PROOF OF CONCEPT STUDY
APPLICATION OF WASTEWATER-BASED SURVEILLANCE TO MONITOR SARS-COV-2 PREVALENCE IN SOUTH AFRICAN COMMUNITIES

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WATER RESEARCH COMMISSION

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Application of wastewater-based surveillance to monitor SARS-CoV-2 prevalence in South African communities

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Coronaviruses (CoVs) are named for the crown-like spikes on their surface. They are enveloped, with a lipid membrane envelope around the surface of the virus. The lipid envelope makes coronaviruses more fragile than other viruses and is relevant to understanding their sensitivity to disinfection and their persistence in the environment and transmission. Coronaviruses mostly infect animals, such as bats, birds and mammals, which act as an intermediate host reservoir. Human coronaviruses (HCoVs) were first identified in the mid-1960s and so far, a total of seven have been reported to be capable of infecting humans. Four of them, the 29E (alpha coronavirus); NL63 (alpha coronavirus); OC43 (beta coronavirus) and HKU1 (beta coronavirus), cause mild to moderate disease. Since the beginning of the 21st century, three more coronaviruses have crossed the animal-human species barrier to cause deadly pneumonia in humans, namely the Severe Acute Respiratory Syndrome (SARS-CoV-1), Middle-East Respiratory Syndrome (MERS), and the current Severe Acute Respiratory Syndrome 2 (SARS-CoV-2). SARS-CoV-2 is the newest of the family of coronaviruses associated with human infections that are grouped into the beta-CoV genus, with 79% genetic similarity to SARS-CoV-1. The outbreak was declared a Public Health Emergency of International Concern on 30 January 2020 and on 11 February 2020 the World Health Organization (WHO) announced a name for the new coronavirus disease: COVID-19. On March 11, WHO upgraded the status of the COVID-19 outbreak from epidemic to pandemic. Many COVID-19 infections are asymptomatic and unless tested, can remain undetected. Likewise, the current total picture of SARS-CoV-2 virus circulation in the population of South Africa is incomplete and the number of COVID-19 patients most likely underestimated, mainly due to the limitations regarding testing.

Once in the body, the virus can be shed through faeces and urine, as well as through saliva and other respiratory discharges. The virus and/or its remnants are introduced into water resources and wastewater environment through the discharge of human waste and bodily fluids containing the virus, e.g. from brushing teeth, mouth washing, coughing and sneezing while bathing or showering, washing of hands or clothes, and discarding tissues and wipes into the toilet. As a complementary approach to monitoring the spread of COVID-19, many countries have since implemented wastewater-based epidemiology (WBE). WBE is a relatively new environmental concept for determining the exposure of populations to substances of concern, and is based on the analysis of target biomarkers related to that substance of concern in raw wastewater in order to obtain qualitative and quantitative data on the health of communities within a given wastewater catchment. WBE has been used to help inform broader infectious disease epidemiological surveillance and mitigation efforts such as the Global Polio Eradication Initiative. Environmental water surveillance has also been used and recommended for monitoring the spread of other infectious disease-causing microorganisms such as typhoid, early warning of hepatitis A and norovirus outbreaks, as well as for antimicrobial resistance.

Thus, the presence / absence of SARS-CoV-2 and/or remnants in wastewater treatment plant influent can determine the presence of infected individuals in a community and can be used as an epidemiological indicator, especially where community testing is not possible. The main aim of this study is to test the feasibility of applying the WBE and environmental water surveillance concept in South Africa as a tool that provides valuable additional information about the spread of the virus as a complement to health surveillance, and also as an early warning system for infection in a community providing a more sensitive and rapid indication of changes in infection rates before such effects become detectable by clinical health surveillance. Critically, this will provide decision support for officials determining the timing and severity of public health interventions to mitigate the overall spread of the disease. This study serves as a short-term, proof of concept study prior to the roll-out of a national surveillance, and also involves preliminary testing, optimisation and validation of sampling and virus analysis methods, as well as results interpretation and reporting in the South African context.

OBJECTIVES OF THE STUDY

The overall aim of this proof of concept study is preliminary testing, optimisation and validation of sampling and virus analysis methods, as well as results interpretation and reporting in the South African context. The study was based on the detection of SARS-CoV-2 signal (RNA) in wastewater and environmental water samples as a means of assessing the presence of infected individuals in a community to consider as an epidemiological surveillance tool. The specific objectives of the study were as follows:

1. Compile state of knowledge reports on SARS-CoV-2 in water and sanitation environments
2. Testing and validation of a sampling protocol for raw sewage
3. Testing and validation of the virus extraction and analysis protocol
4. Testing and validation of a sampling protocol for surface and groundwater, depending on the success of objective 3 above
5. Development of preliminary methodology for quantification of viral load as an indicator of number of infected individuals in a community
6. Guidance on data analysis/interpretation
7. Recommendations for data communication and integration into national reporting platforms

METHODOLOGY

Sampling: Wastewater was collected as 24-hour composite samples from 10 wastewater treatment works (WWTWs) in 5 provinces, over a period of 4 weeks. Additional duplicate grab samples were taken from selected WWTWs during the morning peak flow period. Additional composite samples from wastewater treatment plants at selected power stations, mines and other defined communities were included in the testing. As an indicator for SARS-CoV-2 prevalence in non-sewered communities, four surface water grab samples were also collected from the Jukskei River downstream of Alexandra informal settlement, the Hennops River downstream of Tembisa informal settlement, as well as the Blougatspruit in the Cradle of Humankind and a surface water runoff sample from an informal settlement in Alexandra.

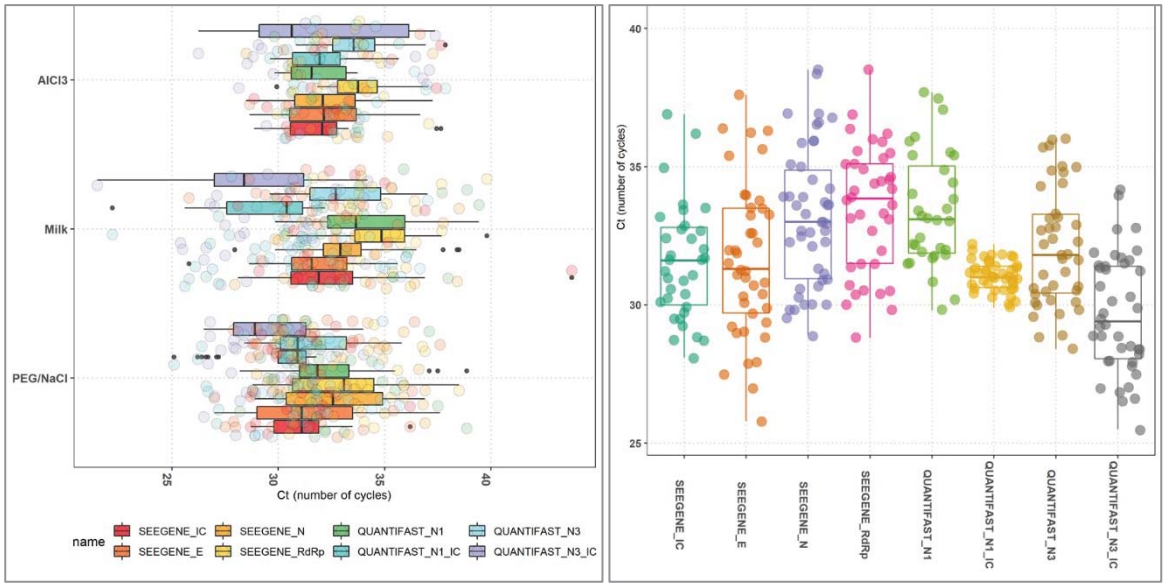
Virus recovery: Three virus recovery methods were tested based on their ease of use and cost effectiveness, namely, PEG 8000/NaCl precipitation, skimmed milk flocculation and aluminium hydroxide adsorption-flocculation. Virus recovery efficiency was determined making use of the mengovirus. 1-2 L sewage samples were received and stored at 4°C until processing. Samples were mixed thoroughly and a 200 mL aliquot was used for each of the three recovery methods. A total of 49 wastewater treatment works samples were collected which included 19 duplicate samples to include a total of 68 wastewater samples, of which 29 samples were recovered with PEG/NaCl, 19 samples were recovered with skimmed milk and 20 samples were recovered with $Al(OH)_3$).

Viral nucleic acid extraction, detection and quantification: Viral nucleic acid extraction was performed with the QIAamp Ultrasens Virus Kit (Qiagen). Screening for SARS-CoV-2 was done with a commercial real time multiplex RT-PCR (Seegene), and repeated with inhouse assays using the QuantiFast Pathogen +IC RT-PCR kit (Qiagen) on the QuantStudio5 real time PCR platform. The Seegene assay targeted the E gene, N gene and RdRp gene as well as an internal control in a single reaction, whereas the QuantiFast duplex assays targeted the N1 or N2 regions of the N gene, as well as an internal control. Ct values below 40 were considered positive. Five gene targets for each recovered sample were tested in three assays, totalling 204 RT-PCR reactions.

RESULTS

SARS-CoV-2 recovery, extraction and detection

All three virus recovery methods tested were effective in the recovery of the SARS-CoV-2 virus and both the Seegene and QuantiFast kits detected the virus RNA.



SARS-CoV-2 analysis in wastewater samples

- Of the total of 68 wastewater samples, 50 were positive for all 5 targets (70.4%), 16 were positive for 4 targets (20.4%), 9 were positive for 3 targets (13%), 2 were positive for 1 target (2.94%) and only 1 (1.47%) was negative.
- Of the 10 defined community wastewater samples tested 8 were positive for all 5 targets (80%), 1 was positive for 4 targets (10%), and 1 was negative.

SARS-CoV-2 analysis in environmental water samples

Of the 4 surface water samples all tested strongly positive with a 200 mL sample volume being sufficient to elicit a response.

SARS-CoV-2 concentration in samples

Virus quantification was successfully carried out with genome copies/mL ranging between 1,2 and 2,7×10^4 for N1 and 4,2-5,5 × 10^4 for N3 target genes.

CONCLUSION AND RECOMMENDATIONS

The detection of SARS-CoV-2 RNA in 98% of wastewater and environmental samples collected, has demonstrated the proof of concept. Raw sewage samples from Gauteng, Western Cape, KwaZulu-Natal, Mpumalanga and Free State provinces with representation over 4-week period were analysed. Composite and grab samples were tested with grab samples being able to detected higher virus signal than 24 h composite samples (92 tested in total).

1. Testing and validation of the virus extraction and analysis methods

Three virus extraction methods were tested based on instrumentation availability and affordability including PEG/NaCl precipitation; skimmed milk flocculation and $Al(OH)_3$ adsorption-flocculation methods illustrating that highly specialised laboratory equipment is not necessary.

Methods need to achieve reproducible high quality and quantitative information. In order to address this, it is recommended that the evaluation and validation of methods includes a minimally acceptable QA/QC including

a) positive control;
b) negative control;
c) estimated limit of detection;
d) reporting of equivalent volume of sample analysed.

Additional validation controls include:

a) inhibition control;
b) initial recovery controls;
c) ongoing precision recovery controls and lastly
d) matrix spike, where a known concentration of target virus is added to the samples before sample preparation and assay

2. Potential for implementing environmental surveillance of SARS-CoV-2 as a proxy for SARS-CoV-2 monitoring in non-sewered communities

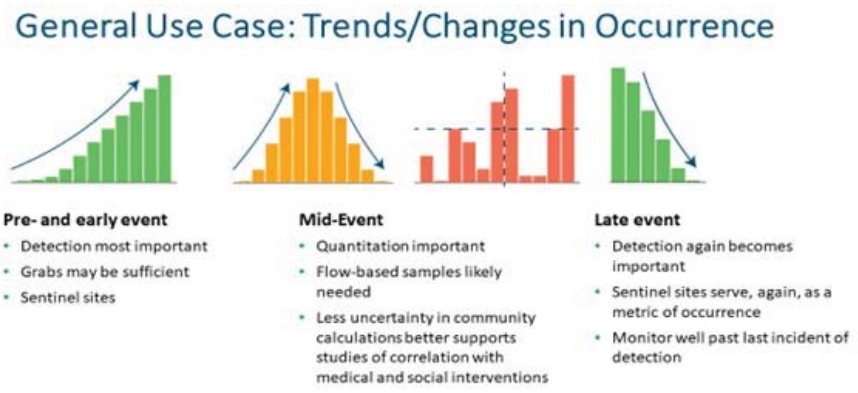
Methods for viral recovery, extraction and detection were tested, optimised and validated for surface water samples. Environmental samples were collected from different locations, with viral RNA detected in all samples.

3. Development of preliminary methodology for quantification of viral load as an indicator of number of infected individuals in a community

Method to quantify the viral load makes use of the Ct number with a proposed categorical data analysis recommended based on the Global Polio Surveillance scheme, and quantification of genome copies/mL was found to range between 1,2-2,7 × 10^4 for N1 and 4,2-5,5 × 10^4 for N3 target genes.

4. Guidance on data analysis/interpretation

International interpretation of data is being followed as described in Objective 5 which was described by the Water Research Foundation webinars held during the first peak of the Covid-19 pandemic.

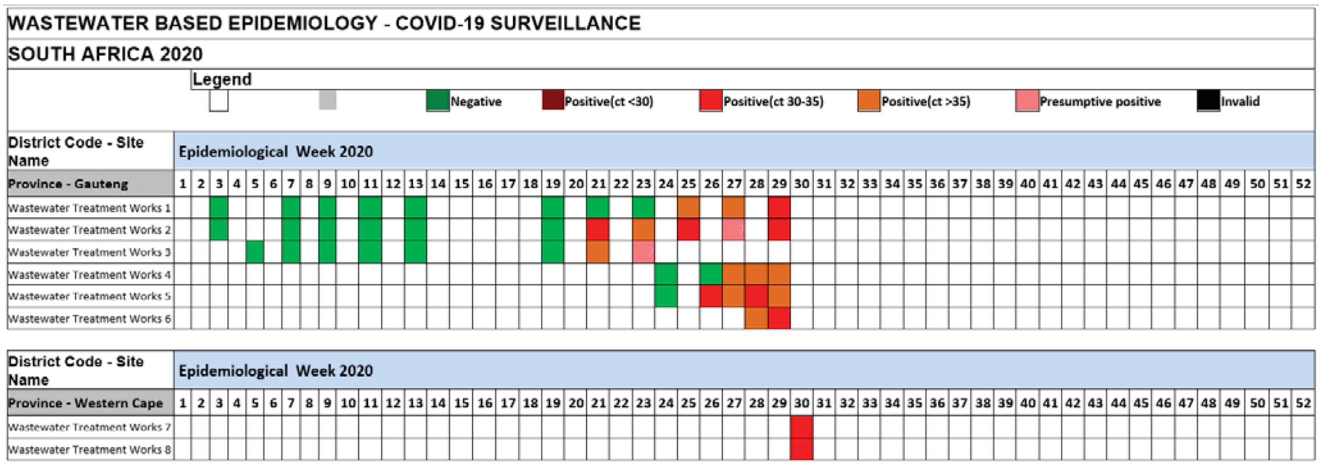


General use case: Source Water Research Foundation 2020

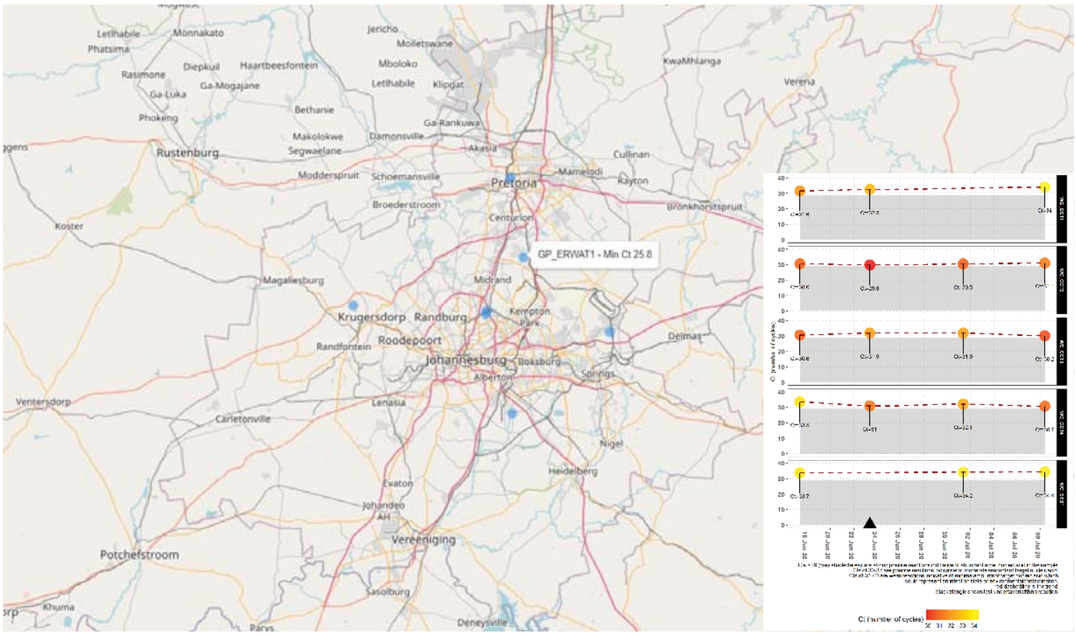
The various use-cases presented highlight the importance of trend monitoring through the various phases of the pandemic. Although translating the viral titres from wastewater into the actual number of cases within a community is highly challenging, if not impossible, monitoring trends in viral load can be used successfully to implement an early warning system.

5. Recommendations for data communication and integration into national reporting platforms

Data communication can take place either as part of Polio Surveillance scheme run by the NICD, following the methodology for reporting based on categorical presentation of Ct value ranges or on a separate Covid-19 Data Centre platform. An example of such is presented below.



Example of proposed Covid-19 surveillance dashboard as per the NICD poliovirus surveillance platform



Mapping of surveillance sites to indicate current status

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CHAPTER 1: INTRODUCTION

1.1 THE SARS-COV-2 VIRUS

Coronaviruses (CoVs) belong to the family of *Coronaviridae* and they are a large and diverse family of viruses. The name 'corona' comes from their round appearance and the spikes on their surface that can be likened to a solar corona (Figure 1-1(a)). Coronaviruses are enveloped, which means that there is a lipid membrane envelope around the surface of the virus, while 'naked' viruses do not have this. The lipid envelope makes coronaviruses more fragile than other viruses (Walls et al., 2020) and is hence relevant to understanding their environmental persistence and transmission and their susceptibility to inactivation by disinfection. The lipidic structure holds the membrane (M), envelope (E) and spike (S) proteins together, with the spike protein protruding from the envelope (Figure 1-1(a)). Since the spike protein is responsible for the connection with the host cells in humans, the virus loses its infectivity if the lipid envelope is destroyed (Figure 1-1(b)) (Walls et al., 2020; Wu et al., 2020a, 2020b, 2020c). Their genome is made up of single-stranded RNA (Figure 1-1(a)), which makes them highly susceptible to UV disinfection. When screening for the virus in wastewater, scientists detect the genetic information that codes for the key proteins in its structure. Eurosurveillance and Centers for Disease Control and Prevention have provided references listing commonly used primers for the detection of SARS-CoV-2 virus. The Eurosurveillance E primers target regions of RNA that code for the envelope (E), while the CDC N1 and N2 primers detect fragments of RNA that code for the nucleocapsid (N) protein (Figure 1-1(a)).

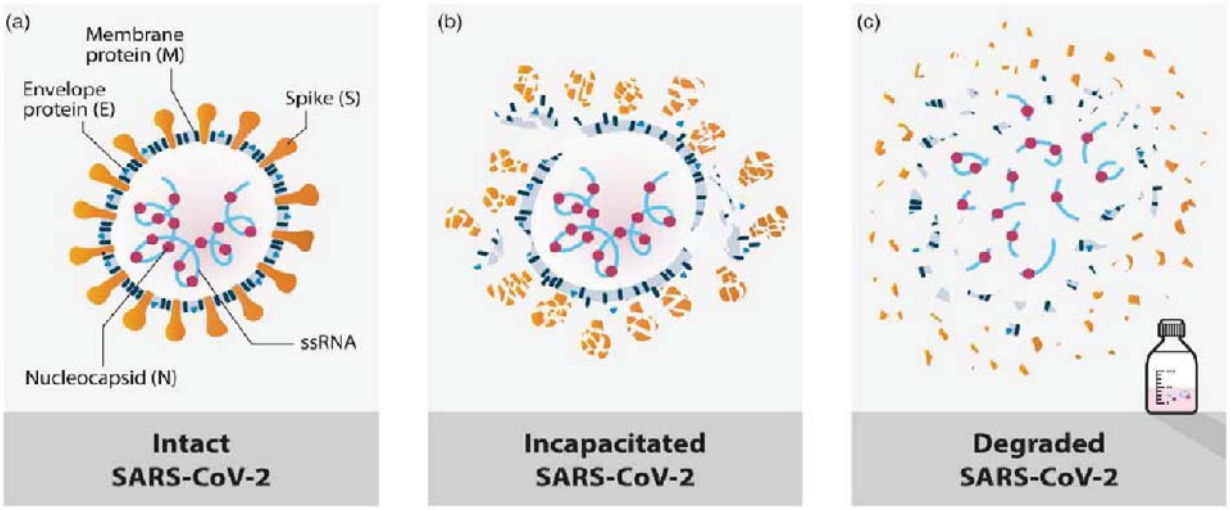


Figure 1-1: SARS-CoV-2 key structure includes S, N, M, E and RNA (a); incapacitation process (b) and degradation (c). The subsequent analysis of SARS-CoV-2 RNA (typically after conversion to DNA) may follow RNA extraction from intact, incapacitated or degraded virus and combinations thereof (Hill et al., 2020).

Coronaviruses mostly infect animals, such as, bats, birds and mammals, which act as an intermediate host reservoir. Human coronaviruses (HCoVs) were first identified in the mid-1960s and so far, a total of seven have been reports to be capable of infecting humans. Four of them, the 29E (alpha coronavirus); NL63 (alpha coronavirus); OC43 (beta coronavirus) and HKU1 (beta coronavirus), cause mild to moderate disease, and may even go unnoticed. However, since the beginning of the 21st century, three more human coronaviruses have been identified and cause deadly pneumonia in humans (Drosten et al., 2003; Zaki

et al., 2012). These include Severe Acute Respiratory Syndrome (SARS-CoV-1), Middle-East Respiratory Syndrome (MERS), and now Severe Acute Respiratory Syndrome 2 (SARS-CoV-2). This CoV is the newest of the family of coronaviruses associated with human infections that are grouped into the beta-CoV genus, with 79% genetic similarity to SARS-CoV-1 (Gorbalenya et al., 2020; Lu et al., 2020).

SARS-CoV-2 was revealed after testing of fluid from a patient's lungs on 3 January 2020, following reports of several patients presenting with a strange pneumonia in November and December 2019 in Wuhan Province, China. The first publications about this virus referred to it as the 'novel coronavirus', and the name 2019-nCoV was used to denote it. Since more has become known about the virus, it has been designated SARS-CoV-2 and is associated with the current pandemic of atypical pneumonia (the disease is designated as COVID-19). SARS-CoV-2 is transmitted from person-to-person via the respiratory system through sneezing, coughing and secretions, and by contact with contaminated surfaces (Huang et al., 2020; Zhu et al., 2020).

1.2 SARS-COV-2 PERSISTENCE AND FATE IN THE ENVIRONMENT

1.2.1 SARS-CoV-2 shedding by infected individuals

Once in this body, the virus will be shed from the upper respiratory and gastrointestinal systems into wastewater and the environment, e.g. through faeces and urine, from brushing teeth, mouth washing, coughing and sneezing while bathing or showering, washing of hands or clothes, and discarding tissues and wipes into the toilet. Wu et al. (2020b) found excretion of the SARS-CoV-2 virus after 3 weeks in phlegm and 4 weeks in stools. The same article noted that there was no association between disease severity and the extended duration of the virus in faeces. The presence of gastrointestinal symptoms was not associated with faecal sample viral RNA positivity.

Other researchers have examined clinical specimens from 73 hospitalised patients infected with SARS-CoV-2. Thirty-nine patients tested positive for SARS-CoV-2 RNA in stool samples and 17 of those patients remained positive for SARS-CoV-2 in stools after becoming negative in respiratory samples, suggesting that viral gastrointestinal infection, or at least shedding, can remain for some time after clearance of the virus in the respiratory tract (Xiao et al., 2020a; Xu et al., 2020). This finding has now been repeated in various studies showing the extended duration of shedding of the virus in faecal samples. Sethuraman et al. (2020) reported that in some cases, viral RNA can be detected in stool samples by RT-PCR 6 weeks after the first positive test.

A recent systematic review on the prevalence of gastrointestinal symptoms and SARS-CoV-2 shedding in faeces analysed data from 23 published and 6 preprint studies with a total of 4805 COVID-19 patients (Parasa et al., 2020). Of these patients 7.4% (95% CI, 4.3%-12.2%) reported diarrhoea and 4.6% (95% CI, 2.6%-8%) reported nausea or vomiting. Eight of the included studies reported SARS-CoV-2 in faeces and shedding was detected in 40.5% (95% CI, 27.4%-55.1%) of patients. Pan and colleagues (2020) evaluated SARS-CoV-2 shedding in different clinical specimens and found virus concentrations ranging between 550 copies per mL to 1.21×10^5 copies per mL in stool. Three studies have been able to confirm infectious SARS-CoV-2 in stool from COVID-19 patients (Wang et al., 2020b, Xiao et al., 2020b, Zhang et al., 2020b). SARS-CoV-2 was cultured from the stool of a patient in China around 15 days after the onset of disease (Zhang et al., 2020b).

The virus was detectable by electron microscopy in the inoculated cell cultures. Xiao and co-workers (2020b) inoculated Vero E6 cells with a faecal specimen with Ct values of 23.34 for the 1lab gene and 20.82 for the N gene and could observe cytopathic effect after a second passage. Virus particles with characteristic coronavirus morphology could be visualised by electron microscopy and the complete

genome was determined from the cultured virus, indicating 5 nucleotide difference with the original Wuhan strain (Xiao et al., 2020b). Wang et al. (2020b) detected SARS-CoV-2 in 44/153 COVID-19 patients with a mean Ct value of 31.4 ± 4.2, ranging from 22.3-38.4. These are limited studies on relatively low numbers of patients, but it indicates that SARS-CoV-2 is shed at relatively high titres in the stool of some individuals and public health measures should take this into account.

Epidemiological investigations conducted by Mizumoto et al. (2020) on the Diamond Princess cruise ship suggested that less than 20% of infected people were asymptomatic. Most of the infected people were reported to exhibit moderate nonspecific symptoms including fever, headache, body aches, intense tiredness and/or dry cough. However, infected people can shed SARS-CoV-2 for a few days before the onset of symptoms and for several days after recovery. Another extensive study based on the Iceland population shows that 43% of SARS-CoV-2 positive patients did not report any symptoms (Gudbjartsson et al., 2020). In this context, a clear majority of infected carriers may silently contaminate susceptible people. Therefore, the contamination of raw wastewaters may occur before the significant appearance of clinical cases. Understanding how the disease affects the human body, and how the virus is shed, can give key insights into the virus shedding rates in wastewater and also on the applicability of complementary wastewater-based surveillance techniques for monitoring COVID-19 infections. One important question is how much of this virus is excreted in faeces, given that viruses, including CoVs, are commonly shed in faeces (Wang et al., 2005a; Wang et al., 2005b, WHO, 2011; Mans et al., 2014; Wang et al., 2020b).

1.2.2 SARS-CoV-2 persistence in the environment and susceptibility to disinfection

Chin et al. (2020) noted that the SARS-CoV-2 virus is susceptible to standard disinfection methods and was undetected after 5 minute contact with household bleach (sodium hypochlorite) at various concentrations (1:49 and 1:99 dilution ratios), ethanol (70%), povidone-iodine (7.5%), chloroxylenol (0.05%) and chlorhexidine (0.05%).

Chin et al. (2020) also reviewed the stability of SARS-CoV-2 within the environment by incubating the virus in virus transport medium at various temperatures for up to 14 days and then tested for infectivity. SARS-CoV-2 virus infectivity was also assayed following incubation on different surfaces, exposure to varying pH values and different disinfectants. The authors found that infectivity was still detectable on day 14 when the virus was incubated at 4°C, whereas at 70°C the virus was inactivated in 5 minutes. In the same article, the stability of the virus on various surfaces was tested by dropping the cultured virus onto surfaces left at room temperature (22°C) and a relative humidity of 65%. They found that treated smooth surfaces, particularly steel and plastic, support the persistence of infective virus more than rougher surfaces such as tissue paper, wood and cloth. The virus was stable at a range of pH values (at room temperature).

Van Doremalen et al. (2020) compared SARS-CoV-2 to the 2005 SARS-CoV-1 in terms of viability in aerosols, finding that, like SARS-CoV-1, SARS-CoV-2 also remains viable in aerosols (testing was for 3 h). Although there is limited data on the survival of SARS-CoV-2 in water, because they behave similarly in aerosols, similar behaviour is likely for SARS-CoV-1 and SARS-CoV-2 in water and wastewater. SARS-CoV-1 was predicted to be very stable at 4°C in filtered tap water, and was found to remain live in stools for 6 days at room temperature, with fragments of SARS-CoV-1 being detected in wastewater for up to 3 days, making it less stable in wastewater than poliovirus (Gundy et al., 2009).

