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- November 2022
WEEK 48 2022

Co-funded by the Water Research Commission, the Bill and Melinda Gates Foundation and the NICD

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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 29 November 2022 (Epidemiological week 48). 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 are generally declining in Gauteng, KwaZulu-Natal and Free State. Omicron sub-lineages are dominant in wastewater across the country. Detailed analyses are described below.

HIGHLIGHTS - week ending 29th November 2022 (Epi week 48)

SARS-CoV-2 levels in wastewater:

- SARS-CoV-2 levels in majority of the wastewater treatment plants across the country have dropped to low levels following increases in the previous weeks.

- These declining levels of SARS-CoV-2 in wastewater have been observed in Tshwane (Daspoort and Rooiwal WWTPs), Johannesburg (Goudkoppies WWTP), Ekurhuleni (Vlakplaats and Hartebeesfontein WWTPS), eThekwini (Central and Northern WWTPs), Bloemfontein (Sterkwater and Bloemspruit WWTPs) and Buffalo City (Potsdam and Eastbank 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 47 (23rd November, 2022) shows that Omicron sub-lineage BE.1.2 is predominantly circulating in South Africa in all plants, followed by Omicron sub-lineage BA.5.3.1, BA.5.2.1, BA.5.3.5 and recombinant XBE.

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. These values are log-transformed before constructing the graphs.

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 –	Grab	None – sample is centrifuged	ZymoBiomics RNA Extraction Kit	RT-qPCR[a] using the Allplex™	QuantStudio 5 (Applied Biosystems)

Tuberculosis Platform (SAMRC-TB Platform)		then supernatant analysed		2019-nCoV 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.

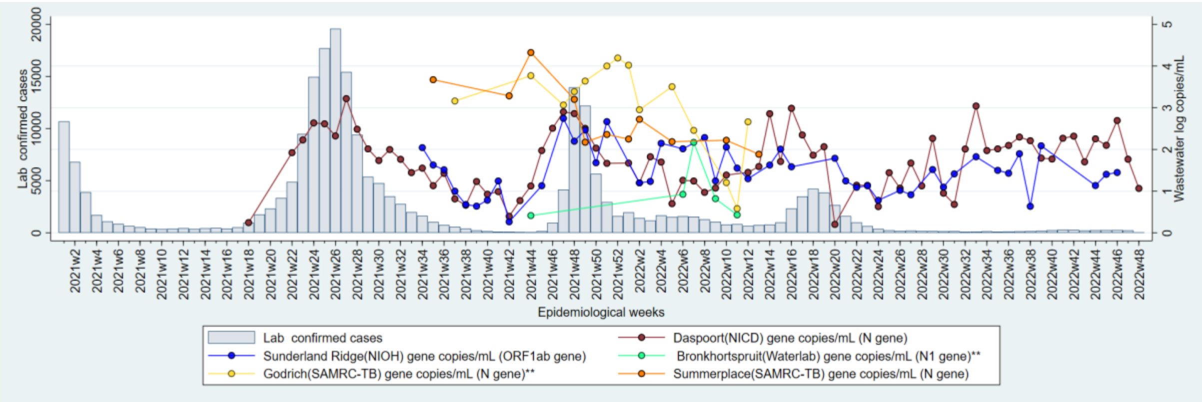
Table 2. Principles of SARS-CoV-2 detection and quantification on influent samples from wastewater treatment plants and interpretive principles to guide application of test results to support COVID-19 public health responses, South Africa.

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
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.

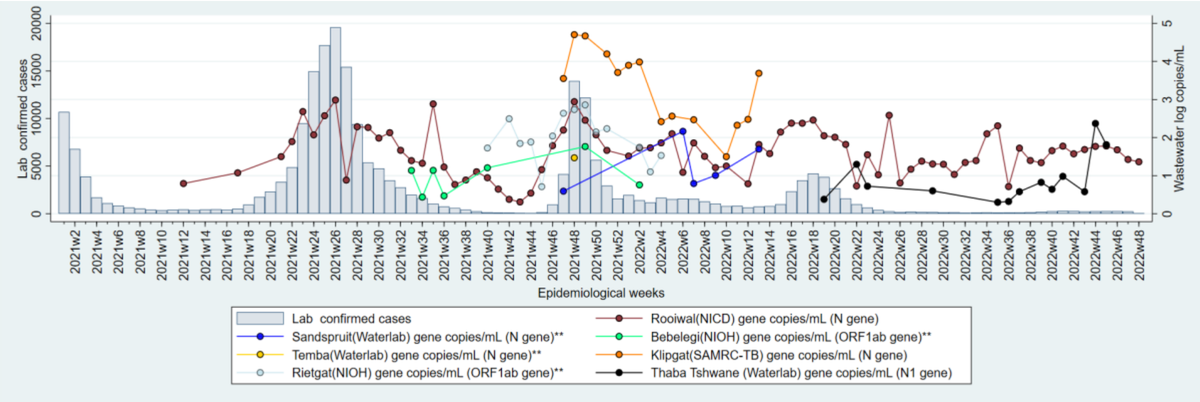
Results

Gauteng Province

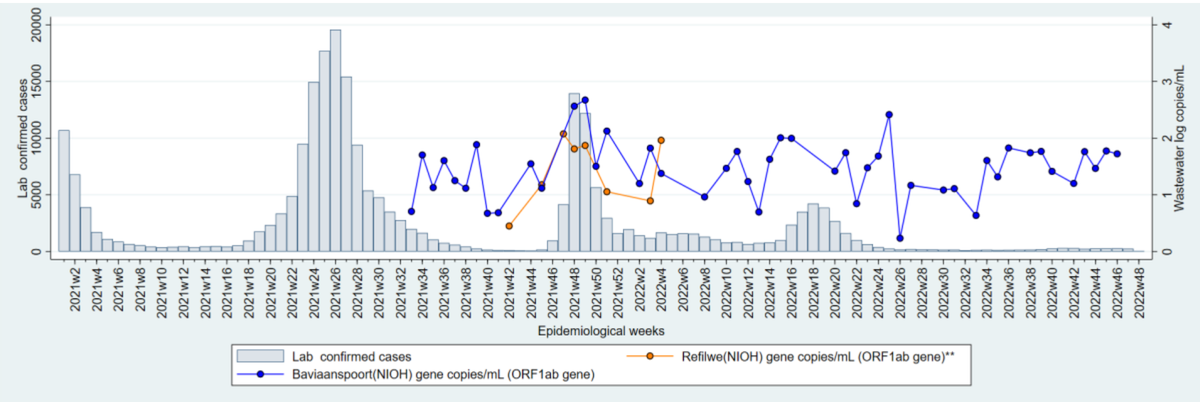
A: City of Tshwane South (sub-districts 3, 4, 6, and 7)



B: City of Tshwane North (sub-districts 1 & 2)



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



Figures 1A-C. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) for selected wastewater treatment plants (WWTP) and metropolitan areas in Tshwane District Municipality (Tshwane South and North), Gauteng Province during epidemiological weeks 1 of 2021 to week 48 of 2022. The testing laboratory and quantified SARS-CoV-2 gene is named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be done for specimens tested in the same laboratory.

**Laboratories where testing has been discontinued

D: City of Johannesburg Metropolitan Municipality

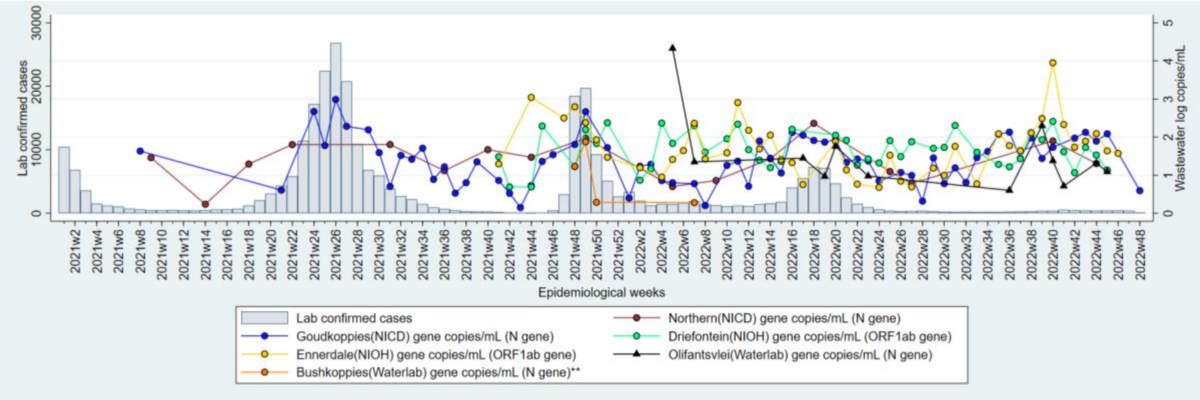
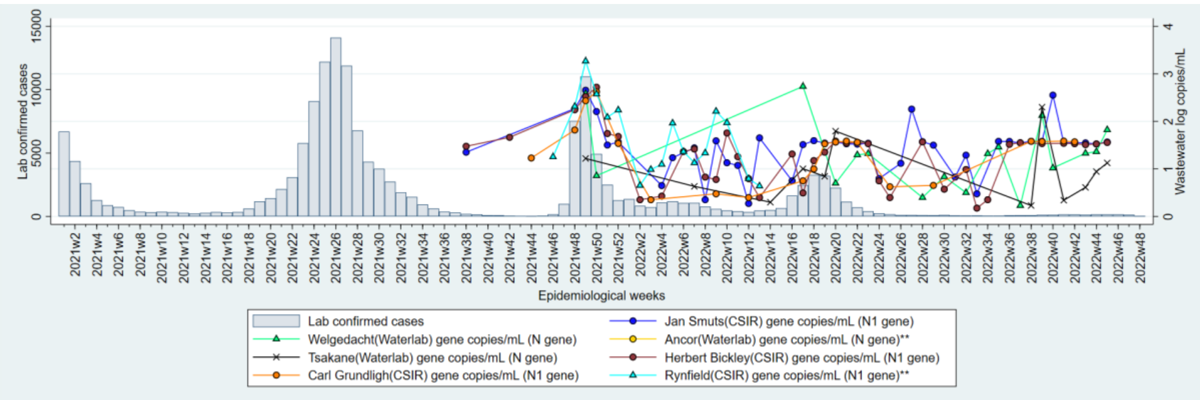


