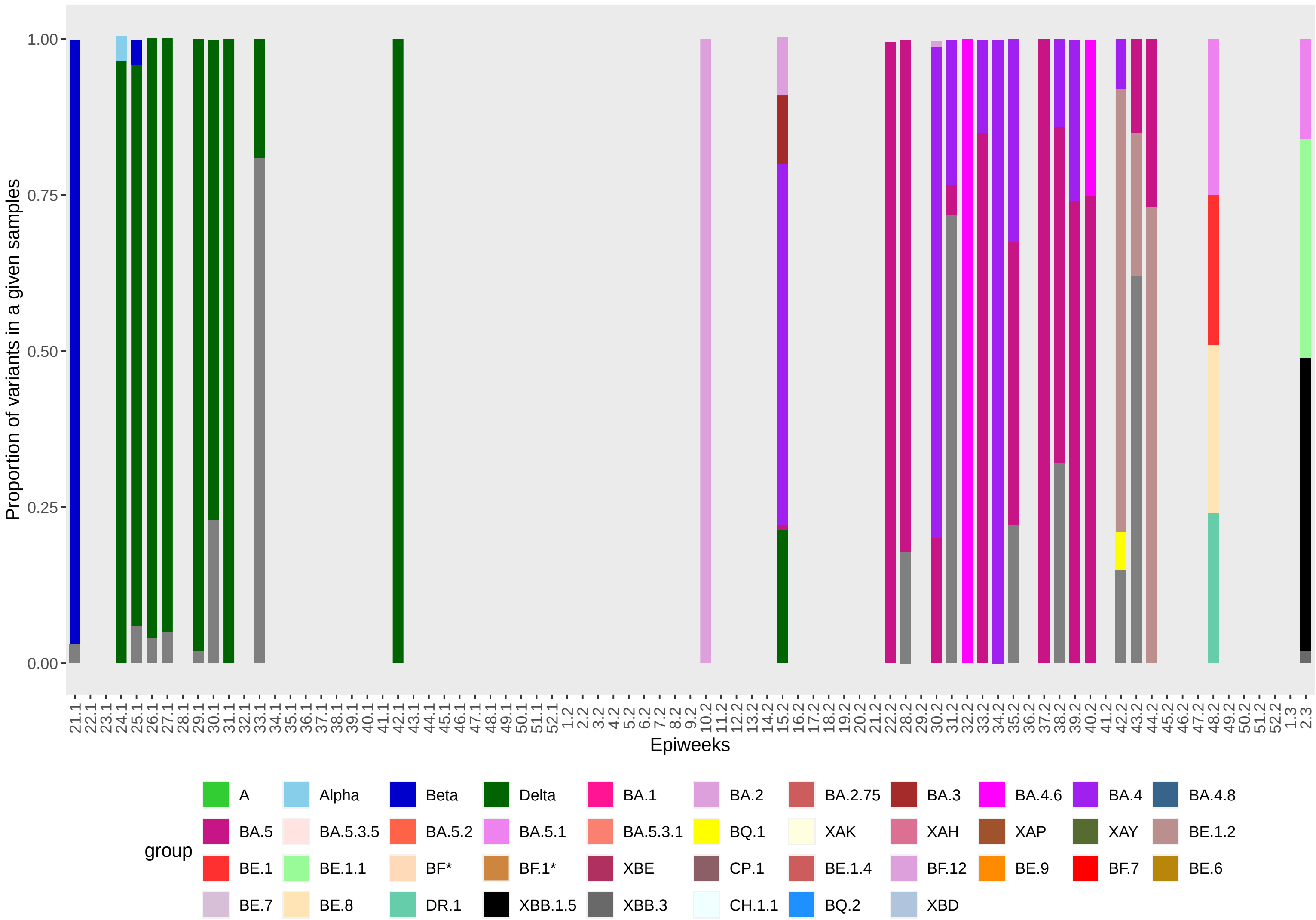


Figure 3: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Goudkoppies, in the Johannesburg region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

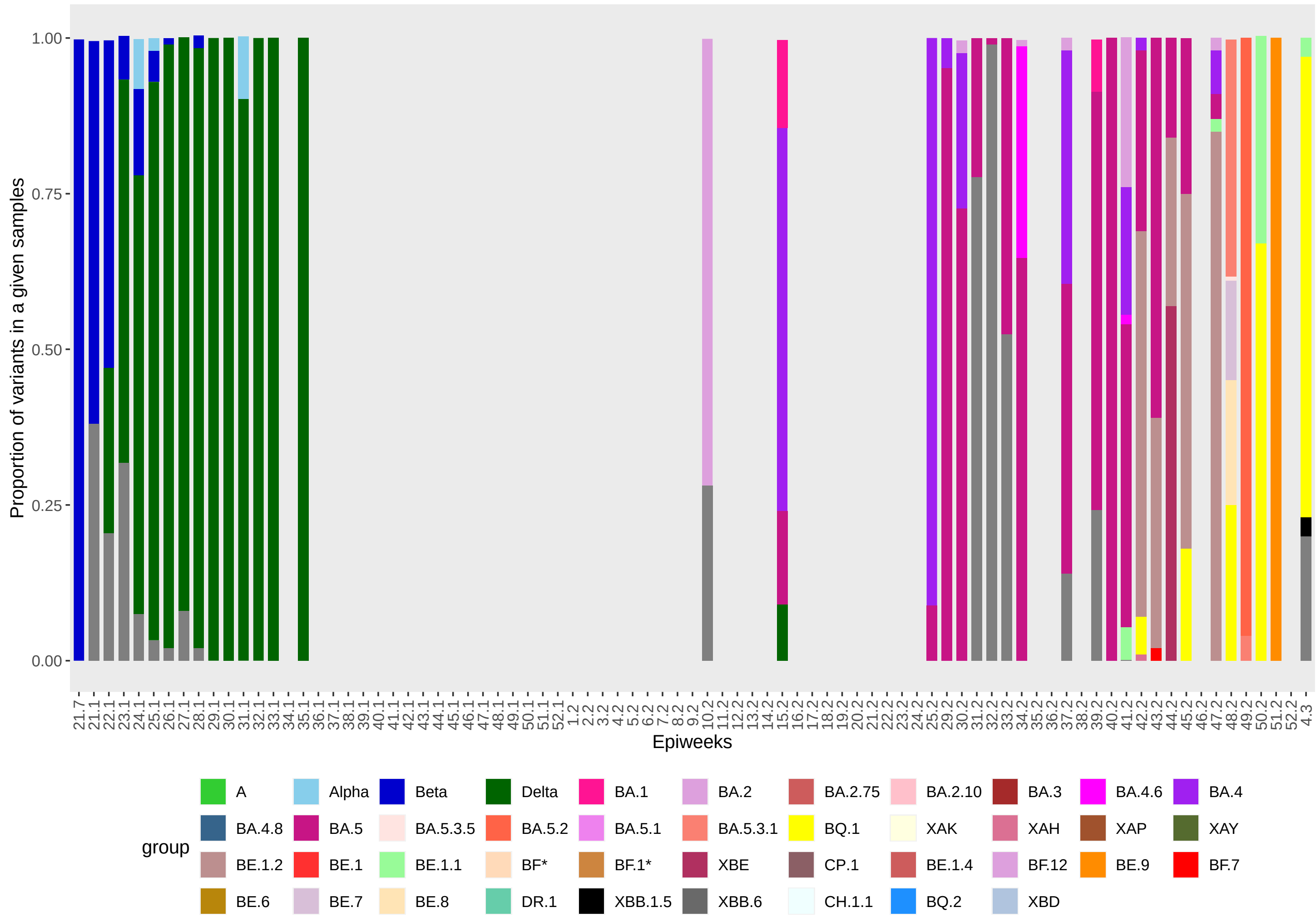


Figure 4: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Rooiwal, in the Tshwane region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

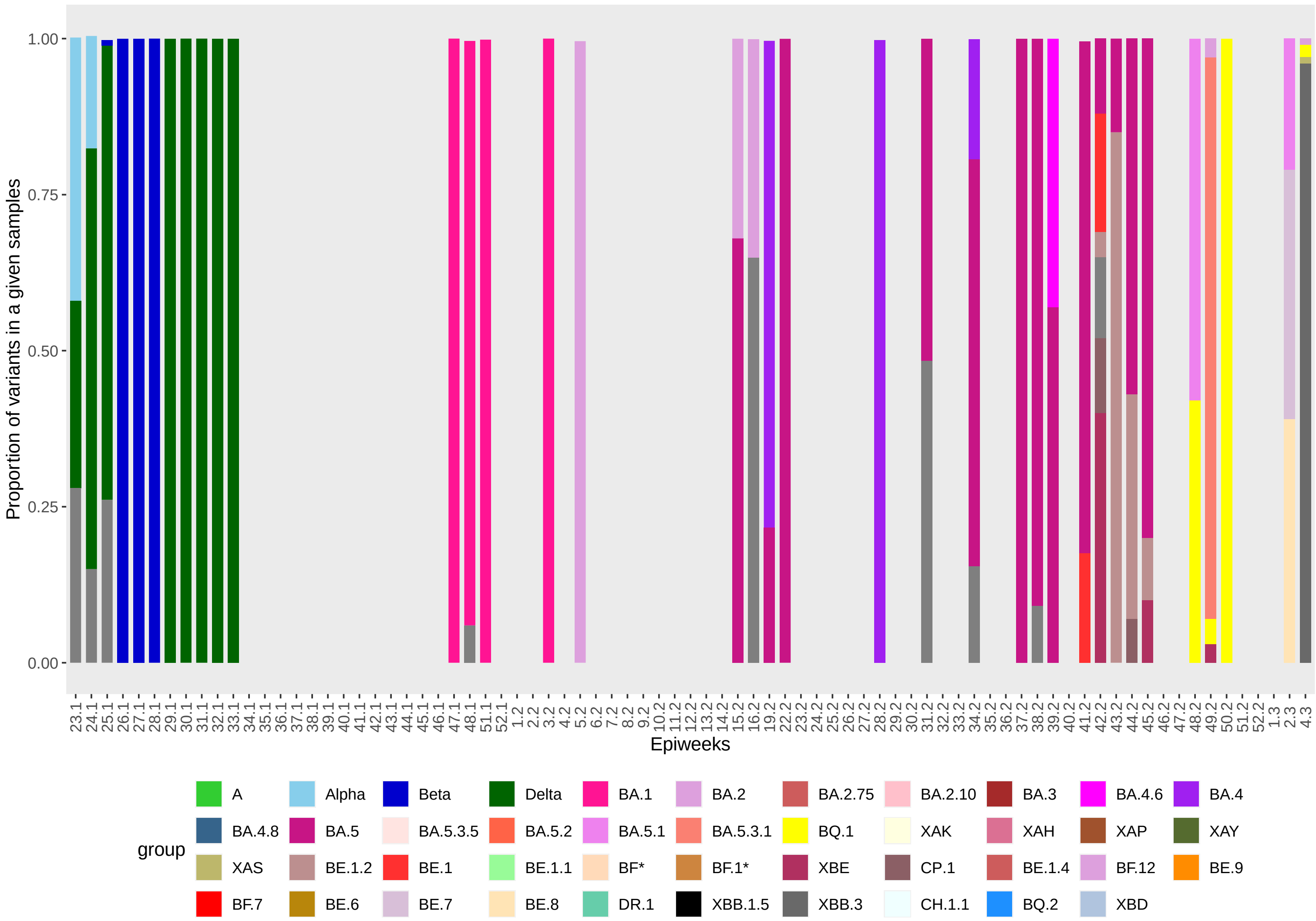


Figure 5: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Vlakplaats, in the Ekhuruleni region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

