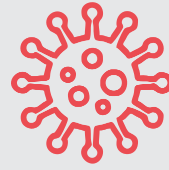

A COMPENDIUM OF EMERGING SOUTH AFRICAN TESTING METHODOLOGIES FOR DETECTING OF SARS-COV 2 RNA IN WASTEWATER SURVEILLANCE

Laboratory Te...
Wastewater
Analysis
Sars-Cov-2

SP 143/20

A COMPENDIUM OF EMERGING SOUTH AFRICAN TESTING METHODOLOGIES FOR DETECTING OF SARS-COV 2 RNA IN WASTEWATER SURVEILLANCE

Compiled and edited by

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FOREWORD

The inputs and submissions contained in this report emanate from contributions made by several research initiatives, individuals, academics and practitioners in South Africa who have responded to the national and global call to action to complement and support the fight against the COVID-19 pandemic.

Surveillance of wastewater treatment plants offers an opportunity for near real-time outbreak data and as an early warning for resurgence of the outbreak. To date, the wastewater-based epidemiology approach has been successfully piloted in developed countries where there is wide coverage of waterborne sanitation, such as the Netherlands, France, United States of America, etc. However, given the varied water and sanitation services delivery mechanisms in South Africa (and lack thereof), the country was in a position to pioneer the development and piloting of an all-encompassing water and sanitation-focused approach for the surveillance of COVID-19 spread in less developed communities. While detection of viral RNA in faeces and urine samples is not an indication that the virus is infectious, further research is required to ascertain if infectious virus particles are excreted in patients' faeces and urine. Furthermore, an investigation of the role of water and sanitation environments in the possible transmission of SARS-CoV-2 through exposure to contaminated surface water sources, poorly treated municipal wastewater, poorly managed domestic greywater, urine and faeces of infected people is necessary.

The South African science community has made remarkable progress in detecting coronavirus RNA signals in wastewater. One of the key developments was the establishment of the South African Collaboration COVID-19 Environmental Surveillance System (SACCESS). The SACCESS partners include Amanzi-4-all, Bath University UK, City of Cape Town, Council for Scientific and Industrial Research (CSIR), Durban University of Technology (DUT), Ekurhuleni Water Care Company (ERWAT), eThekwini Municipality, Instru-Serve, Lumegen Laboratories, Magalies Water, National Department of Health, National Health Laboratory Service (NHLS), National Institute for Communicable Diseases (NICD), National Institute for Occupational Health (NIOH), Nelson Mandela Bay Metro Health, North-West University (NWU), Praecautio, South African Local Government Association (SALGA), South African Medical Research Council (SAMRC), Stellenbosch University, University of Cape Town, University of KwaZulu-Natal (UKZN), Waterlab, Water Research Commission (WRC), Western Cape Department of Health, World Health Organization (WHO) and World Health Organization South Africa (WHO-SA).

ACKNOWLEDGEMENTS

This compendium is generated by the individual efforts of members who have led the SACCESS consortium and is being shared in the interest to advance the fight against the COVID-19 pandemic.

SACCESS

The South African Collaboration COVID-19 Environmental Surveillance System (SACCESS) was initially established by a collective of researchers in the Western Cape. Through a spontaneous and organic process, a network was established which rapidly expanded to involve a wide range of actors including university researchers, municipal sanitation and public health officials, provincial health departments, the NICD, private laboratories and research councils. Driven by a common goal of harnessing science to generate public health tools for better control of the epidemic, participants developed locally appropriate methods, shared experiences, refined tools and built common approaches across 4 provinces and multiple locales. All of this used existing resources or limited dedicated funding. Achievements thus far include the establishment of a national dashboard at the NICD for results from 21 wastewater sites, timeous detection of spikes in the Western Cape, and piloting of protocols for surveillance of high-risk populations (prisons, old age homes, residences).

SOUTH AFRICAN MEDICAL RESEACH COUNCIL

The South African Medical Research Council (SAMRC) was established in 1969 with a mandate to improve the health of the country's population, through research, development and technology transfer, so that people can enjoy a better quality of life. The scope of the organisation's research projects includes tuberculosis, HIV/AIDS, cardiovascular and non-communicable diseases, gender and health, and alcohol and other drug abuse. With a strategic objective to help strengthen the health systems of the country – in line with that of the Department of Health, the SAMRC constantly identifies the main causes of death in South Africa.

THE NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES (NICD)

The NICD is a national public health institute of South Africa, providing reference microbiology, virology, epidemiology, surveillance and public health research to support the government's response to communicable disease threats. The NICD serves as a resource of knowledge and expertise of communicable diseases to the South African Government, Southern African Development Community countries and the African continent. The institution assists in the planning of policies and programmes to support and respond to communicable diseases.

LUMEGEN LABORATORIES

In 2013, Lumegen Laboratories opened its doors in Potchefstroom. The laboratory originated from services previously provided by the North West University's Mitochondrial Research Laboratory at the Centre for Human Metabolomics. The laboratory is still collaborating with the university on various projects to ensure the development of new molecular diagnostic tests. In 2016 the laboratory moved to a new facility to expand and include cattle and wildlife tests.

WATERLAB (Pty) Ltd

Waterlab (Pty) Ltd was established in 1983 as a service company specialising in analytical chemistry, providing services in the various disciplines of water, being potable water supply, sewage treatment, industrial effluents, acid mine drainage or underground water. With the arrival of legislation governing environmental impacts by industry, mining and other activities, the range of services supplied by Waterlab were expanded.

UNIVERSITY OF PRETORIA – Department of Medical Virology

The Department of Medical Virology at the University of Pretoria was established as an independent Department in 1983 after previously resorting within the Department of Medical Microbiology as the "Section: Virology". In October 2004 the Department of Medical Virology became part of the Tshwane Academic Division, Northern Branch of the National Health Laboratory Services (NHLS). The Department is located in the Pathology building, Prinshof Campus, Faculty of Health Sciences, University of Pretoria and is involved in teaching and training, research and the provision of a viral diagnostic service to the public and private sector. The teaching and training component services pre- and post-graduate medical students and post-graduate medical science students specializing in basic and medical virology. Research focuses include Enteric viruses, Environmental Virology, Respiratory viruses and Arboviruses such as West Nile virus, Wesselsbron virus, Shuni virus, Alphaviruses, etc.

CSIR

The Council for Scientific and Industrial Research (CSIR) is a leading scientific and technology research organisation that researches, develops, localises and diffuses technologies to accelerate socioeconomic prosperity in South Africa. The organisation's work contributes to industrial development and supports a capable state. The CSIR was established through an Act of Parliament in 1945 and the organisation's executive authority is the Minister of Higher Education, Science and Technology. The organisation plays a key role in supporting public and private sectors through directed research that is aligned with the country's priorities, the organisation's mandate and its science, engineering and technology competences.

WATER RESEARCH COMMISSION

The WRC was established in terms of the Water Research Act (Act No 34 of 1971), following a period of serious water shortage. It was deemed to be of national importance to generate new knowledge and to promote the country's water research purposefully, owing to the view held that water would be one of South Africa's most limiting factors in the 21st century.

DURBAN UNIVERSITY OF TECHNOLOGY – Institute for Water and Wastewater Technology

The Institute for Water and Wastewater Technology (IWWT) was founded as the Centre for Water Research in mid-90s and subsequently became the Centre for Water and Wastewater Technology. It is one of 13 NRF-recognized research niche areas and was granted institute status in 2011. This organization, based at Durban University of Technology, has over the years developed into a "Centre of Excellence" and proficiency. The focus of the institute is largely based on developing and optimizing technology for the treatment of water and wastewater, and green energy to satisfy the needs of industry and the community. Research projects are selected and designed in close consultation with industrial partners with the aim to help industries maintain acceptable levels of effluent discharges and to meet increasing energy requirements. This plays an essential role in reducing negative environmental impact and commercialization of products generated from waste streams.

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CASE 1

WRC Proof of Concept Study: Sampling of wastewater treatment plant influent and rivers as a non-invasive, preliminary surveillance method to establish the spread of SARS-CoV-2 in South African communities: Summary of methodologies applied for wastewater analysis

J. Mans[1], M.B. Taylor[1], B. Genthe[2], L. Coetzee[3], G. Pocock[3]

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1 Introduction

The concept of screening municipal sewage as an epidemiological tool for viruses is not a new concept, and has been used to help inform broader infectious disease epidemiological surveillance and mitigation efforts such as the Global Polio Eradication Initiative (Hovi el al., 2012, Humayan et al., 2014). Environmental surveillance has also been used and recommended for other infections, such as typhoid (WHO, 2018), early warning of hepatitis A and norovirus outbreaks (Hellmér et al., 2014) as well as for antimicrobial resistance (Hendriksen, 2019) with modelling techniques used to assist both the design and interpretation of those efforts (Wang et al., 2020a, Wang et al., 2020b). Wastewater based epidemiology (WBE) is also commonly used in the surveillance of licit and illicit drugs and various chemical contaminants which may impact human health (Choi et al., 2018).

In laboratories, identification of SARS-CoV-2, the virus responsible for the Covid-19 disease, mainly includes viral isolation and viral nucleic acid detection. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) assays targeting small regions of the SARS-CoV-2 genome have been developed and are routinely applied in clinical testing (Corman et al., 2020). SARS-CoV-2 is shed at relatively high titres in the stool of some individuals. Viral gastrointestinal infection, or at least shedding, can remain for some time after clearance of the virus in the respiratory tract (Xiao et al., 2020, Xu 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). The detection of SARS-CoV-2 in sewage is relevant not necessarily because of the potential risk of disease spread, but rather because of the potential to determine the presence of infected individuals in a community. SARS-CoV-2 screening in raw sewage water using RT-PCR can therefore 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. 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.

If we can rapidly replicate the WBE programmes gaining traction internationally 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, the Water Research Commission commissioned this short-term, preliminary proof of concept study prior to the roll-out of a pilot and finally a national wastewater surveillance programme. This will require a coordinated effort by numerous laboratories. The purpose of this document is to review the methodologies used by the various laboratories in South Africa that are currently analysing wastewater for the presence of SARS-CoV-2, with the aim of identifying those methods that are most practical and cost effective for use in a national surveillance programme. It will also be important to determine whether it is necessary for all laboratories to follow the same protocol, or whether it is possible for the methods to be used interchangeably.

The sampling methodology used by the research team appointed by the Water Research Commission for the proof of concept study is presented and discussed below.

2 Sampling methodology

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. For the proof of concept samples were taken for a period of 4 weeks using automated composite samplers installed at the wastewater treatment works. In addition to the composite samples, grab samples were taken from three of the WWTW during the morning peak at 9am, in order to compare the viral recovery efficiency with the composite samples.

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.

3 Methodology for sample processing and analysis

3.1 Viral recovery

3.1.1 Sample clarification

Samples were first clarified prior to viral recovery. The 1-2 L sewage samples were shaken and mixed thoroughly prior to a 200 mL aliquot being poured off for further processing. The aliquot was clarified by centrifugation (Sorvall T20, du Pont) for 30 minutes at 1180 x g at 4°C after which the supernatants 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 extraction were applied, as discussed below, and illustrated in Figure 1.

3.1.2 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 then divided into 4 x 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 x g at 4°C. The supernatant was carefully poured off and discarded into biohazardous waste drum 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 inactivation 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.

3.1.3 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. In brief, 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 x 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.

3.1.4 Aluminium hydroxide adsorption-precipitation

The aluminium hydroxide method is an adsorption-precipitation method previously described for concentrating enteric viruses from wastewater and effluent water, modified for this study from AAVV, 2011; Randazzo et al., 2019, Randazzo et al., 2020a, Randazzo et al., 2020b. In brief, 200 mL of wastewater samples had the pH adjusted to 6.0 before adding 1 part 0.9 N $AlCl_3$ solution to 100 parts sample and readjusting the pH to 6.0. Samples were mixed using an orbital shaker at 150 rpm for 15 minutes at room temperature. Viruses were concentrated by centrifugation at 1,700 × g for 20 minutes and the pellet resuspended in 1 mL Trizol and stored at -20°C until nucleic acid extraction took place.

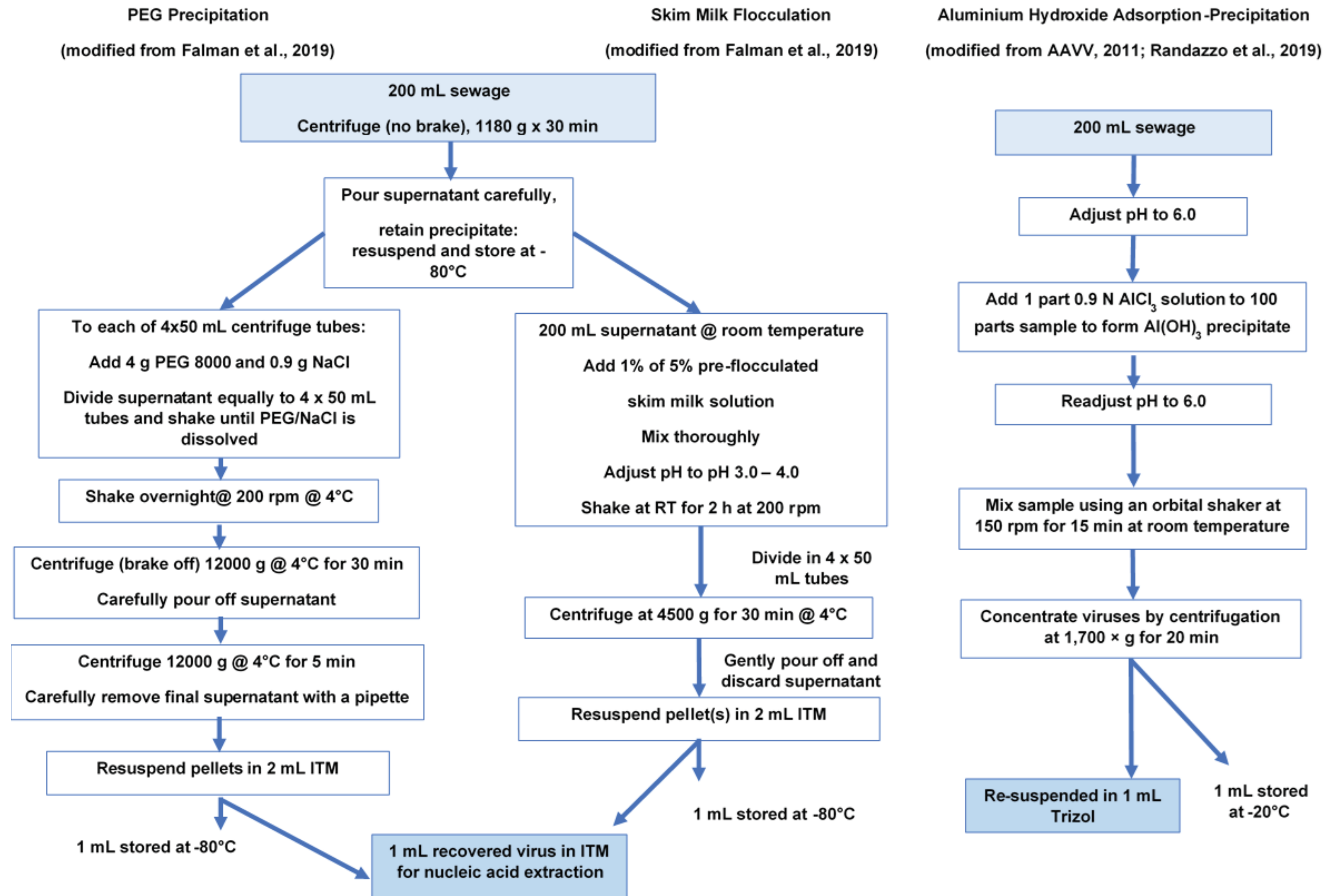


Figure 1: Workflow for virus recovery from wastewater samples, comparing the PEG precipitation, skim milk flocculation and Aluminium Hydroxide Adsorption-Precipitation methods

3.2 Viral detection

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

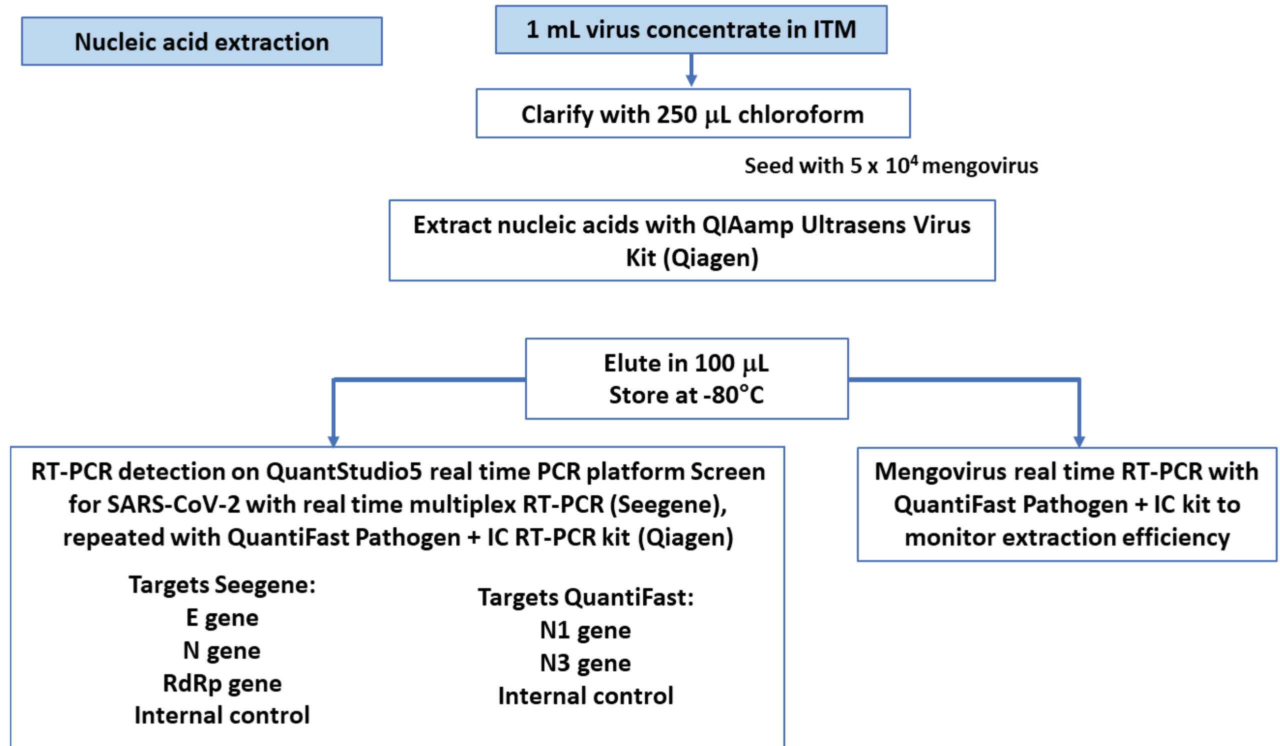


Figure 2: Workflow for virus extraction and detection in wastewater samples.