1.3 ENVIRONMENTAL SURVEILLANCE OF SARS-COV-2

1.3.1 Overview

Many COVID-19 infections are asymptomatic and unless tested, can remain undetected. As a complementary approach to monitoring the spread of COVID-19, many countries have since implemented wastewater-based surveillance of COVID-19 infections by monitoring the absence and presence and concentration of SARS-CoV-2 viral particles in wastewater and contaminated environmental water sources. Environmental surveillance has also been used and recommended for other infectious disease-causing microorganisms such as typhoid (WHO, 2018), early warning of hepatitis A and norovirus outbreaks (Hellmér et al., 2014), as well as for antimicrobial resistance (Hendriksen et al., 2019), with modelling techniques used to assist both the design and interpretation of those efforts (Wang et al., 2020a, 2020b).

A compartmental epidemiological model developed by Danchin et al. (2020) suggested that contaminated natural water bodies could become environmental reservoirs of SARS-CoVs, which would require the enforcement of strict post-epidemic measures to prevent re-infection. Currently however, the minimal infectious dose (MID) of SARS-CoV-2, that is, the number of viral particles that causes an infection, for humans is unknown (Kitajima et al., 2020) and while SARS-CoV-2 has been detected in sewage and has been described to survive for 14 days in sewage at 4°C, and 2 days at 20°C, no faecal-oral transmission has yet been described for COVID-19. Due to its lipid envelope, it is expected that the new CoV will be less abundant as an infectious virus in wastewater when compared to other known enteric viruses, and less stable when exposed to water treatment processes in water and wastewater treatment plants. In the context of surveillance, the presence or absence of the virus in wastewater is relevant, not due to the potential risk of infection spread, but because of the potential to determine the presence of infected individuals in a community. More research is required to determine the potential for infection due to exposure to untreated wastewater or environmental sources contaminated with untreated wastewater.

1.3.2 Methods for SARS-CoV-2 analysis in environmental samples

In laboratories, identification of SARS-CoV-2 mainly includes viral isolation and viral nucleic acid detection. Accurate detection of SARS-CoV-2 RNA is of notable value. Reverse transcriptase polymerase chain reaction (RT-PCR) assays targeting small regions of the SARS-CoV-2 genome have now been developed and are routinely applied in clinical testing (Corman et al., 2020). The detection of SARS-CoV-2 RNA in untreated domestic wastewater has been reported in Australia (Ahmed et al., 2020a), the Netherlands (Medema et al., 2020), USA (Wu et al., 2020d, Nemudryi et al., 2020, Peccia et al., 2020), France (Wuertzer et al., 2020a; 2020b), China (Zhang et al., 2020a), Israel (Bar-Or et al., 2020), Turkey (Kocamemi et al., 2020), Spain (Randazzo et al., 2020a, 2020b), Italy (La Rosa et al., 2020), and Japan (Haramoto et al., 2020). A review of the methods used is presented below.

1.3.2.1 Virus recovery from wastewater

Due to the stringent biosafety requirements of working with SARS-CoV-2, a model virus with similar structural and morphological characteristics can be used as a surrogate for estimating the recovery efficiency of SARS-CoV-2 concentration methods. A handful of non-human CoVs, porcine epidemic diarrhoea virus (Randazzo et al., 2020b) and avian infectious bronchitis virus (Kocamemi et al., 2020) have been used to estimate human CoV recoveries. Randazzo et al., 2020b found that aluminium flocculation-based concentration methods recovered approximately 11% and 3% of the seeded porcine epidemic diarrhoea virus from untreated and treated wastewater, respectively. These CoV recoveries were similar to the recoveries of the nonenveloped mengovirus, which is often used as a process control for enteric virus detection in environmental samples (da Silva et al., 2007; Sima et al., 2011; Farkas et al., 2018).

Ahmed et al., 2020b evaluated the efficiencies of murine hepatitis virus (MHV) recovery from wastewater using various virus concentration methods previously used to detect SARS-CoV-2 in wastewater (Ahmed et al., 2020a; Medema et al., 2020; Wu et al., 2020b). MHV is an enveloped and positive-sense single-stranded RNA Beta-CoVs, which belongs to the same genus as SARS-CoV-2, and is responsible for a number of diseases in mice and rats (Roth-Cross et al., 2008). The performance of seven virus concentration methods was estimated and compared by seeding MHV in untreated sewage samples, and using RT-qPCR assays to measure MHV concentrations to identify the relative performance of each method for CoV recovery. Methods A, B and C were derived from virus adsorption concentration methods, where samples were passed through 0.45-µm pore-size, 47-mm diameter electronegative membranes via a magnetic filter funnel and filter flask (Ahmed et al., 2015).

Method A began with acidification of sample to pH 4 using 2 N HCl, Method B did not manipulate the sample, only measuring the initial pH (pH = 6.9), Method C began with the addition of $MgCl_2$ to the sample to achieve a final concentration of 25 mM $MgCl_2$. The membrane was immediately inserted into a 2 mL bead beating tube followed by RNA extraction. Methods D and E were ultrafiltration methods using centrifugation. Both methods began with the centrifugation of the sample at 4,500 g for 10 min at 4°C to obtain a pellet. For Method D, the supernatant was concentrated using an Amicon® Ultra-15 (30 K) Centrifugal Filter Devices (Merck Millipore Ltd.), which was centrifuged at 4,750 g for 10 min at 4°C. The concentrated sample (400 µL) was collected from the sample reservoir with a pipette and transferred into a 2 mL-bead beating tube. For Method E, the supernatant was further centrifuged at 3,500 g for 30 min at 4°C through the Centricon Plus-70 centrifugal filter with a molecular weight cut-off of 10 kDa (Merck Millipore). The concentrated sample (300 µL) was collected and mixed with 100 µL of DNase and RNase free water and transferred into a 2 mL-bead beating tube (Ahmed et al., 2020a; Medema et al., 2020).

Method F employed PEG precipitation, which is commonly used to concentrate viruses from water matrices (Mull & Hill, 2012; Gyawali et al., 2019; Wu et al., 2020b). The method started with sample centrifugation at 10,000 g for 20 min at 4°C to remove larger particles and debris. The resulting supernatant was then transferred to a fresh centrifuge tube and stored at 4°C, while MHV was isolated from the pellet. The pellet was re-suspended in beef extract (3% w/v) in 0.05 M glycine (pH 9.0) at a ratio of 1:5. The pellet was agitated on a shaking incubator at 200 rpm for 30 min at room temperature. The pellet suspension was then centrifuged at 10,000 g for 10 min at 4°C and the supernatant was transferred into the centrifuge tube containing supernatant from the initial centrifugation step. The pH of the supernatant mixture was neutralized by the addition of 2 M HCl. PEG 8000 and NaCl were added to the supernatant at ratios of 10% and 2% w/v, respectively. The centrifuge tubes were then incubated at 4°C for 2 h on an orbital shaker set to 120 rpm. Following incubation, the sample was centrifuged at 10,000 g for 30 min at 4°C to obtain a pellet. The supernatant was discarded, and the pellet was resuspended in 800 µL Trizol. Finally, 400 µL of the concentrated sample was transferred to a 2-mL bead beating tube.

Method G used ultracentrifugation, which is frequently used to concentrate viruses from water and wastewater (Fumian et al., 2010; Ammersbach & Bienzle, 2011; Ye et al., 2016). It began with sample centrifugation at 100,000 g for 1 h at 4°C. Supernatant was removed carefully, and the pellet was suspended in 3.5 mL of 0.25 N glycine buffer (pH 9.5). The sample was incubated on ice for 30 min. The sample was neutralized by the addition of 3 mL of 2 × PBS (pH 7.2). The supernatant was clarified by centrifugation (12,000 g for 15 min at 4°C). The virus was recovered by ultracentrifugation at 100,000 g for 1 h at 4°C (Fumian et al., 2010). The pellet was resuspended in 400 µL of 1 × PBS (pH 7.2) and transferred into a 2-mL bead beating tube. The authors found that the mean MHV recoveries ranged from 26.7 to 65.7%. Method C (adsorption-extraction method, supplemented with $MgCl_2$) provided the highest mean MHV recovery of 65.7 ± 23.0%. The second highest mean recovery was for Method B, the adsorption-extraction method without pre-treatment. Method D (Amicon Ultra-15 centrifugal filter device) yielded the third-highest mean recovery (56.0 ± 32.3%) of MHV. While Method E (Centricon Plus-70 ultrafilter centrifugal device) was similar to that of Method D, it produced approximately 50% less recovery

(28.0 ± 9.10%) of MHV from untreated wastewater samples compared to Method D. Method F (PEG precipitation) provided greater recoveries (44.0 ± 27.7%) than Methods A and E, however, it recovered significantly less MHV in comparison to Methods B and C. The authors concluded that PEG precipitation appeared to be a promising approach for MHV concentration because it incorporated the concentration of viruses from both the liquid and solid fractions of wastewater. Ye et al. (2016) reported MHV recovery of approximately 5% using PEG precipitation, which was much lower than the value obtained by Ahmed et al. (2020b), although Ye et al. (2016) only concentrated MHV from the liquid phase. Different versions of PEG precipitation have been used for the assessment of SARS-CoV-2 in sewage, but the efficiencies were not reported (Wu et al., 2020b; Kocamemi et al., 2020; Zhang et al., 2020a; Bar-or et al., 2020).

The virus concentration methods used in these studies to recover SARS-CoV-2 RNA from wastewater included ultrafiltration, polyethylene glycol (PEG) precipitation, ultracentrifugation, and filtration with an electronegative membrane. Rapid, efficient (high recovery), and cost-effective virus concentration methods are needed to monitor SARS-CoV-2 and its nucleic acid in untreated wastewater samples for the successful application of WBE for COVID-19 surveillance.

1.3.2.2 Virus detection and quantification

Accurate estimates of viral concentration in untreated wastewater require that the concentration observed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays be adjusted using the recovery efficiency of a particular combination of virus and concentration method. The concentration methods used in each of the above studies were originally developed for the detection of nonenveloped enteric viruses, such as adenovirus, norovirus, enterovirus in water/wastewater samples. Little is known about the recovery efficiency in wastewater of each method for an enveloped virus such as SARS-CoV-2. The virus concentration recovery efficiencies of SARS-CoV-2 may be different from those of nonenveloped enteric viruses because of significant structural differences between enveloped viruses and nonenveloped enteric viruses. Haramoto et al. (2009) demonstrated differences in virus recovery efficiencies for enveloped and non-enveloped viruses in lake water in Japan. Such discrepancies could lead to large errors of an order of magnitude in the estimated concentration of SARS-CoV-2 in untreated wastewater.

The most widely used methods for quantification of DNA and RNA viruses in wastewater are quantitative PCR (qPCR) and quantitative reverse transcription PCR (RT-qPCR), respectively (Haramoto et al., 2018, Farkas et al., 2020a). These methods detect a small segment of the viral genome, enabling rapid, sensitive and accurate strain-level detection of up to five targets in one assay (Jiang et al., 2014). Several qRT-PCR assays have been designed for the detection of SARS-CoV-2 (Vogels et al., 2020, Corman et al., 2020, Chan et al., 2020 & Nalla et al., 2020) which are suitable for wastewater monitoring. Substantial differences in viral detection rates were observed when different primer/probes were used for quantification. For example, the ‘N2’ assay did not detect SARS-CoV-2 in wastewater samples which were positive for the ‘N1’ and ‘N3’ genes (Medema et al., 2020), hence the use of multiple primer/probe sets is recommended (Farkas et al., 2020b). A limitation of qPCR-based approaches is that the reverse transcription and polymerase enzymes are often inhibited by organic co-contaminants, which are concentrated and extracted together with the targets. Recently, digital PCR (dPCR) -based approaches have also been used for viral detection in environmental samples (Farkas et al., 2020a). These methods enable the absolute quantification of the targets and are less sensitive to inhibition, however more expensive than qPCR-based assays.

1.3.3 Using wastewater based epidemiology for monitoring COVID-19 infections

Wastewater based epidemiology (WBE) is a relatively new environmental concept for determining the exposure of populations to substances of concern, and is based on the analysis of target biomarkers related to that substance of concern in raw wastewater in order to obtain qualitative and quantitative data on the health of communities within a given wastewater catchment. The concept of screening municipal sewage as an epidemiological tool for viruses has been used to help inform broader infectious disease epidemiological surveillance and mitigation efforts such as the Global Polio Eradication Initiative (Hovi et al., 2012, Humayun et al. 2014). Wastewater based epidemiology (WBE) has also been commonly used in the surveillance of licit and illicit drugs and various chemical contaminants which may impact human health (Choi et al., 2018).

SARS-CoV-2 screening in raw sewage water using RT-PCR can be used as a tool to measure the virus circulation in a defined population, for example a city or a smaller municipality feeding to the same wastewater treatment works (WWTW). This was carried out earlier in 2020 in the Netherlands by KWR (an independent knowledge generating entity with the Dutch water companies as its shareholders) (https://www.kwrwater.nl/en/actueel/what-can-we-learn-about-the-corona-virus-through-waste-water-research/). Similarly, Biobot in the US have also initiated sewage testing for SARS-CoV-2 (www.biobot.io). In Australia, a national wastewater monitoring project known as ColoSSoS (Collaboration on Sewage Surveillance of SARS-CoV-2) is being coordinated by Water Research Australia (Water Research Australia, 2020). In Canada, the Canadian Water Network is leading a coalition of municipalities, utilities, researchers, public health organisations and governments supporting public health decisions through wastewater surveillance for COVID-19 (Canadian Water Network, 2020). These projects are linked to a global research effort managed by the US-based Water Research Foundation which is developing a coordinated approach to data collection, method development and data interpretation in order to promote best practices, save resources and accelerate progress on SARS-CoV-2 research in the water sector (Water Research Foundation, 2020).

According to the Water Research Foundation (2020), environmental surveillance has three uses: (i) trend detection (one direction, up- or downward), (ii) changes in trend (two directions) and (iii) assessment of community infection (tracking disease prevalence) (Figure 1-2). While it was felt that current knowledge is sufficient to advance uses (i) and (ii) by supporting decision-making relating to medical and social interventions, the ultimate objective is to use back-calculation methods to assess infection prevalence.

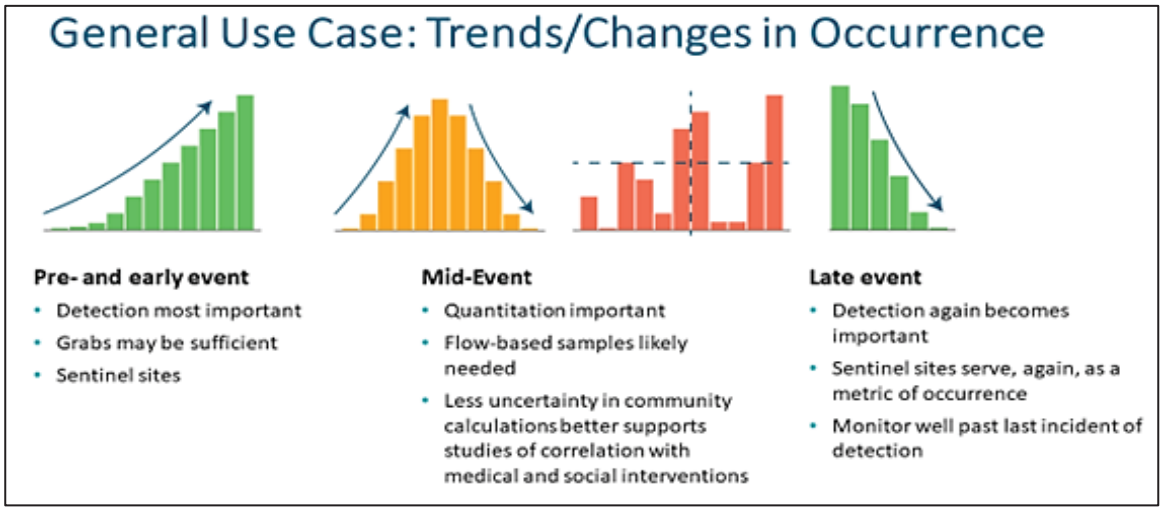


Figure 1-2: General use case: Source Water Research Foundation 2020

For SARS-CoV-2, considerable knowledge still needs to be gathered, especially regarding shedding rates and duration, links between the genetic signal and the infection prevalence and the fate within wastewater and how this changes with wastewater characteristics (e.g. dilution, temperature, retention time, percentage trade waste, etc.) that may vary with time and season. Models, however, may already be very helpful now for uses (i) and (ii) to normalise the genetic signals for spatial (comparing between wastewater catchments) and temporal (seasonality of fate-affecting conditions) variability in order to maximise the power of the signals obtained in supporting COVID-19 management decisions.

1.4 ENVIRONMENTAL SURVEILLANCE OF SARS-COV-2 IN THE SOUTH AFRICAN CONTEXT

The current total picture of SARS-CoV-2 virus circulation in the population of South Africa is incomplete and the number of COVID-19 patients most likely underestimated, mainly due to the limitations regarding testing. Most people who have experienced mild symptoms have not been tested, since testing is mainly (and rightly so) reserved for use in hospitals for patients with serious medical conditions. If the WBE programmes gaining traction internationally can be replicated in South Africa, the water sector will have a tool that provides valuable additional information about the spread of the virus as a complement to health surveillance, but also acting as an early warning system for infection in a community providing a more sensitive and rapid indication of changes in infection rates before such effects become detectable by clinical health surveillance. Critically, this will provide decision support for officials determining the timing and severity of public health interventions to mitigate the overall spread of the disease. When the current peak is over, sewage screening will also be useful to help early detection of re-emergence of the virus. Because of the need to validate sampling and analysis methods in the South African context, this study aims to serve as a short-term, preliminary proof of concept study prior to the roll-out of a pilot and finally a national surveillance programme.

In the studies done in the Netherlands by KWR, SARS-CoV-2 was not found in the effluent of wastewater treatment works, indicating that conventional sewage treatment may be sufficient to reduce the viral load. However, in communities with poorly functioning wastewater treatment plants, or in communities lacking any formal sewerage networks, such as is the case in many regions of South Africa, raw sewage or poorly treated sewage enters our rivers. Rimoldi et al. (2020) surveyed raw and treated samples from three wastewater treatment plants, and two river samples in the Milano Metropolitan Area, Italy, for SARS-CoV-2 RNA presence and infectivity. Positive PCR results were found for raw wastewater samples, while treated water samples were always negative (four and two samples, respectively, sampled on two different days). Samples from receiving rivers (two sites, sampled on the same dates as the wastewaters) showed in some cases a positive PCR result, which the authors attributed to non-treated discharges, or the combined sewage overflows in mixed sewage-stormwater systems. Viral vitality was found to be negligible in the river samples. In Quito (Ecuador) where wastewater is directly discharged into natural waters, SARS-CoV-2 was detected in all samples from three urban river locations (Guerrero-Latorre et al., 2020). Based on these studies, the likelihood of detecting viral particles, whether infectious or not, in South African river systems is therefore very good. It was therefore proposed that, in addition to sampling of wastewater treatment works, South Africa's rivers also be sampled at defined points, particularly where known non-point sources of sewage contamination are occurring as a result of unsewered informal housing communities.

Greywater polluted by sewage in unsewered communities can also be sampled as a potential epidemiological indicator. This may give an early warning of the presence of COVID 19 infections in these communities, where there is the risk of both rapid spread and low likelihood of conventional testing. This will enable deployment of rapid response teams into these areas to conduct more intensive testing and quarantining of infected individuals to curb the spread of the virus. Similarly, in areas where pit latrines are

used instead of sewer networks, sampling of groundwater may give an indication of sewage contamination, and therefore a source of SARS-CoV-2 epidemiological information as well.

Primary sludge samples were used for SARS-CoV-2 analysis and compared with the local hospital admission data and community Covid-19 testing data in New Haven, Connecticut, USA (Peccia et al., 2020). This study uniquely utilized primary sewage sludge (gravity thickened and composed of solids removed during the primary sedimentation step) instead of raw wastewater for virus RNA measurements. Due to the greater solids content of primary sludge (2-5%) in comparison to raw wastewater (0.01 to 0.05%) and the high case load observed during the outbreak (~1,200 per 100,000 population), the concentrations of SARS-CoV-2 RNA reported here ranged from two to three orders of magnitude greater than raw wastewater SARS-CoV-2 values previously reported. Of interest to this study is the comparisons of the hospital admissions and the SARS-CoV-2 RNA concentrations in the sewage sludge which correlated with the COVID 19 testing data when adjusted 7 days forward with a correlation coefficient of R=0.994. Therefore, the SARS-CoV-2 RNA concentrations in sewage sludge were a leading indicator of community outbreak dynamics over hospitalization and compiled COVID-19 testing data. In this study, SARS-CoV-2 RNA concentrations led hospital admissions by 3 days and COVID-19 cases by 7 days. Hospital admissions to Yale New Haven Hospital from the four towns served by the wastewater treatment facility both rose and fell more slowly than the observed RNA concentrations. Raw wastewater and sludge-based surveillance is of value for low- and middle-income countries where clinical testing capacity is limited (Peccia et al., 2020).

Based on the experience in the Netherlands, while the RT-PCR method is not yet quantitative, the concentration level of the virus can be an indicator for the number of virus infections in the population. It could possibly provide an early warning signal in advance of a new outbreak, for instance when a lockdown is lifted. Similarly, these analyses can help monitor the effect of measures put in place to mitigate the spreading of the pandemic.

1.5 AIMS AND OBJECTIVES OF THE STUDY

The main aim of this study is to test the feasibility of applying the WBE and environmental water surveillance concept in South Africa as a tool that provides valuable additional information about the spread of the virus as a complement to health surveillance, and also as an early warning system for infection in a community providing a more sensitive and rapid indication of changes in infection rates before such effects become detectable by clinical health surveillance. Critically, this will provide decision support for officials determining the timing and severity of public health interventions to mitigate the overall spread of the disease. This study serves as a short-term, proof of concept study prior to the roll-out of a national surveillance, and also involves preliminary testing, optimisation and validation of sampling and virus analysis methods, as well as results interpretation and reporting in the South African context.

The specific objectives of the study were as follows:

1. Compile state of knowledge reports on SARS-CoV-2 in water and sanitation environments
2. Testing and validation of a sampling protocol for raw sewage
3. Testing and validation of the virus extraction and analysis protocol
4. Testing and validation of a sampling protocol for surface and groundwater, depending on the success of objective (3) above
5. Development of preliminary methodology for quantification of viral load as an indicator of number of infected individuals in a community
6. Guidance on data analysis/interpretation
7. Recommendations for data communication and integration into national reporting platforms

CHAPTER 2: METHODOLOGY

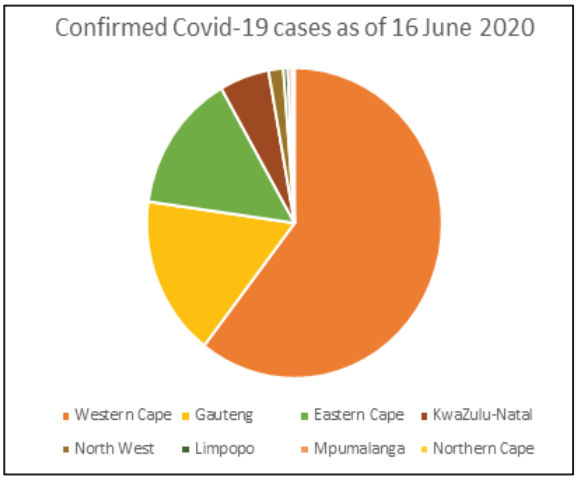
2.1 ETHICS APPROVAL

The study was reviewed and approved by the University of Pretoria (UP) Faculty of Health Sciences Research Ethics Committee (Ethics Reference no.: 374/2020).

2.2 SAMPLING SITES

2.2.1 Selection of sampling sites

Due to this study beings a proof of concept, COVID-19 infection hotspots (based on the number of infections) were selected at the start of the study to give the best chance of finding positives in the samples. According to the NICD and Department of Health National Covid-19 daily report, as of the 16th of June 2020 South Africa had 76 334 confirmed cases of Covid-19 (https://www.nicd.ac.za/diseases-a-z-index/covid-19/surveillance-reports/). The provincial breakdown of these numbers is presented in Figure 2-1. For a more detailed breakdown of case number per province, please refer to Appendix A. The Western Cape had the highest number of confirmed cases at 45 357, followed by Gauteng at 13 023 confirmed cases, the Eastern Cape at 11 039 cases and KwaZulu-Natal at 4 048 cases.



Location	Confirmed Covid-19 cases as of 16 June 2020
Western Cape	45 357
Gauteng	13 023
Eastern Cape	11 039
KwaZulu-Natal	4048
North West	1 281
Limpopo	391
Mpumalanga	343
Northern Cape	211
Unknown	63
Total	**76 334**

Figure 2-1: Provincial breakdown of confirmed Covid-19 cases as of 16 June 2020
https://www.nicd.ac.za/diseases-a-z-index/covid-19/surveillance-reports/

A map of all the sampling sites selected for this study is presented in Figure 2-2, showing a spread of 20 sampling sites located in five provinces representing different sites including; wastewater treatment works, package wastewater treatment works serving industry and mines, hospital, prison and surface water.

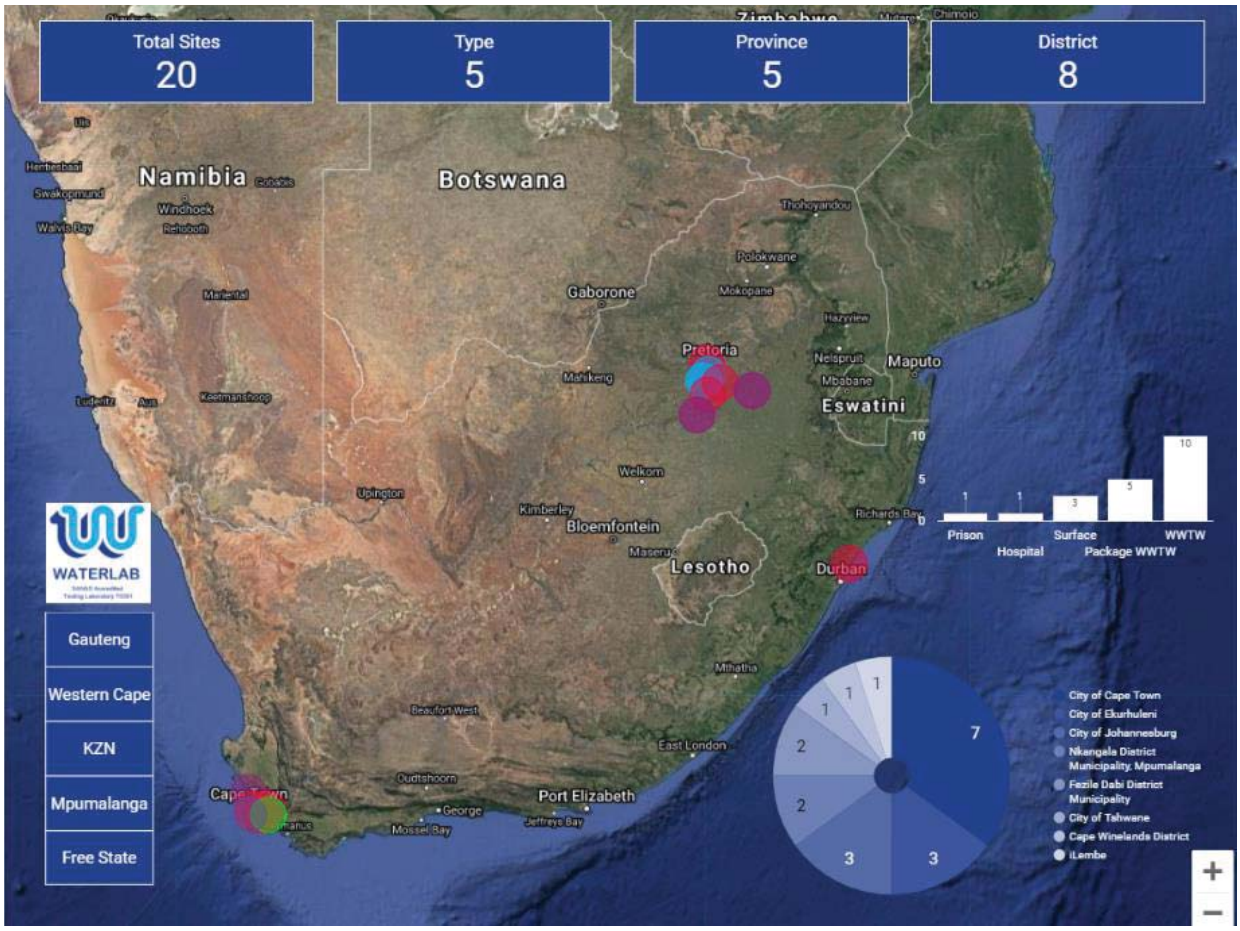


Figure 2-2: Map showing the selected sites for sampling

2.2.2 Western Cape

A total of 8 sites representing 4 types of sampling locations were selected in the Western Cape, as shown in Figure 2-3.

Municipal wastewater treatment works – A total of five WWTWs were selected in the Western Cape, four from the City of Cape Town and one from the Cape Winelands District were selected for sampling. The names of the WWTW selected in the Western Cape were coded as follows:

- City of Cape Town:
  - WC_CCT1(105 Ml/d)
  - WC_CCT2 (200 Ml/d)
  - WC_CCT3 957 Ml/d)
  - WC_CCT4 (72 Ml/d)
- Cape Winelands District
  - WC_STB1 (20 Ml/d)

Other – Additionally, a hospital (WC_HHOS) and 2 community wastewater treatment works each serving a prison (WC_CCT5) and a power generation facility (WC_KPS) were selected as sites.