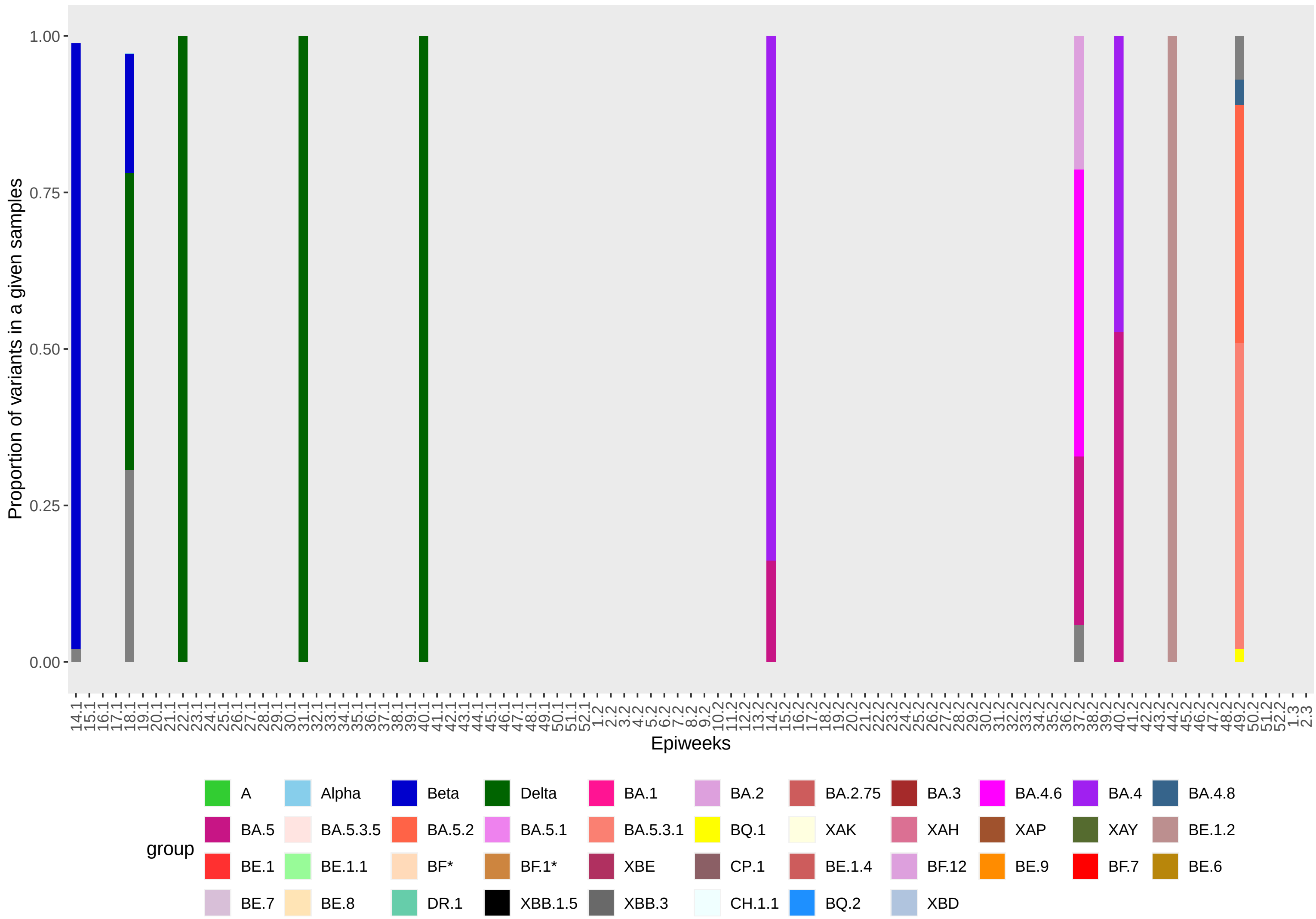


Figure 6: The proportion of SARS-CoV-2 variants and lineages in environmental samples collected from Northern Johannesburg, in the Johannesburg region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

KwaZulu- Natal province

In KwaZulu-Natal province, **53** samples yielded good sequences and were included in the analysis by Frejya. Results are represented in Figure 7 and 8. The Beta variant was detected in a single sample from Central eThekwini plant in week 24, 2021. Subsequently, Delta was first detected after week 22, 2021 in Central eThekwini, followed by Northern eThekwini, in week 24, 2021. As in the Gauteng Province, during the interwave period (weeks 34-44) most samples submitted for sequencing failed to yield good quality sequence data, most likely due to low or absent SARS-CoV-2 RNA fragments. Omicron lineage BA.2 was first detected in wastewater in week 41, 2021 in central eThekwini and week 9, 2022 in central eThekwini and continued to be present up to week 11 of 2022. Omicron lineage BA.4 and BA.4.6 was then detected from week 14, 2022, in both plants. The low levels or absence of BA.2, BA.3 and BA.4 in both sites was due to either no sampling or low sequence coverage in that period of sampling. Gaps in the graph are due to either low coverage or samples were not received during that week. Due to the nature of the wastewater matrix, the genome of certain enveloped RNA viruses like SARS-CoV-2 degrade faster than nonenveloped enteric viruses and therefore have very low coverage. Omicron lineage BA.5 was found to be detected earlier (week 41, 2021) than BA.1, 2 and 3 in central eThekwini. BA.5 then re-emerged in week 9, 2022 and continues to circulate to date. BQ.1 has recently been detected in central eThekwini, along with lineage XAY, at a low proportion in week 48, 2022. In week 2, omicron lineages; BE.1.4, BQ.1 and CH.1.1 were detected in central eThekwini. In the recent (week 4), BQ.1 is consistently emerging with XBB.1.5 showing up fir the first time in wastewater in Northern eThewkini.

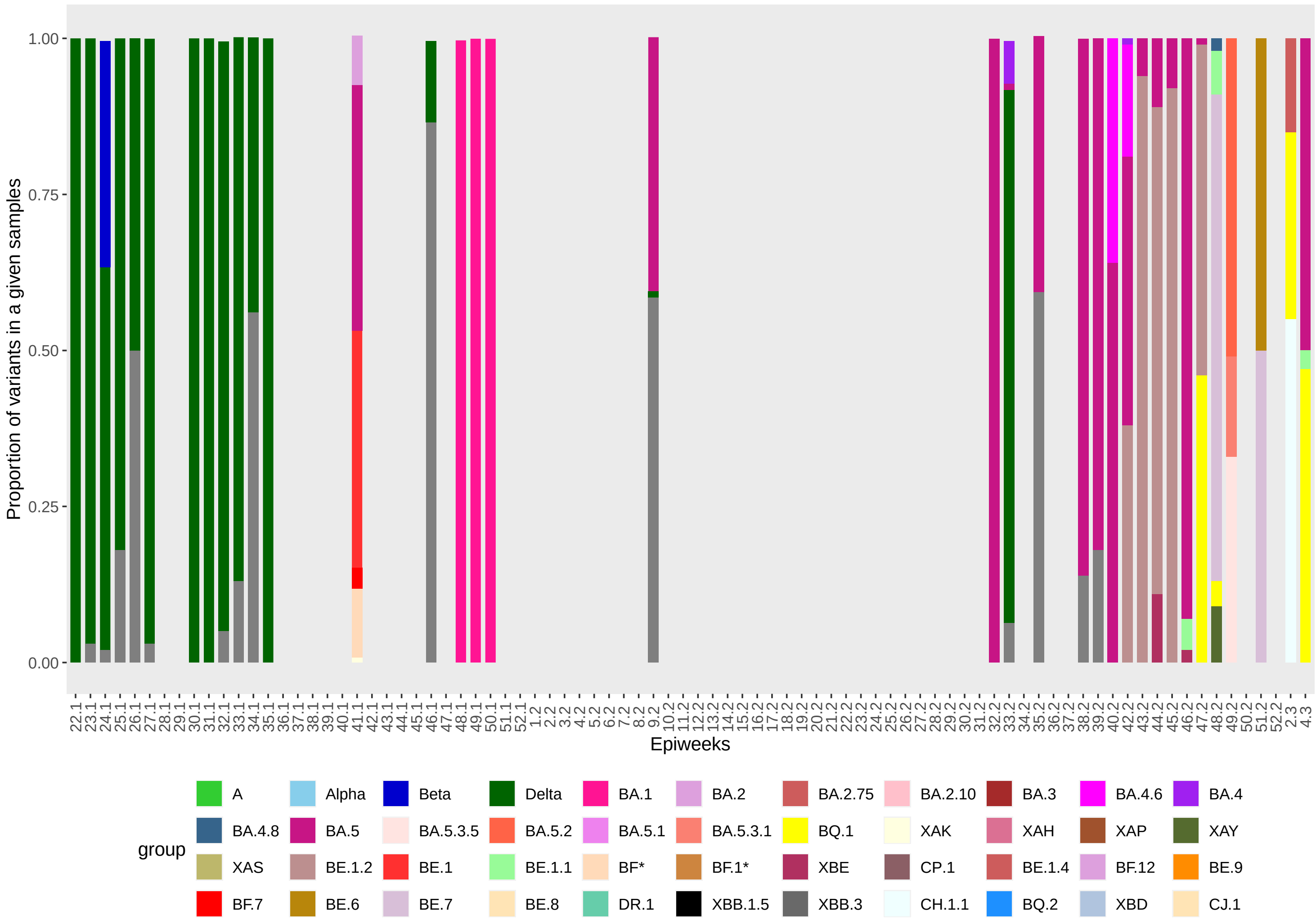


Figure 7: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Central eThewkini, in the eThekwini region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

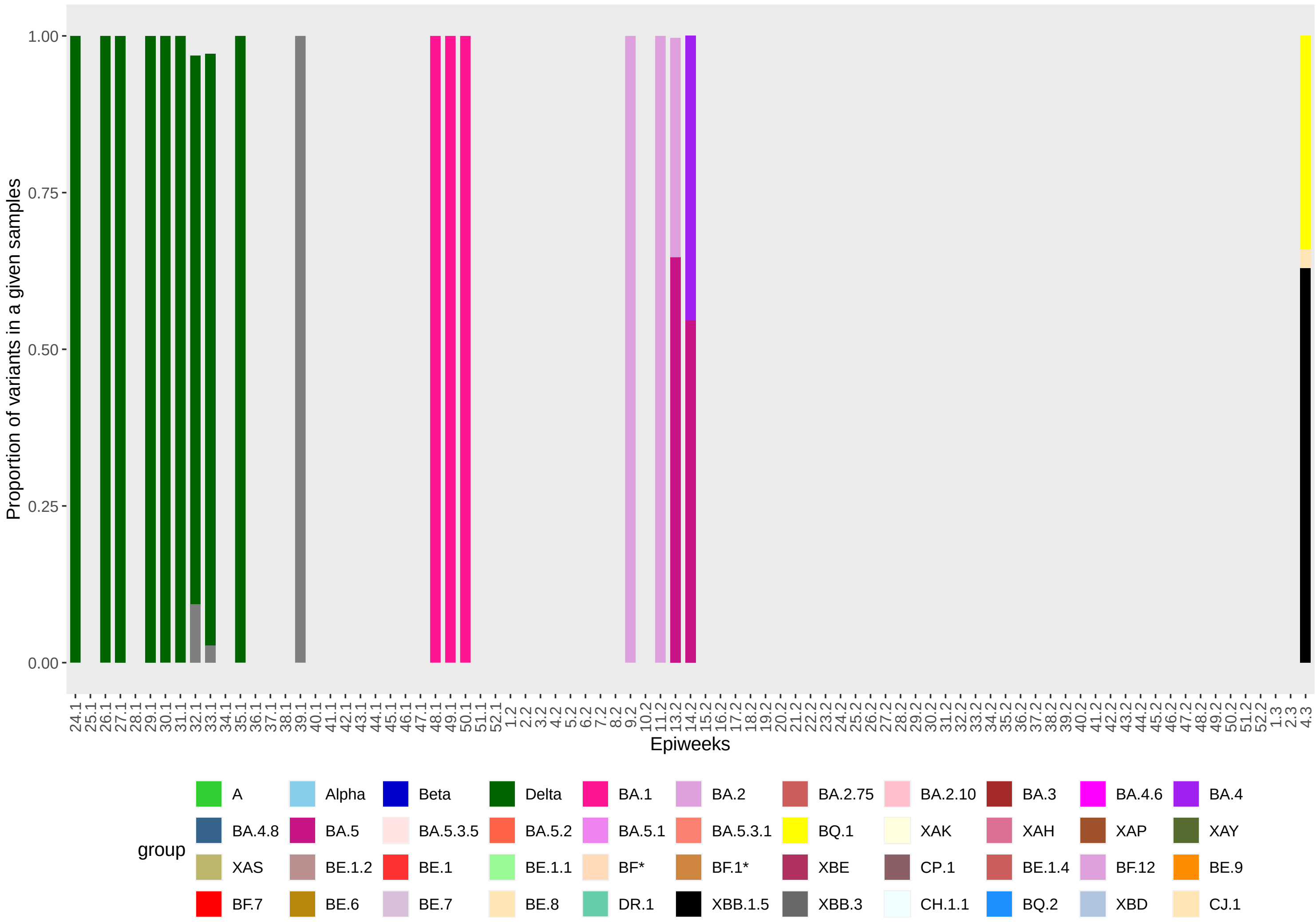


Figure 8: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Northern eThewkini, in the eThekwini region, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