3.2.1 Nucleic acid extraction

All samples were pre-treated with chloroform prior to extraction. Chloroform (250 mL) was added to 1 mL recovered virus concentrate and the mixture was vortexed 3 x 15 seconds and then incubated at room temperature for 5 minutes before centrifugation at 1500 x g for 10 minutes. The upper phase (~ 1 mL) was transferred to a 2 mL microcentrifuge tube and spiked with 5 x 10^4 mengovirus to enable monitoring of extraction efficiency. Mengovirus strain MC0 was kindly provided by Professor Albert Bosch, Department of Microbiology, Facultat de Biologia, University of Barcelona, Barcelona, Spain.

Viral nucleic acids were extracted from the spiked sample using the QIAamp® Ultrasens® Virus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleic acids were eluted in 100 µL buffer AVE and stored at -80°C.

3.2.2 Viral amplification

3.2.2.1 *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 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 mL RNA were added to each reaction. The real time RT-PCR was performed on a QuantStudio™ 5 Real Time PCR System (Applied Biosystems, Foster City, CA). The target/reporter combinations were E gene (FAM), N gene (CY5), RdRp gene (ROX) and the internal control (VIC). QuantStudio™ 5 Design and Analysis Software v 1.5.1 was used to analyse data. Samples with cycle threshold (Ct) values <40 were considered positive. In the event that the internal control amplification failed and no SARS-CoV-2 targets were amplified, the assay was repeated with a 1 in 10 dilution of the nucleic acids.

3.2.2.2 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 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 x QuantiFast® Pathogen Master Mix, 400 nM forward and reverse N1 or N3 primers, 160 nM N1 or N3 probes, 1 x Internal Control Assay mix, 1 x Internal Control RNA and 0,25 mL QuantiFast® Pathogen RT mix in 20 mL. 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.

3.2.2.3 Mengovirus QuantiFast® Pathogen RT-PCR + IC assay

Mengovirus was detected in each sample to determine nucleic acid extraction efficiency. Published primers and probe (Table 1) (Pinto et al., 2009) were used with the QuantiFast® Pathogen RT-PCR + IC kit (Qiagen). The reaction mix consisted of 1 x QuantiFast® Pathogen Master Mix, 400 nM Mengo110F and Mengo209R primers, 160 nM Mengo147 probe, 1 x Internal Control Assay mix, 1 x Internal Control RNA and 0,25 mL QuantiFast® Pathogen RT mix in 20 mL. 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 1: Primers and probes for SARS-CoV-2 and mengovirus detection

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

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

3.2.3 Construction of standard curves

3.2.3.1 *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 copies/mL. The plasmid was diluted to 100 000 copies/mL 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.

3.2.3.2 *Mengovirus*

Mengovirus with a TCID50 titre of 1.4 x 10^6 was used to construct a standard curve. Serial ten-fold dilutions 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.

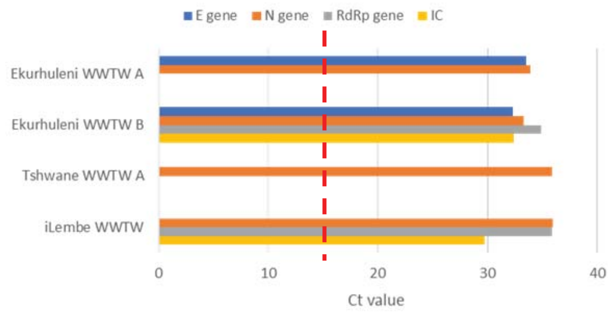
3.3 Viral quantification

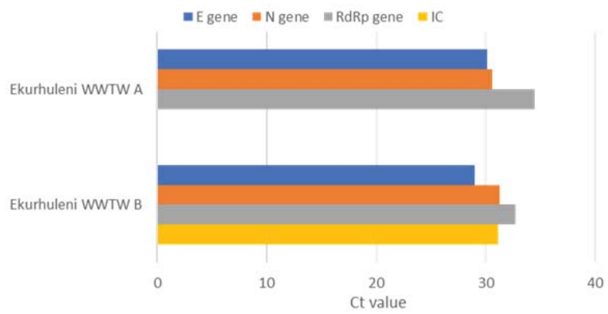
The SARS-CoV-2 genome copies (GC) per reaction for each tested sample was calculated based on the N1 or N3 standard curves and then further converted to GC/mL. Nucleic acid extraction efficiency was assessed using mengovirus. The mengovirus TCID50 titre representing 100% extraction efficiency was determined from 5 x 10^4 mengovirus spiked in 1 mL PBS and extracted with the QIAamp Ultrasens kit. 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.

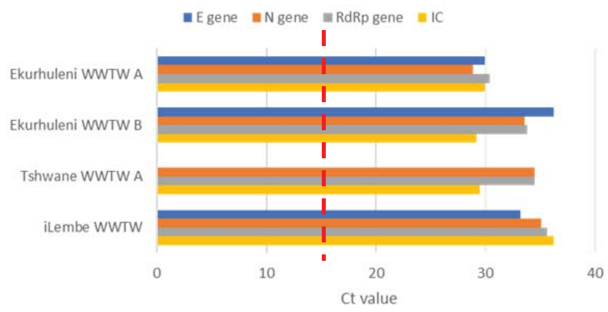
4 Comparison of methods

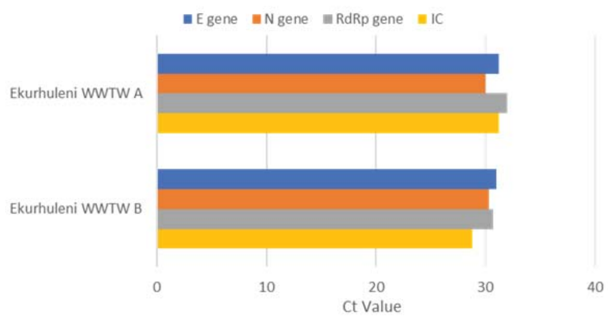
4.1 Composite versus grab sampling

Results of 24-hour composites and grab samples taken during peak flow, using the PEG precipitation recovery method, are presented in Figure 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.


Week 1, Composite sample, PEG recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
Ekurhuleni WWTW B
Tshwane WWTW A
iLembe WWTW
0
10
20
30
40
Ct value


Week 1, Grab sample, PEG recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
Ekurhuleni WWTW B
0
10
20
30
40
Ct value


Week 2, Composite sample, PEG recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
Ekurhuleni WWTW B
Tshwane WWTW A
iLembe WWTW
0
10
20
30
40
Ct value


Week 2, Grab sample, PEG recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
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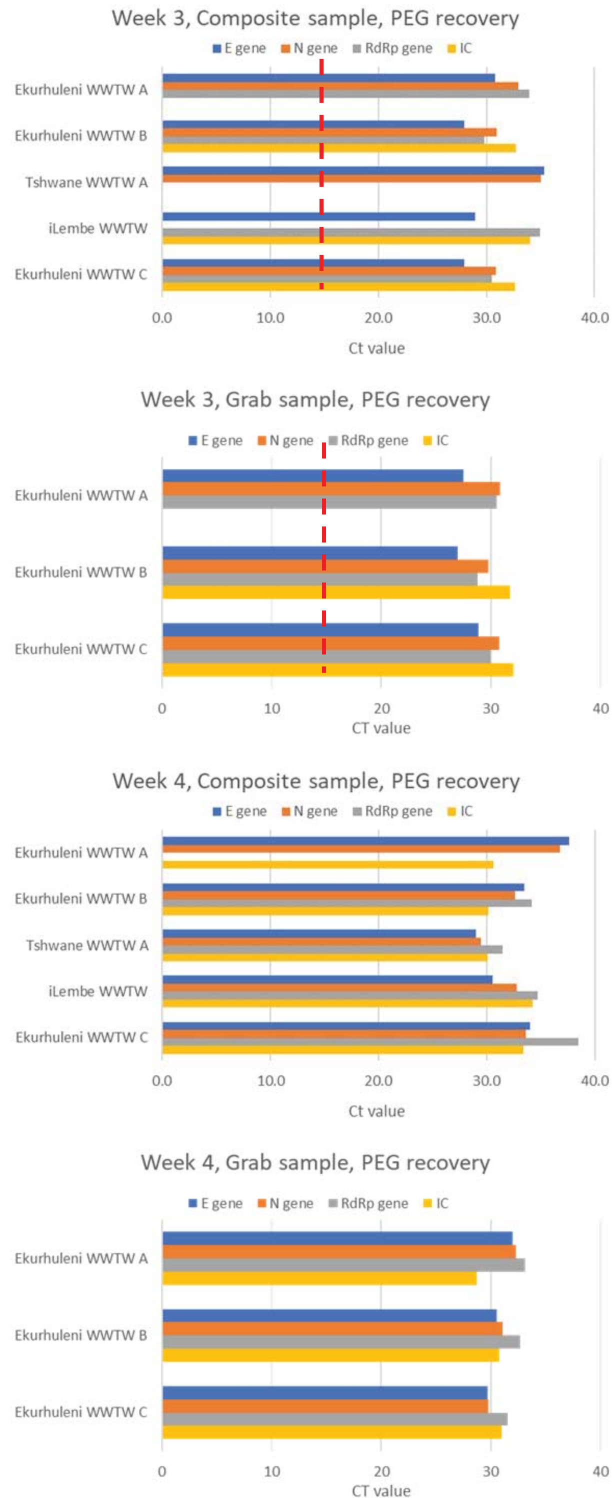
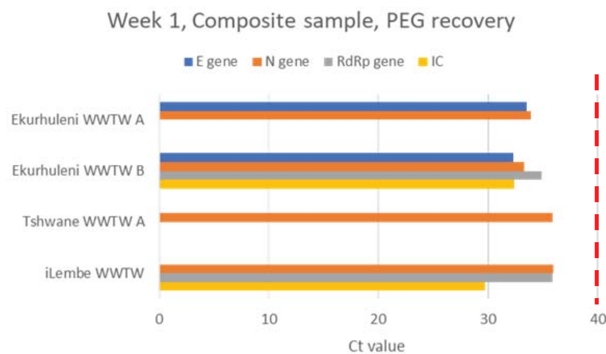


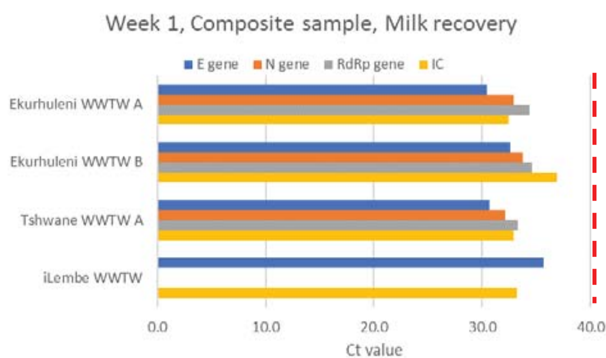
Figure 3: SARS-CoV-2 gene amplification and internal control (IC) for composite and grab samples for weeks 1-4, with PEG recovery. Ct value of 40 and below were considered positive

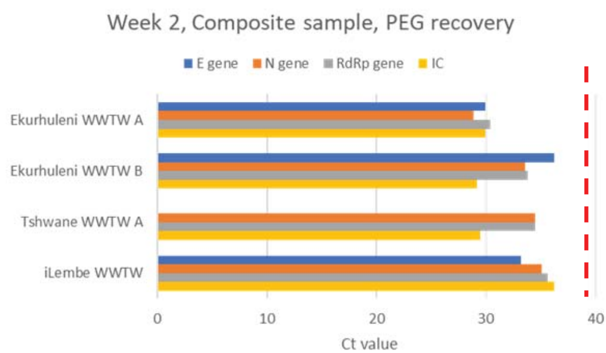
4.2 Comparison of virus recovery methods

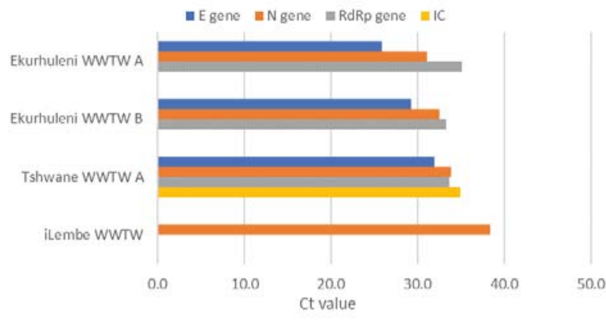
A comparison between the results achieved with the PEG precipitation recovery method when compared with the skim milk flocculation method are presented in Figure 4. These two recovery methods were applied to all the samples from the City of Ekurhuleni, City of Tshwane

and the iLembe District. The success of the milk recovery method was variable between different sites. In the week 1 samples it clearly improved recovery from the Ekurhuleni WWTW A and Tshwane WWTW A samples. However, the PEG recovery method performed better for the samples from the iLembe WWTW, as well as the Ekurhuleni WWTW C sampled in week 3. In week 4 the skim milk method improved recovery at several of the sites, notably the Ekurhuleni WWTW A and iLembe WWTW and Ekurhuleni WWTW C. The effect of the skim 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. It should be noted that the method is successful, and could be used interchangeably or even preferentially to the PEG method. As a cheaper and faster method to employ this is advantageous.








Week 2, Composite sample, Milk recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
Ekurhuleni WWTW B
Tshwane WWTW A
iLembe WWTW
0.0
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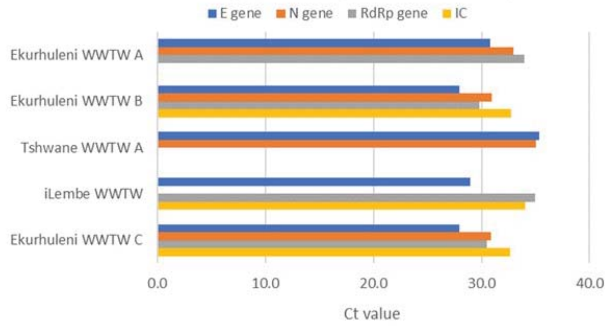
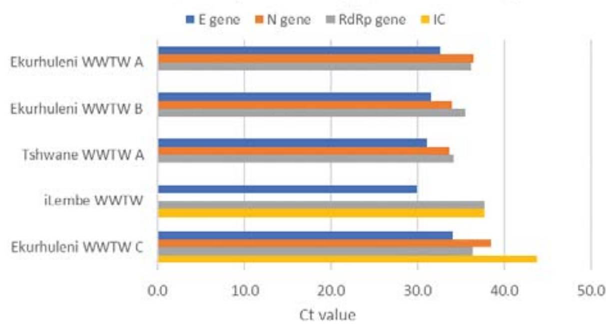
Ct value


Week 3, Composite sample, PEG recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
Ekurhuleni WWTW B
Tshwane WWTW A
iLembe WWTW
Ekurhuleni WWTW C
0.0
10.0
20.0
30.0
40.0
Ct value


Week 3, Composite sample, Milk recovery
E gene
N gene
RdRp gene
IC
Ekurhuleni WWTW A
Ekurhuleni WWTW B
Tshwane WWTW A
iLembe WWTW
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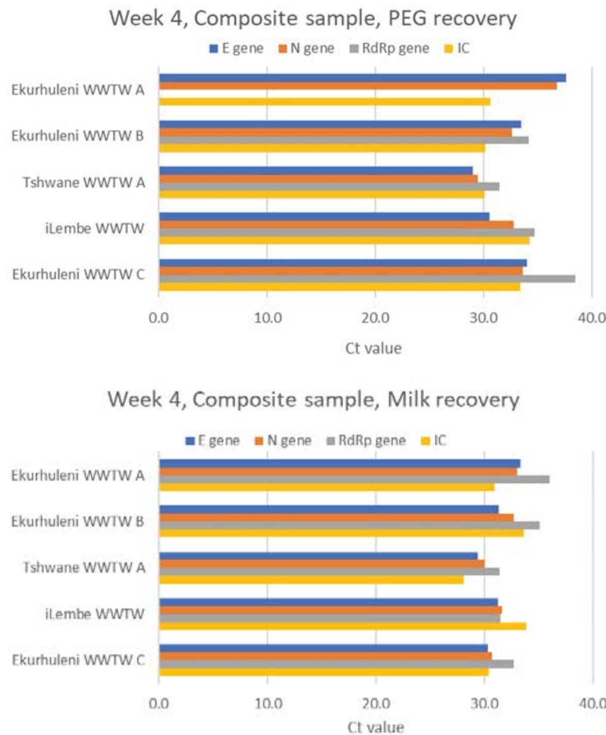

Figure 4: SARS-CoV-2 gene amplification and internal control (IC) for weeks 1-4, comparing PEG and SKIM MILK recovery. Ct value of 40 and below were considered positive

The Aluminium hydroxide adsorption-precipitation method was applied to all the samples from the Western Cape. Figure 5 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 skim 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 skim 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 skim 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.

