DEVELOPMENT OF A FRAMEWORK FOR WATER QUALITY-BASED COVID-19 EPIDEMIOLOGY SURVEILLANCE FOR NON-SEWERED COMMUNITIES

Report to the
Water Research Commission

by

Waterlab (Pty) Ltd

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EXECUTIVE SUMMARY

The Coronavirus Disease 2019 (COVID-19) pandemic is caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus. The virus was declared a Public Health Emergency of International Concern on the 30th of January 2020 by the World Health Organisation (WHO) (WHO, 2020a) and subsequently elevated to pandemic status on 11 March 2020 (WHO, 2020b). Since then, COVID-19 has swept across the world infecting 433,358,400 people and causing 5,940,413 deaths globally as of 26 February 2021 (https://coronavirus.jhu.edu/) (John Hopkins University & Medicine, 2022). As part of the efforts to stop the spread of this virus, the detection of SARS-CoV-2 in municipal sewage was successfully been proven both internationally (Medema et al., 2020) and in South Africa (Pocock, et al., 2020; NICD, 2021). Environmental surveillance of municipal sewage offers the benefit on population-level data for monitoring COVID-19. In certain cases, researchers have shown the presence of SARS-CoV-2 virus in municipal sewage before the first clinical detection in a country (Medema, et al., 2020; La Rosa, et al., 2020), and the benefits of .WBE has led to many developed countries, including the Netherlands, Finland France, Italy, Portugal, Spain, to establish programmes at various levels to complement clinical data cases The WHO lists the following benefits of detection of SARS-CoV-2 in municipal sewage – also known as Wastewater Based Epidemiology (WBE) (WHO, 2020c):

- early warning and hotspots for COVID-19 cases in a community;
- detection of COVID-19 in locations with less developed clinical surveillance;
- monitoring circulation of the virus and its variants during outbreaks; or
- to trigger case-finding in targeted locations where there are or may be suspected cases. This includes quarantine hotels, university campuses, fitness centres, airplanes or prisons.

Developing countries which have lower sewerage coverage are not able to access this useful tool in their pandemic response. A third of the global population (2.4 billion people) have access to private sanitation facilities connected to sewers from which wastewater is treated (WHO, 2019). Around 2 billion people have no access to any sanitation facility (WHO, 2019). In South Africa, around two thirds of the population have access to a flush toilet connected to the public sewerage system (Statistics South Africa, 2019). Therefore, whilst WBE of communities for SARS-CoV-2 viral prevalence by sampling from wastewater treatment works (WWTWs) is a powerful complementary epidemiological tool, in South Africa almost 40% of the population will not be covered with this method. These are also usually the most vulnerable communities who do not have access to sufficient health care or financial resources. In communities lacking any formal sewerage networks, with no sanitation facilities, as well as those connected to non-functional or poorly performing communal WWTWs, poorly or partially treated human excreta, greywater and / or sewage (from WWTWs) is disposed into the environment. The likelihood of detecting viral particles in our river and tributary systems is therefore very good.

At a municipal WWTWs, a largely homogenous influent sample can be taken at the head of the works or sludge sampled at the front end of the works. Non-sewered settlements are more challenging to the variety of systems and wastes that be generated. This includes fresh human excreta, faecal sludge accumulating in non-sewered sanitation systems which range from flushing toilets connected to latrines or septic tanks to dry latrine-based systems, blackwater (faeces, urine and flush water) and greywater (from washing activities). Non-sewered sanitation systems are known to produce samples that are more highly variable and can be magnitudes higher in concentration than domestic wastewater from sewered systems. The sampling methodology in this study was guided by an earlier Proof of Concept WBE study in which a few non sewered settlements were also included (Pocock et al., 2020), where it was demonstrated that surface waters could potentially be used for an early warning system for non-sewered communities in densely-populated non sewered settlements. It was these sampling points that were first explored as reliable sampling points to understand the exposure of these communities to COVID-19.

Due to the transient nature of greywater sample sites, it was recommended that rivers are used as the more stable and reliable sample point for detection of COVID-19 exposure within the community. It was proposed that rivers and streams were sampled at defined points, particularly where known non-point sources of greywater and / or blackwater / sewage / human excreta contamination were occurring as a result of informal housing with no connection to sewers. Greywater polluted by blackwater / human excreta in non-sewered communities could also be sampled as a potential epidemiological indicator when available. Similarly, in areas where non sewered sanitation systems such as pit latrines were used instead of sewered systems, sampling of the faecal sludge may give an indication of SARS-CoV-2 community-level epidemiological information.

The project aims were as follows:

1. Develop a sampling framework for COVID-19 surveillance in non-sewered communities. The sampling framework will be based on field observations of non-sewered environments and include aspects such ideal sampling points, sampling method (random vs. systematic), sample types and potential areas of virus concentration that would be later correlated to virus detection in the laboratory. This includes, for example, standing pools, greywater plumes and communal stand-pipe pools. The sampling framework will serve as standardized operating procedure for SARS-CoV-2 sampling and subsequent detection.
2. Develop and optimise the methodology for SARS-CoV-2 detection, quantification and monitoring in different types of samples from non-sewered environments. This aspect will include appropriate viral concentration methods.
3. Target a minimum of four (4) provinces and up to twenty (20) settlements/sample sites from targeted provinces.
4. Screening for additional pathogens as indicators of public health.
5. Assessment of crAssphage as an indicator of human faecal contamination for data normalisation.
6. Sequencing of a subset of positive samples for possible variant tracking.
7. Provide the data and recommendations for the development of a surveillance reporting platform and undertake mapping and trend analysis.
8. Support capability building for water quality-based SARS-CoV-2 epidemiology.

The data from this study indicated that COVID-19 could be identified in non-sewered community run-off, surface water and in the rivers which lie downstream of these communities. The incidence of COVID-19 in these communities was reflected in the Cycle Threshold (Ct) values (the number of cycles required for the fluorescent signal to cross the threshold or background level and is inversely proportional to the amount of target nucleic acid in sample) obtained in the rivers and surface run-off samples. During the third wave from May to July 2021 the incidence of COVID-19 at detectable levels in these environmental samples increased with a corresponding increase in daily cases reported, and a similar increase was observed for the fourth wave in November and December 2021. In Gauteng province where the informal settlements are dense and the rivers are highly polluted by faecal matter from these communities, the trends were even more evident. It should be noted that the incidence of COVID-19 infections in the unsewered communities was very likely underreported as the cost of testing was prohibitive to these individuals and any free government testing would result in long queues and consequently time off work (if employed) would be required. Peaks in COVID-19 detection in the rivers were noted in March 2021, although these were not reflected in the clinical case load data, possibly due to unreported or untested infections.

Passive sampling of rivers has shown to be generally more sensitive that grab samples for the sites tested, with the passive samplers detecting SARS-CoV-2 earlier than the grab samples, and for longer into the inter-wave periods following the wave peaks. The use of passive samplers for detection of low viral loads will be particularly applicable during the rainy season when the dilution factor is high.

Quantification of the human impact on a river is challenging as the number of individuals contributing to the viral load in the river is unknown. Therefore, it is necessary where possible to monitor indicators of human faecal contamination in these environmental samples. Use of the crAssphage as an indicator of faecal pollution has shown promise. Initial screening of the Jukskei River downstream of Alexandra showed crAssphage trends in the river samples similar but not identical to that of the ammonia concentration and *E. coli* counts in the water quality. crAssphage screening on the Kaalspruit in Gauteng, as well as the run-off into the Plankenbrug River in the Western Cape, and the Palmiet River in KwaZulu-Natal (KZN), showed that crAssphage trend more closely followed that of the ammonia concentration in the less polluted rivers where the informal settlement had an obvious impact on the downstream water quality, such as the Palmiet River downstream of the Quarry Road Informal Settlement. This study illustrated the potential application of crAssphage for normalisation of environmental sampling data to prevent misinterpretation of low Ct positive SARS-CoV-2 results. Low Cts indicating a higher case load may in fact be due to more concentrated sewage or a higher faecal load in the river or run-off water, perhaps due to dumping or a pollution event. CrAssphage may also serve as an indicator of stormwater dilution due to rainfall where river flow rates cannot be determined.

While this non-sewered surveillance programme focused on SARS-CoV-2, it was clearly illustrated that the same sample collection, recovery and extraction techniques could be successfully applied to collect contextual and public health information on other pathogens and indicators. Norovirus is an enteric virus that is very commonly present in South Africa's population and shed via stool, often used as reference viruses for sewage surveillance. Norovirus was found to be almost ubiquitously present in all four provinces (Gauteng, Mpumalanga, KZN and he Western Cape) across sites sampled, with norovirus genogroup I (GI)

detected in 48% and norovirus genogroup II (GII) in 53% of 87 samples tested across eight sites. Both genogroups were present in 34% of samples. Similarly, twenty three percent of 44 samples tested positive for Hepatitis E, and positive samples were found in all four provinces tested. While not as prevalent in the samples assayed as the norovirus, the presence of Hepatitis E in 23% of the limited number of samples screened across the four provinces indicated that incidence of the virus is widespread. CrAssphage as an indicator of sewage contamination was also successfully isolated using these techniques.

A subset of 29 positive samples were selected for sequencing to confirm the SARS-CoV-2 assay and identify the specific variants present in those samples and potentially allow for tracking of variants over time. It was possible to generate successful sequencing libraries for 28 samples, with an average coverage of 92.9% when compared with the reference genome. 22 of these samples matched with other SARS-CoV-2 sequences in the Basic Local Alignment Search Tool (BLAST) database by >99%, confirming the positive assay results. Both grab samples and passive samples yielded successful libraries. Variant lineages were assigned to 12 of the 28 successful libraries (43%), and the shift in dominance from the Delta variant to the Omicron variant was apparent in samples taken in November 2021 from the Jukskei Source and the Jukskei downstream of Alexandra. Tracking of variants in environmental samples is therefore a viable method for determining the incidence of these variants in the non-sewered communities impacting on these water sources. More samples could be assigned to lineages in the Jukskei Source than other sites, indicating that the ribonucleic acid (RNA) may have been less fragmented and more like would be expected in a wastewater sample taken from a wastewater treatment works inlet. The same was seen in tanker effluent which may be a useful source of variant tracking in non-sewered areas with conservancy tanks.

This program initiated from this study provided valuable additional information about the spread of the virus in non-sewered settlements across South Africa. It has the potential to complement clinical health surveillance as well as the conventional Wastewater Based Epidemiology (WBE) being undertaken through the South African Collaborative COVID-19 Environmental Surveillance System (SACCESS) network (NICD, 2021) and together serve as an early warning system for COVID-19 infections. Critically, this information will provide decision support to officials determining the timing and severity of public health interventions and to mitigate the overall spread of the disease. Regular screening of these sample points will also be useful to assist in early detection of the re-emergence of the virus.

COVID-19 is most likely not going to be the only pandemic we will be facing in years to come. Urban water streams represent a rich and highly relevant source of information about exposure to pathogens as well as the opportunity to monitor emerging contaminants, lifestyle indicators, and antimicrobial resistance. This study demonstrated that the same methodologies used for the isolation and extraction of genetic material from the SARS-CoV-2 virus could be applied to other enteric pathogens as well as crAssphage as an indicator virus. This could be used to build a strategy and envision various scenarios about how this information can be used to prevent, mitigate, or even predict future outbreaks, as well as monitor human health on a broad scale, turning data into actionable insights for public health authorities and policy makers. It is important to consider how best to ethically and legally balance public health with civil liberties when handling this type of information (Gostin et al., 2020). One of the benefits of wastewater is that it has limited sociological bias with few if any ethical issues.

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TABLE OF CONTENTS

ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CBD	Central Business District
COD	Chemical Oxygen Demand
COVID-19	Coronavirus Disease 2019
EC	Electrical Conductivity
ERWAT	Ekurhuleni Water Care Company
GDP	Gross Domestic Product
GI	Genogroup I
GII	Genogroup II
KZN	KwaZulu-Natal
NGS	Next Generation Sequencing
NICD	National Institute for Communicable Diseases
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
ROL	Rivers of Life Aquatic Health Services
RPM	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SACCESS	South African Collaborative COVID-19 Environmental Surveillance System
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SS	Suspended Solids
TDS	Total Dissolved Solids
TOC	Total Organic Carbon
TS	Total Solids
UDDT	Urine Diversion Dehydration Toilet
UKZN	University of KwaZulu-Natal
UNDP	United Nations Development Programme
UP	University of Pretoria
VoC	Variant of Concern
VOC	Volatile Organic Compounds (VOC)
WBE	Wastewater Based Epidemiology
WHO	World Health Organisation
WRC	Water Research Commission
WWTW	Wastewater Treatment Works

UNITS

CFU	Colony Forming Units
Ct	Threshold Cycle
°C	degree Celsius
L	Litre
mg	Milligrams
mL	Millilitre

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

The Coronavirus Disease 2019 (COVID-19) pandemic is caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus. The virus was declared a Public Health Emergency of International Concern on the 30 January 2020 by the World Health Organisation (WHO) (WHO, 2020a) and subsequently elevated to pandemic status on 11 March 2020 (WHO, 2020b). Since then, COVID-19 has swept across the world infecting 433,358,400 people and causing 5,940,413 deaths globally as of 26 February 2021 (https://coronavirus.jhu.edu/) (John Hopkins University & Medicine, 2022). It has also had a severe impact on the world economy and international trade (Pak et al., 2020). South Africa is expected to Gross Domestic Product (GDP) decline and no recovery by 2024 (UNDP, 2020). As part of the efforts to stop the spread of this virus, the detection of SARS-CoV-2 in municipal sewage has successfully been proven both internationally (Medema et al., 2020) and in South Africa (Pocock et al., 2020; NICD, 2021). Environmental surveillance of municipal sewage offers the benefit on population-level data for monitoring COVID-19. In certain cases, researchers have shown the presence of SARS-CoV-2 virus in municipal sewage before the first clinical detection in a country (Medema, 2020; La Rosa, et al., 2020). The WHO lists the following benefits of detection of SARS-CoV-2 in municipal sewage – also known as Wastewater Based Epidemiology (WBE) (WHO, 2020c):

- early warning and hotspots for COVID-19 cases in a community;
- detection of COVID-19 in locations with less developed clinical surveillance;
- monitoring circulation of the virus and its variants during outbreaks; or
- to trigger case-finding in targeted locations where there are or may be suspected cases. This includes quarantine hotels, university campuses, fitness centres, airplanes or prisons.

The benefits of WBE has led to many developed countries, including the Netherlands, Finland, France, Italy, Portugal, Spain, to establish programmes at various levels to complement clinical data cases (evidence in Medema et al., 2020). Developing countries which have lower sewerage coverage are not able to access this useful tool in their pandemic response. A third of the global population (2.4 billion people) have access to private sanitation facilities connected to sewers from which wastewater is treated (WHO, 2019). Around 2 billion people have no access to any sanitation facility (WHO, 2019). In South Africa, around two-thirds of the population have access to a flush toilet connected to the public sewerage system (Statistics South Africa, 2019). This sewerage infrastructure distribution is not uniform across provinces. Further, within a province, the distribution may be higher around urbanised metropolitan municipalities and lower in local municipalities which may be less developed. Flush toilets connected to public sewerage systems were most common in the most urbanised provinces, namely the Western Cape province (87%) and Gauteng province (84%) (Statistics South Africa, 2019). Only 20% of households in the Limpopo province had access to any type of flush toilet, the lowest of any province. In the absence of flush toilets, 69% of households in the Limpopo province used pit latrines, most (54%) without ventilation pipes which can be considered as below the minimum sanitation standard for the country. In the Mpumalanga province, 42% of households have access to flush toilets connected to the public sewer system. Around 45% of households use latrines of which 34% do not have ventilation pipes. The KwaZulu-Natal (KZN) province has a similar number of households with a flush toilet connected to public sewerage system (1,047,538 households in KZN versus 1,607,420 households in the Western Cape) but the KwaZulu-Natal province population size is around a third larger and has a large rural component. Around 42% of households in the KZN province are connected to the public sewerage system while 37% have access to a pit latrine, 5% to flush toilet connected to a septic tank and 9% to temporary sanitation facility in the form of a chemical toilet (Statistics South Africa, 2019). These results highlight the geographical differences in the type of toilet facilities that can occur across and with provinces. Most municipalities located with all provinces in South Africa are not fully sewered and can have a diverse range of sanitation technologies.

Therefore, whilst WBE of communities for SARS-CoV-2 viral prevalence by sampling from Wastewater Treatment Works (WWTWs) is a powerful complementary epidemiological tool, in South Africa almost 40% of the population will not be covered with this method. These are also usually the most vulnerable communities who do not have access to sufficient health care or financial resources. In communities lacking any formal sewerage networks, no sanitation facilities, as well as those connected to non-functional or poorly-performing communal WWTWs, poorly or partially treated human excreta, greywater and / or sewage (from WWTWs) is disposed into the environment. With communities with no sanitation facilities or facilities in poor condition, this disposal could be to the ground or into a nearby stream or water source that has the potential to enter streams and rivers. The likelihood of detecting viral particles, whether infectious or not, in our river and tributary systems is therefore very good.

The current picture of SARS-CoV-2 virus circulation in the population of South Africa is incomplete and the number of COVID-19 patients most likely underestimated, mainly due to the limitations regarding individual testing. At the start of the pandemic, most people who experienced mild symptoms were not tested due to the shortage of test kits. Pricing for individual COVID-19 PCR tests at private laboratories was probably not

affordable to indigent populations (around ZAR1200 at the start of the pandemic to around ZAR850 in December 2021) with the Competition Commission of South Africa agreeing with settlements with private laboratories to drop their persistently high and unchanged pricing despite greater volumes and availability of tests (Competition Commission South Africa, 2021). Further testing is mainly reserved for symptomatic patients, close contact cases and for use in hospitals for patients with serious medical conditions.

An adaption of the WBE approach for SARS-CoV-2 detection in non-sewered settlements would be highly beneficial to developing countries which do not have significant sewer coverage. Community-level data and hot spot detection can potentially be achieved to overcome the burden of individual testing in these areas. It is therefore vital to develop a framework and methods for sampling and surveillance of grey and wastewaters within the non-sewered community to ensure a timeous response to an upsurge in SARS-CoV-2 within these vulnerable communities.

The challenge that the research team were faced with at the beginning of the project was what to sample and how often. At a municipal WWTWs, a largely homogenous influent sample can be taken at the head of the works or sludge sampled at the front end of the works. Non-sewered settlements are more challenging to the variety of systems and wastes that be generated. This includes fresh human excreta from no toilet facilities, faecal sludge accumulating in non-sewered sanitation systems which range from flushing toilets connected to latrines or septic tanks to dry latrine-based systems, blackwater (faeces, urine and flush water) and greywater (from washing activities). Non-sewered sanitation systems are known to produce samples that are more highly variable and can be magnitudes higher in concentration than domestic wastewater from sewered systems.

The sampling methodology in this study was guided by an earlier Proof-of-Concept WBE study in which a few non-sewered settlements were also included (Pocock, et al., 2020). Greywater run-off and the nearest stream or river near non-sewered settlements were sampled. Similar studies to this Proof-of-Concept study have been performed in Italy (Rimoldi, et al., 2020) and in Ecuador (Guerrero-Latorre, et al., 2020) where wastewater was discharged into the natural waters. In the Proof-of-Concept study, surface water grab samples were collected at four different sites within Gauteng, 3 rivers downstream of informal settlements as well as surface run-off within an unsewered community, and all four surface water samples tested positive for the SARS-CoV-2 virus, indicating that these surface waters could potentially be used for an early warning system for non-sewered communities in densely-populated non-sewered settlements. It was these sampling points that were first explored as reliable sampling points to understand the exposure of these communities to COVID-19.

Due to the transient nature of greywater sample sites, it was recommended that rivers are used as the more stable and reliable sample point for detection of COVID-19 exposure within the community. It was proposed that rivers and streams were sampled at defined points, particularly where known non-point sources of greywater and / or blackwater / sewage / human excreta contamination were occurring as a result of informal housing with no connection to sewers. Greywater polluted by blackwater / human excreta in non-sewered communities could also be sampled as a potential epidemiological indicator when available. Similarly, in areas where non-sewered sanitation systems such as pit latrines were used instead of sewered systems, sampling of the faecal sludge may give an indication of SARS-CoV-2 community-level epidemiological information. While it is not necessarily possible to relate viral loads in surface water to a defined population or possible case numbers, sampling of rivers and streams may provide a means to monitor the spread of SARS-CoV-2 within informal settlements by monitoring river or stream quality over time, as well as monitoring trends in viral loads to identify possible infection spikes in communities upstream of the sample point. This may give an early warning of the presence of COVID-19 infections in these communities, where there is both the risk of rapid spread and low likelihood of conventional testing. This will enable deployment of rapid response teams into these areas to conduct more intensive testing and quarantining of infected individuals to curb the spread of the virus.

This programme initiated from this study provided valuable additional information about the spread of the virus in non-sewered settlements across South Africa. It has the potential to complement clinical health surveillance as well as the conventional WBE being undertaken through the South African Collaborative COVID-19 Environmental Surveillance System (SACCESS) network (NICD, 2021) and together serve as an early warning system for COVID-19 infections. Critically, this information will provide decision support to officials determining the timing and severity of public health interventions and to mitigate the overall spread of the disease. Regular screening of these sample points will also be useful to assist in early detection of the re-emergence of the virus. As this is a novel virus, this project aimed to build capacity in the testing and identification of COVID-19 virus within Southern Africa and within this project, for non-sewered surveillance.

The project aims were as follows:

1. Develop a sampling framework for COVID-19 surveillance in non-sewered communities. The sampling framework will be based on field observations of non-sewered environments and include aspects such ideal sampling points, sampling method (random vs. systematic), sample types and potential areas of virus concentration that would be later correlated to virus detection in the laboratory. This includes, for example, standing pools, greywater plumes and communal stand-pipe pools. The sampling framework will serve as standardized operating procedure for SARS-CoV-2 sampling and subsequent detection.
2. Develop and optimise the methodology for SARS-CoV-2 detection, quantification and monitoring in different types of samples from non-sewered environments. This aspect will include appropriate viral concentration methods.
3. Target a minimum of four (4) provinces and up to twenty (20) settlements/sample sites from targeted provinces.
4. Screening for additional pathogens as indicators of public health.
5. Assessment of crAssphage as an indicator of human faecal contamination for data normalisation.
6. Sequencing of a subset of positive samples for possible variant tracking.
7. Provide the data and recommendations for the development of a surveillance reporting platform and undertake mapping and trend analysis.
8. Support capability building for water quality-based SARS-CoV-2 epidemiology.

The project methodology is detailed in the following section.

2 METHODS

2.1 METHODOLOGY OVERVIEW

Testing and validation of various virus recovery, extraction assay methods has already been established as part of the Proof-of-Concept Study performed by this team (Pococket al., 2020). Polyethylene Glycol (PEG)/Sodium Chloride (NaCl) precipitation; skimmed milk flocculation and Aluminium Hydroxide ($Al(OH)_3$) adsorption/ flocculation virus recovery methods have all proven to be robust and relatively inexpensive methods which provide sufficient sensitivity to detect COVID-19 in both sewage and surface waters (Pocock et al,. 2020).

To determine the trends within a river or other contaminated water sources, a sampling point both up- and downstream of a selected informal settlement was identified to ensure that the impact of the community could be assessed based on the background upstream contamination. As part of the study, standing pools of water were also identified as sampling points especially in the KZN area where these are semi-permanent fixtures within the community. Grab samples were taken at all selected sample sites, and passive sampling was undertaken at a subset of sample sites.

Samples were either collected by Waterlab staff or couriered to Waterlab in Pretoria when sampled by others for sample preparation and analysis of water quality parameters. Samples were clarified by low-speed centrifugation for 10 minutes at 4 degrees Celsius (°C), and the virus recovered from 200 millilitres (mL) samples using skimmed milk flocculation recovery. The final pellet was dissolved in Phosphate Buffered Saline (PBS) and samples transferred to the Department of Medical Virology, University of Pretoria (UP) for ribonucleic acid (RNA) extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) detection. In addition, a 1:10 dilution of the extracted RNA was routinely performed as inhibition was often observed in the internal controls for the surface samples, as was observed in the proof-of-concept study.

A multiplex assay (Seegene) was used for this environmental work to enable detection of multiple gene targets due to the amount of variability observed in these waters. Samples with a cycle threshold (Ct) value of <40 were considered positive. Extraction and reagent negative controls were included in each run. RNA extracted from SARS-CoV-2 clinical samples were used for positive controls. A database was compiled for the project and plotted onto maps to visualise the trends and compared to the National Institute for Communicable Diseases (NICD) database of COVID-19 patients at the same date and wastewater treatment works surveillance programmes to establish trends within the data set. As part of the development of a non-sewered community sampling framework, the research team further attempted to develop methodologies to correlate the water quality data in terms of the level of pollutant contamination to better correlate viral loads to potential infection numbers.

In addition to SARS-CoV-2 screening, Next generation sequencing (NGS) was performed on a subset of samples positive for SARS-CoV-2 on a MiSeq using a NEBNext® ARTIC SARS-CoV-2 kit, to confirm the presence of SARS-CoV-2 and to attempt to identify the specific variants present in those samples.

2.2 SITE SELECTION

Collaborative engagements were made with various river action groups, community leaders, Universities and research facilitators to enable the collection of samples from identified sites.

Samples from identified sample sites were collected from March 2021 to September 2021, as presented in **Table 1**.

Table 1: List of sample site locations

No.	Pro-vince	City/Town	District	Site name	Type	Co-ordinates	Sample frequency	Sample duration
1	Gauteng	City of Johannesburg	Johannesburg MM	Jukskei upstream Alex	River	-26.109573, 28.113083	Monthly	20 weeks
2*		City of Johannesburg	Johannesburg MM	Jukskei Source	River	-26.19338, 28.07110	bi-weekly	20 weeks
2		City of Johannesburg	Johannesburg MM	Jukskei downstream Alex	River	-26.079089, 28.108555	bi-weekly	20 weeks
3		City of Johannesburg	Johannesburg MM	Silvertown, Alexandra standpipe/wetland	Surface	-26.087994, 28.107073	bi-weekly	20 weeks

No.	Pro-vince	City/Town	District	Site name	Type	Co-ordinates	Sample frequency	Sample duration
4		Kliptown, Soweto	Johannesburg MM	Klipspruit (K5) at Kliptown	River	-26.290033, 27.885617	bi-weekly	20 weeks
5		Sebokeng, Emfuleni LM	Sedibeng	Rietspruit (RV1) at Sebokeng	River	-26.728650, 27.717950	Monthly	20 weeks
6		City of Tshwane	Tshwane MM	Rietspruit (Hennops tributary) at Thatchfield	River	-25.895948, 28.119746	bi-weekly	20 weeks
7		City of Tshwane	Tshwane MM	Kaalspruit (Hennops tributary) downstream Tembisa	River	-25.957542, 28.206773	bi-weekly	20 weeks
8		City of Ekurhuleni	Ekurhuleni MM	Glenshaft Pan (DS Benoni WWTW at informal settlement)	Surface	-26.215597, 28.314323	bi-weekly	20 weeks
9		City of Ekurhuleni	Ekurhuleni MM	Klip River (Upstream Waterval WWTW)	River	-26.435323, 28.092954	bi-weekly	20 weeks
10	Western Cape	Stellenbosch	Cape Winelands	Plankenbrug River upstream informal settlement	River	-33.891337, 18.828423	bi-weekly	20 weeks
11		Stellenbosch	Cape Winelands	Plankenbrug/Krom River at run-off	River	-33.934110, 18.851063	bi-weekly	20 weeks
12		Franschhoek/ Langrug	Cape Winelands	Franschhoek River upstream Langrug settlement	River	-33.905828, 19.101960	bi-weekly	20 weeks
13		Franschhoek/ Langrug	Cape Winelands	Langrug settlement run-off into Stiebeuelrivier	Surface	-33.896159, 19.100807	bi-weekly	20 weeks
14	KZN	eThekwini	eThekwini	Palmiet river upstream Quarry Road West informal settlement	River	-29.804962, 30.965609	bi-weekly	20 weeks
15		eThekwini	eThekwini	Palmiet river downstream Quarry Road West informal settlement	River	-29.802616, 30.970598	bi-weekly	20 weeks
16		eThekwini	eThekwini	Quarry Road West informal settlement	Surface	-29.804122, 30.966965	bi-weekly	20 weeks
17		eThekwini	eThekwini	Umhlangane River upstream Johanna Road informal settlement	River	-29.796499, 30.993136	bi-weekly	20 weeks
18		eThekwini	eThekwini	Umhlangane River Downstream Johanna Road informal settlement	River	-29.799888, 30.991315	bi-weekly	20 weeks
19		eThekwini	eThekwini	Johanna Road informal settlement	Surface	-29.797288, 30.994522	bi-weekly	20 weeks
20		eThekwini	eThekwini	Urine Diversion Toilet composite samples in peri-urban eThekwini (TBC)	Unsewered on site sanitation	Various	5 samples	May to August
21	Mpumalanga	Kanyamazane	Mbombela	Crocodile River at Kanyamazane Upstream	River	-25.483761, 31.147015	bi-weekly	20 weeks

No.	Pro-vince	City/Town	District	Site name	Type	Co-ordinates	Sample frequency	Sample duration
22		Kanyamazane	Mbombela	Crocodile River at Kanyamazane Downstream	River	-25.488409, 31.171980	bi-weekly	20 weeks
* See discussion in 2.1.1								

In September 2021, the sites and results were reviewed and based on preliminary study findings some sites were retained for expansion of longitudinal data for historical trend analysis, sampling at some sites was stopped, and additional sites were added, to be sampled for an additional 12 weeks.