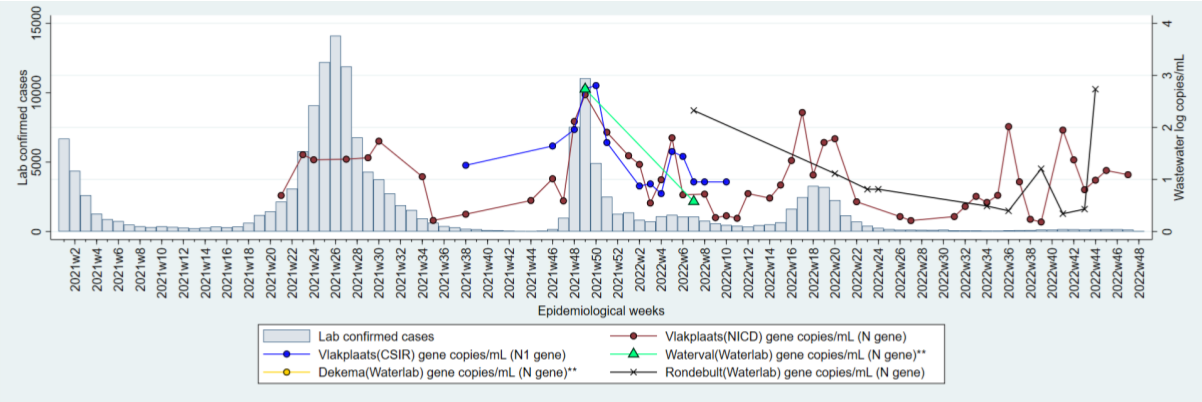
Figure 1D. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) for selected wastewater treatment plants (WWTPs) in the City of Johannesburg Metropolitan Municipality, Gauteng Province during epidemiological weeks 1 of 2021 to week 48 of 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels overtime should only be made for specimens tested in the same laboratory.

**Laboratories where testing has been discontinued

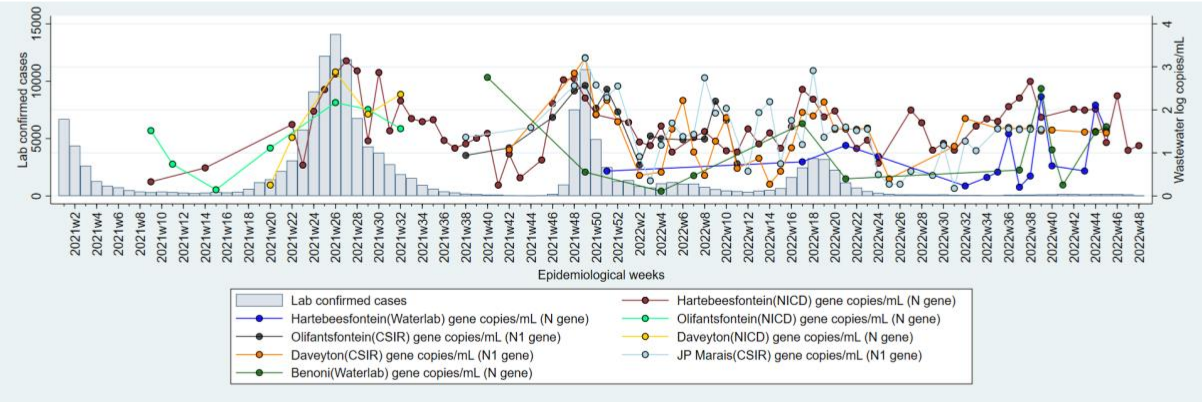
E: **Ekurhuleni East (sub-districts D, E or E1, E2)**



F: Ekurhuleni South (sub-districts A, F or S1, S2)



G: Ekurhuleni North (sub-districts B, C or N1, N2)



Figures 1E-G. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) for selected wastewater treatment plants (WWTP) in Ekurhuleni Metropolitan Municipality, Gauteng Province during epidemiological weeks 1 of 2021 to week 48 of 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory.

**Laboratories where testing has been discontinued

SARS-CoV-2 levels in majority of the wastewater treatment plants in Gauteng have dropped to minimal levels. These plants are Daspoort and Rooiwal WWTP in Tshwane district, Goudkoppies in Johannesburg, Vlakplaats and Hartebeesfontein in Ekurhuleni South and North respectively.

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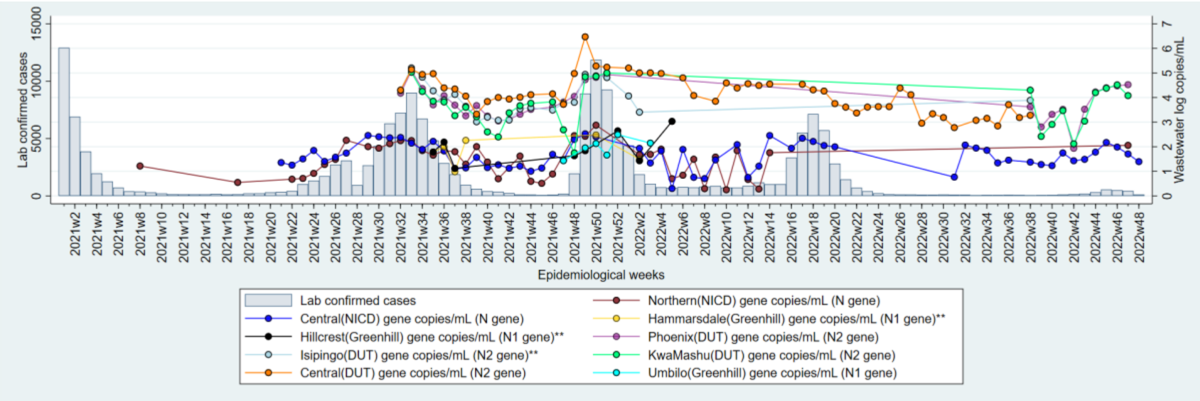
KwaZulu-Natal Province