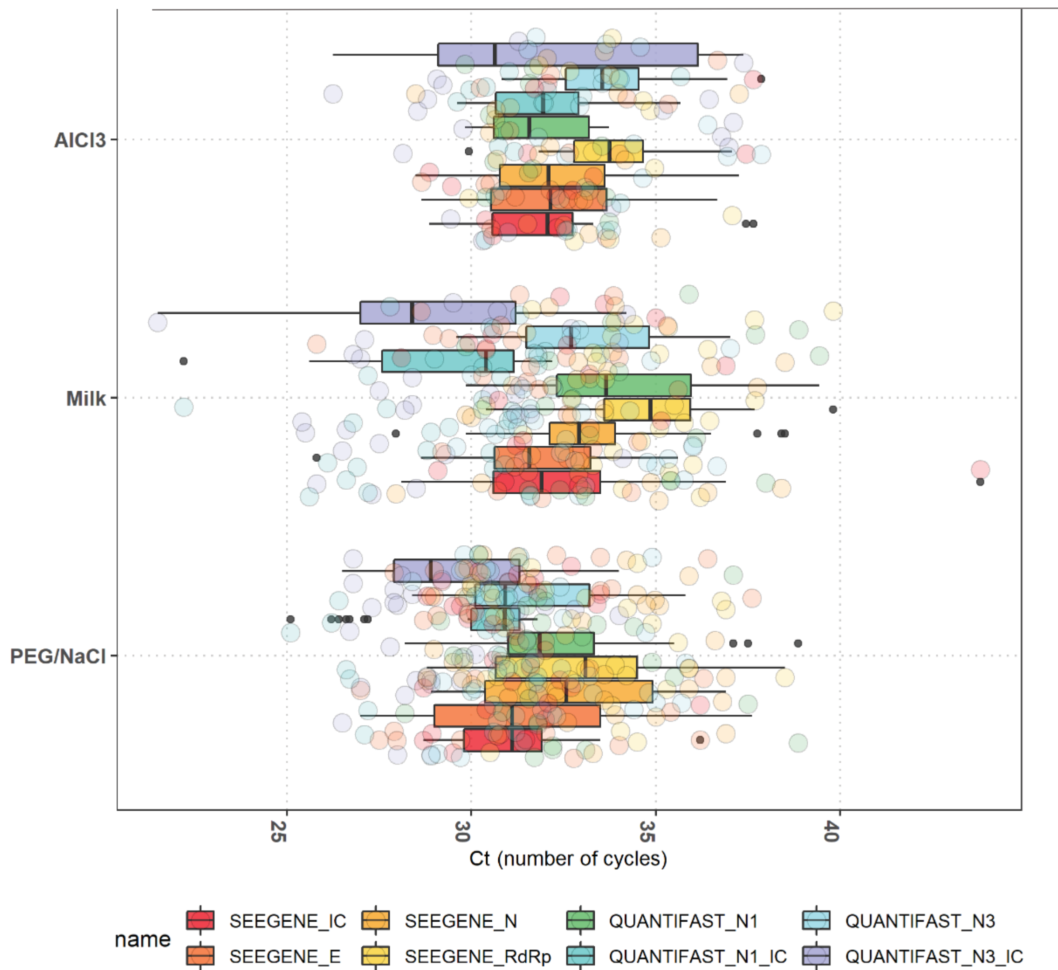

Figure 5: Comparison of Ct values by recovery method and assay

A comparison between the resuspension/inactivation reagents and their impact on the assays is presented in Figure 6. Only the samples from the Western Cape were re-suspended and inactivated in Trizol; the remaining samples were either inactivated in ITM or PBS for comparison. It was possible to extract and amplify the virus from all three suspension media.

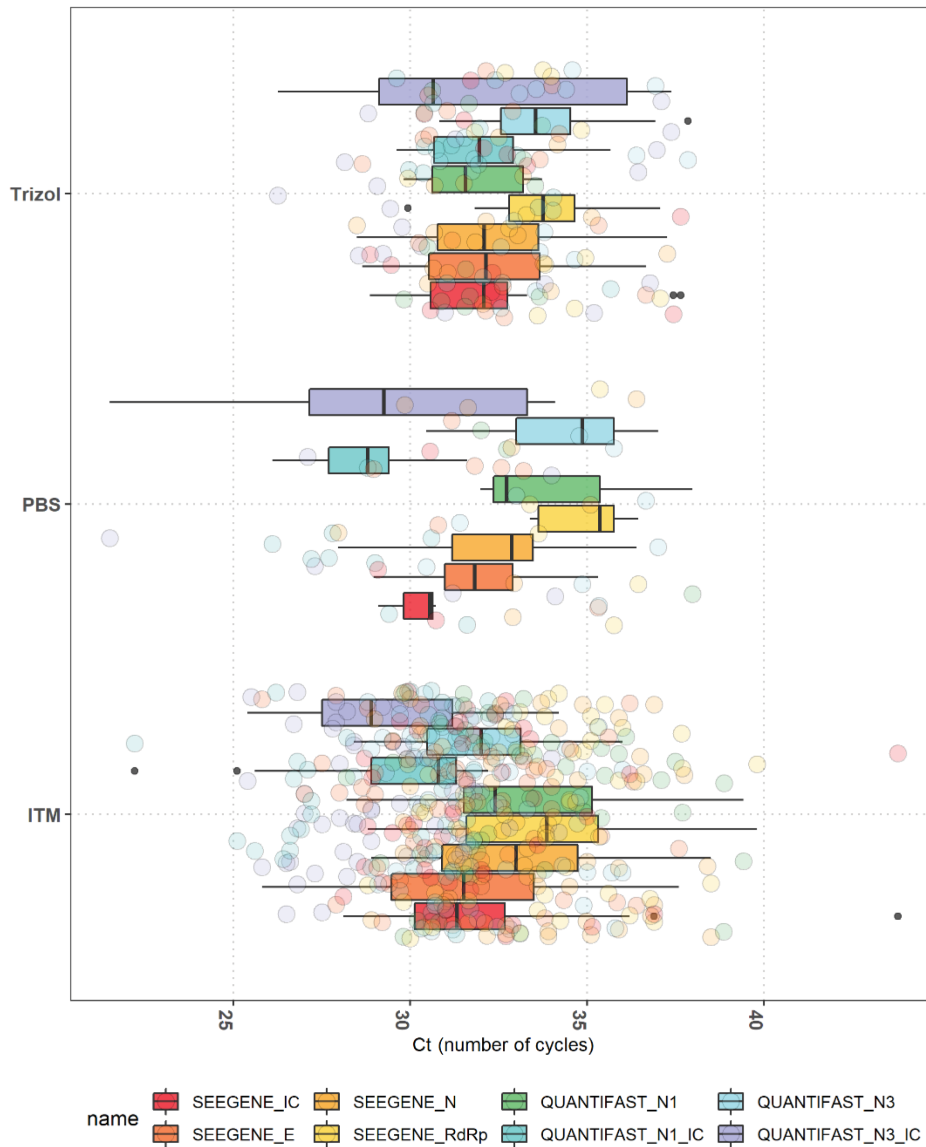

Figure 6: Comparison of Ct values by inactivation/ re-suspension method and assay method

4.3 Comparison between assays: QuantiFast SARS-CoV-2 N1 and N3 and Seegene 2019 nCoV assays

A comparison of the Quantifast N1 and N3 assays with the Seegene 2019 nCoV N gene assay is illustrated with samples from 5 sample points in the Western Cape (Figure 7). Virus extraction for these samples was done with the aluminium hydroxide flocculation-precipitation method. 13/20 samples were positive with all three assays, 4/20 detected by two assays, 3/20 one assay and 0/20 negative by all assays. When considering only the Western Cape samples, the Seegene 2019 nCoV N gene assay appeared to be the most sensitive, followed by the Quantifast N3 assay.

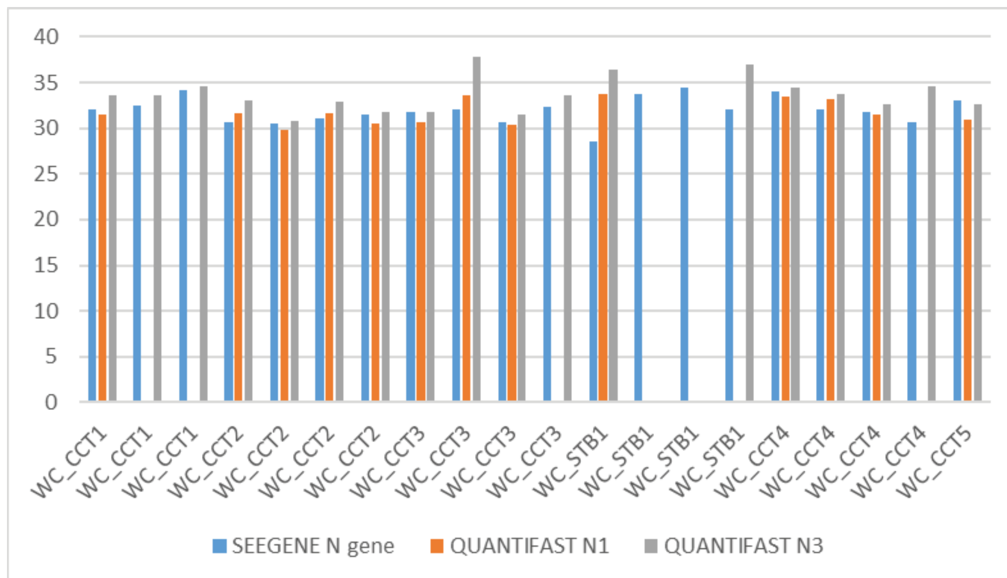

Figure 7: A comparison of the Quantifast N1 and N3 assays with the Seegene 2019 nCoV assay for Western Cape samples

While the same extraction methods were not used for all sites, the Seegene 2019 nCoV and Quantifast N1 and N3 assays were all applied to all samples. Figure 8 shows a comparison of 56 different samples of either grab or composite sewage influent for 86 RT-PCR assays. For the WWTW sewage influent, the Seegene N (95%) and QuantiFast N3 (96.5%) detected most consistently. 58% of samples came up positive for all 5 targets, 21% were positive for 4 targets, and 13% of samples were positive for 3 targets.

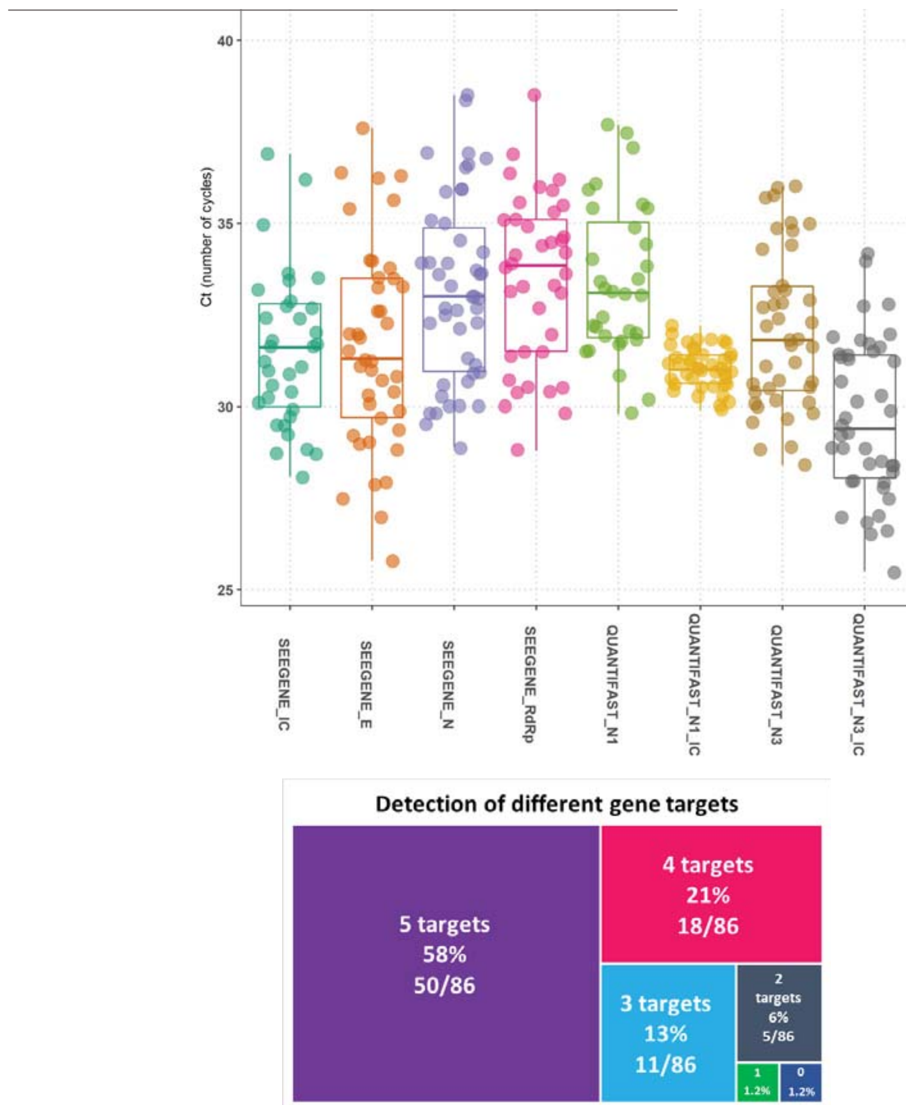

Figure 8: Comparison of Ct values for sewage influent samples per assay method

4.4 Viral quantification

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). The assay was 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.

SARS-CoV-2 quantification based on N1 gene showed 68% (59/86) of samples were SARS-CoV-2 N1 positive. The Ct range was 28.2-39.4, and the Genome copies/mL ranged from 0.2-581.4 gc/mL.

All samples were screened for mengovirus to calculate the extraction efficiency. It is intended that the number of N gene copies will be quantified in the sewage samples, by adjusting the concentrations for extraction efficiency. It was noted that in some cases, even though the SARS-CoV-2 gene targets amplified, there was inhibition in the mengovirus assay. This was possible to resolve in most cases by making serial dilutions and repeating the assays, but

assaying multiple dilutions for the purpose of copy number correction when conducting routine analysis may not be feasible or economically viable.

5 Evaluation of methods and recommendations

Based on limited results, grab sampling of the WWTW influent during the morning peak 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.

All three of the virus recovery methods tested (PEG flocculation, skim milk precipitation and aluminium hydroxide adsorption-precipitation) worked well. Recovery was variable and is likely dependent on sample composition. The skim 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 two resuspension buffers, ITM and PBS used for the PEG and skim milk methods both performed well, as did Trizol which was used for re-suspension of the pellet when using the aluminium hydroxide precipitation method. The three methods could therefore be used interchangeably between laboratories, although due to the slight variation in results it is recommended that one method be used consistently when monitoring a site to enable the visualisation of trends.

The commercial multiplex Seegene assay and QuantiFast Pathogen Kit in-house N1 and N3 assays all detected SARS-CoV-2. The Seegene kit is 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.

When samples were tested for mengovirus as a measure of virus recovery efficiency it was found that there was inhibition in some samples, where the mengovirus assay showed no amplification when there was a positive result for one or more SARS-CoV-2 targets. Repeating these samples with a 1 in 10 dilution of nucleic acids cleared the inhibition in some cases, but this makes for a costly analysis process that is not feasible for routine analysis. Use of a more appropriate surrogate virus, such as coronavirus NL63 could be tested as an alternative to mengovirus should a stock of this virus become available The use of the minimum Ct value of the assayed targets per sample as an indicator of viral load appears to be sufficient for trend analysis, which could be managed in a central database and visualised on a national dashboard.