Passive sampling of environmental sites showed promise in the preliminary results, overcoming issues of low yield during high dilution periods. Passive samplers also had the advantage of allowing for easier and cheaper transport of samples, and sample processing was also much quicker. Therefore, passive sampling was elected to continue at 4 sample sites in parallel to the grab samples so as to compare more extensive data sets.

Three additional sites in the Western Cape were included as the study progressed; the Kuils River which is impacted by the un-serviced Khayelitsha informal settlement, the Black River impacted by various informal settlements, and passive sampling of pumped faecal sludge from on-site septic and conservancy tanks that were emptied at a WWTWs. A single proof of concept sampling event in this regard showed a successful outcome and there was value to continue this sampling protocol for a longer period. Only passive sampling was conducted at this site.

A list of the sample sites for sampling from September 2021 to February 2022 are presented in **Table 2**.

Table 2: List of new sample sites and sites for continued longitudinal monitoring to project conclusion

No.	Pro-vince	City/Town	District	Site name	Type	Co-ordinates	Sample frequency	Sample duration
1	Gauteng	City of Johannesburg	Johannesburg MM	Jukskei Source*	River	-26.193562, 28.070964	bi-weekly	12 weeks
2		City of Johannesburg	Johannesburg MM	Jukskei downstream Alex*	River	-26.079089, 28.108555	bi-weekly	12 weeks
3		City of Johannesburg	Johannesburg MM	Silvertown, Alexandra standpipe/wetland*	Surface	-26.087994, 28.107073	bi-weekly	12 weeks
4		City of Tshwane	Tshwane MM	Rietspruit (Hennops tributary) at Thatchfield	River	-25.895948, 28.119746	bi-weekly	12 weeks
5		City of Tshwane	Tshwane MM	Kaalspruit (Hennops tributary) downstream Tembisa	River	-25.957542, 28.206773	bi-weekly	12 weeks
6	Western Cape	Stellenbosch	Cape Winelands	Plankenbrug River upstream informal settlement	River	-33.891337, 18.828423	bi-weekly	12 weeks
7		Stellenbosch	Cape Winelands	Plankenbrug/Krom River at run-off*	River	-33.934110, 18.851063	bi-weekly	12 weeks
8		City of Cape Town	City of Cape Town	Borcherds Quarry WWTW**	WWTW tanker trucks	-33.961374, 18.589610	bi-weekly	12 weeks
9		Khayelitsha, Cape Town	City of Cape Town	Kuils River at Khayelitsha*	River	-34.042778, 18.721389	bi-weekly	12 weeks
10		City of Cape Town	City of Cape Town	Black River at Athlone WWTW	River	-33.950907, 18.514221	bi-weekly	12 weeks
11	KZN	eThekwini	eThekwini	Palmiet river upstream Quarry Road West informal settlement	River	-29.804962, 30.965609	bi-weekly	12 weeks

No.	Pro-vince	City/Town	District	Site name	Type	Co-ordinates	Sample frequency	Sample duration
12		eThekwini	eThekwini	Palmiet river downstream Quarry Road West informal settlement	River	-29.802616, 30.970598	bi-weekly	12 weeks
13		eThekwini	eThekwini	Quarry Road West informal settlement	Surface	-29.804122, 30.966965	bi-weekly	12 weeks
14		eThekwini	eThekwini	Umhlangane River upstream Johanna Road informal settlement	River	-29.796499, 30.993136	bi-weekly	12 weeks
15		eThekwini	eThekwini	Umhlangane River Downstream Johanna Road informal settlement	River	-29.799888, 30.991315	bi-weekly	12 weeks
16		eThekwini	eThekwini	Johanna Road informal settlement	Surface	-29.797288, 30.994522	bi-weekly	12 weeks

*passive sample to be collected in parallel to grab
** passive sample only

A description of each sample site is provided in the following sections, together with maps for visualisation.

2.2.1 Gauteng

Nine sample points were identified in the Gauteng province, including rivers up- and down-stream from informal settlements and contaminated surface run-off water (greywater) from within these settlements (**Figure 1)**.

In the City of Johannesburg, the Jukskei River was sampled at source and downstream of Alexandra, and surface run-off samples taken from within the Silvertown Township in Alexandra (**Figure 2)**. The Jukskei Source is situated in an area within the Johannesburg Central Business District (CBD) where a significant amount of sewage is entering the groundwater due to leaks and discharge from hijacked buildings (
Figure 3). These sites were sampled by the project team, with assistance from local community members in Alexandra by agreement with community leaders (
Figure 4). Initially samples were also received from the Jukskei River upstream of Alexandra but sampling was discontinued due to logistical challenges.

The Klipspruit River, which is a tributary of the Vaal River, was sampled at the Kliptown suburb of Soweto, downstream of an informal settlement (indicated in red in **Figure 5**), and the Rietspruit was sampled downstream of low cost housing and informal settlements at Sebokeng (**Figure 6**). Collection of these samples was facilitated by Rand Water.

In the City of Tshwane, the Rietspruit, a tributary of the Hennops River, was sampled at Thatchfield, downstream of informal settlements (indicated in red in **Figure 7**). The Kaalspruit, also a tributary of the Hennops River was sampled downstream from Tembisa (**Figure 8**). The City of Tshwane assisted with the collection of these samples.

In the City of Ekurhuleni, samples were taken from the Glenshaft Pan, which receives treated effluent from the Benoni WWTW as well as contaminated run-off from an informal settlement (**Figure 9**). The Klip River was sampled upstream from Waterval WWTW, where the river is receiving run-off from an informal settlement (indicated in red in **Figure 10**). Collection of these samples was facilitated by the Ekurhuleni Water Care Company (ERWAT).

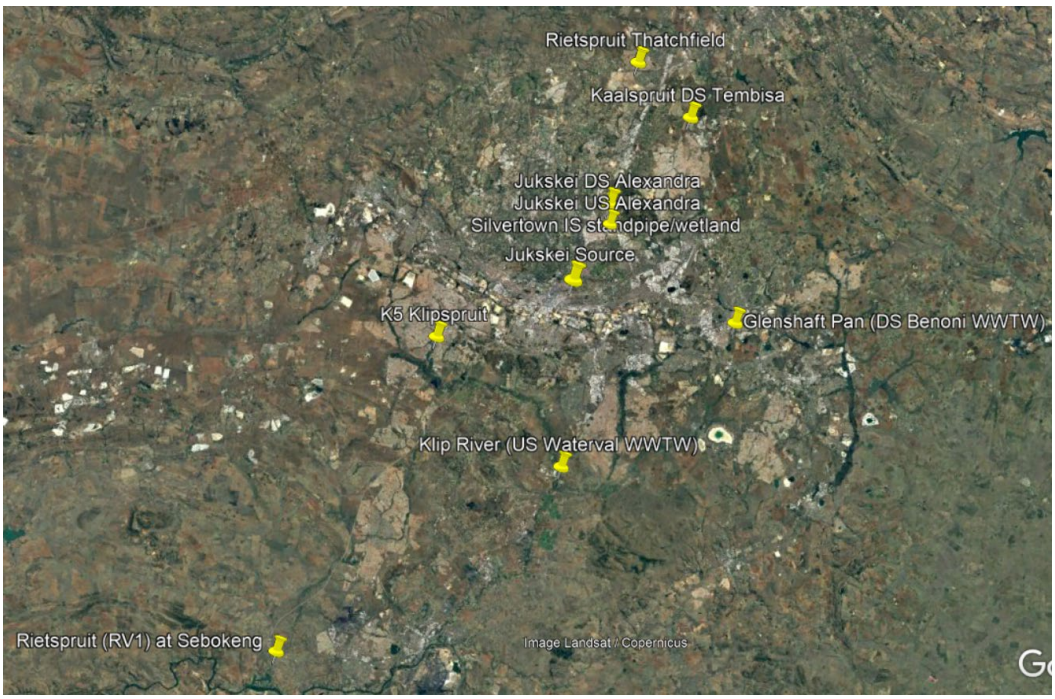


Figure 1: Distribution of Gauteng sample points. Source: Google Maps

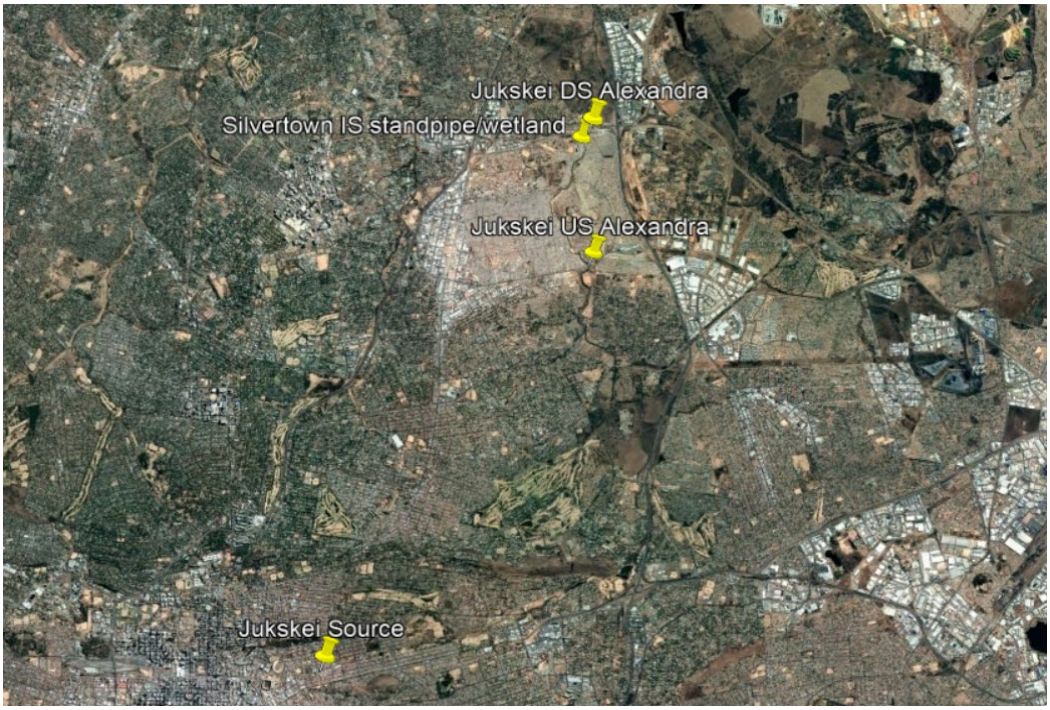


Figure 2: Sample points in the City of Johannesburg. Source: Google Maps

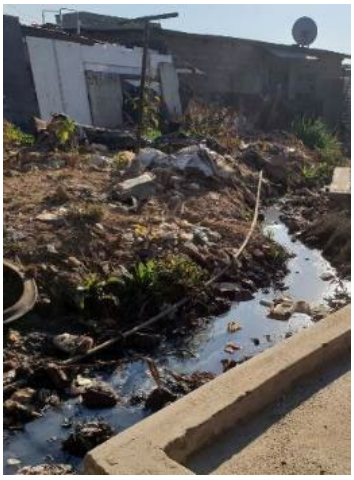
Juksei source. Source: Google Maps

Source of the Jukskei River contaminated by sewage entering groundwater

Deployment of passive sampler at the Jukskei River source.

Figure 3: Sampling point at the Jukskei Source in the Johannesburg CBD

Silvertown Informal Settlement and the Jukskei River. Source: Google Maps



Grey water and sewage draining from the unsewered community in Silvertown Informal Settlement, Alexandra

Sampling from the Jukskei River in Alexandra downstream of the Silvertown Informal Settlement

Sampling from a greywater drain within the community

Typical wastewater stream flowing through the Alexandra area

Figure 4: Sampling wastewater run-off from a drain in the Silvertown Informal Settlement in Alexandra, and the Jukskei River downstream of Alexandra

Figure 5: Klipspruit River at Kliptown, Soweto, Johannesburg Metro Municipality, downstream of informal settlement marked in red. Source: Google Maps

Figure 6: Rietspruit at Sebokeng, Sedibeng Municipality, downstream of informal settlements marked in red. Source: Google Maps

Figure 7: Rietspruit (Hennops tributary) at Thatchfield, City of Tshwane. Source: Google Maps

Figure 8: Kaalspruit (Hennops tributary) downstream from Tembisa, City of Tshwane. Source: Google Maps

Figure 9: Glenshaft Pan, downstream of Benoni WWTW receiving run-off from informal settlement, City of Ekurhuleni. Source: Google Maps

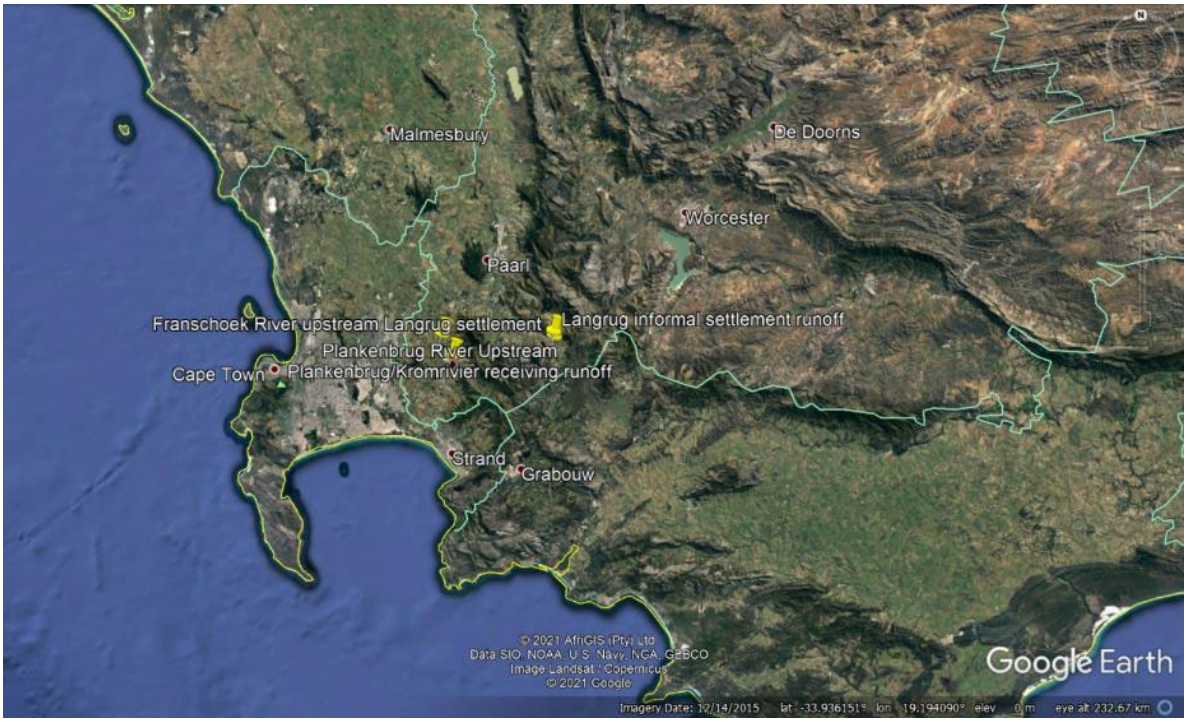
Figure 10: Klip River, upstream from Waterval WWTW receiving run-off from informal settlement, City of Ekurhuleni. Source: Google Maps

2.2.2 Western Cape

Two areas were identified for sampling within the Cape Winelands District of the Western Cape province (**Figure** 11). These sites were sampled by the project team. The Plankenbrug River was sampled upstream of informal settlements at Kayamandi, Stellenbosch (indicated in red in **Figure 12**), and the run-off from these settlements was sampled as it entered the Plankenbrug River after confluence with the Kromrivier (**Figure 12**). The Franschhoek River was sampled upstream of the Langrug settlement outside of

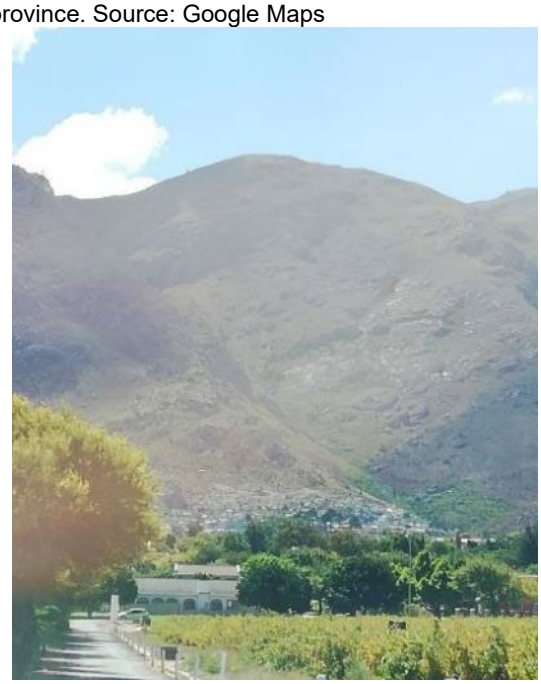
Franschhoek, and run-off from informal settlements within Langrug was sampled as it entered the Stiebeuelrivier (**Figure 13**).

Two rivers were sampled in the City of Cape Town (**Figure 14**), the Kuils River which is impacted by the un-serviced Khayelitsha informal settlement, and the Black River impacted by various informal settlements and contaminated run-off (**Figure 15**).



Sampling sites in the Western Cape province. Source: Google Maps

Plankenbrug Upstream of the informal settlements at Kayamandi



Informal Settlement on mountainside towards Siebiel (from Franschhoek river site)

Figure 11: Distribution of sample sites in the Cape Winelands, Western Cape

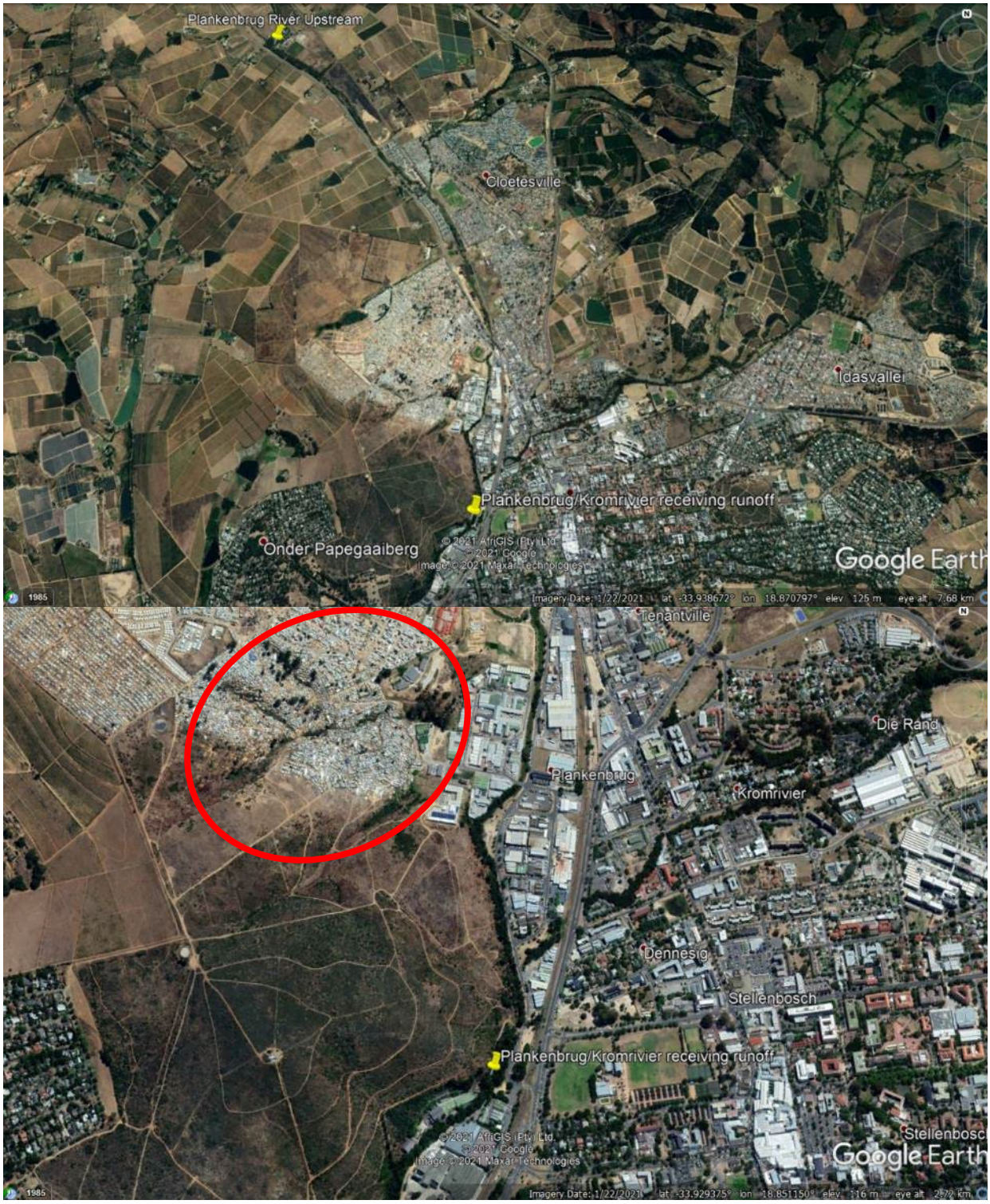


Figure 12: Plankenbrug River upstream informal settlement at Kayamandi, and Plankenbrug River/Kromrivier at run-off from informal settlement, Stellenbosch. Source: Google Maps

Figure 13: Franschhoek River upstream Langrug settlement and Langrug informal settlement run-off into the Stiebeuelrivier, Franschhoek/Langrug, Cape Winelands. Source: Google Maps

Figure 14: River sample sites in the City of Cape Town. Source: Google Maps

Black River at Athlone WWTW. Source: Google Maps

Kuils River at Khayelitsha. Source: Google Maps

Black River sampling point

Passive sampler from Black River after 24h deployment

Figure 15: Black River upstream of the Athlone WWTW discharge point, and the Kuils River at Khayelitsha

2.2.3 KwaZulu-Natal

In KZN, two informal settlements were identified on the Palmiet and Umhlangane Rivers in eThekwini, both tributaries of the Umgeni River (**Figure 16**). Samples were taken of the Palmiet River upstream and downstream of the Quarry Road West Informal Settlement as well as a contaminated surface run-off sample from within the settlement around standpipes and ablutions (**Figure 17**).

The Umhlangane River was sampled upstream and downstream of the Johanna Road Informal Settlement as well as surface water from within the community (**Figure 18)**. The site is downstream of the effluent discharge from the Northern WWTW; the upstream sample is intended to identify background pollution from the works.

Collection of samples from these sites is facilitated by Durban Green Corridor in collaboration with Adopt-a-River. Durban Green Corridor made use of unemployed youth from the informal settlements, who are currently being trained in waste sorting and beneficiation initiatives, for collection of the samples. They were trained in the correct sampling techniques including data collection methods.

In addition to these sites, sampling of Urine Diversion Dehydrating Toilets (UDDTs) in peri-urban Maphephetheni area in the eThekwini Municipality was collected on behalf of the project team by Khanyisa Projects, with assistance from the WASH R&D Centre at the University of KwaZulu-Natal (UKZN) as a different sample type which can potentially be used to determine community COVID-19 infection. Various toilets from 4-5 single households were sampled by Khanyisa Projects, and a composite sample prepared by the UKZN WASH R&D Centre for processing. Access to these samples required significant engagement with community members, as well as formal informed consent and ethics clearance. It is the intention that the preparation of composite samples and anonymity of the samples would give sufficient peace of mind to the community members to avoid the social stigma associated with a positive COVID-19 test result. The aim was to test the practicality and viability of the method for community COVID-19 exposure. Costs for sampling from these UDDT's was found to be significant and this, along with the exhaustive permissions require from ethics committees and the community as well as the time required to obtain the relevant permission from all the stakeholders, appears to make this option too expensive and time consuming to allow it to be a practical option. Three (3) monthly composite samples were prepared and couriered to Waterlab by UKZN.

