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**77**  
**PROOF OF CONCEPT STUDY  
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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iii  
**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.  
iv  
**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.  
v  
**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**x 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.  
x 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 2,7×104  
for N1 and 4,2-5,5 × 104 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).  
vi  
**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 × 104 for N1 and 4,2-5,5 × 104 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*  
vii  
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**  
viii  
**ACKNOWLEDGEMENTS**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
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POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
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ix  
**CONTENTS**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
**EXECUTIVE SUMMARY ..............................................................................................................................iii  
ACKNOWLEDGEMENTS ..........................................................................................................................viii  
CONTENTS .................................................................................................................................................. ix  
LIST OF FIGURES .......................................................................................................................................xi  
LIST OF TABLES.......................................................................................................................................xiii  
CHAPTER 1: INTRODUCTION .................................................................................................................1**1.1 THE SARS-COV-2 VIRUS ................................................................................................................. 1  
1.2 SARS-COV-2 PERSISTENCE AND FATE IN THE ENVIRONMENT ............................................... 2  
1.2.1 SARS-CoV-2 shedding by infected individuals .................................................................... 2  
1.2.2 SARS-CoV-2 persistence in the environment and susceptibility to disinfection ..................3  
1.3 ENVIRONMENTAL SURVEILLANCE OF SARS-COV-2 .................................................................. 4  
1.3.1 Overview .............................................................................................................................. 4  
1.3.2 Methods for SARS-CoV-2 analysis in environmental samples............................................ 4  
1.3.2.1 Virus recovery from wastewater ......................................................................... 4  
1.3.2.2 Virus detection and quantification....................................................................... 6  
1.3.3 Using wastewater based epidemiology for monitoring COVID-19 infections ......................7  
1.4 ENVIRONMENTAL SURVEILLANCE OF SARS-COV-2 IN THE SOUTH AFRICAN CONTEXT ....8  
1.5 AIMS AND OBJECTIVES OF THE STUDY....................................................................................... 9  
**CHAPTER 2: METHODOLOGY ..............................................................................................................10**2.1 ETHICS APPROVAL .......................................................................................................................10  
2.2 SAMPLING SITES ...........................................................................................................................10  
2.2.1 Selection of sampling sites ................................................................................................10  
2.2.2 Western Cape ....................................................................................................................11  
2.2.3 Gauteng .............................................................................................................................12  
2.2.4 KwaZulu-Natal....................................................................................................................14  
2.2.5 Mpumalanga and Free State .............................................................................................14  
2.3 SAMPLING METHODOLOGY .........................................................................................................16  
2.3.1 Sampling of wastewater treatment plant influent ...............................................................16  
2.3.2 Sampling of industry sewage package plants, prison and hospital ...................................16  
2.3.3 Sampling of surface environmental samples .....................................................................17  
2.4 METHODS FOR SAMPLE PROCESSING AND ANALYSIS ..........................................................17  
2.4.1 Viral recovery .....................................................................................................................17  
2.4.1.1 Sample clarification ..........................................................................................17  
2.4.1.2 Polyethylene glycol 8000/sodium chloride precipitation ...................................17  
2.4.1.3 Skimmed-Milk flocculation ................................................................................18  
2.4.1.4 Aluminium hydroxide adsorption-precipitation..................................................18  
2.4.2 Viral detection ....................................................................................................................20  
2.4.2.1 Nucleic acid extraction......................................................................................20  
2.4.2.2 Viral detection using the Allplex™ 2019 nCoV assay ......................................20  
2.4.2.3 Viral detection using the QuantiFast® Pathogen RT-PCR + IC N1 and N3  
assays...............................................................................................................21  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯  
x  
2.4.2.4 Mengovirus QuantiFast® Pathogen RT-PCR + IC assay ................................21  
2.4.3 Viral quantification ..............................................................................................................21  
2.4.4 Construction of standard curves ........................................................................................22  
2.4.4.1 Mengovirus .......................................................................................................22  
2.4.4.2 SARS-CoV-2 N1 and N3 ..................................................................................22  
2.5 DATA INTERPRETATION AND VISUALIZATION ..........................................................................22  
**CHAPTER 3: RESULTS AND DISCUSSION .........................................................................................23**3.1 SARS-COV-2 ANALYSIS IN MUNICIPAL WASTEWATER SAMPLES ..........................................23  
3.1.1 Determining viral extraction efficiency using Mengovirus recovery from wastewater .......23  