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CASE 2

Detection and quantification of SARS-CoV-2 in wastewater from Durban and Pietermaritzburg

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1 BACKGROUND

The concept of wastewater-based epidemiology (WBE) has been used extensively for the detection of drug use patterns and disease incidence from a population perspective (Xagoraraki & O'Brien, 2020). The WBE principle postulates that the identified and quantified human biomarkers in untreated wastewater can give an indication of diet, health, diseases and exposure to contaminants within the wastewater treatment plant (WWTP) catchment of the community in real time (Gracia-Lor *et al*., 2017; Hernández *et al*., 2018). With the economic and practical limits of medical screening for COVID-19 coming sharply into focus worldwide, authorities are turning now to WBE as a potential tool for assessing and managing the pandemic. This approach is built on the knowledge that some infected persons shed viral particles/RNA in their stool as a result of gastrointestinal infections (GI) and other excreta. Early reports from Wuhan, China, showed that abdominal pain (an indication of GI infections) was reported more frequently in patients admitted into intensive care, than individuals not requiring intensive care (Yang et al., 2020; Wang et al., 2020; Chen et al., 2020). The proportion of patients with diarrhoea varied between regions, in China about 10% of the patients had diarrhoea, 1-2 days before the development of fever and respiratory symptoms (Wang et al., 2020). Studies have reported 2-35% of the patients having GI symptoms, such as diarrhoea, abdominal pain, and vomiting, although it is less frequent compared to respiratory symptoms (Yeo et al., 2020; Wang et al., 2020). It is estimated that infected individuals with Coronavirus will shed about 10^6 g c/g of faeces and 10^1 gc/mL of urine (Woelfel et al., 2020; Hung et al., 2004). The relative infection level for a population could, therefore, be estimated using the information on RNA copies shed per gram of faeces and litre of urine and viral titer in wastewater per day (Ahmed et al., 2020). These findings support the potential of stool, urine and wastewater analysis as an early warning system, since viral RNA can be detected in faeces, and subsequently wastewater, weeks before the onset of illness Currently, several countries have reported SARS-CoV-2 detection in wastewater, using it as a data source to determine the circulation of the virus in human population. These include Australia, China, France, Israel, Italy, Japan, Netherlands, Spain, the United States, India, and Mexico (Ahmed *et al*., 2020a, Lodder and de Roda Husman, 2020, Medema *et al*., 2020; Wu *et al*., 2020; Kumar *et al*., 2020; Randazzo *et al*., 2020; Rimoldi *et al*., 2020; La Rosa *et al*., 2020; Wurtzer *et al*., 2020). Therefore, WBE may provide a better resolution of the infection rate at community level since all classes of COVID-19 patience, i.e. symptomatic, asymptomatic and pre-symptomatic, which may not be captured

by clinical surveillance are represented. Despite the reports of possible applicability there are a number of challenges that need to be addressed. These include optimization of sampling approaches, viral concentration and RNA extraction methods and improvement of the models for the prediction of infection numbers. The work done by the Institute for Water and Wastewater Technology, Durban University of Technology in collaboration with Umgeni waters and eThekwini municipality is presented here.

2 METHODOLOGY

2.1 Sampling protocol

Four domestic wastewater treatment plants (WWTPs) in KwaZulu-Natal province were selected for the method optimization study. The Darvill and Howick plants are located in Pietermaritzburg and treat an average of 70 ML/d and 6 ML/d of wastewater per day respectively, while the Isipingo and Central WWTPs situated in Durban treat an average of 80 ML/d and 14 ML/d of wastewater respectively. Grab samples (2 L) of raw wastewater were collected at the head of works (post-primary screening) for each of the works. Samples were collected between 07:00-11:00 on a weekly basis and transported to the laboratory within two hours. Full PPE was worn during sampling events (Face shield, FFP2 face mask, waterproof coveralls and safety boots). Upon arrival at the laboratory, samples were pasteurised at 60°C for 90 min.

2.2 Viral recovery

After pasteurization, samples were left to cool to room temperature. Thereafter, samples were mixed thoroughly and 250 ml aliquots were removed for processing and the rest of the sample stored at -80°C. The 250 ml collected from each plant was then equally divided into 50 ml centrifuge tubes and clarified by centrifugation at 4750 x g for 30 min. The supernatants from each tube were then pooled and used for viral concentration while the pellets were stored at -80°C for future analysis. For viral concentration methods such as PEG 6000/NaCl, PEG 8000/NaCl and Centricon Plus 70 10K were employed as described below.

2.2.1 PEG 6000/NaCl and PEG 8000/NaCl precipitation

Two different molecular weights of PEG was used to determine the effect of the molecular weight on viral recovery. The method described by Kocamemi et al. (2020) was slightly modified and used in this study. Briefly, the supernatant of each sample was filtered with a 0.45 µm nitrocellulose membrane filter to remove any remaining particles. Thereafter, 5X stock solutions of PEG 6000/NaCl and PEG 8000/NaCl were prepared by dissolving 100 g of PEG (Merck, Germany) and 17.5 g of Sodium Chloride (Merck, Germany) in 200 ml of distilled water. The pH of each solution was adjusted to 7.0-7.2 and the resulting solution filtered with a 0.2 µm filter. Supernatant from each sample was then mixed with PEG 6000/NaCl and PEG 8000/NaCl (10% w/v) and incubated overnight at 4ºC at 60 rpm. Following incubation, each sample was centrifuged at 5700 x g for 2 h. The resulting supernatant was discarded while the pellet was resuspended in 140 µl phosphate buffer saline (PBS) and stored at -80ºC.

2.2.2 Centricon® Plus-70

The method of ultrafiltration was used to concentrate the virus particles as previously described by Medema et al. (2020). Briefly, 70 ml of supernatant was filtered through a Centricon® Plus-70 centrifugal ultrafilter with a cut-off of 10 kDa at 1500 x g for 15 minutes. The volume of the resulting concentrate for each sample varied due to the composition of the sample matrix. In instances where the concentrate was less than 140 µl, (the minimum amount required for RNA extraction) the concentrate was topped up to 140 µl using PBS. The volumes of the concentrate recovered as well as the volume of PBS added (if required) were recorded to account for the dilution effect.

2.3 Nucleic acid extraction and quantification

Nucleic acid was extracted from 140 µl of viral concentrate using the QiAmp Viral RNA MiniKit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was eluted in 80 µl of sterile nuclease free water and then quantified using the Implen Nanophotometer®. In the absence of a viral control strain, the suitability of a concentration method to be used in our study was determined by assessing the quality and quantity of extracted RNA obtained from each concentration method. The RNA was then stored at -80ºC for further analysis.

2.4 Viral detection and quantification

2.4.1 RT-qPCR

The real time RT-PCR technique was used for the detection and the absolute quantification of the viral nucleic materials in the wastewater. Standard curve was prepared using the 2019-nCoV_N_Positive Control supplied at 2 x 10^5 genome copies (gc)/ µL) (Integrated DNA Technologies, Leuven, Belgium). The standard curve was prepared from a tenfold serial dilution of the target gene (10^5 to 10^0 copy numbers). The real-time PCR quantification was carried out according to modification of the method described by Medema et al. (2020) using Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems, Grand Island, NY). In a singleplex one-step RT-qPCR reaction, 2 ng RNA template, 5 µl of One Step PrimeScript™ III RT-PCR Kit [Takara Bio Inc, Japan], 0.02 µl Rox Dye II, 0.4 µM forward and reverse N2 primer set, 0.2 µM N2 gene Taq-probe were combined, and the reaction volume was adjusted to a final volume of 10 µl with RT-PCR grade water (delivered with the One Step PrimeScript™ III RT-PCR Kit). The primer set used were synthesised at Inqaba Biotechnical Industries (Pty) Ltd (Table 1). All reactions were carried out in duplicates with the thermal cycling condition including the reverse transcription at 55°C for 5 minutes, preheating at 95°C for 10 seconds followed by 45 cycles of amplification at 95°C for 10 seconds and 55°C for 30 seconds.

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

Assay	Target gene	Primer/Probe	Sequence (5'→3')	Reference
N2	Nucleocapsid (N)	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	(CDC 2020)
		2019-nCoV_N2-R	GCGCGACATTCCGAAGAA	
		2019-nCoV_N2-P	HEX-ACAATTTGCCCCCAGCGCTTCAG-BHQ1	

2.4.2 Droplet digital PCR

Detection of SARS-CoV-2 in wastewater samples was carried out using the One-Step RT-ddPCR Advanced Kit for Probes (Biorad, USA) in conjunction with the 2019-nCoV CDC ddPCR Triplex Probe Assay (Biorad, USA) which simultaneously targets the N1 (FAM labelled) and N2 (FAM and HEX labelled) region of the SARS-CoV-2 genome. The triplex probe assay also targets the human RPP30 (HEX labelled) gene for use as an internal extraction control. Extracted RNA was diluted to 1 ng and 5 µl of this was used as a template in ddPCR reactions. The ddPCR reaction mix was prepared according to manufacturer's instructions. Thereafter, droplet generation was carried out using the QXDx Automated Droplet Generator (Biorad, USA), and the plates were then heat sealed with a pierceable foil. A C1000 Touch Thermal Cycler (Biorad, USA) was then used to perform PCR under the following conditions: Reverse transcription at 50ºC for 1 h, enzyme activation at 95ºC for 10 min, 40 cycles of denaturation at 94ºC for 30 s and annealing at 55ºC for 60 s. This was followed by enzyme deactivation at 98ºC for 10 min and droplet stabilization at 4ºC for 30 min with a ramp rate of 2ºC/second. The sealed droplet plate was then transferred to the QX200 Droplet Reader (Biorad, USA). The distribution of positive and negative droplets in each sample well was read using the QuantaSoft 1.7 software (Biorad, USA) while further analysis was carried out using the QuantaSoft Analysis Pro 1.0 software (Biorad, USA).

2.4.3 Control materials

Each sample plate contained positive, negative and no template control wells. For ddPCR, the SARS-CoV-2 positive and negative controls (Exact Diagnostics) were utilized. The positive control contained synthetic RNA transcripts of 5 gene targets (E, N, ORF1ab, RdRP and S) of SARS-CoV-2 while the negative control contained human genomic DNA and RNA spiked into a synthetic matrix. Sterile nuclease free water was used in place of RNA for the no template control. If all controls exhibit the expected performance, a sample is considered positive if it has any or both of the 2 SARS-CoV-2 markers even in the absence of the RPP30 gene. Similarly, a sample is considered negative if it does not contain any of the SARS-CoV-2 markers even if it contains RPP30.

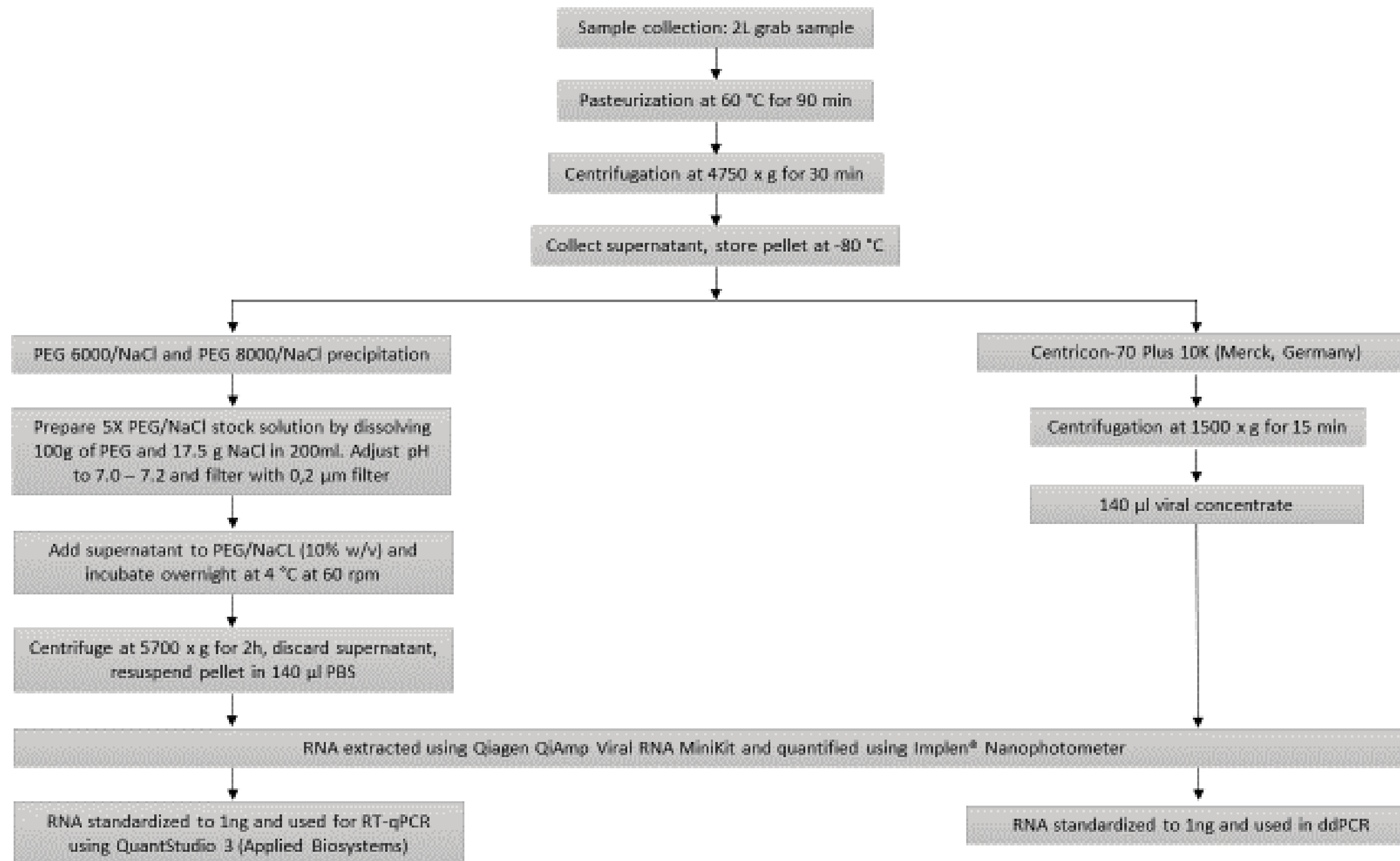


Figure 1. Workflow for viral recovery, extraction and detection of SARS-CoV-2 from wastewater samples

3 RESULTS

3.1 RT-qPCR

The results presented here are for samples taken from 14th July to 14th August, 2020. The efficiencies of the RT-qPCR runs (N2 primer) were between 109.3%, and the standard curves were linear over five orders of magnitude (r^2>0.99) (Figure 2). The results shown in Figure 3 indicates the changes in the viral N2 gene quantified in the four plants during the four-week period. All the samples investigated were positive for the N2 gene with Ct values ranging from 35.5-38.8. However, when quantifying the exact copy numbers samples from the Darvill wastewater treatment plant for week 3, Horwick for weeks 2 and 4 had a lower copy number that fell out of the standard curve range. Hence, their copy number were excluded for those periods (Figure 3).

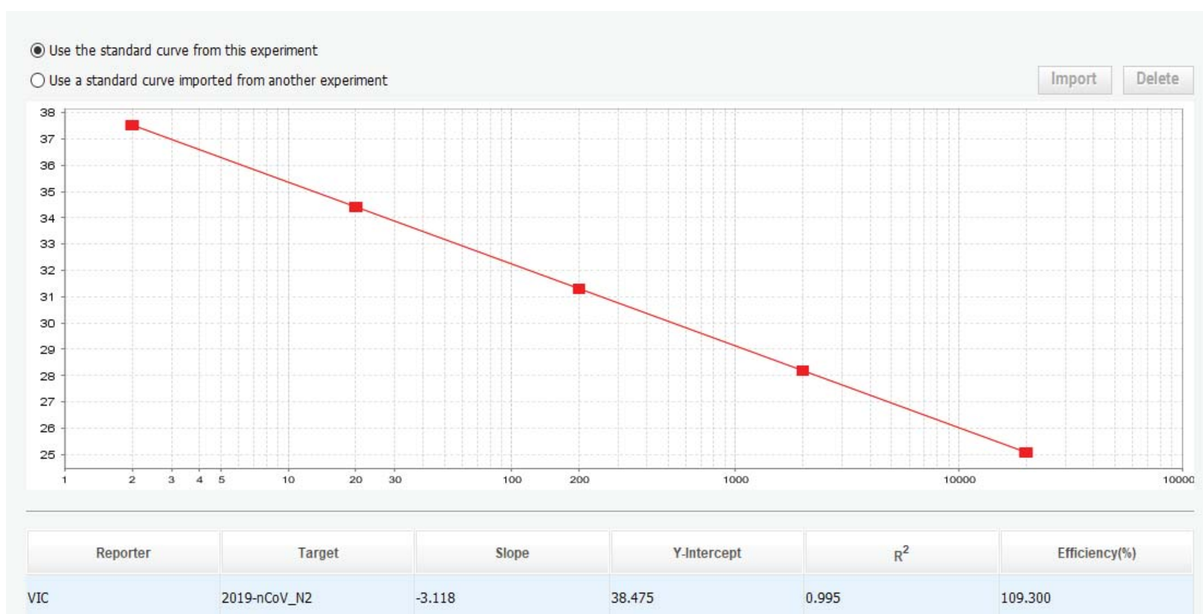
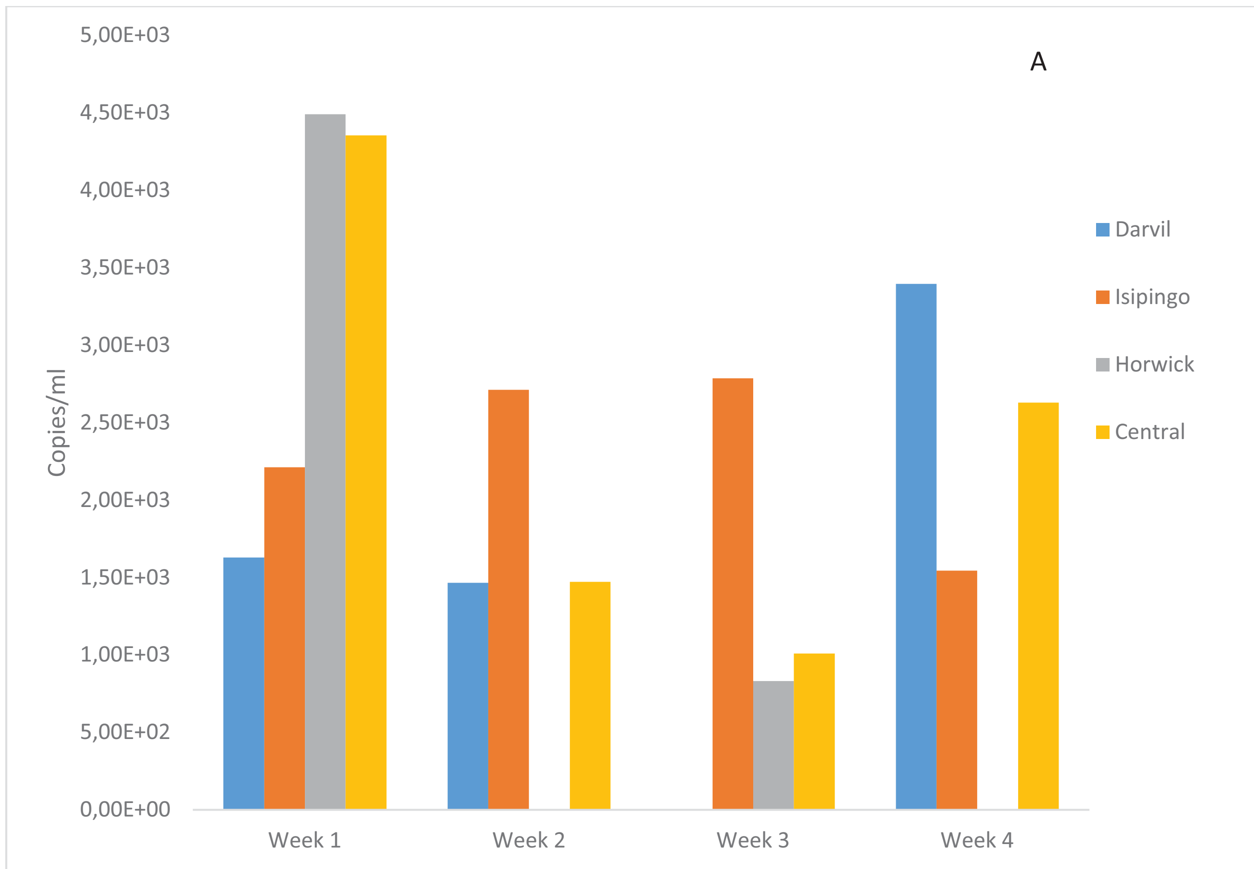


Figure 2: The standard curve used for estimating the gene copy numbers of the N2 gene and its parameters.