2A: eThekwini Metropolitan Municipality



B: uMgungundlovu District Municipality

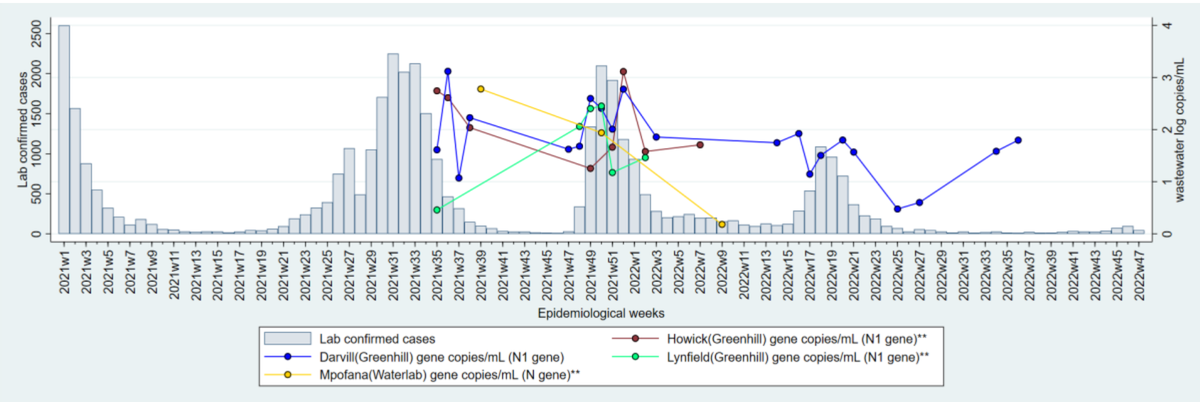


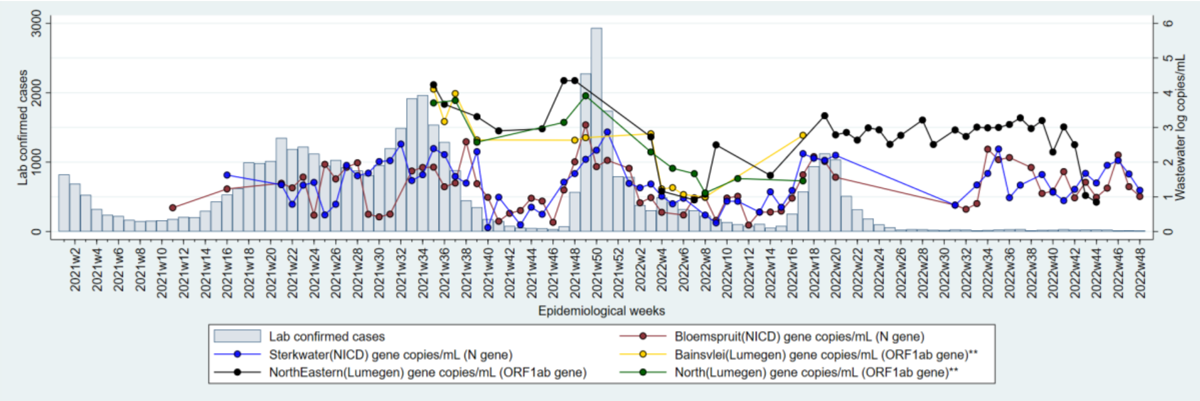
Figure 2A-B. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) from wastewater treatment plants (WWTP) in eThekwini, (A-B) and uMgungundlovu Metro (C), Kwa-Zulu Natal Province during epidemiological weeks 1-52, 2021 and week 48, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory.

**Laboratories where testing has been discontinued

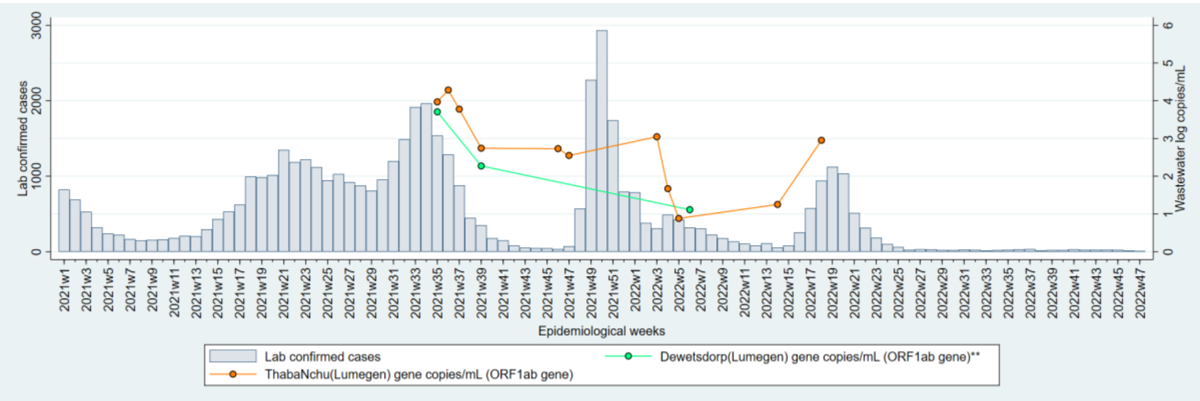
In week 48, a decline to minimal levels have also been observed at Central and Northern WWTPs in eThekwini. While KwaMashu and phoenix have remained high as of week 48.

Free State Province - Mangaung

A: Bloemfontein sub-district



B. Naledi & ThabaNchu sub-districts



Figures 3A-B. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) from wastewater treatment plants (WWTPs) in Mangaung, Free State Province (Bloemfontein, Botshabelo, Naledi and ThabaNchu) during epidemiological weeks 1, 2021 to 48, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory.

**Laboratories where testing has been discontinued

SARS-CoV-2 levels in Sterkwater and Bloemspruit decreased to minimal levels in week 48.

Eastern Cape Province

A: Nelson Mandela Metropolitan Municipality

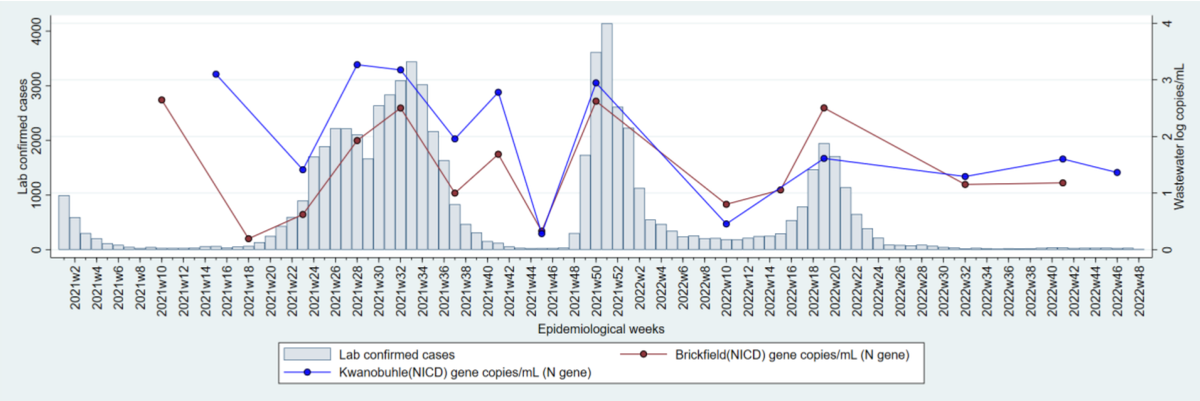


Figure 4A. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) from wastewater treatment plants (WWTPs) in Nelson Mandela Metro, Eastern Cape Province during epidemiological weeks 1, 2021 to 47, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory

B Buffalo City Metropolitan Municipality

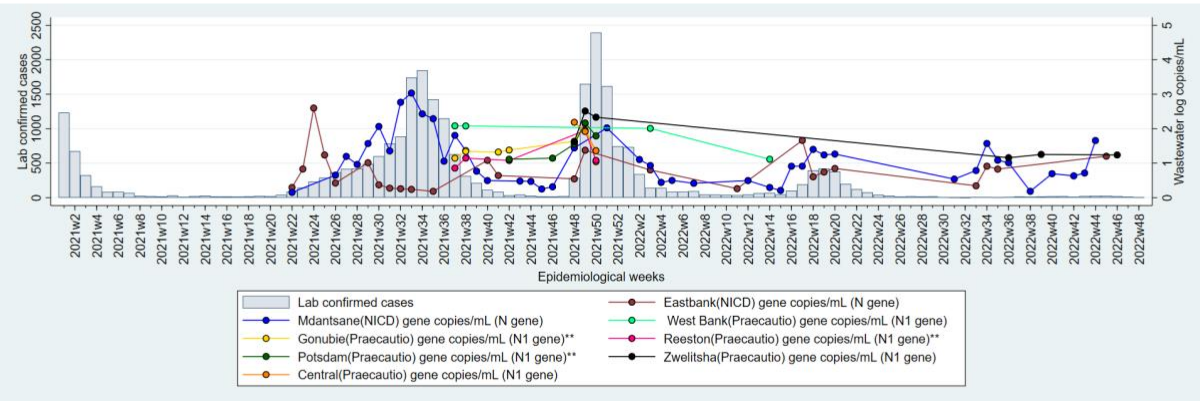


Figure 4B. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) from wastewater treatment plants (WWTPs) in Nelson Mandela Metro, Eastern Cape Province during epidemiological weeks 1, 2021 to 48, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory

**Laboratories where testing has been discontinued

As of week 47, SARS-CoV-2 levels have remained at minimal levels at Potsdam and Eastbank WWTPs in Buffalo City. 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

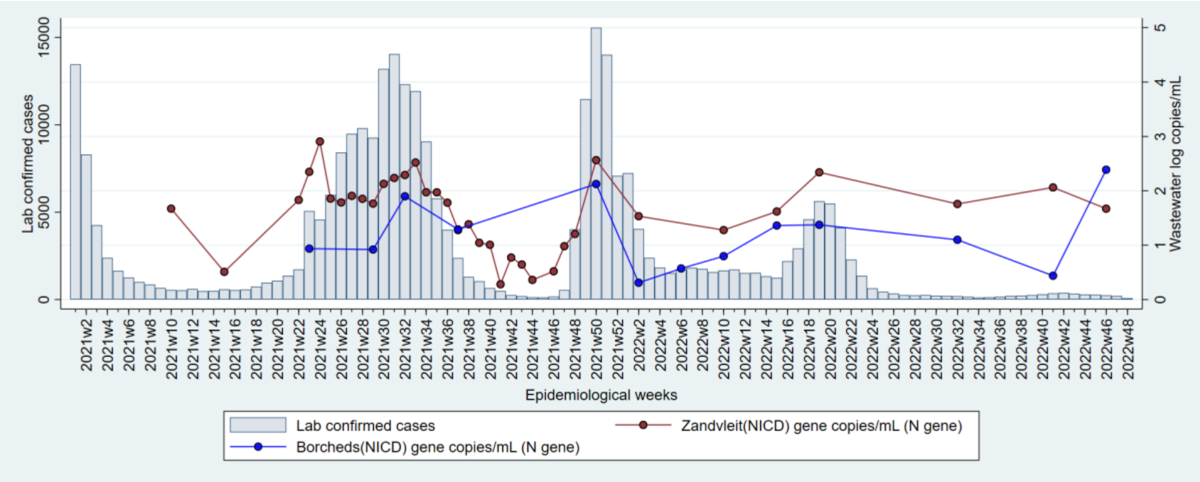


Figure 5. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) from wastewater treatment plants (WWTPs) in the City of Cape Town, Western Cape Province during epidemiological weeks 1, 2021 to 48, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP.