3.1.2 SARS-CoV-2 recovery using PEG precipitation and skimmed milk methods ...................25  
3.1.3 SARS-CoV-2 detection in wastewater ...............................................................................28  
3.1.4 Trends in Ct values ............................................................................................................31  
3.1.5 Viral quantification ..............................................................................................................34  
3.2 SARS-COV-2 ANALYSIS IN WASTEWATER SAMPLES FROM DEFINED POPULATIONS:  
MINES, POWER STATIONS AND PRISON SAMPLE RESULTS ..............................................................35  
3.2.1 Power stations, mines and prison ......................................................................................35  
3.2.2 Hospital samples ................................................................................................................36  
3.3 SARS-COV-2 ANALYSIS IN SURFACE WATER ...........................................................................38  
3.3.1 Water quality ......................................................................................................................38  
3.3.2 Detection of SARS-CoV-2 in surface water .......................................................................39  
3.4 DATA VISUALIZATION AND RESULTS INTERPRETATION ........................................................41  
3.4.1 Visualising trends in Ct values and data interpretation ......................................................41  
3.4.2 Data reporting ....................................................................................................................47  
3.5 SUMMARY OF FINDINGS ..............................................................................................................47  
3.5.1 Grab vs. composite wastewater samples ..........................................................................47  
3.5.2 Use of primary sludge for SARS-CoV-2 monitoring...........................................................47  
3.5.3 Efficiency of virus recovery ................................................................................................48  
3.5.4 Methods for viral detection .................................................................................................49  
3.5.5 SARS-CoV-2 detection in non-sewered samples ..............................................................49  
3.5.6 Data interpretation and reporting .......................................................................................49  
**CHAPTER 4: CONCLUSION AND RECOMMENDATIONS ...................................................................50**4.1 CONCLUSIONS...............................................................................................................................50  
4.2 RECOMMENDATIONS FOR SCALING UP INTO NATIONAL SURVEILLANCE PROGRAMME .51  
4.2.1 Sampling methodology ......................................................................................................51  
4.2.2 SARS-CoV-2 detection ......................................................................................................51  
4.2.3 Recovery methods .............................................................................................................51  
4.2.4 SARS-CoV-2 gene assays.................................................................................................52  
4.2.5 Viral quantification ..............................................................................................................52  
4.2.6 Upstream sampling and monitoring of smaller defined populations ..................................52  
4.2.7 Analysis of non-sewered environmental water samples ....................................................52  
4.2.8 Data visualisation and trend monitoring .............................................................................53  
**REFERENCES ............................................................................................................................................54  
APPENDIX A: COVID-19 CASES IN SELECTED PROVINCES AS OF 16TH JUNE 2020 ......................60**  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯  
xi  
**LIST OF FIGURES**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
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). ............................................................................................................................................................ 1  
Figure 1-2: General use case: Source Water Research Foundation 2020 .................................................. 7  
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/ ................................................ 10  
Figure 2-2: Map showing the selected sites for sampling .......................................................................... 11  
Figure 2-3: Map of sampling locations in the Western Cape by type ......................................................... 12  
Figure 2-4: Map of sampling locations in Gauteng by type ........................................................................ 13  
Figure 2-5: Map of sampling locations in KwaZulu-Natal by type .............................................................. 14  
Figure 2-6: Map of sampling locations in Mpumalanga by type ................................................................. 15  
Figure 2-7: Map of sampling locations in Free State by type ..................................................................... 15  
Figure 2-8: Sampling and sample preparation methodology flow chart representation ............................. 16  
Figure 2-9: Workflow for virus recovery from wastewater samples, comparing the PEG precipitation,  
skimmed milk flocculation and Aluminium Hydroxide Adsorption-Precipitation methods .......................... 19  
Figure 2-10: Workflow for virus extraction and detection in wastewater samples...................................... 20  
Figure 3-1: A) Real time RT-PCR amplification curves of 10-fold serial dilutions of mengovirus RNA  
starting at 1.4 × 105. 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). .......................................................... 23  
Figure 3-2: Internal control amplification during 60 RT-PCR reactions in the mengovirus recovery  
experiment .................................................................................................................................................. 25  
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 ................................. 26  
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 ............................................ 27  
Figure 3-5: A comparison of the Quantifast N1 and N3 assays with the Seegene 2019 nCoV assay for  
Western Cape samples .............................................................................................................................. 28  
Figure 3-6: Comparison of Ct values for sewage influent samples per assay method .............................. 29  