Free State province

In Mangaung, Free State province, **77** samples yielded sequencing results and were analysed by Frejya (Figure 9 and 10). The Beta variant was detected in week 16, 2021 and present until week 25, 2021 in both plants. Alpha variant re-emerged in week 24, 2021 in Bloemspruit. The Beta variant was then replaced by Delta in week 22, 2021 in Sterkwater and week 24, 2021, in Bloemspruit and continued to circulate until week 49, 2021. Gaps in the graph are due to either low coverage or samples were not received during that week. Due to the nature of the wastewater matrix, the genome of certain enveloped RNA viruses like SARS-CoV-2 degrade faster than nonenveloped enteric viruses and therefore have very low coverage. Omicron lineage BA.1 was first detected in week 48 and 51, 2021 at both plants and continued to be present up until week 51, 2021. Lineage BA.2 was detected in week 8, 2022, in Bloemspruit and BA.4 from week 15, 2022 in both plants. Shortly after the emergence of BA.4, BA.5 emerged in both plants after week 31, 2022 and continues to circulate to date, along with BE.1.1 and BE.9 emerging from week 44, 2022. In the recent week (week 2 and 4, 2023), omicron lineage BQ.1 is circulating in both Bloemspruit and Sterkwater.

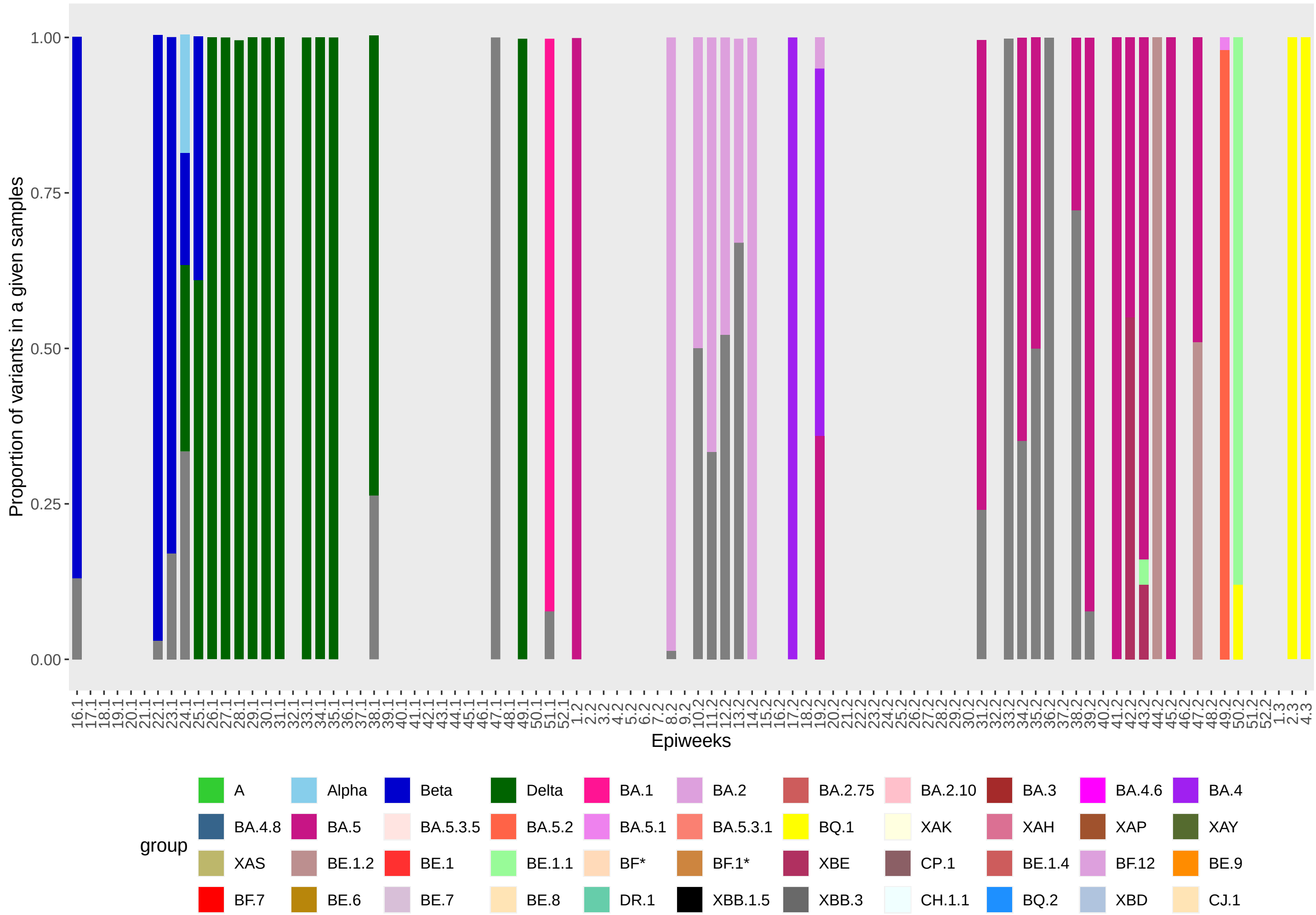


Figure 9: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Bloemspruit, in the Free State, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

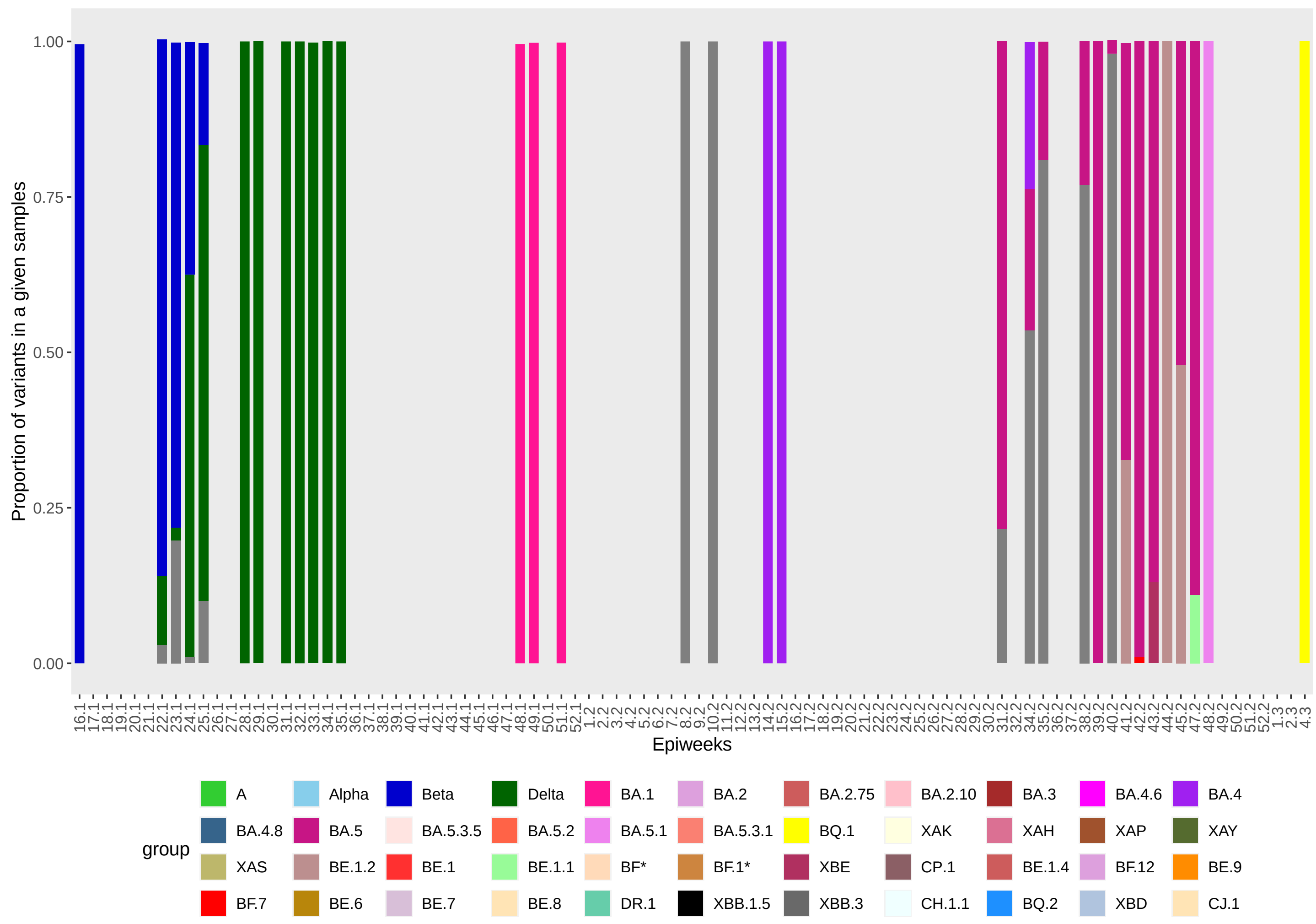


Figure 10: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Sterkwater, in the Free State, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

Western Cape province

In the Western Cape Province, **26** samples yielded sequencing results displayed in Figure 11 and 12. The Beta variant emerged in week 15, 2021 in Borcherd's Quarry and week 22, 2021 in Zandvliet. Beta variant was then replaced by the Delta variant from weeks 23 to 35, 2021. Gaps in the graph are due to either low coverage or samples were not received during that week. Due to the nature of the wastewater matrix, the genome of certain enveloped RNA viruses like SARS-CoV-2 degrade faster than nonenveloped enteric viruses and therefore have very low coverage. Omicron BA.1 was first observed in week 47, 2021 in Zandvliet, followed by BA.2 and BA.3 in week 10, 2022 and week 10, 2022 in Borcherd's Quarry. At both sites, majority of the samples yielded low quality sequence data from week 34, 2021 to week 2, 2022 and week 15, 2022 to week 40, 2022. Omicron lineage BA.5 was first detected in week 41, 2022 and continues to circulate to date with BQ.1and BE.1.1.