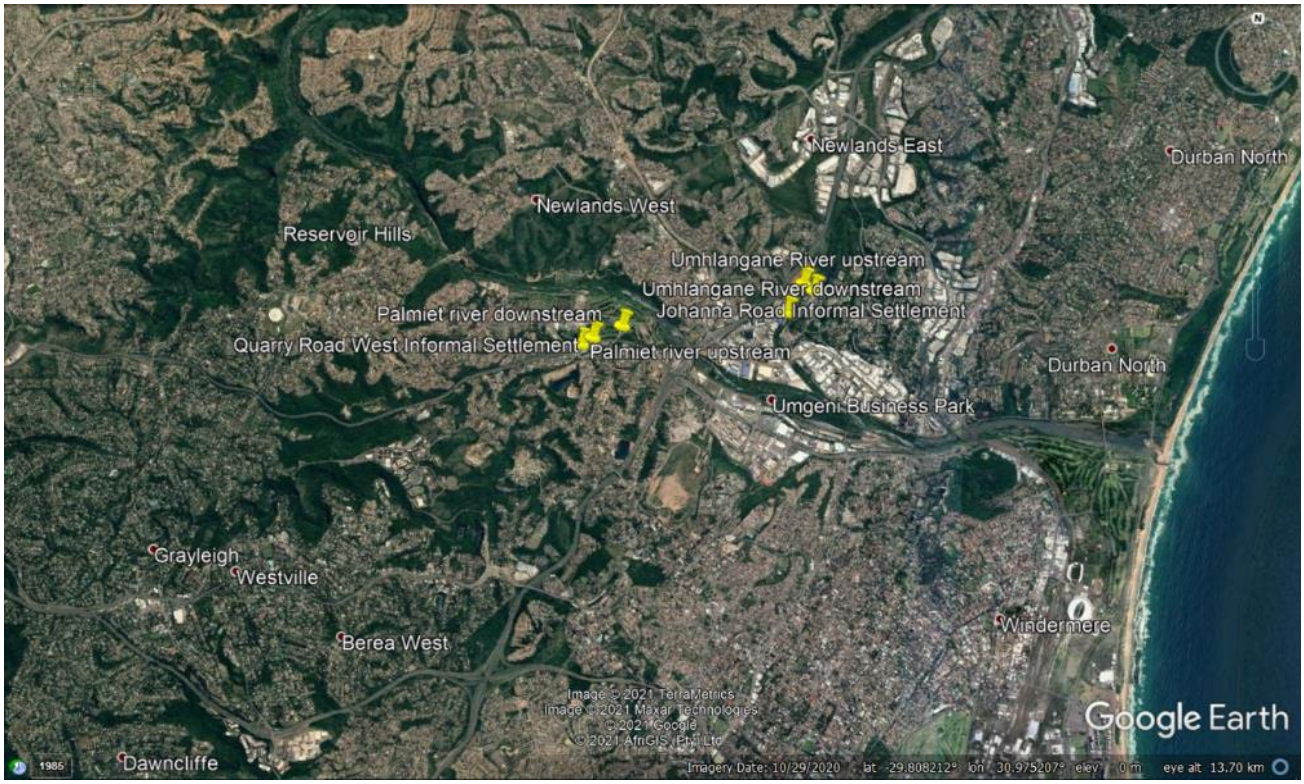


Figure 16: Distribution of surface water sample sites in KwaZulu-Natal. Source: Google Maps

Figure 17: Palmiet River upstream of, Palmiet River downstream of Quarry Road West Informal Settlement and surface run-off within Quarry Road West informal settlement, eThekwini. Source: Google Maps

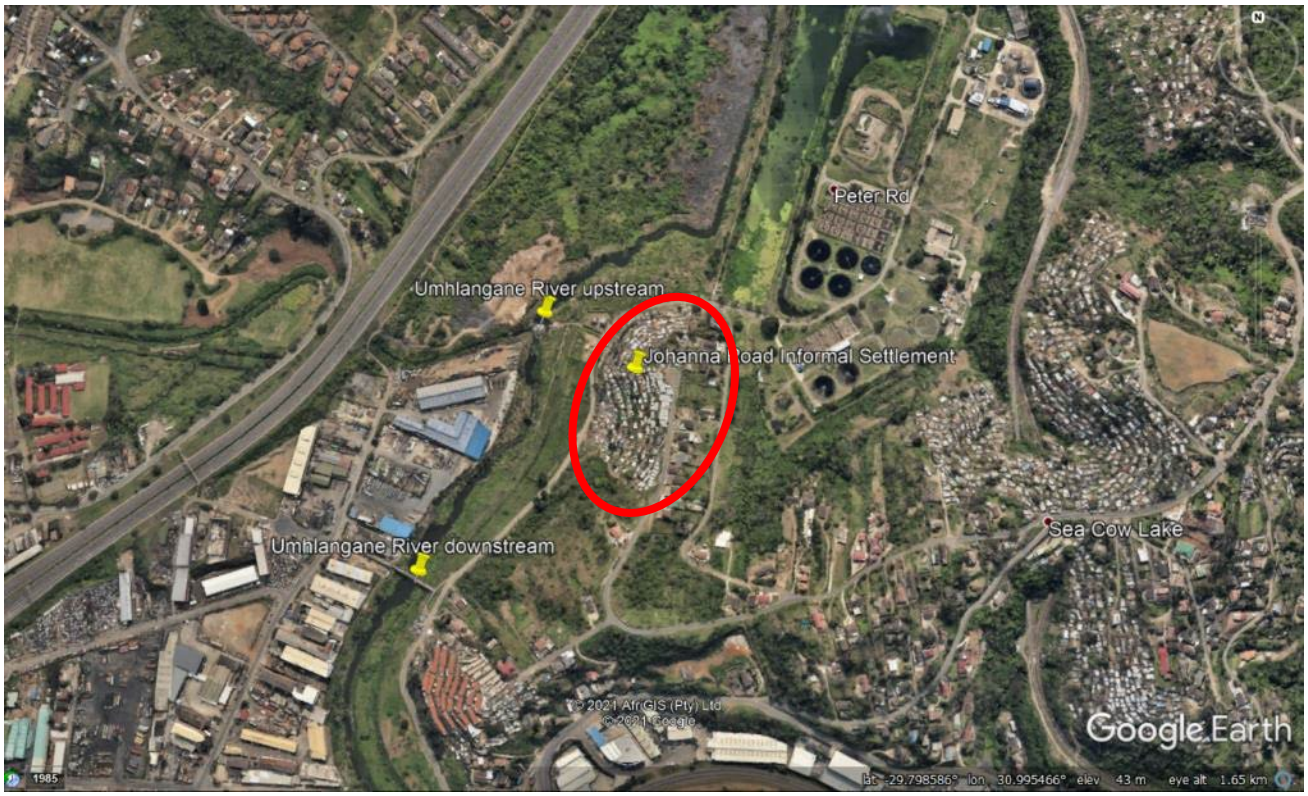


Figure 18: Umhlangane River upstream of Johanna Road informal settlement, Umhlangane River downstream of Johanna Road informal settlement and surface run-off within Johanna Rd informal settlement, eThekwini. Source: Google Maps

Sampling of grey water standing pools

Collection of river samples next to the river

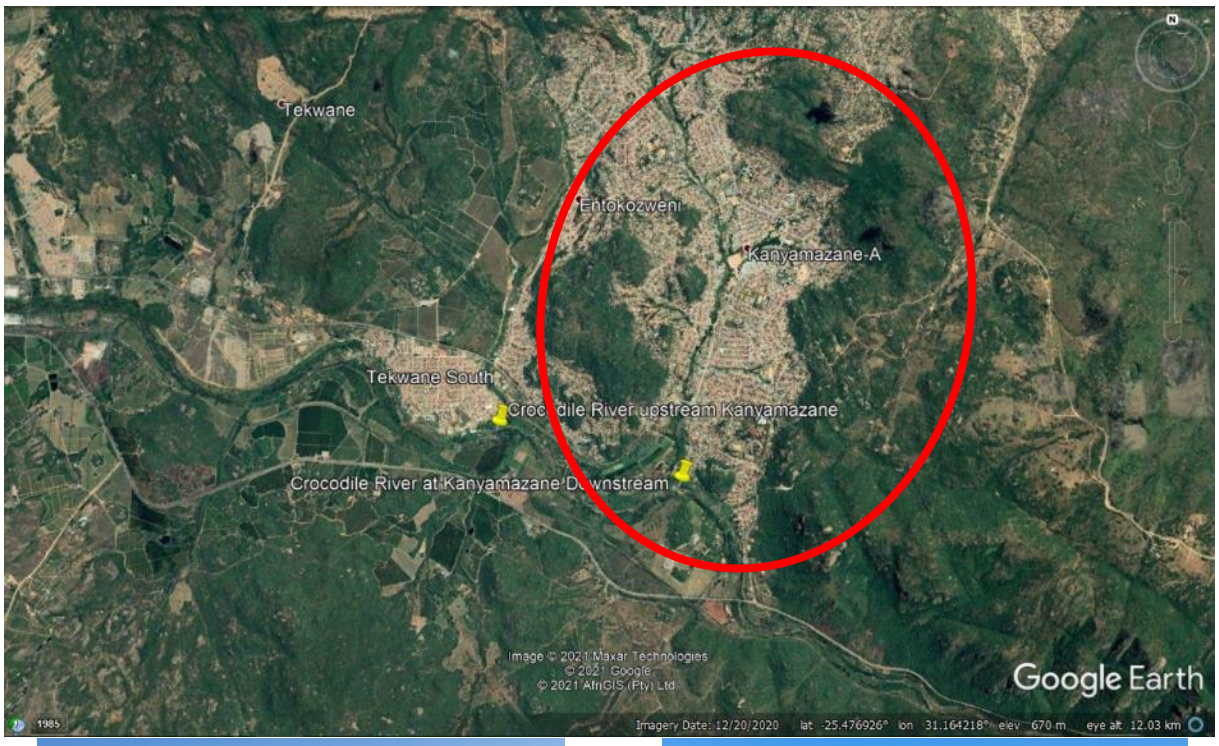
Greywater sampling sites

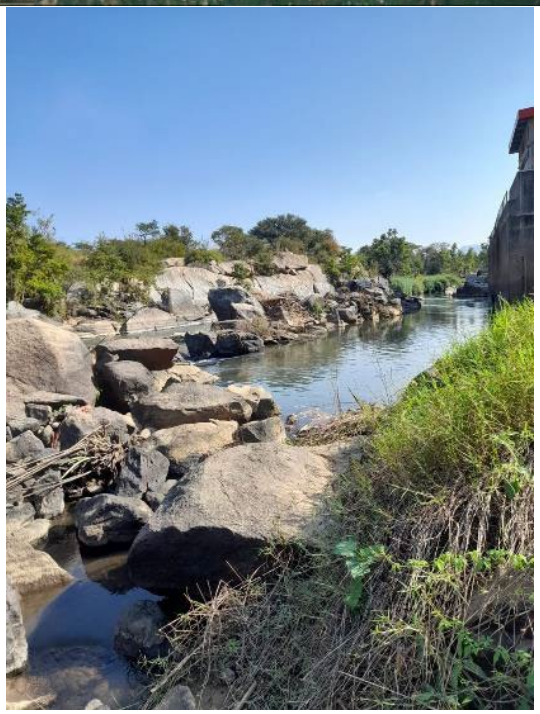
Sample collection below the toilets

Figure 19: Sampling from the Johanna Road and Quarry Road West Informal Settlements in KwaZulu-Natal

2.2.4 Mpumalanga

In Mpumalanga, samples were taken from the Crocodile River upstream and downstream of Kanyamazane, which consist of both low cost and informal housing (**Figure 20**). Sampling in this area was facilitated by Rivers of Life Aquatic Health Services (ROL), an applied research organisation providing water resources management and conservation services, operated from the University of Mpumalanga and UKZN.





Crocodile River sample site upstream of Kanyamanzane

Crocodile River sample site downstream of Kanyamanzane

Figure 20: Crocodile River upstream and downstream of Kanyamazane, Mbombela (Map source Google maps)

2.3 SAMPLE COLLECTION

Bi-weekly samples were taken from the initial sample sites presented in **Table 1** for a period of 5 months, with a sub-set of these samples continued for an additional 16 weeks as presented in **Table 2**. One exception is the Rietspruit sample at Sebokeng, which was sampled monthly for the first 5 months as per Rand Water's sample schedule. Collection of samples required a co-ordinated logistical effort, with assistance from local authorities in the selected areas, as well as river action groups and research organisations as described in Section 2.2. Samples were analysed for water quality parameters including Chemical Oxygen Demand (COD), ammonia, faecal coliforms and *E. coli*, indicative of pollution, as well as SARS-CoV-2 viral RNA in order to determine trends and patterns within the data set.

Grab samples were found to be sufficient for surveillance monitoring in surface waters from the Proof-of-Concept study (Pocock et al., 2020). Sampling kits complete with the correct sampling equipment were couriered to the identified areas, with detailed instructions for sampling to project collaborators. Three litres (L) of water per sampling location was collected to allow for sufficient water to provide for both SARS-CoV-2 recovery and assay as well as analysis of supporting water quality parameters. For virus recovery, 200 mL of water proved to be sufficient from previous work, even in surface water. In addition to the grab samples, passive samplers were deployed for viral recovery comparison at a sub-set of the sample sites, discussed further in Section 2.5.1.

Due to the difficulty and cost associated with the collection of faecal sludge from the on-site UDDTs in the eThekwini Municipality, the sample frequency was less than for the other sites. It was initially intended to be monthly for 5 months, but due to unrest in the sampling areas this was reduced to three samples.

In addition, sampling from tankers pumping from conservancy tanks was investigated as a viable option, where the source of the tanker was known to assist in determining the spread of COVID-19 in that area of the community. Only passive samplers were used for this sample matrix.

2.4 SAMPLE PROCESSING AND ANALYSIS

Samples were received at Waterlab in Pretoria and were concentrated as described below prior to RNA extraction.

2.4.1 Sample clarification

Samples with a high suspended solids load were first clarified prior to viral recovery. The 1-2 L samples were shaken and mixed thoroughly before a 200 mL aliquot was poured off for further processing. The aliquot was clarified by centrifugation (Sorvall® Super T20, Thermo Fisher Scientific, Waltham, MA) for 30 minutes at 1180 g at 4°C after which the supernatant was retained for further viral recovery and the pellet saved and stored at -80°C.

2.4.2 Skimmed-milk flocculation

Virus recovery was done using the skimmed-milk flocculation method as described by (Falman & Meschke, 2019), using a 5% w/v skimmed-milk solution (Oxoid Ltd., Basingstoke, UK) and 2 hour shaking protocol. A 5% pre-flocculated skimmed-milk solution (2 mL) was added to 200 mL clarified water samples. The pH was adjusted to pH 3.0-4.0 with 1 M hydrochloric acid (Merck, Darmstadt, Germany) followed by shaking for 2 hours at 200 rpm at room temperature (20-25°C). The sample was then centrifuged (Sorvall® Super T20) at 4500 x g for 30 minutes at 4°C, the supernatant carefully removed, and the pellet was resuspended in 2 mL PBS pH 7.4 (Sigma-Aldrich, St. Louis, MO). The recovered virus concentrate was aliquoted, with 1 mL stored at -20°C until analysis and the remainder stored at -80°C.

2.4.3 Processing of passive samplers

A discussion on the deployment of passive samplers is presented in Section 2.5.1. Elution of potential viral nucleic acids was carried out using a modified methodology described by Schang et al. (2020). PBS with 0.05% Tween 20 (10 mL) was added to the gauze samples, which were then massaged for 3 minutes to elute the virus. The anti-foaming agent used by Schang et al. (2020) was found to be unnecessary and was excluded. The eluted material was then directly extracted as per the viral nucleic acid extraction methodology described in Section 2.4.4.

2.4.4 Nucleic acid extraction

All samples were pre-treated with chloroform prior to extraction. Chloroform (250 µL) (Merck) was added to 1 mL recovered virus concentrate and the mixture vortexed for 3 x 15 seconds and then incubated at room temperature for 5 minutes before centrifugation at 3500 x g for 3 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.

2.4.5 Viral amplification

2.4.5.1 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 envelope (E), nucleocapsid (N) and RNA dependent RNA polymerase (RdRp) genes of SARS-CoV-2 and contains an internal control to monitor inhibition. The RT-PCR reactions were prepared according to the manufacturer's instructions and 8 µL RNA 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.

The disadvantage of this assay is that it cannot be used for quantification, as that requires a singleplex assay. Due to the increased sensitivity of the multiplex Allplex™ assay however, and the complexity of the matrix, it was intended that only this assay will be used for the surface water sample assays.

2.4.5.2 Mengovirus QuantiFast® Pathogen RT-PCR + IC assay

Mengovirus was detected in each SARS-CoV-2-positive sample to determine nucleic acid extraction efficiency for quantification purposes. Published primers and probe (Table 2) (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 µL QuantiFast® Pathogen RT mix in 20 µL. Five microlitres of RNA was 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.

2.4.5.3 Construction of Mengovirus standard curves

Mengovirus is a small non-enveloped virus that is used as process control for virus recovery from environmental samples. To determine the recovery rate of mengovirus from wastewater samples, 3 x 200 mL wastewater samples were spiked with 1.5 x 10^5 TCID50 mengovirus after clarification. The viruses were recovered with skimmed milk flocculation as detailed in Section 2.4.2. The mean recovery efficiency of the mengovirus from the triplicate samples was 5.9% (SD = 1.25).

Mengovirus with a TCID50 titre of 1.4 x 10^6 was used to generate a standard curve to quantify the mengovirus. Serial ten-fold dilutions of the cell culture stock were run in triplicate in the QuantiFast® Pathogen RT-PCR + IC assay and the QuantStudio™ 5 Design and Analysis Software v 1.5.1 was used to generate a standard curve.

2.4.5.4 Detection of enteric norovirus and hepatitis E virus in wastewater

Methods that have been optimised in the South African setting (Mabasa et al., 2018) were used to detect norovirus in the environmental samples. The norovirus GI and GII targets were screened with the QuantiFast® Pathogen RT-PCR + IC assay, making use of published primers and probes; QNIF4, NV1LCR primers and NVGGIp Probe for GI (da Silva et al., 2007; Svraka et al., 2007) and QNIF2 and COG2R primers and QNIFS probe for GII (Kageyama et al., 2003; Loisy et al., 2005) (
Table 3).

In addition, samples were screened for Hepatitis E virus, a virus spread by the faecal-oral route, using the QuantiFast® Pathogen RT-PCR+IC assay and published primers JVHEVF and JVHEVR, and probe JVHEVP (Garson et al., 2012) (**Table 3**).

This data will contribute to the database needed to extend the application of WBE to other diseases.

2.4.5.5 PCR for CrAssphage quantification in wastewater

A previously described CrAssphage CPQ_064 specific PCR (Stachler et al. 2017) was used to quantify this DNA-virus that is ubiquitously present in human intestinal tracts in high concentrations (see discussion

below in Section 2.5.2), using the QuantiFast Pathogen PCR+IC kit (Qiagen), 064F1 and 064R1 primers and 064P1 probe (**Table 3**). Quantification was performed based on a standard curve generated with a dilution series of a synthetic quantified gBlock (obtained from IDT, Leuven, Belgium) containing the CPQ_064 gene fragment. The crAssphage concentration was applied to the surface water samples to determine the extent of human faecal pollution in the rivers and visualize the effects of dilution due to stormwater or illustrate incidences of sewage dumping or spills.

Table 3: Primers and probes for SARS-CoV-2, mengovirus, norovirus, hepatitis E virus and crAssphage detection

Target	Kit	Primers/Probes	PCR conditions	Reference
SARS-CoV-2 E gene N gene RdRp gene	Allplex-nCoV-2019 (Seegene)	Proprietary	RT: 50°C, 20 min PCR: 95°C, 15 min 94°C, 15 sec 58°C, 30 sec 45 cycles	Not applicable
Mengovirus	QuantiFast Pathogen RT-PCR +IC kit (Qiagen)	Mengo 110F Mengo 209R Probe Mengo 147	RT: 50°C, 20 min PCR: 95°C, 5 min 95°C, 15 sec 60°C, 30 sec 45 cycles 65°C, 30 sec	Pinto et al., 2009
Norovirus GI and GII	QuantiFast Pathogen RT-PCR +IC kit (Qiagen)	GI QNIF4 NV1LCR Probe NVGGIp GII QNIF2 COG2R Probe QNIFS	RT: 50°C, 20 min PCR: 95°C, 5 min 95°C, 15 sec 60°C, 30 sec 45 cycles 65°C, 30 sec	GI Da Silva et al., 2007; Svraka et al., 2007 GII Kageyama et al., 2003; Loisy et al., 2005
Hepatitis E virus	QuantiFast Pathogen RT-PCR +IC kit (Qiagen)	JVHEVF JVHEVR Probe JVHEVP	RT: 50°C, 30 min PCR: 95°C, 5 min 95°C, 15 sec 60°C, 1 min 45 cycles	Garson et al., 2012
crAssPhage	QuantiFast Pathogen PCR +IC kit (Qiagen)	064F1 064R1 Probe 064P1	PCR: 95°C, 10 min 95°C, 15 sec 60°C, 1 min 40 cycles	Stachler et al., 2017

RT = Reverse transcription
PCR = Polymerase chain reaction

2.5 DEVELOPMENT AND OPTIMISATION OF SARS-COV-2 SAMPLING AND ANALYSIS METHODOLOGY

As part of the study objectives, it was required to develop and optimise the methodology for SARS-CoV-2 detection, quantification, and monitoring in samples from both sewered and non-sewered settlements.

2.5.1 Passive sampling

Passive samplers were validated in a study from Australia (Schang, et al., 2021). Passive sampler units made from readily available consumables were shown to be at least as sensitive as the grab/composite wastewater sampling method and had a higher viral recovery than these traditional methods. Although Moore's swabs are often used to collect samples these are prone to high rates of fouling and therefore this passive sampler was developed to minimise fouling. These samplers can potentially be used for WBE for a broader scope than only SARS-CoV-2.

Torpedo shaped passive samplers were 3D printed as per the design provided by Schang et al. (2021). The plastic housing of the sampler was used to house medical gauze swabs, and the unit was tied to a rope / line and suspended in the sample medium at various locations (
Figure 21).

Six standard 75mm x 75 mm medical gauze swabs were used, and the passive sampler was wrapped in 50% shade cloth (

Figure 21). Passive samplers were deployed using a 3 mm nylon rope or fishing line as per availability and suitability at site. Schang et al. (2021) found that exposure of the passive sampler to wastewater in the sewer for 8 hours was sufficient. However, due to the more diluted sample medium expected in river and surface samples, additional time was expected to be required *in situ*. As such, 48- and 24-hour exposure times were assessed, depending on the sampling environment. The advantage of these passive samplers was that deployment was relatively easy and rapid and reduced sampling errors which can exist when taking water samples using the conventional grab or composite sampling methodologies.

Elution and extraction of potential viral nucleic acids was carried out as previously described in Sections 2.4.3 and 2.4.4.

Torpedo style passive sampling devices and two pipe sampling devices (above)

Torpedo filled with gauze and wrapped in shade cloth ready for deployment.

Figure 21: Passive sampling devices

Passive samplers were deployed at the Source of the Jukskei river in Braamfontein, Johannesburg, the Silvertown informal settlement in Alexandra, the Jukskei River downstream of Silvertown, and in the Plankenbrug River in Stellenbosch downstream of the informal settlements at Kayamandi.

The Jukskei river is already significantly contaminated at the point of daylight due to sewage contamination of stormwater and groundwater from hijacked and un-serviced buildings and blocked and overflowing sewer lines in the Johannesburg CBD. Deployment of the passive sampler is presented in
Figure 22. The initial deployment was for a 48h period, with significant fouling of the device and gauze observed. A second deployment of 24h was undertaken with less fouling observed, and as such a 24h passive sampling period was applied for this site.

Passive sampler deployed in the source of the Jukskei River

After 48 hours, sample swab is significantly fouled

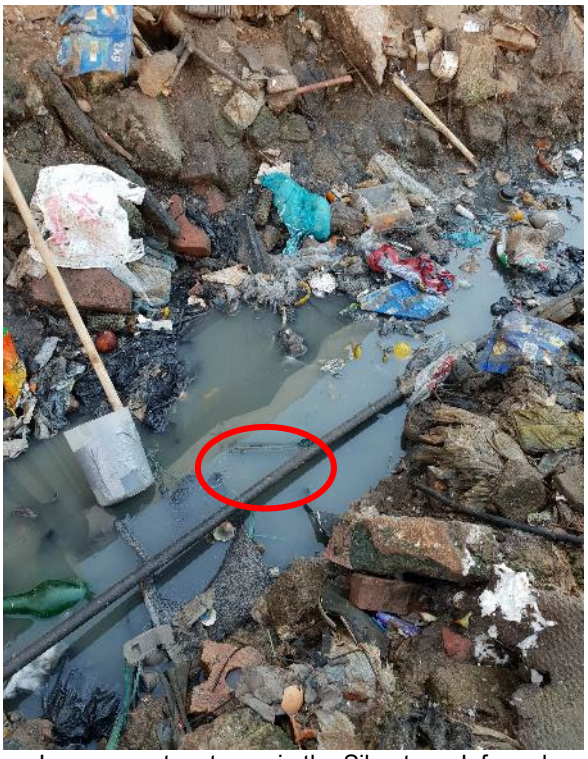
Figure 22: Passive sampling device installed in the source of the Jukskei river in Braamfontein, Johannesburg

Deployment of the samplers in Silvertown Informal Settlement and the Jukskei River downstream of Silvertown is presented in
Figure 23. These samplers were recovered 24h after installation with low fouling of the device and gauze observed. Comparative 48h samples were also deployed, to compare viral recovery especially when viral load was expected to be low in between infection waves.

The 24h deployment was also undertaken in the Plankenbrug River in Stellenbosch downstream of the Kayamandi Informal Settlement (
Figure 24). The conventional Moore swab method was also applied to observe the degree of fouling and determine whether it is prohibitive in an environmental sampling context.

The addition of tampons as sampling devices was also undertaken as this provides an easily accessible and cheap method of standardised sampling technique. The level of surface fouling versus absorption was found to be too great and this method was discarded.

Second deployment in Alexandra in the Jukskei River downstream of Silvertown Informal Settlement



In a greywater stream in the Silvertown Informal Settlement in Alexandra

Torpedos retrieved 24 after installation

Gauze ready for processing

Figure 23: Passive sampling in the Silvertown Informal Settlement in Alexandra, Gauteng, and in the Jukskei River downstream of the Silvertown Settlement

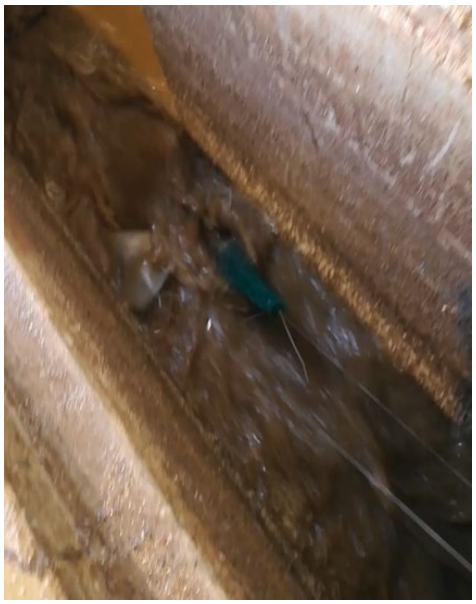
Deployment of sampler in the Plankenbrug River downstream of Kayamandi informal settlements

Recovered gauze swabs

Figure 24: Passive sampling in the Plankenbrug River in Stellenbosch downstream of the Kayamandi Informal Settlement

Also investigated was the deployment of a passive samplers in effluent from honeysucker tankers which were used to collect blackwater / faecal sludge from chemical toilets or septic tanks from areas within the City of Cape Town that were not connected to the sewage network (
Figure 25). The tankers keep manifests of the areas served so it is possible to relate a passive sampler to a specific community.

Tanker trucks collecting waste from septic tanks and chemical toilets discharging to the wastewater treatment works



Passive sampler in the tanker effluent discharge.

Figure 25: Passive sampling of tanker effluent discharge to wastewater treatment works

2.5.2 Data normalization and visualization

According to the Water Research Foundation (2020), environmental surveillance has three uses: (i) trend detection (one direction, up- or downward), (ii) changes in trend (two directions) and (iii) assessment of community infection (tracking disease prevalence). While the current knowledge is sufficient to advance uses (i) and (ii) by supporting decision-making relating to medical and social interventions, the ultimate objective is to use back-calculation methods to assess infection prevalence. For SARS-CoV-2, considerable knowledge still needs to be gathered, especially regarding shedding rates and duration, links between the genetic signal and the infection prevalence and the fate within wastewater and how this changes with wastewater characteristics (e.g. dilution, temperature, retention time, percentage trade waste, etc.) that may vary with time and season. These variables will be even more pronounces in environmental samples such as those to be analysed in this study. Existing models may be very helpful for uses (i) and (ii) to normalise the genetic signals for spatial (comparing between wastewater catchments or rivers) and temporal (seasonality of fate-affecting conditions) variability in order to maximise the power of the signals obtained in supporting COVID-19 management decisions.

The various use-cases presented by the Water Research Foundation (2020) (**Figure 26)** highlight the importance of trend monitoring through the various phases of the pandemic. Although translating the viral titres from wastewater into the actual number of cases within a community is highly challenging, if not impossible, monitoring trends in viral load can be used successfully to implement an early warning system.

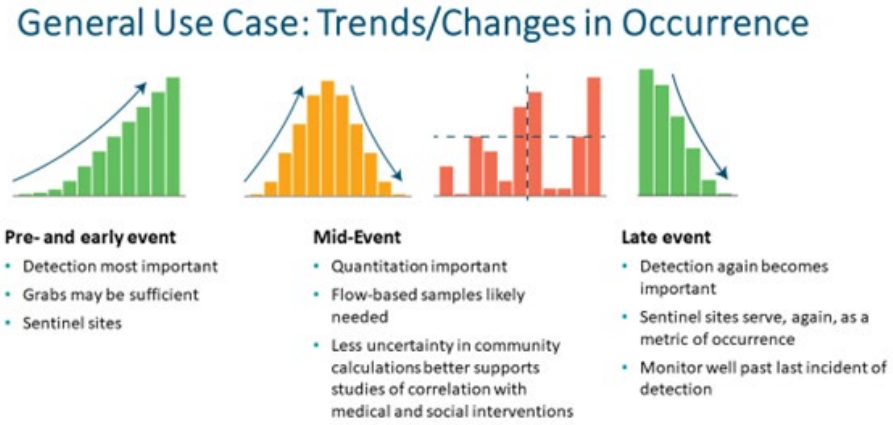


Figure 26: General use case: Source Water Research Foundation (2020)

While it is difficult to directly relate viral loads in environmental samples to a case load in the unsewered community, the importance of trend monitoring should be emphasised, and tools developed to facilitate this.