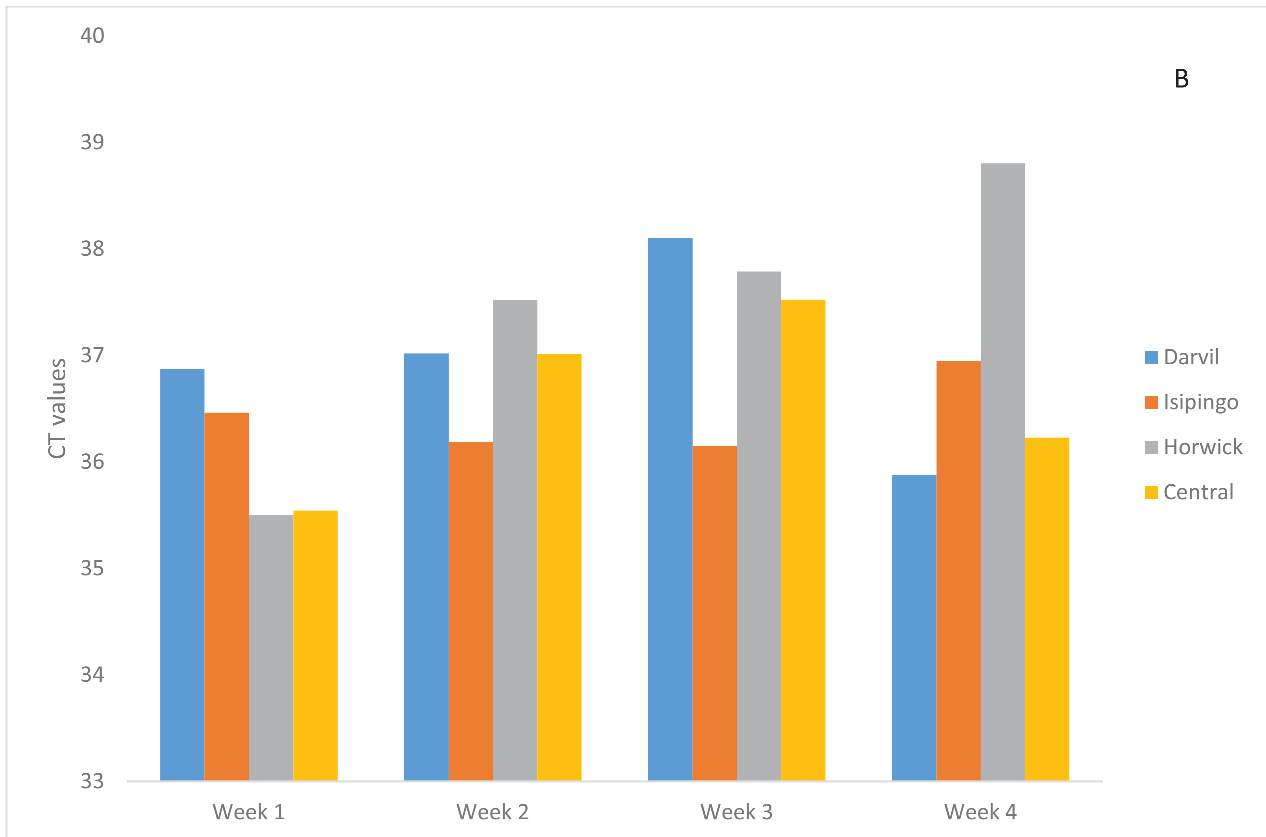


Figure 3: SARS-CoV-2 N2 gene amplification for grab samples for weeks 1-4. [A]: Indicates the copy numbers estimated with the standard curve; [B]: indicates the corresponding Ct values for the samples. Ct values of 40 and below were considered positive

3.2 ddPCR standard and limit of detection

Figure 4 below shows the amplification of the SARS-CoV-2 positive standard on our ddPCR platform. In the standard, N1 was quantified at 96.6 copies/µl, N2 at 95.1 copies/µl and RPP30 at 61.3 copies/µl. The N2 gene was selected as the gene of interest for SARS-CoV-2 as it amplifies best on our comparative RT-qPCR system, QuantStudio 3 (Applied Biosystems). Therefore, serial dilutions of the standard were prepared (10^{-0}-10^{-7}) and the limit of detection for the N2 gene was recorded. For N2, the limit of detection of 0.102 copies/µl was obtained.

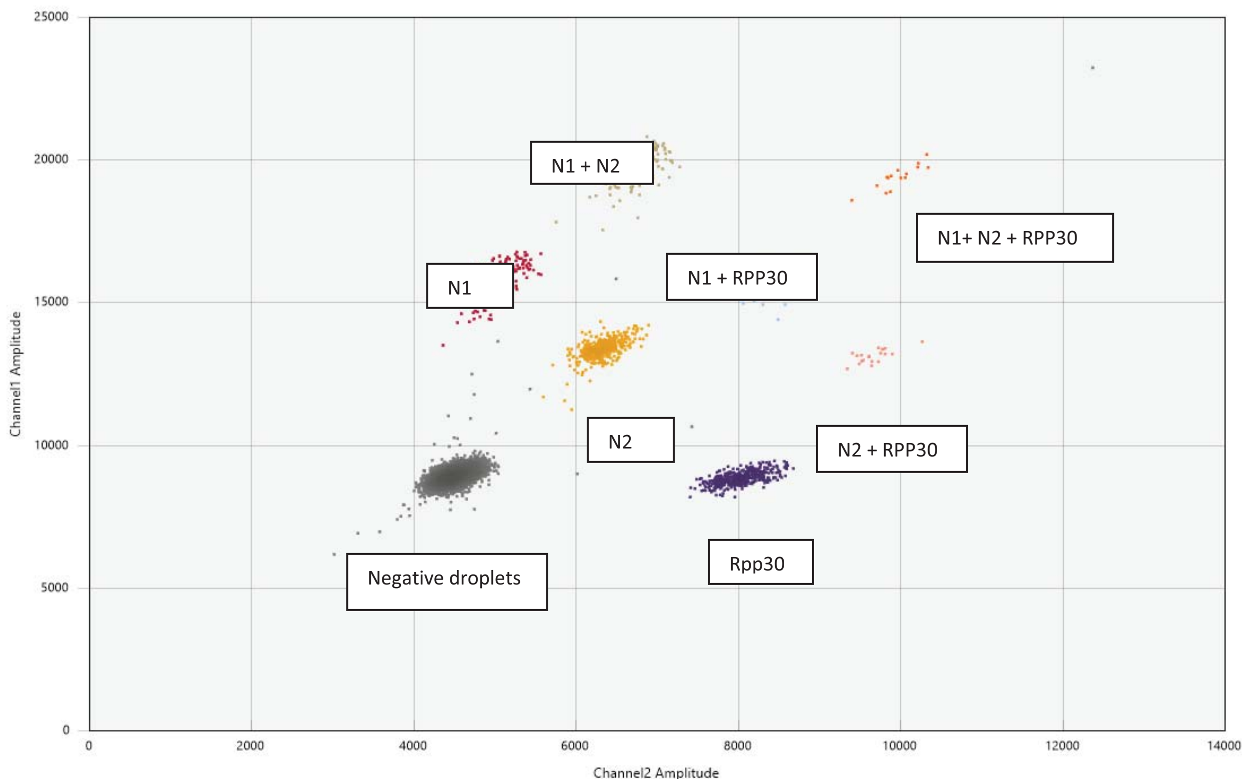


Figure 4: SARS-CoV-2 positive control with expected amplification clusters

3.3 Viral amplification and quantification using Biorad Triplex Probe Assay

Sixteen wastewater samples collected over a 4-week period was screened in duplicate for the presence of the SARS-CoV-2 targeting the genetic marker N2. The results obtained for each WWTP are presented below in Figure 5, which shows a similar trend with the results obtained with the RT-qPCR.

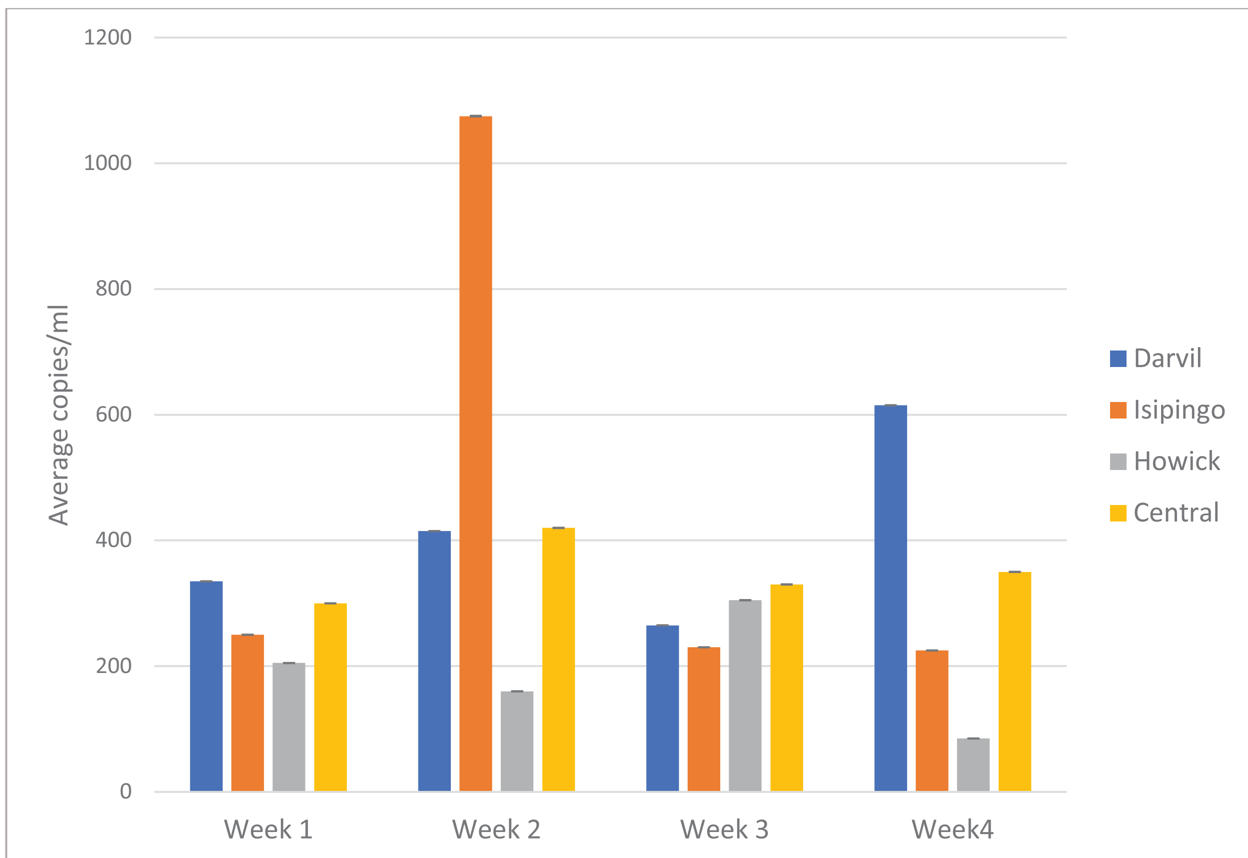


Figure 5: Presence of SARS-CoV-2 in wastewater treatment plants over a 4-week period

4 Challenges of using WBE for SARS-CoV-2 monitoring: Lesson learned and recommendations

4.1 Measurement of wastewater characteristics: We have observed that the characteristics of the wastewater varied between and within the wastewater treatment plants across the sampling period and time. Therefore, for an optimized WBE approach for the detection and quantification of SARS-CoV-2, the characteristics (such as pH, temperatures, COD, total suspended solids, etc.) of each sample must be analyzed and considered.

4.2 Optimization of sampling approach: The distribution of the viral particles or RNA in the wastewater could vary from time to time within the sample plant, depending on the flow rate. Therefore, for the effective application of WBE, there is a need for an optimized and unified sampling approach. Grab sampling has been used extensively, others have also reported the use of autosamplers to get a much more representative sample. These composite samples are considered to be a better representation of the viral load in wastewater, therefore may give an efficient WBE in monitoring the infection rate in the community.

4.3 Viral concentration and RNA extraction: Several viral concentration and RNA extraction methods have been used and reported. However, there are few comparative studies and quality control data. This is important especially taking into consideration the significant variation in the wastewater characteristics between and within the plants. This may be achieved using a surrogate virus to determine the efficiency of this viral concentration and RNA extraction methods. However, this is not always practical for every lab due to the complexity of obtaining and maintaining a true surrogate virus for SARS-CoV-2.

5 ACKNOWLEDGEMENTS

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CASE 3

Concentration and detection of SARS-CoV-2 in wastewater samples at the NICD

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At NICD, the preferred concentration method is ultrafiltration using the Centricon® Plus-70 centrifugal ultrafilter device. The alternative method is the PEG/dextran two-phase separation method, used in the absence of the Centricon ultrafilters.

A. Concentration method 1: Ultrafiltration using the Centricon® Plus-70 centrifugal ultrafilter device.

1. Equipment and Materials

Labels or a permanent marker for labelling

Biological safety cabinet (class II)

Conical centrifuge tubes (250ml)

Refrigerated centrifuge (+2-8°C), accommodating 250 centrifuge tubes (also needed for the Centricon® Plus-70 centrifugal ultrafilter device)

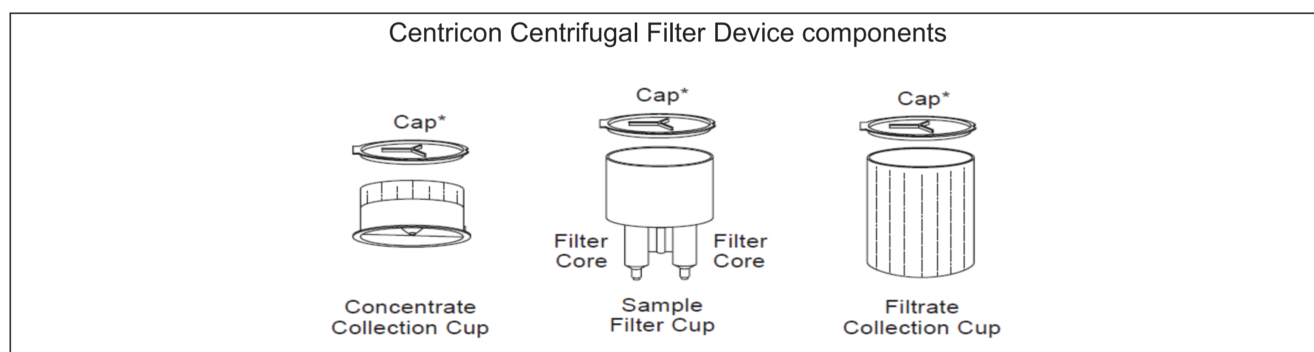
Appropriate serological pipettes and pipette aid

Discard container

200-1000 µl pipette and appropriate pipette tips

Centricon® Plus-70 centrifugal ultrafilter device

Laboratory disinfectants (e.g. Virkon solution and 70% ethanol)



2. Procedure

2.1 Begin by labelling of the sample, the sample form, the 250 ml centrifuge tube and all items of the Centricon ultrafilter device (e.g. a cap, filtrate collection cup, sample filter cup and concentrate collection cup).