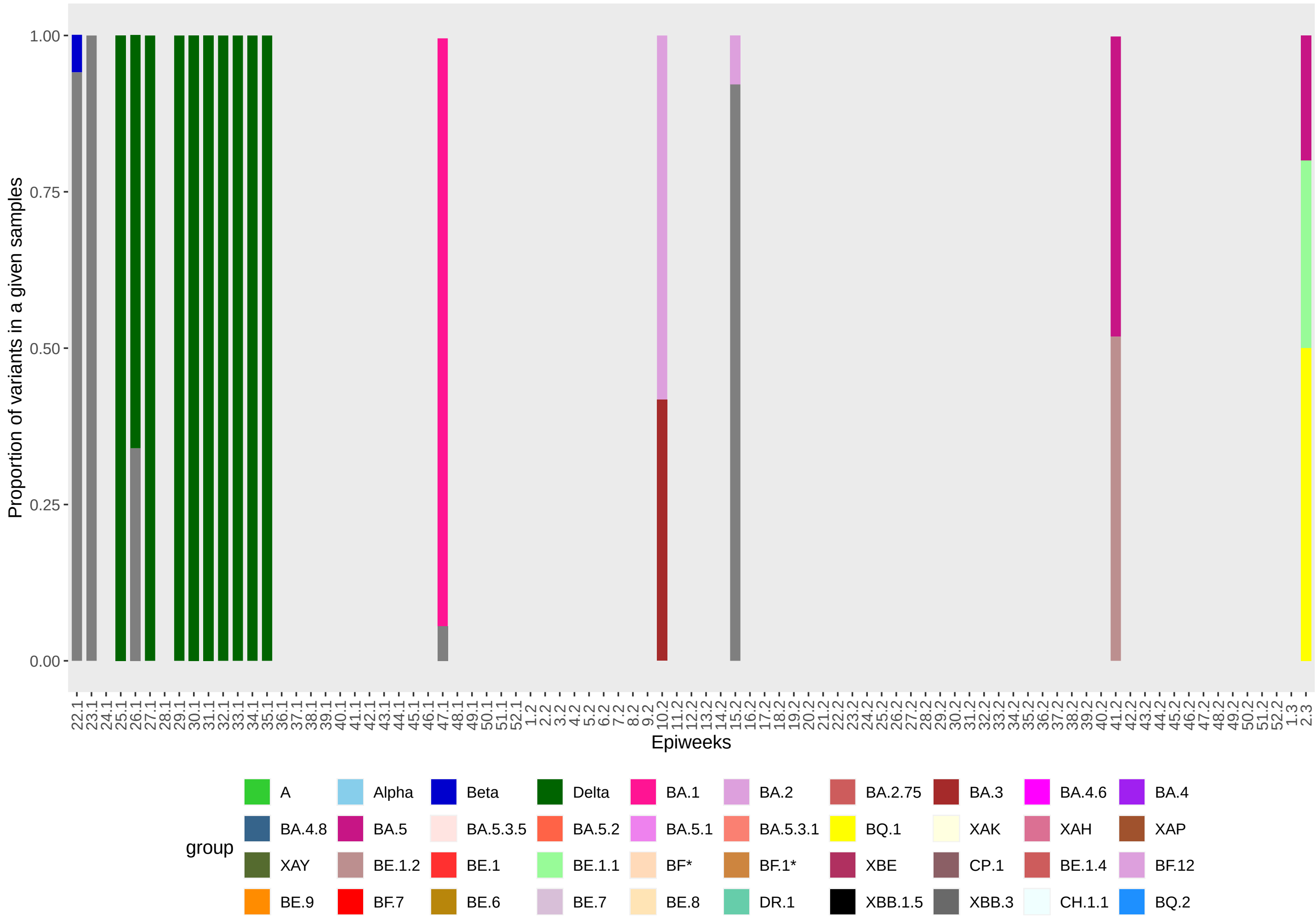


Figure 11: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Zandvliet, in the Western Cape, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

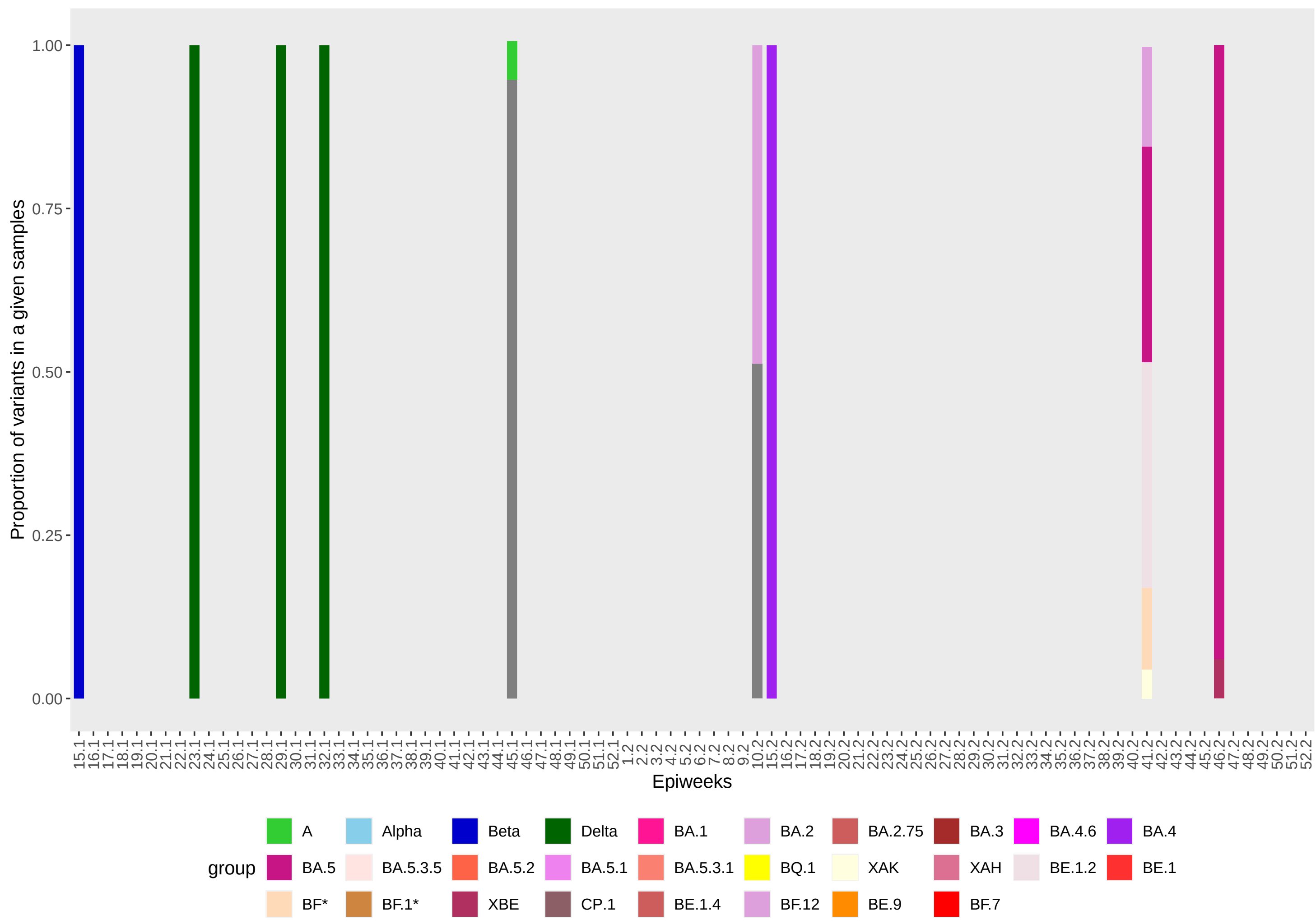


Figure 12: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Borcherd's Quarry, in the Western Cape, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

Eastern Cape province

In the Eastern Cape Province, **55** samples yielded sequencing results displayed in Figures 13,14,15 and 16. The Alpha variant was detected in week 22, 2021 in Eastbank. Delta was first observed in week 23, 2021, in Kwanobuhle and by week 27, 2021, this variant was circulating in all other sites in the Eastern Cape. Gaps in the graph are due to either low coverage or samples were not received during that week. Due to the nature of the wastewater matrix, the genome of certain enveloped RNA viruses like SARS-CoV-2 degrade faster than nonenveloped enteric viruses and therefore have very low coverage. Omicron lineage BA.1 was first detected in week 48 at the Mdantsane site and week 50, 2021 at the Kwanobuhle and Brickfield sites. BA.2 was then detected from week 10, 2022 in Mdantsane, Brickfield and Eastbank.. BA.2 was subsequently replaced by BA.4 in week 16, 2022 in all sites except for Brickfield. BA.5 sub-lineages were then detected from week 34, 2022 and continue to circulate in all sites to date, with BE.1, BE.9, CH.1.1 and XBD.

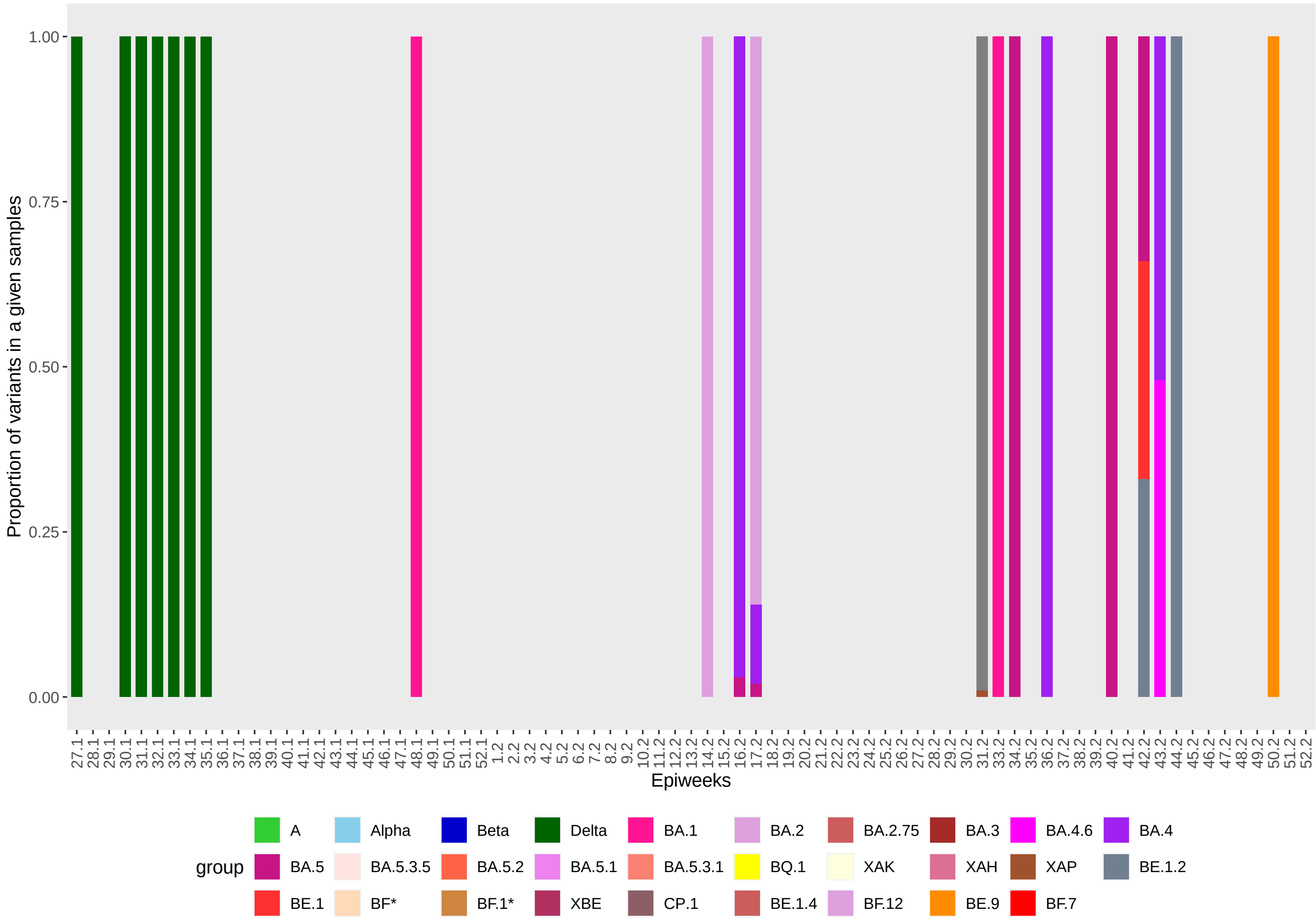


Figure 13: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Mdantsane, in the Eastern Cape, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