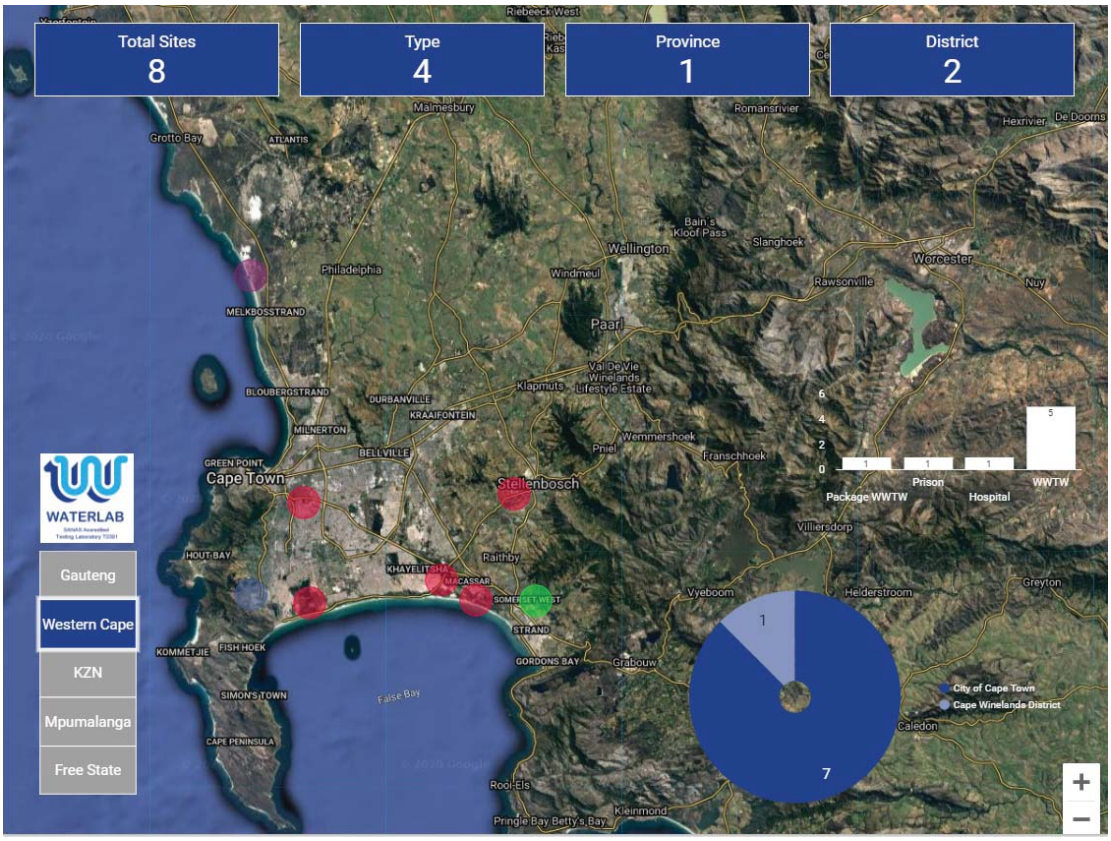


Figure 2-3: Map of sampling locations in the Western Cape by type

2.2.3 Gauteng

A total of 8 sampling sites, representing WWTWs and environmental waters were selected in the Gauteng province (Figure 2-4).

Municipal wastewater treatment works at the City of Ekurhuleni – For the proof of concept study, samples were taken from two WWTWs from the City of Ekurhuleni, operated by ERWAT, for a period of four weeks, and a third plant for the final two weeks of sampling. The first WWTW in the north serves communities and industries in Tembisa, Olifantsfontein and Ivory Park, as well as sections of Kempton Park and Midrand, falling within the City of Ekurhuleni North 1 Sub-District. The second WWTW sampled was located in the south-west in Vosloorus, treating effluent from Boksburg and Vosloorus as well as areas of Tsakane, Duduza and Brakpan. Finally, the third plant to be sampled for the final two weeks was located in the south east of Ekurhuleni, in sub-district Ekurhuleni East 1, treating domestic effluent from Daveyton and Etwatwa. The names of the selected WWTWs were coded as follows:

- City of Ekurhuleni:
  - GP_ERWAT1 (105 Ml/d)
  - GP_ERWAT2 (83 Ml/d)
  - GP_ERWAT3 (2 weeks) (19 Ml/d)

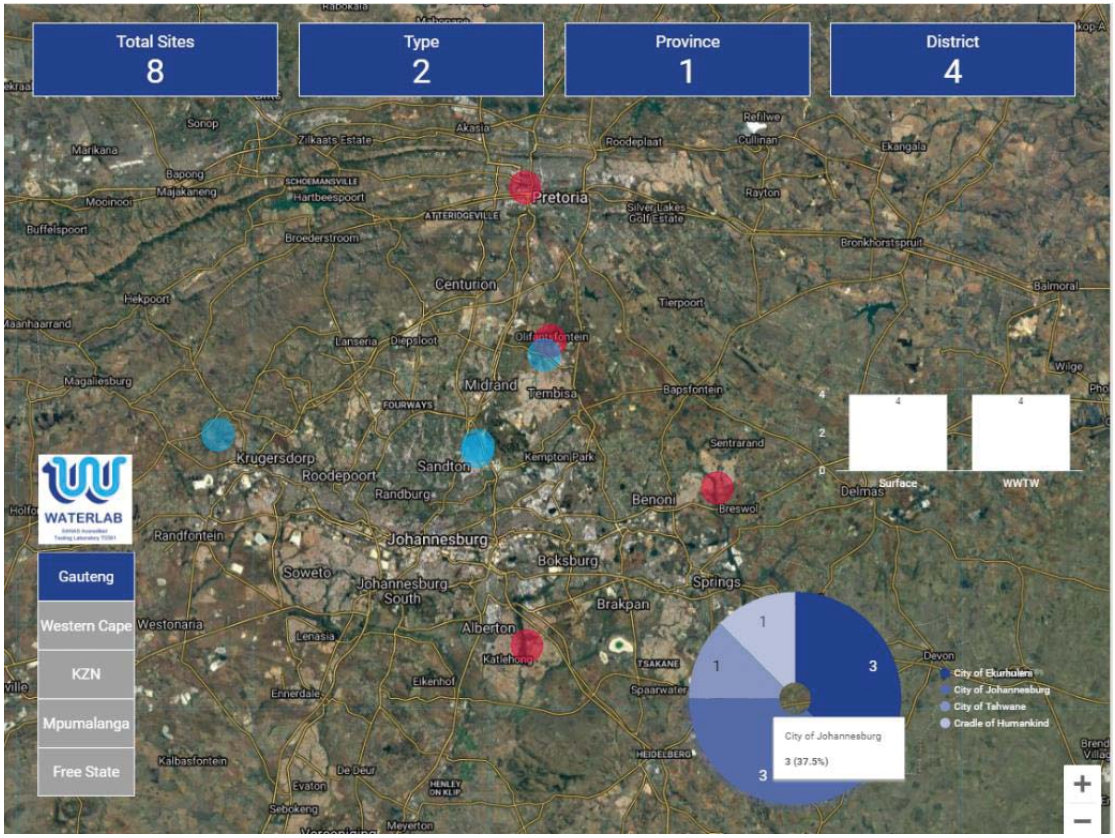


Figure 2-4: Map of sampling locations in Gauteng by type

Municipal wastewater treatment works at the City of Tshwane – A central WWTW that serves the populations located in Tshwane Region 3 was selected for sampling. The name of the selected WWTW was coded as follows:

- City of Tshwane:
    - GP_TSHWN1 (55 Ml/d)

Surface water sampling sites – As an indicator for SARS-CoV-2 prevalence in non-sewered communities, four surface water grab samples were also collected from the Jukskei River downstream of Alexandra informal settlement, the Hennops River downstream of Tembisa informal settlement, as well as the Blougatspruit in the Cradle of Humankind and a surface water runoff sample from an informal settlement in Alexandra. The surface water sampling sites were coded as follows:

- Contaminated greywater runoff sample from Alexandra, Johannesburg (GP_JUKS1)
- River sample from the Jukskei River downstream of Alexandra (GP_ALEXIN)
- River sample from the Blougatspruit in the Cradle of Humankind, downstream of WWTW discharge (GP_COHK)
- River sample from Hennops River downstream of Tembisa, upstream of WWTW discharge (GP_HENN1)

2.2.4 KwaZulu-Natal

The City of eThekwini and the iLembe District of KZN were the province's hotspots, with iLembe, located to the north of eThekwini, showing 0.9% of the total national infections. Only one site, a municipal WWTW from the iLembe District Municipality was selected for sampling (Figure 2-5) and was coded as follows:

- iLembe District (KwaZulu-Natal):
    - KZN_ILEBE1 (12 Ml/d)

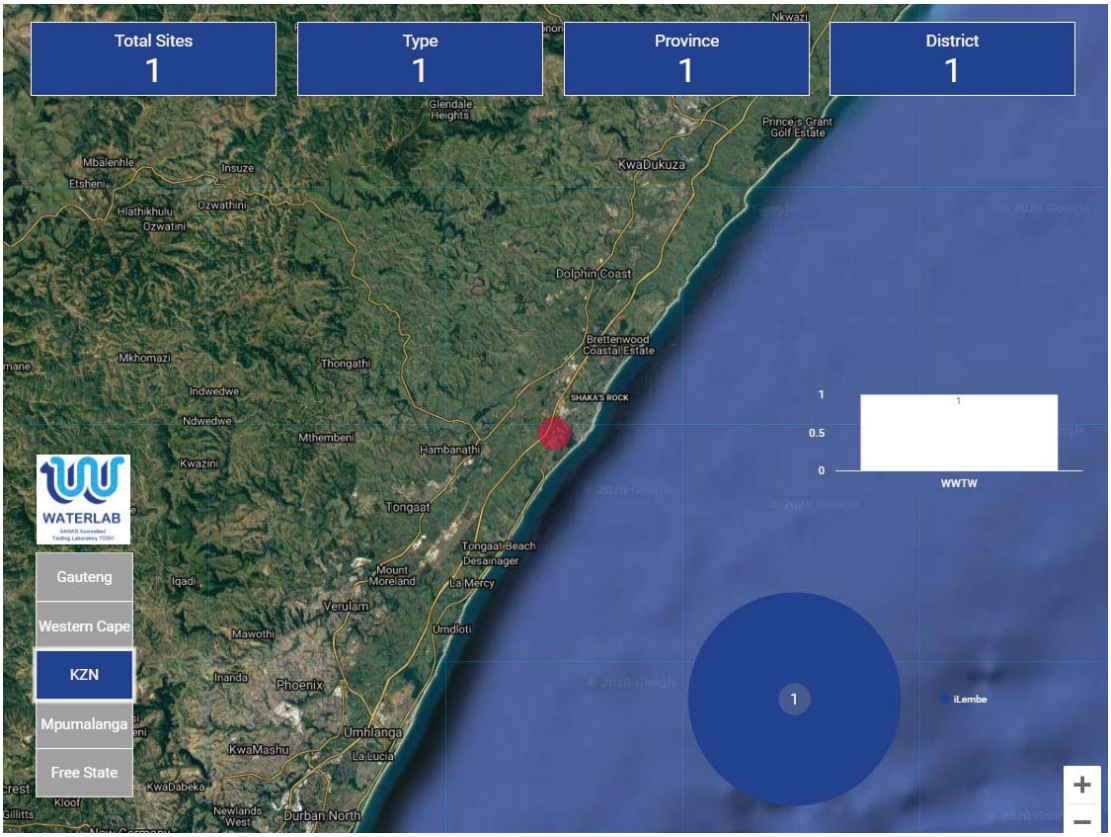


Figure 2-5: Map of sampling locations in KwaZulu-Natal by type

2.2.5 Mpumalanga and Free State

Figures 2-6 and 2-7 show the selected sampling sites in Mpumalanga and Free State provinces. Both locations are package plant WWTWs serving communities within a mine and power generating station. The locations were coded as follows:

- Power stations and mines:
    - MP_MPS1
    - MP_MM1
    - FS_LPS1
    - FS_NVM1

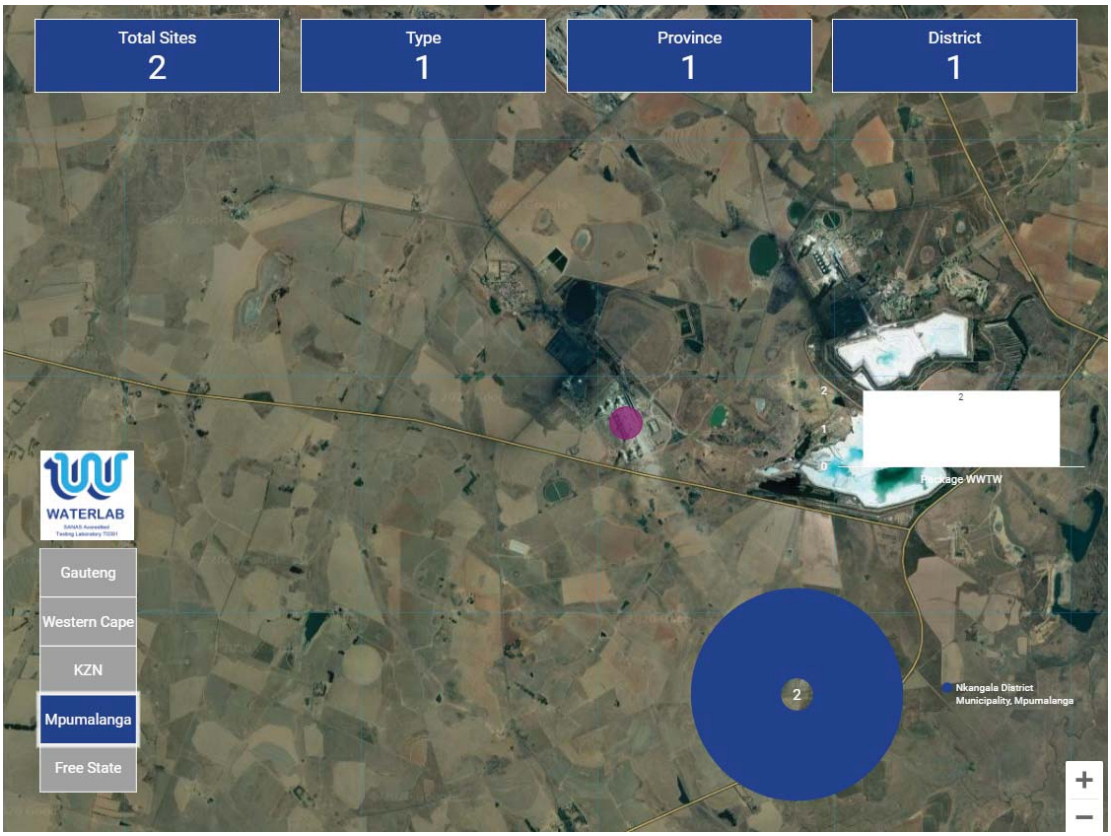


Figure 2-6: Map of sampling locations in Mpumalanga by type

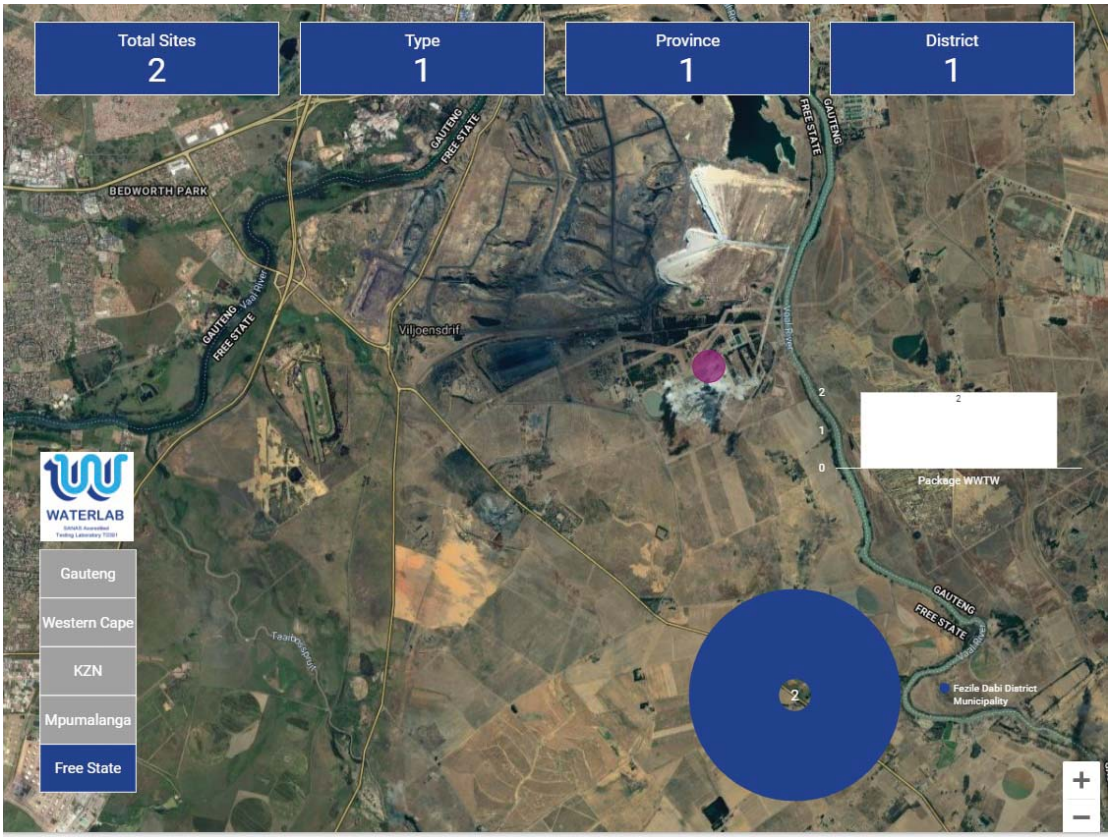


Figure 2-7: Map of sampling locations in Free State by type

2.3 SAMPLING METHODOLOGY

2.3.1 Sampling of wastewater treatment plant influent

In order to demonstrate proof of concept, 1 L 24-hour composite samples were taken from the influent of 9 wastewater treatment works (WWTW) from the City of Ekurhuleni, the City of Tshwane, the Western Cape, and the iLembe District Municipality in KwaZulu-Natal, as identified in the sample site selection process. In addition to the composite samples, 1 L grab samples were taken from three of the WWTW in the City of Ekurhuleni during the morning flow peak at 9am, in order to compare the viral recovery efficiency with the composite samples. Primary sludge grab samples were also tested, with the aim of evaluating the potentially higher virus recovery rate and RNA extraction methodology. Samples were kept cold and delivered to the laboratory on the same day as sampling. Virus recovery was done within 24 hours of delivery of sample to the laboratory.

2.3.2 Sampling of industry sewage package plants, prison and hospital

In addition to the large WWTW, 2 L grab samples were received from three package WWTW serving three Eskom power stations and their associated mines and staff housing developments. These samples were taken during the morning flow peak between 8 and 10am. The benefit of sampling from these WWTW is that they serve a defined population. A 1 L grab sample was also taken from a prison in the City of Cape Town from the sewer manhole downstream of the prison, as well as three 1 L grab samples over a period of 4 weeks from a sewer manhole receiving sewage from the Covid ward of a Hospital in the City of Cape Town that had active cases at the start of sampling. The hospital and prison samples were all recovered using the aluminium hydroxide adsorption-precipitation method, and the Seegene multiplex assay and QuantiFast N1 and N3 assays were used for detection of SARS-Co-V-2 genes in the hospital and prison samples.

A flow diagram indicating the sewage sampling methodology is presented in Figure 2-8.

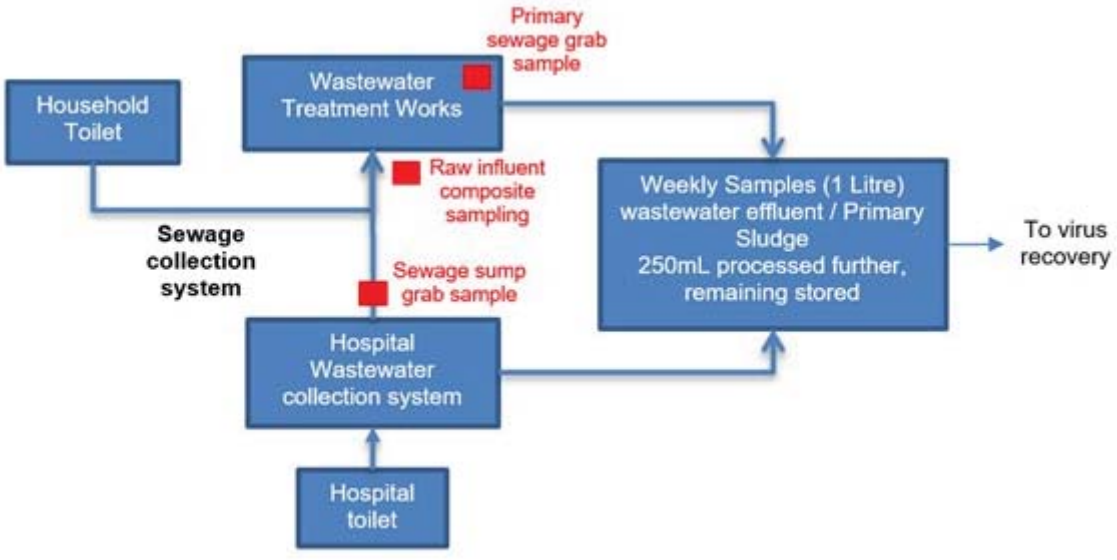


Figure 2-8: Sampling and sample preparation methodology flow chart representation

NOTE: There is some risk of exposure for wastewater treatment plant workers to the aerosol, especially during periods of high incidence of COVID-19 and at low temperatures, but standard PPE is sufficient to prevent infections. SARS CoV-2 remained viable in aerosols throughout a 3-hour experiment, with a

reduction in infectious titre from 10E3.5 to 10E2.7 TCID50 per litre of air. This reduction was similar to that observed with SARS-CoV-1 (van Doremalen et al., 2020). Operators at the WWTW that assisted with sample collection were aware of the risks associated with the handling of raw sewage and appropriate PPE was worn at all times.

2.3.3 Sampling of surface environmental samples

Following proof of concept being demonstrated with the sewage samples, samples were taken from various surface water sources. 10 L of river water was sampled from each source for virus recovery. An additional 1 L of sample was taken for chemical and microbiological analysis. Appropriate PPE was worn during sampling. For the surface water samples, additional analysis was done to indicate the level of untreated sewage contamination in the samples, which included chemical oxygen demand (COD), ammonia, suspended solids, orthophosphates, *E. coli* and total coliforms.

2.4 METHODS FOR SAMPLE PROCESSING AND ANALYSIS

2.4.1 Viral recovery

The SARS-CoV-2 viruses were recovered from the sewage and surface river water samples at two independent laboratories, namely Department of Medical Virology, University of Pretoria (UP) for the samples from Gauteng, KwaZulu-Natal, Mpumalanga and Free State and the CSIR, Natural Resources and the Environment, Stellenbosch, for the samples from the Western Cape.

2.4.1.1 Sample clarification

Samples referred to UP were first clarified prior to viral recovery. The 1-2 L sewage samples were shaken and mixed thoroughly before a 200 mL aliquot was poured off for further processing. The aliquot was clarified by centrifugation (Sorvall® Super T20, du Pont) for 30 minutes at 1180 g at 4°C after which the supernatant was retained for further viral recovery and the pellet saved and stored at -80°C. The 10-20 L surface river water samples were mixed thoroughly by shaking and a 200 mL aliquot was clarified as for the sewage samples. Additional aliquots (1 L and 2 L) we also clarified by centrifugation as described for the 200 mL aliquot except that the pellets were chloroform extracted and the aqueous phase was added back to the supernatants of the 1 L and 2 L samples. Three methods for virus recovery were applied, illustrated in Figure 2-9.

2.4.1.2 Polyethylene glycol 8000/sodium chloride precipitation

The PEG 8000/NaCl precipitation method as described by Falman et al. (2019) was adapted for the study. A total of 16 g PEG 8000 (Amresco, Solon, OH) and 3.6 g NaCl (Merck KGaA, Darmstadt, Germany) was added to 200 mL clarified sewage sample and shaken vigorously for 5 minutes to dissolve the PEG 8000. The sample was the divided into 4 × 50 mL centrifuge tubes and shaken overnight (16-18 hours) at 200 rpm at 4-10°C after which the sample was centrifuged (Sorvall T-20) for 30 minutes at 18500 × g at 4°C. The supernatant was discarded and the precipitate was subjected to a second round of centrifugation at 12 000 rpm for 5 minutes at 4°C after which the remaining supernatant was carefully drawn off with a Pasteur pipette. The final pellet was resuspended in 2 ml inactivated transport medium (ITM) (Nest Biotechnology, Jiangsu, China) or 2 mL PBS pH 7.4 (Sigma-Aldrich, St. Louis, MO). The recovered virus concentrate was aliquoted with 1 mL stored at -20°C until analysis and the remainder stored at -80°C.

2.4.1.3 Skimmed-Milk flocculation

The skimmed-milk flocculation method as described by Falman et al. (2019) was applied to the study using the 5% w/v skimmed-milk solution (Oxoid Ltd., Basingstoke, UK) and 2 hour shaking protocol. 2 mL 5% pre-flocculated skimmed-milk solution was added to 200 mL clarified sewage or river water sample. The pH was adjusted to pH 3.0-4.0 with 1 M hydrochloric acid (Merck) followed by shaking for 2 hours at 200 rpm at room temperature (20-25°C). The sample was then centrifuged (Sorvall T20, du Pont) at 4500 × g for 30 minutes at 4°C, the supernatant carefully removed and for the 200 mL samples the pellet was resuspended in 2 ml ITM (Nest Biotechnology) or 2 mL PBS pH 7.4 (Sigma-Aldrich) while for the 1 L and 2 L river water samples the pellet was resuspended in 10 mL PBS pH 7.4 (Sigma-Aldrich). The recovered virus concentrate was aliquoted with 1 mL stored at -20°C until analysis and the remainder stored at -80°C.

2.4.1.4 Aluminium hydroxide adsorption-precipitation

The aluminium hydroxide method is an adsorption-precipitation method previously described for concentrating enteric viruses from wastewater and effluent water, modified for this study from AAVV, 2011; Randazzo et al., 2019, Randazzo et al., 2020a, Randazzo et al., 2020b. In brief, 200 mL of wastewater samples had the pH adjusted to 6.0 before adding 1 part 0.9 N $AlCl_3$ solution to 100 parts sample and readjusting the pH to 6.0. Samples were mixed using an orbital shaker at 150 rpm for 15 minutes at room temperature. Viruses were concentrated by centrifugation at 1700 × g for 20 minutes and the pellet resuspended in 1 mL Trizol® reagent (Invitrogen Life Technologies, Paisley, UK) and stored at -20°C until nucleic acid extraction took place.

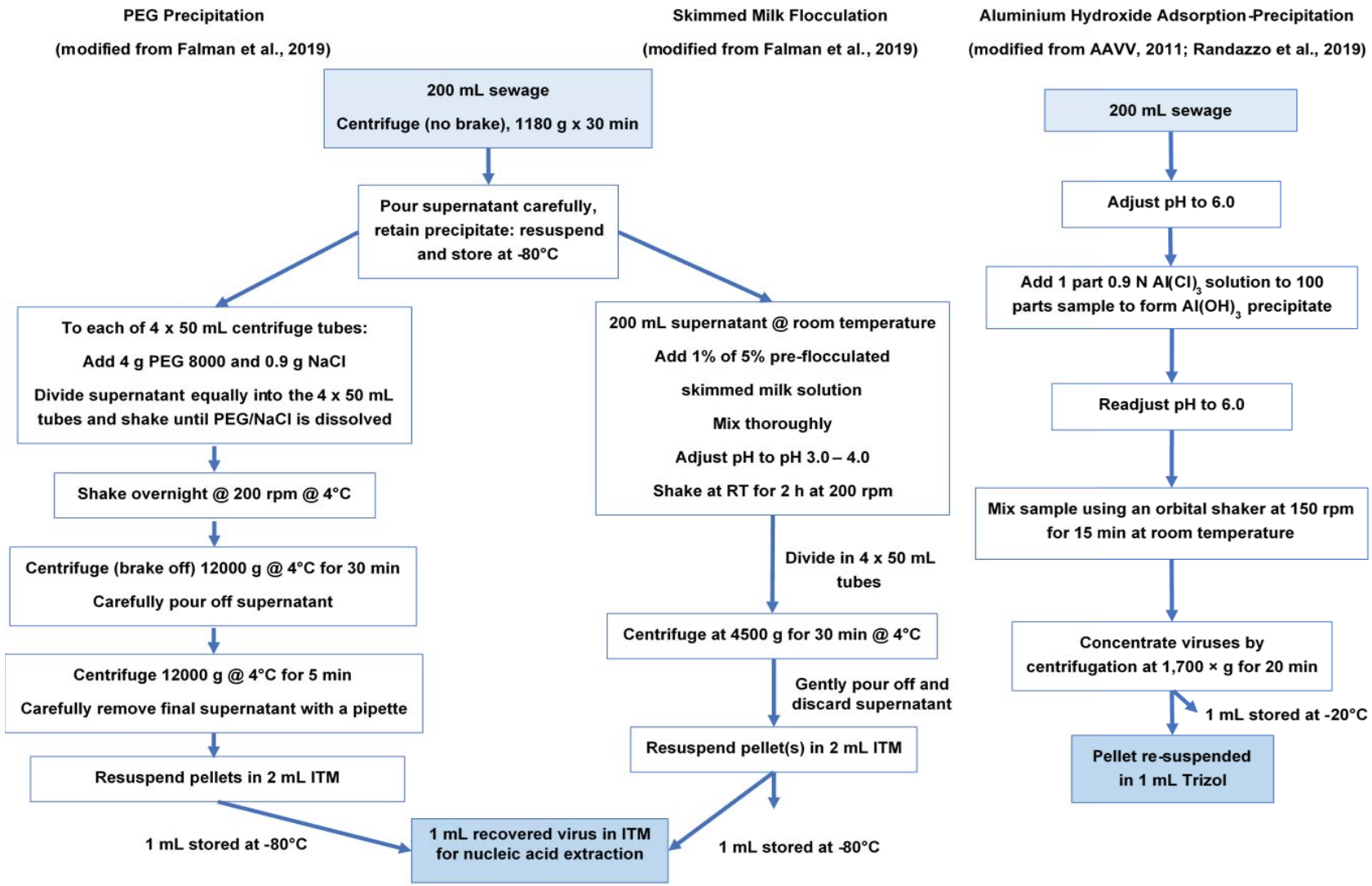


Figure 2-9: Workflow for virus recovery from wastewater samples, comparing the PEG precipitation, skimmed milk flocculation and Aluminium Hydroxide Adsorption-Precipitation methods

2.4.2 Viral detection

A flow diagram of the virus extraction and testing methodology is presented in Figure 2-10.