While levels at Zandvleit and Borcherd's Quarry are at intermediate levels, with Borcherd's Quarry showed signs of increases in week 46.

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.

Northern Cape Province

Frances Baard District Municipality

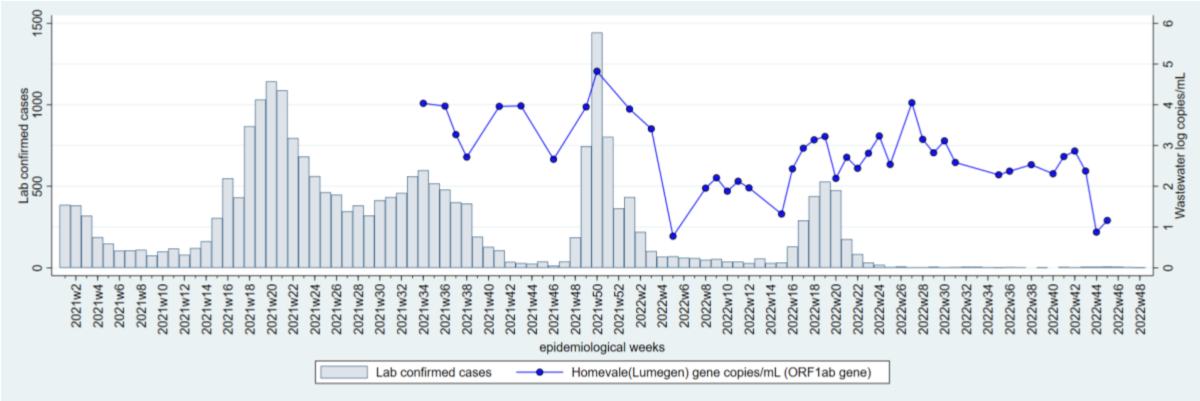


Figure 6. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) in wastewater treatment plants (WWTP) from Kimberly in Frances Baard District, Northern Cape Province during epidemiological weeks 1, 2021 to week 47, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory.

The SARS-CoV-2 levels in wastewater at Homevale WWTP in France Baard district declined in week 46.

Northwest Province

JB Marks Local Municipality

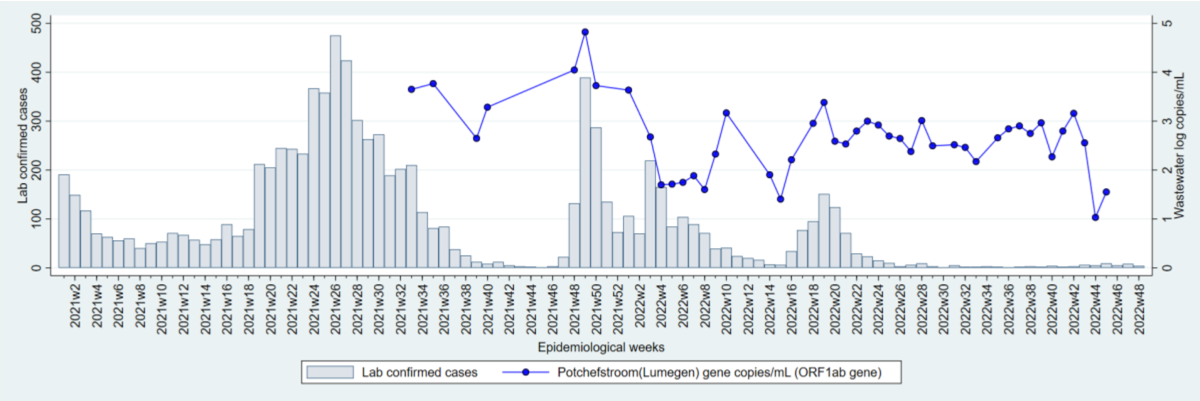


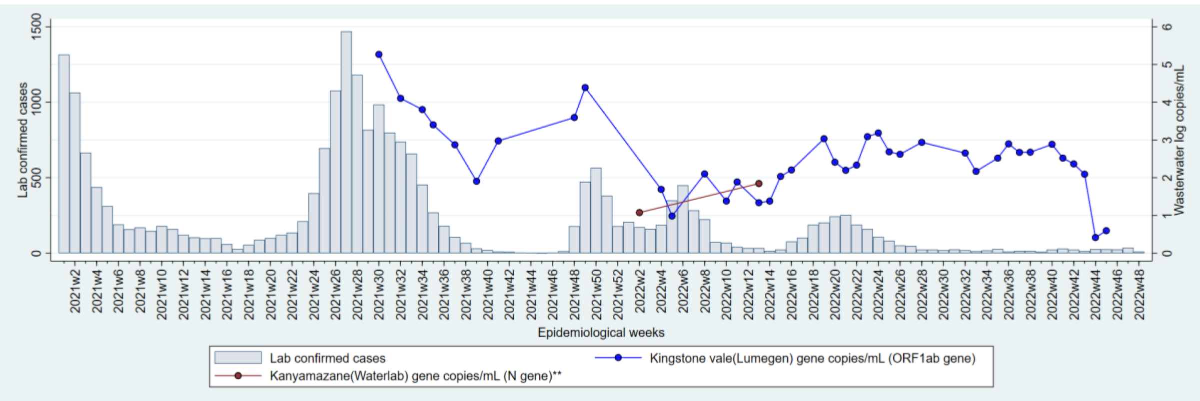
Figure 7. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) in wastewater treatment plants (WWTPs) from Potchefstroom, JB Marks District (A) Rustenberg, Bojanala District (B), and City of Matlosana, Northwest Province during epidemiological weeks 1, 2021 to 46, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory.

**Laboratories where testing has been discontinued

SARS-CoV-2 levels declined at Potchefstroom as of week 46.

Mpumalanga Province

A: Mbombela Local Municipality



B: Emalahleni Local Municipality

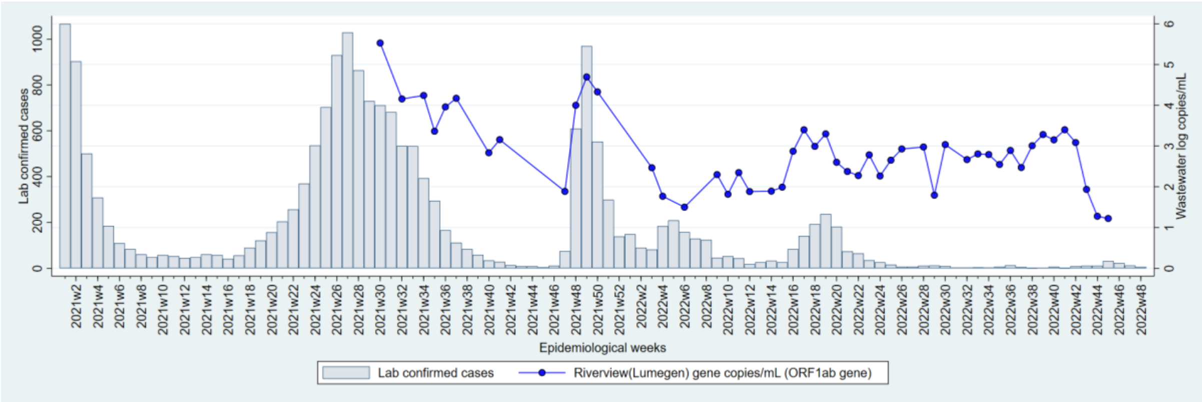


Figure 8A-B: Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) in wastewater treatment plants (WWTPs) from Mbombela and Emalahleni Local Municipality, Mpumalanga Province during epidemiological weeks 1, 2021 to 47, 2022. The testing laboratory and quantified SARS-CoV-2 gene are named in brackets after the name of the WWTP. Note that comparisons of levels over time should only be made for specimens tested in the same laboratory.

SARS-CoV-2 levels at Kingstone vale and Riverview WWTPs were low in weeks 45 and 46 respectively.