2.5.2.1 Analytical method to assess faecal loads in urban water streams

CrAssphage is a ubiquitous phage found in humans and excreted by most humans, although in varying amounts. As part of the sampling framework, water quality data was collected at sites both upstream and downstream of the communities. The use of crAssphage assay as a method of estimating the volume of human faecal matter within the sample was evaluated as suggested by Park et al. (2020), and a trend analysis conducted to determine how the viral load correlated with the various water quality parameters tested. It is envisaged that the crAssphage can serve as an indicator of human faecal pollution and has been applied in normalisation studies for SARS-CoV-2 WBE (Wilder et al., 2021; Heijnen et al., 2021).

2.6 SEQUENCING OF SAMPLES POSITIVE FOR SARS-COV-2

A subset of 29 samples across different provinces that were found to be positive for SARS-CoV-2 with Ct values of 34 or less on one or more of the assay gene targets were selected for sequencing (**Table 4**).

Table 4: SARS-CoV-2 positive samples submitted for sequencing

No.	SAMPLE SITE	Province	SAMPLE TYPE	DATE SAMPLED START	DATE SAMPLED END	RT-PCR SEEGENE E GENE	RT-PCR SEEGENE N GENE	RT-PCR SEEGENE RdRp GENE
1	Jukskei Source	Gauteng	Passive (24h)	2021/06/21	2021/06/22	29.6	30.2	35.5
2	Jukskei Source	Gauteng	Grab	2021/06/22	2021/06/22	30.7	31.1	-
3	Jukskei Source	Gauteng	Passive (24h)	2021/07/06	2021/07/07	-	32.4	-
4	Jukskei Source	Gauteng	Grab	2021/07/07	2021/07/07	31.8	32.6	-
5	Jukskei Source	Gauteng	Passive (24h)	2021/08/03	2021/08/04	30.6	32.7	-
6	Jukskei Source	Gauteng	Passive (24h)	2021/11/22	2021/11/23	31.1	32.8	35.6
7	Jukskei Source	Gauteng	Grab	2021/12/07	2021/12/07	30.8	32.2	32.0
8	Jukskei Source	Gauteng	Passive (24h)	2021/12/06	2021/12/07	29.3	32.1	31.3
9	Alex Silvertown	Gauteng	Grab	2021/07/13	2021/07/13	32.2	31.2	31.2
10	Jukskei Downstream Alexandra	Gauteng	Grab	2021/03/09	2021/03/09	-	33.1	-
11	Jukskei Downstream Alexandra	Gauteng	Grab	2021/03/16	2021/03/16	-	33.1	-
12	Jukskei Downstream Alexandra	Gauteng	Grab	2021/06/15	2021/06/15	30.0	30.6	-
13	Jukskei Downstream Alexandra	Gauteng	Passive (24h)	2021/06/28	2021/06/29	29.3	31.1	35.6
14	Jukskei Downstream Alexandra	Gauteng	Grab	2021/07/13	2021/07/13	32.8	32.9	-
15	Jukskei Downstream Alexandra	Gauteng	Grab	2021/07/27	2021/07/27	32.8	35.5	-
16	Jukskei Downstream Alexandra	Gauteng	Grab	2021/08/10	2021/08/10	33.1	33.3	-
17	Jukskei Downstream Alexandra	Gauteng	Passive (48h)	2021/09/06	2021/09/08	33.9	-	-
18	Jukskei Downstream Alexandra	Gauteng	Grab	2021/11/24	2021/11/24	33.3	34.3	34.9
19	Jukskei Downstream Alexandra	Gauteng	Passive (48h)	2022/01/10	2022/01/12	32.7	34.9	-
20	Kaalspruit Downstream Tembisa	Gauteng	Grab	2021/06/29	2021/06/29	29.0	31.0	-
21	Kaalspruit Downstream Tembisa	Gauteng	Grab	2021/07/07	2021/07/07	30.7	31.2	-
22	Kaalspruit Downstream Tembisa	Gauteng	Grab	2021/11/29	2021/11/29	30.6	34.1	-
23	Rietspruit at Thatchfield	Gauteng	Grab	2021/05/10	2021/05/10	-	34.6	-
24	Rietspruit at Thatchfield	Gauteng	Grab	2021/07/22	2021/07/22	34.5	-	-
25	Klip River	Gauteng	Grab	2021/07/15	2021/07/15	34.9	33.7	-
26	Tanker waste	Western Cape	Passive (10ml)	2021/07/30	2021/07/31	30.4	31.6	-
27	Tanker waste	Western Cape	Passive (50ml)	2021/07/30	2021/07/31	28.6	33.7	34.7
28	Plankenbrug River Downstream Kayamandi	Western Cape	Grab	2021/07/20	2021/07/20	-	34.2	-
29	Umhlangane River upstream Johanna Road informal settlement	KZN	Grab	2021/12/14	2021/12/14	33.9	33.8	-

NGS was performed on a MiSeq using a NEBNext® ARTIC SARS-CoV-2 kit, to confirm the presence of SARS-CoV-2 and to attempt to identify the specific variants present in those samples. The percentage coverage of the successful libraries was determined by comparison with the reference genome, SARS-CoV-2 isolate Wuhan-Hu-1, complete genome (GenBank reference: MN908947.3 S). Scaffolds were generated by means of *de novo* assembly of the gene fragments in the libraries, and a Basic Local Alignment Search Tool (BLAST) analysis done to determine whether they matched with other SARS-CoV-2 sequences in the database.

Currently, the tool most commonly used for the assignment of newly isolated SARS-CoV-2 genomes to lineages is Pangolin, which offers pangoLEARN (the default) and UShER modes. Pangolin was developed to implement the dynamic nomenclature of SARS-CoV-2 lineages, known as the Pango nomenclature, allows a user to assign a SARS-CoV-2 genome sequence to the most likely lineage (Pango lineage) as described in Rambaut et al. (2020). The Pango nomenclature is used by researchers and public health agencies worldwide to track the transmission and spread of SARS-CoV-2, including variants of concern (O'Toole et al., 2021). The assignment of Pango lineages to newly sequenced SARS-CoV-2 genomes has been central in aiding health officials to trace the spread of the virus locally and globally and identifying differences among viral lineages.

Where possible the most likely Pango lineage was assigned to a given sequence based on the designated SARS-CoV-2 diversity. Scorpio (serious constellations of reoccurring phylogenetically-independent origin) was used in conjunction with UShER/ pangoLEARN to curate variant of concern (VoC)-related lineage calls.

3 RESULTS

3.1 WATER QUALITY RESULTS

As per the scope of work, the following parameters were measured: -

- pH
- Electrical Conductivity
- Chemical Oxygen Demand (COD)
- Suspended Solids (SS)
- Ammonia
- Faecal Coliforms
- *E. coli*

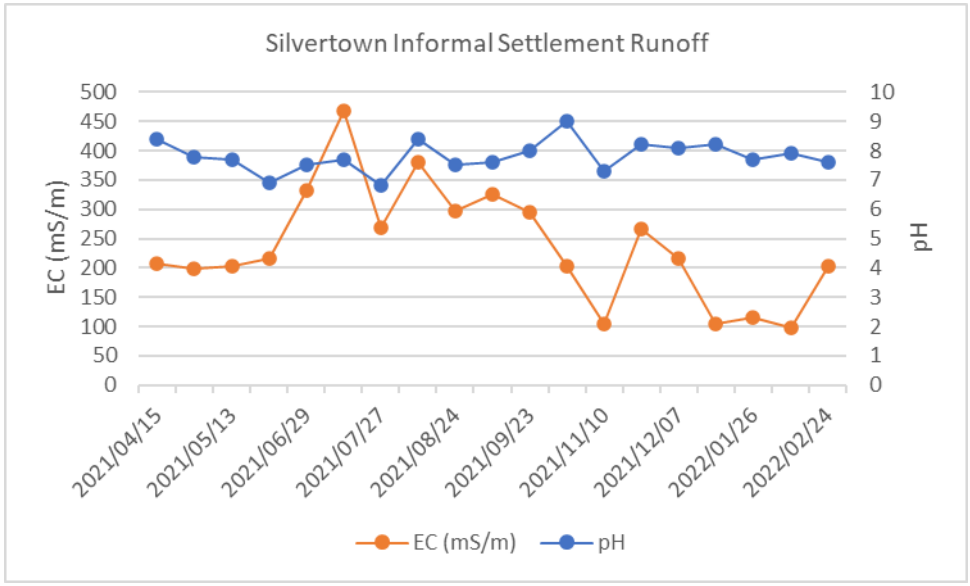
The typical values for untreated domestic wastewater are presented in **Table 5** (Nozaic & Freese, 2009) as an indicator of wastewater contamination in the samples tested. The strength of domestic sewage is dependent on the presence of industrial effluent, the water use in the catchment area (affluent areas typically using more water than non-affluent areas), the amount of stormwater ingress into the sewer system and whether vacuum tankers servicing septic tanks are disposing to the WWTWs. Similarly, the extent of sewage contamination in a river or other surface water source depends on the availability of sanitation in the area, whether there are leaking sewers and pumpstations in the catchment and whether any illegal dumping is taking place.

Table 5:Typical Values for Untreated Domestic Wastewater. Source: Nozaic and Freese (2009)

Contaminants	Units	Low Strength	Medium Strength	High Strength
Total Solids (TS)	mg/L	390	720	1230
Total Dissolved Solids (TDS) - Fixed - Volatile	mg/L	270 160 110	500 300 200	860 520 340
Suspended solids (SS) - Fixed - Volatile	mg/L	120 25 95	210 50 160	400 85 315
Settleable solids	mg/L	5	10	20
Biochemical Oxygen Demand (BOD) 5 days @ 20°C	mg/L	110	190	350
Total Organic Carbon (TOC)	mg/L	80	140	260
Chemical Oxygen Demand (COD)	mg/L	250	430	800
Nitrogen (total as N) - Organic - Free ammonia - Nitrites - Nitrate	mg/L	20 8 12 0 0	40 15 25 0 0	70 25 45 0 0
Phosphorous (total as P) - Organic - Inorganic	mg/L	4 1 3	7 2 5	12 4 10
Chlorides	mg/L	30	50	90
Sulphate	mg/L	20	30	50
Oil and grease	mg/L	50	90	100
Volatile Organic Compounds (VOC)	mg/L	<100	100-400	>400
Total Coliforms	No./100mL	10^5 - 10^8	10^7 - 10^9	10^7 - 10^{10}
Faecal Coliforms	No./100mL	10^3 - 10^5	10^4 - 10^6	10^5 - 10^8

3.1.1 Gauteng water quality

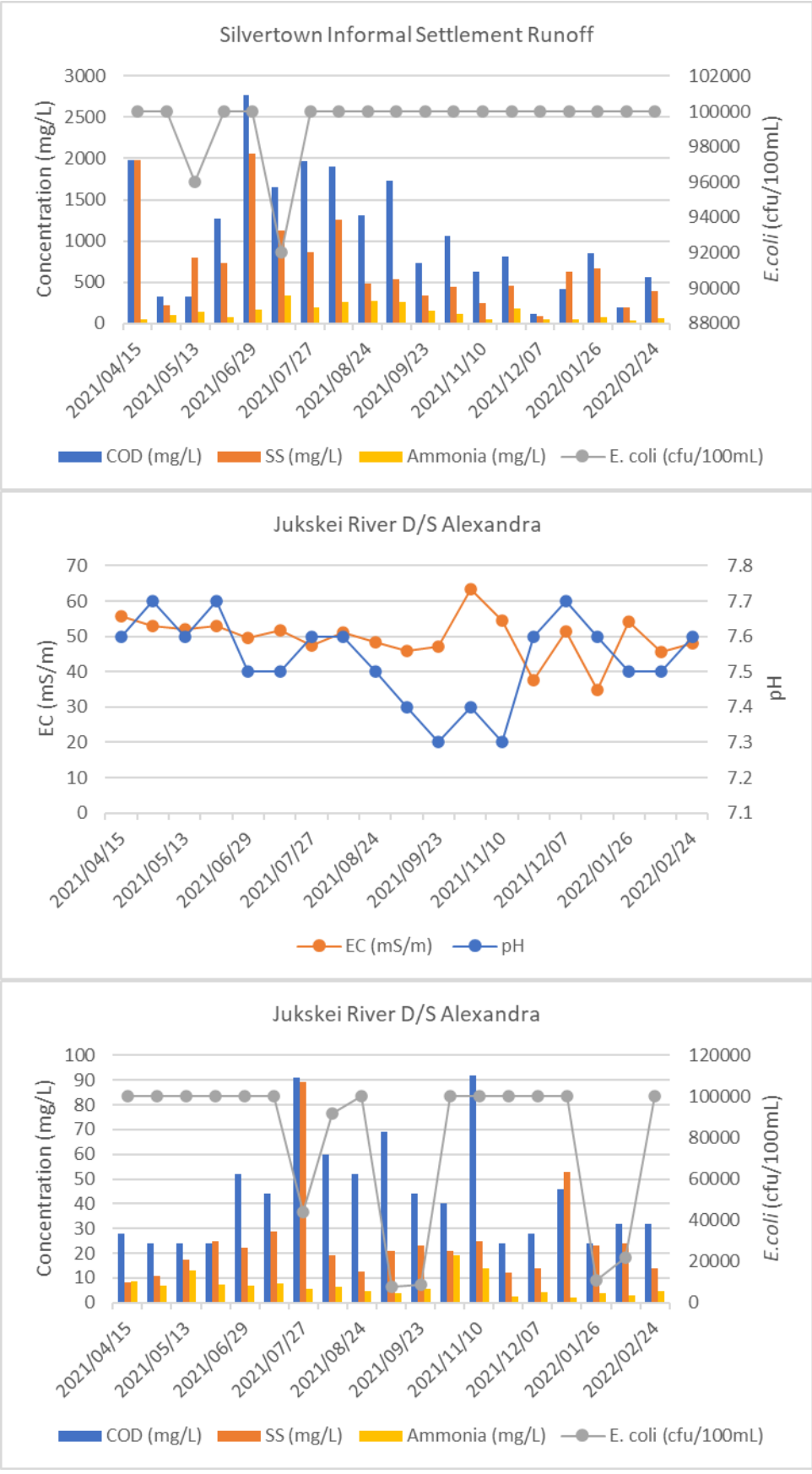
The water quality data collected to date for grab samples taken from the Jukskei River downstream of Alexandra and in the surface wastewater run-off from the Silvertown Informal Settlement in Alexandra are presented in **Figure 27** below. Water quality for the Jukskei source site, which daylights in the Johannesburg CBD, is presented in **Figure 28**.



Suspended solids are unsurprisingly high in the surface water samples in the Silvertown informal settlement.

With the exception of two samples in April and May 2021, all samples showed *E. coli* counts in excess of 100 000 per 100ml indicating significant pollution contamination (similar to medium- to high-strength sewage).

The concentration of COD, SS and ammonia appeared to be impacted by rainfall, with more concentrated samples in the winter months. COD concentrations in excess of 800 mg/L



through winter were characteristic of high strength sewage.

The informal settlement does not appear to have a significant impact on the downstream water quality, which is already highly polluted from upstream activities.

The concentrations of COD and ammonia in the river increased during the winter months when there was no rainfall. Two spikes in COD concentration in July and November 2021 may indicate spillage events in the river.

Figure 27: Water quality of the run-off from the Silvertown informal settlement and the impact of Alexandra Informal Settlement on the Jukskei river quality

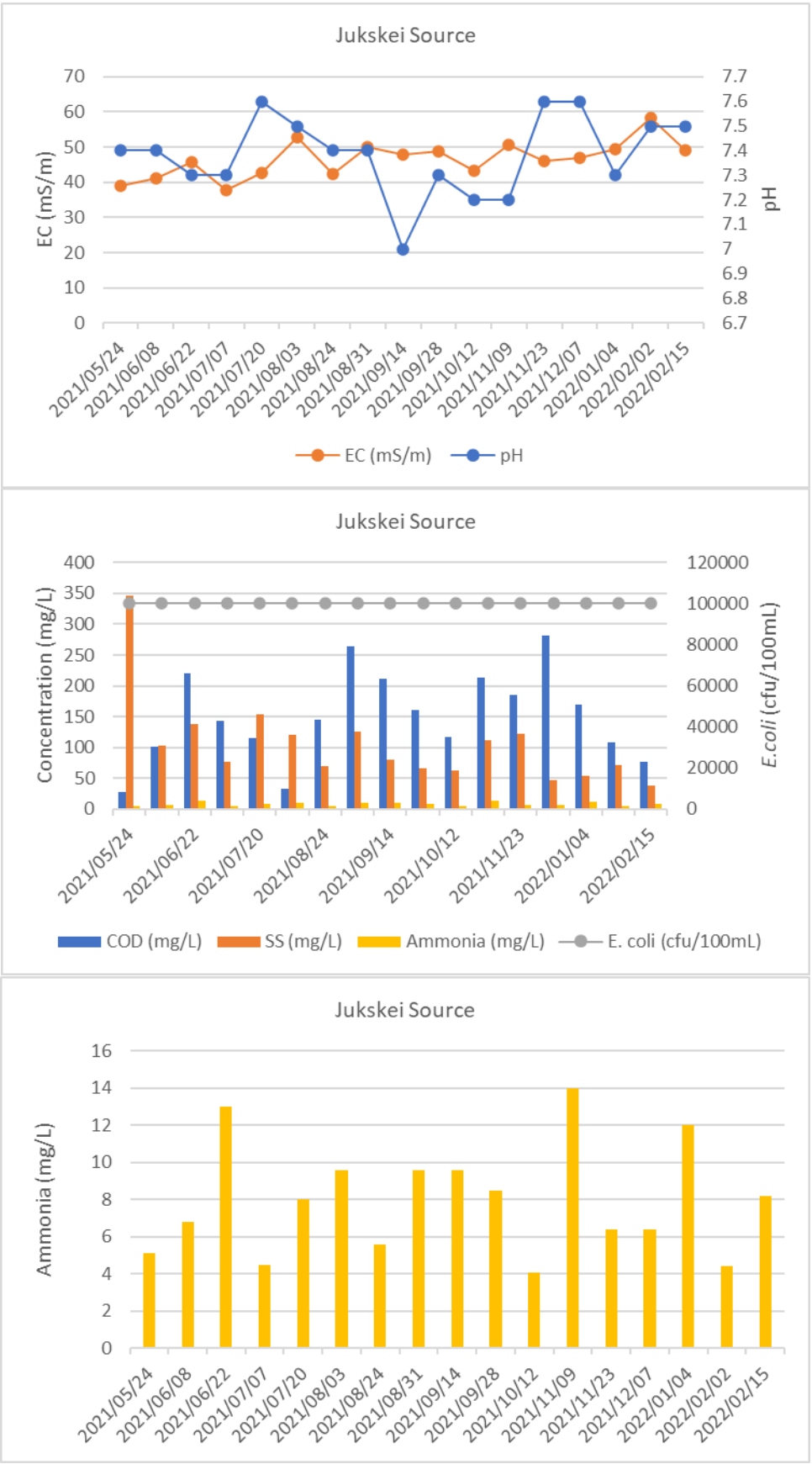
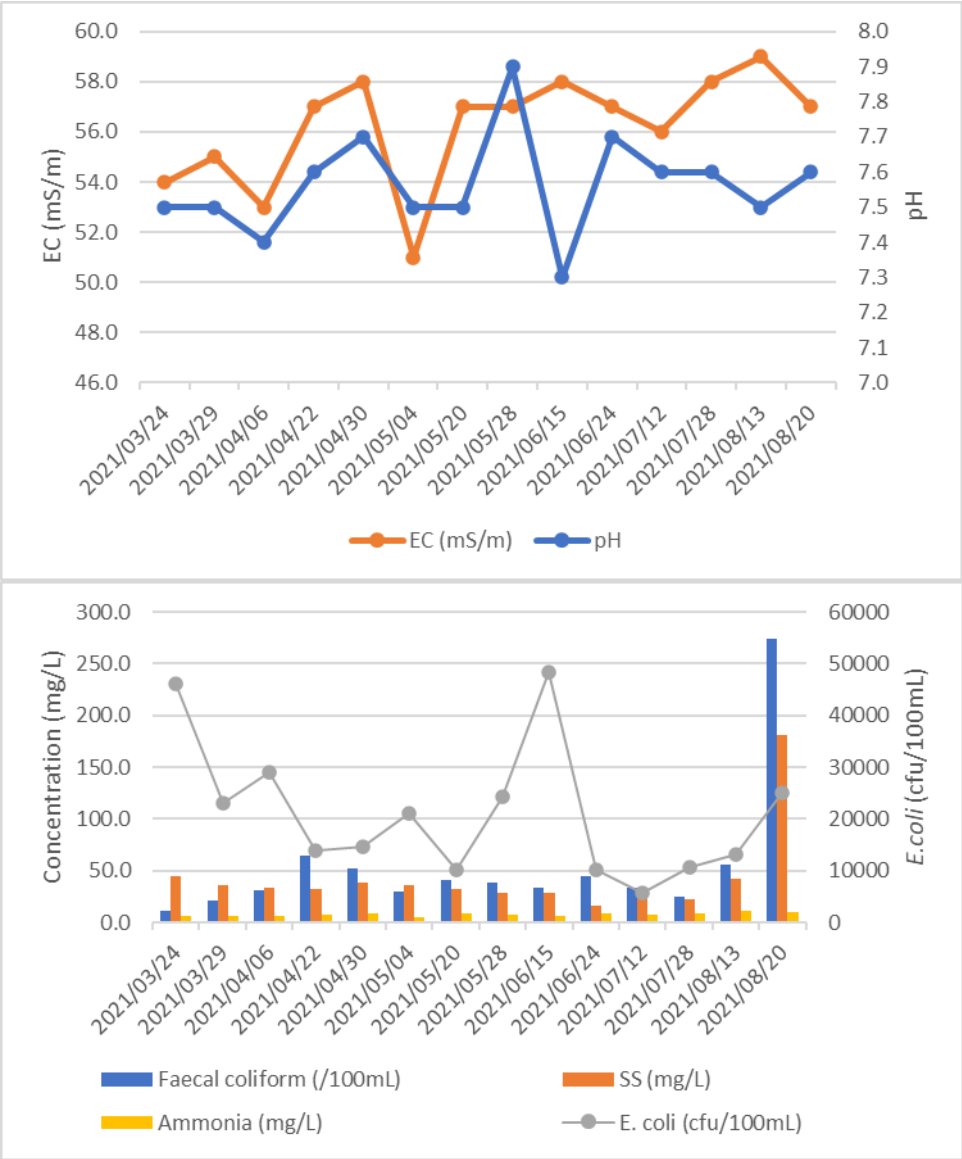


Figure 28: Water quality of the Jukskei Source

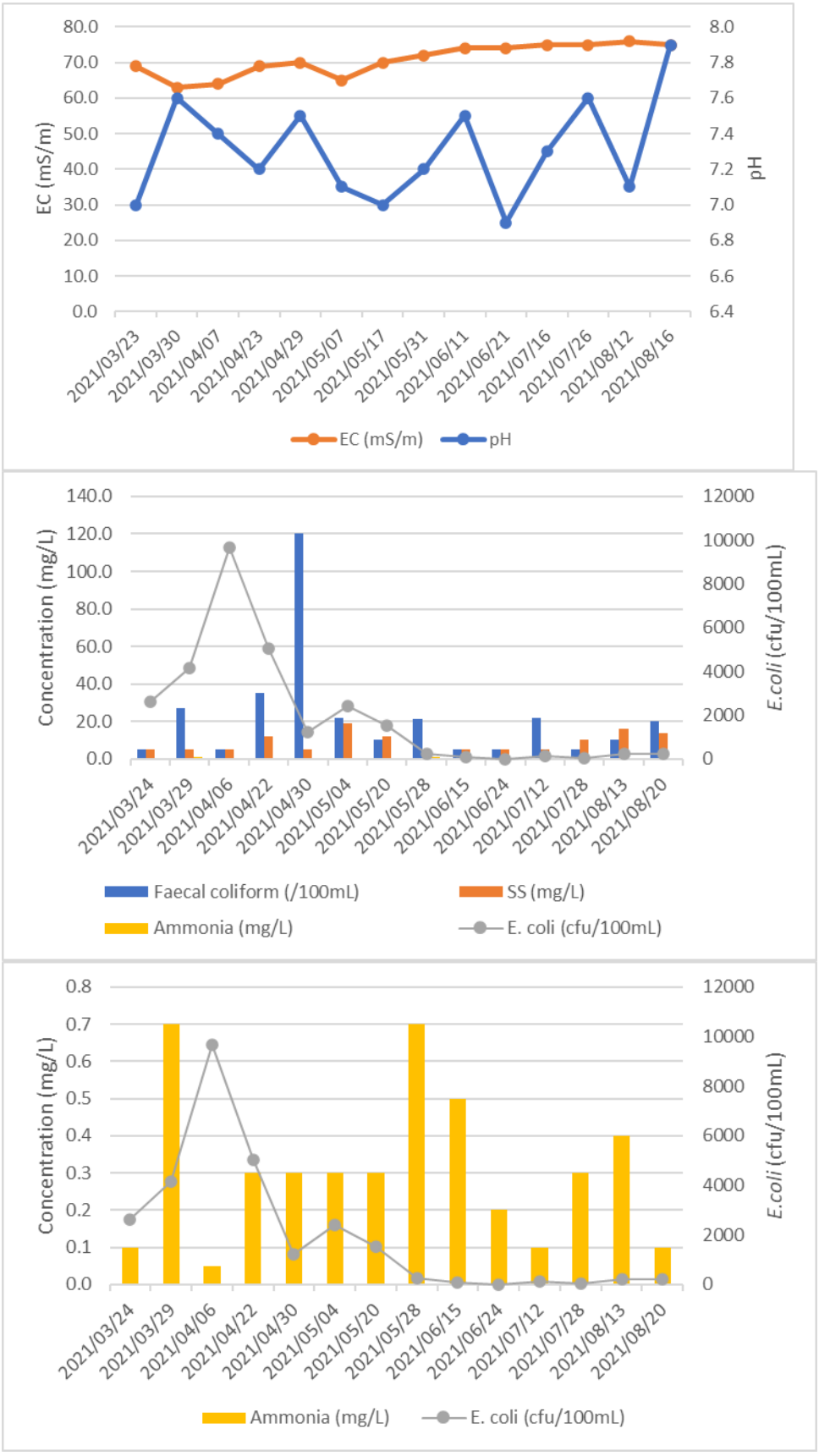
The COD concentration of the Jukskei source was significantly higher than expected at 100 mg/L or more. The downstream samples showed some recovery from these concentrations (**Figure 27**). *E. coli* counts were in excess of 100 000 cfu/100 mL in all samples tested.

The Jukskei source had high concentrations of ammonia which indicated probable groundwater contamination.



The water quality of the Klip River upstream of the Waterval WWTW showed COD concentrations below 50 mg/L for the sample period, with the exception of the 20-08-2021 when the COD concentration spiked to in excess of 250 mg/L in tandem with raised *E. coli* counts and suspended solids, indicating a possible sewage spill. *E. coli* counts were variable with the highest count on 15 June 2021 at 48 300 CFU/100mL.

Figure 29: Water quality of the Klip River (Upstream Waterval WWTW)



The water quality of the Glenshaft Pan downstream of the Benoni WWTW is good, with COD concentrations below 40 mg/L and ammonia concentrations below 0.7 mg/L. The with the exception of a spike in COD concentration in April 2021 which may have been due to a spill.