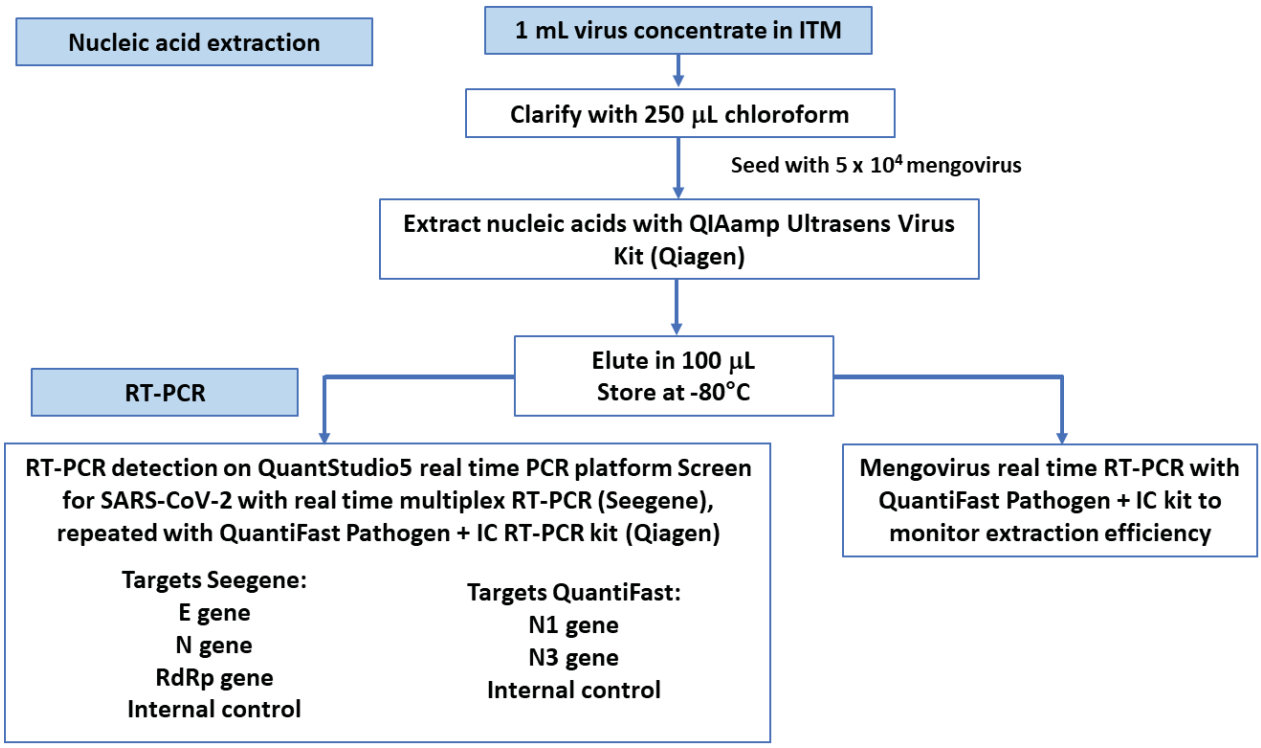


Figure 2-10: Workflow for virus extraction and detection in wastewater samples.

2.4.2.1 Nucleic acid extraction

All samples were pre-treated with chloroform prior to extraction. Chloroform (250 µL) (Merck) was added to 1 mL recovered virus concentrate and the mixture was vortexed 3 × 15 seconds and then incubated at room temperature for 5 minutes before centrifugation at 1500 × g for 10 minutes. The upper phase (~ 1 mL) was transferred to a 2 mL microcentrifuge tube and spiked with 5 × 10^4 mengovirus to enable monitoring of extraction efficiency. Mengovirus strain MC0 was kindly provided by Professor Albert Bosch, Department of Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain. Viral nucleic acids were extracted from the spiked sample using the QIAamp® Ultrasens® Virus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleic acids were eluted in 100 µL buffer AVE and stored at -80°C.

2.4.2.2 Viral detection using the Allplex™ 2019 nCoV assay

The Allplex™ 2019 nCoV assay (Seegene Inc. Seoul, South Korea) was used to detect SARS-CoV-2 RNA in virus concentrates from wastewater samples. The assay targets the envelope (E), nucleocapsid (N) and RNA dependent RNA polymerase (RdRp) genes of SARS-CoV-2 and contains an internal control to monitor inhibition. The RT-PCR reactions were prepared according to the manufacturer's instructions and 8 µL RNA were added to each reaction. The real time RT-PCR was performed on a QuantStudio™ 5 Real Time PCR System (Applied Biosystems, Foster City, CA). The target/reporter combinations were E gene (FAM), N gene (CY5), RdRp gene (ROX) and the internal control (VIC). QuantStudio™ 5 Design and Analysis

Software v 1.5.1 was used to analyse data. Samples with cycle threshold (Ct) values <40 were considered positive. In the event that the internal control amplification failed and no SARS-CoV-2 targets were amplified, the assay was repeated with a 1 in 10 dilution of the nucleic acids.

2.4.2.3 Viral detection using the QuantiFast® Pathogen RT-PCR + IC N1 and N3 assays

Singleplex RT-PCR assays with N1 or N3-specific primer/probe sets (Table 1) and the QuantiFast® Pathogen RT-PCR + IC kit (Qiagen) were used to detect the SARS-CoV-2 nucleocapsid gene. The primers and probes were based on assays developed by the CDC (CDC, 2020) and applied by Medema and colleagues (Medema et al., 2020). The reaction mix consisted of 1 × QuantiFast® Pathogen Master Mix, 400 nM forward and reverse N1 or N3 primers, 160 nM N1 or N3 probes, 1 × Internal Control Assay mix, 1 × Internal Control RNA and 0,25 µL QuantiFast® Pathogen RT mix in 20 µL. Five microlitres of RNA were added to the reaction mix and the one step RT-PCR reaction was performed with the following protocol: Reverse transcription for 20 minutes at 50°C, enzyme activation for 5 minutes at 95°C and 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. Fluorescence was recorded during the annealing/extension step. Samples with a cycle threshold (Ct) value of <40 were considered positive.

2.4.2.4 Mengovirus QuantiFast® Pathogen RT-PCR + IC assay

Mengovirus was detected in each sample to determine nucleic acid extraction efficiency. Published primers and probe (Table 2-1) (Pinto et al., 2009) were used with the QuantiFast® Pathogen RT-PCR + IC kit (Qiagen). The reaction mix consisted of 1 × QuantiFast® Pathogen Master Mix, 400 nM Mengo110F and Mengo209R primers, 160 nM Mengo147 probe, 1 × Internal Control Assay mix, 1 × Internal Control RNA and 0,25 µL QuantiFast® Pathogen RT mix in 20 µL. Five microlitres of RNA were added to the reaction mix and the one step RT-PCR reaction was performed with the following protocol: Reverse transcription for 20 minutes at 50°C, enzyme activation for 5 minutes at 95°C and 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 65°C for 30 seconds. Fluorescence was recorded during the extension step. Samples with a cycle threshold (Ct) value of <40 were considered positive.

Table 2-1: Primers and probes for SARS-CoV-2 and mengovirus detection

Assay	Target gene	Primer/Probe	Sequence	Reference
SARS-CoV-2 N1	Nucleocapsid (N)	2019-nCoV_N1-F 2019-nCoV_N1-R 2019-nCoV_N1-P	5'-GACCCCAAAATCAGCGAAAT-3' 5'-TCTGGTTACTGCCAGTTGAATCTG-3' 5'-FAM-ACCCCGCAT/abNFQ/TACGTTTGGTGGACC-NFQ-3'*	Medema et al., 2020
SARS-CoV-2 N3	Nucleocapsid (N)	2019-nCoV_N3-F 2019-nCoV_N3-R 2019-nCoV_N3-P	5'-GGGAGCCTTGAATACACCAAAA-3' 5'-TGTAGCACGATTGCAGCATTG-3' 5'-FAM AYCACATTG/abNFQ/GCACCCGCAATCCTG-NFQ -3'	Medema et al., 2020
Mengo		Mengo110F Mengo209R Mengo147	5'-GCGGGTCCTGCCGAAAGT-3' 5'-GAAGTAACATATAGACAGACGCACAC -3' *5' MGB*-ATCACATTACTGGCCGAAGC-*TAMRA-3'*	Pinto et al., 2009

*abNFQ – an abasic non fluorescent quencher placed internally between the 9th and 10th bases from the 5' end; NFQ – non fluorescent quencher at 3'end.

2.4.3 Viral quantification

The SARS-CoV-2 genome copies (GC) per reaction for each tested sample was calculated based on the N1 or N3 standard curves and then further converted to gc/mL. Nucleic acid extraction efficiency was assessed using mengovirus. The mengovirus TCID50 titre representing 100% extraction efficiency was determined from 5×10^4 mengovirus spiked in 1 mL PBS and extracted with the QIAamp Ultrasens kit (Qiagen). The mengovirus in each sample was quantified based on the TCID50 standard curve and extraction efficiency was calculated. [TCID50 copies test sample/TCID50 copies PBS control)*100]. The GC/mL in the SARS-CoV-2 positive samples were then adjusted based on the percentage extraction efficiency for each reaction.

2.4.4 Construction of standard curves

2.4.4.1 Mengovirus

Three types of raw sewage samples (early grab, late grab and 24 h composite) were collected in the week of 8 June 2020, in order to validate the virus recovery method using mengovirus. Mengovirus is a small non-enveloped virus that is used as process control for virus recovery from environmental samples. In order to determine the recovery rate of mengovirus from different wastewater samples the wastewater was spiked with 2.8×10^6 TCID50 mengovirus either before the first clarification step (composite S) or after clarification (composite, early and late grab samples). The viruses were recovered with PEG8000/NaCl precipitation as detailed in Section 2.4.1.2. Mengovirus with a TCID50 titre of 1.4×10^6 was used to generate a standard curve in order to quantify the mengovirus. Serial ten-fold dilutions of the cell culture stock were run in triplicate in the QuantiFast® Pathogen RT-PCR + IC assay and the QuantStudio™ 5 Design and Analysis Software v 1.5.1 was used to generate a standard curve.

2.4.4.2 SARS-CoV-2 N1 and N3

Standard curves were constructed using the 2019_nCoV_N positive control plasmid (Integrated DNA Technologies, Inc, Coralville, IA) which is provided at a concentration of 200 000 gc/μL. The plasmid was diluted to 100 000 copies/μL and a serial ten-fold dilution was prepared. A standard curve was generated in triplicate at 6 dilutions for the QuantiFast N1 and QuantiFast N3 assays as described in 2.4.3. QuantStudio™ 5 Design and Analysis Software v 1.5.1 was used to generate the standard curves.

2.5 DATA INTERPRETATION AND VISUALIZATION

Ct values were used as a valuable tool for determining the presence and absence of SARS-CoV-2 RNA in samples, and for establishing trends in viral load and identifying either new occurrences in areas previously unaffected, or for early warning of second waves of infection. Ct values below 40 were considered positive.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 SARS-COV-2 ANALYSIS IN MUNICIPAL WASTEWATER SAMPLES

3.1.1 Determining viral extraction efficiency using Mengovirus recovery from wastewater

The mengovirus amplification curves at each dilution (1.4×10^5 to 0.0014) and the resulting standard curve are shown in Figure 3-1 A and B. The quantification results of the recovery experiment are summarised in Table 3-1. The mengovirus recovery ranged between 0.49% and 8.4%. The 24 h composite sample, spiked after clarification gave the highest recovery of 8.4%.

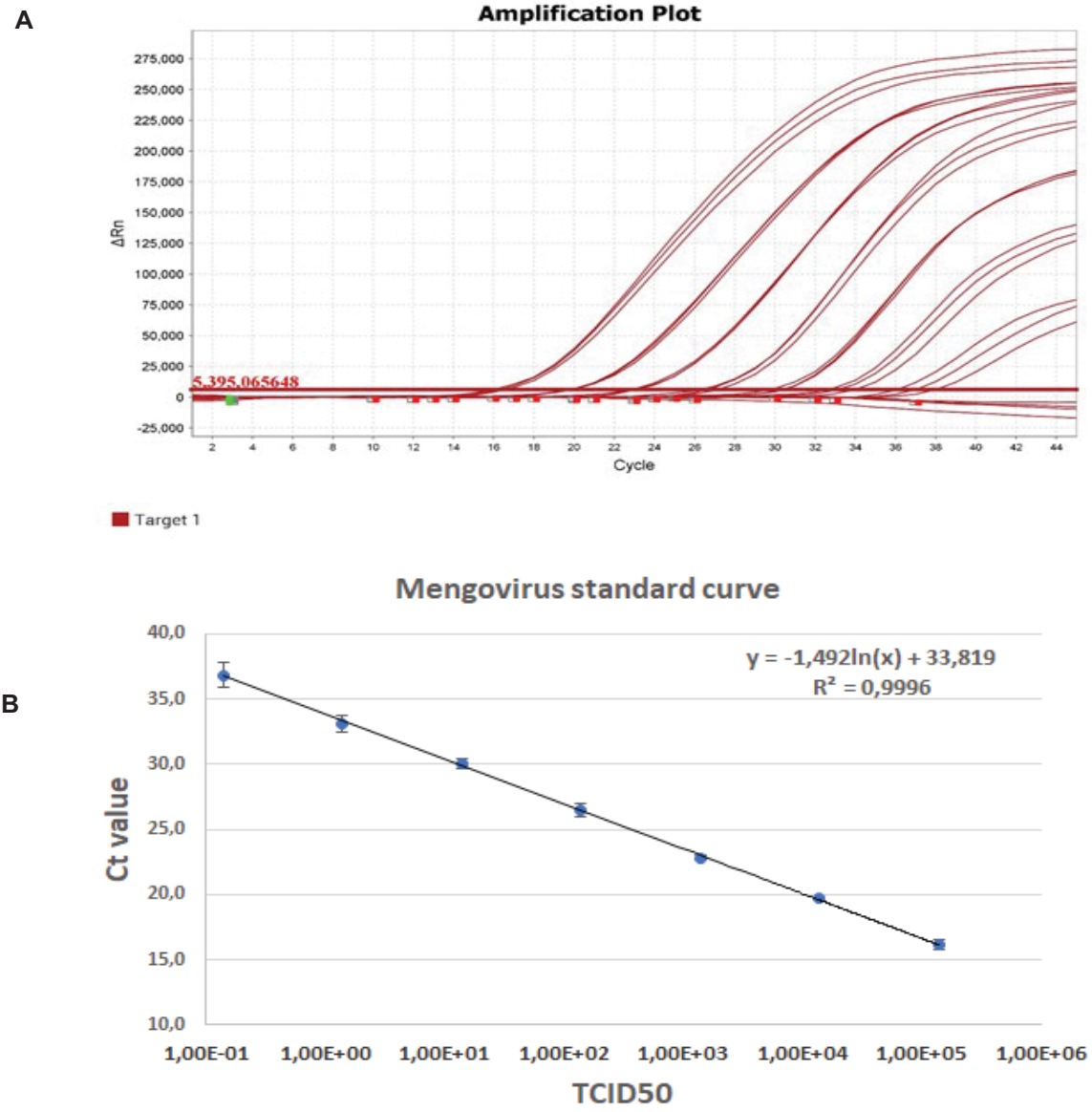


Figure 3-1: A) Real time RT-PCR amplification curves of 10-fold serial dilutions of mengovirus RNA starting at 1.4×10^5. B) Standard curve generated from the dilution series shown in A. The standard curve ranges from Ct 16.1 (140 000 TCID50) to Ct 36.9 (0.014 TCID50).

Table 3-1: Mengovirus recovery efficiency from grab and composite raw wastewater samples.

Sample type	Early grab	Late grab	24 h composite	24 h composite
Theoretical mengovirus concentration in each spiked sample based on cell culture TCID50*	2.8×10^6	2.8×10^6	2.8×10^6	2.8×10^6
Step at which sample was spiked	After clarification	After clarification	After clarification	Before clarification
Actual mengovirus TCID50# before clarification	NA	NA	NA	933 500,0
Actual mengovirus TCID50 in 200 mL before PEG precipitation	3 718 488,0	1 050 800,0	753 600,0	1 098 355,5
Mengovirus TCID50 in supernatant after PEG precipitation (discarded)	102 677,1	205 167,7	215 475,0	690 900,1
Mengovirus TCID50 in final concentrate (2 mL)	18 256,0	62 768,0	61 980,0	39 552,0
% Recovery	**0,49**	**5,97**	**8,4**	**3,6**

*TCID50 = median tissue culture infectious dose 50
TCID50 calculated from comparative standard curve linking Ct to TCID50

The internal control (IC) that forms part of the QuantiFast Pathogen + IC RT-PCR kit was not significantly inhibited by the RNA extracted from the various sewage samples. Figure 3-2 shows the amplification of the internal control in reactions with RNA extracted at different stages of the recovery experiment well as those in the final recovered virus concentrate RNA. Internal Control Ct values in control reactions (No template control, extraction negative control and positive standards) ranged from 30.7 to 31.6 with a median of 30.98 whereas the IC Ct values in reactions with sewage derived RNA ranged from 30.5 to 32.2 with a median of 31.5. Thus, no significant inhibition was observed due to the sewage samples when 5 uL of RNA was used in the detection assay.

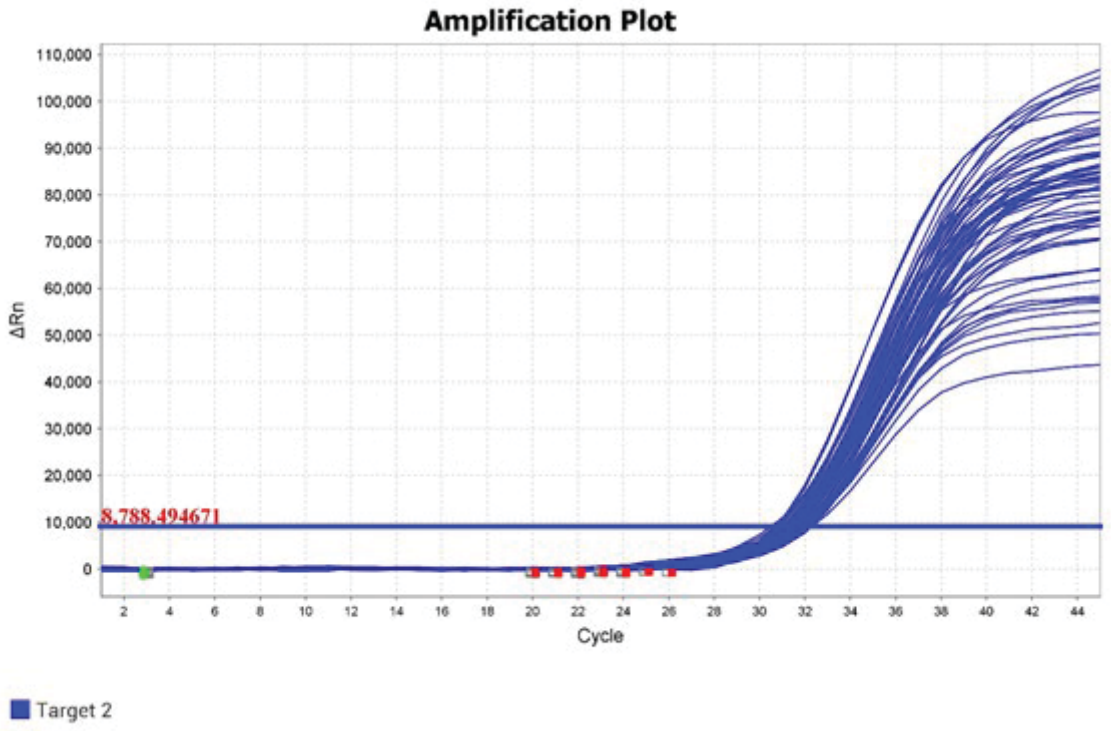


Figure 3-2: Internal control amplification during 60 RT-PCR reactions in the mengovirus recovery experiment

3.1.2 SARS-CoV-2 recovery using PEG precipitation and skimmed milk methods

Results show proof of concept in terms of both virus recovery methods and positive gene amplification of SARS-CoV-2 at all nine WWTW sampled. Results of 24-hour composites and grab samples taken during morning peak flow, using the PEG precipitation recovery method, are presented in Figure 3-3. SARS-CoV-2 was detected at all sites tested, and in all samples (Ct values below 40 for at least one target), but not all targets were positive. Initially, better virus recovery was found in composite wastewater samples when compared to grab samples taken during peak flow times. However, by weeks 2 and 3 comparable results were found for the grab and composite samples. This is possibly due to an increase in the viral load over time. By week 4, the grab samples from the City of Ekurhuleni were giving more consistent results. Two primary sludge samples from both the GP_ERWAT2 WWTW and from GP_TSHWN1 WWTW were analysed. Extractions were performed on 50% and 10% dilutions of the sludge samples. The GP_ERWAT2 samples were completely inhibited at both extraction dilutions. The GP_TSHWN1 50% sample was inhibited but the 10% sample tested positive for the N (Ct=36.2) and RdRp (Ct=35) targets. The internal control amplified very late (Ct=38.3) indicating that there was still considerable inhibition in these samples. Due to the strong likelihood of inhibition no further primary sludge samples were analysed.

A comparison between the results achieved with the PEG precipitation recovery method when compared with the skimmed milk flocculation method are presented in Figure 3-4. The success of the skimmed milk recovery method was variable between different sites. In the week 1 samples it clearly improved recovery from the GP_ERWAT1 and GP_TSHWN1 samples. However, the PEG recovery method performed better for the samples from KZN_ILEBE1 for weeks 1-3, as well as the samples from GP_ERWAT3 sampled in week 3. In week 4 the skimmed milk method improved recovery at several of the sites, notably GP_ERWAT1, KZN_ILEBE1 and GP_ERWAT3.

Figure 3-3: SARS-CoV-2 gene amplification and internal control (IC) for composite and grab samples for weeks 1-4, with PEG recovery. Ct value of 40 and below were considered positive

The effect of the skimmed milk method on recovery therefore seems to be quite variable. This may be due to differing inhibitors present in the wastewater received at the various sites. Both methods were successful, and the skimmed milk method could be used interchangeably or even preferentially to the PEG method. As a cheaper and faster method to employ this is advantageous.

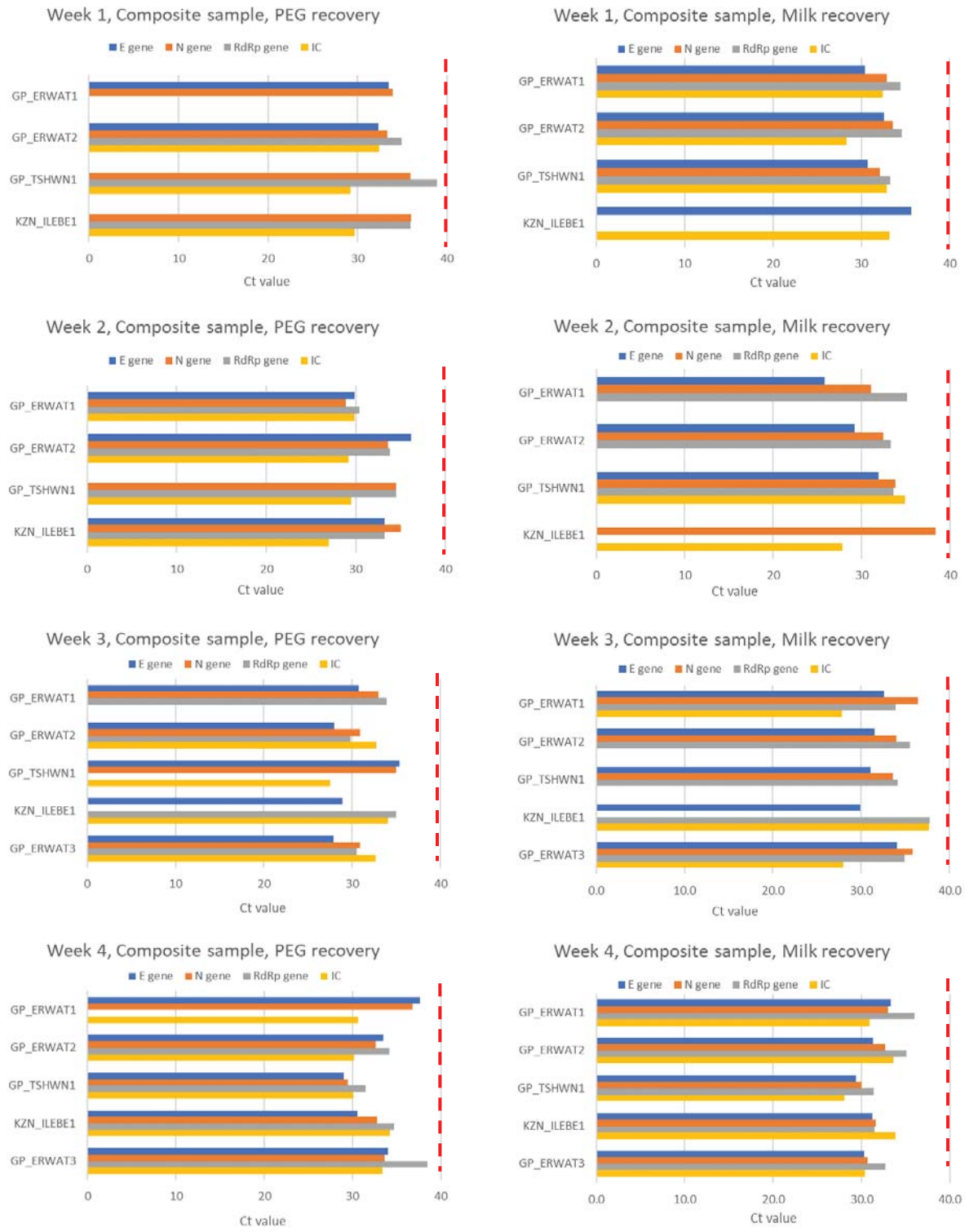


Figure 3-4: SARS-CoV-2 gene amplification and internal control (IC) for weeks 1-4, comparing PEG and skimmed milk recovery. Ct value of 40 and below were considered positive

3.1.3 SARS-CoV-2 detection in wastewater

A comparison of the Quantifast N1 and N3 assays with the Seegene 2019 nCoV assay is illustrated with samples from 5 WWTW in the Western Cape (Figure 3-5). Virus extraction for these samples was done with the aluminium hydroxide flocculation-precipitation method. 13/20 samples were positive with all three assays, 5/20 were detected by two assays, 1/20 was detected by one assay and 1/20 was negative by all assays. The same extraction methods were not used for all sites, with PEG precipitation and skimmed milk flocculation recovery methods being applied to the samples from Ekurhuleni, Tshwane and iLembe, and the aluminium hydroxide method being applied for samples from the Western Cape. However, the Seegene and Quantifast N1 and N3 assays were all applied to all samples. It is therefore useful to compare all the sample results by recovery method, suspension media and assay.

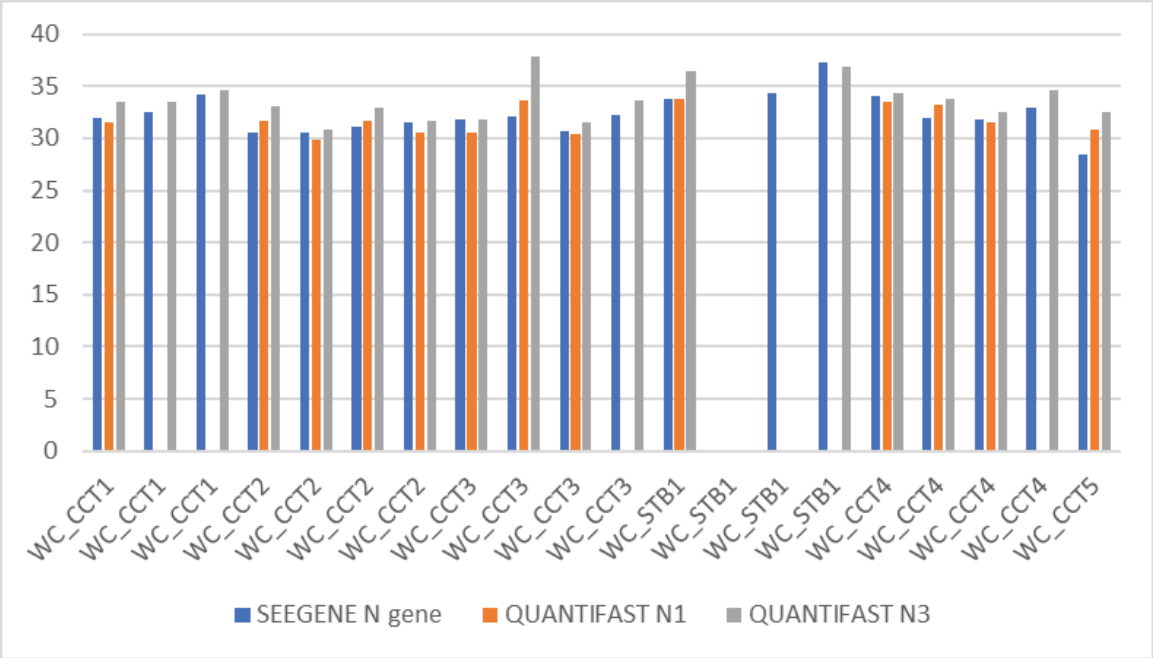


Figure 3-5: A comparison of the Quantifast N1 and N3 assays with the Seegene 2019 nCoV assay for Western Cape samples

Figure 3-6 shows a comparison of 56 different samples of either grab or composite sewage influent for 86 RT-PCR assays. For the WWTW sewage influent, the Seegene N (95%) and QuantiFast N3 (96.5%) detected most consistently. 58% of samples came up positive for all 5 targets, 21% were positive for 4 targets, and 13% of samples were positive for 3 targets.