Limpopo Province

Polokwane Local Municipality

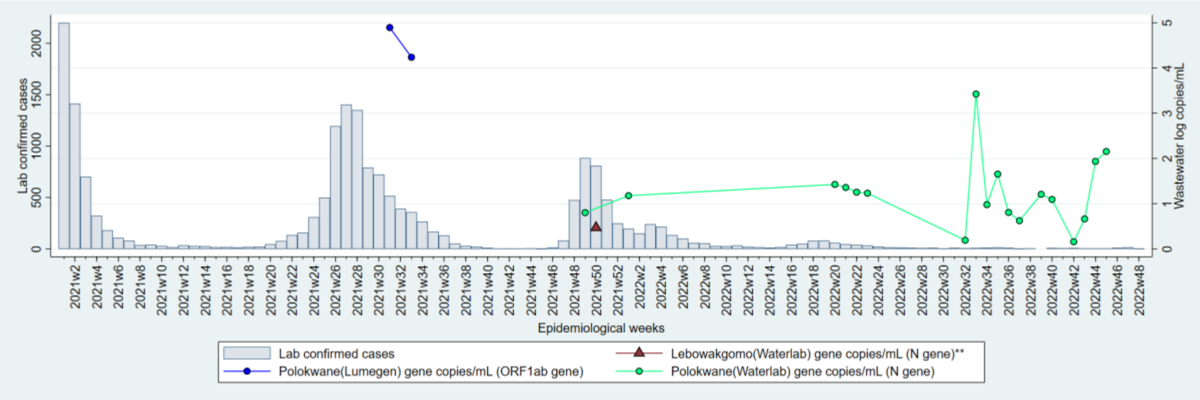


Figure 9. Laboratory confirmed cases of SARS-CoV-2 (bars) and levels of SARS-CoV-2 in log copies/ml of wastewater (coloured lines) in wastewater treatment plants (WWTPs) from Polokwane Local Municipality, Limpopo Province during epidemiological weeks 1, 2021 to 47, 2022.

**Laboratories where testing has been discontinued

SARS-Cov-2 levels increased from low to intermediate the the Polokwane WWTP in week 44-45

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 quantitative testing 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 quantitative SARS-CoV-2 results, 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 47 of 2022.

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, 2021-2022

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	18	38,30
	Nelson Mandela Metro	Brickfield	40000	15	12	6	50,00
		KwaNobuhle	100320	15	12	4	33,33
Free State	Mangaung	Sterkwater	200000	16	57	32	56,14
		Bloemspruit	350000	16	60	31	51,67
Gauteng	Ekurhuleni Metro	Daveyton	100000	20	5	2	40,00
		Hartebeesfontain	100000	14	63	43	68,25
		Vlakplaats	200000	21	54	34	62,96
	Johannesburg Metro	Northern	1200000	14	15	9	60,00
		Goudkoppies	500000	21	56	38	67,86
	Tshwane Metro	Rooiwal	unknown	17	71	50	70,42
		Daspoort	unknown	14	67	46	68,66
KwaZulu-Natal	eThekwini Metro	Northern	316425	17	36	17	47,22
		Central	350000	17	56	35	62,50
Western Cape	City of Cape Town Metro	Borcherd's Quarry	380000	15	13	5	38,46
		Zandvliet	460000	15	32	13	40,63
Total					689	394	

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. We included all mutations, including low frequency mutations and recorded the proportion of reads where that mutation was detected (the 'read frequency') as a percentage of total reads. Using the list of unique mutations for each VOC and VOI in the spike protein region (Table 3) we interrogated the matrix for the presence or absence of known signature mutations in each sample using STATA software (Table 3). As new variants were detected and identified in clinical specimens, we added signature mutations to the STATA code, allowing us to identify the presence of new variants both retrospectively and prospectively.

Table 3: List of signature mutations which was used to identify VOC and VOI present in wastewater samples from week 14 in 2021 to week 47 of 2022

Omicron	Alpha	Beta	Delta	C.1.2	Gamma	Lambda	Mu
V213G	A570D	D80A	R145H	P9L	T20N	G75V	Y144S
G339D	S982A			C136F	P26S	T76I	Y145N
S371L	D1118H			Y449H	T1027I	D253N	
S373P						L452Q	
S375F						F490S	
T376A							
D405N							
F486V*							
Q493R							
G496S							
Y505H							
T547K							
N764K							
N856K							
Q954H							
N969K							
L981F							
N658S*							
V3G*							
R21G#							
W152L#							
F186L#							
V486P#							
P621S#							
A706V#							
T1117I#							

*associated with Omicron variant BA.4/5

associated with Omicron variant XAY/XBA

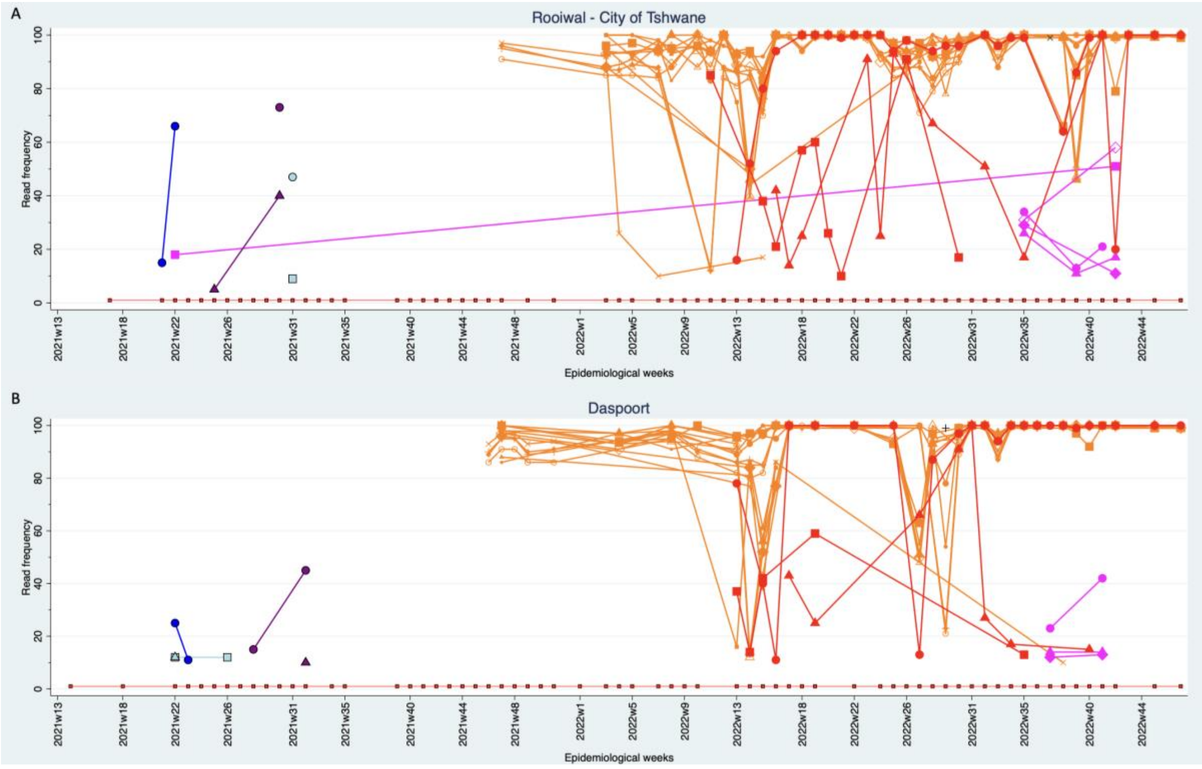
Results and discussion

Up to the **23rd November, 2022**, a total of **689** wastewater samples from sites listed in Table 1 underwent RNA extraction, amplification and sequencing. Of these **689** samples, **394 (57.18%)** yielded SARS-CoV-2 RNA sequences.

Detection of SARS-CoV-2 variants from wastewater samples using signature mutations analysis

Gauteng province

In the Gauteng province**, 223** samples yielded sequencing results displayed in Figure 1, which illustrates how beta variant was present in north and southern Gauteng province in week 21-22 but was replaced by delta and mutations associated with C.1.2 (a variant first detected in South Africa), which were simultaneously present at Rooiwal, Daspoort and Goudkoppies. 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 variant was first detected in week 46 and by week 47 was found to be present at all plants across the province. The F486V mutation (see Figure 1 below, represented as a red circle) was found in Rooiwal, Daspoort, Goudkoppies WWTP, Hartebeesfontein, Vlakplaats after week 10. Mutation N658S (see Figure 1 below, represented as a red square) was found in all sites, after week 13. Mutation V3G (see Figure 1 below, represented as a red triangle) was found in Rooiwal, Goudkoppies WWTP and Vlakplaats after week 13. Mutations (G21R, W152L, F186L, P621S A706V and T1117I) associated with XAY/XBA (lineages first detected in South Africa) were first detected week 22 - 2021 and continued to circulate up until week 41 - 2022, in Rooiwal, Daspoort and Vlakplaats. This suggests that the lineages XAY/XBA and sub-lineages BA.4 and BA.5 of Omicron are circulating in Johannesburg, Ekurhuleni and City of Tshwane. In week 41, mutations associated with BA.4 and BA.5 are consistently circulating at a high read frequency, with V3G and N658S consistently decreasing or falling off in all plants in Gauteng, suggesting that the mutations may be disappearing from population. Mutations (T1117I and P621S), associated with XAY/XBA were found in Goudkoppies in week 43 and 44. Additionally, omicron mutations continue to be present in week 45, including mutations associated with BA.4/BA.5, at high read frequency, in all plants in Gauteng. Mutations signature to Omicron continue to circulate in Vlakplaats, in week 46. Omicron mutations continue to circulate in all plants and mutation associated with BA.4/BA.5 (F486V) continues to circulate in week 47, at a high read frequency, in the Rooiwal, Hartbeesfontein and Daspoort plant.