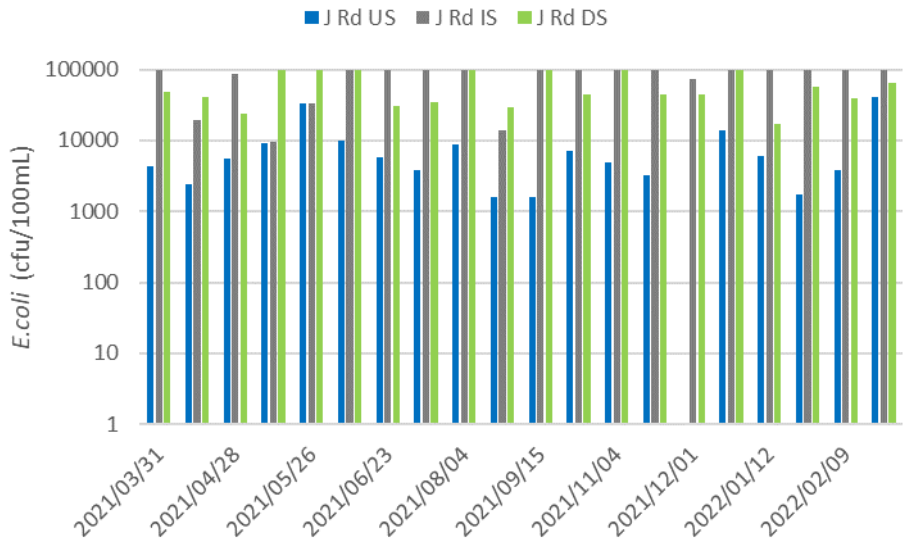
The *E. coli* counts peaked at 10 000 CFU/mL in April 2021, prior to the spike in COD concentration, and counts were below 200 CFU/mL from May 2021 to the end of sampling.

Figure 30: Water quality for Glenshaft Pan (DS Benoni WWTW at informal settlement)

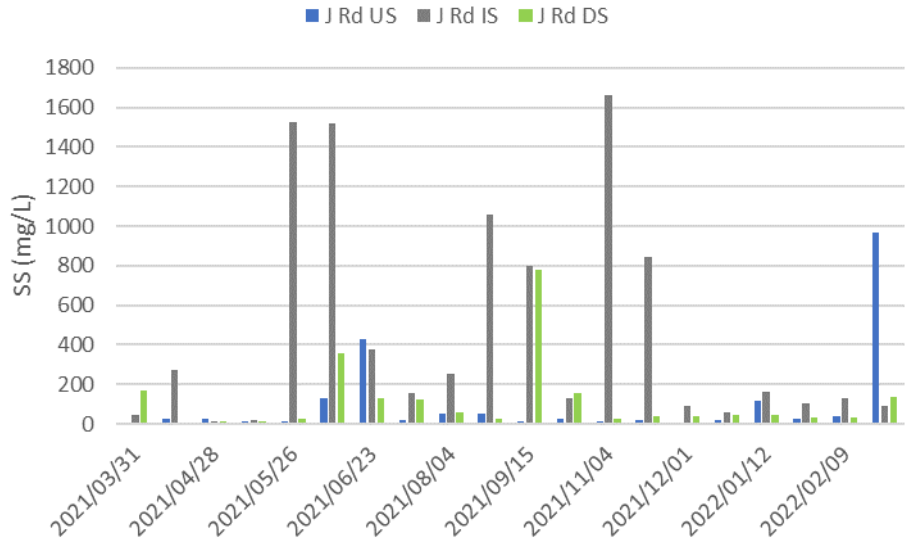
3.1.2 KwaZulu-Natal water quality

The water quality data collected to date for the Umhlangane River upstream and downstream of the Johanna Road Informal Settlement and in the surface wastewater run-off from the Johanna Road Informal Settlement in eThekwini, KZN is presented in **Figure 31** below. Note that the results for COD, suspended

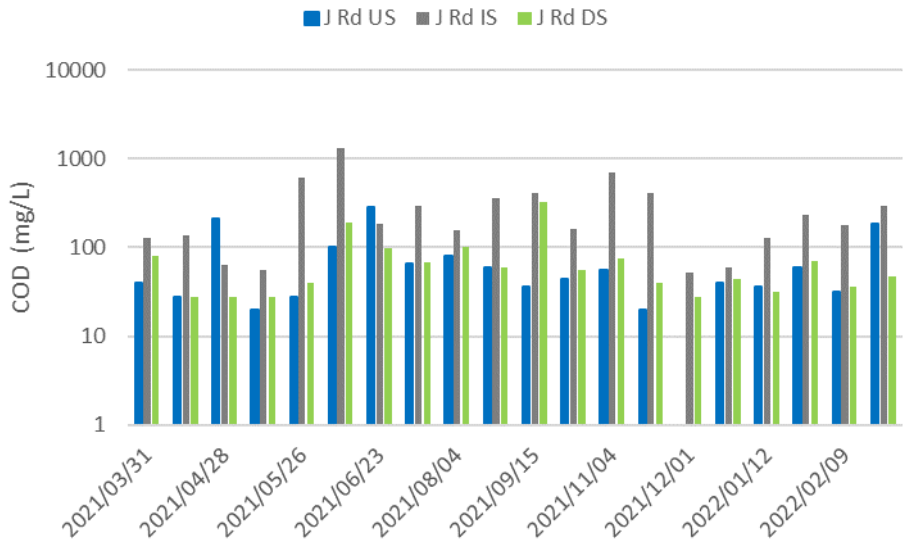
solids and *E. coli* are presented with a log scale to allow all sites to be represented on the same graph for comparative visualization.



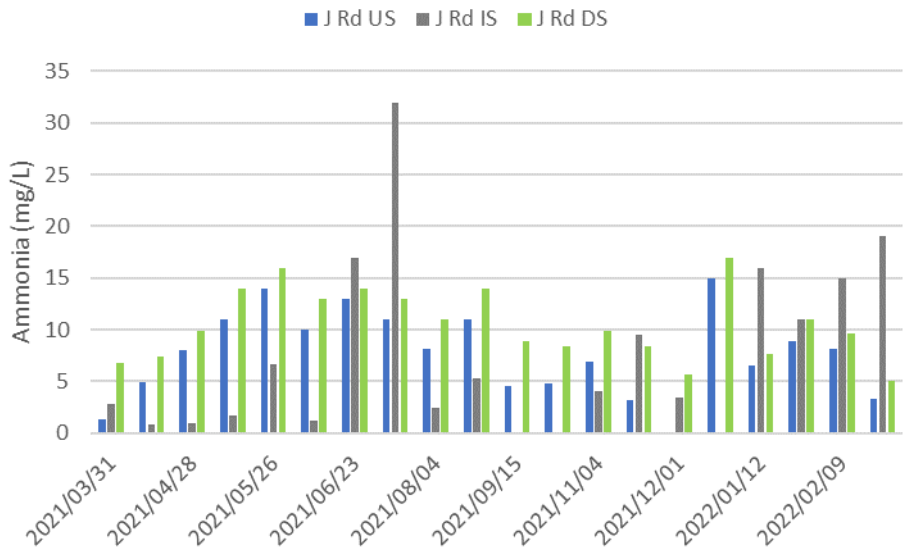
There was a clear distinction between the microbiological quality of the upstream sample site and the informal settlement and downstream sampling site which indicated significant contamination in the settlement run-off and impact on the river quality



Very low levels of suspended solids were evident in the upstream sampling site while the influence of the informal settlement on the suspended solids concentration was evident.



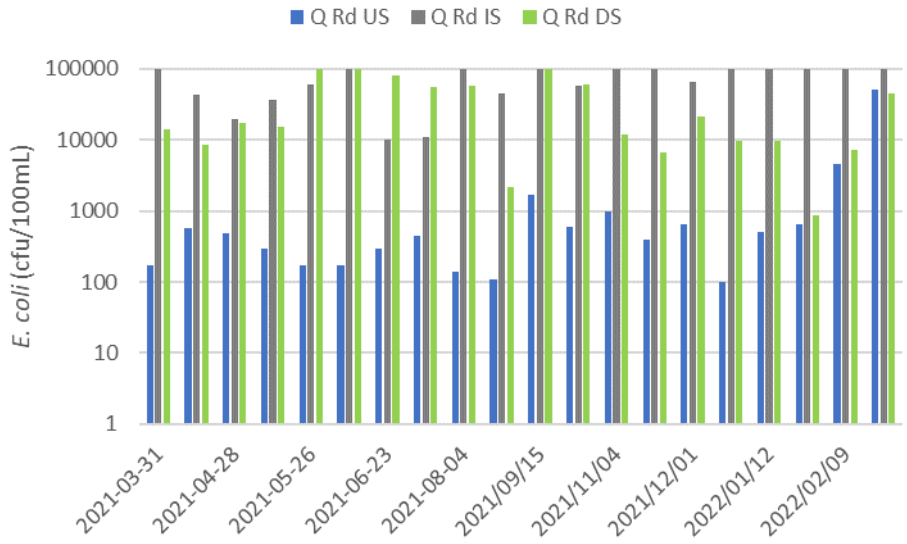
COD concentrations increased during winter and decreased during summer which was expected due to the lack of rainfall during winter to provide a dilution effect. Concentrations in the upstream site remained low throughout the period.



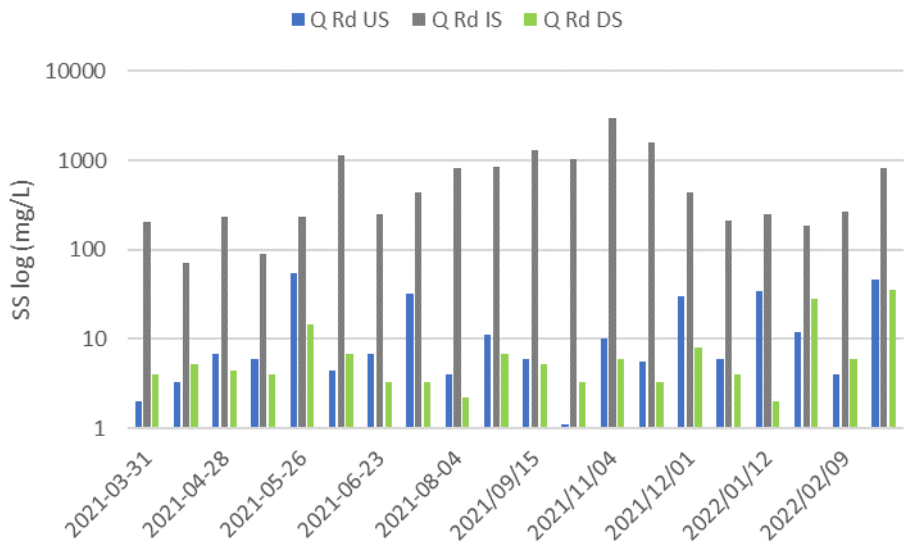
Ammonia followed a similar trend to COD, increasing in winter and decreasing in summer with low concentrations of ammonia present in the upstream sample site. The impact on the downstream site was evident. The informal settlement run-off showed high concentrations of ammonia present.

Figure 31: Water Quality data for Johanna Road Sites

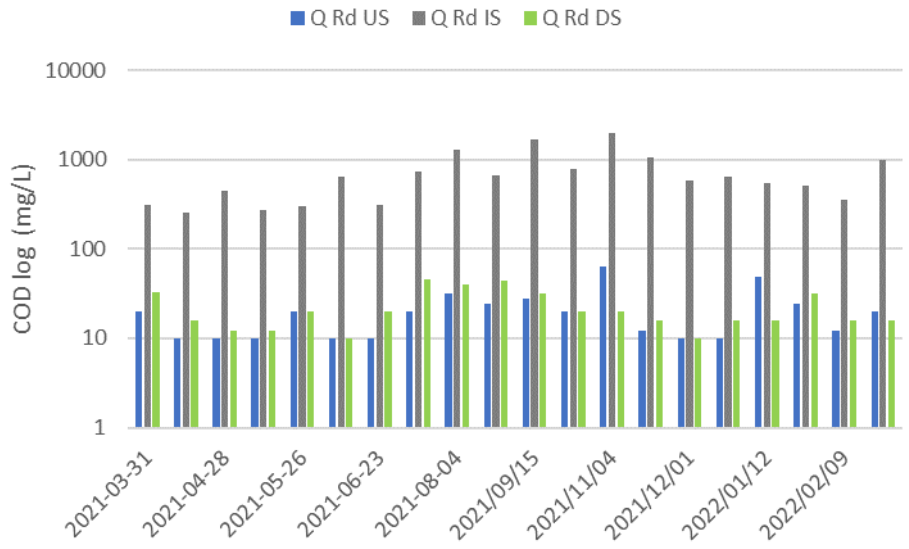
The water quality data collected to date for the Palmiet River upstream and downstream of the Quarry Road West Informal Settlement and in the surface wastewater run-off from the Quarry Road West Informal Settlement in eThekwini, KZN province is presented in **Figure 32** below. Note that the results for COD, suspended solids and *E. coli* are presented with a log scale to allow all sites to be represented on the same graph for comparative visualization.



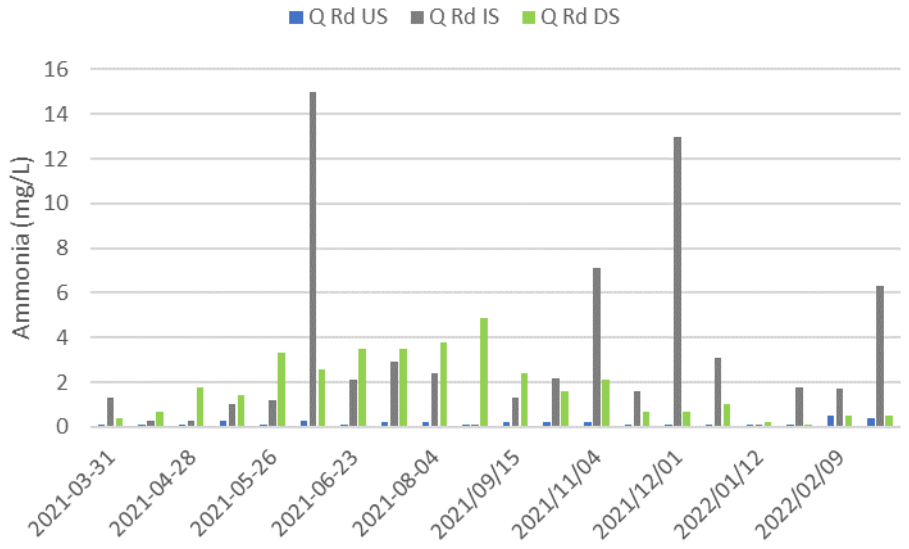
The upstream site did not appear to be significantly polluted; the downstream site had a significant microbiological load. The run-off from the informal settlement was highly polluted.



The upstream site was not significantly polluted and had low suspended solids concentrations. The informal settlement run-off was very high in suspended solids, impacting on the downstream quality.



COD concentration of the informal settlement run off was very high. The upstream site was not significantly polluted. While the downstream site concentrations were higher than upstream in some cases they were below 75mg/L, and the COD impact appeared negligible.

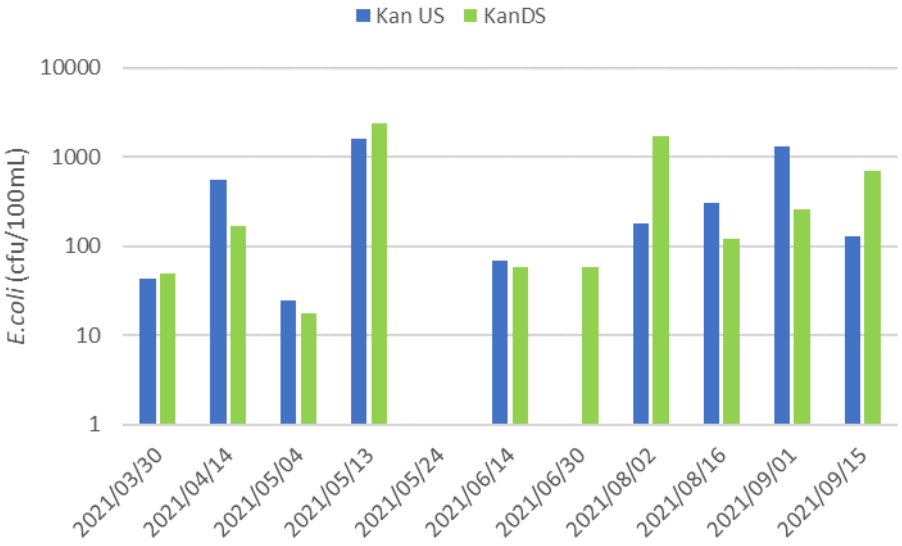


Ammonia was evident in the run-off from the informal settlement indicative of sewage pollution. The upstream site had very low concentrations and the downstream site showed a significant increase which was evidence of the impact of the settlement on the river.

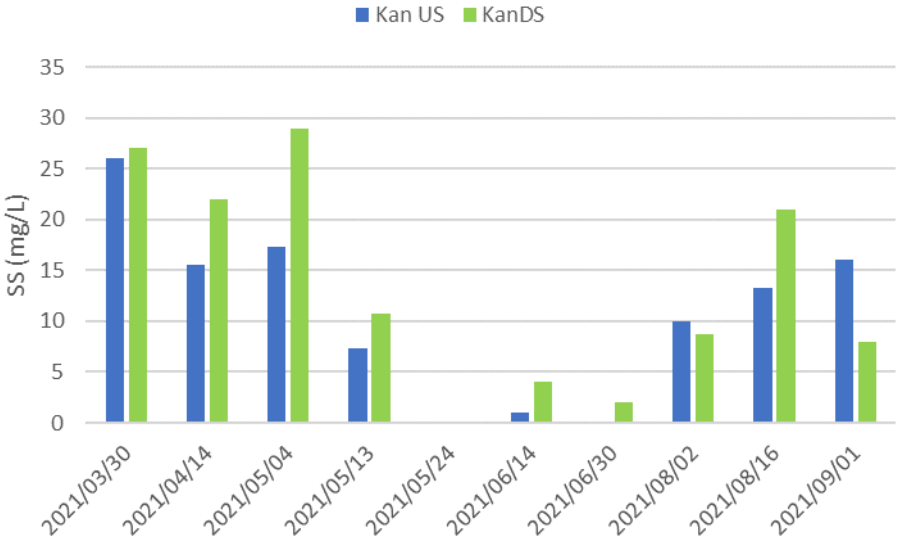
Figure 32: Water quality data for Quarry Road Sites

3.1.3 Mpumalanga water quality

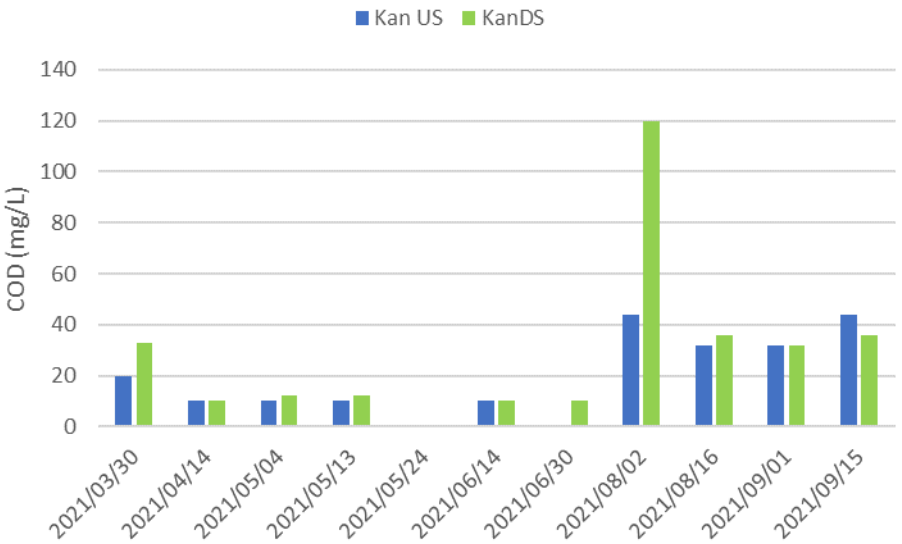
The water quality data collected to date for the Crocodile River upstream and downstream of Kanyamazane, Mpumalanga is presented in **Figure 33**. Note that the results for COD and *E. coli* are presented with a log scale to allow all sites to be represented on the same graph for comparative visualization.



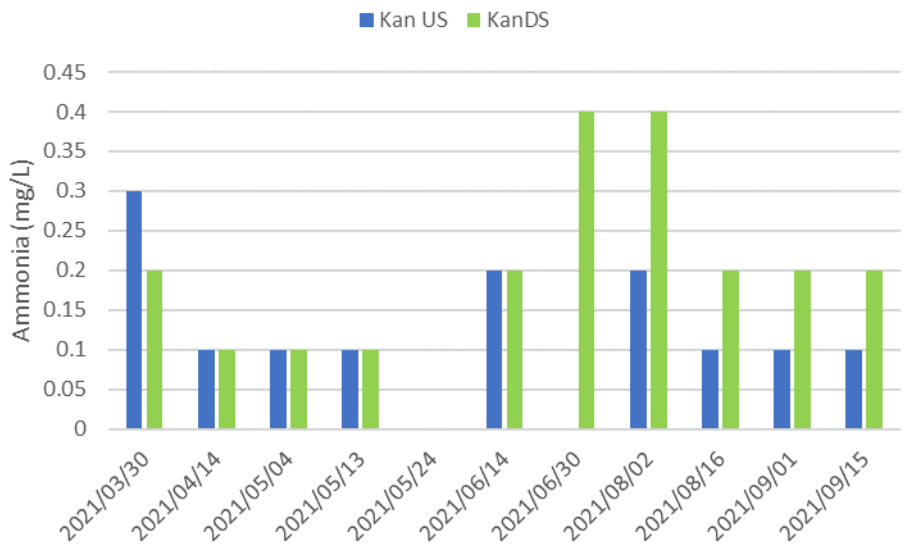
The data indicated that there was an increase in *E. coli* per 100 mL downstream of the informal settlement in some samples but not in others, with the higher impact seen in August and September 2021. The high upstream counts were likely a result of the WWTW discharging upstream.



The suspended solids concentrations were higher downstream of the informal settlement, with the exception of two samples taken in August and September 2021, indicating the impact of the informal settlement.



COD concentrations for this stream remained low with only one sample measuring above 50 mg/L. In general, the data showed a slight increase in COD downstream of Kanyamanzane settlement.

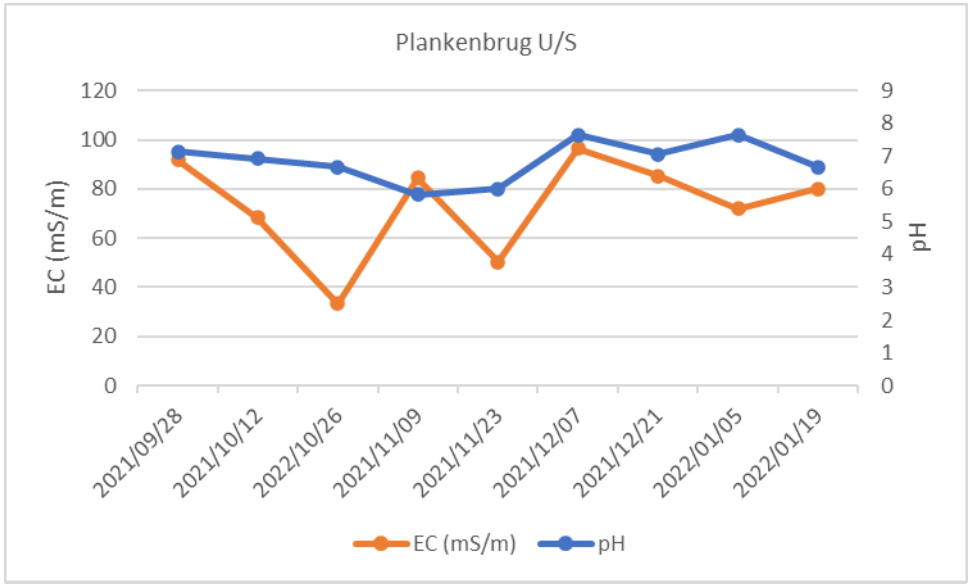


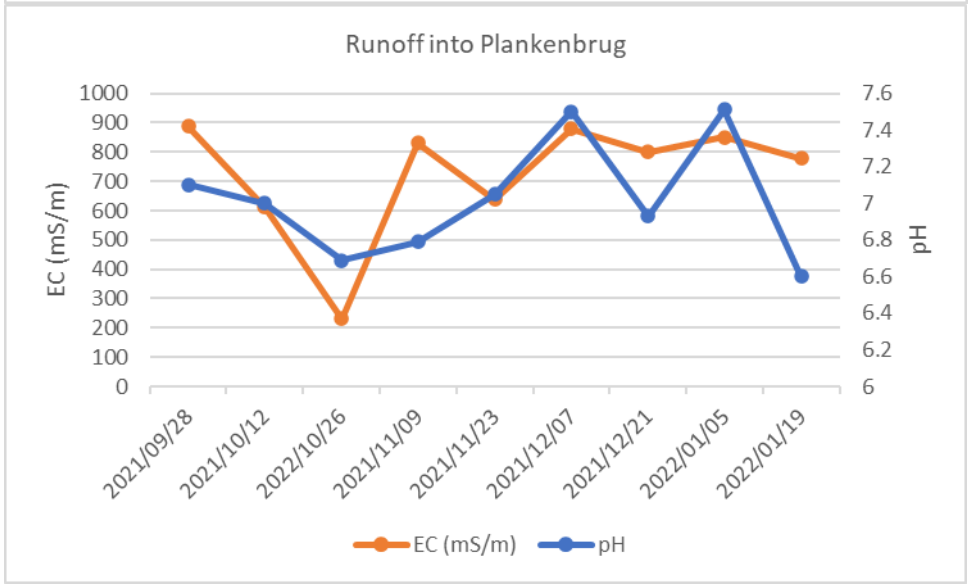
Ammonia concentrations were low. While they did increase downstream of the Kanyamanzane settlement the concentrations remain below 1 mg/L.

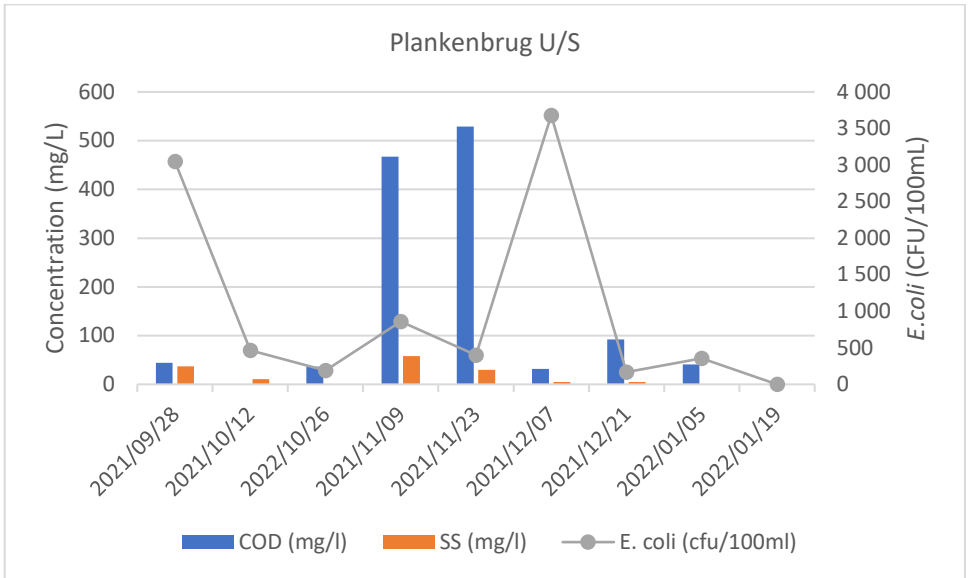
Figure 33: Water quality data for Kanyamanzane Crocodile River

3.1.4 Western Cape water quality

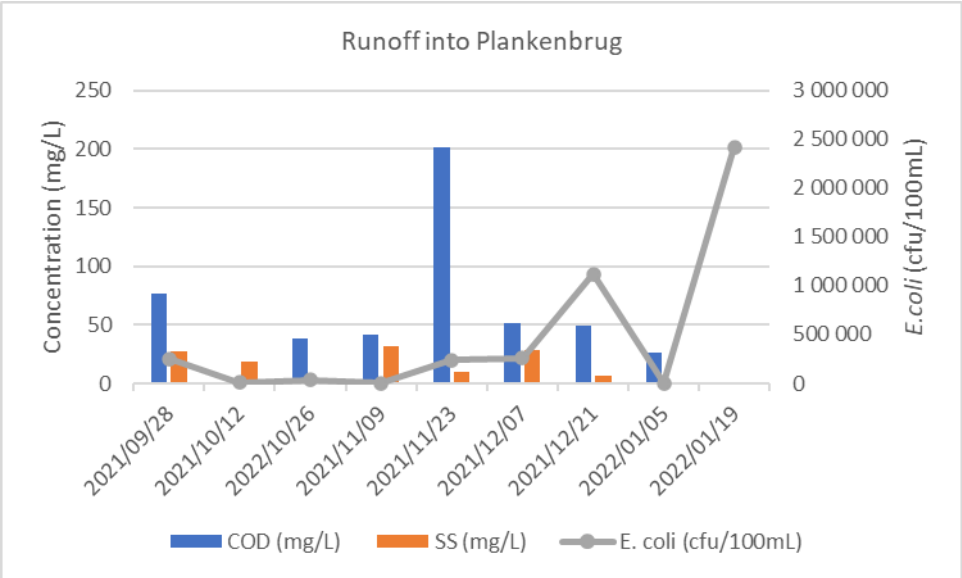
Water quality data for the Plankenbrug River at the point of informal settlement run-off, the Kuils River and the Black River for September 2021 to January 2022 are presented in **Figure 34**, **Figure 35** and **Figure 36** respectively. Note that the results for COD, suspended solids and *E. coli* are presented with a log scale to allow all sites to be represented on the same graph for comparative visualization.