2.2 Working in a biosafety cabinet, one sample at a time, mix sample gently by inverting it a few times and leave to stand for ±5 minutes to allow coarse material to settle.

2.3 Carefully transfer 250 ml of the sample into a 250 ml conical centrifuge tube.

2.4 Centrifuge at 4650 g at +4°C for 30 minutes with no brake to remove debris.

2.5 Transfer 70 ml of the supernatant to a Centricon sample filter cup contained within a Centricon filtrate collection cup.

2.6 Cap the sample filter cup and centrifuge at 1500 g at +4°C for 15 minutes.

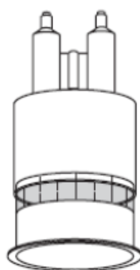
2.7 Remove the cap from the sample filter cup and check if any volume of sample is retained in the sample filter cup.

2.7.1 If this is the case, transfer the remaining sample volume to the filtrate collection cup which should already contain the volume of sample that was filtered. Use the cap to cap the filtrate collection cup.

2.7.2 If this is not the case, proceed to 2.8 below.

2.8 Remove the cap and place the sample filter cup upside down into the Centricon concentrate collection cup. Use the cap to cap the filtrate collection cup and keep aside as this may be needed for a further centrifugation if the concentrate volume obtained is less than 300 µl.

2.9 Centrifuge the sample filter cup/concentrate collection cup assembly at 1000 g at +4°C for 2 minutes in the assembled position as shown below.



2.10 Remove and place the sample filter cup aside as this may be needed for a further centrifugation if the concentrate volume obtained is less than 300 µl.

2.11 Use a suitable pipette and tip to transfer the concentrate from the concentrate collection cup into a 2ml cryovial to obtain the final concentrate.

2.12 Once a volume of 300 µl or more (maximum of 1.5 ml) of concentrate is obtained, store the original wastewater sample and concentrate, and dispose of all other items appropriately.

NB: If the concentrate volume is less than 300 µl, repeat steps 2.5 to 2.11 above reusing the same Centricon device after carefully discarding the sample contained within the filtrate collection cup. This can only be reused for the same sample once. Add the concentrate obtained from the second centrifugation process to the vial of concentrate that was less than 300 µl.

B. Concentration method 2: PEG/dextran two-phase separation method

3. Equipment and Materials

Analytical balance

Measuring cylinders of various sizes

Magnetic stirrer

Magnetic stirrer bars

Laboratory beakers of various sizes

Aluminium foil

Biological safety cabinet (class 11)

Centrifuge tubes (50ml and 250ml)

Refrigerated centrifuge (+2-8°C), accommodating 50ml and 250 centrifuge tubes \

Refrigerator (+2-8°C)

pH paper with 0.5 unit (or tighter) scale for pH adjustment

1 litre separation funnels with conical bottom

Laboratory stands with a ring holder or other means to steadily keep the separation funnel in a vertical position.

Grease

Deionised water

Vortex mixer

Minimum 1-2 litre glass/plastic beakers

4. Chemicals / Reagents

22% (w/w) Dextran

- Note: Technical quality Dextran (molecular weight of 40,000)

29% (w/w) PEG 6000

- Polyethylene glycol (PEG), average molecular weight 6000

5N sodium chloride

- Sodium chloride (NaCl, molecular weight 58.44)

1N sodium hydroxide (NaOH)**

1N hydrochloric acid (HCl)**

** For pH adjustment.

5. Procedure

Advance preparation:

- Prepare dextran, PEG and NaCl for the number of samples required (refer to appendix 1)
- Reagents expire two weeks from date of preparation, and therefore volumes prepared should be based on the expected number of samples as per the sample collection schedule.

Day 1 – Day of initiation of sample processing:

5.1 Working in a biosafety cabinet, one sample at a time, mix sample gently by inverting it a few times and leave to stand for ±5 minutes to allow coarse material to settle.

5.2 Carefully transfer 500 ml of the sample into a 500 ml centrifuge tube.

5.3 Centrifuge for 20 min at 1500 g in a refrigerated centrifuge (+2-8°C).

5.4 Transfer all of the supernatant to a 1-2 litre beaker equipped with a magnetic stirrer bar.

5.5 Store the pellet/s at +4°C.

5.6 Check the pH using pH strips and adjust the pH of the supernatant to neutral (pH 7-7.5) using NaOH and/or HCl and checking after a brief period of stirring. A pH of <7 must be neutralized with NaOH and >7.5 with HCl.

5.7 To the 500 ml of supernatant, add 39.5 ml of 22% dextran, 287 ml 29% PEG, and 35 ml 5N NaCl in this order.

5.8 Mix thoroughly on a magnetic stirrer and keep in constant agitation for 1 hour, preferably at +2-8°C or at room temperature.

5.9 Prepare a sterile conical 1 litre separation funnel per sample being evaluated and attach the funnel to a stand. Spread a small amount of grease (do not over grease) on the gliding glass/Teflon surface of the valve but do not obstruct the holes. Place a suitable container below the funnel to contain any leakage. Check water tightness with a small volume of deionised water which must be discarded on confirmation. Discard any water that might have dripped at this stage and replace container. A small amount of grease should also be spread on the stopper to facilitate its removal following the overnight incubation.

5.10 Pour the mixture into the funnel (take extra care not to let the magnetic stirrer bar fall into the funnel) and leave overnight at +2-8°C.

Day 2 – Obtaining the concentrate:

5.11 Transfer the funnel carefully, to not disturb the layers, from +2-8°C to the biosafety cabinet. Observe the lower part of the funnel. Usually a small lower phase and a fuzzy interphase with aggregated solid materials can be seen.

5.12 Release the pressure from the funnel before opening the valve by carefully removing and replacing the stopper. Open the valve with caution, collecting the entire lower layer and the interphase slowly drop-wise, into a sterile 50 ml centrifuge tube.

5.13 When the interphase has been collected, close the valve and disinfect the funnel and upper phase by adding a suitable disinfectant to the fluid in the funnel.

5.14 Add the pellet stored in the fridge from day 1 to the collected lower phase and interphase.

5.15 Vortex intermittently for ±1 minute to obtain the final concentrate for RNA extraction and SARS-CoV-2 PCR.

Appendix 1: Reagent preparation guide

22% (w/w) Dextran (Preparation per number of samples)						
No. of samples	1	2	3	4	5	6
Dextran (g)	10	20	30	40	50	60
Distilled Water (ml)	35.5	71	106.5	142	177.5	213
Procedure	• Switch on the balance. • Place a suitable sized clean beaker on the balance, add a clean magnetic bar to the beaker and zero the balance • Weigh the required amount of dextran directly into the beaker very carefully using a clean spatula or tongue depressor. If extra added, return the extra amount to the original container. • Place the beaker with the magnetic bar and dextran on the magnetic stirrer. • Measure the required volume of distilled water (preferably pre-warmed) in a measuring cylinder and add to the beaker. • Cover the beaker with aluminium foil. • Turn the power of the magnetic stirrer on and increase the speed very slowly to ensure the powder does not disperse. You may need to facilitate the stirring by swirling the beaker very carefully a few times or using a clean wooden spatula to loosen the powder. • Once the stirring is continuous, leave to stir until a clear, colourless solution is achieved. Keep an eye on the stirring process to ensure that the magnetic bar does not get dislodged from the centre. • Store at +4°C for up to two weeks maximum, labelling the beaker accordingly with the date of prep, initial and date of expiration.						

29%(w/w) PEG 6000 (Preparation per number of samples)						
No. of samples	1	2	3	4	5	6
PEG (g)	92	184	276	368	460	552
Distilled Water (ml)	225	450	675	900	1125	1350
Procedure	• Switch on the balance. • Place a suitable sized clean beaker on the balance, add a clean magnetic bar to the beaker and zero the balance • Weigh the required amount of PEG directly into the beaker very carefully using a clean spatula or tongue depressor. If extra added, return the extra amount to the original container. • Place the beaker with the magnetic bar and PEG on the magnetic stirrer. • Measure the required volume of distilled water (preferably pre-warmed) in a measuring cylinder and add to the beaker. • Cover the beaker with aluminium foil. • Turn the power of the magnetic stirrer on and increase the speed very slowly to ensure the powder does not disperse. You may need to facilitate the stirring by swirling the beaker very carefully a few times or using a clean wooden spatula to loosen the powder. • Once the stirring is continuous, leave to stir until a clear, colourless solution is achieved. Keep an eye on the stirring process to ensure that the magnetic bar does not get dislodged from the centre. • Store at +4°C for up to two weeks maximum, labelling the beaker accordingly with the date of prep, initial and date of expiration.						

<table>
<tr><td colspan="3">5N sodium chloride (NaCl) solution (Preparation per number of samples)</td></tr>
<tr><th>No. of samples</th><th>Up to 7</th><th>Up to 14</th></tr>
<tr><td>NaCl (58.44) (g)</td><td>73.05</td><td>146.10</td></tr>
<tr><td>Distilled Water</td><td>250</td><td>500</td></tr>
<tr><td>Procedure</td><td colspan="2">
Switch on the balance.
Place a suitable sized clean beaker on the balance, add a sterilized magnetic bar to the beaker and zero the balance
Weigh the required amount of NaCl directly into the beaker very carefully. If extra added, use a suitable clean spatula or tongue depressor to return the extra amount to the original container.
Place the beaker with the magnetic bar and NaCl on the magnetic stirrer.
Measure the required volume of distilled water in a measuring cylinder and add to the beaker.
Turn the power of the magnetic stirrer on and increase the speed very slowly to ensure the powder does not disperse. You may need to facilitate the stirring by swirling the beaker very carefully
Once the stirring is continuous, leave to stir until a clear, colourless solution is achieved. Keep an eye on the stirring process to ensure that the magnetic bar does not get dislodged from the centre
Store at +4°C until all used up
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6. Nucleic acid Extraction for PCR.

Kit: Qlamp Viral RNA Mini Kit (Separation Scientific Catalogue #: 52906)

6.1 Pipet 560 µl prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube. Note: If the sample volume is larger than 140 µl, increase the amount of Buffer AVL-carrier RNA proportionally (e.g. a 280 µl sample will require 1120 µl Buffer AVL-carrier RNA) and use a larger tube.

6.2 Add 140 µl plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s. Note: To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used. Add 5 µl of Seegene Allplex 2019-nCoV PCR Internal Control to the microcentrifuge tube containing the AVL-sample mix

6.3 Incubate at room temperature for 10 min. Note: Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA. 28 QIAamp Viral RNA Mini Handbook 07/2020

6.4 Briefly centrifuge the tube to remove drops from the inside of the lid.

6.5 Add 560 µl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid. Note: Use only ethanol, since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances, such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g. a 280 µl sample will require 1120 µl ethanol). To ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6.6 Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate. Note: Close each spin column to avoid cross-contamination during centrifugation. Note: Centrifugation is performed at 6000 x g (8000 rpm) to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

6.7 Carefully open the QIAamp Mini column, and repeat step 6. If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

6.8 Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Note: It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl. QIAamp Viral RNA Mini Handbook 07/2020 29

6.9 Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 11; or to eliminate possible Buffer AW2 carryover, perform step 10 and then continue with step 11. Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flowthrough, containing Buffer AW2, contacting the QIAamp Mini column. Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.

6.10 Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

6.11 Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

6.12 Centrifuge at 6000 x g (8000 rpm) for 1 min. A single elution with 60 µl Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double

elution using 2 x 40 µl Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate

7. SARS-CoV-2 PCR

Kit: Seegene Allplex 2019-nCoV PCR Kit. (Inqaba Catalogue #: SG RP10243X)

①Prepare following reagents in a labelled sterile 1.5 mL tube. Set up all reagents on ice. NOTE: Be sure to centrifuge the PCR tube before running PCR reaction in order to set the liquid to the bottom and to eliminate air bubbles.

No. of Reactions	1	2	3	4	5
2019-nCoV MOM	5	10	15	20	25
RNase-free Water	5	10	15	20	25
5X Real-time One-step Buffer	5	10	15	20	25
Real-time One-step Enzyme	2	4	6	8	10

②

Mix by inverting the tube 5 times or quick vortex, and briefly centrifuge.

③ Aliquot 17 µL of the One-step RT-PCR Mastermix into PCR tubes*.

④ Add 8 µL of each sample's nucleic acids, 2019-nCoV PC and NC (RNase-free Water) into the tube containing an aliquot of the Onestep RT-PCR Mastermix.

⑤ Close the cap, and briefly centrifuge the PCR tubes.

⑥ Verify that the liquid containing all PCR components is at the bottom of each PCR tube. If not, centrifuge again at a higher rpm and for a longer time.

⑦ Immediately initiate PCR.

Step	No. of cycles	Temperature	Duration
1	1	50℃	20 min
2	1	95℃	15 min
3	45	94℃	15 sec
4*		58℃	30 sec
5	GOTO Step 3, 44 more times		

* **Plate Read at Step 4**. Fluorescence is detected at 58°C.

CASE 4

A novel method for processing and detection of SARS-CoV-2 in wastewater samples – TFF approach

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1 Introduction

SARS-CoV-2 (Severe Acute Respiratory Syndrome Corona Virus 2) has been classified as a novel virus as of 2019. It has been leading to the ongoing global pandemic of coronavirus disease 2019 (COVID-19) (World Health Organization, 2020a, World Health Organization, 2020b). The WHO (World Health Organisation) has classified COVID-19 as a public health emergency of international concern and as a global pandemic, as of 11 March 2020. Due to an increase in positive cases of COVID-19, many countries have taken steps to fight this global pandemic through flattening the curve of positive cases by means of flight restrictions, the closure of international borders and national lockdowns.

According to recent literature, SARS-CoV-2 RNA has been detected in human bodily waste of symptomatic as well as asymptomatic patients (Ahmed et al., 2020, Gao et al., 2020, Holshue et al., 2020, Cai et al., 2020, Liu et al., 2020, Wölfel et al., 2020, Zhang et al., 2020a, Zhang et al., 2020b). Several studies have reported that the presence of viral SARS-CoV-2 RNA in human waste of COVID-19 patients vary in concentration with up to 6.8 log_{10} genome copies/g of human waste (La Rosa et al., 2020, Chen et al., 2020, Lo et al., 2020, Lescure et al., 2020, Han et al., 2020). Although viral SARS-CoV-2 RNA has been detected in human waste as such, this does not necessarily entail the presence of the virus is in its infectious state, nor does it indicate whether the virus is transmissible in this state (Holshue et al., 2020, Cahill and Morris, 2020). The WHO (2003) has reported that the SARS-CoV-1 virus can remain viable in human waste samples for up to 17 days at 4°C. The survival rate is significantly lower at higher temperatures of about 20°C at around three to four days (Wang et al., 2005).

Efficient and effective wastewater-based epidemiology studies and methods are essential for monitoring the prevalence of SARS-CoV-2 in wastewater. This increases the chances of identifying potential hotspots that, in turn, holds the crucial potential of rapidly treating the affected population (Ahmed et al., 2020). Studies world-wide have already reported the presence of SARS-CoV-2 in wastewater. Countries where this has been confirmed include France (Wurtzer et al., 2020), Australia (Ahmed et al., 2020), The Netherlands (Medema et al., 2020) and the USA (Wu et al., 2020). To date, no study has been published that provide insight into the presence of SARS-CoV-2 in wastewater in South Africa.

There are various techniques to be used to concentrate and isolate viruses from complex media such as wastewater. Some of the most frequently used techniques include adsorption-elution, chemical flocculation, ultracentrifugation and ultrafiltration such as the method known as TFF (Sun et al., 2014). This method is one of the most efficient, as it overcomes numerous flaws encountered when using other methods. These flaws include selective adsorption of viruses, interference of elution buffers in downstream applications and low viral concentration (Sun et al., 2014).

Also known as crossflow filtration, TFF is a rapid and efficient method for the separation and purification of biomolecules (Hirsjärvi et al., 2009). It is based on a size separation principle, where a solution continuously passes over a membrane and all particles smaller than the pore size of the filter are removed from the sample. The solution passes parallel to the filter and is not pushed through as in other filtration methods. This preserves the integrity of viral particles to a greater degree than the other methods mentioned above (Hirsjärvi et al., 2009).

The application of TFF is widely used in the areas of biotechnology, mainly for the purification of cells, virus and bacterial proteins (Charcosset, 2006) as well as nanoparticles (Hirsjärvi et al., 2009, Limayem et al., 2004, Tishchenko et al., 2003). In the field of liposomes, cationic solid lipid nanoparticles (Guichardon et al., 2005) and toxicity reduction (Heydenreich et al., 2003), TFF has also been successfully extended in the separation of gold nanoparticles from small impurities (Sweeney et al., 2006),. Although SARS-CoV-2 has a molecular weight of 33796.64 Dalton (Ul Qamar et al., 2020) and has a size of 65-125 nm (Shereen et al., 2020), the complex medium of wastewater will almost certainly fragment the virus.