A
Rooiwal - City of Tshwane
Read frequency
Epidemiological weeks
B
Daspoort
Read frequency
Epidemiological weeks

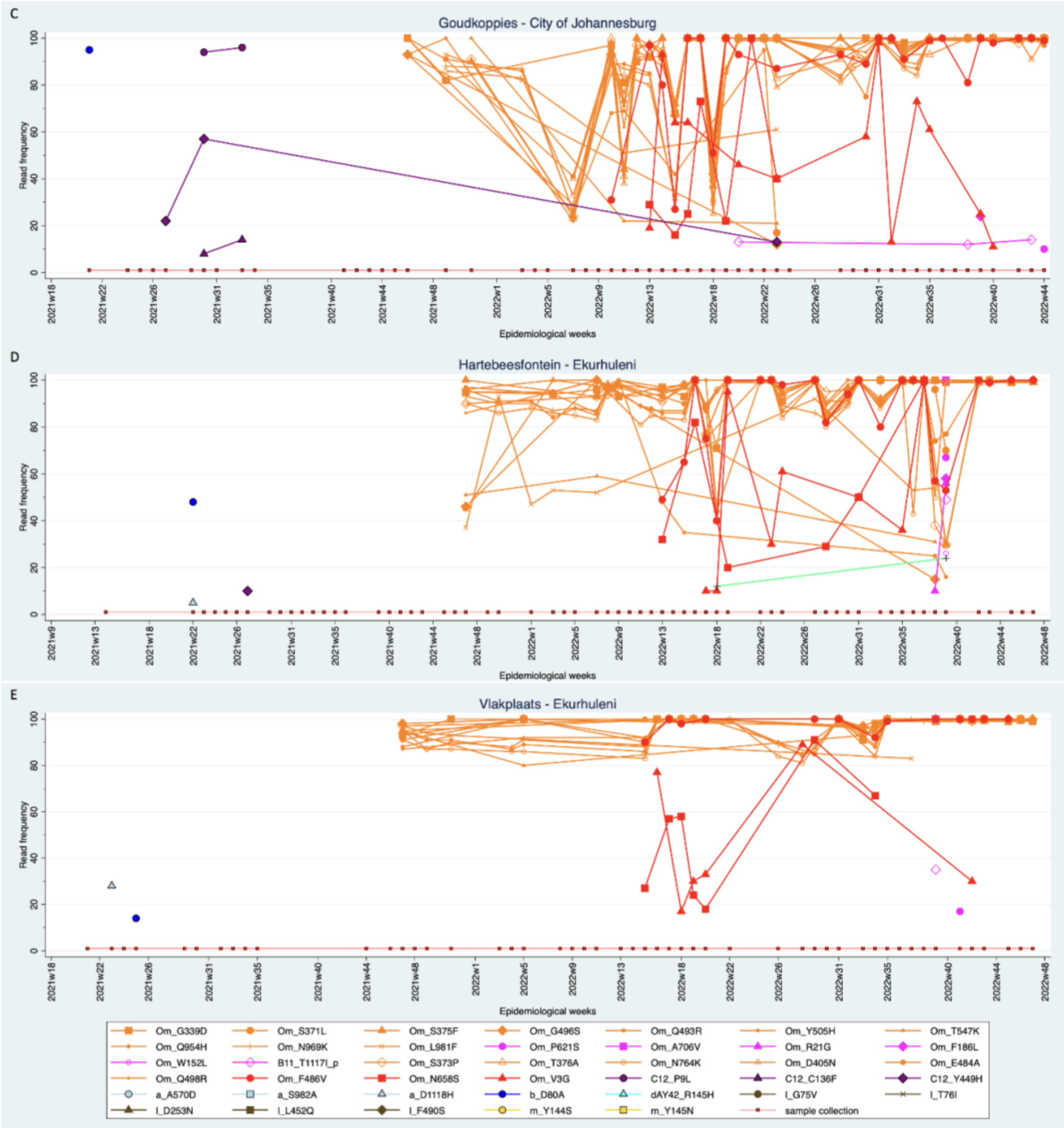


Figure 1: Beta mutation (D80A, dark blue circle). Delta mutations: R145H (pale blue triangle), d_R158G (green cross), A222V (pale blue diamond), C.1.2 mutations: P9L (purple circle), C136F (purple triangle), Y449H purple diamond. Omicron mutations: G339D (orange square), S371L (orange circle), S375F (orange triangle), G496S (orange diamond), Q493R (orange dot), Y505H (medium point orange circle), T547K (orange small triangle), Q954H (orange small diamond), N969K (orange small line), L981F (orange small x), S373P (orange hollow diamond), T376A (orange hollow triangle), D405N (small orange hollow triangle), E484A (small orange solid circle), Q498R (point orange circle), F486V (red circle), N658S (red square), V3G (red triangle), R21G (pink triangle), W152L (open pink circle), F186L (pink diamond), P621S (pink circle) A706V (pink square), T1117I (open pink diamond). Dots on the red line shows the timepoints of sample collection, absence of specific coloured lines means the mutation was not detected at that timepoint. A (Rooiwal plant), B (Daspoort plant), C (Goudkoppies plant), D (Hartebeesfontein plant), and E (Vlakplaats plant).

KwaZulu- Natal province

In KwaZulu-Natal province, **52** samples yielded good sequences and were included in Figure 2. Beta variant was detected in a single sample from Central plant in week 24. As in the Gauteng Province, C.1.2 was present at lower read frequencies from weeks 35, 2021 and 4, 2022 in central eThekwini. 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 variant was first detected in week 39, 2021 in Northern eThekwini and week 48, 2021 in central eThekwini and continues to be present up to week 40 of 2022. Mutation F486V was found in both central and northern eThekwini from Epiweek 13 (see Figure 2 below, represented as a red circle) and mutation V3G was found in central eThekwini after Epiweek 15. Mutations (G21R, W152L, F186L, P621S and A706V) associated with XAY/XBA (lineages first detected in South Africa) were first detected in week 35 - 2021, week 4 – 2022 and re-emerged in week 34 and 41 - 2022, in central eThekwini. This may suggest that the lineages XAY/XBA are circulating in Central eThekwini. In week 41, mutations associated with BA.4 and BA.5 continued to circulate up until week 40 in central eThekwini at a high read frequency with V3G consistently decreasing in read frequency and falling off in week 41. This may suggest that the mutations are disappearing from population. Omicron mutations and mutations associated with BA.4/BA.5 continue to circulate in week 45, with some at a relatively low read frequency, in previous weeks, in central eThekwini. Omicron mutations and mutation associated with BA.4/BA.5 (F486V) continues to circulate in week 47, at a high read frequency, in the both plant.

Figure 2: Beta mutation (D80A, dark blue circle). Delta mutations: R145H (pale blue triangle), E156del (green square), R158 (green cross), A222V (pale blue diamond). C.1.2 mutations: P9L (purple circle), C136F (purple triangle), Y449H purple diamond. Omicron mutations: G339D (orange square), S371L (orange circle), S375F (orange triangle), G496S (orange diamond), Q493R (orange dot), Y505H (medium point orange circle), T547K (orange small triangle), Q954H (orange small diamond), N969K (orange small line), L981F (orange small x), S373P (orange hollow diamond), T376A (orange hollow triangle), D405N (small orange hollow triangle), E484A (small orange solid circle), Q498R (point orange circle), F486V (red circle), N658S (red square), V3G (red triangle), R21G (pink triangle), W152L (open pink circle), F186L (pink diamond), P621S (pink circle) A706V (pink square), T1117I (open pink diamond). Dots on the red line shows the timepoints of sample collection, absence of specific coloured lines means the mutation was not detected at that timepoint. A (Northern eThekwini plant) and B (Central eThekwini plant).

Free State province

In Mangaung, Free State province, **62** samples yielded sequencing results displayed in Figure 3. The Beta variant was present until week 23 (Sterkwater plant) and 25 (Bloemspruit). Variant C.1.2 was detected in week 31 in Bloemspruit plant. No samples yield quality sequence data from weeks 35-46. Omicron was first detected in week 48 at both plants and continues to be present up until week 40. Mutation V3G was found in both Sterkwater and Bloemspruit in 2021, week 43 and 2022, week 16, respectively. Mutations F486V and N658S (see Figure 3 below, represented as a red circle and square) were found in the Sterkwater and Bloemspruit plant in week 14 and 16, respectively, and they continue to circulate at a high read frequency in week 41 with V3G having fallen off, indicating that the BA.4, and BA.5 sub-variants are circulating in Mangaung. Mutation (F186L) associated with XAY/XBA (lineages first detected in South Africa) was first detected in week 36 – 2022 in Bloemspruit and continues to circulate in week 41 in both Sterkwater and Bloemspruit. This may suggest that the lineages XAY/XBA are circulating in Free State. Omicron mutations and mutations associated with BA.4/BA.5 continue to circulate in week 43, at a high read frequency, in both plants in the Free State. Omicron mutations and mutation associated with BA.4/BA.5 (F486V) continues to circulate in week 47, at a high read frequency, in the both plant.