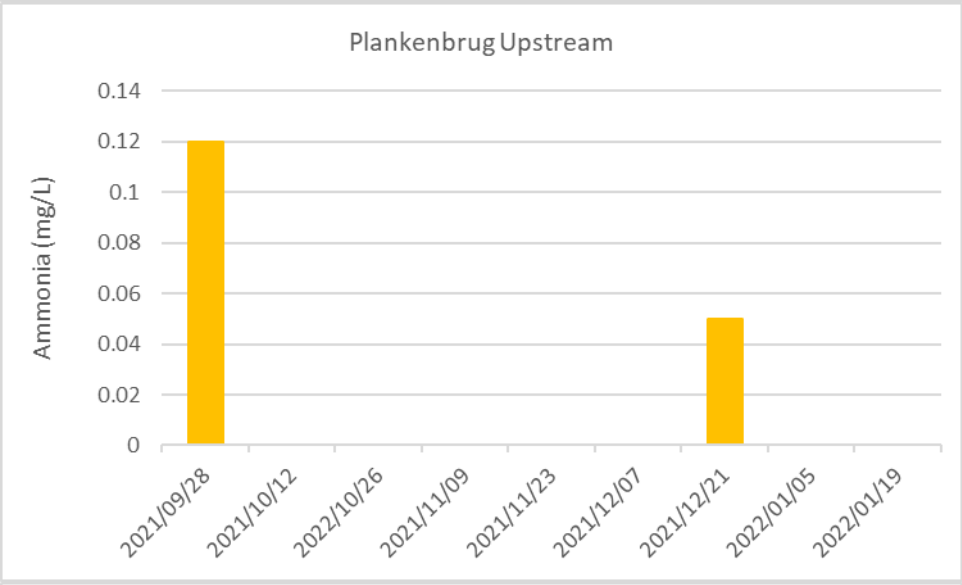


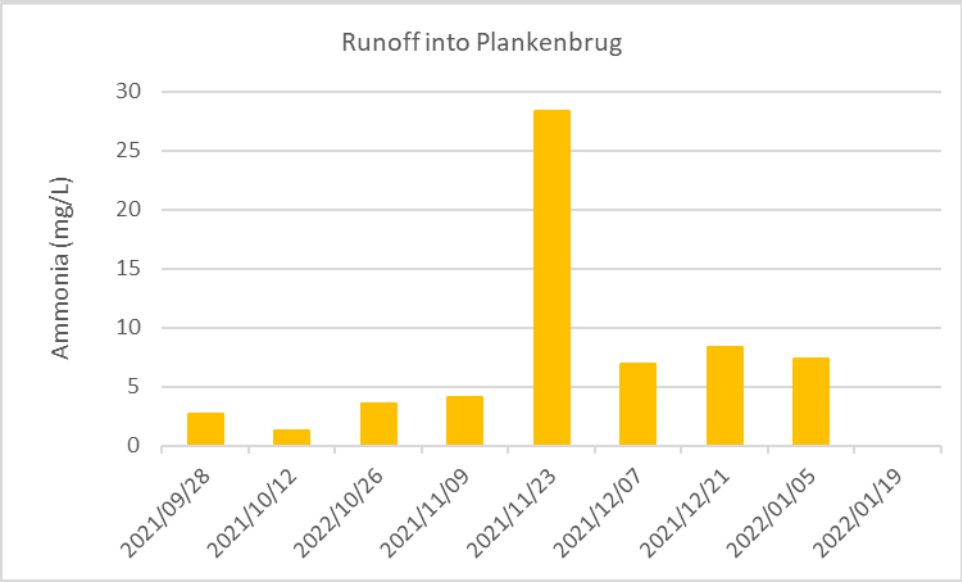




The Plankenbrug was generally very impacted, with COD concentrations in excess of 75 mg/L even in the upstream sample. The human impact in the run-off from the informal settlement into the river could be seen in the very high *E. coli* counts, which increased through the summer months with reduced rainfall in the Western Cape region.







The ammonia concentration in the upstream samples was negligible, but the impact of human pollution in the run-off into the Plankenbrug from the informal settlement was clearly evident in the high ammonia concentrations seen, especially in November. This spike in ammonia coincided with a spike in COD and *E. coli*.

Figure 34: Water quality data for Plankenbrug River

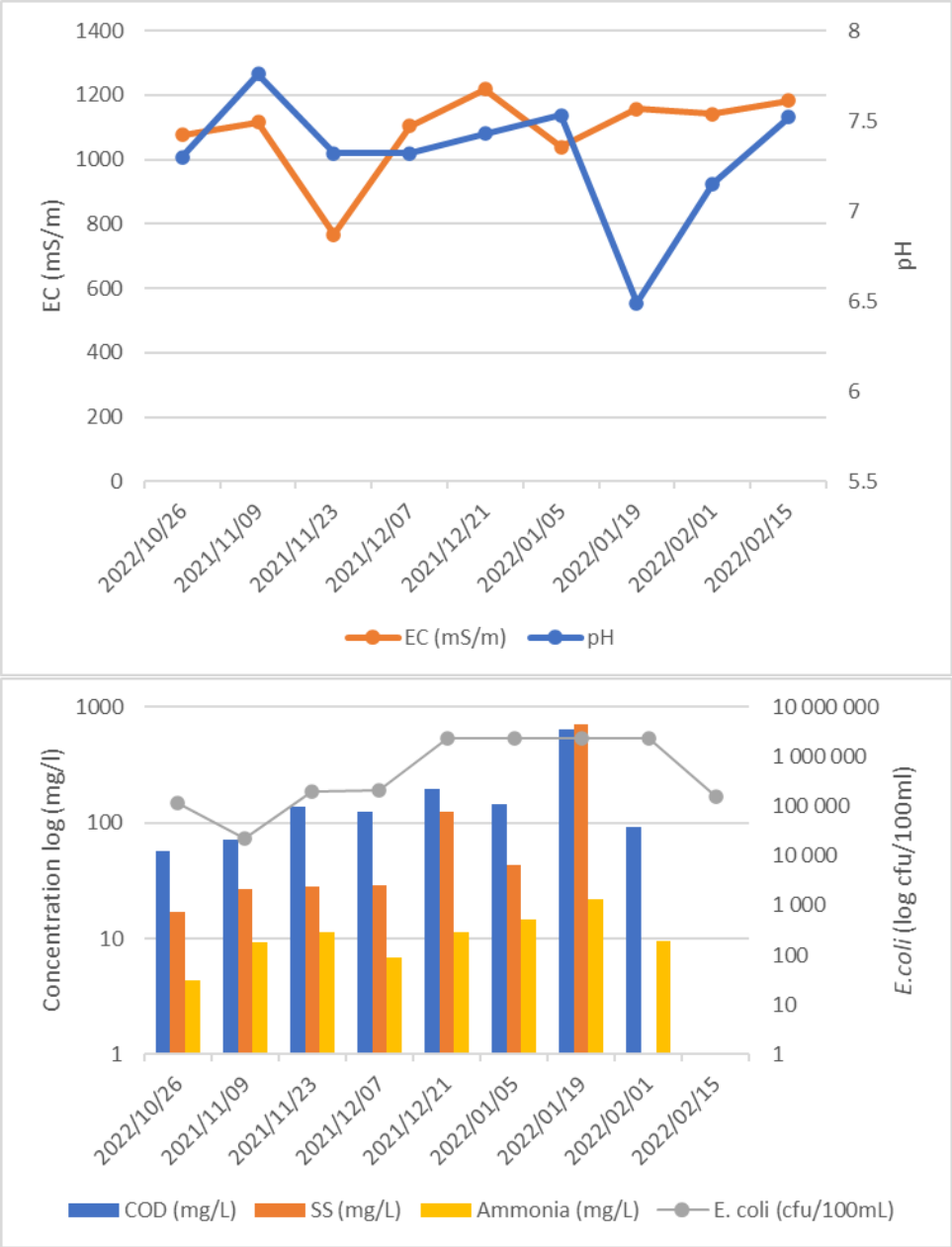


Figure 35: Water quality data for the Black River

The Black River was highly polluted, with COD concentrations in excess of 75 mg/L in all samples, increasing through the summer months. There were also increases in the ammonia concentration and *E. coli* counts through summer.

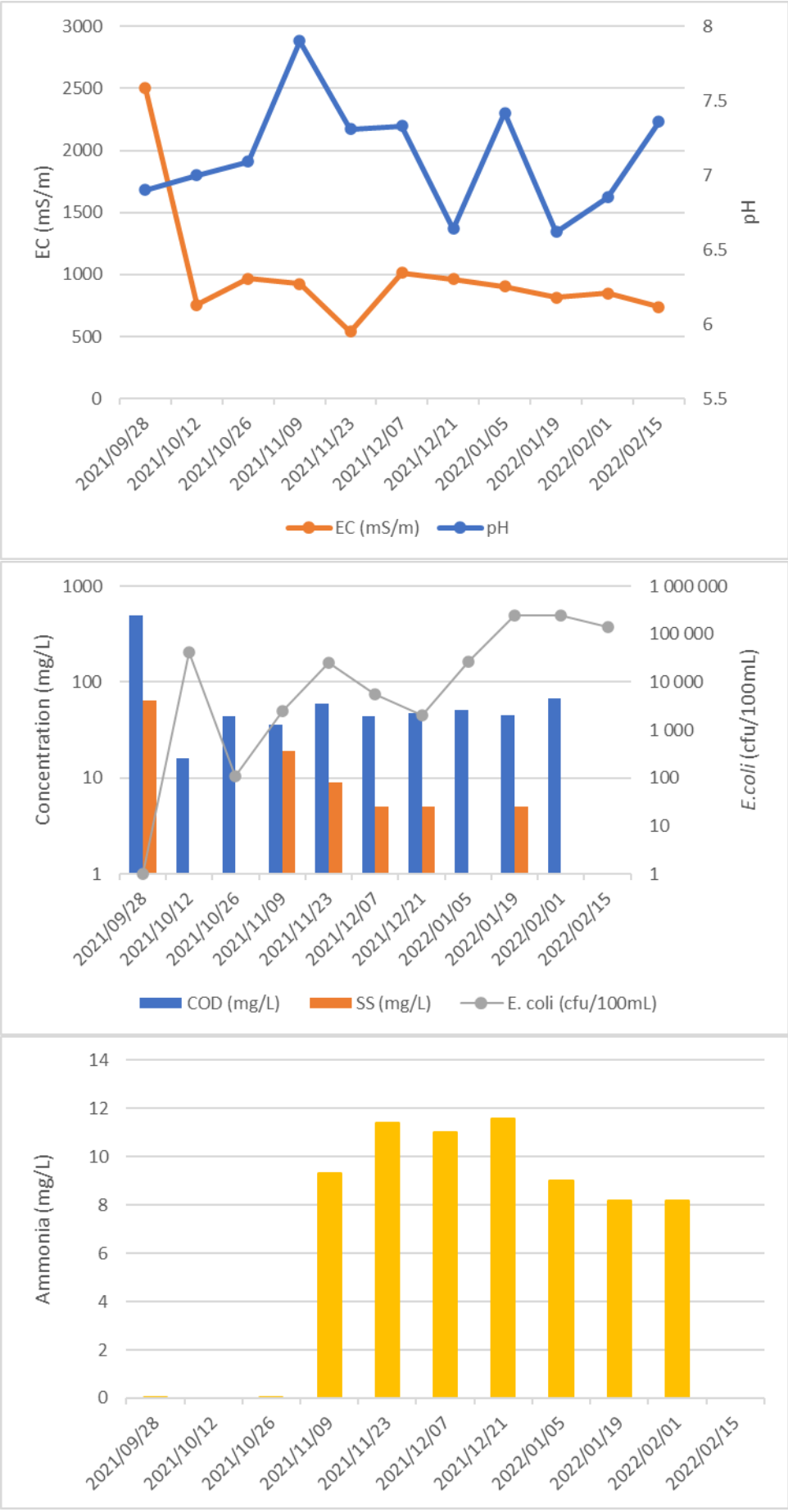


Figure 36: Water quality data for the Kuils River

The water quality of the Kuils River was also severely impacted by pollution. Interestingly, while the COD concentration remained fairly constant throughout the test period at around 50 mg/L, both the ammonia concentration and *E. coli* counts increased from October 2021 to February 2022.

3.1.5 Water quality results discussion

The results indicated a significant change in the quality of the river water as the summer rainfall came to an end in the summer rainfall areas with a significant decline in water quality evident at some sites. This included an increase in ammonia as well as faecal coliform and *E. coli* counts which indicated that the dilution caused by rainwater during the summer months had stopped. This dilution of river water with rain and stormwater flows may impact on the detection of SARS-CoV-2 in these diluted waters during summer (in the summer rainfall areas) and in winter in the Western Cape. There was a clear deterioration in the water quality in the Western Cape samples through the summer months.

The data shows that many sites had significant faecal pollution with *E coli* counts exceeding 100 000 CFU/mL (KZN informal settlements and Alexandra Silvertown Informal Settlement) and ammonia levels above 40 mg/L in the Alexandra Silvertown informal settlement, indicative of significant faecal pollution present.

Based on the data it appears that *E. coli* counts and ammonia concentration typically follow similar trends, that do not necessarily match the trends of the COD concentrations. Ammonia and *E. coli* may therefore be better indicators of human impact on the environmental samples than the COD concentration, which may be more affected by external influences such as industrial effluent or chemicals.

3.2 RESULTS OF SARS-COV-2 SCREENING

The number of COVID19 cases in South Africa reached 3,645,269 people as of 16 February 2022 with the number of deaths at a total of 97,431 (https://www.worldometers.info/coronavirus/country/south-africa/). The four waves of COVID-19 infection are provided in the graph below. The second wave peaked at the end of January 2021 and the third wave, caused mainly by the Delta variant, peaked in July 2021 with a longer and fatter peak than the previous two waves, stretching through August and September 2021 before significant decline was noted in the daily case load. The fourth wave peaked in December 2021, with a sharp incline in cases as a result of the Omicron variant. While South Africa technically exited the fourth wave in mid-January 2022, as of mid-February 2022 the case numbers had not yet reached the lows of the previous inter-wave periods.

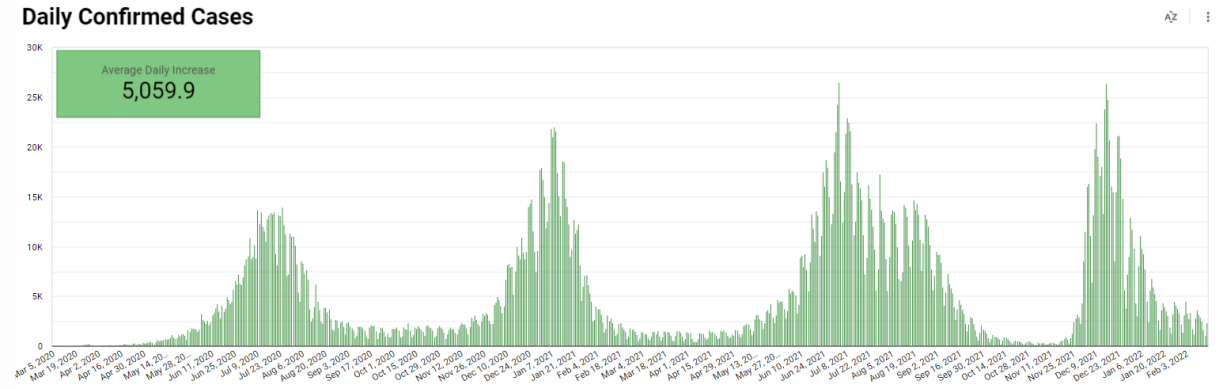


Figure 37: Daily New Cases in South Africa. Source: https://www.covid19sa.org/

Samples were collected from the sample points presented in **Table 1** from mid-March 2021, although one sample site (Jukskei Downstream) was continuously sampled from January 2021. As discussed in Section 2.2, a subset of sample sites were then selected for continued sampling from September 2021, with the addition of new sampling sites in the Western Cape, as presented in **Table 2**. RT-PCR assay results with any target with a Ct value below 40 were considered positive. The results of all sites are presented below, per Province. Yellow triangles represent negative results.

3.2.1 Gauteng

COVID-19 results for the Johannesburg metropolitan area are shown below (**Figure 38**).

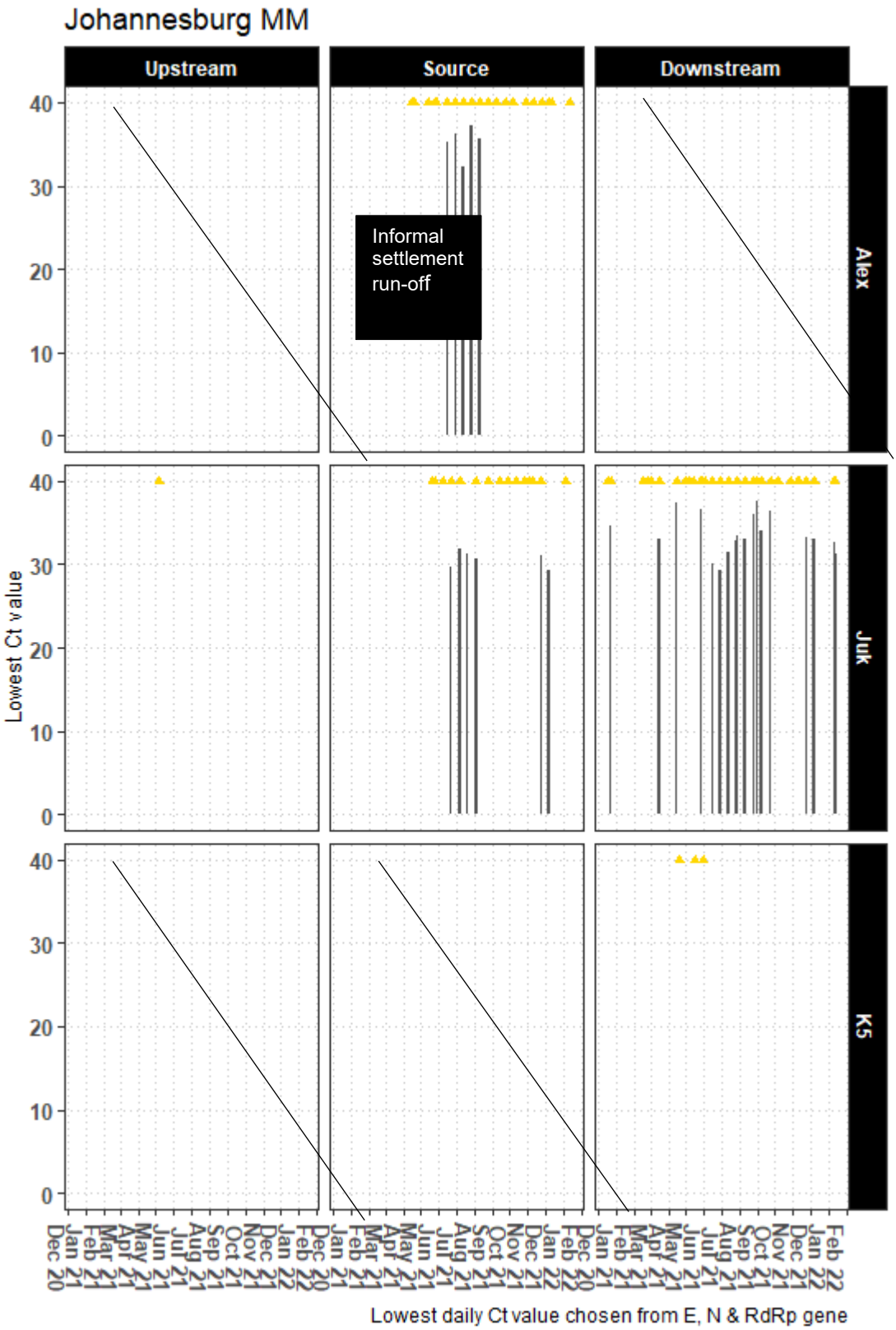


Figure 38: Jukskei and Klip Rivers and Alexandra informal settlement run off

Sites in Johannesburg are very densely populated, and the pollution of the downstream site was clear which provided several positive COVID-19 results in the downstream sites. Due to resource challenges, the Jukskei River site upstream of Alexander was not sampled regularly and was substituted for the Jukskei Source site which tested positive for COVID-19 four times during the third wave. The Jukskei Downstream site provided positive results during both the second and the third wave and did appear to show that infection rates within the community appeared to be higher than the cases reported during that time. The trend in Ct values also showed lower Ct values, indicative of higher viral loads during the third wave (June-July 2021). The Klipriver site which is upstream of a large Wastewater Treatment Works

(See **Figure 39**) and downstream of an informal settlement also provided positive results with lower Ct values measured in July 2021 and August 2021 during the third wave. Some evidence of community infection was evident in the results from May 2021, which suggests that, due to cost of testing, many of the infected community members did not confirm COVID-19 infections.

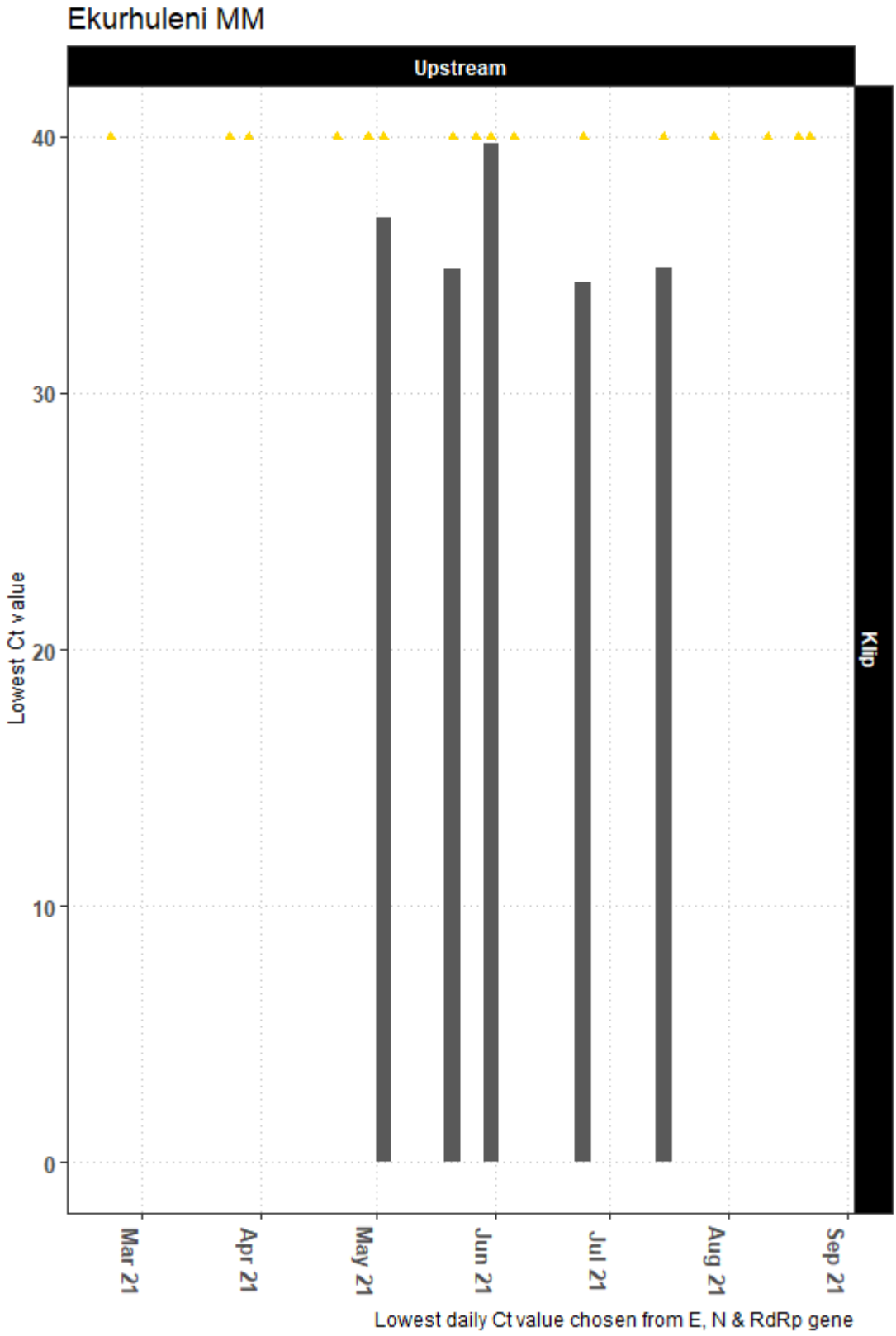


Figure 39: Sites in the Klipriver in Ekurhuleni downstream of Informal Settlements

The trend in the City of Tshwane sites (**Figure 40**) was similar to that of Johannesburg with lower Ct values measured during July 2021 (in the Hennops River downstream of Tembisa) and at the end of July 2021 in the Rietspruit sample (measured near Thatchfield) in Centurion. This follows the same trend as the daily case load data as seen in **Figure 37**.