A summary of assay results for all samples tested is presented in Figure 3-7. When including all samples, the Quantifast N3 still detected the most consistently, followed by the Seegene E and Seegene N.

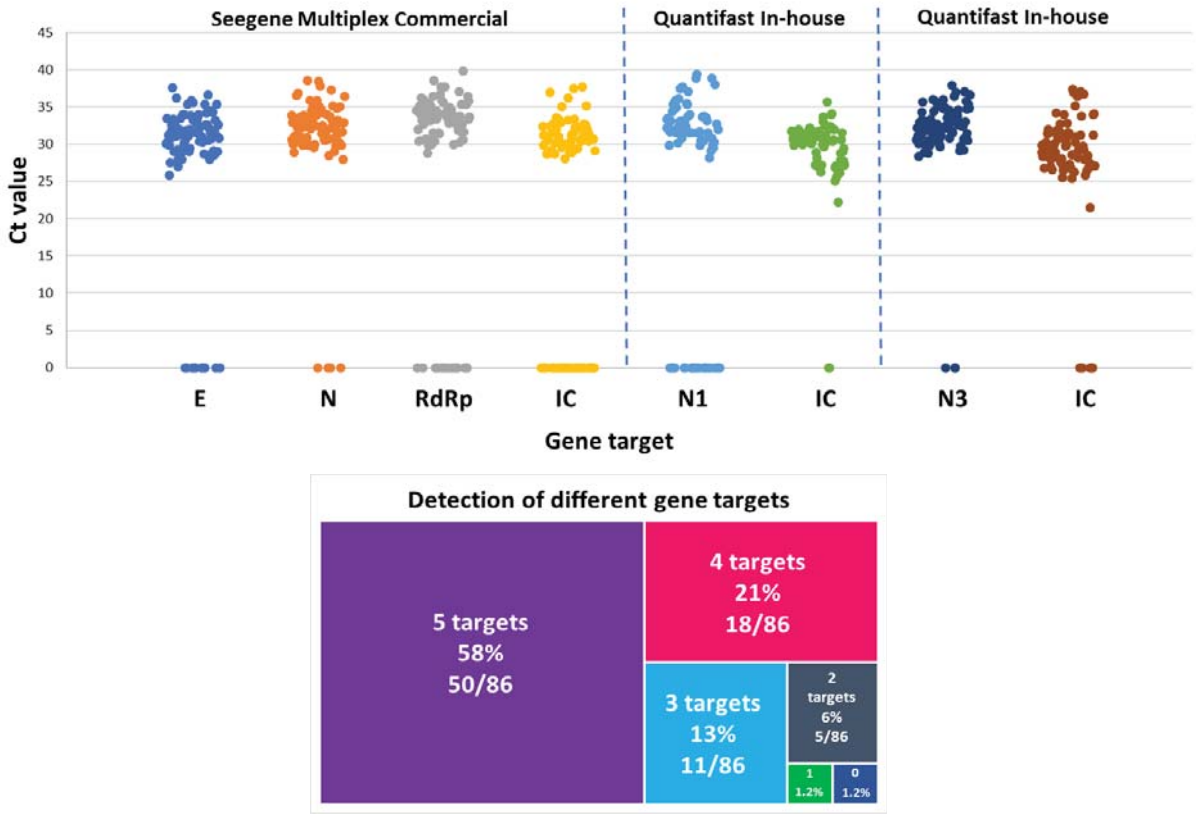


Figure 3-6: Comparison of Ct values for sewage influent samples per assay method

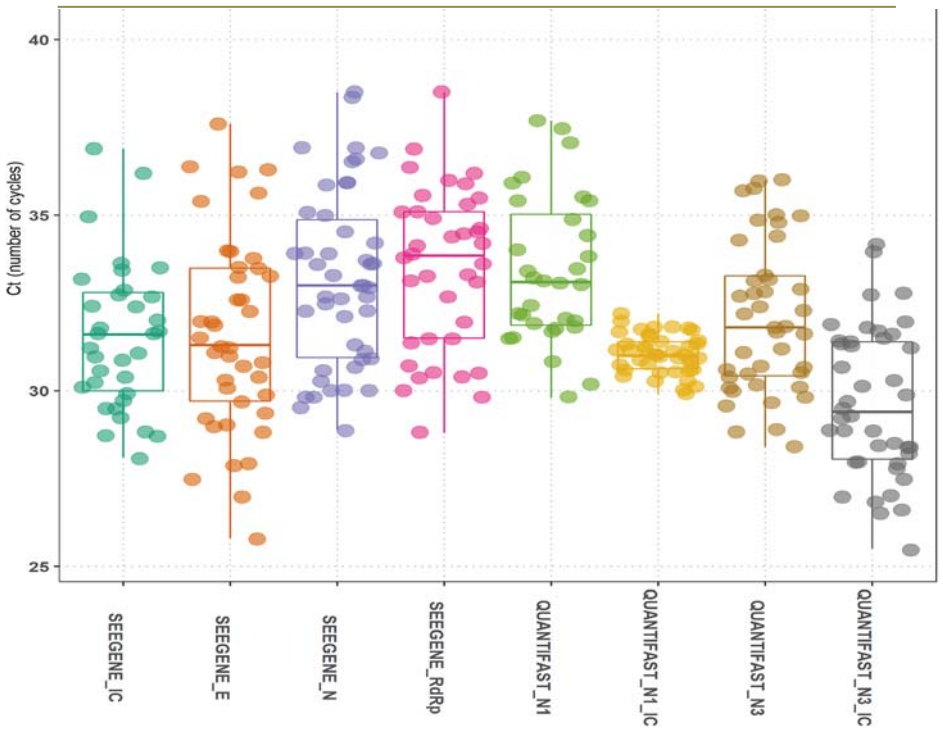


Figure 3-7: Comparison of Ct values for all samples per assay method

Figure 3-8 shows a comparison of Ct values by recovery method and assay. While the three methods cannot be directly compared as they were applied to different sites, it can be seen that all three methods were effective in the recovery of the SARS-CoV-2 virus. As the PEG and skimmed milk methods were applied to the same sites, these can be more directly compared. It can be seen that there is much variability between targets, with some showing a narrower spread and lower Ct mean for skimmed milk, and others for PEG. It appears that the methods can be used interchangeably between laboratories, but because of inherent variability it is recommended that the same method be applied to the same site when monitoring trends over time. The skimmed milk method and aluminium hydroxide adsorption-precipitation methods are preferred, as they are both faster and cheaper than the PEG method, and only require low speed centrifugation.

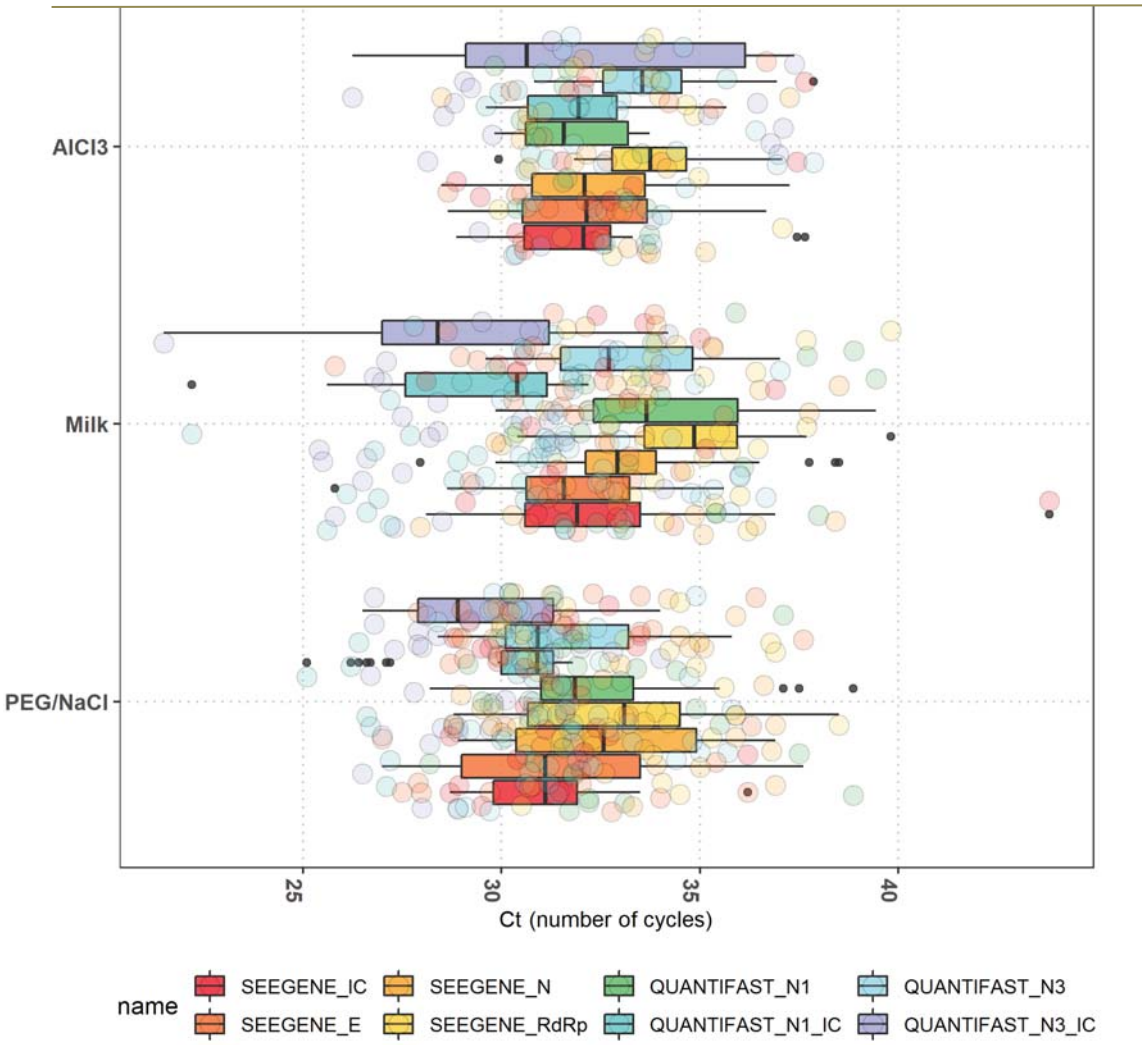


Figure 3-8: Comparison of Ct values by recovery method and assay

A comparison between the resuspension/inactivation reagents and their impact on the assays is presented in Figure 3-9. The samples from the Western Cape were re-suspended and inactivated in Trizol; the remaining samples were either inactivated in ITM or PBS for comparison. It was possible to extract and amplify the virus from all three suspension media.

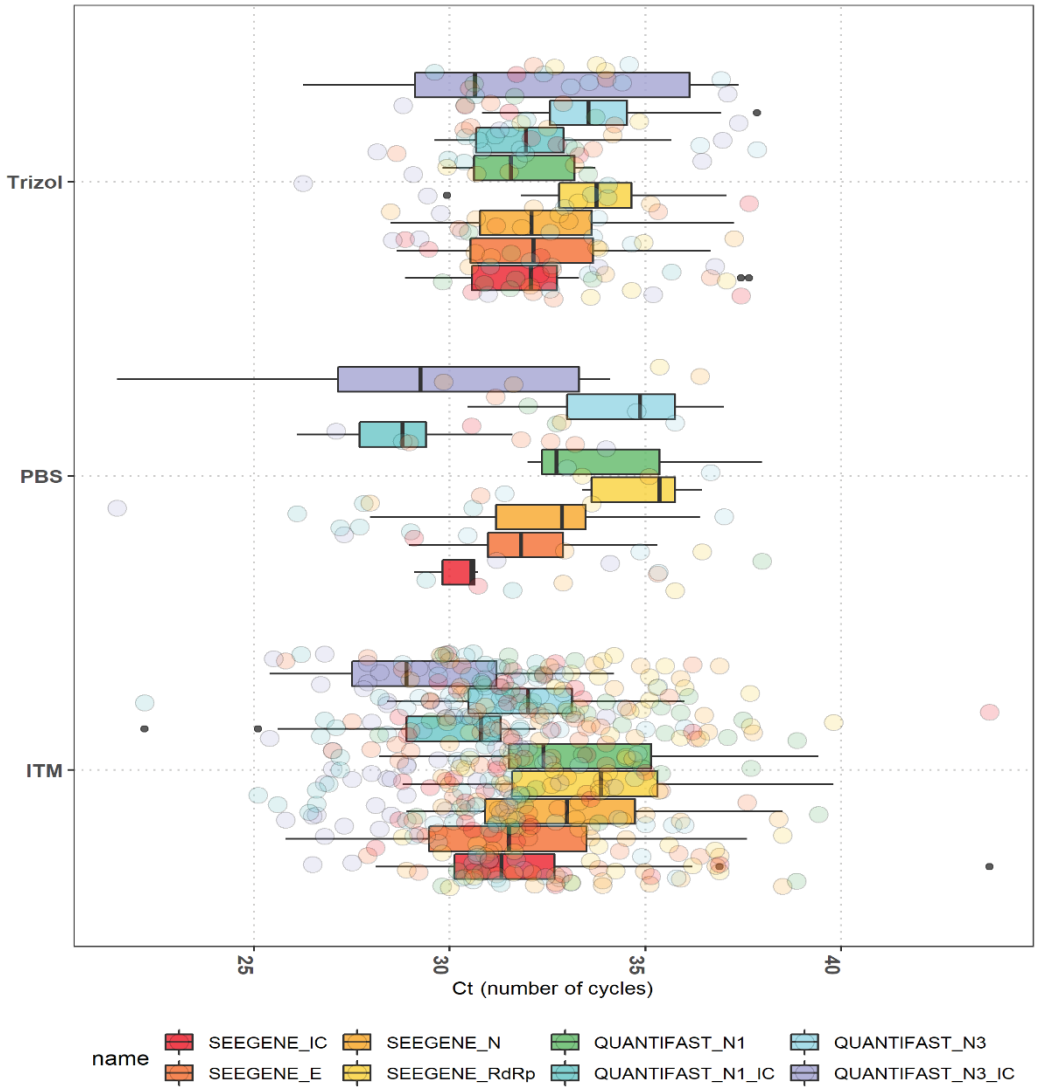


Figure 3-9: Comparison of Ct values by inactivation/ re-suspension method and assay method

3.1.4 Trends in Ct values

The Ct values for the N gene assay for samples recovered with PEG from the Gauteng WWTW (the City of Ekurhuleni and City of Tshwane) are presented Figure 3-10, including initial grab and composite test samples for GP_ERWAT1 and GP_ERWAT2. The Ct trends were overlaid with active case numbers in the province for the period of sampling to indicate the stage of the pandemic. There was a downward trend in the Ct values for most samples for the first three weeks, except for week 3 for GP_ERWAT1, which appeared to indicate an increasing viral load in the samples, corresponding to the increase in case numbers in the province as the peak infection phase of the pandemic was entered. Week 4 showed an increase in Ct values for all sites as the case numbers started to plateau, with the exception of the grab sample from the GP_ERWAT1, and GP_TSHWN1. This may be as a result of less optimal recovery and is indicative of the impacts of the complex matrix. Figure 3-11 shows only the results from the City of Ekurhuleni, overlaid with daily increase in cases.

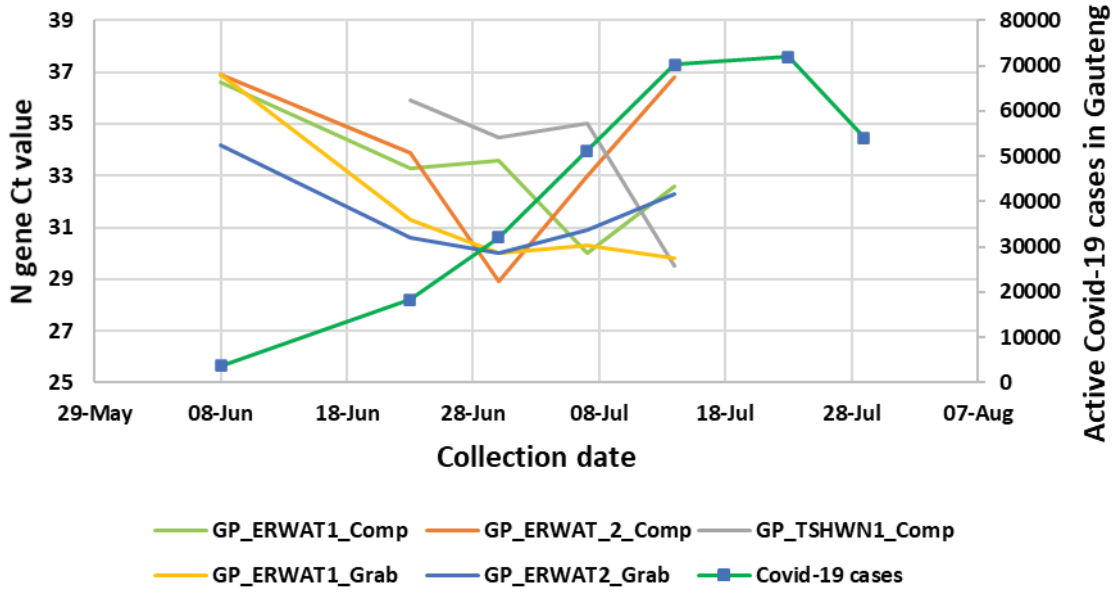


Figure 3-10: N gene Ct values over time for the composite and grab samples from Gauteng; two WWTW from the City of Ekurhuleni (GP_ERWAT1, GP_ERWAT2) and one WWTW from the City of Tshwane (GP_TSHWN1), overlaid with the active case number for the province at the time of sampling (Source: https://www.covid19sa.org/provincial-breakdown)

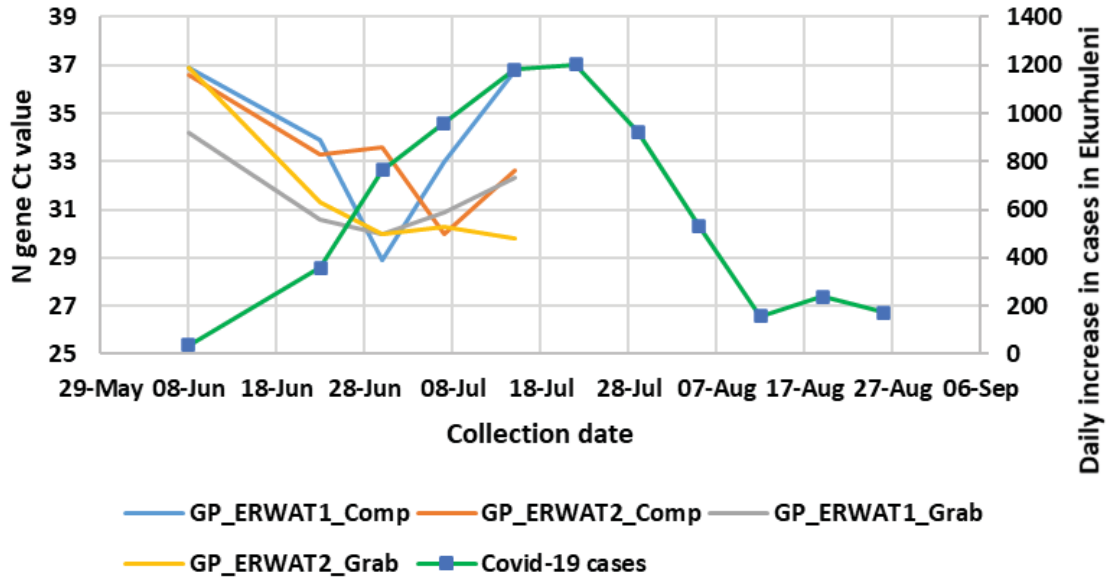


Figure 3-11: N gene Ct values over time for the composite and grab samples from the City of Ekurhuleni; overlaid with the daily increases in cases for the City at the time of sampling https://github.com/dsfsi/covid19za/blob/master/data/district_data/gp_ekurhuleni.csv

The Ct values for the N gene assay recovered with both PEG and skimmed milk from the composite samples taken from the iLembe District Municipality are presented in Figure 3-12. There is a downward trend in the Ct values, indicating an increased viral load, which corresponds to the increase in active case numbers in KwaZulu-Natal during the period of sampling. The Ct values for the N gene assay for samples from the Western Cape are presented in Figure 3-13, overlaid with active case numbers in the province for the period of sampling. WC_STB1 showed a strong downward trend in Ct values over the four-week period, but the

remaining works either remaining consistent or showed slight increasing trends. It can be seen that the Province reached its peak number of active cases during the period of sampling, which explains the relatively similar Ct values for the period.

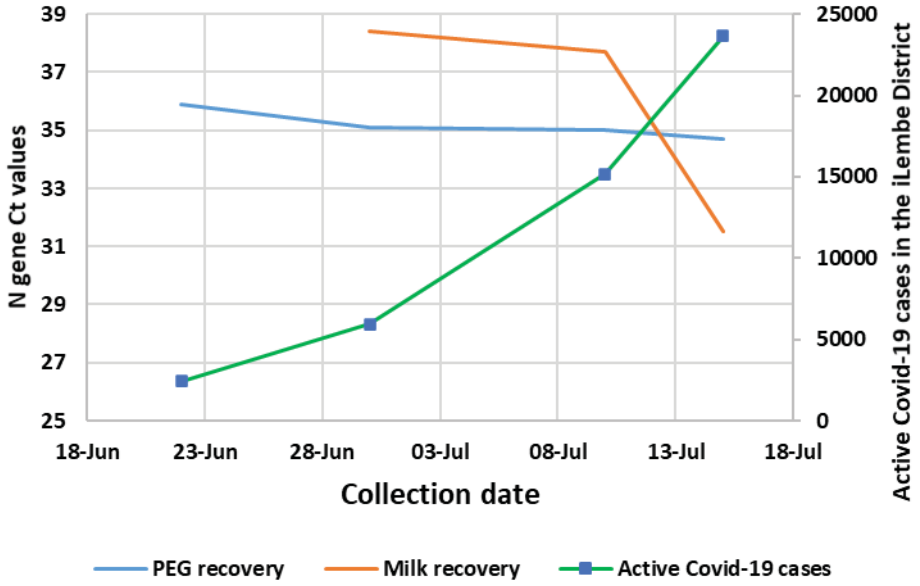


Figure 3-12: N gene Ct values over time for the composite samples from the iLembe District Municipality recovered with skimmed milk and PEG; overlaid with the active case number for the province at the time of sampling (Source: https://www.covid19sa.org/provincial-breakdown)

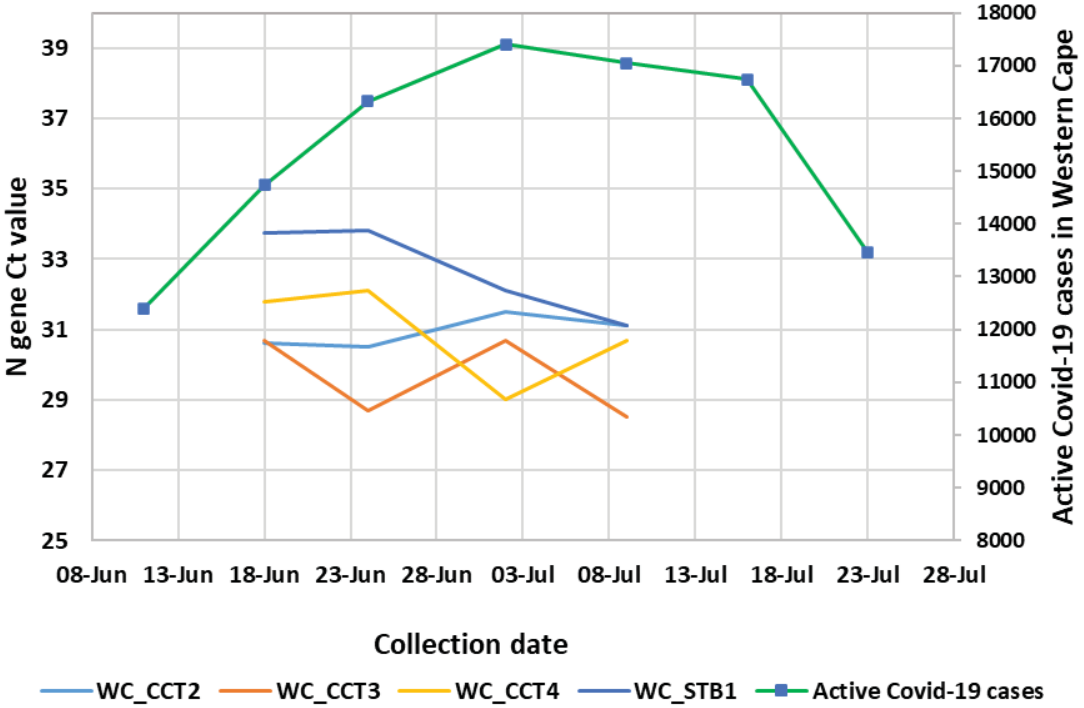


Figure 3-13: N gene Ct values over time for the composite and grab samples from the Western Cape; three WWTW from the City of Cape Town (WC_CCT2, WCCT3, WC_CCT4) and one WWTW from the Cape Winelands District (WC_STB1), overlaid with the active case number for the province at the time of sampling (Source: https://www.covid19sa.org/provincial-breakdown)

3.1.5 Viral quantification

Standard curves were generated and assays optimised for SARS-CoV-2 N1 and N3 using a commercial SARS-CoV-2 N gene plasmid to validate a method for quantification of the virus, as the ultimate aim of the study is to develop infection trends within communities. Both primer and probe sets were tested with the QuantiFast Pathogen + IC RT-PCR kit and both SARS-CoV-2 assays were compatible with the internal control in the Qiagen kit. Standard curves were generated for the N1 and N3 assays using the 2019-nCoV-N plasmid (IDT) (Figure 3-14). The assays are linear over a wide range and 5 gene copies per reaction could be detected, but reliable detection in all replicates was only observed at 50 copies per reaction for both assays. SARS-CoV-2 detection based on the N1 gene showed 65% (60/92) of samples were SARS-CoV-2 N1 positive. The Ct range was 28.2-39.4, with a median of 32.2 and the genome copies/mL ranged from 0.2-918 gc/mL. The N3 assay detected SARS-CoV-2 RNA in 91% (84/92) of samples and Ct values ranged from 28.2-37.9, median 32.6. SARS-CoV-2 concentrations ranged from 1.2-707 gc/mL. These concentrations have not been adjusted to account for differences in extraction efficiencies.

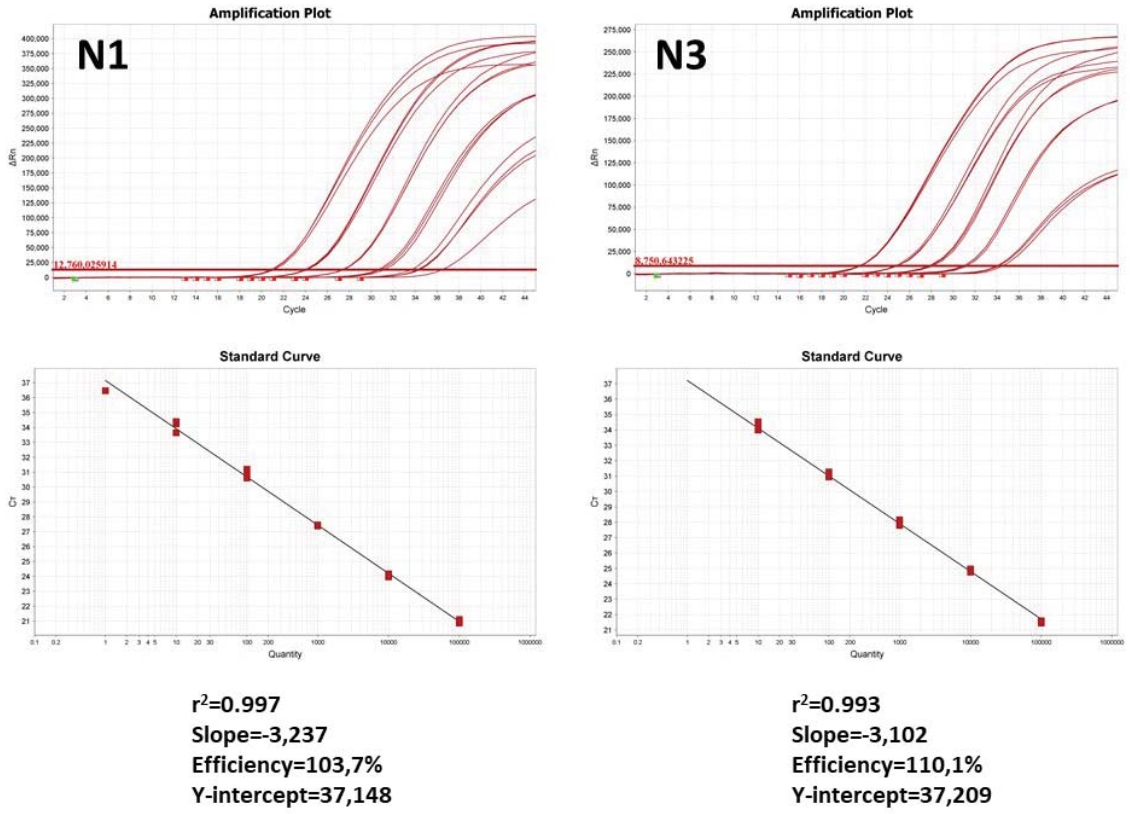


Figure 3-14: Detection of SARS-CoV-2 N-gene plasmid in serial ten-fold dilution range using the QuantiFast Pathogen + IC RT-PCR kit, as well as the standard curve generated from the amplification data. Slope -3.237, r^2=0.997, Efficiency= 103.6% for the N1 target and Slope -3.102, r^2=0.993, Efficiency= 110.1% for the N3 target

All SARS-CoV-2 positive samples were screened for mengovirus to calculate the extraction efficiency. In the initial round of screening, mengovirus RNA amplification was successful in 69% (60/87) of samples. Ct values ranged from 27.9-38.7, median 32.9. Reactions that failed were repeated at a 1 in 10 dilution of RNA and mengovirus RNA could be amplified in eight additional samples yielding a total of 78% (68/87) positives. The extraction efficiencies were calculated and ranged from 0,3-100%. The mengovirus positive rate did not correlate with the SARS-CoV-2 positive rate, since 17/19 mengovirus negative samples tested positive for SARS-CoV-2 by the Seegene, N1 or N3 assays. After adjustment of N1 and N3 concentrations based on

extraction efficiency there was a weak correlation between Ct value and log genome copies/mL (Figure 3-15). Genome copies/mL ranged between 1,2-2,7 × 10^4 for N1 and 4,2-5,5 × 10^4 for N3.