Figure 3: Beta mutation (D80A, dark blue circle). Delta mutations: R145H (pale blue triangle), E156del (green square), R158 (green cross), A222V (pale blue diamond). C.1.2 mutations: P9L (purple circle), C136F (purple triangle), Y449H purple diamond. Omicron mutations: G339D (orange square), S371L (orange circle), S375F (orange triangle), G496S (orange diamond), Q493R (orange dot), Y505H (medium point orange circle), T547K (orange small triangle), Q954H (orange small diamond), N969K (orange small line), L981F (orange small x), S373P (orange hollow diamond), T376A (orange hollow triangle), D405N (small orange hollow triangle), E484A (small orange solid circle), Q498R (small point orange circle), F486V (red circle), N658S (red square), V3G (red triangle), R21G (pink triangle), W152L (open pink circle), F186L (pink diamond), P621S (pink circle) A706V (pink square), T1117I (open pink diamond). Dots on the red line shows the timepoints of sample collection, absence of specific coloured lines means the mutation was not detected at that timepoint. A (Sterkwater plant) and B (Bloemspruit plant).

Western Cape province

In the Western Cape Province, **18** samples yielded sequencing results displayed in Figure 4. At the Zandvliet plant, Delta variant along with sub-lineages were detected from week 25-35. Evidence of C1.2 was found in week 22. A single mutation associated with Omicron was observed in week 24, 2021 (G339D) and re-emerged in week 2, 2022. Other omicron mutations were detected in week 47, 2021 and continue until week 41, 2022. At the Borcherd's Quarry plant, no samples yielded quality sequence data from week 34, 2021 to week 2, 2022 and 2 mutations associated with omicron were detected in week 15, and continues until week 41, 2022.

The F486V, N658S and V3G mutations (see Figure 4 below, represented as a red circle and square, respectively) were, in addition, found in week 19 in Zandvliet and week 41 in Borcherds Quarry, indicating that the new BA.4, and BA.5 sub-variants was circulating in parts of City of Cape Town. Mutations (R21G, F186L and P621S) associated with XAY/XBA were also found to be circulating in week 41, in Borcherds Quarry, suggesting that the lineages may be circulating in some parts of city of Cape Town. Mutations signature to Omicron and BA.4/BA.5 continue to circulate in Borcherds Quarry, in week 46, however the sampling rate in both plants is not consistent.

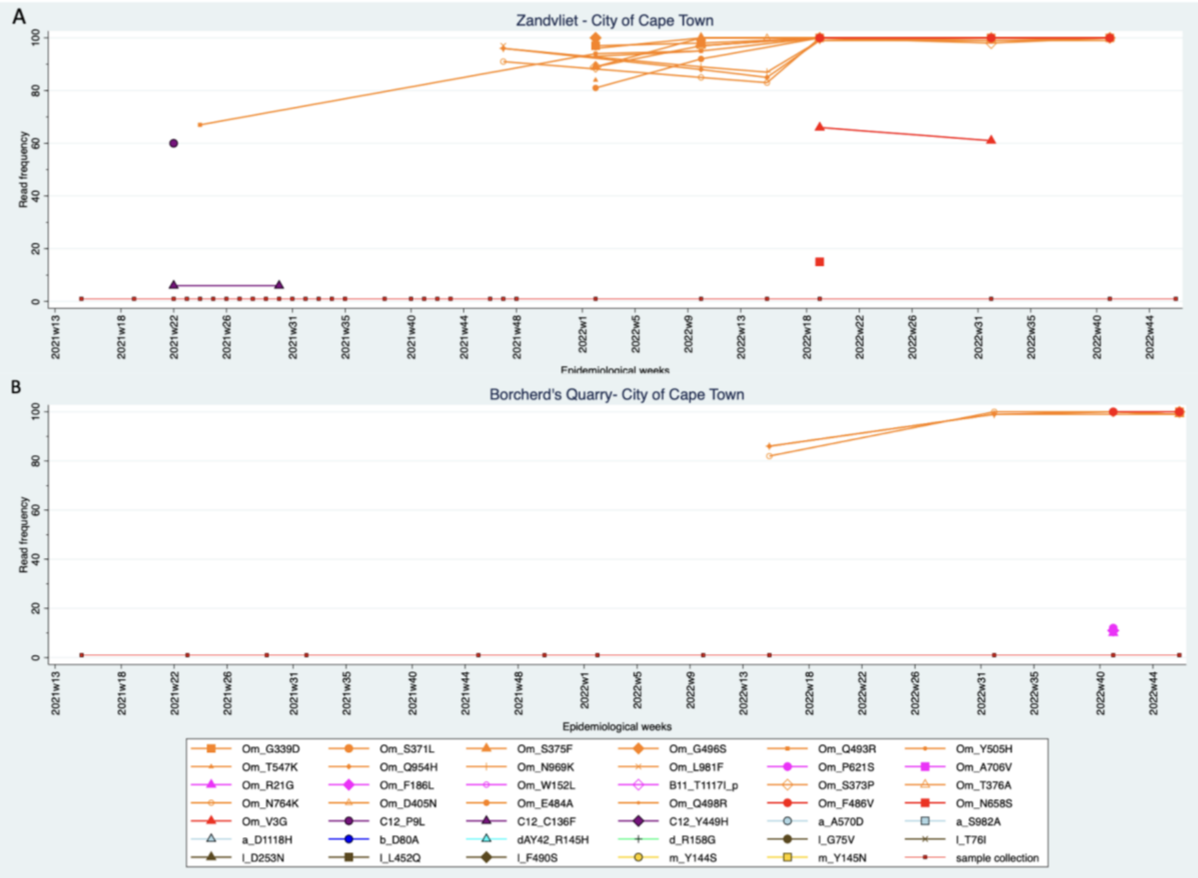


Figure 4: Beta mutation (D80A, dark blue circle). Delta mutations: R145H (pale blue triangle), E156del (green square), R158 (green cross), A222V (pale blue diamond). C.1.2 mutations: P9L (purple circle), C136F (purple triangle), Y449H purple diamond. Omicron mutations: G339D (orange square), S371L (orange circle), S375F (orange triangle), G496S (orange diamond), Q493R (orange dot), Y505H (medium point orange circle), T547K (orange small triangle), Q954H (orange small diamond), N969K (orange small line), L981F (orange small x), S373P (orange hollow diamond), T376A (orange hollow triangle), D405N (small orange hollow triangle), E484A (small orange solid circle), Q498R (small point orange circle), F486V (red circle), N658S (red square), V3G (red triangle), R21G (pink triangle), W152L (open pink circle), F186L (pink diamond), P621S (pink circle) A706V (pink square), T1117I (open pink diamond). Dots on the red line shows the timepoints of sample collection, absence of specific coloured lines means the mutation was not detected at that timepoint. A (Zandvliet plant) and B (Borcherd's Quarry plant).

Eastern Cape province

In the Eastern Cape Province, **40** samples yielded sequencing results displayed in figure 5. Omicron variant was first detected in week 48 at the Mdantsane plant, week 50 at the Kwanobuhle plant and Brickfield plants and week 10, 2022 at the Eastbank plant. No C1.2. variants were detected at all plants. Mutations F486V, N658S and V3G (see Figure 5 below, represented as a red circle, square and triangle, respectively) were found in Epiweek 16, 2022, indicating that BA.4 and BA.5 sub-lineages are circulating and continue to circulate in parts of the Eastern Cape, at a high read frequency, in week 41. Mutations (R21G, F186L and P621S) associated with XAY/XBA were also found to be circulating in week 41, in Brickfield and Kwanobuhle, suggesting that the lineages may be circulating in some parts of the Eastern Cape. Omicron mutation (N658S) associated with BA.4/BA.5 continues to circulate in week 43 and 44, at a high read frequency, in Mdantsane and mutation F486V continues to circulate in Eastbank in week 45, at a high read frequency.