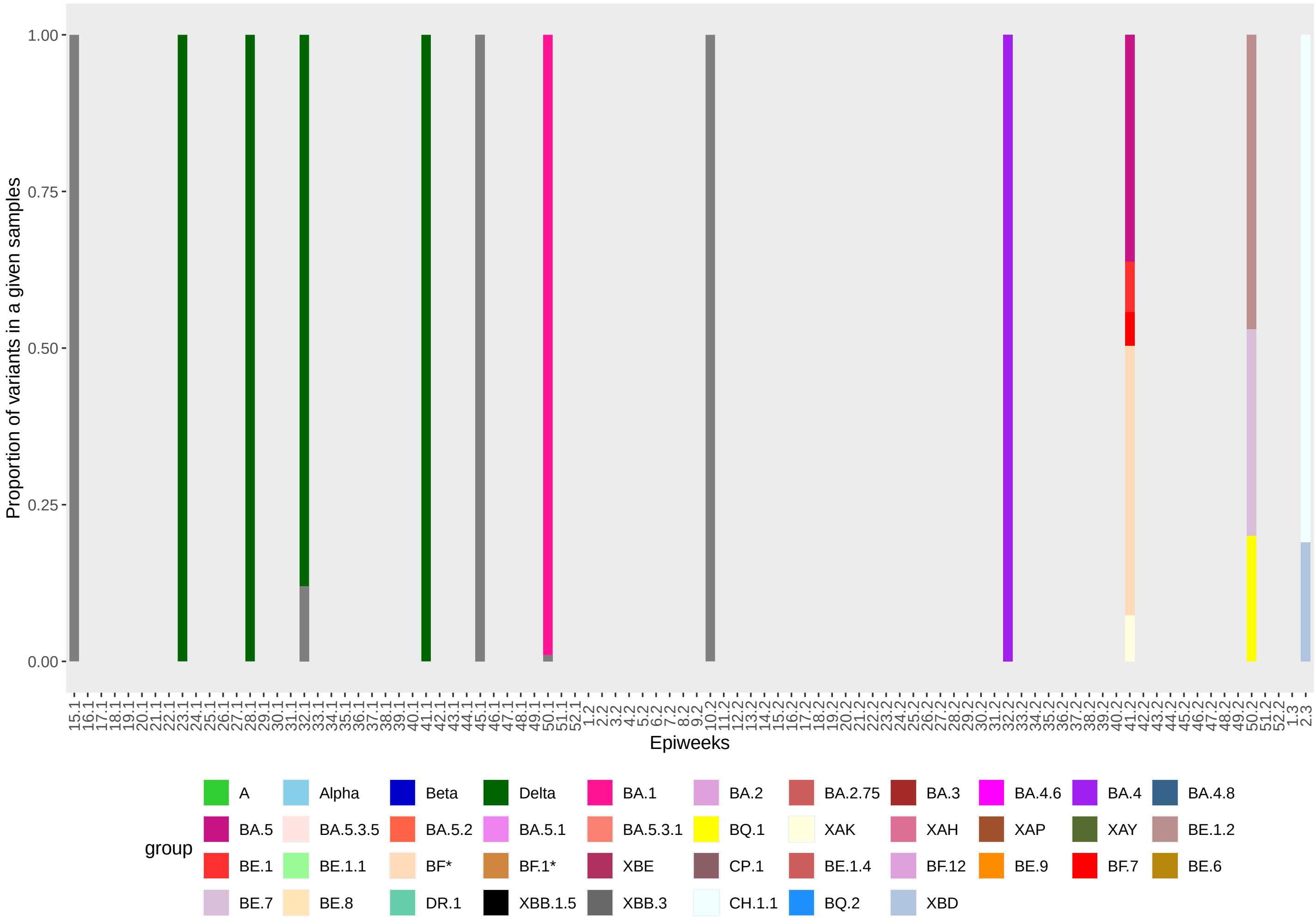


Figure 14: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Kwanobuhle, in the Eastern Cape, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

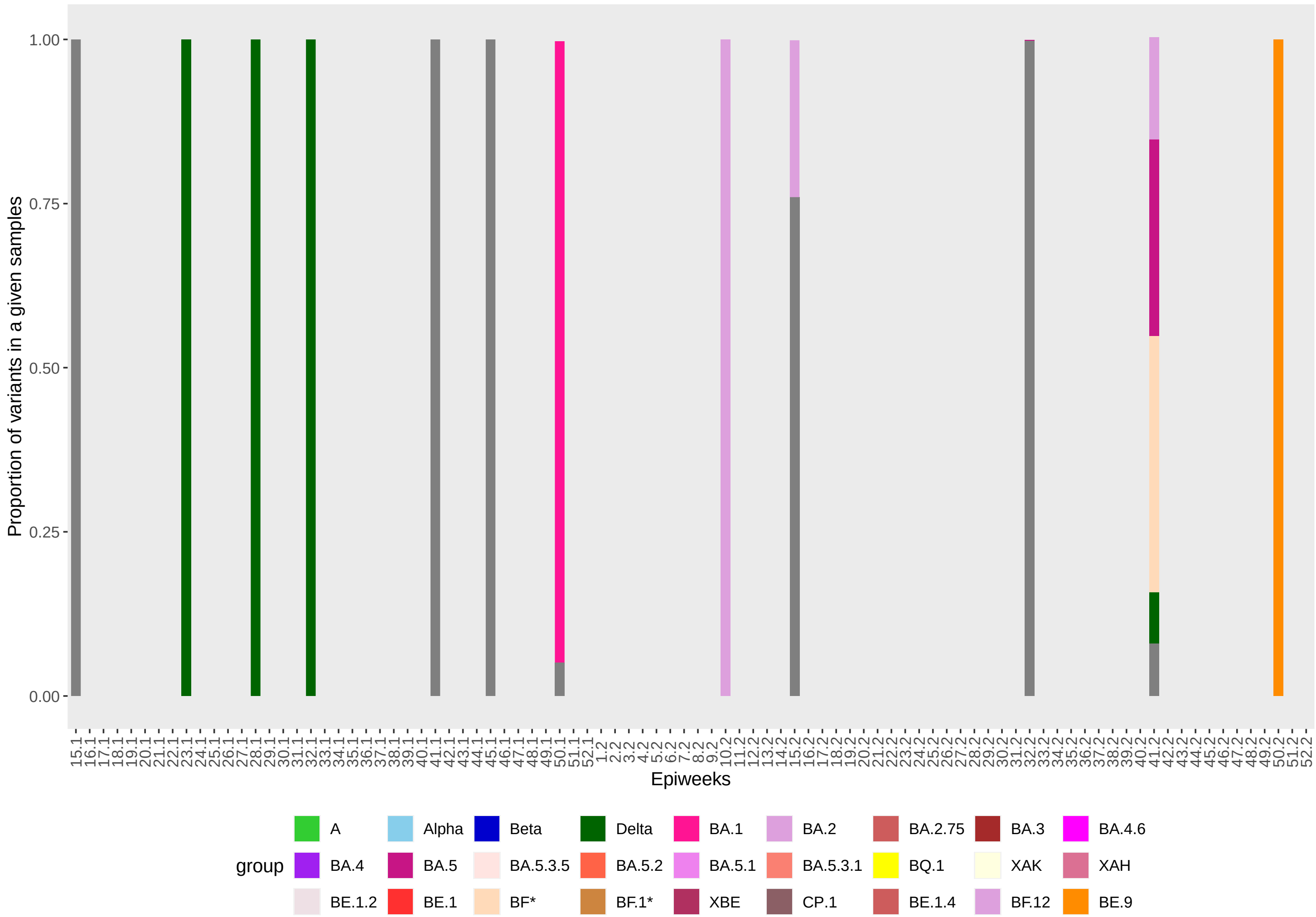


Figure 15: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Brickfield, in the Eastern Cape, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

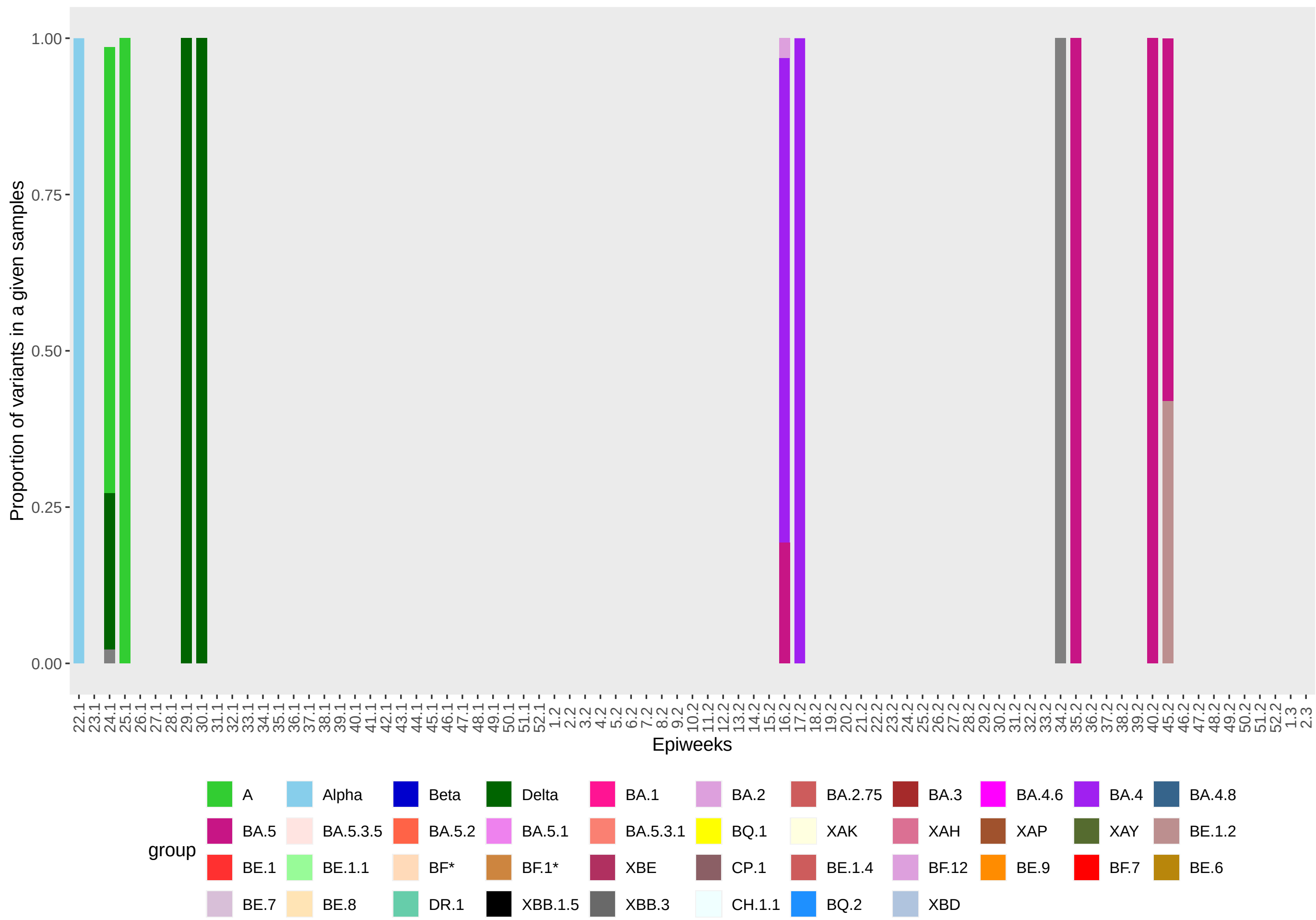


Figure 16: The proportion of SARS-CoV-2 variants and lineages in the environmental samples collected from Eastbank, in the Eastern Cape, arranged chronologically by epidemiological week (i.e. 22.1 is epidemiological 22, year 2021). Only samples that had a coverage of >50% were included in the analysis.