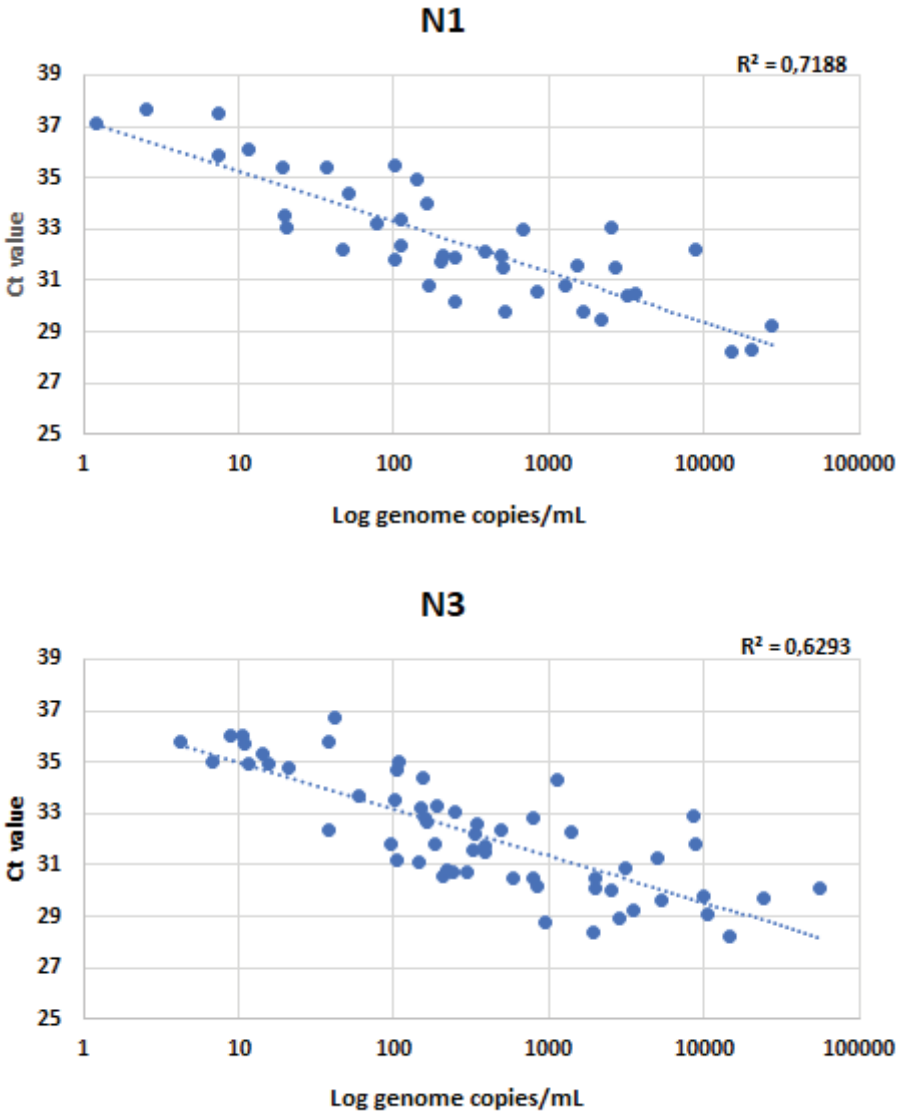


Figure 3-15: Correlation between Ct values obtained for N1 (n=41) and N3 (n=59) targets using the QuantiFast assay and concentrations (genome copies/mL) obtained after adjusting for extraction efficiency based on mengovirus

3.2 SARS-COV-2 ANALYSIS IN WASTEWATER SAMPLES FROM DEFINED POPULATIONS: MINES, POWER STATIONS AND PRISON SAMPLE RESULTS

3.2.1 Power stations, mines and prison

Results from the samples taken at the wastewater treatment plants serving two mines (MP_MM1 and FS_NVM1), their two associated coal fired power stations (MPMPS1 and FS_LPS1), a third power station wastewater treatment plant in the Western Cape receiving water from the power station only (WC_KPS), and a prison in the Western Cape (WC_CCT5) are presented in Figure 3-16, indicating the recovery method applied. The Western Cape samples were both recovered using the aluminium hydroxide precipitation method, and the remaining samples were processed with both the PEG method and skimmed milk method for comparison.

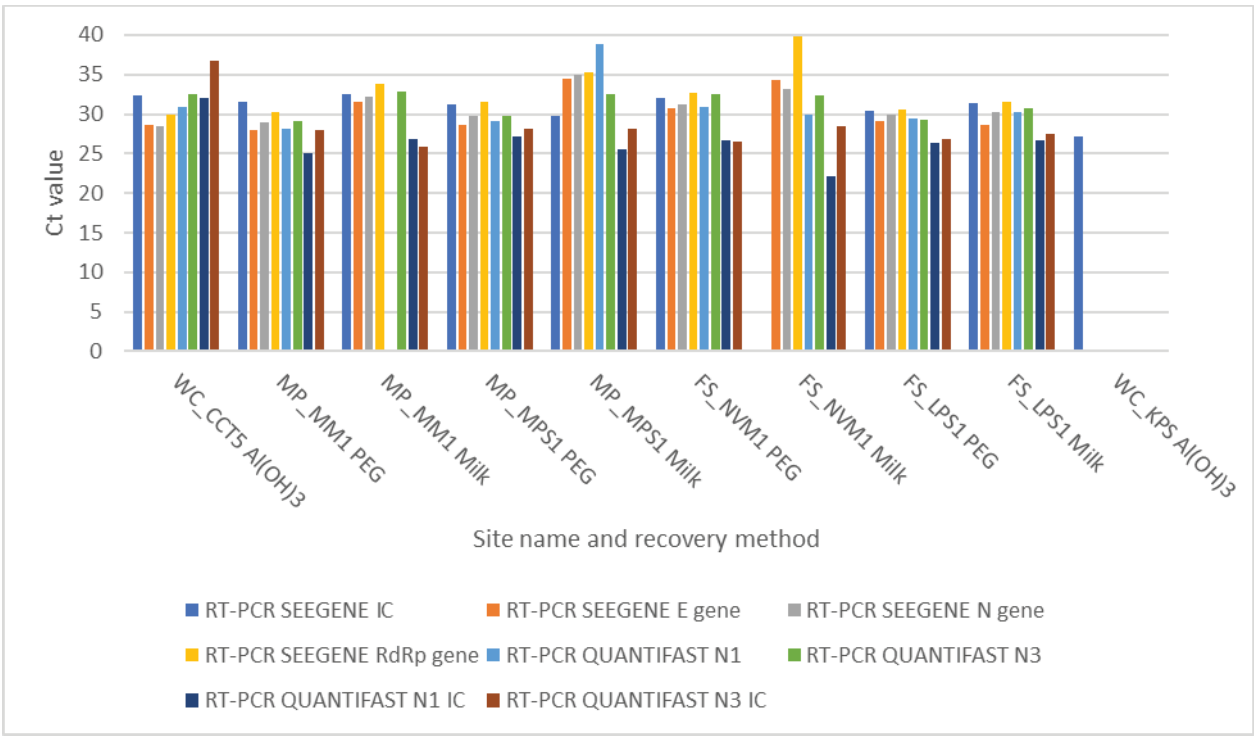


Figure 3-16: SARS-CoV-2 gene detection for prison, power station and mine

With the exception of the Western Cape power station WWTW (WC_KPS), which showed amplification of the internal control but was negative for all SARS-CoV-2 samples, all other sites showed amplification of the SARS-CoV-2 gene targets. Only the QuantiFast N1 assay for MP_MM1 with the milk recovery and the Seegene assay IC for FS_NVM1 with milk recovery did not amplify. Only one grab sample was taken from each site for proof of concept. The consistent amplification of most gene targets for the sites indicate that there were active cases at these sites, with the exception of the Western Cape Power Station. However, a negative assay result did not necessarily mean that there were no cases, just that the genes were not detected in the limited grab sample that was taken. Sampling of combined sewage for a defined population can be useful for surveillance of increased viral load to give early warning of a possible surge in infections. It is important however that regular samples be taken over time to establish trends and baselines, due to the inherent variability of sampling from smaller populations compared to a regional WWTW.

3.2.2 Hospital samples

After approaching major hospitals in the hot-spot provinces to access wastewater samples and numerous attempts at obtaining permission to collect samples, permission was obtained to gain access to one wastewater system at a hospital in the Western Cape. Three samples were taken over a period of four weeks from a hospital in the Eastern District of the City of Cape Town with a Covid ward that had active Covid cases at the start of sampling. Samples were all recovered using the aluminium hydroxide adsorption-precipitation method, and the Seegene multiplex assay was used for detection of SARS-CoV-2 genes. The results for the detection of the SARS-CoV-2 genes with the Seegene assay are presented in Figure 3-17. In the first week of sampling all three targets amplified, as well as the IC. The second two samples were negative for all targets, with amplification only being observed for the IC in those samples. The period of sampling is indicated on the graph in Figure 3-18. It can be seen that week 1 of sampling was during the peak in case numbers, which was steadily declining through the sample period. The hospital had 23 Covid cases in the Covid ward at the start of the testing, and by week four the Covid ward was once again being used as a general ward.

Due to the difficulty in obtaining representative samples for hospital sewage (a grab sample will only contain the toilet flushes of one or a few patients), and the difficulty in extracting the virus from what is essentially a stool sample, hospital sampling is not recommended for surveillance purposes. Proof of concept was however demonstrated.

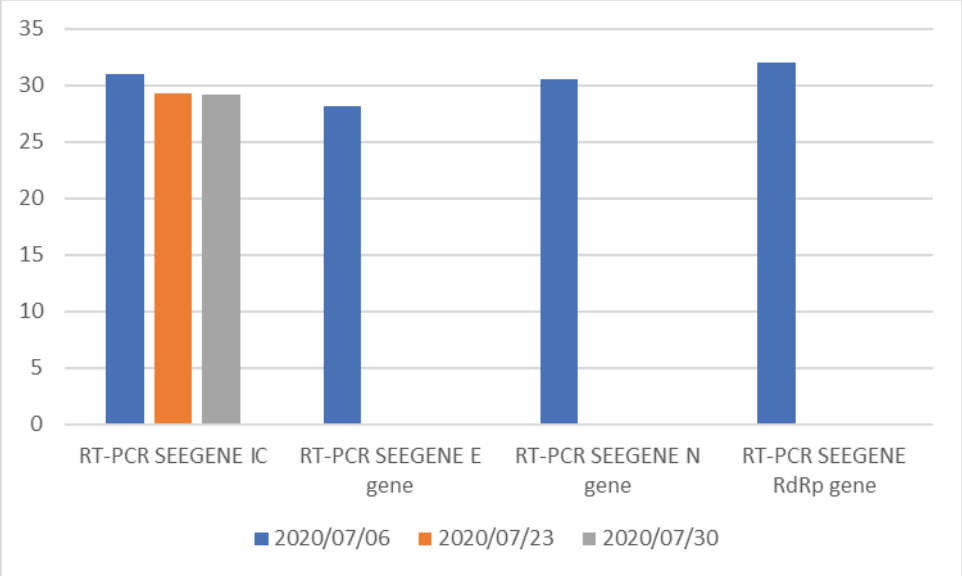


Figure 3-17: Detection of SARS-CoV-2 samples in hospital wastewater over a 4 week period

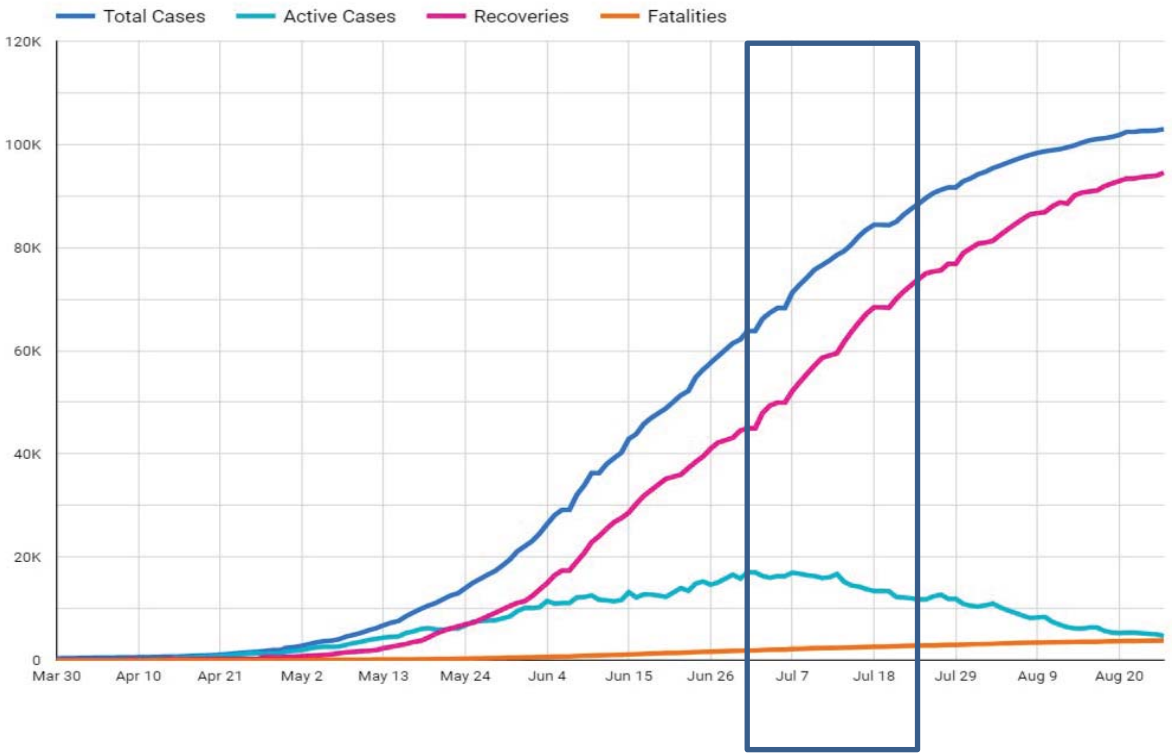


Figure 3-18: Covid case numbers for the Western Cape, with period of hospital sampling indicated in the block

3.3 SARS-COV-2 ANALYSIS IN SURFACE WATER

3.3.1 Water quality

The water quality results for the surface water samples taken are presented in Table 3-2. All sources were contaminated with untreated or poorly treated sewage. The quality of the surface water runoff from Alexandra was characteristic of raw high strength sewage (Table 3-3), as was indicated by an overflowing sewer manhole in the area. The water quality for the Jukskei River, Blougatspruit and Hennops River was also poor. All three river samples had ammonia, suspended solids and *E. coli* concentrations in excess of the general wastewater discharge limits, and the Blougatspruit and Hennops River exceeded the COD general limit as well. All rivers displayed qualities similar to that of low strength domestic wastewater (Table 3-3).

Table 3-2: Surface water quality analysis results

Parameter (in mg/l unless stated otherwise)	Alexander surface (GP_ALEXIN)	Jukskei River (GP_JUKS1)	Blougatspruit (GP_COHK)	Hennops River (GP_HENN1)	Wastewater discharge Limits*	
					General Limit	Special Limit
Chemical oxygen demand	1076	56	197	353	<75	<30
Free and saline ammonia	63	12	24	22	<6	<2
Orthophosphate as P	4.8	0.7	3.5	0.8	<10	<1
Suspended solids	284	96	151	37	<25	<10
Total coliform bacteria / (100 ml)	>100000	>100000	>100000	>100000		-
E. coli / (100 ml)	>100000	>100000	>100000	>100000	1000	0

*Revision of General Authorisation in terms of Section 39 of the National Water Act, 1998, Act No. 36 of 1998).

Table 3-3: Typical Values for Untreated Domestic Wastewater (Nozaic & Freese, 2009)

Contaminants	Units	Low Strength	Medium Strength	High Strength
Suspended solids (SS)	mg/l	120	210	400
Chemical Oxygen Demand (COD)	mg/l	250	430	800
Free ammonia nitrogen	mg/l	12	25	45
Phosphorous (total as P)	mg/l	4	7	12
Total Coliforms	count100 ml	10^5-10^8	10^7-10^9	10^7-10^{10}
Faecal Coliforms	count/100 ml	10^3-10^5	10^4-10^6	10^5- 0^8

3.3.2 Detection of SARS-CoV-2 in surface water

In order to determine the recovery efficiency from the surface samples which were expected to be more dilute than the wastewater treatment plant influent samples, samples were recovered in triplicate, from 200 mL, 1 L and 2 L volumes using the skimmed milk recovery method as described in the methodology in Section 2.4. SARS-CoV-2 was detected in all surface water samples tested, but not all targets amplified. Figure 3-19 illustrates the Ct values of the positive gene amplification of the gene targets for the various assays, for the three volumes recovered for each sample (two for the Hennops river sample). 1/11 samples assayed was positive for all 5 targets (9.09%), 5/11 were positive for 4 targets (45.5%) 3/11 were positive for 3 targets (27.3%) and 2/11 (18.2%) were positive for one target. Only one sample assay, the 1 L Hennops sample, was negative for all targets (Seegene assay only).

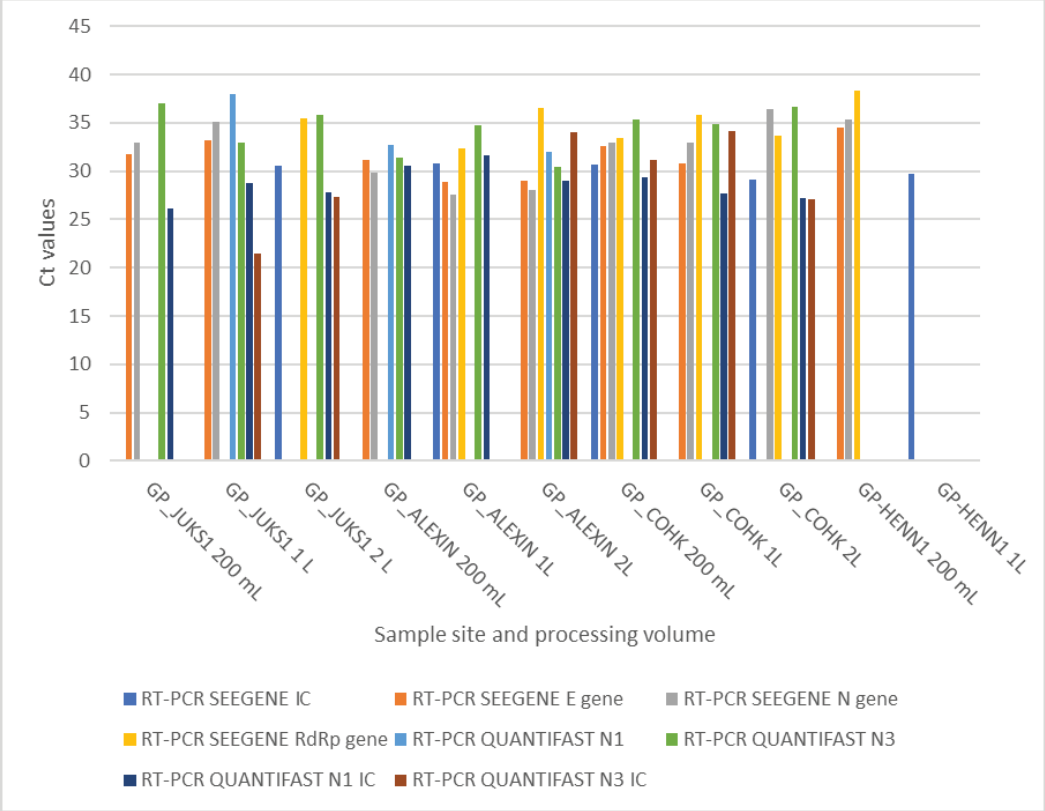


Figure 3-19: Detection of SARS-CoV-2 in surface water

In some cases, there was inhibition of the internal control, while gene targets still amplified. For the Jukskei River sample assayed with the Seegene multiplex assay, the internal control was negative for both the 200 mL and 1 L recovery volumes, but amplified for the 2 L volume. Positive amplifications were seen for the E gene and N gene for the 200 mL and 1 L recoveries, but not for the 2 L recovery. The inverse was true of the RdRp gene where the IC was inhibited at 200 mL and 1 L, but amplified for 2 L, and the RdRp gene target only amplified in the 2 L volume recovery. For the QuantiFast assay for the Jukskei River sample, the IC for N1 gene assay amplified for all recovery volumes, and the N3 IC only for 1 L and 2 L. The N3 gene assay amplified for all volumes, whereas the N1 assay only amplified for the 1 L volume. From the Alexandra surface water sample, the Seegene IC only amplified for the 1 L sample, and all Seegene targets amplified as well. There was amplification for the E gene and N gene in the 200 ml recovery volume, but not the RdRp gene or IC. For the 2 L volume recovery all targets amplified but the IC did not. For the QuantiFast assay for the Alexandra surface water sample, the N3 gene again amplified for all recovery volumes, even though the IC for the 1 L volume was inhibited. The N1 IC amplified in all volumes but was inhibited for the 1 L volume. For the

Blougatspruit all targets for the Seegene assay amplified with the exception of the IC for the 1 L recovery volume and the E gene assay for the 2 L recovery volume. The QuantiFast assay showed amplification of both the N1 and N3 ICs at all recovery volumes, and again amplification of the N3 gene target in all recovery volumes. None of the N1 gene targets amplified. Finally, for the Hennops River samples, where only the Seegene kit was applied based on the previous findings, the IC failed to amplify in the 200 mL sample volume recovery, although all three targets amplified. In the 1 L sample volume the IC amplified but all other targets were negative. It is interesting to note that recovery from the surface waters was sufficient even in the 200 mL volumes to enable gene detection. The number of targets that amplified for each sample volume recovery is presented in Figure 3-20. For the Jukskei River samples the most targets amplified in the 1 L recovery volume. For the Alexandra surface the 2 L recovery had the most targets amplify, and the Blougatspruit and Hennops had the most targets amplify in the 200 mL sample recovery volume.

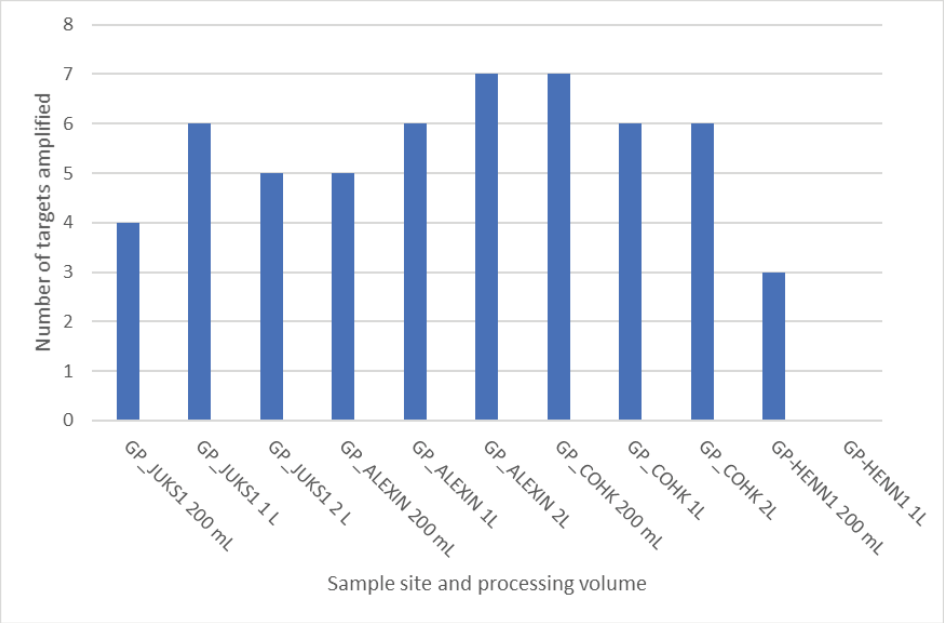


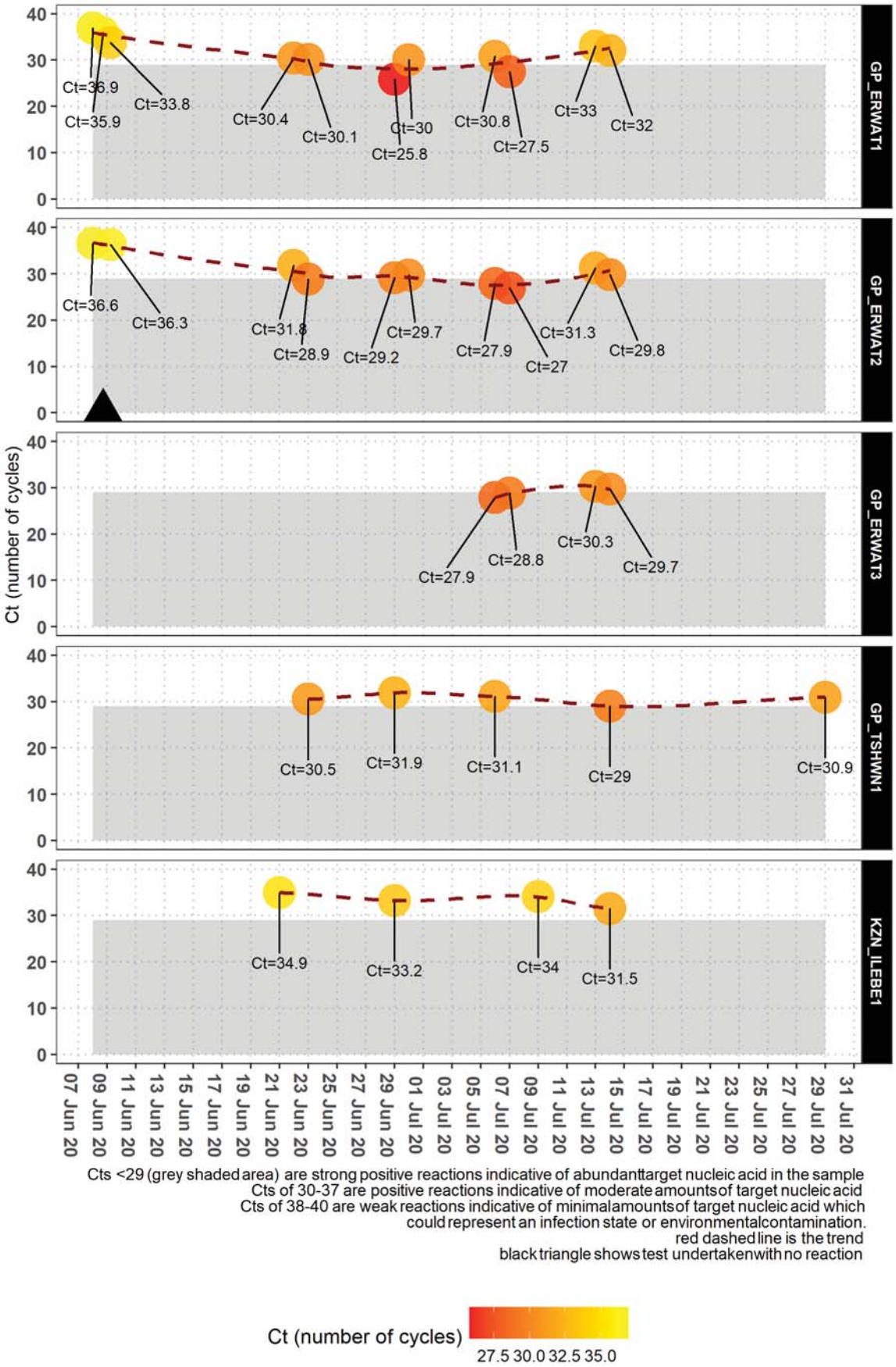
Figure 3-20: Number of SARS-CoV-2 gene targets amplified per sample site and recovery volume

It can be seen that there is a variable inhibitory effect on the internal controls for the surface water samples. The Quantifast N3 assay detected the most consistently of the assays, and the N1 assay the least consistently. Interestingly, the N1 IC amplified in all cases but the N3 IC did not. Because it is easier to process 200 mL than 1 L or 2 L of sample, and since at least 2 targets came up in every 200 mL sample, it can be recommended that only 200 mL of river sample be processed. While the RdRp gene did not amplify, this is not an unusual finding as it was the target that failed to amplify the most often in the wastewater samples. Because of the inhibition seen in the IC, it is recommended that 1:10 dilutions of the extracted RNA also be tested. Because of the variability observed it is also recommended that a multiplex assay such as the Seegene assay be used for the environmental samples to enable detection of multiple targets.