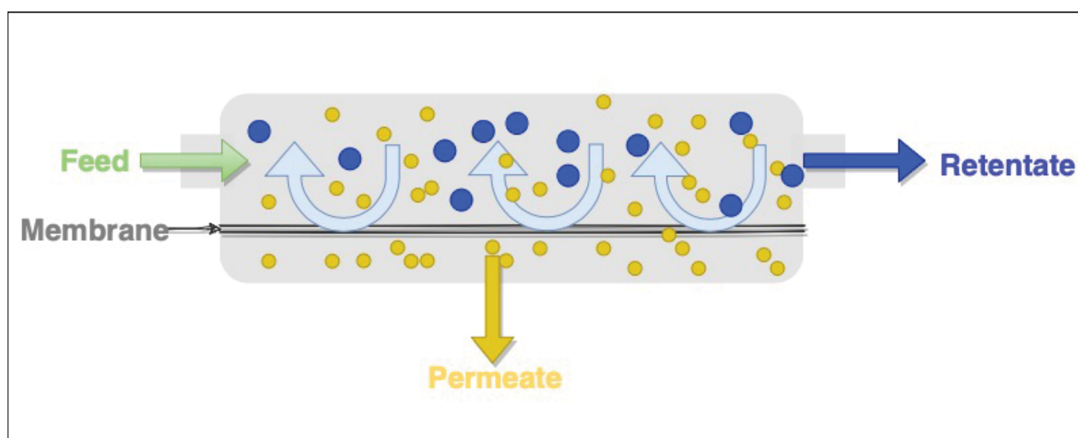


Figure 1. Basic principle of TFF (tangential flow filtration), where the solution passes parallel to the membrane. Smaller particles pass through the membrane while the rest of the sample is recirculated (Shwartz, 2013)

Extant studies conducted by Ahmed et al. (2020), Wu et al. (2020), Medema et al. (2020) and Wurtzer et al. (2020), illustrated that the methods used proved helpful for the detection of SARS-CoV-2 in wastewater. The aim of the present study has been to test a novel method to concentrate wastewater samples to identify SARS-CoV-2 within the samples, which could be used in future

outbreaks for pathogenic monitoring. In this study, we explored the use of the Minimate^{TM} TFF Capsule as an effective method for processing wastewater for the detection of SARS-CoV-2.

2 Sampling methodology

A 1000 ml volume of untreated wastewater samples were collected by means of a grab or composite sampling technique at various WWTPs (wastewater treatment plants) in the Gauteng and North-West provinces of South Africa. Grab sampling was performed between 08:00 and 10:00 (~ when the first flush reaches the WWTP). These samples were collected by placing a 1000 ml container directly into the water, immediately after the first screening phase, at each of the treatment plants. Auto-samplers were used to collect 24-hour composite samples and the contents added to 1000 ml containers. The filled containers were then sealed properly, and the outside sterilized with either 70% ethanol or a chlorine-based cleaning solution. Samples were immediately placed on ice or stored at -20°C until transported to the laboratory.

3 Sample processing and analysis

3.1 Viral recovery

3.1.1 Sample clarification method

The pre-filtration step was conducted with a 1 µm PP Melt Blown filter Cartridge (Darlly,® China) and thereafter with a 0.5 µm PP Melt Blown filter Cartridge (Darlly®, China) where the wastewater sample was flushed through each filter three times. Initially a 1000 ml wastewater sample was used to pre-filter and approximately 70-80% of the initial volume was retrained as the final volume to be used for concentration.

3.1.2 Virus recovery method(s)

The pre-filtered wastewater samples were then concentrated with the use of the Minimate^{TM} TFF Capsule w/10 kDa Omega (PALL® Life Science, USA), while the sample was kept on ice. Figure is a schematic representation for the Minimate^{TM} TFF Capsule setup. In this setup, we used three Minimate^{TM} TFF Capsules simultaneously to speed up the concentration process. Among the various wastewater samples that were received, initial samples were concentrated 20x. By changing the setup, we concentrated the subsequent samples 100x to generate an even more concentrated sample for RNA isolation. Optimisations included shortening the tubing setup, monitoring permeate volume, adding more Minimate^{TM} TFF Capsules, and using a better peristaltic pump. After each sample had been concentrated, the setup was cleaned before next use, in accordance with the user manual of the Minimate^{TM} TFF Capsule w/10 kDa Omega (PALL® Life Science, USA), as described under the post-treatment section below. The flushing and cleaning section of the user manual was followed to prevent contamination between samples.

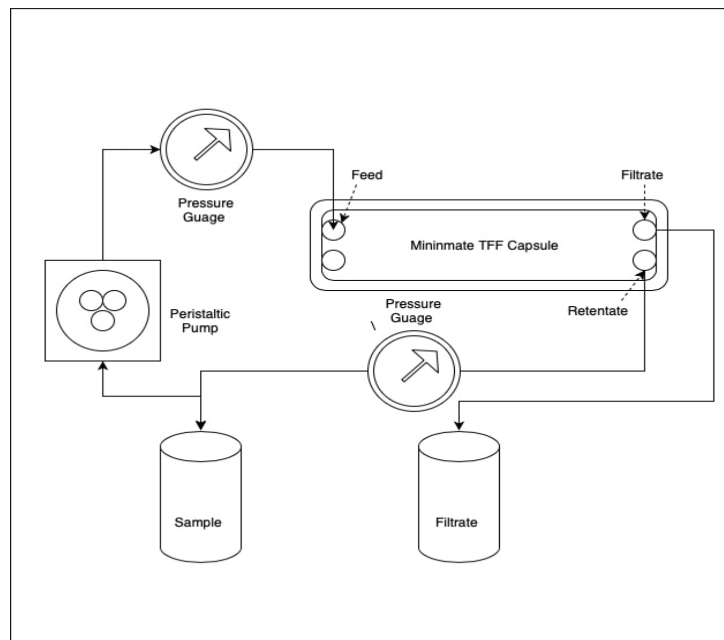


Figure 2. Schematic representation of the Minimate™ TFF Capsule setup that was used during the concentration of the wastewater samples

3.2 Viral detection

3.2.1 Nucleic acid extraction

The isolation of viral RNA was attained by using Macherey-Nagel RNA and DNA pathogen isolation kit (744_210; Macherey-Nagel in Düren, Germany) and the samples were run on the KingFisher Duo Prime (Thermo Fisher Scientific). The plate-setup was conducted according to the 96-deep well format as per the Macherey-Nagel RNA and DNA pathogen isolation kit (744_210 by Macherey-Nagel in Düren, Germany), as found in appendix A below. The script for the KingFisher Duo Prime was supplied by Machery-Nagel, and was run without any optimisation.

3.2.2 Viral amplification

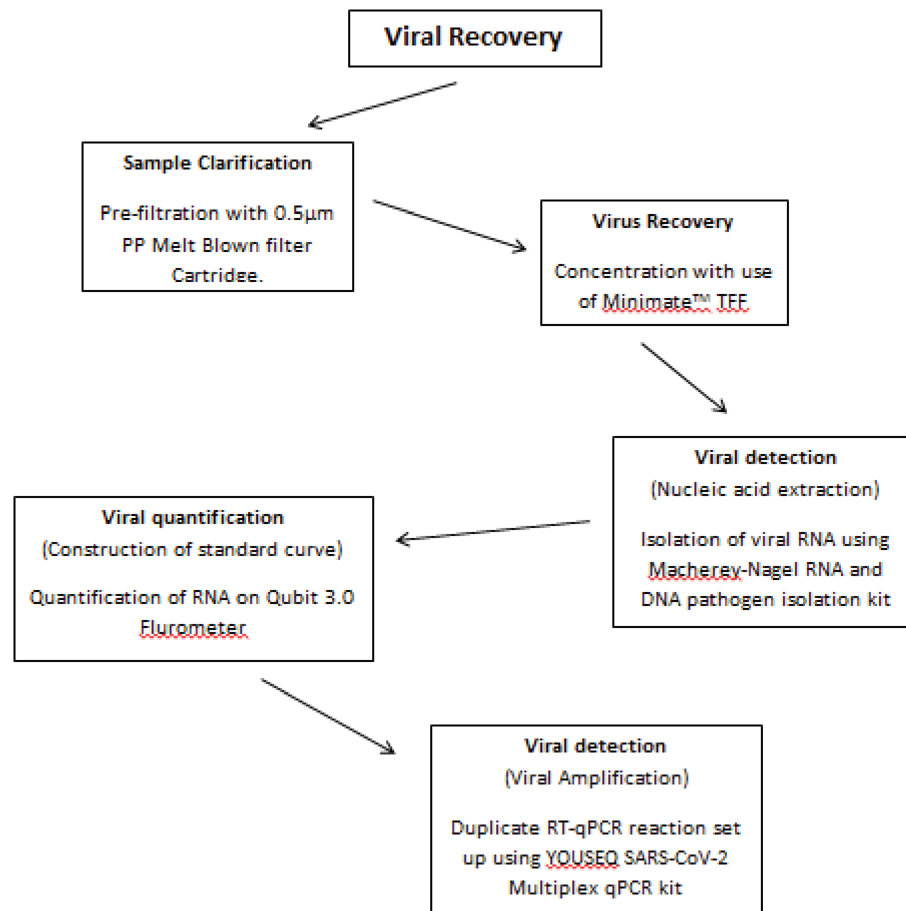
Quantification of RNA was done on the Qubit 3.0 Fluorometer with the Qubit® RNA HS Assay Kit (Life Technologies, Q32852) as per the manufacturer protocol. 1 µl of isolated RNA was used for quantification. Where RNA concentrations were too low for detection, up to 6 µl was used.

3.3 Viral quantification

3.3.1 Construction of standard curves

Duplicate 20 µl RT-qPCR reactions were set up using the SARS-CoV-2 Multiplex qPCR kit (YOUSEQ Ltd, United Kingdom). This multiplex kit contains primer and probe sets for the *E*, *N* and *RdRp* viral genes. The internal control primer and probe was set for the RNaseP human gene. Samples were run on the QuantStudio 5 (Applied Biosystems) and each run contained an additional PC (positive control) and NTC (non-template control). Thermal cycling conditions were carried out at 55°C for 10 minutes, followed by 95°C for 3 minutes and 45 cycles of 95°C for 15 seconds each as well as 60°C for 60 seconds. Data was collected at this stage after every cycle.

3.3.2 Calculation of copy numbers per sample volume



4 Evaluation of methods

4.1 Collection

With a view to our RT-qPCR results we consistently amplified both grab and composite samples with only one grab comparison sample that did not amplify in both duplicate RT-qPCR reactions.

4.2 Pre-filtration

A pre-filtration of wastewater samples was conducted to remove debris and solid particulates that could affect the efficacy of the TFF. After pre-filtration, between 70%-80% of the sample was retrieved. Although viral retrieval studies have not been conducted in this study, it is believed that most of the volume lost was due to solid matter found in the wastewater matrix. This will be tested in future studies. Alternative, less dense pre-filters may also be employed to ensure higher retrieval rates of the total sample volume. Because of the complex sample matrix of wastewater, 0.5 µm pre-filtration is not sufficient for sustainable TFF. A 0.2 µm pre-filtration might need to be considered, although this might lead to additional loss of viral particles, as they might get trapped with the particles in the filter.

4.3 TFF

Because of the small surface area and small particles still present after 0.5 µm pre-filtration, the concentration of the samples took much longer than anticipated. Some of these samples took up to 12 hours. It is inferred that it is essential first to allow the sample to pass through a smaller pre-filtration filter where excess solids are filtered out, as this will ensure a longer life span of the Minimate™ capsule and increase the efficacy of filtration. The Minimate™ capsule is also one of the smaller versions of TFF filters by PALL® Life Science. A filter with a larger surface area will drastically improve processing time of each sample. Shortening the tubing of the TFF setup ensured that we had less "dead volume" inside of the system. Monitoring the permeate ensured we knew exactly how much of our retentate was left, which effectively told us how concentrated our sample was. It also showed us how quickly a sample had been concentrated and when this decreased, so that we knew further the amount of Minimate™ TFF Capsule w/10 kDa Omega (PALL® Life Science, USA) needed to be washed before continuing. We added up to three Minimate™ TFF Capsules w/10 kDa Omega (PALL® Life Science, USA) at a time, which significantly sped up our concentration/ filtering process.

4.4 RNA isolation

Initially, our 20x concentration of the samples showed very low RNA yields after isolation, with some occurring below the detection limit of the Qubit 3.0. However, after some optimisation, we could concentrate the samples faster and up to 100x with the TFF setup. This ensured consistent RNA yields and showed that the pre-filtration and concentration methods were not detrimental to otherwise fragile RNA. Because the method preserved the virus, it could be used for various other pathogenic monitoring applications in the future. After these optimisations, we consistently enjoyed RNA yields higher than 30 ng/µl. However, because of the complex sample matrix, we could not determine the fraction of SARS-CoV-2 viral RNA present with the equipment at our disposal. Furthermore, this would be highly variable in each sample. As soon as we were confident that we could consistently recover RNA efficiently, we no longer incorporated the quantification step.

4.5 RT-qPCR

Table 1 Showing the RT-qPCR amplification data from three different treatment plants. Results include the various Ct values of each gene.

Treatment Plant	Sample type	E assay	E gene Ct	RdRP assay	RdRP gene Ct	N assay	N Gene Ct	SARS-CoV 2 PCR results
A	Grab	Pos	39	Pos	38	Pos	37	Positive
B	Grab	Pos	32	Pos	32	Pos	33	Positive
C	Grab	Pos	33	Pos	32	Pos	34	Positive
C	Composite	Pos	35	Pos	34	Pos	35	Positive

Treatment Plant	Sample type	E assay	E gene Ct	RdRP assay	RdRP gene Ct	N assay	N Gene Ct	SARS-CoV 2 PCR results
D	Grab	Neg	N/A	Neg	N/A	Neg	N/A	Negative
D	Composite	Neg	N/A	Neg	N/A	Neg	N/A	Negative
C	Grab	Neg	43	Pos	34	Neg	44	Positive
C	Composite	Pos	33	Pos	34	Pos	34	Positive
D	Composite	Pos	31	Pos	33	Neg	N/A	Positive

It is important to note that these results were obtained when infection rates were relatively low in the areas that serviced the WWTPs where the samples were collected. Although exact infection rates were not available, these areas did not experience a spike in infections at the time of sample collection.

5 Recommendations

With some optimisation, the sensitivity and effectivity of this method could be improved considerably with a view to future samples and effective surveillance. An added benefit of the method is the preservation effect around viral particles of the TFF concentration. The sample matrix is suitable for various other pathogens could be used, with some alterations, in broader water-based epidemiology (WBE) studies.

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CASE 5

Qualitative and quantitative detection of SARS-CoV-2 RNA from untreated wastewater in the Western Cape, South Africa

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Recent studies have shown that the detection of SARS-CoV-2 genetic material in wastewater may provide the basis for a surveillance system to track the environmental dissemination of this virus in communities. An effective wastewater-based epidemiology (WBE) system may prove critical in South Africa (SA), where health systems infrastructure, testing capacity, personal protective equipment and human resource capacity are constrained. In this proof-of-concept study, we investigated the potential of SARS-CoV-2 RNA surveillance in untreated wastewater as the basis for a system to monitor COVID-19 prevalence in the population, an early warning system for increased

transmission, and a monitoring system to assess the effectiveness of interventions. The laboratory confirmed the presence (qualitative analysis) and determined the RNA copy number of SARS-CoV-2 viral RNA by reverse transcription polymerase chain reaction (quantitative) analysis from 24-hour composite samples collected on 18 June 2020 from five wastewater treatment plants in Western Cape Province, SA. The study has shown that a WBE system for monitoring the status and trends of COVID-19 mass infection in SA is viable, and its development and implementation may facilitate the rapid identification of hot spots for evidence-informed interventions.

Wastewater-based epidemiology (WBE) has been used effectively to monitor the circulation of viral pathogens in specific populations. WBE was originally defined as a non-intrusive tool to measure biomarkers of effect at community level. Although this approach has been extensively utilised for estimating substance use and abuse, it has recently been extended to include public health biomarkers.[1] To date, WBE has played a key role in the development of early warning systems (EWSs) for a range of enteric viruses including poliovirus, norovirus and hepatitis E.[2-4] Scientific evidence emerged recently that SARS-CoV-2, the virus that causes the disease COVID-19, is shed in faeces of infected symptomatic and asymptomatic individuals (5, 6), with detectable RNA, ending up in wastewater.[7,8] The detection of SARS-CoV-2 RNA in faeces has stimulated rapid research into wastewater surveillance. An increasing number of countries, including The Netherlands, Spain, Australia, the UK and the USA, have detected SARS-CoV-2 RNA in untreated wastewater.[7-12] Based on these studies and the success of previous wastewater surveillance strategies, the tracking of SARS-CoV-2 in wastewater has been proposed as a potentially important public health strategy to combat the current COVID-19 pandemic.

The first case of COVID-19 in South Africa (SA) was reported on 5 March 2020 and was followed by a national lockdown 3 weeks later. By early July 2020, over 1.5 million COVID-19 tests had been conducted, with over 130 000 confirmed cases disproportionally spread across the country.[13] Currently, COVID-19 hotspots in SA are determined by human testing data which, owing to numerous factors including undetected asymptomatic cases and operational issues, may not accurately represent case numbers.[14] In SA, and other socioeconomically vulnerable countries, under-reporting of COVID-19 cases may pose a challenge to the management of COVID-19, and an effective, complementary EWS that can be used to identify COVID-19 hot spots and emerging outbreaks will therefore be a valuable tool to guide action and the distribution of resources for containment and mitigation strategies. As viral shedding may occur before COVID-19 cases are reported,[10] WBE by molecular detection of viral RNA in wastewater can be used as a potential tool for public health monitoring at a community level. As such, this study aimed to evaluate an RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR) as a means to qualitatively and quantitatively detect the presence or absence of SARS-CoV-2 viral RNA from 24-hour composite wastewater samples from wastewater treatment plants (WWTPs) in Western Cape Province, SA.