Detection of patterns of emerging SARS-CoV-2 mutations from wastewater samples using a mutational heatmap and mutational profile

A total of **714** wastewater samples from sites listed in Table 1 were used to create a heatmap of patterns of amino acid mutations, starting from epidemiological week 1, 2021 (at the top of the heatmap) to recent week 4, 2023 (at the bottom of the heatmap). In the recent (week 4, 2023), sequencing results and mutations from 8 new samples (from Hartbeesfontein – Gauteng, Vlakplaats – Gauteng, Daspoort – Gauteng, central eThekwini – Kwa-Zulu Natal, northern eThekwini – Kwa-Zulu Natal, Bloemspruit – Free State, Sterkwater – Free State), have been added the heatmap (Figure 19) and the mutational profile (Figure 20).

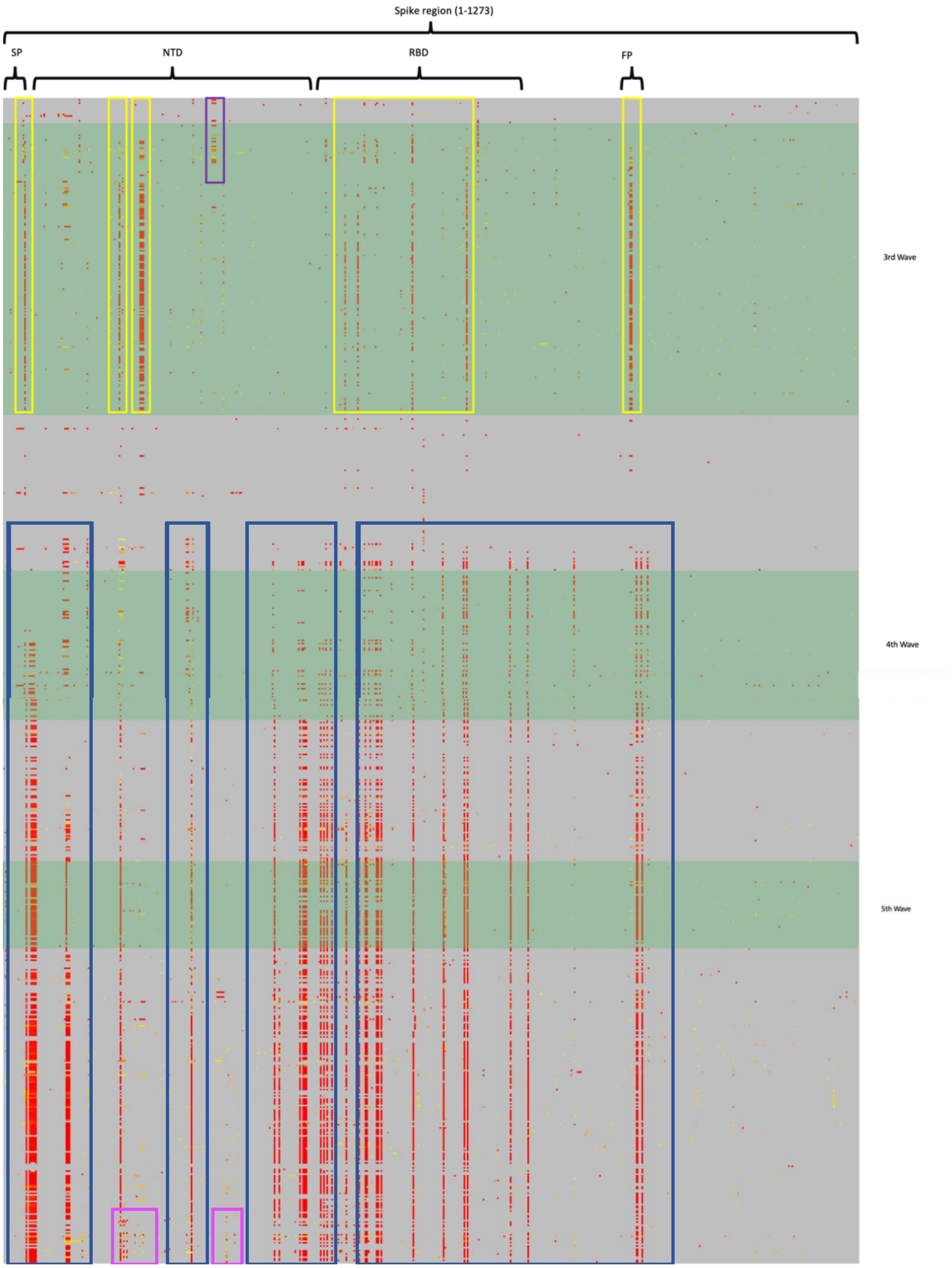


Figure 19: Heatmap of amino acid mutations distributed across the SARS-CoV2 spike protein in comparison with the Wuhan reference strain, arranged vertically in chronological order. Each row represents a sample, organized by the date of sample collection (From April, 2021 to January, 2023). Each column represents an amino acid position of the spike protein. Regions with no mutations or low occurrences are represented in grey (0%) and light yellow (1-34%). Regions with mutations that have a 50% read frequency are represented in dark yellow. Regions with mutations with a read frequency between 60-80% are represented in orange and very high occurring mutations (89-100%) are represented in red. Regions that are highlighted with green represent the time period in which South Africa experienced a wave. Yellow boxes indicate mutations that had emerged that lead to the Delta wave, the blue

boxes indicate mutations that had emerged that lead to the Omicron wave and pink bow indicate recent emerging mutations.

The alignment and ordering of the spike amino acid positions in Figure 19 demonstrate characteristic patterns of emerging mutations in epidemiological week. In week 16, 2021, the Delta variant was characterized by the emergence of mutations in the N-terminal domain (NTD) region (G142D, E156del, F157del, and R158G) highlighted in the yellow box, followed by the loss of the N-terminal domain (NTD) region mutations after week 35. This signified the transition from the Delta variant to the Omicron variant. The Omicron variant (highlighted in blue box) was characterized by the emergence of mutations in the receptor binding (RBD) domain (G339D, S371L, 373, N440K, S477N, E484A, Q493R, G496S, Q498R), and fusion peptide (FP) region (N764K, D796Y), and the heptad repeat 1 (HR1) region (Q954H, N969K, L9811F), in week 45, 2021, highlighted in the blue box. Between the third and fourth wave of infection low sequence coverage of spike was observed, likely due to low levels of virus in wastewater because of low clinical caseloads, and few mutations were detected. Mutations (G21R, W152L, F186L, P621S A706V and T1117I) associated with XAY (a lineage first detected in South Africa), were first detected in wastewaterin week 20, 2022, and continue to emerge sporadically in specific weeks. In the recent week, (week 4, 2023) mutations Y144del, H146Q, W152R, R156del, F157del, R158G and I210V are consistently re-emerging in the heatmap (highlighted in pink). The significance of this occurrence is not yet known, but Y144del and H146Q mutations are associated with XBB.1.5 and W152R, F157L and I210V are mutations associated with BA.2.75.

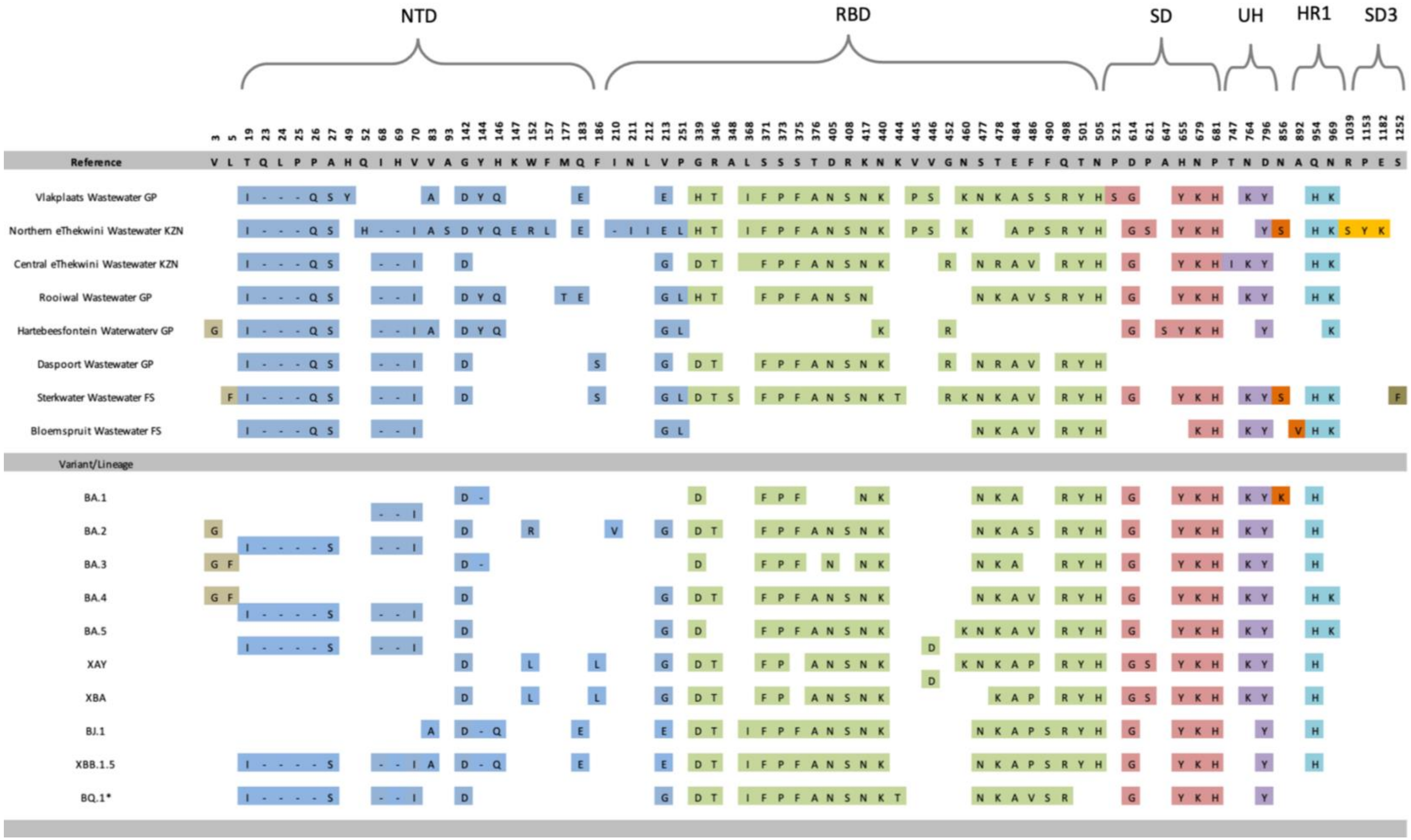


Figure 20: SARS-CoV-2 spike protein mutational profile of samples collected from wastewater sites across South Africa (Zandvliet – Western Cape, Kwanobuhle – Eastern Cape, Brickfield – Eastern Cape, Hartbeesfontein – Gauteng, Vlakplaats – Gauteng, Goudkoppies – Gauteng, Daspoort – Gauteng, central eThekwini – Kwa-Zulu Natal, northern eThekwini – Kwa-Zulu Natal, Bloemspruit – Free State, Sterkwater – Free State) with the respective associated lineage or variant. Each row represents the site in which a sample was collected (top half) and the mutations that are associated with lineages or variants of concern (bottom half). Each column represents an amino acid position of the spike protein, with the wildtype represented below. Mutations are listed within the plot and are colour coded according to the spike region they are found in (NTD – N-terminal domain (blue), RBD – Receptor binding domain (green), SD – Subdomain (pink), UH – Upstream helix (purple), HR1 – Heptad repeat (powder blue), SD3 – Subdomain 3 (dark orange).