3.4 DATA VISUALIZATION AND RESULTS INTERPRETATION

3.4.1 Visualising trends in Ct values and data interpretation

As shown in Section 3.1.4, following the trends in Ct values may be a valuable tool for determining trends in viral load and identifying either new occurrences in areas previously unaffected, or for early warning of second waves of infection. Either a specific target could be selected as the indicator, such as the N gene as was presented earlier, or preferably, the minimum Ct value for the targets assayed could be reported for surveillance purposes. This will allow for the use of various assays by different laboratories. A COVID-specific dashboard could also be developed, where the Ct values can be illustrated in trend graphs per site. This is illustrated in Figure 3-22 for the wastewater treatment works, Figure 3-23 for hospital and prison sites, Figure 3-24 for power stations and mines, and Figure 3-25 for surface water samples. Where there is more than one bubble for a specific date that indicates the result of the different recovery methods tested in this study. For routine reporting only one Ct value would be reported, the minimum Ct value for the targets assayed. A black triangle on the graph indicates a negative result (Ct value greater than 40). An interactive map is recommended where surveillance sites can be mapped and current status visualised (Figure 3-26).


GP_ERWAT1
Ct=36.9
Ct=35.9
Ct=33.8
Ct=30.4
Ct=30.1
Ct=30
Ct=25.8
Ct=30.8
Ct=27.5
Ct=33
Ct=32
GP_ERWAT2
Ct=36.6
Ct=36.3
Ct=31.8
Ct=28.9
Ct=29.2
Ct=29.7
Ct=27.9
Ct=27
Ct=31.3
Ct=29.8
GP_ERWAT3
Ct=27.9
Ct=28.8
Ct=30.3
Ct=29.7
GP_TSHWN1
Ct=30.5
Ct=31.9
Ct=31.1
Ct=29
Ct=30.9
KZN_ILEBE1
Ct=34.9
Ct=33.2
Ct=34
Ct=31.5
Ct (number of cycles)
0
10
20
30
40
07 Jun 20
09 Jun 20
11 Jun 20
13 Jun 20
15 Jun 20
17 Jun 20
19 Jun 20
21 Jun 20
23 Jun 20
25 Jun 20
27 Jun 20
29 Jun 20
01 Jul 20
03 Jul 20
05 Jul 20
07 Jul 20
09 Jul 20
11 Jul 20
13 Jul 20
15 Jul 20
17 Jul 20
19 Jul 20
21 Jul 20
23 Jul 20
25 Jul 20
27 Jul 20
29 Jul 20
31 Jul 20
Cts <29 (grey shaded area) are strong positive reactions indicative of abundant target nucleic acid in the sample
Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid
Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.
red dashed line is the trend
black triangle shows test undertaken with no reaction
Ct (number of cycles)
27.5 30.0 32.5 35.0

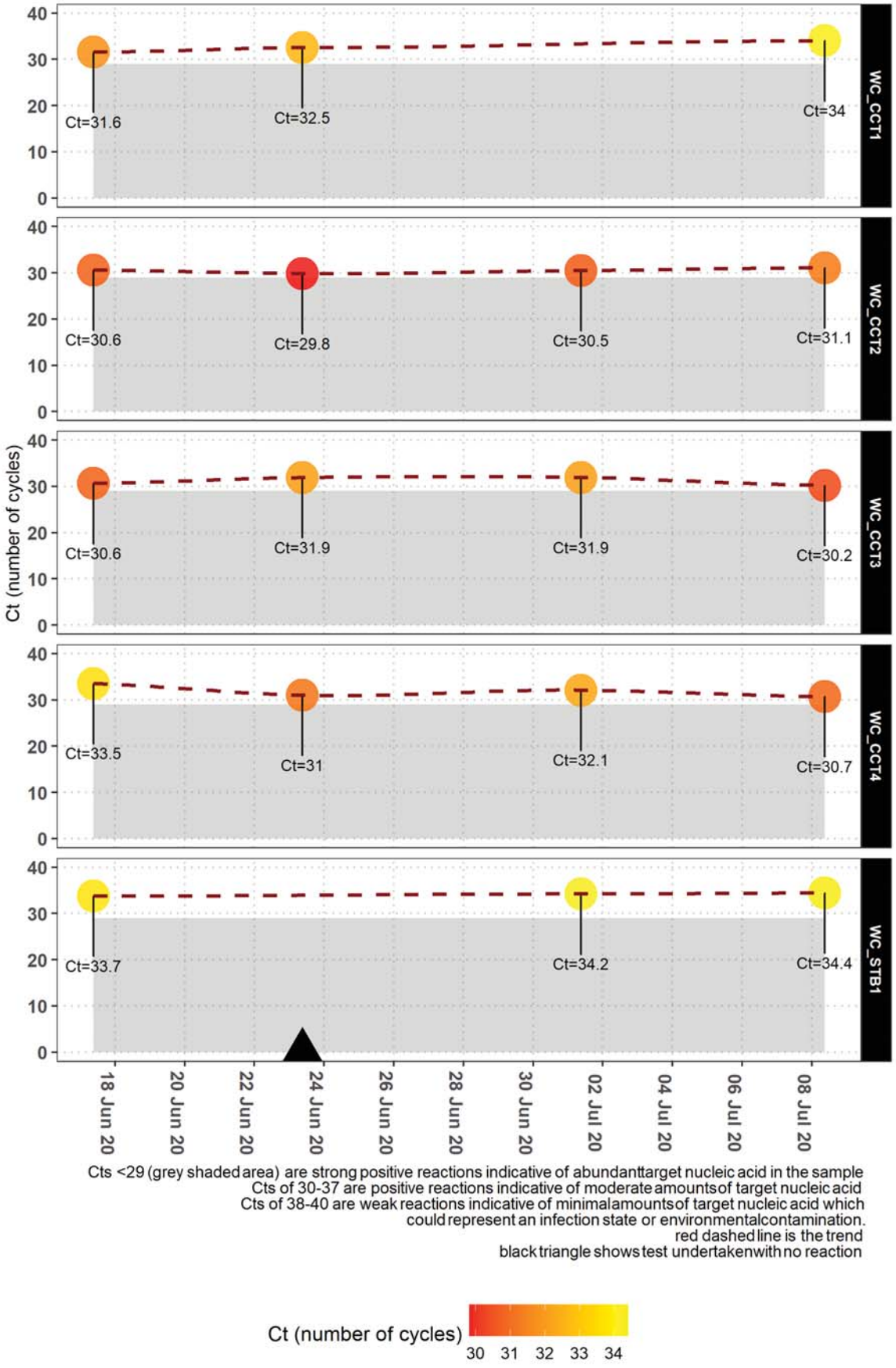


Figure 3-21: Trends in Ct values, by minimum Ct value per site for wastewater treatment works. A black triangle is indicative of a negative result (Ct > 40)

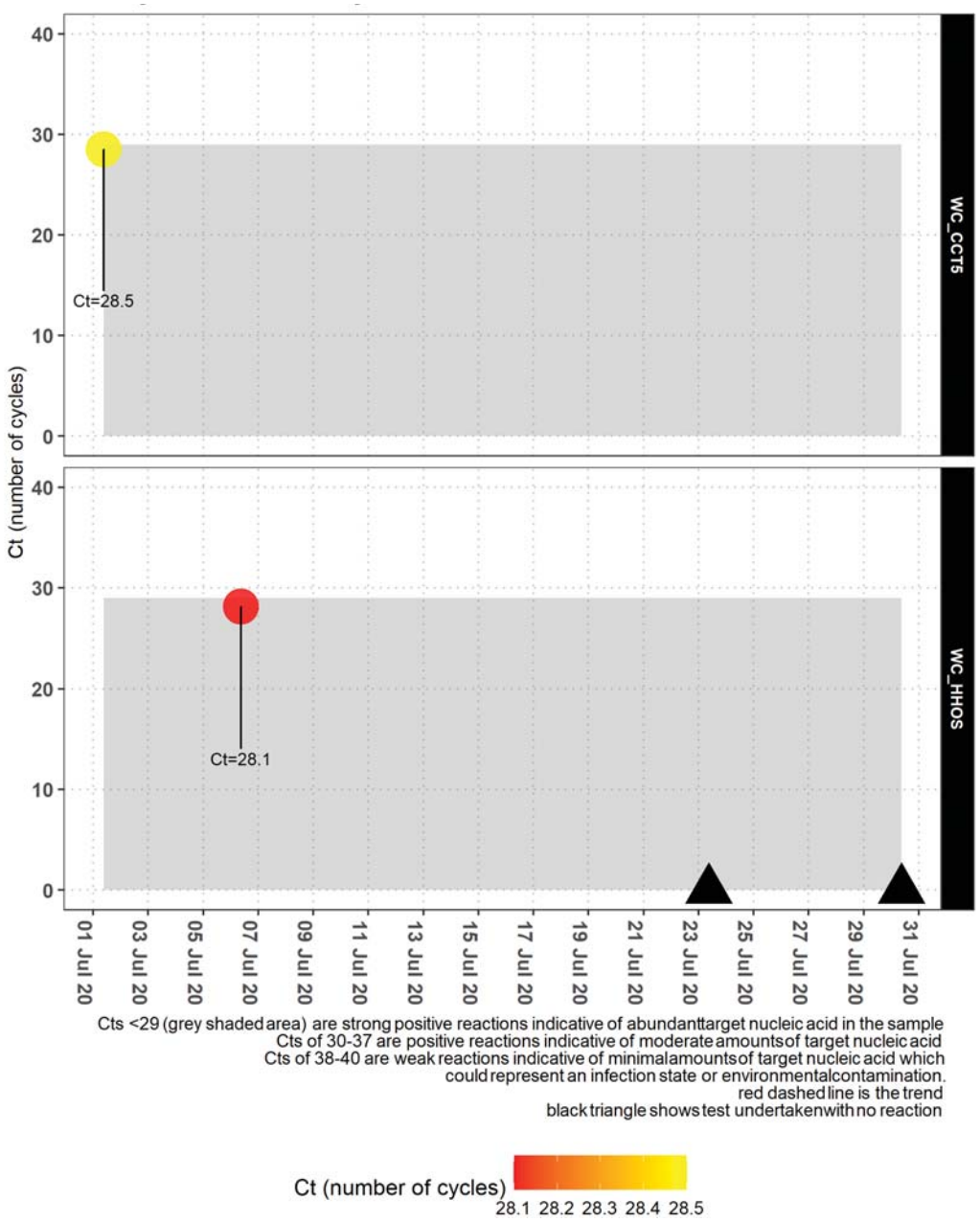


Figure 3-22: Trends in Ct values, by minimum Ct value per site for prison and hospital sites. A black triangle is indicative of a negative result (Ct > 40)

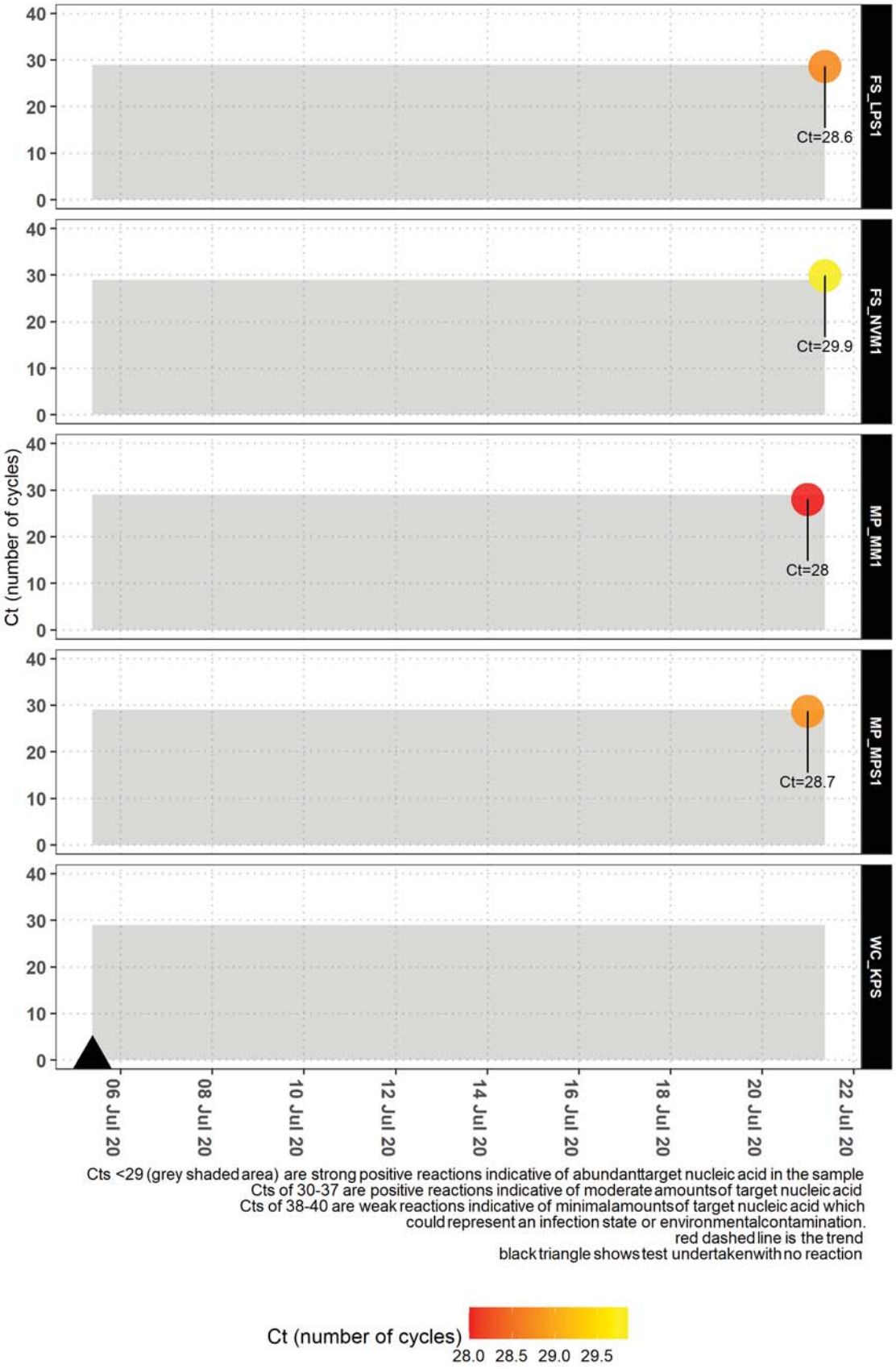


Figure 3-23: Trends in Ct values, by minimum Ct value per site for the power station and mine sites A black triangle is indicative of a negative result (Ct > 40)

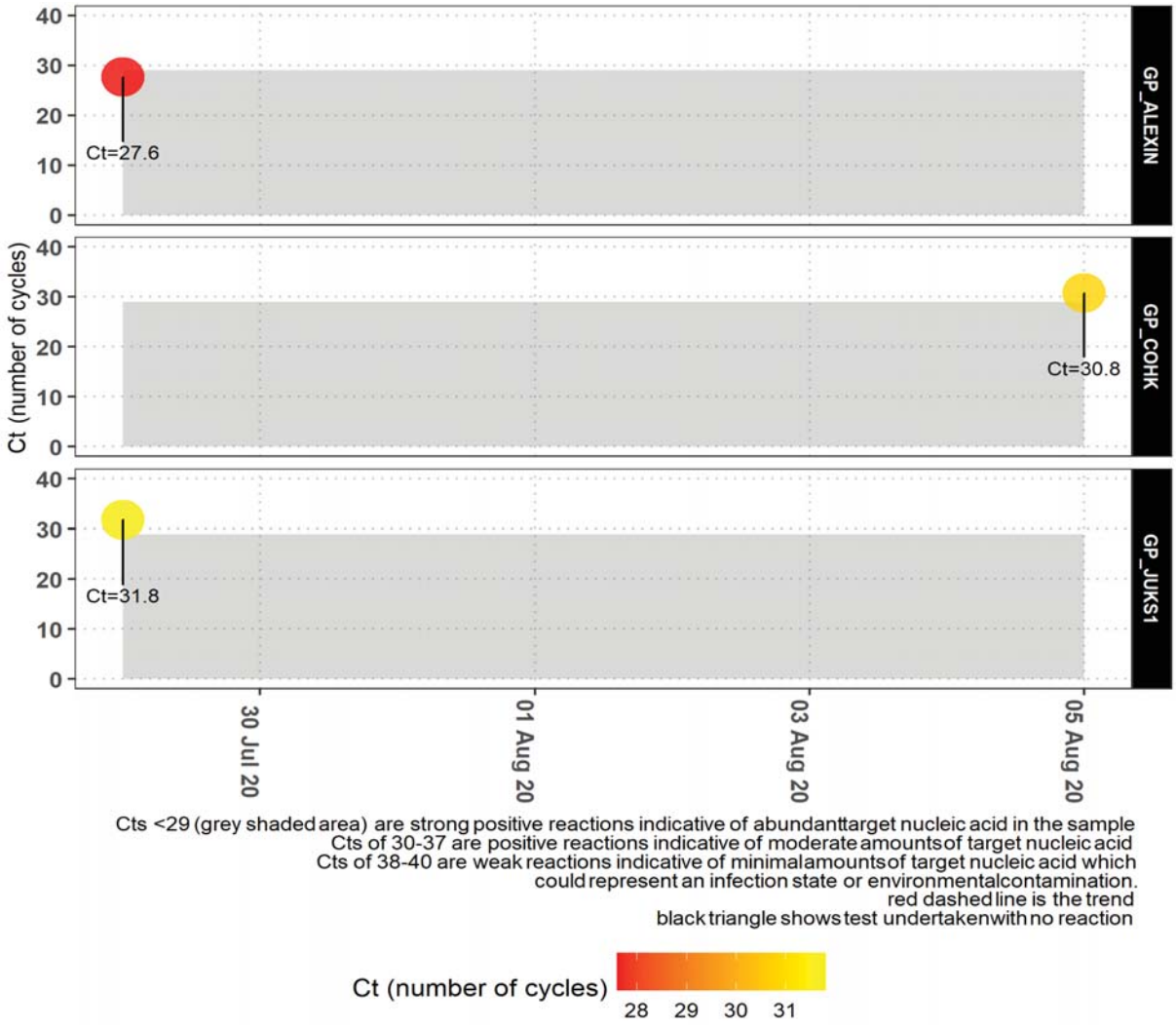


Figure 3-24: Trends in Ct values, by minimum Ct value per site for surface water samples

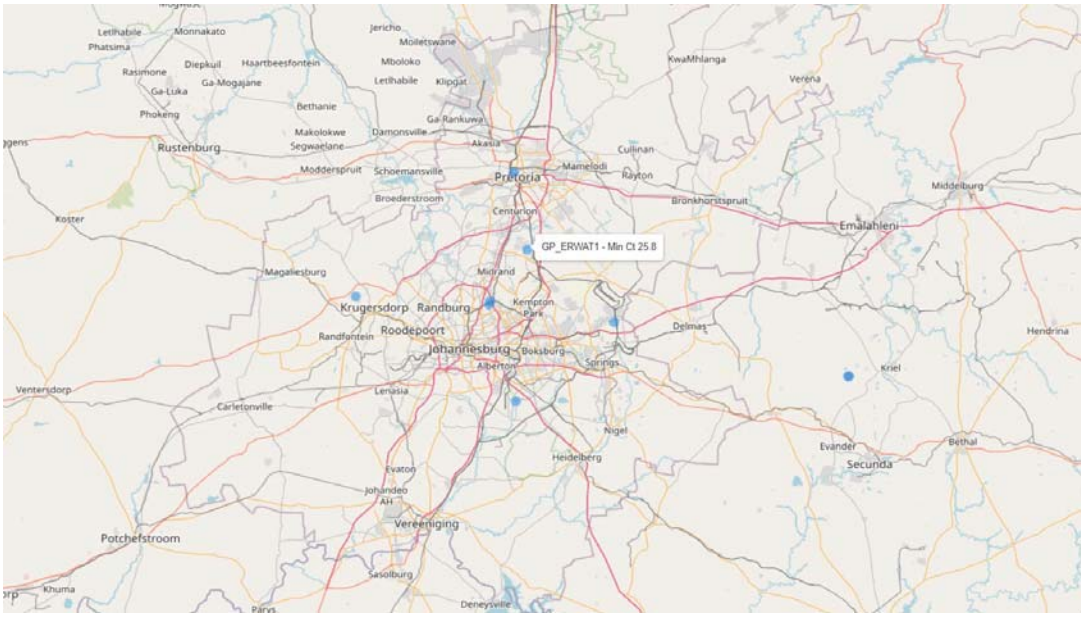


Figure 3-25: Mapping of surveillance sites to indicate current status

3.4.2 Data reporting

Ct values can be assigned a quantitative value, based on the Ct range, as is currently the internationally accepted practice for poliovirus surveillance in wastewater. This methodology for quantitative reporting of Ct values on a weekly basis per site has been recommended by the NICD for a national reporting dashboard as per the poliovirus dashboard that is currently in place. An example of what this dashboard could look like is presented in Figure 3-26.

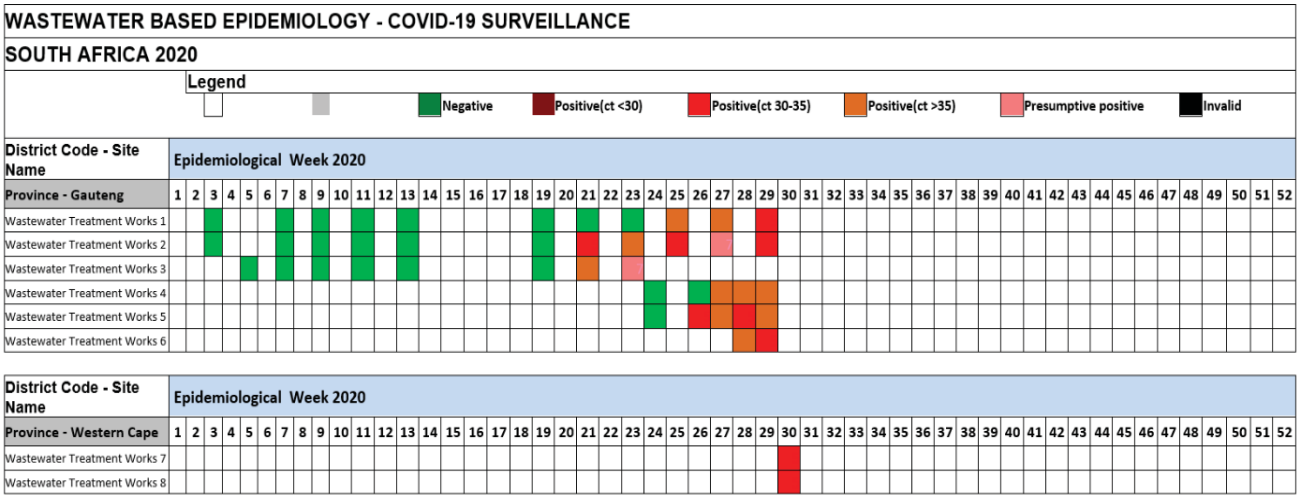


Figure 3-26: Example of proposed Covid-19 surveillance dashboard as per the NICD poliovirus surveillance platform

The COVID-19 Information Centre is a data centre set up to monitor and track the spread of the coronavirus (COVID-19) in the country. The centre, which is housed at the CSIR provides close to real-time analytics and dashboards on the Coronavirus outbreak per province, district, local municipality and ward. Based on the findings of this study, recommendations will be made for data integration into national reporting. It is envisaged that in the following phases of the project, during a national roll out, a method for estimating number of infections in communities can be tested and compared with reported values. It is not possible to do this in the initial proof of concept phase as supporting data is required using modelling and Monte Carlo simulations.

3.5 SUMMARY OF FINDINGS

3.5.1 Grab vs. composite wastewater samples

For the wastewater treatment plants grab sampling during the morning peak flow period performs as well as or better than composite sampling. This is an advantage when sampling from plants where no composite sampler is available, as is the case for most wastewater treatment works in South Africa.

3.5.2 Use of primary sludge for SARS-CoV-2 monitoring

Peccia et al. (2020) utilized primary sewage sludge (gravity thickened and composed of solids removed during the primary sedimentation step) instead of raw wastewater for SARS-CoV-2 analysis in Connecticut, USA. The authors found that due to the greater solids content of primary sludge (2-5%) in comparison to raw wastewater (0.01 to 0.05%) and the high case load observed during the outbreak (~1,200 per 100,000 population), the

concentrations of SARS-CoV-2 RNA ranged from two to three orders of magnitude greater than raw wastewater SARS-CoV-2 values previously reported. D'Aoust et al. (2020) found that RT-qPCR showed higher frequency of detection of N1 and N2 genes in primary sludge (92.7, 90.6%) when compared to influent samples taken post grit removal (79.2, 82.3%). For the influent samples the authors applied prefiltration through a 1.5 µm glass fibre filter (GFF) followed by a 0.45 µm GF6 mixed cellulose ester (MCE) filter (filtrate fraction), after which the virus was eluted with an elution buffer, followed by PEG concentration.

For the sludge samples, only PEG precipitation could be applied due to the incompatibility of the matrix with ultrafiltration due to complication associated with membrane clogging. The authors noted that when analysing high solids-containing samples, PEG precipitation or other flocculation approaches may be more effective, however the advantages of using sludge, which may have a greater and more consistent RNA signal, should be balanced against the apparent lower recovery of PEG precipitation. Sampling of primary settled sludge may however be an effective approach for SARS-CoV-2 viral quantification during periods of declining and low COVID-19 incidence in the community. In this study, based on limited sludge sampling from two WWTW, one from the City of Ekurhuleni and one from the City of Tshwane, the same success in terms of virus recovery was not experienced, with a high level of inhibition present in the samples, even when testing 1:10 and 1:1 dilutions of the sludge. The higher solids concentration in the sludge also made virus recovery more difficult, more time consuming and more costly. Further sludge samples were not analysed and based on these findings is not recommended that primary sewage sludge be used as the source for surveillance monitoring. In addition to the difficulties experience in recovery, when it comes to data interpretation, more detail will be required for back calculation to population numbers, due to the different operating conditions that will be implemented at different plants in terms of sludge retention time and desludging rates that are not easily correlated to plant inflow volumes.

3.5.3 Efficiency of virus recovery

In this study, initial recovery tests were conducted using mengovirus as a surrogate in order to determine the recovery rate of mengovirus from different wastewater samples. The wastewater was spiked with 2.8×10^6 TCID50 mengovirus either before the first clarification step (composite only) or after clarification (composite, early and late grab samples). The viruses were recovered with the PEG/NaCl precipitation method, and the mengovirus recovery ranged between 0.49% and 8.4%. All three of the virus recovery methods tested in this study (PEG flocculation, skimmed milk precipitation and aluminium hydroxide adsorption-precipitation) were able to recover the SARS-CoV-2 virus. Recovery was variable as expected due to the inherent variability of the sample matrix. Preliminary data in a study by Rusinol et al. (2020) analysing different concentration methods for the detection of SARS-CoV-2 in wastewater from Catalonia, Spain, showed no statistically significant differences (p-value of the ANOVA test: 0,332) between the quantitative data (RT-qPCR) produced by the Skimmed Milk Flocculation protocol, the centrifugal ultrafiltration of the samples with Centricon® Plus-70 100 kDa or an ultrafiltration protocol using the automatic Concentrating Pipette (CP-Select™) both for SARS-CoV-2 and for MS2 which was used as a process control.

The mouse hepatitis (MHV), a surrogate for human CoV, has been used by other researchers for studying persistence, survival and method comparison studies. Ye et al. (2016) compared three methodologies by means of MHV recoveries to concentrate enveloped viruses from wastewater samples, PEG precipitation, ultracentrifugation, and ultrafiltration with pre-filtration. PEG precipitation and ultracentrifugation recovered approximately 5% of the spiked viruses, whereas with prefiltration with ultrafiltration followed by Centricon® Plus-70 10 kDa filtration protocol the concentration was significantly higher (25%). Ahmed et al. (2015) evaluated six virus concentration strategies using MHV as a surrogate. The three filtration methods assayed provided the highest mean recoveries: when $MgCl_2$ pre-treatment was included, 65% of the MHV were recovered, when sample was directly filtered through 0.45-µm pore-size electronegative membranes, MHV recoveries were 60%, but when pre-acidifying the sample the mean recovery decreased to 27%. Between the two centrifuge ultrafiltration methods tested, the Amicon® Ultra-15 30KDa recovered 56% of the spiked

surrogate and Centricon® Plus-70 10KDa recovered 28%. Finally, by means of PEG precipitation and ultracentrifugation, MHV recoveries were 44% and 33% respectively.

3.5.4 Methods for viral detection

Both the commercial Seegene assay and QuantiFast Pathogen Kit inhouse N1 and N3 (QF N1 or N3) assays detected SARS-CoV-2. The Seegene kit was found to be more sensitive than the Quantifast N1 assay, and has the advantage of amplifying 3 targets in one reaction, and is more likely to be more consistent across laboratories. The disadvantage is that the Ct value must then be used to approximate viral load, as the copy number cannot be determined by comparison with a standard curve. The N target was detected most frequently, then E, then RdRp. All SARS-CoV-2 positive samples were screened for mengovirus to calculate the extraction efficiency. In the initial round of screening, mengovirus RNA amplification was successful in 69% of samples, with Ct values ranging from 27.9-38.7, median 32.9. Reactions that failed were repeated at a 1 in 10 dilution of RNA, and mengovirus RNA was amplified in eight additional samples yielding a total of 78% positive. The extraction efficiencies were calculated and ranged from 0.3-100%. The mengovirus positive rate did not correlate with the SARS-CoV-2 positive rate, since 17 of the 19 mengovirus negative samples tested positive for SARS-CoV-2 by the Seegene, N1 or N3 assays.