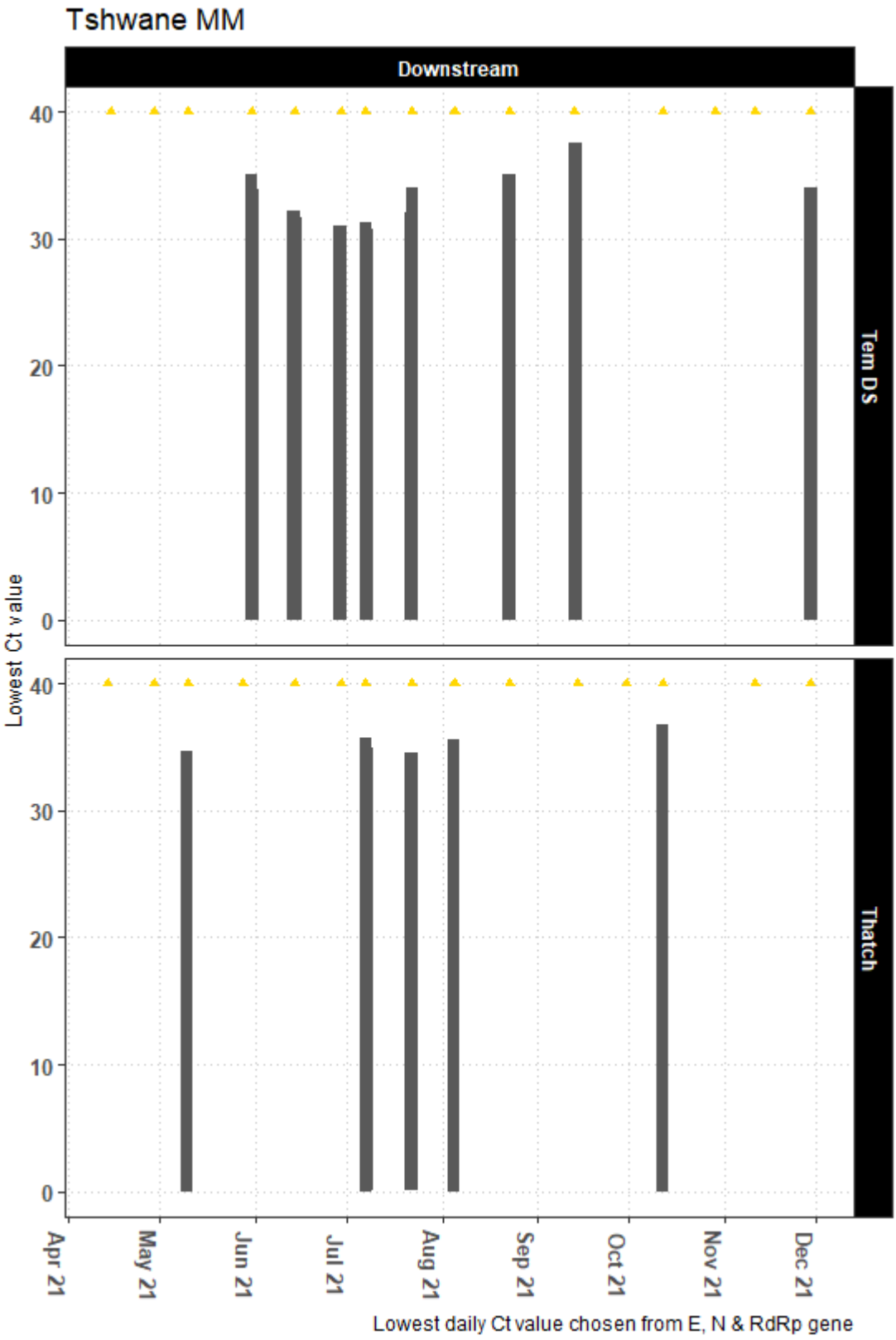


Figure 40: City of Tshwane Sites downstream of informal settlements

The site downstream of Sebokeng in Sedibeng also provided some positive results although these were sporadic (**Figure 41**).

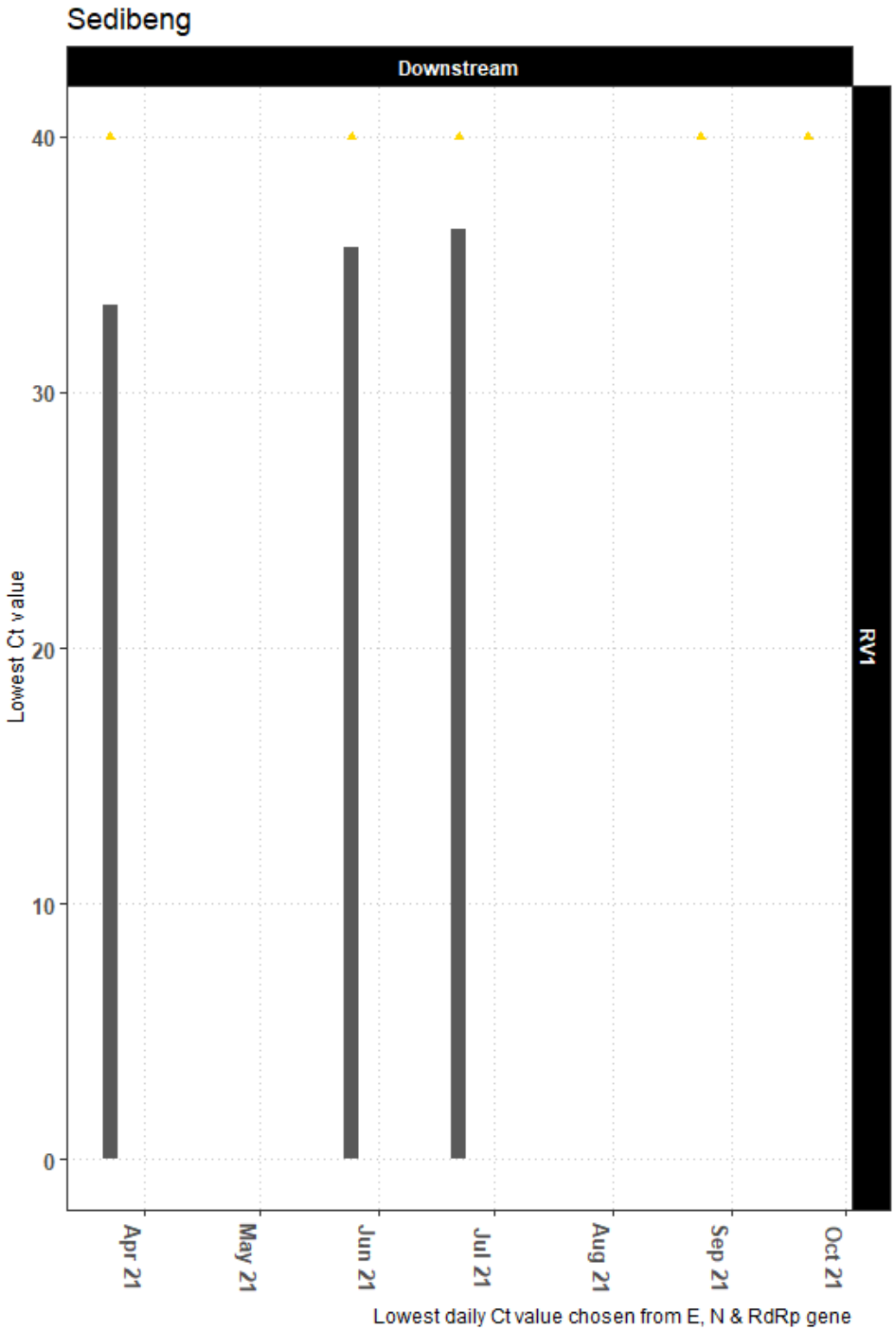


Figure 41: Rietspruit downstream of an informal settlement in Sedibeng

3.2.2 Western Cape

The Western Cape did not experience the third wave with as much intensity as Gauteng. This was evident in the results below where only one site tested positive. The informal settlements were also some distance from the streams which could also reduce the likelihood of run-off entering the stream. It may also be due to the rainfall experienced during the winter rainfall period which diluted the receiving body to the extent that SARS-CoV-2 RNA was not detected.

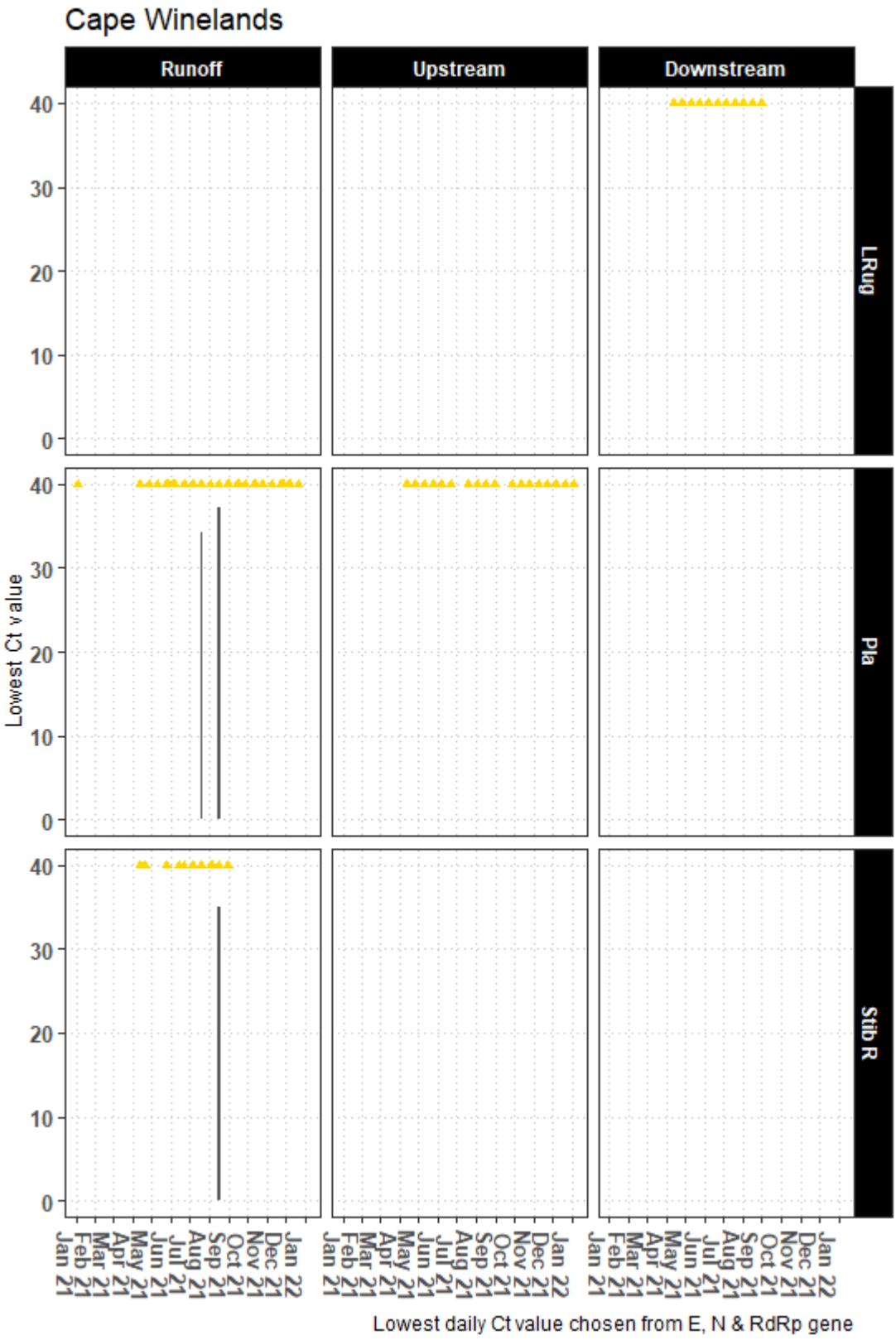


Figure 42: Cape Winelands sample sites

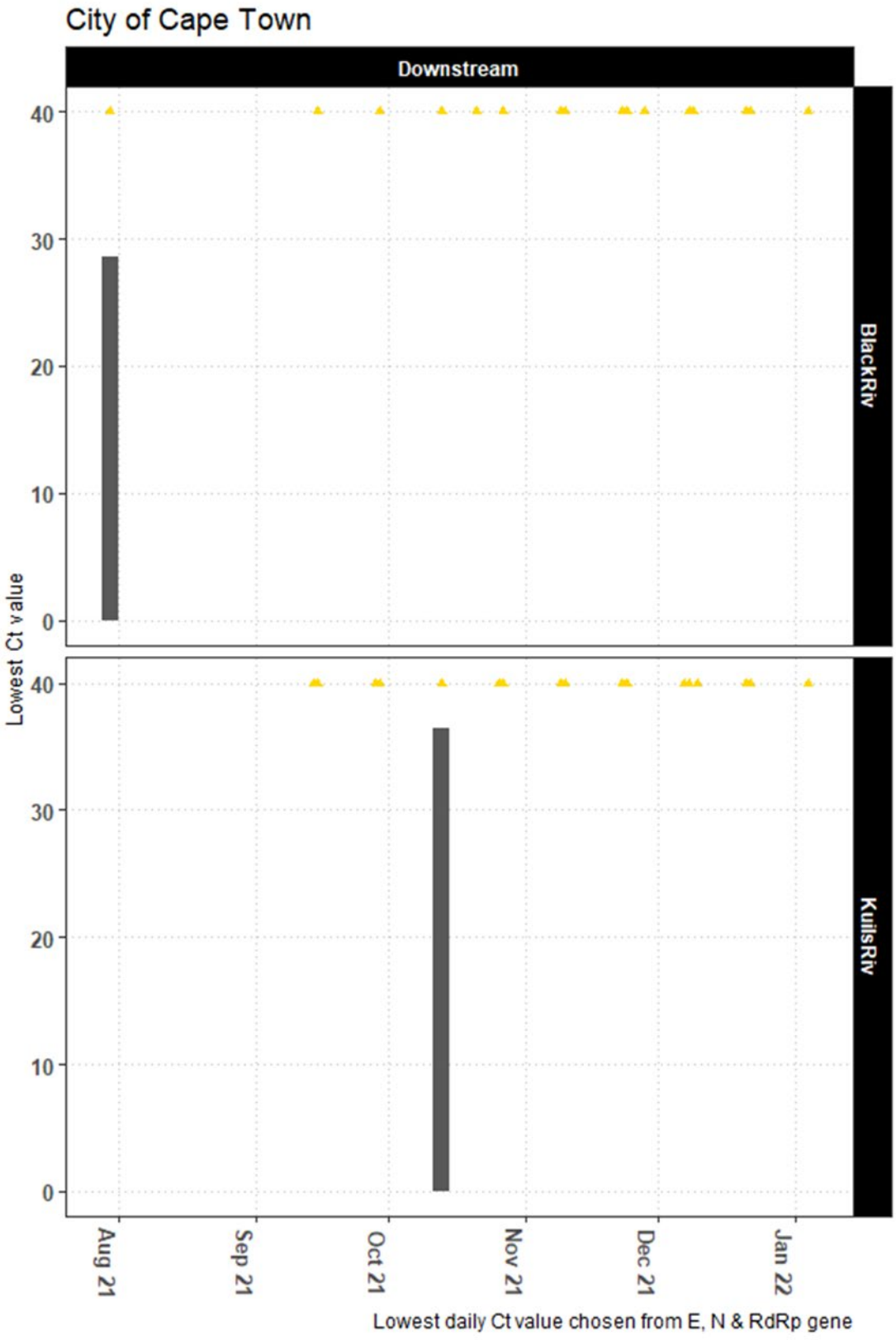


Figure 43: City of Cape Town sample sites

3.2.3 KwaZulu-Natal

The data that was obtained from the eThekwini sites shows that both the Johanna Road sites (upstream and downstream) provided positive COVID-19 results, indicative of pollution upstream of the upstream site, most likely from the discharge of the wastewater treatment works located upstream of the informal settlement. The data seemed to follow the pattern evident in the daily cases in KZN where the third wave was later than in Gauteng, with positive samples present in August 2021.

In the case of Quarry Road, although negative COVID-19 results in the upstream sample site, the downstream site had positive results during the corresponding third wave in KZN. This data corresponds well with the water quality data which showed that the upstream Quarry Road site was in a good condition compared to the downstream sample site.

Samples from two sampling runs from UDDT sanitation sites were tested for SARS-CoV-2 and all samples tested returned negative results. This method of sampling is not recommended due to the significant costs of sampling, laborious sample compilation and privacy of the homeowners. It is also felt that this does not provide community wide surveillance data but only focuses on a few households which is not indicative of the community health in general. As such, these samples will not be considered as a viable sample option in future surveillance programmes.

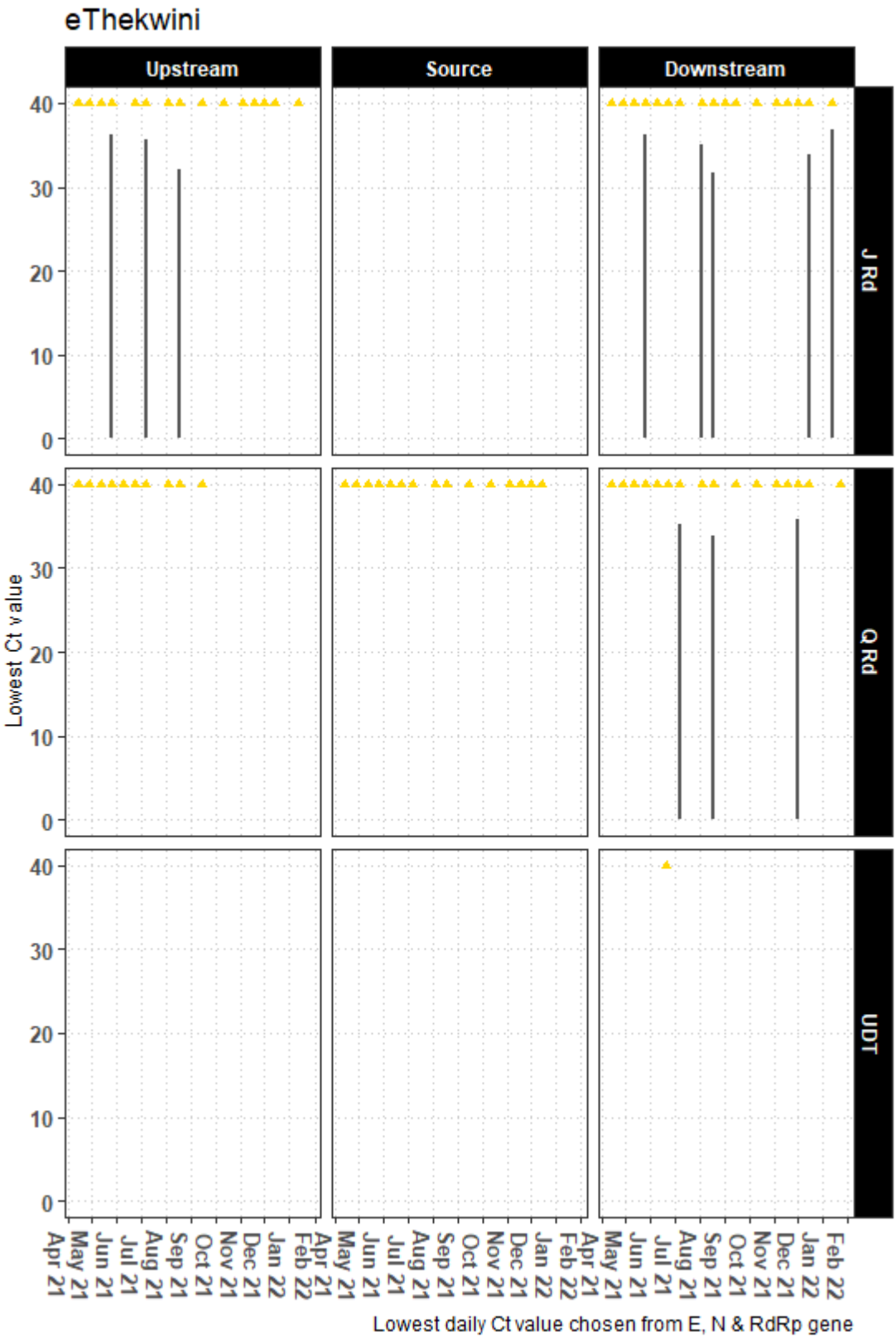


Figure 44: Presence of COVID-19 at eThekwini Sites

3.2.4 Mpumalanga

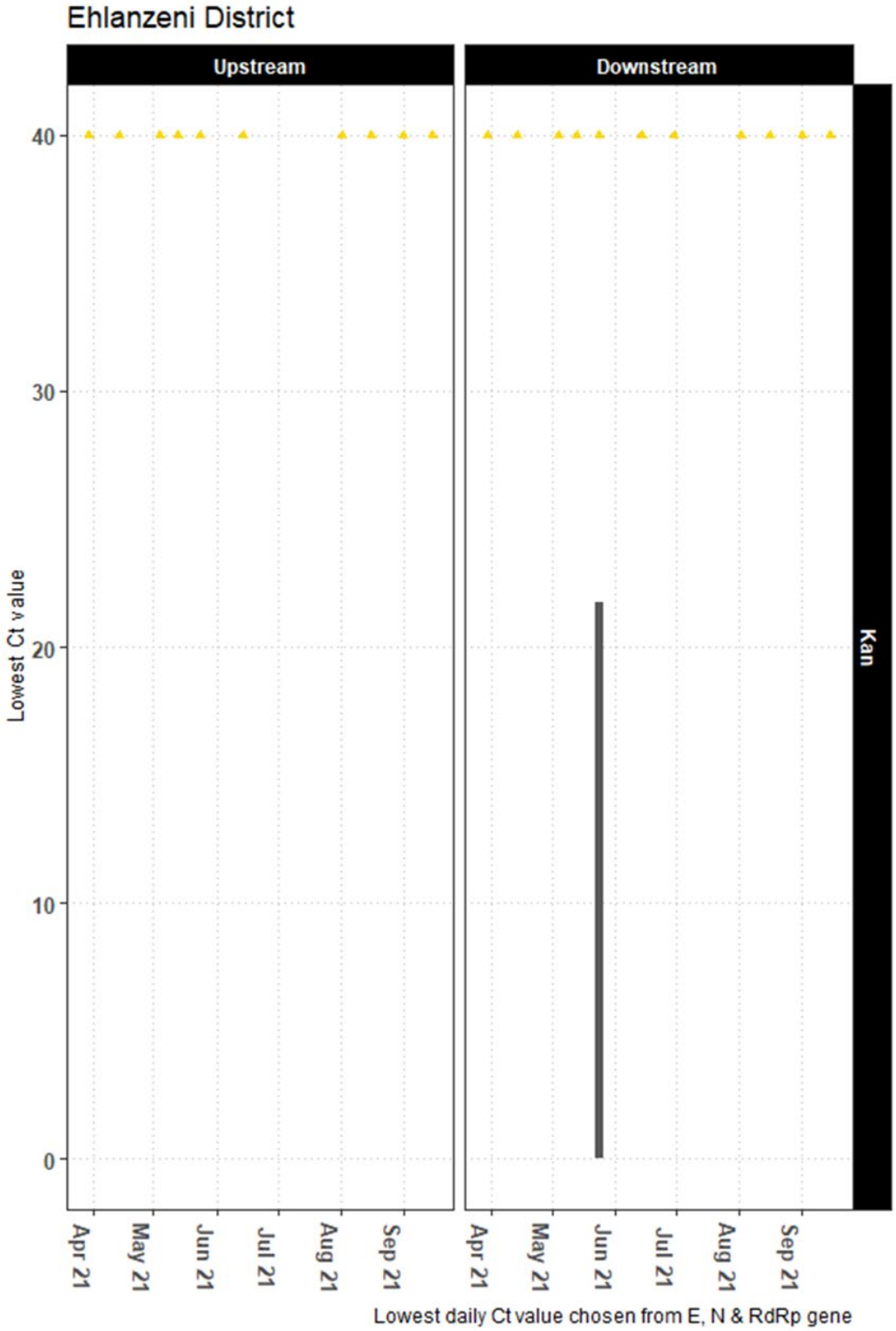


Figure 45: Crocodile River, up and downstream of Kanyamanzane settlement

The water quality of the Mpumalanga sites was shown to be in a good condition with limited pollution evident. The COVID-19 analysis of these two sites showed that the downstream site did test positive during the third wave, however the upstream site did not show any evidence of viral load being present.

3.2.5 Passive sampler results

As described in section 2.5.1, passive samplers were deployed at the Source of the Jukskei river in Braamfontein, Johannesburg, the Silvertown informal settlement in Alexandra, the Jukskei River downstream of the Silvertown informal settlement in Alexandra, and in the Plankenbrug River in Stellenbosch downstream of the informal settlements at Kayamandi.

Also investigated was the deployment of a passive samplers in disposed blackwater / faecal sludge from honeysucker tankers which were used to collect sewage from chemical toilets or septic tanks from areas within the City of Cape Town that were not connected to the sewage network. The tankers keep manifests of the areas served so it is possible to relate a passive sampler to a specific community. A comparison between the results obtained with the grab and 24h passive samples are presented in
Figure 46 for the Jukskei Source, and
Figure 48 for the Jukskei River downstream of Alexandra.
Figure 49 shows a comparison between a 24h and 48h passive sampler deployment in the Jukskei River downstream of Alexandra.
Figure 50 illustrates the SARS-CoV-2 results for grab samples versus 24h passive samples in the run-off into the Plankenbrug River in the Western Cape. Finally, SARS-CoV-2 results for passive samples taken from discharge from tankers emptying septic tanks into a wastewater treatment works using a small torpedo sampler are presented in **Figure 51**, with a comparison between the small torpedo sampler with the standard 10 mL elution compared to a larger sieve sampler with a 50 mL elution presented in **Figure 52**.

Blue bars on the charts indicate positive results for the assay internal control, with other colours representing positive results (Ct value <40) for the SARS-CoV-2 gene targets.

In general, the passive sampling was more effective at detecting SARS-CoV-2 RNA in the rivers than the grab sampling method, particularly at the start and end of the wave when the viral load was low. This was likely due to the longer exposure time in a dilute sample matrix. For the samples taken at the Source of the Jukskei (**Figure 46**), the passive samplers detected SARS-CoV-2 two weeks prior to the grab sampling in June 2021 at the start of the third wave in Gauteng, and in November 2021 at the start of the 4th wave. In the Jukskei River downstream of Alexandra (**Figure 48**), the passive sampler continued to detect SARS-CoV-2 in the river for two weeks longer than the grab samples at the end of the third wave, although did not show improved early detection in comparison with the grab samples at this site. When comparing passive samplers deployed in the Jukskei River downstream of Alexandra for 24h and 48h periods (**Figure 49**) from August to December 2021, between the waning 3rd wave and start of the 4th wave, the 48h sampler detected SARS-CoV-2 for two weeks longer than the 24h sampler as the 3rd wave ended, although both samplers detected the start of the 4th wave at the same time at the beginning of December. In the Plankenbrug River in the Western Cape (**Figure 50**), neither the passive nor the grab samples detected SARS-CoV-2 between August 2021 and December 2021, despite reduced rainfall through this period, and evidence of faecal pollution in the river.

SARS-CoV-2 was detected in the first passive samples taken from the tanker discharge in the Western Cape in July at the peak of the 3rd wave (**Figure 51**), but there was no further virus detection in samples taken until December 2021. The tankers collect from specific small communities, and it is possible that there were not high case numbers in these communities into the 4th wave. Comparing a small passive sampler with a 10 mL elution with a large sieve sampler with a 50 mL elution (**Figure 52**), showed better virus recovery from the larger sampler, with a larger surface area for adsorption of the virus in the high flow from the tanker into the plant inlet works. Sample processing from the larger sampler was however very unpleasant and is not viewed as a sustainable long term sampling method going forward. The remaining samples were taken with the small torpedo samplers.