In week 4, 2023, a combination of spike mutations (V83A, Y144-, H146Q, Q183E, R346T, L368I, F486P, F490S) associated with XBB.1.5 were identified in Northern eThekwini site, KZN (Figure 20), corroborating with the findings from the Freyja tool, which identified the presence of XBB.1.5 in the same sample (Figure 8). The same mutations were found in the Vlakplaats sites, Gauteng, however mutation F486S was detected here instead of F486P. In the Rooiwal site, Gauteng, a combination of spike mutations (Y144-, H146Q, Q183E) associated with either XBB.1.5 or BJ.1 were detected and in the Hartbeesfontein site, Gauteng a combination of spike mutations (V83A, Y144-, H146Q) were also detected. XBB.1.5 was first isolated in South African clinical samples in December, in the Western Cape and continues to emerge in the province and all the other provinces. Wastewater data has detected spike mutations associated with XBB1.5 however the same mutations (V83A, Y144-, H146Q, Q183E, R346T, L368I, F490S) are also associated with BJ.1 except for F486P and mutations; T19I, Q23del, L24del, P25del, P26del, I68del, H69del and V70I. Therefore, due to the presence of the other mutations (T19I, Q23del, L24del, P25del, P26del, I68del, H69del, V70I) in the recent wastewater samples and considering that BJ.1 is a sub-lineage of BA.2 that was only circulating in October, 2022, XBB.1.5 may be the lineage that is currently circulating.

Limitations

The ability to identify variants in wastewater relies on the presence of non-degraded SARS-CoV-2 fragments in wastewater. Our amplicon-based sequencing approach requires binding of primers across the entire SARS-CoV-2 genome. Differential decay of certain portions of the SARS-CoV-2 virus, and disruption of RNA fragments through environmental or chemical pressure leads to imperfect and absent primer binding. In this case, coverage of the genome and the number of reads will be poor or low, and our ability to interpret sequence results and therefore to infer lineages will be impacted.

Conclusion

Qualitative wastewater data from epidemiologic week 06, 2023 demonstrate declining levels of SARS-CoV-2 in Gauteng, Mangaung and KwaZulu-Natal (eThekwini). Sequencing data from week 04, 2023 show that Omicron lineages XBB.3, BQ.1, BQ.1.1.28, BE.8, XBB.1.5, BE.1.1 and CH.1.1, BQ.1.1.20, BE.7, BA.5.11 and delta lineage AY.120.2 are circulating in January in South Africa. The qualitative and sequencing results must be read along with the SARS-CoV-2 reports generated by the Centre for Respiratory Diseases and Meningitis found at (https://www.nicd.ac.za/wp-content/uploads/2022/03/Update-of-SA-sequencing-data-from-GISAID-18-Mar-2022_2.pdf).

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Supplementary Table: Data for all wastewater treatment plants tested by SACCESS network

S/No	Wastewater plant name	Province	Metro or District	Official subdistrict SD or Local municipality	Subdistrict as represented on the graphs	Water service authority	Suburbs in drainage reticulation	Testing laboratory	Date quantitative testing started
1	Central	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Wisonia, Dawn, Summer Pride, Amalinda Forest, Haven Hills, Buffalo flats ext, Scenery Park	Praecautio	20-10-2021
2	East Bank	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Blue Bend, Bonza Bay, Nahoon, Beacon Bay	NICD	13-04-2021
3	Gonubie	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Sunrise-on-Sea, Gonubie Manor, Thorn Ridge, Cyprus Dale, Bay View, Donny-brook, Gonubie, Gonubie Park	Praecautio	15-09-2021
4	Mdantsane	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Mdantsane Newlands	NICD	01-06-2021

5	Potsdam	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Zone 12 to Zone 18, Unit P, Potsdam, Khayelitsha, WSU Potsdam, Campus, Mbekweni	Praecautio	20-10-2021
6	Reeston	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Reeston, Chicken Farm, Newlife	Praecautio	15-09-2021
7	West Bank	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Duncan Village, Leach Bay, Nahoon	Praecautio	15-09-2021
8	Zwelitsha	Eastern Cape	Buffalo City Local Municipality	Amathole district municipality	No subdistrict	Buffalo City Local Municipality	Sweet Waters, Zwelitsha, Phakamisa, Ilitha Park	Praecautio	15-09-2021
9	Brickfield	Eastern Cape	Nelson Mandela Metropolitan Municipality	Nelson Mandela A SD	No subdistrict	Nelson Mandela Metropolitan Municipality	KwaNobuhle, Uitenhage, Van Riebeekhoogte	NICD	13-04-2021
10	KwaNobuhle	Eastern Cape	Nelson Mandela Metropolitan Municipality	Nelson Mandela A SD	No subdistrict	Nelson Mandela Metropolitan Municipality	KwaNobuhle, Uitenhage	NICD	13-04-2021
11	Bainsvlei	Free State	Mangaung	Bloemfontein SD	Bloemfontein	Mangaung	Bloemfontein, Bain’s Vlei	Lumegen	01-09-2021
12	Bloemspruit	Free State	Mangaung	Bloemfontein SD	Bloemfontein	Mangaung	Langenhoven Park, Bloemfontein	NICD	16-03-2021

13	Northeastern works	Free State	Mangaung	Bloemfontein SD	Bloemfontein	Mangaung	Bloemfontein Maselspoort, Rustig	Lumegen	01-09-2021
14	Sterkwater	Free State	Mangaung	Bloemfontein SD	Bloemfontein	Mangaung	Fonteintjie, Rooidam	NICD	16-03-2021
15	Botshabelo	Free State	Mangaung	Botshabelo SD	Botshabelo	Mangaung	Bonolo, Botshabelo, Poklenberg, Dankbaar, Roodekop	Lumegen	04-10-2021
16	Welvaart	Free State	Mangaung	Botshabelo SD	Botshabelo	Mangaung	Kagisanong, Fichardtpark, Bochebela, Phahameng, Generaal deWet, Willows, Batho, Rocklands, Universitas	Lumegen	09-09-2021
17	Northern Works	Free State	Mangaung	Bloemfontein SD	Bloemfontein	Mangaung	Midway, Bloemspruit, Grasslands	Lumegen	01-09-2021
18	Dewetsdorp	Free State	Mangaung	Naledi SD	Naledi and Thabanchu	Mangaung	Dewetsdorp, Frankfort, Glengary	Lumegen	01-09-2021
19	Thaba Nchu	Free State	Mangaung	Thaba N'chu SD	Naledi and Thabanchu	Mangaung	Thaba Nchu, Mokwena, Selosesha, Abramskraal, Roodekop, Strydom College, Bultfontein Number Three, Ratlau, Serwalo, Bultfontein Number One, Bultfontein Number Two, Motlala, Lusaka	Lumegen	01-09-2021

20	Ennerdale	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg G SD	No subdistrict	City of Johannesburg Metropolitan Municipality	Walkerville, Hartsenbergfontein, Althea, Golfview, Blignautrus	NIOH	04-10-2021
21	Northern Wastewater Treatment Works	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg A SD	No subdistrict	City of Johannesburg Metropolitan Municipality	Strydompark, Olivedale, Rivonia, Jukskei Park, Douglasdale, Ferndale, Lone Hill, Sandton, North Riding, Fourways, Paulshof	NICD	06-04-2021
22	Goudkoppies	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg D SD	No subdistrict	City of Johannesburg Metropolitan Municipality	Soweto, Rivasdale	NICD	24-05-2021
23	Bushkoppies	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg G SD	No subdistrict	City of Johannesburg Metropolitan Municipality	Baragwanath, Pimville, Johannesburg South, Dube, Willowdene, Nancefield	Waterlab/UP	11-10-2021
24	Olifantsvlei	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg G SD	No subdistrict	City of Johannesburg Metropolitan Municipality	Soweto, Eldorado, Lenasia	Waterlab/UP	11-10-2021
25	Driefontein	Gauteng	City of Johannesburg	Mogale City LM	No subdistrict	City of Johannesbur	Kelvin, Morningside Manor, Edenburg, Lone	NIOH	04-10-2021

			Metropolitan Municipality			g Metropolitan Municipality	Hill, Rivonia, Sandton, Nort hdene, Fourways, Paulshof		
26	Bronkhortspr uit	Gauteng	City of Tshwane Metropolitan Municipality	Thembisile Hani LM	Tshwane North (sub-districts 3,4,6,7)	City of Tshwane Metropolitan Municipality	Wilgerivier, Wonderfontein, Graley Crown Douglas, Bronkhorst	Waterlab/UP	04-10-2021
27	Klipgat	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 1 SD	Tshwane North (sub-districts 1,2)	City of Tshwane Metropolitan Municipality	KlipgatBoekenhoutfontein, Soshanguve, Mabopane, Honeyvale, Boekenhoutfontein, Lebaleng	SAMRC-TB Platform	02-11-2021
28	Sandspruit	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 1 SD	Tshwane North (sub-districts 1,2)	City of Tshwane Metropolitan Municipality	Medunsa, Hebron, Rosslyn, Strydfontein, Hornsnek, Kruisfontein	Waterlab/UP	11-09-2021
29	Rooiwal Eastern Works	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 2 SD	Tshwane North (sub-districts 1,2)	City of Tshwane Metropolitan Municipality	Rooiwal, Pylpunt, Pyramid, Vasfontein, Petronella, Stil Gelee	NICD	23-03-2021
30	Temba	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 2 SD	Tshwane North (sub-districts 1,2)	City of Tshwane Metropolitan Municipality	Majanen,Hammanskraal, Mabopane, Soshanguve, Pyramid, Doornpoort	Waterlab/UP	26-09-2021
31	Daspoort Wastewater	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 3 SD	Tshwane North (sub-districts 3,4,6,7)	City of Tshwane	Groenkloof, Arcadia, Pretoria South, Gezina, Hercules, Rietfontein,	NICD	02-03-2021

	Treatment Works					Metropolitan Municipality	Pretoria Central, Sunnyside, Pretoria East, Prinshof, Daspoort, Villieria, Capital Park, Pretoria West, Wonderboom South, Pretoria-Wes, Innesdale		
32	Sunderland Ridge	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 4 SD	Tshwane North (sub-districts 3,4,6,7)	City of Tshwane Metropolitan Municipality	Centurion, Olivenhoutbosch and some parts of Midrand.	NIOH	18-08-2021
33	Babelegi	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 5 SD	Tshwane North (sub-districts 5)	City of Tshwane Metropolitan Municipality	Industrial sites	NIOH	18-08-2021
34	Baviaanspoo rt	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 5 SD	Tshwane North (sub-districts 5)	City of Tshwane Metropolitan Municipality	Elandsfontein, Cullinan, So nderwater	NIOH	18-08-2021
35	Refilwe	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 5 SD	Tshwane North (sub-districts 5)	City of Tshwane Metropolitan Municipality	Cullinan	NIOH	05-10-2021
36	Zeekoegat	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 5 SD	Tshwane North (sub-districts 5)	City of Tshwane Metropolitan Municipality	Zeekoegat, Magalies Water, Buffelsdrif	Waterlab/UP	04-10-2021