Wastewater sampling

Untreated influent wastewater samples were collected on 18 June 2020 from four WWTPs in the City of Cape Town metropole and one in the neighboring municipality of Stellenbosch, using 24-hour time-proportional composite samplers (100 mL every 10 minutes, 09h00-09h00). The samples were collected at the raw influent of each WWTP after coarse screens and de-gritting (prior to discharge

to primary settlement reactors and/or addition of chemical flocculants). The WWTPs (and the populations they serve) were Athlone (population ~385 000), Macassar (~200 000), Cape Flats (~855 000), Zandvliet (~755 000) and Stellenbosch (~180 000) (Fig. 1). At the time of sampling, the Western Cape was the epicentre of the SARS-CoV-2 outbreak in SA. All samples were transported on ice to the laboratory and stored at -80ºC before subsequent viral RNA extraction, within 24 hours, using a modified method described by Peccia *et al*.[10]

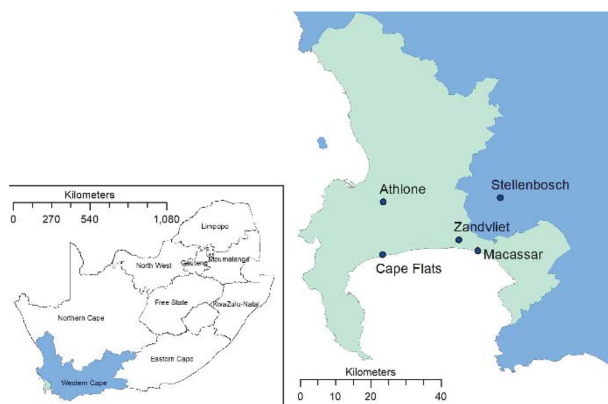

Figure 1: Location of wastewater treatment plants, Western Cape

Viral RNA extraction from wastewater

SARS-CoV-2 viral RNA was extracted from the wastewater samples using the RNeasy PowerSoil Kit as per the manufacturer's instructions (Qiagen, Germany).[10] Briefly, 50-100 mL influent wastewater was spun down at 3 500 *g* for 20 minutes, and 5-10 mL of the pellet was added to a 15 mL PowerBead Tube containing lysis buffer, provided with the kit, to stabilise viral RNA. Thereafter, the sample was homogenised and phase-separated using an equal volume of phenol/chloroform and the upper aqueous phase was transferred to a new 15 mL tube and mixed with buffers supplied with the RNeasy PowerSoil Kit. The aqueous phase was then transferred to the RNeasy JetStar Mini Column to elute out the bound RNA before centrifugation at 13 000 *g* for 10 minutes. The resultant pellet was dried and dissolved in ribonuclease-free water to a final volume of 70 µL. Total RNA was measured by spectrophotometry using a NanoDrop Spectrophotometer (ThermoFisher Scientific, USA). It should be noted that attempts to extract RNA from the supernatant yielded no detectable RNA.

Amplification of SARS-CoV-2 viral RNA using RT-qPCR analysis

Primer and probe sets, approved by the Centers for Disease Control and Prevention, that target two sequences from the SARS-CoV-2 nucleocapsid gene (N1 and N2 primer/probe) were purchased from Whitehead Scientific (2019-nCov CDC EUA Kit, Integrated DNA Technologies, USA). The N-gene primer/probe was used to assess the presence of SARS-CoV-2 viral titres. Both the N1 and N2 primer/probe sets aligned 100% to the N protein of the SARS-CoV-2 strain. The sequences of the primers and probes targeted to the N gene, including the thermocycling conditions used to amplify these regions for detection, are shown in Table 1.

Viral load quantification was assessed using the standard curve method. For RT-qPCR positive control and viral RNA copy number quantification, a 10-fold serial dilution was made using the 2019-nCoV-N-positive plasmid control as a standard, which was supplied at 200 000 copies/µL (Qauntabio, USA).[15] For the qualitative detection of SARS-CoV-2 viral RNA, a one-step qRT-PCR reaction was performed using iTaq Universal Probes One-Step Reaction Mix (Bio-Rad Laboratories, USA) in a final reaction volume of 10 µL using 1 µL of 0.1 µg/µL of total RNA. The RT-qPCR reactions were conducted on the Applied Biosystems QuantStudio 7 Flex Real-Time PCR System (ABI Technologies, SA) using universal cycling conditions as per the manufacturer's instructions (Table 1). All reactions were done in duplicate and a reagent blank was included for each sample. To minimise potential contamination, RNA extraction and qRT-PCR were performed in separate laboratories.

Table 1. Thermal cycling protocol and primers and probes used in the study

Organism	Target	Assay name	Target	Sequence (5′-3′)	Cycling parameters	References
SARS-CoV-2	N-protein	2019-nCoV CDC	N1 primer/probe	F 5′-GAC CCC AAA ATC AGC GAA AT-3′ R 5′-TCT GGT TAC TGC CAG TTG AAT CTG-3′ P-FAM-ACC CCG CAT TAC GTT TGG ACC-BHQ1	Reverse transcription: 50º for 10 min; 95º for 1 min Amplification: 95º for 15 s; 60º for 1 min (40 cycles)	10, 15
			N2 primer/probe	F 5′-TTA CAA ACA TTG GCC GCA AA-3′ T 5′-GCG CGA CAT TCC GAA GAA-3′ P-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1		

Qualitative detection of SARS-CoV-2 viral RNA extraction using RT-qPCR analysis. During the peak of the COVID-19 outbreak in the Western Cape, total RNA ranging between 500 and 1 000 ng/mL was extracted from either 50 or 100 mL of influent wastewater using the Qiagen RNeasy PowerSoil Kit. Quantitative PCR (qPCR) analysis revealed that all samples were amplified above the quantification cycle threshold (Ct) and ranged between 29 and 32.8 cycles (samples diluted 1:10) for either the N1 or N2 primer/probe assays. A viral load of <35 cycles can be considered as a wastewater sample with low SARS-CoV-2 RNA. The 2019-nCoV N-positive control was 10-fold serially diluted and used to determine copy numbers between the unknown wastewater sample and the calibrator.

The slope for the N1 and N2 assays were -3.4 and -3.5, respectively and y-intercept values were -37.47 (for the N1 assay) and -37.81 (for the N2 assay). The correlation (r^2) values for the N1 and N2 assays were both 0.99, while the amplification efficiency for the two assays was 94% and 95%, respectively.

Quantitative and qualitative detection of SARS-CoV-2. Quantitative and qualitative analysis using either N1 or N2 primer/probes confirmed the presence of SARS-CoV-2 viral RNA in all five wastewater samples, with a viral RNA copy number ranging between 4.6×10^3 and 454×10^3 virus RNA copies/mL (Fig. 2A). The lowest concentration of viral RNA copy numbers was from the Stellenbosch WWTP, with a Ct value of 32, while the highest was detected in the Zandvliet WWTP, with a Ct value of 29 (Fig. 1). Based on the Western Cape government COVID-19 dashboard[16], the number of confirmed COVID-19 cases in the Western Cape on 18 June 2020 in the Khayelitsha subdistrict (one of the areas served by the Zandvliet WWTP) had a higher number of COVID-19 cases (*n*=80) compared with the Stellenbosch municipality (*n*=43). Our Zandvliet and Stellenbosch WWTP SARS-CoV-2 data corresponded with this difference in higher and lower case numbers, respectively. The COVID-19 dashboard showed that 1 week before the wastewater collection date, there were 82 positive cases in Khayelitsha and 17 in Stellenbosch.

Wastewater-based epidemiology-based calculations

The calculated viral RNA copy numbers (viral copy numbers/mL) for the N1 and N2 fragments from the various WWTPs were normalised to account for the variable treatment size of the WWTPs used in this study and the variable populations that are connected to the sewer works. The average daily flow rate (in mL/day) at the raw inlet of the WWTPs was used to calculate an estimated daily load of viral RNA copy numbers for each location (in viral copy numbers/day; Fig. 2B). The population estimates from each WWTP were then used to generate an estimated per capita daily load (copy numbers/day/1 000 inhabitants) of the viral RNA markers at the respective WWTPs (Fig. 2C). In this way, the RT-qPCR quantitative results for SARS-CoV-2 RNA markers were normalised to compensate for the variation in WWTP flow conditions ~~that is~~ received by each plant, as well as to compensate for the variable population size in each community that is served by the respective WWTPs for more accurate comparisons between locations.

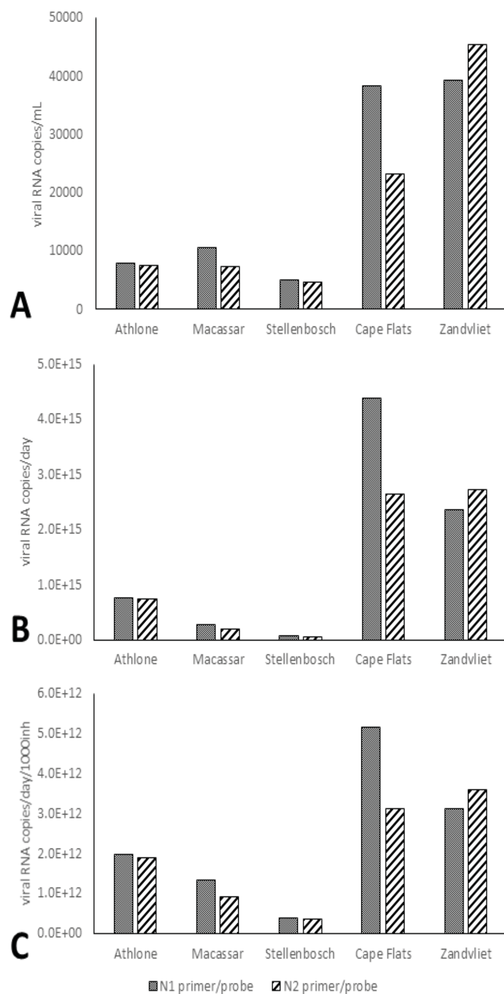

Figure 2A: SARS-CoV-2 Viral RNA detected in wastewater samples from wastewater treatment plants (WWTPs) in Cape Towns and Stellenbosch, Western Cape, South Africa. SARS-CoV-2 viral RNA copy number of 24hr composite samples collected on the 18th of June from Athlone, Macassar, Stellenbosch, Cape Flats and Zandvliet WWTPs (Western Cape). Figure 2B – normalised with the variable daily flow rate. Figure 2C is normalised with both the variable flow rate and population size (inh=inhabitants)

What does this mean for a COVID-19 EWS?

In this proof-of-concept study, we set out to establish whether SARS-CoV-2 viral RNA could be detected both quantitatively and qualitatively in 24-hour composite wastewater samples as the basis for an early warning system for COVID-19 infection in communities in urban settings of SA. The study confirmed that the Qiagen RNeasy PowerSoil Kit is a robust extraction method for the detection of SARS-CoV-2 viral RNA in wastewater. For quantitative analysis, the 2019-nCoV EUA N-gene assays produced positive results for all samples tested. The CDC-approved primer/probe was specifically designed for the detection of SARS-CoV-2 in wastewater.[15,17] Our study confirmed previous findings showing that the N gene can be used to detect SARS-Cov-2 viral RNA in wastewater samples in the USA,[11] The Netherlands,[8] the UK,[10] Spain,[9] Italy[12] and Australia.[7] Furthermore, to the best of our knowledge, this is the first published study on the viral load copy numbers from wastewater obtained from WWTPs in SA, and the first report to quantitatively detect SARS-CoV-2 viral RNA in wastewater samples from five WWTPs using the CDC N1 and N2 assays. The study further demonstrated that the viral load in the wastewater samples from Stellenbosch was

much lower than that in the samples from Zandvliet (Fig. 2B and C, respectively), and this finding corresponded with the number of positive COVID-19 cases detected in the areas at the time. This is important, as tracking of SARS-CoV-2 viral RNA in wastewater could complement surveillance efforts in the development of an EWS for local COVID-19 outbreaks. As these wastewater samples were collected at the peak of the epicentre in the Western Cape, longer-term wastewater surveillance will be required to establish a baseline to distinguish between high-risk and low-risk events. Furthermore, while normalisation of data provided some insight, data on normalisation factors may be lacking in resource-constrained settings. Having baseline temporal data from which trends can be identified is therefore crucial. In this case, comparing standardised volumes (copies/mL) between sites is in fact an adequate endpoint.

An effective COVID-19 EWS may prove critical in SA and other low- and middle-income countries with inadequate health systems infrastructure, resources and human capacity. Furthermore, it can be argued that benefits of investment in a WBE system in SA will reach beyond the current COVID-19 wave, as it may forewarn of future resurgences. To date, the screening of wastewater is recognised as an important tool for monitoring wild poliovirus and vaccine-derived polioviruses,[18,19] and there are opportunities to learn from polio wastewater surveillance to overcome potential logistic and operational issues for WBE of SARS-CoV-2. As such, our future planned studies will include regular sampling coupled with SARS-CoV-2 prevalence estimation. Currently, WBE is best suited to a converging sewer network serving a target population. However, in SA, 40% of households are not connected to the sewage system.[20] Although wastewater surveillance for SARS-CoV-2 has the potential to be a powerful public health tool, especially in resource-constrained settings, alternatives to sewage from wastewater treatment plants therefore need to be supported by resolutions for more locally relevant situations (such as wastewater runoff in informal settlements) considering sanitation diversity.[21] We acknowledge that this study did not include a spike RNA control to investigate the percent viral recovery, and this will be included in a follow-up study.

Conclusions

This proof-of-concept study confirms that qualitative and quantitative detection of SARS-CoV-2 viral RNA from wastewater influent samples can be used as a surveillance tool in the current SA context, thereby establishing the potential for an EWS for monitoring status and trends of COVID-19 mass infections in SA that could facilitate the rapid identification of hot spots for evidence-informed interventions. An important yet unexpected outcome was that this study brought together professionals and experts spanning skill sets, disciplines and sectors including public health, microbiology, town planning and wastewater treatment facilities, and formed the basis for a comprehensive weekly follow-up study that includes a larger number of wastewater treatment plants being sampled over consecutive days with the overall agenda of advancing equity and health.

Declaration. None.
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Author contributions. RJ, RS, AM, CM, GG, MM and JL conceptualized, designed, wrote and edited the manuscript. RJ and SG conducted and analysed the experiments, LB collected the samples. CM, JL, EA, SN, NB, MV, LB, GW, MW, GG, HG, LB, SM, AM and MM provided scientific input. All authors read and approved final manuscript.

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Conflicts of interest. None.

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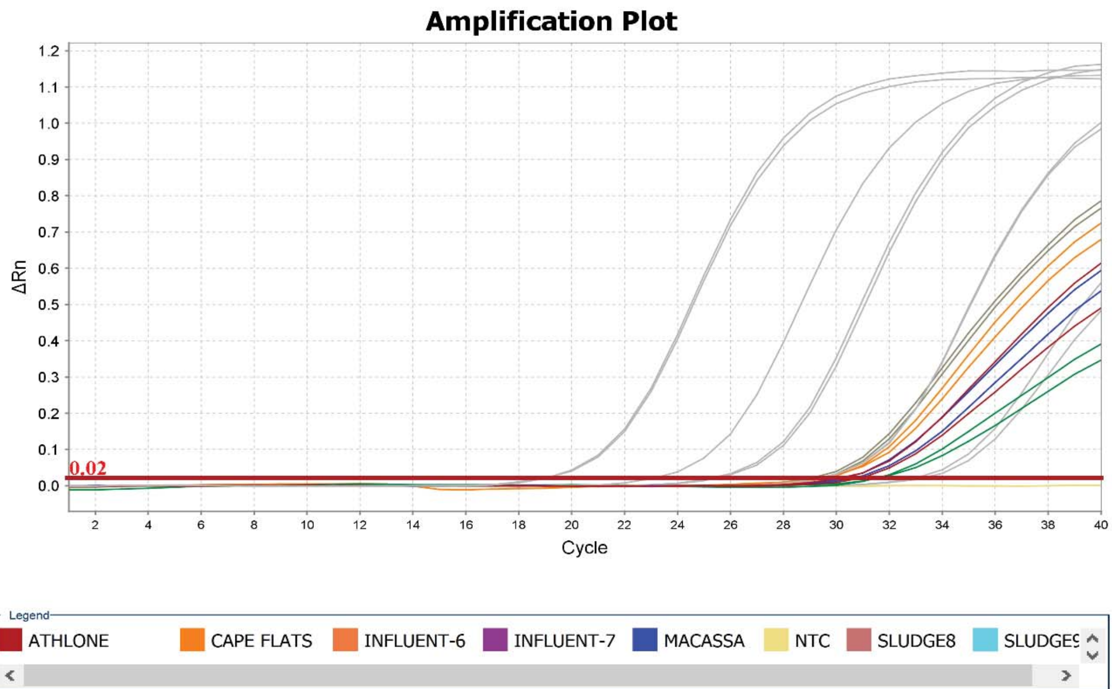

Supplementary Figure 1. Amplification plot

Supplementary Table 1. Ct values

N1 primer	Ct
Athlone	30.497
Macassar	30.408
Stellenbosch	31.521
Cape Flats	29.511
Zandvliet	29.263

N2 primer	Ct value
Athlone	31.22
Macassar	31.304
Stellenbosch	34.142
Cape Flats	30.588
Zandvliet	29.785