3.5.5 SARS-CoV-2 detection in non-sewered samples

This study also looked at sampling of three rivers and one contaminated surface run-off sample as a means to apply wastewater-based epidemiology principles to non-sewered communities in South Africa. Supplementary analysis of the water indicated very high levels of sewage contamination in these samples. The quality of the surface run-off sample was characteristic of high strength domestic wastewater, and the water quality for the Jukskei River, Blougatspruit and Hennops River was also poor. All three river samples had ammonia, suspended solids and *E. coli* concentrations in excess of the general wastewater discharge limits, and the Blougatspruit and Hennops River exceeded the COD general limit as well. All rivers displayed qualities similar to that of low strength domestic wastewater. The skimmed milk recovery method was applied to all river and surface water samples in this study. When comparing recoveries from 200 mL, 1 L and 2 L volumes, recovery from the surface waters was sufficient in 200 mL sample volumes for all samples to enable gene detection. For the Jukskei River samples the most targets amplified in 1 L recovery volume. For the Alexandra surface the 2 L recovery had the most targets amplify, and the Blougatspruit and Hennops River samples had the most targets amplified in the 200 mL sample recovery volume. When testing two river samples from the Milano Metropolitan Area, Italy, Rimoldi et al. (2020) pre-filtered 500 mL of sample on Whatman 0.7 µm nominal pore size 145 mm glass fibre filters, then on Millipore 0.2 µm nominal pore size, 145 mm diameter filters, before extraction, and found positive SARS-CoV-2 gene amplification. Guerrero-Latorre et al. (2020) applied an adapted skimmed milk flocculation recovery method to river samples from three rivers in Quito, Ecuador, using 2 L sample volumes, and detected SARS-Co-V-2 for N1 and N2 target regions in all samples.

3.5.6 Data interpretation and reporting

Trends in Ct values may be a valuable tool for determining trends in viral load and identifying either new occurrences in areas previously unaffected, or for early warning of second waves of infection. Either a specific target could be selected as the indicator, such as the N gene as was presented earlier, or preferably, the minimum Ct value for the targets assayed could be reported for surveillance purposes. This will allow for the use of various assays by different laboratories. A COVID-specific dashboard could also be developed, where the Ct values can be illustrated in trend graphs per site. Ct values can be assigned a quantitative value, based on the Ct range, as is currently the internationally accepted practice for poliovirus surveillance in wastewater.

CHAPTER 4: CONCLUSION AND RECOMMENDATIONS

4.1 CONCLUSIONS

This study illustrates clear proof of concept for the use of wastewater-based epidemiology as a complementary surveillance tool for management of the Covid-19 pandemic, for both wastewater and environmental samples. Continued sampling of those sites already involved will allow for the expansion of trend monitoring, and it is recommended that more WWTWs be added to the sampling protocol so as to move to a pilot phase study. South Africa is through its first peak of the pandemic, but the experience of other countries teaches us that second and even third waves of infection are likely, if not inevitable, as the economy and intra- and international borders re-open.

Translating the viral titres from wastewater into the actual number of cases within a community is highly challenging, if not impossible. This type of calculation relies on many assumptions, which still remain poorly quantified, for example the amount and dynamics of viral shedding in faeces, viral persistence in the sewer network and variation in wastewater flow and temperature due to climatic conditions. Although wastewater surveillance of SARS-CoV-2 provides a powerful tool to evaluate disease incidence at the community level, it is clear that they also need to be integrated into other public health initiatives, for example campaign-based and randomised testing of individuals (presence of pathogen or antibodies), clinical case reporting, and mobile-based contact-tracing and self-reporting systems (Boulos & Geraghty, 2020). It may also require a harmonization of approaches. It is important to consider how best to ethically and legally balance public health with civil liberties when handling this information (Gostin et al., 2020). One of the benefits of wastewater, is that it has limited sociological bias with few if any ethical issues.

All objectives of the study were addressed as listed below.

1) *Compile state of knowledge reports on SARS-CoV-2 in water and sanitation environments*

Literature was reported and summarized up to date as of submission of report.

2) *Testing and validation of a sampling protocol for raw sewage*

Raw sewage samples from Gauteng, Western Cape, KwaZulu-Natal, Mpumalanga and Free State provinces with representation over 4-week period were analysed. Composite and grab samples were tested with grab samples being able to detected higher virus signal than 24 h composite samples (92 tested in total).

3) *Testing and validation of the virus extraction and analysis*

Three virus extraction methods were tested based on instrumentation availability and affordability including PEG/NaCl precipitation; skimmed milk flocculation and $Al(OH)_3$ adsorption-flocculation methods illustrating that highly specialised laboratory equipment is not necessary.

4) *Testing and validation of a sampling protocol for surface and groundwater*

Environmental samples were tested from different locations, with viral RNA detected in all samples.

5) *Development of preliminary methodology for quantification of viral load as an indicator of number of infected individuals in a community*

Method to quantify the viral load makes use of the Ct number with a proposed categorical data analysis recommended based on the Global Polio Surveillance scheme, and quantification of genome copies/mL was found to range between 1,2-2,7 × 10^4 for N1 and 4,2-5,5 × 10^4 for N3 target genes.

6) Guidance on data analysis/interpretation

International interpretation of data is being followed as described in Objective 5 which was described by the Water Research Foundation webinars held during the first peak of the Covid-19 pandemic. The various use-cases presented highlight the importance of trend monitoring through the various phases of the pandemic. Although translating the viral titres from wastewater into the actual number of cases within a community is highly challenging, if not impossible, monitoring trends in viral load can be used successfully to implement an early warning system.

7) Recommendations for data communication and integration into national reporting platforms

Data communication can take place either as part of Polio Surveillance scheme run by the NICD, following the methodology for reporting based on categorical presentation of Ct value ranges or on a separate Covid-19 Data Centre platform.

4.2 RECOMMENDATIONS FOR SCALING UP INTO NATIONAL SURVEILLANCE PROGRAMME

4.2.1 Sampling methodology

- **Wastewater treatment works influent** – Based on the findings of this study it is recommended that 1 L grab samples be taken at the WWTW during the morning peak flow period between 8 and 10 am. These samples should be kept cool and transported to the relevant laboratory on the day of sampling, stored at 4°C and viral recovery performed within 24 h of sampling.

- **Rivers and surface water** – It is recommended that 3 L river samples be selected to allow for analysis of supporting water quality data.

4.2.2 SARS-CoV-2 detection

Methods need to achieve reproducible high quality and quantitative information. In order to address this, it is recommended that the evaluation and validation of methods includes a minimally acceptable QA/QC including:

1. positive control;
2. negative control;
3. estimated limit of detection;
4. reporting of equivalent volume of sample analysed.

Additional validation controls include:

5. inhibition control;
6. initial recovery controls;
7. ongoing precision recovery controls; and lastly
8. matrix spike, where a known concentration of target virus is added to the samples before sample preparation and assay.

4.2.3 Recovery methods

It is recommended that 200 mL of both wastewater and surface water be used for recovery. For wastewater, of the three recovery methods applied in this study, the skimmed milk method and aluminium hydroxide adsorption-precipitation methods are preferred, as they are both faster and cheaper than the PEG method, and only require low speed centrifugation. The inactivation buffer ITM and PBS used for the PEG and skimmed milk methods both performed well, as did Trizol which was used for re-suspension of the pellet when using the

aluminium hydroxide precipitation method. The three methods could therefore be used interchangeably between laboratories, although due to the slight variation in results it is recommended that one method be used consistently when monitoring a site to enable the visualisation of trends. For surface water samples the skimmed milk flocculation method is recommended for recovery.

4.2.4 SARS-CoV-2 gene assays

Although both the commercial Seegene assay and the QuantiFast Pathogen Kit inhouse N1 and N3 (QF N1 or N3) assays detected SARS-CoV-2, because the Seegene assay was found to be more sensitive than the QuantiFast N1 assay, has the advantage of amplifying 3 targets in one reaction, and is more likely to be more consistent across laboratories, this assay is recommended for further work. The disadvantage is that the Ct value must then be used to approximate viral load, as the copy number cannot be determined by comparison with a standard curve (refer to 4.2.5). The N target was detected most frequently, then E, then RdRp.

4.2.5 Viral quantification

The mengovirus positive rate did not correlate with the SARS-CoV-2 positive rate, since 17 of the 19 mengovirus negative samples tested positive for SARS-CoV-2 by the Seegene, N1 or N3 assays. While repeating negative samples with dilutions cleared the inhibition in some cases, this makes for a costly analysis process that is not feasible for routine analysis. The use of the minimum Ct value of the assayed targets per sample as an indicator of viral load appears from this study to be sufficient for trend analysis, which could be managed in a central database and visualised on a national dashboard.

4.2.6 Upstream sampling and monitoring of smaller defined populations

The potential to use this methodology for testing the wastewater of smaller, defined communities, such a prisons and mines, has been demonstrated, with positive results found at wastewater treatment works serving mines and industries, as well as sewer sampling downstream of a prison and hospital. Sampling of combined sewage for a defined population can be useful for surveillance of increased viral load to give early warning of a possible surge in infections. It is important however that regular samples be taken over time to establish trends and baselines, due to the inherent variability of sampling from smaller populations than a regional WWTW. This could provide a cost effective and less invasive means of continuous screening. Where increasing trends in viral load are noted then additional clinical test methods could be rolled out based on an early warning system.

4.2.7 Analysis of non-sewered environmental water samples

It is recommended that a 1:10 dilution of the extracted RNA be routinely performed to clear inhibition often observed in the internal controls for the surface samples. Because of the variability observed it is also recommended that a multiplex assay such as the Seegene assay be used for the environmental samples to enable detection of multiple targets. While it is not necessarily possible to relate viral loads in surface water to a defined population or possible case numbers, sampling of rivers may provide a means to monitor the spread of SARS-CoV-2 to areas previously unaffected by monitoring river quality over time, as well as monitoring trends in viral loads to identify possible infection spikes in communities upstream of the sample point. This is of high value for low- to middle-income countries such as South Africa, where many communities are not connected to formal sewer networks, or where sewage is allowed to discharge into rivers untreated.

4.2.8 Data visualisation and trend monitoring

Based on the limited dataset, plotting of weekly sample results appeared to be sufficient to indicate trends, as such weekly sampling of identified sites for national surveillance is recommended. It is recommended that viral load be quantified making use of the Ct number with a proposed categorical data analysis recommended based on the Global Polio Surveillance scheme. Interpretation of data should be done according to the various use cases suggested by the Water Research Foundation (2020) (Figure 4-1), highlighting the importance of trend monitoring through the various phases of the pandemic.

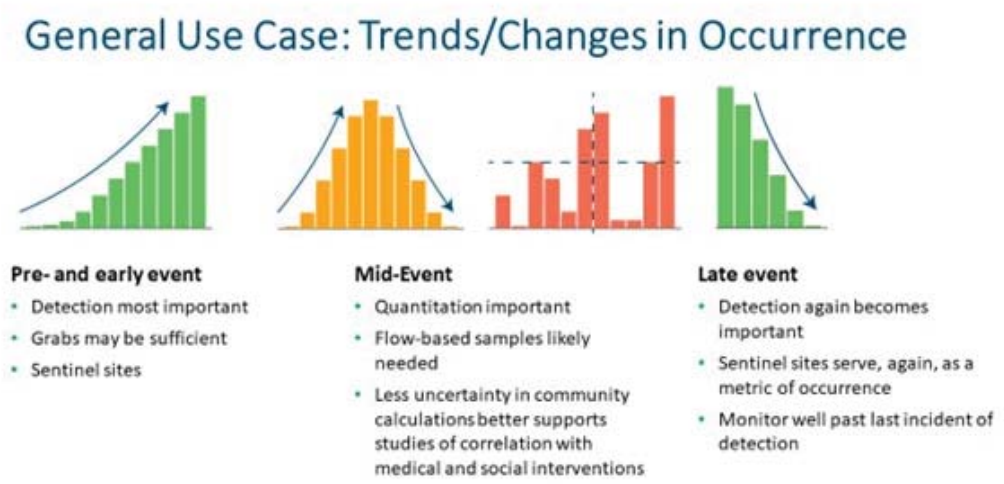


Figure 4-1: General use case: Source Water Research Foundation 2020

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APPENDIX A: COVID-19 CASES IN SELECTED PROVINCES AS OF 16TH JUNE 2020

A1 – Western Cape

A Subdistrict breakdown in the number of Covid-19 cases in the Western Cape as of the 17th of June is presented in Figure A1 below, according to the Western Cape Government Covid-19 dashboard (https://coronavirus.westerncape.gov.za/covid-19-dashboard). Tygerberg, Khayelitsha and Klipfontein Subdistricts had the highest number of confirmed cases, at 6 406, 5 490 and 4 911 respectively.

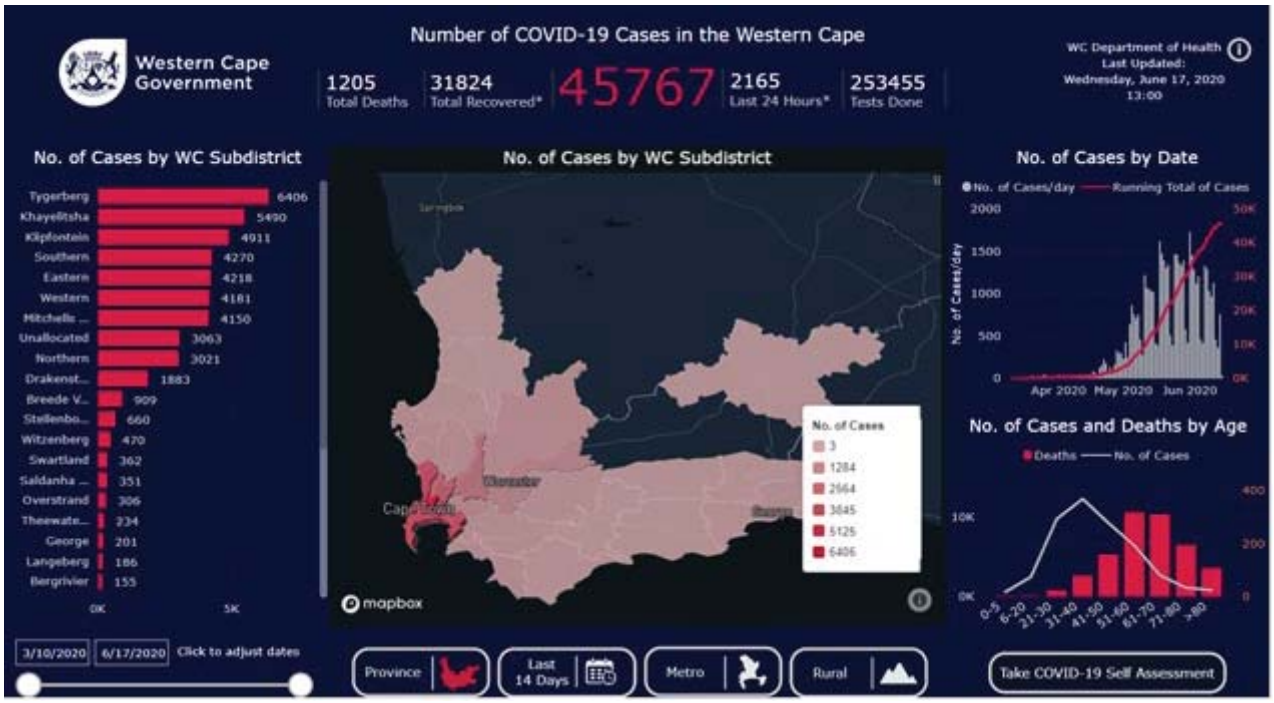

Figure A1: Number of Covid-19 cases in the Western Cape by Sub-district as of 17 June 2020

A2: Gauteng

As at 13 June 2020, Gauteng accounted for 9 897 COVID-19 cases (Gauteng Provincial Government). At that stage 565 people were hospitalised due to the virus. A breakdown in case number per district as of the 13th of June 2020 is presented in Figure A2 and Table A1. Regionally, the City of Johannesburg accounted for the biggest portion of cases in the province, with the district data showing the metro accounted for 5 545 cases – half of all confirmed cases in the province at that stage. This was followed by the City of Ekurhuleni (2 234 cases), and the City of Tshwane (1 543 cases). For the proof of concept study, samples were taken from two WWTW from the City of Ekurhuleni, operated by ERWAT, for a period of four weeks, and a third plant for the final two weeks of sampling. The first WWTW in the north serves communities and industries in Tembisa, Olifantsfontein and Ivory Park, as well as sections of Kempton Park and Midrand, falling within the City of Ekurhuleni North 1 Sub-District where there were 553 confirmed cases as of the 13th of June 2020. The second WWTW sampled was located in the south-west in Vosloorus, treating effluent from Boksburg and Vosloorus as well as areas of Tsakane, Duduza and Brakpan. This falls within the Ekurhuleni South 1 Sub-district with 422 confirmed cases. Finally, the third plant to be sampled for the final two weeks was located in the south

east of Ekurhuleni, in sub-district Ekurhuleni East 1, treating domestic effluent from Daveyton and Etwatwa, where there were 151 confirmed cases as of the 13th of June 2020.

Figure 0A2: District breakdown of cases in Gauteng as of 13 June 2020

Table A1: Covid-19 cases per District in Gauteng as of 13 June 2020

District	Total District	Total Recoveries	Sub-District	Total Sub-District	Recoveries Sub-District
City of Johannesburg	4 885	1708	**City of Johannesburg A:** Diepsloot, Kya Sands, Dainfern, Midrand, Lanseria, Fourways, Ivory Park	436	245
			City of Johannesburg B: Randburg, Rosebank, Emmarentia, Greenside, Melville, Mayfair, Northcliff, Parktown, Parktown North	649	222
			City of Johannesburg C: Roodepoort, Constantia Kloof, Northgate, Florida, Bram Fishersville	520	161
			City of Johannesburg D: Doornkop, Soweto, Dobsonville, Protea Glen	786	263
			City of Johannesburg E: Alexandra, Wynberg, Sandton, Orange Grove, Houghton	773	334
			City of Johannesburg F: Inner City, Johannesburg South	1142	270
			City of Johannesburg G: Orange Farm, Weilers Farm, Ennerdale, Lenasia, Eldorado Park, Protea South	343	129
			Unallocated	236	84
City of Tshwane	1 385	405	**City of Tshwane 1:** Ga-Rankuwa, Mabopane Winterveldt, Soshanguve, Rosslyn, Karenpark, Wonderboom, Akasia, Nina Park, Orchads, Amandasig, Thereza Park, Pretoria North	198	69
			City of Tshwane 2: Hammanskraal, Temba, Suurman, Dilopye, Stinkwater, Ramotse, New Eesterus, Kameeldrit, Pyramid/Rooiwal, Doornpoort, Kekana Gardens, KekansStad, Marokolong, Randstown, Kanana	85	18
			City of Tshwane 3: Atteridgeville, Laudium, Pretoria CBD, Hercules, Danville, Saulsville, Lotus, Pretoria West	496	119
			City of Tshwane 4: Lyttelton, Eldoraigne , Waterkloof, Olievenhoutbosch, Rooihuiskraal, Lyttelton, Silverton, Centurion, Brooklyn, Hatfield	301	74
			City of Tshwane 5: East Lynne, Rayton, Cullinan, Dewagensdrift, Refilwe, Silverton, Onverwacht	30	16
			City of Tshwane 6: Eesterus, Lethabong, Mamelodi, Silverlakes, Garsfontein, Lynnwood, Queenswood, Wilgers, Watloo, Equestria, Mooikloof, Brummeria	209	84
			City of Tshwane 7: Ekangala, Sokhulumi, Dark City, Zithobeni, Bronkhorstspruit, Kanana, Rethabiseng	25	15
			Unallocated	41	10
City of Ekurhuleni	1 961	766	**Ekurhuleni East 1:** Etwatwa, Daveyton, Brakpan, Tsakane	151	78
			Ekurhuleni East 2: Springs, Kwa-Thema, Duduza, Nigel	120	45
			Ekurhuleni North1: Birchleigh, Birchleigh North, Bonaero Park, Crystal Park, Erin, Ethafeni, Kempton Park, Olifantsfontein, Tembisa, Winnie Mandela	478	224
			Ekurhuleni North 2: Bedfordview, Boksburg, Chief Albert Luthuli, Dan Kubheka, Edenvale, Endayeni, Esangweni, Itireleng, Kemston, Lethabong, Ramaphosa, Reiger Park, Van Dyk Park	553	206
			Ekurhuleni South 1: Alberton, Brackenhurst, Eden Park, Primrose, Germiston, Leondale, Dawn Park, Tswelopele, Vosloorus, Villa Liza	422	161
			Ekurhuleni South 2: Thokoza, Greenfields, Katlehong, Moleleki, Zonkizizwe, Palm Ridge, Moleleki, Tsietsi Sunrise View, Tamaho, Khumalo, Motsamai	166	44
			Unallocated	71	8
Sedibeng	235	65	**Lesedi**	36	6
			Emfuleni	159	48
			Midvaal	24	11
			Unallocated	16	
West Rand	797	263	**Mogale City**	277	50
			Rand West City	134	16
			Merafong City	304	197
			Unallocated	82	
Unallocated	634				
Total	9897	3207			
Deaths	81				

GAUTENG
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A map of the Tshwane regional areas is presented in Figure A3, together with a map of the WWTW serving the City of Tshwane (CoT). Region 3, consisting of Atteridgeville, Laudium, Pretoria CBD, Hercules, Danville, Saulsville, Lotus and Pretoria West, was the area with the highest number of confirmed positive cases as of 9 June 2020 (268 cases and 104 recoveries). A central WWTW that serves the populations located in Tshwane Region 3 was selected.

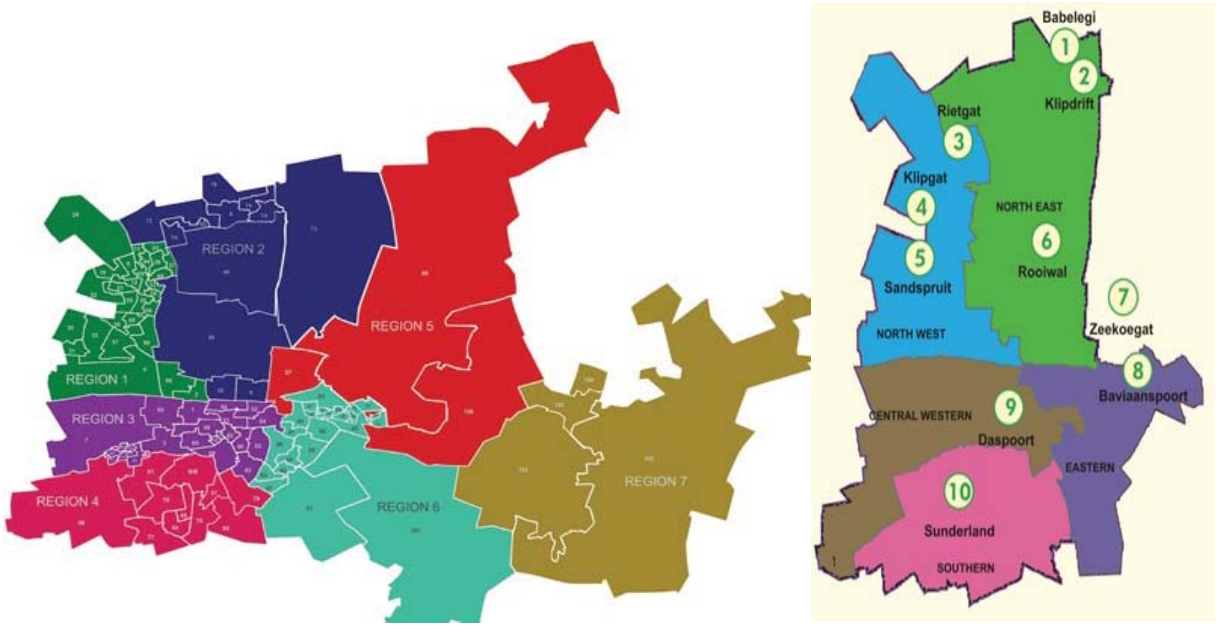

Figure A3: City of Tshwane Regional demarcations (left) and location of the City of Tshwane WWTW (right)

A3: Eastern Cape

The total number of confirmed cases as of the16th of June for the Eastern Cape was 11 039. While District based data was available for the Eastern Cape for May 2020 (Figure A4), there was no more recent information published at the time of sample selection. Although the Eastern Cape had the third highest number of positive cases in the country at the start of the study, for the purposes of the proof of concept study the decision was taken to exclude WWTW from this area due to the difficulty of sample collection and transport logistics as well as a lack of detailed case distribution information.

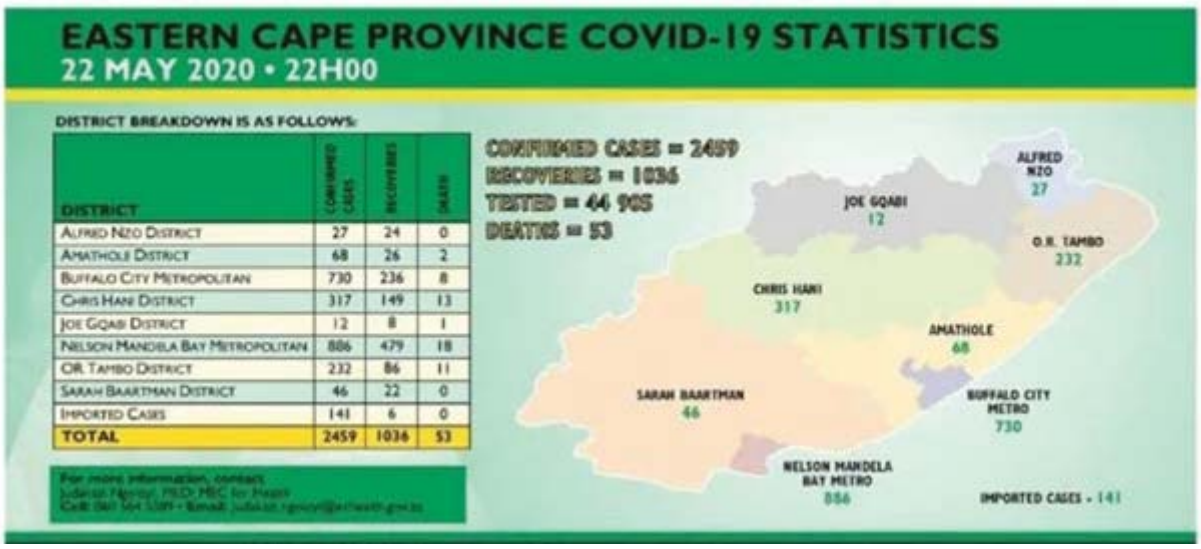

Figure A4: Covid-19 cases per District in the Eastern Cape (May 2020)

A4: KwaZulu-Natal

As of the 16th of June, KZN had 4048 positive cases. eThekwini and the iLembe District of KZN were the province's hotspots, with iLembe, located to the north of eThekwini, showing 0.9% of the total national infections (Figure A5). A WWTW from the iLembe District Municipality was selected for sampling.

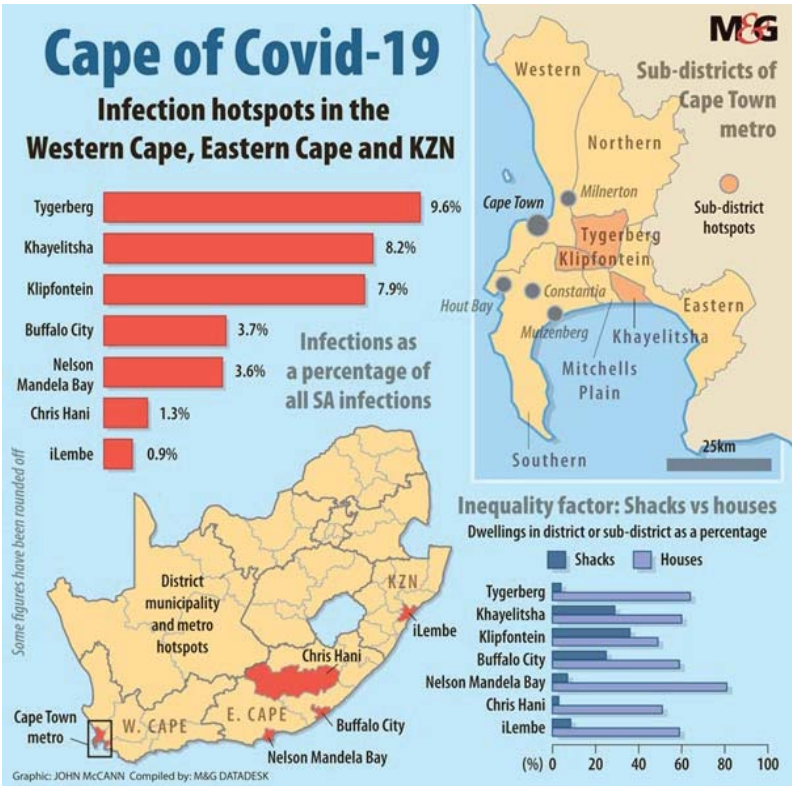

Figure A5: Infection hotspots in the Western Cape, Eastern Cape and KwaZulu-Natal