37	Godrich	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 7 SD	Tshwane North (sub-districts 3,4,6,7)	City of Tshwane Metropolitan Municipality	Bronkspruit town Rhema Park Caltura park Venster Park Zithobeni	SAMRC-TB Platform	13-09-2021
38	Summer Place Package Plant	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 7 SD	Tshwane North (sub-districts 3,4,6,7)	City of Tshwane Metropolitan Municipality	Summerplace	SAMRC-TB Platform	01-09-2021
39	Rietgat	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 2 SD	Tshwane North (sub-districts 1,2)	City of Tshwane Metropolitan Municipality	Soshanguve	NIOH	05-10-2021
40	Thaba Tshwane	Gauteng	City of Tshwane Metropolitan Municipality	Tshwane 2 SD	Tshwane North (sub-districts 1,2)	City of Tshwane Metropolitan Municipality	Thaba Tshwane, Generaal Kemp Heuwel Radio Uitkyk	Waterlab/UP	05-01-2022
41	Daveyton WasteWater Treatment Works	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E1 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	Welgedag, Persida	NICD and CSIR	NICD:02-03-2021 CSIR: 21-09-2021
42	Rynfield	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E1 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	New Modder, Lakefield, Benoni, Boksburg, Northmead, Atl asville	CSIR	21-09-2021

43	Ancor	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E2 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	Welgedag, Payneville, Selcourt, Casseldale, Springs	Waterlab/UP	21-09-2021
44	Carl Grundlingh	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E2 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	Nigel, Bultfontein, Laversburg	CSIR	21-09-2021
45	Jan Smuts	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E2 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	Dalpark, Brakpan, Dersley, Dalview, Benoni, New Modder, Schapenrust	CSIR	21-09-2021
46	Tsakane	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E2 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	Benoni, Dersley, Dalpark, Brakpan, Dalview, Schapenrust	Waterlab/UP	05-10-2021
47	Welgedacht	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni E2 SD	Ekurhuleni East (E1, E2)	Ekurhuleni Metropolitan Municipality	KwaThema, Brakpan, Dersley, Schapenrust	Waterlab/UP	21-09-2021
48	Hartebeesfontein WasteWater Treatment Works	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni N1 SD	Ekurhuleni North (N1, N2)	Ekurhuleni Metropolitan Municipality	Mid-Ennerdale, Althea, Grasmere, Elandsfontein	NICD Waterlab/UP	NICD:02-03-2021 Waterlab/UP : 05-10-2021
49	Herbert Bickley	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni N1 SD	Ekurhuleni North (N1, N2)	Ekurhuleni Metropolitan Municipality	Jameson Park	CSIR	21-09-2021
50	Olifantsfontein WasteWater	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni N1 SD	Ekurhuleni North (N1, N2)	Ekurhuleni Metropolitan Municipality	Pinedene, Clayville, Tembisa, Midstream Estates, Olifantsfontein	CSIR and NICD	CSIR: 21-09-2021

	Treatment Works								NICD: 02-03-2021
51	Benoni	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni N2 SD	Ekurhuleni North (N1, N2)	Ekurhuleni Metropolitan Municipality	Northmead, Dalpark, Dalvi ew, Lakefield, Benoni, New Modder	Waterlab/UP	05-10-2021
52	J.P. Marais	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni N2 SD	Ekurhuleni North (N1, N2)	Ekurhuleni Metropolitan Municipality	Northmead, Atlasville, Ne w Modder, Lakefield, Benoni	CSIR	21-09-2021
53	Dekema	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni S1 SD	Ekurhuleni South (S1, S2)	Ekurhuleni Metropolitan Municipality	Katlehong, Natalspruit, Randhart, Alrode	Waterlab/UP	05-10-2021
54	Rondebult	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni S1 SD	Ekurhuleni South (S1, S2)	Ekurhuleni Metropolitan Municipality	Bartlett, Atlasville, Boksburg North, Lakefield, Bonaero Park, Ravenswood, Witfield, Boksburg	Waterlab/UP	21-09-2021
55	Vlakplaats WasteWater Treatment Works	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni S2 SD	Ekurhuleni South (S1, S2)	Ekurhuleni Metropolitan Municipality	Vosloorus	NICD and CSIR	NICD: 22-02-2021 CSIR: 21-09-2021
56	Waterval WWTW	Gauteng	Ekurhuleni Metropolitan Municipality	Ekurhuleni S2 SD	Ekurhuleni South (S1, S2)	Ekurhuleni Metropolitan Municipality	Kliprivier, Henley on Klip, Ophir, Glen Donald, Chrissiefontein, Ro	Waterlab/UP	21-09-2021

							thdene, Riversdale, Meyerton Farms		
57	Flip Human	Gauteng	West Rand	West Rand *(Johannesburg C SD)	No Subdistrict	Mogale City Local Municipality	Rietvallei, Bhongwem, Brink's Vlakfontein	Waterlab/UP	12-10-2021
58	Magaliesburg	Gauteng	West Rand	West Rand (Mogale City LM)	No Subdistrict	Mogale City Local Municipality	Magaliesburg, Mogale City	Waterlab/UP	12-10-2021
59	Percy Steward	Gauteng	West Rand	West Rand (Mogale City LM)	No Subdistrict	Mogale City Local Municipality	Lewisham, Krugersdorp North	Waterlab/UP	12-10-2021
60	Hammarsdale	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini West	eThekwini Metropolitan Municipality	Hammarsdale, Elangeni, Mpumalanga	GreenHill	02-09-2021
61	Hillcrest	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini West	eThekwini Metropolitan Municipality	New Germany, Pinetown, Clermont, Pinelands, KwaDabeka	GreenHill	02-09-2021
62	Isipingo	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini South	eThekwini Metropolitan Municipality	Umbogintwini, Malukaze, Egolokodo, KwaMakhutha, Umlazi	DUT	10-08-2021
63	Central	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini North	eThekwini Metropolitan Municipality	Brighton Beach, Grosvenor, King's Rest, Ocean View, Fynnland and Treasure Beach	NICD and DUT	NICD: 22-02-2021 DUT: 10-08-2021 for

64	KwaMashu	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini South	eThekwini Metropolitan Municipality	La Lucia, Umhlanga, Prestondale, Phoenix, Duff's Road, Glen Ashley, Mount Edgecombe	DUT	10-08-2021
65	Northern	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini North	eThekwini Metropolitan Municipality	Newlands, KwaMashu, Greenwood Park, Park Hill	NICD	22-02-2021
66	Phoenix	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini South	eThekwini Metropolitan Municipality	Rietrivier, KwaMashu, Duff's Road, Mount Edgecombe, Phoenix, Richmond, Inanda	DUT	10-08-2021
67	Frasers	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini North	iLembe District municipality	Salt Rock, Ballitoville, Umhlali, Fraser, Zimbali, Shaka's Rock, Ballito	Waterlab/UP	11-10-2021
68	Umbilo	KwaZulu-Natal	eThekwini Metropolitan Municipality	eThekwini MM Sub	eThekwini North	eThekwini Metropolitan Municipality	Acorn, Albany, Alexander Park	GreenHill	28-10-2021
69	Darvill	KwaZulu-Natal	uMgungundlovu District municipality	Msunduzi LM	No subdistrict	The Msunduzi Local Municipality	Pelham, Hayfields, New England, Northdale, Hay Paddock, Scottsville, Cleland, Bishopstowe, Sobantu	GreenHill	02-09-2021
70	Lynfield Park	KwaZulu-Natal	uMgungundlovu	Msunduzi LM	No subdistrict	The Msunduzi Local Municipality	Thornville, Hayfields, Hay Paddock, Lynnfield Park, Cleland	GreenHill	02-09-2021

			District municipality						
71	Mpofana	KwaZulu-Natal	Umgungundlovu District municipality	Mpofana LM	No subdistrict	Mpofana Local Municipality	Bruntville, Brown Stones, Windy, Weston, Mooirivier	Waterlab/UP	28-09-2021
72	Howick	KwaZulu-Natal	Umgungundlovu District municipality	uMngeni LM	No subdistrict	UMgungundlovu District municipality	Riversdale, Merrivale, Cedara	GreenHill	02-09-2021
73	Polokwane	Limpopo	Capricorn District municipality	Polokwane LM	No subdistrict	Polokwane Local Municipality	Westenburg, Nirvana, Bendor, Welgelegen, Moregloed, Annadale, Ivydale, Flora Park, Fauna Park, Penina Park, Ivy Park, Ster Park, Dalmada, Broadlands, Woodlands, and Thornhill	Lumegen: /Waterlab/UP	Lumegen: 02-08-2021 Waterlab/UP: 11-10-2021
74	Lebowakgomo	Limpopo	Capricorn District municipality	Lepelle-Nkumpi LM	No subdistrict	Lepelle-Nkumpi LM	Thabamoopo, Vaalboschlaagte, Lekhuswaneng, Moepeng, Makurung, Sekurwaneng, Ga-Matshele, Makurun	Waterlab/UP	12-10-2021
75	Emalahleni (Riverview)	Mpumalanga	Nkangala District Municipality	Emalahleni LM	No subdistrict	Emalahleni LM	Lynnville, Duvhapark, Paxton, Klipfontein	Lumegen	26-07-2021

76	Kanyamazane	Mpumalanga	Ehlanzeni District	City of Mbombela LM	No subdistrict	Mbombela/ Umjindi	Daantjielokasie, eNyamazaneni	Waterlab/UP	30-03-2021
77	Mbombela (Kingstonvale)	Mpumalanga	Ehlanzeni District	Mbombela/Umjindi	No subdistrict	Mbombela/ Umjindi	Gutshwa, eMpumalanga, eNyalungu, Dwaleni, Hlauhlau, Phasha, Ngodini	Lumegen	26-07-2021
78	Calvinia	Northern Cape	Namakwa	Hantam Local Municipality	No subdistrict	Hantam Local Municipality	Calvinia	NICD	06-07-2021
79	Homevale Kimberley	Northern Cape	Frances Baard	Sol Plaatjie Local Municipality	No subdistrict	Sol Plaatjie Local Municipality	Remount Camp, Kenilworth, Homevale	Lumegen	28-08-2021
80	Potchefstroom	NorthWest	JB Marks Local Municipality	JB Marks LM	No subdistrict	JB Marks Local Municipality	Harpington, Vyfhoek, Mooi bank, Wilgeboom	Lumegen	17-08-2021
81	Klerksdorp main	NorthWest	Matlosana Local Municipality	City of Matlosana LM	No subdistrict	Matlosana Local Municipality	Boetrand, Wilkoppies	Lumegen	02-08-2021
82	Rustenburg	NorthWest	Bojanala Platinum	Rustenburg Local Municipality	No subdistrict	Rustenburg Local Municipality	Rustenburg	Lumegen and Waterlab/UP	Lumgen: 26-07-2021 Waterlab: 11-10-2021
83	Bellville	Western Cape	City of Cape Town Metropolitan Municipality	CT Northern SD	No subdistrict	City of Cape Town Metropolitan Municipality	Parow, Belhar, Brackenfell	MRC-BRIP	Not testing quantitatively

84	Cape Flats	Western Cape	City of Cape Town Metropolitan Municipality	CT Southern SD	No subdistrict	City of Cape Town Metropolitan Municipality	Khayelitsha, Mitchells Plain	Waterlab/UP	06-10-2022
85	Athlone	Western Cape	City of Cape Town Metropolitan Municipality	CT Tygerberg	No subdistrict	City of Cape Town Metropolitan Municipality	Crawford, Gleemore, Rondenbosch East	Waterlab/UP	06-10-2021
86	Borcherd's Quarry	Western Cape	City of Cape Town Metropolitan Municipality	CT Tygerberg SD	No subdistrict	City of Cape Town Metropolitan Municipality	Belhar, Elsiesrivier	NICD	09-03-2021
87	Zandvliet	Western Cape	City of Cape Town Metropolitan Municipality	Stellenbosch LM	No subdistrict	City of Cape Town Metropolitan Municipality	Faure, Macassar, Somerset West	NICD	09-03-2021