Figure 3-7: Comparison of Ct values for all samples per assay method .................................................... 29  
Figure 3-8: Comparison of Ct values by recovery method and assay ....................................................... 30  
Figure 3-9: Comparison of Ct values by inactivation/ re-suspension method and assay method ............. 31  
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) ..................................................................................... 32  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯  
xii  
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 ............................... 32  
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) ................................................... 33  
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) ....................................................... 33  
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, r2=0.997, Efficiency= 103.6% for the N1 target and Slope -3.102, r2=0.993, Efficiency=  
110.1% for the N3 target............................................................................................................................. 34  
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 ................................................................................................................. 35  
Figure 3-16: SARS-CoV-2 gene detection for prison, power station and mine .......................................... 36  
Figure 3-17: Detection of SARS-CoV-2 samples in hospital wastewater over a 4 week period ................ 37  
Figure 3-18: Covid case numbers for the Western Cape, with period of hospital sampling indicated in the  
block............................................................................................................................................................ 37  
Figure 3-19: Detection of SARS-CoV-2 in surface water ........................................................................... 39  
Figure 3-20: Number of SARS-CoV-2 gene targets amplified per sample site and recovery volume ....... 40  
Figure 3-22: 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)...................................................................................... 43  
Figure 3-23: 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)...................................................................................... 44  
Figure 3-24: 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) ............................................................................ 45  
Figure 3-25: Trends in Ct values, by minimum Ct value per site for surface water samples ..................... 46  
Figure 3-25: Mapping of surveillance sites to indicate current status ........................................................ 46  
Figure 3-26: Example of proposed Covid-19 surveillance dashboard as per the NICD poliovirus  
surveillance platform ................................................................................................................................... 47  
Figure 4-1: General use case: Source Water Research Foundation 2020 ................................................ 53  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯  
xiii  
**LIST OF TABLES**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
Table 2-1: Primers and probes for SARS-CoV-2 and mengovirus detection ............................................. 21  
Table 3-1: Mengovirus recovery efficiency from grab and composite raw wastewater samples. .............. 24  
Table 3-2: Surface water quality analysis results ....................................................................................... 38  
Table 3-3:Typical Values for Untreated Domestic Wastewater (Nozaic & Freese, 2009) ......................... 38  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯  
1  
**CHAPTER 1: INTRODUCTION**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
**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  
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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).  
**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 SARSCoV-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  
FRSLHVSHUP/WR௖î௖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  
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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. SARSCoV-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. SARSCoV-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).  
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**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 flocculationbased 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).  
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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 singlestranded 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-ȝP SRUH-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 MgCl2 to the sample  
to achieve a final concentration of 25 mM MgCl2. 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  
FRQFHQWUDWHGVDPSOH  
ȝ/ZDVFROOHFWHGIURPWKHVDPSOHUHVHUYRLUZLWKDSLSHWWHDQGWUDQVIHUUHGLQWR  
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  
0LOOLSRUH7KHFRQFHQWUDWHGVDPSOH  
ȝ/ZDVFROOHFWHGDQGPL[HGZLWKȝ/RI'1DVHDQG51DVH  
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 ZDVUHVXVSHQGHGLQȝ/7UL]RO)LQDOO\ȝ/RI  
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). 7KHSHOOHWZDVUHVXVSHQGHGLQȝ/RIî3%6  
S+DQGWUDQVIHUUHG  
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 MgCl2) provided the highest mean  
MHV recovery of 65.7 ± 23.0%. The second highest mean recovery was for Method B, the adsorptionextraction 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  
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(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.  
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**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-waterresearch/). 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**  
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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.  
**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, SARSCoV-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 nonpoint 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  
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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.  
**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  
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**CHAPTER 2: METHODOLOGY**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
**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).  
**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.  
**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.

|  |  |
| --- | --- |
| **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** |

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**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:  
x City of Cape Town:  
o WC\_CCT1(105 Ml/d)  
o WC\_CCT2 (200 Ml/d)  
o WC\_CCT3 957 Ml/d)  
o WC\_CCT4 (72 Ml/d)  
x Cape Winelands District  
o 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.  