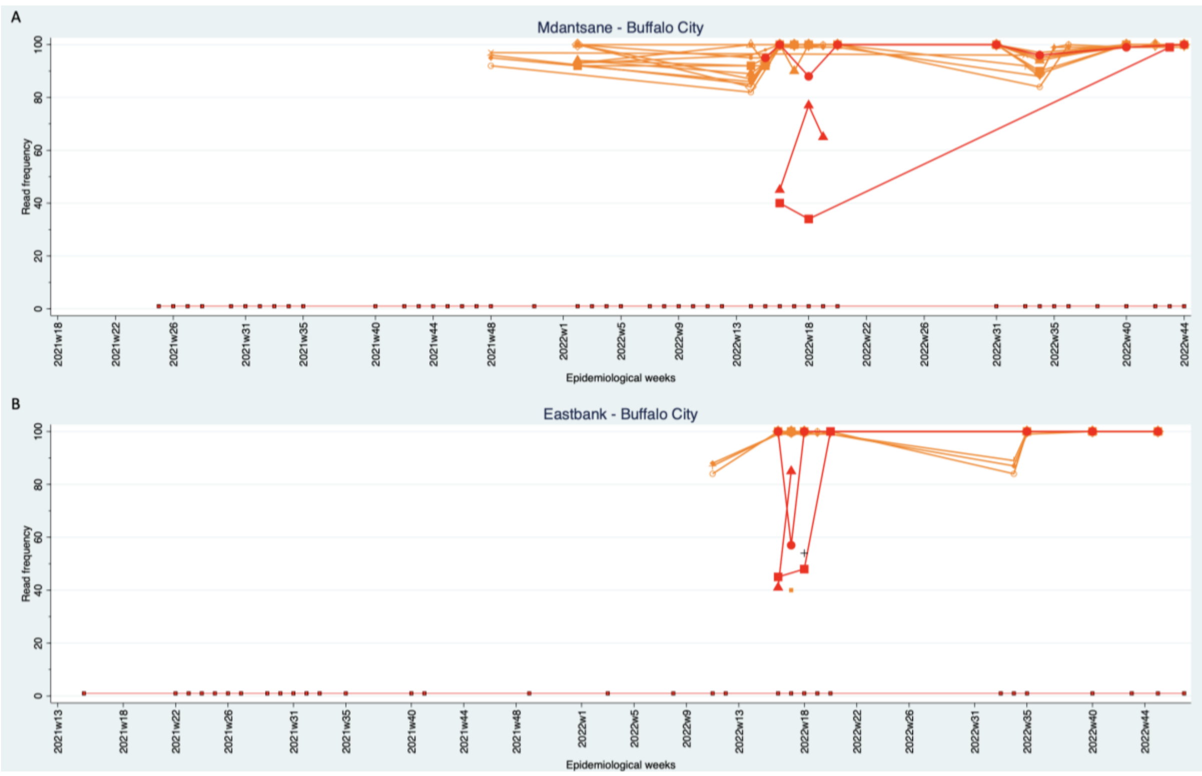


Figure 5: Beta mutation (D80A, dark blue circle). Delta mutations: R145H (pale blue triangle), E156del (green square), R158 (green cross), A222V (pale blue diamond). C.1.2 mutations: P9L (purple circle), C136F (purple triangle), Y449H purple diamond. Omicron mutations: G339D (orange square), S371L (orange circle), S375F (orange triangle), G496S (orange diamond), Q493R (orange dot), Y505H (medium point orange circle), T547K (orange small triangle), Q954H (orange small diamond), N969K (orange small line), L981F (orange small x), S373P (orange hollow diamond), T376A (orange hollow triangle), D405N (small orange hollow triangle), E484A (small orange solid circle), Q498R (point orange circle), F486V (red circle), N658S (red square), V3G (red triangle), R21G (pink triangle), W152L (open pink circle), F186L (pink diamond), P621S (pink circle) A706V (pink square), T1117I (open pink diamond). Dots on the red line shows the timepoints of sample collection, absence of specific coloured lines means the mutation was not detected at that timepoint. A (Mdantsane plant), B (Eastbank plant) C (Brickfield plant) and D (Kwanobuhle plant).

The distribution of SARS-CoV-2 variants 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 6). Furthermore, Omicron sub-lineage BE.1.2 is predominantly circulating in South Africa in all plants, followed by Omicron sub-lineage BA.5.3.1, BA.5.2.1, BA.5.3.5 and recombinant XBE, as of week 47 (Figure 7).

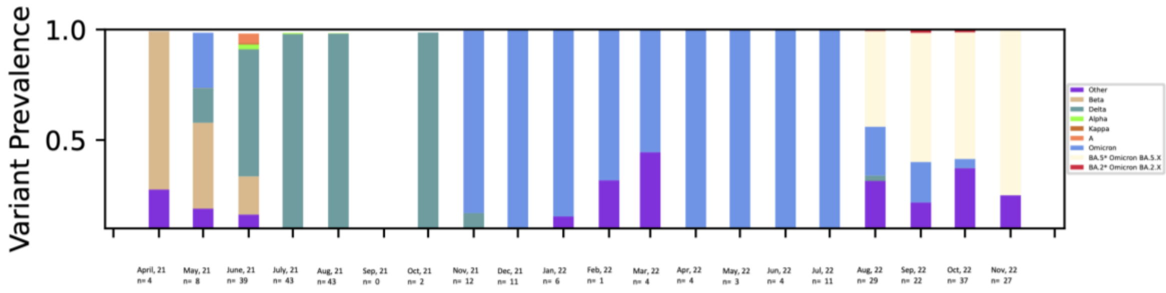


Figure 6. The proportion of SARS-CoV-2 variants in the environmental samples sorted by month and year (January 2021-October 2022) from all South African provinces. The number of samples processed each month, with a coverage >50% are indicated as n.

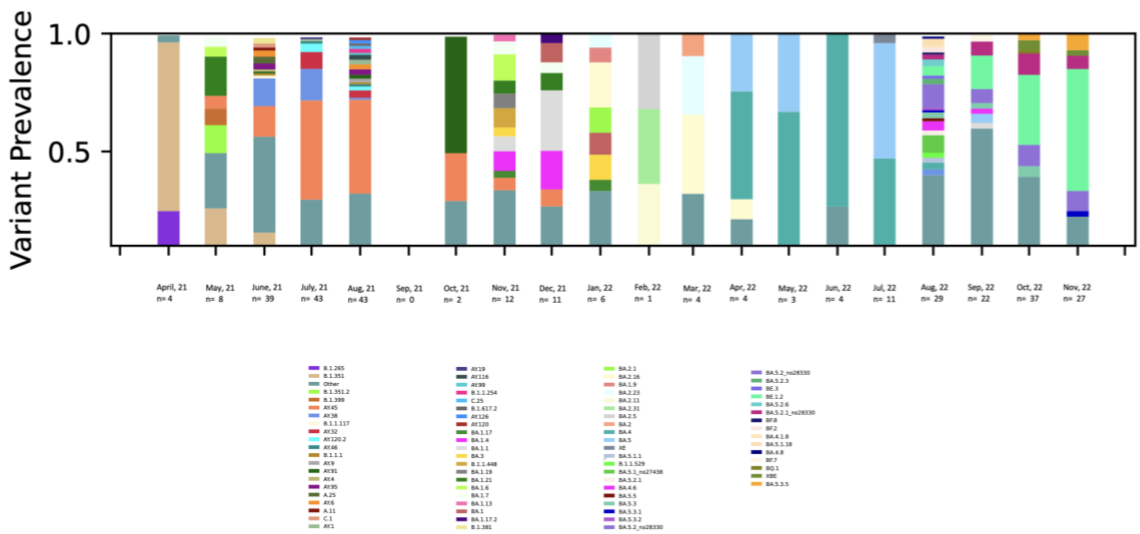


Figure 7. The proportion of SARS-CoV-2 lineages in the environmental samples sorted by month and year (January 2021-August 2022) from all South African provinces. The number of samples processed each month, with a coverage >50% are indicated as n.

Limitations

The ability to identify variants in wastewater relies on the identification of single nucleotide polymorphisms found in clinical strains and which are uniquely associated with these variants. We are not yet able to detect new variants. Sequencing of SARS-CoV-2 from wastewater may not yield good quality sequence data when viral concentration in wastewater is low. However, SARS-CoV-2 data from wastewater at South African sentinel sites do show concordance with clinical, epidemiologic curves and sequencing data (not shown) in the respective locations, illustrating the potential of the SACCESS network to provide descriptive epidemiological data pertaining to geographic variation, burden and variants of SARS-CoV-2.

Conclusion

The SACCESS network of laboratories is able to provide population-level data regarding the distribution in time, place and burden of disease of SARS-CoV-2 and to identify currently circulating variants. These data from epidemiologic week 14 demonstrate the increased circulation of SARS-CoV-2 in Gauteng, Mangaung and KwaZulu-Natal (eThekwini) suggestive of a new variant. Sequencing data available up to week 47 shows that Omicron sub-lineage BE.1.2 is predominantly circulating in South Africa in all plants, followed by Omicron sub-lineage BA.5.3.1, BA.5.2.1, BA.5.3.5 and recombinant XBE. The quantitative 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 Johannesbur g 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 Johannesbur g Metropolitan Municipality	Strydompark, Olivedale, Ri vonia, Jukskei Park, Douglasdale, Ferndal e, 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 Johannesbur g Metropolitan Municipality	Soweto, Rivasdale	NICD	24-05-2021
23	Bushkoppies	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg G SD	No subdistrict	City of Johannesbur g Metropolitan Municipality	Baragwanath, Pimville, Joh annesburg South, Dube, Willowdene, Nancefield	Waterlab/UP	11-10-2021
24	Olifantsvlei	Gauteng	City of Johannesburg Metropolitan Municipality	Johannesburg G SD	No subdistrict	City of Johannesbur g 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