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**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:  
x City of Ekurhuleni:  
o GP\_ERWAT1 (105 Ml/d)  
o GP\_ERWAT2 (83 Ml/d)  
o GP\_ERWAT3 (2 weeks) (19 Ml/d)  
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**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:  
x City of Tshwane:  
o 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:  
o Contaminated greywater runoff sample from Alexandra, Johannesburg (GP\_JUKS1)  
o River sample from the Jukskei River downstream of Alexandra (GP\_ALEXIN)  
o River sample from the Blougatspruit in the Cradle of Humankind, downstream of WWTW  
discharge (GP\_COHK)  
o River sample from Hennops River downstream of Tembisa, upstream of WWTW discharge  
(GP\_HENN1)  
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**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:  
x iLembe District (KwaZulu-Natal):  
o 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:  
x Power stations and mines:  
o MP\_MPS1  
o MP\_MM1  
o FS\_LPS1  
o FS\_NVM1  
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**Figure 2-6: Map of sampling locations in Mpumalanga by type  
Figure 2-7: Map of sampling locations in Free State by type**  
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**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  
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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.  
**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.  
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*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 AlCl3 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.  
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**Figure 2-9: Workflow for virus recovery from wastewater samples, comparing the PEG  
precipitation, skimmed milk flocculation and Aluminium Hydroxide Adsorption-Precipitation  
methods**  
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**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 PL) (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 × 104 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 PL 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  
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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) Medema et al., 2020 |
| SARS-CoV- 2 N3 Nucleocapsid (N) Medema et al., 2020 |
| Mengo Mengo110F Mengo209R Mengo147 5’-GCGGGTCCTGCCGAAAGT-3’ 5’-GAAGTAACATATAGACAGACGCACAC -3’ *5’ MGB*-ATCACATTACTGGCCGAAGC-*TAMRA-3’* Pinto et al., 2009 |

2019-nCoV\_N1-F 5’-GACCCCAAAATCAGCGAAAT-3’ 2019-nCoV\_N1-R 5’-TCTGGTTACTGCCAGTTGAATCTG-3’ 2019-nCoV\_N1-P 5’-FAM-ACCCCGCAT/abNFQ/TACGTTTGGTGGACCNFQ-3’\*  
2019-nCoV\_N3-F 5’-GGGAGCCTTGAATACACCAAAA-3’ 2019-nCoV\_N3-R 5’-TGTAGCACGATTGCAGCATTG-3’ 2019-nCoV\_N3-P 5’-FAM AYCACATTG/abNFQ/GCACCCGCAATCCTGNFQ -3’  
\*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 × 104 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.  
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**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 nonenveloped 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 × 106 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 × 106 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/PL. The plasmid was  
diluted to 100 000 copies/PL 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.  
**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.  
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**CHAPTER 3: RESULTS AND DISCUSSION**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
**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 × 105 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 × 105. 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).  
A  
B**  
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24  
**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 × 106 | 2.8 × 106 | 2.8 × 106 | 2.8 × 106 |
| **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.  
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**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.  
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26  
**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.  
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**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**  
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**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 RTPCR 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.  
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**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**  
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30  
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.  
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31  
**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.  
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32  
**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  
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33  
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)**  
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**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, r2=0.997, Efficiency= 103.6% for the N1 target and Slope -3.102, r2=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  
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35  
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 × 104 for N1 and 4,2-5,5 × 104 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  
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.  
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36  
**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.  
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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**  
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38  
**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 | 105-108 | 107-109 | 107-1010 |
| Faecal Coliforms | count/100 ml | 103-105 | 104-106 | 105- 08 |

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**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  
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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.  
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**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).  
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**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)**  
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**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)**  
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**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)**  
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**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**  
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**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.  
**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  
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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 × 106  
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 MgCl2 pre-treatment was included, 65% of the MHV were  
recovered, when sample was directly filtered through 0.45-ȝPSRUH-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  
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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 ȝPQRPLQDOSRUHVL]HPPGLDPHWHU  
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.  
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**CHAPTER 4: CONCLUSION AND RECOMMENDATIONS**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  
**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 mobilebased 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 × 104 for N1 and 4,2-5,5 × 104 for N3 target genes.  
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*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 usecases 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.  
**RECOMMENDATIONS FOR SCALING UP INTO NATIONAL SURVEILLANCE  
PROGRAMME  
4.2.1 Sampling methodology**x **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.  
x **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  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
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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.  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
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**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**  
POC – Wastewater-based surveillance of SARS-CoV-2 in South Africa  
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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  
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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  
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Table A1: Covid-19 cases per District in Gauteng as of 13 June 2020  
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.  
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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)  
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**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