Figure 46: SARS-CoV-2 results for grab samples versus passive samples in the Jukskei Source, indicating the daily provincial case number for Gauteng, with the sample period indicated in red

Figure 47: SARS-CoV-2 results for grab samples versus passive samples from the Silvertown Informal Settlement, indicating the daily provincial case number for Gauteng, with the sample period indicated in red

Figure 48: SARS-CoV-2 results for grab samples versus 24h passive samples in the Jukskei River downstream of Alexandra, indicating the daily provincial case number for Gauteng, with the sample period indicated in red

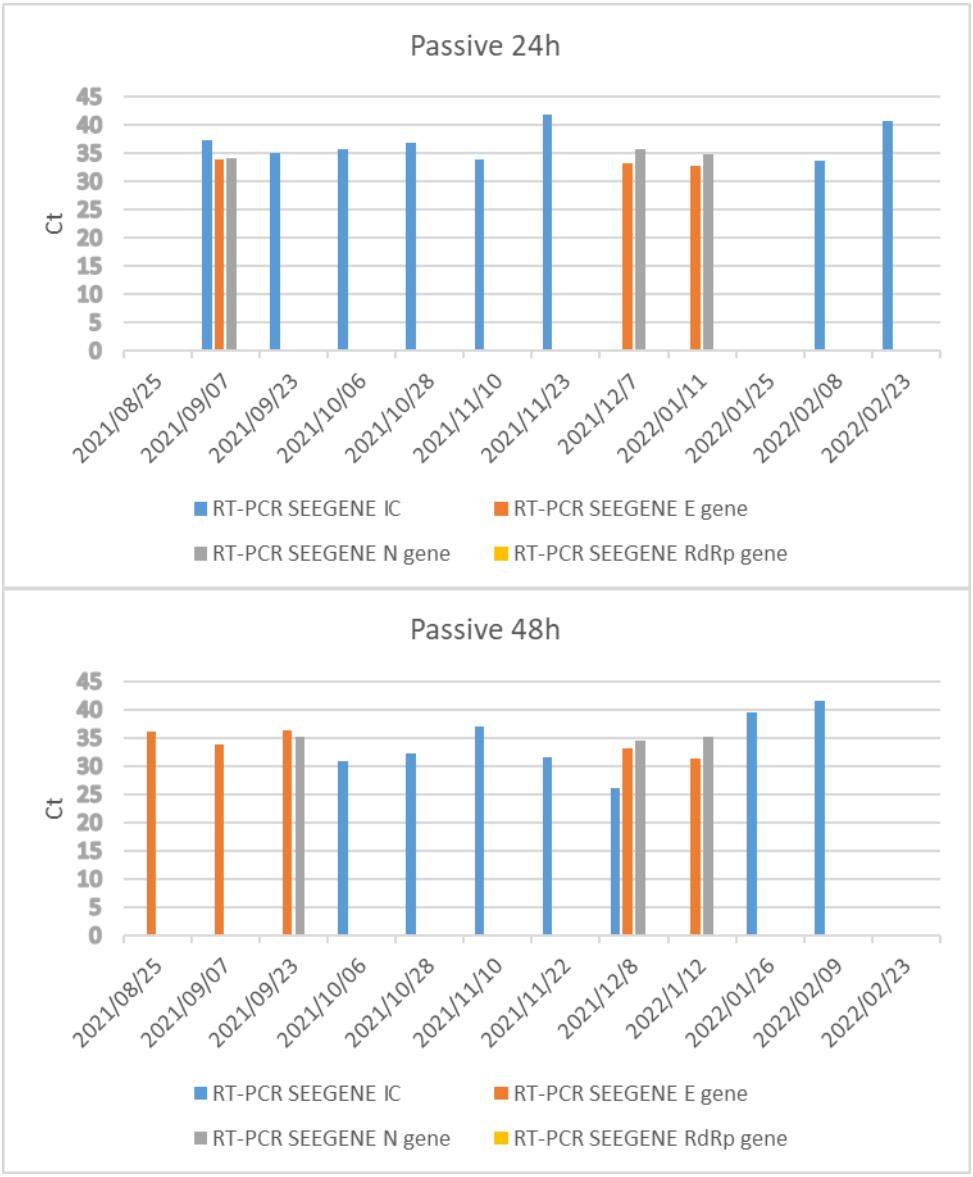


Figure 49: SARS-CoV-2 results for 24h vs 48h passive samples in the Jukskei River downstream of Alexandra

Figure 50: SARS-CoV-2 results for grab samples versus 24h passive samples in the run-off into the Plankenbrug River, indicating the daily provincial case number for the Western Cape, with the sample period indicated in red

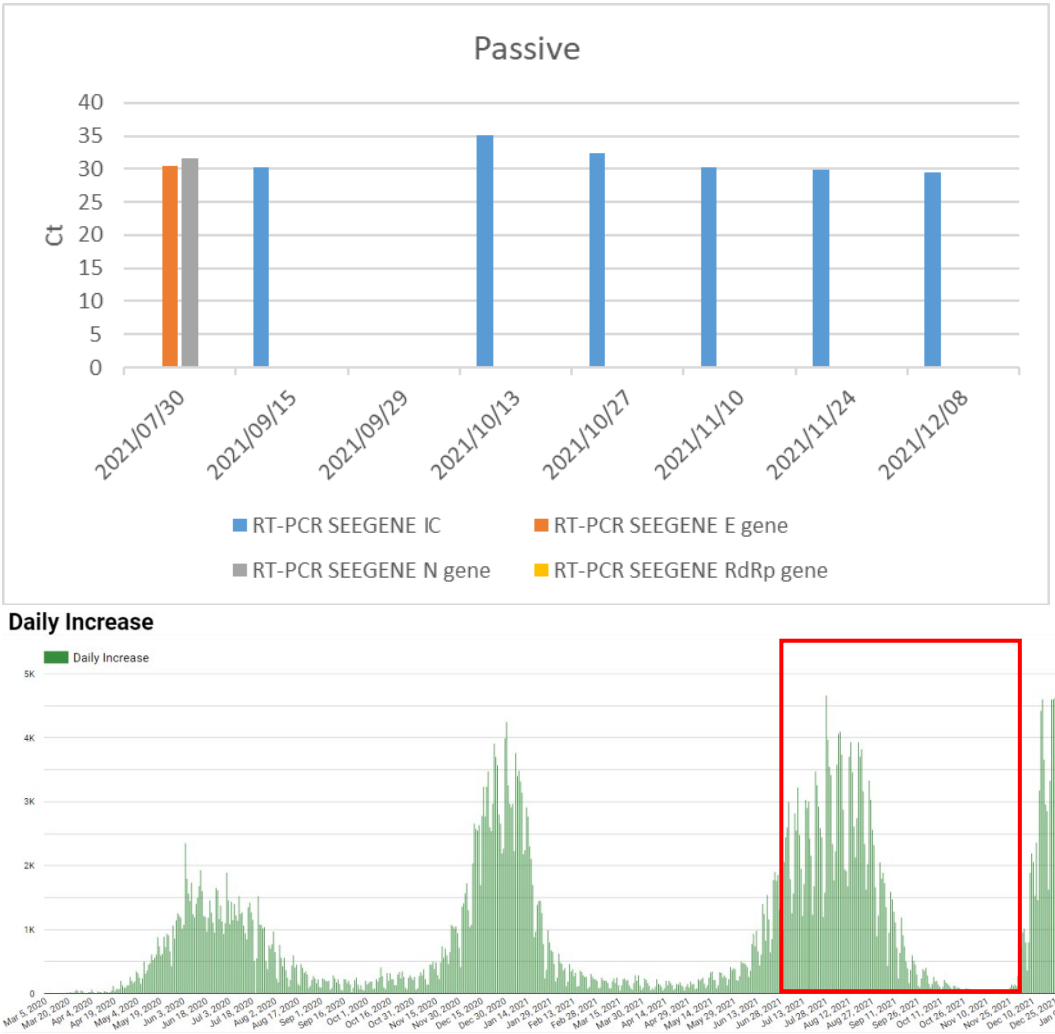


Figure 51: SARS-CoV-2 results for passive samples taken from tanker discharge from septic tanks

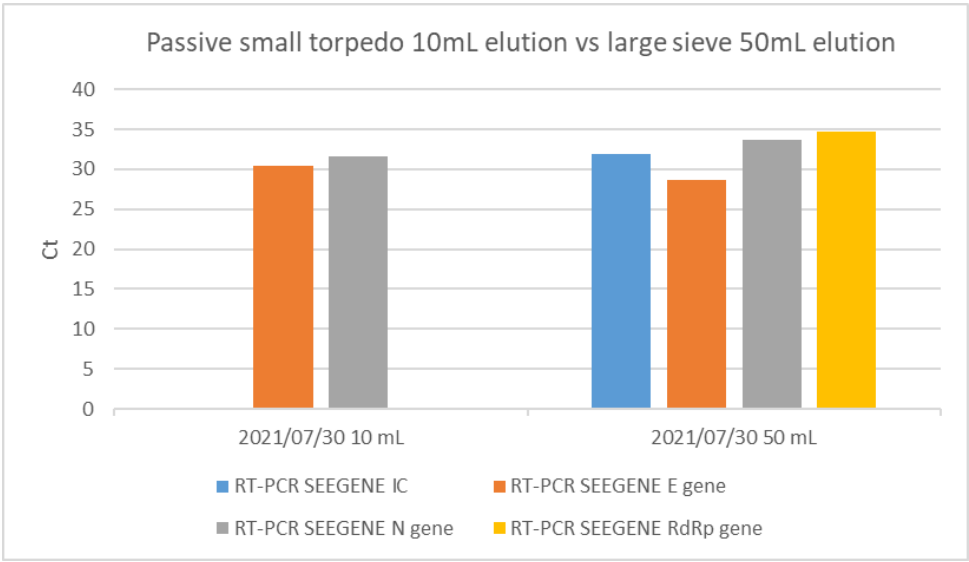
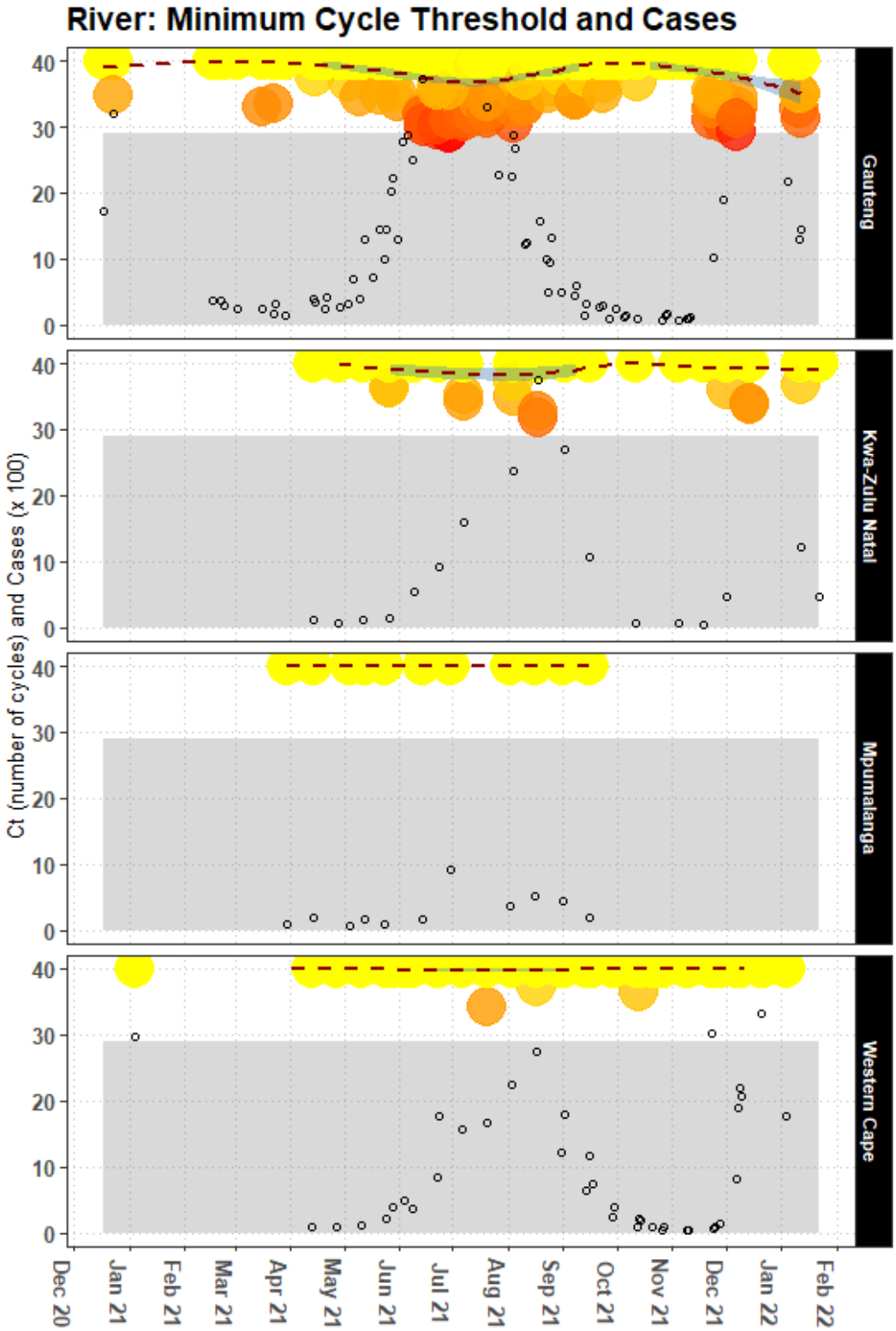


Figure 52: A comparison between a 10 mL elution from small passive sampler compared to large sieve sampler with 50 mL elution

3.2.6 Summary of COVID-19 results in non-sewered communities

The data indicated that COVID-19 could be identified in non-sewered community run-off, surface water and in the rivers which lie downstream of these communities. The incidence of COVID-19 in these communities was reflected in the Ct values obtained in the rivers and surface run-off samples. In Gauteng province where the informal settlements are dense and the rivers are highly polluted by faecal matter from these communities, the trends were even more evident.



Cts <29 (grey shaded area) are strong positive reactions indicative of abundant target nucleic acid in the sample
Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid
Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.
Red dashed line is the trend
Shaded blue area give 95% confidence of the trend line
Circle point is the number of cases (x100)

Figure 53: Daily cases and Ct values in rivers downstream of unsewered communities

In **Figure** 53, the data shows that as the cases rose, the Ct values drop accordingly, indicative of a higher viral load. Note that in Gauteng, where testing has been more regular since the start of 2021, the second wave was also captured (in January 2021). Interestingly, another peak in COVID-19 detection in the rivers was noted in March although these were not reflecting in the clinical case load data This may be due to a level of infection within the community which is unreported and untested due to financial constraints.

A similar figure for each province (**Figure 55**) provides a clearer indication that the infection within the community can be monitored utilizing rivers and run off from informal settlements.

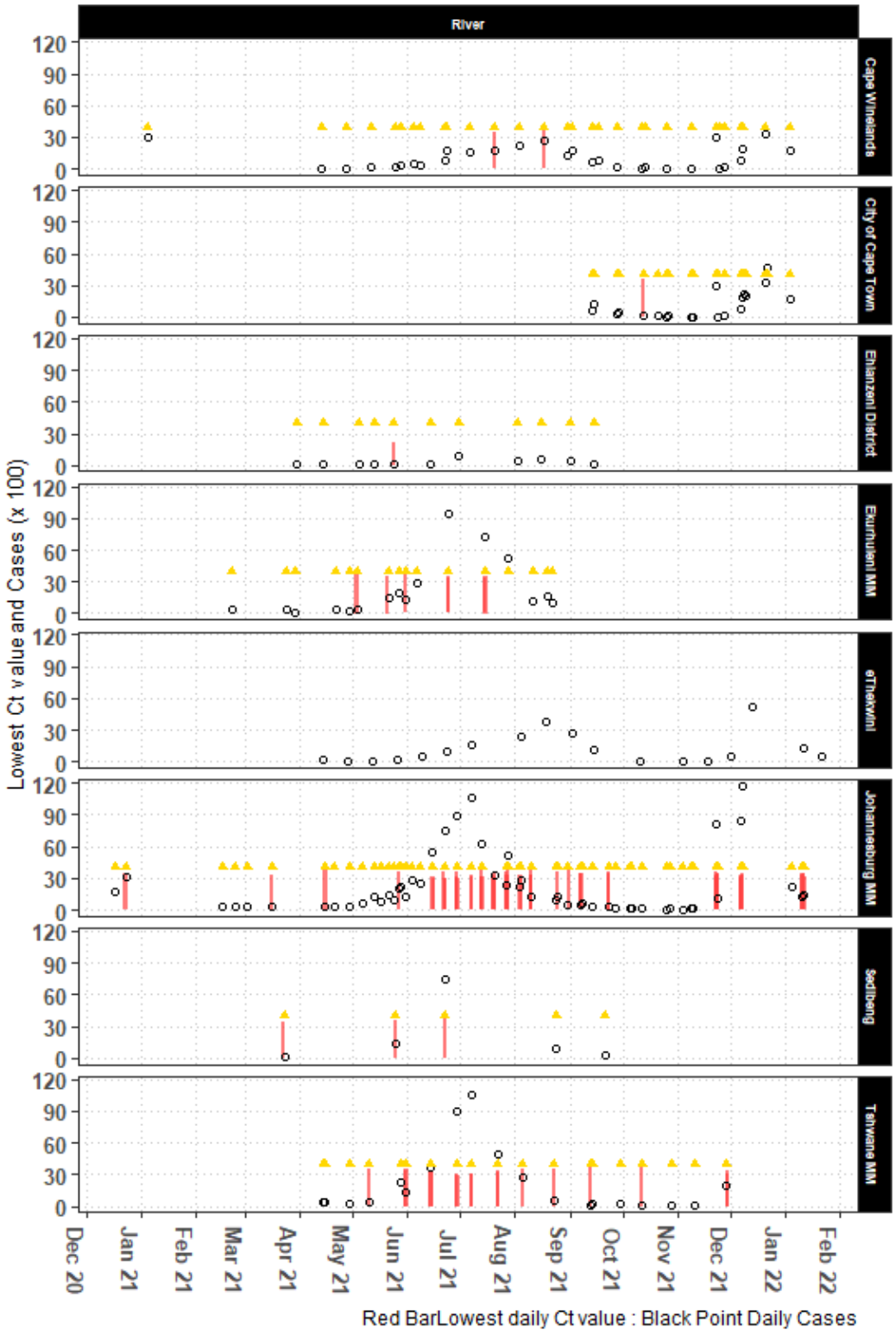


Figure 54: Ct values and case numbers per municipal area

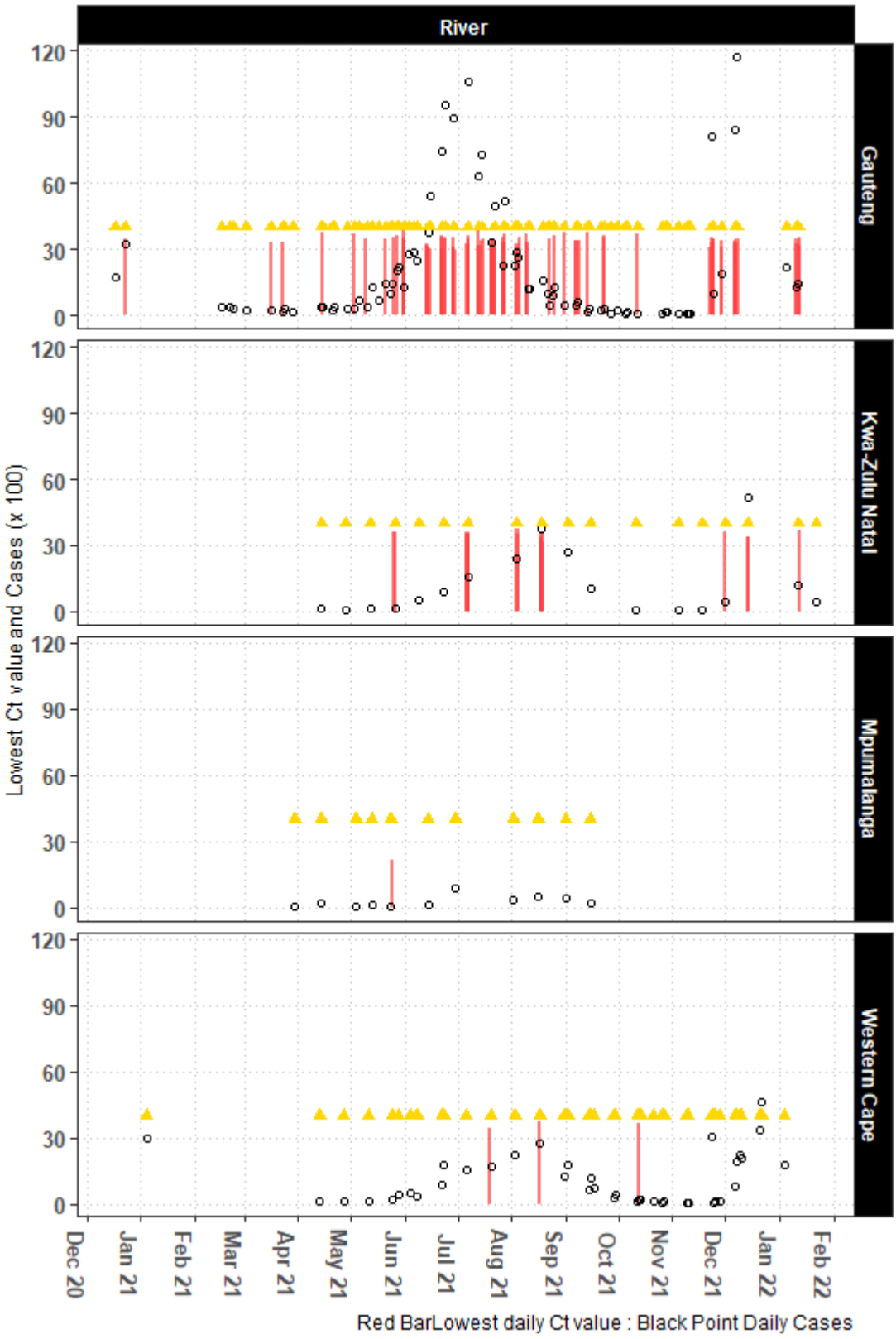


Figure 55: Provincial daily case numbers and prevalence of COVID in unsewered communities

3.3 RESULTS OF SEQUENCING

Of the 29 positive samples submitted for sequencing, sequencing libraries were successfully generated for 28 samples. An average coverage of 92.9% was achieved across all successful libraries when compared with the reference genome (SARS-CoV-2 isolate Wuhan-Hu-1, complete genome, GenBank reference: MN908947.3). The BLAST (basic local alignment search tool) top hits of 22 of the 28 scaffolds generated matched with other SARS-CoV-2 sequences in the database by >99%, confirming the positive assay results (**Table 6**).

Pango Lineages were successfully assigned to 12 samples across 5 sample sites, and VoC-related Scorpio lineage calls were also assigned for these (**Table 6**).

Table 6: Sequencing results for SARS-CoV-2 positive samples showing % coverage when compared to the reference genome, BLAST hit results and Pango Lineages

No.	SAMPLE SITE	Province	SAMPLE TYPE	DATE SAMPLED START	DATE SAMPLED END	% COVER-AGE	BLAST HIT	BLAST % MATCH	PANGO LINEAGE	SCORPIO CALL
1	Jukskei Source	Gauteng	Passive (24h)	2021/06/21	2021/06/22	99.97	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/ZAF/NHLS-UCT-GS-D158/2021, complete genome	100.00	**B.1.617.2**	**Delta (B.1.617.2-like)**
2	Jukskei Source	Gauteng	Grab	2021/06/22	2021/06/22	100.00	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/ZAF/NHLS-UCT-GS-D114/2021	100.00	**B.1.617.2**	**Delta (B.1.617.2-like)**
3	Jukskei Source	Gauteng	Passive (24h)	2021/07/06	2021/07/07	100.00	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/WA-S54/2020, complete genome	100.00	**B.1.617.2**	**Delta (B.1.617.2-like)**
4	Jukskei Source	Gauteng	Grab	2021/07/07	2021/07/07	98.81	Severe acute respiratory syndrome coronavirus 2 isolate hCoV-19/Switzerland/AG-ETHZ-35351569/2021 genome assembly, chromosome: 1	100.00	**B.1.617.2**	**Delta (B.1.617.2-like)**
5	Jukskei Source	Gauteng	Passive (24h)	2021/08/03	2021/08/04	78.60	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/WA-S859/2020, complete genome	100.00	Unassigned	
6	Jukskei Source	Gauteng	Passive (24h)	2021/11/22	2021/11/23	99.93	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/UT-UPHL-220324929749/2022, complete genome	100.00	**BA.1**	**Probable Omicron (BA.1-like)**
7	Jukskei Source	Gauteng	Grab	2021/12/07	2021/12/07	99.78	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/BGD/BCSIR_NILMRC_115/2020, complete genome	100.00	Unassigned	
8	Jukskei Source	Gauteng	Passive (24h)	2021/12/06	2021/12/07	99.97	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/SD-SDAIHG-1461/2022, complete genome	100.00	**BA.1.1**	**Probable Omicron (BA.1-like)**
9	Alex Silvertown	Gauteng	Grab	2021/07/13	2021/07/13	86.92	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/TX-DSHS-16155/2022	99.51	Unassigned	
11	Jukskei Downstream Alexandra	Gauteng	Grab	2021/03/16	2021/03/16	83.70	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/CA-CZB-1776/2020	100.00	Unassigned	
12	Jukskei Downstream Alexandra	Gauteng	Grab	2021/06/15	2021/06/15	100.00	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/ZAF/NHLS-UCT-GS-D114/2021	100.00	**B.1.617.2**	**Delta (B.1.617.2-like)**
13	Jukskei Downstream Alexandra	Gauteng	Passive (24h)	2021/06/28	2021/06/29	98.98	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/Human/USA/UT-01572/2020, complete genome	100.00	Unassigned	

14	Jukskei Downstream Alexandra	Gauteng	Grab	2021/07/13	2021/07/13	68.44			Unassigned	
15	Jukskei Downstream Alexandra	Gauteng	Grab	2021/07/27	2021/07/27	85.62			Unassigned	
16	Jukskei Downstream Alexandra	Gauteng	Grab	2021/08/10	2021/08/10	80.15	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/CA-CDPH-3000042683/2021, complete genome	100.00	Unassigned	
17	Jukskei Downstream Alexandra	Gauteng	Passive (48h)	2021/09/06	2021/09/08	92.08	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/MA_MGH_00705/2020, complete genome	100.00	Unassigned	
18	Jukskei Downstream Alexandra	Gauteng	Grab	2021/11/24	2021/11/24	99.84	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/CA-CDC-LC0721546/2022, complete genome	100.00	**BA.1**	**Probable Omicron (BA.1-like)**
19	Jukskei Downstream Alexandra	Gauteng	Passive (48h)	2022/01/10	2022/01/12	77.81		100.00	Unassigned	
20	Kaalspruit Downstream Tembisa	Gauteng	Grab	2021/06/29	2021/06/29	99.78	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/ZAF/NHLS-UCT-GS-C368/2021 ORF1ab	99.75	**B.1.617.2**	**Delta (B.1.617.2-like)**
21	Kaalspruit Downstream Tembisa	Gauteng	Grab	2021/07/07	2021/07/07	95.95	Severe acute respiratory syndrome coronavirus 2 isolate hCoV-19/Switzerland/BL-ETHZ-35863499/2022 genome assembly, chromosome: 1	100.00	Unassigned	
22	Kaalspruit Downstream Tembisa	Gauteng	Grab	2021/11/29	2021/11/29	94.08			Unassigned	
23	Rietspruit at Thatchfield	Gauteng	Grab	2021/05/10	2021/05/10	78.56			Unassigned	
24	Rietspruit at Thatchfield	Gauteng	Grab	2021/07/22	2021/07/22	96.95	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/LA-BIE-LSUH000922/2021	100.00	Unassigned	
25	Klip River	Gauteng	Grab	2021/07/15	2021/07/15	6.09	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/MA_MGH_00705/2020, complete genome	100.00	Unassigned	
26	Tanker waste	Western Cape	Passive (10ml)	2021/07/30	2021/07/31	99.86	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/ZAF/NHLS-UCT-GS-D146/2021, complete genome	99.98	**AY.32**	**Delta (B.1.617.2-like)**
27	Tanker waste	Western Cape	Passive (50ml)	2021/07/30	2021/07/31	99.90	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/ZAF/NHLS-UCT-GS-D146/2021, complete genome	99.91	**B.1.617.2**	**Delta (B.1.617.2-like)**
29	Umhlanganae River upstream Johanna Road informal settlement	KZN	Grab	2021/12/14	2021/12/14	99.78	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/CA-CZB-1775/2020, complete genome	100.00	**B.1.617.3**	**Delta (B.1.617.2-like)**

Both grab samples and passive samples yielded successful libraries. Of the samples sequenced, those sampled at the source of the Jukskei River provided the most Pango-lineage assignments and Scorpio calls when compared to the other surface water samples. Because the samples were taken shortly after the river daylights, these samples had minimal exposure to the environment in terms of sunlight and heat and the SARS-CoV-2 RNA was possibly less fragmented than RNA extracted from other surface waters. Both passive samples taken from the tanker waste disposal to the wastewater treatment works in the Western Cape also yielded good coverage and Pango-lineages were assigned to both. It is possible that these samples also yielded better quality RNA, but this will need to be confirmed with a larger number of samples.

The World Health Organization designated the new variant as a variant of concern and named it Omicron (B.1.1.529) on the 26th of November 2021 The first Omicron identified samples were collected on 8 November 2021 from a 34 year old man and a 23 year old man in Johannesburg (Al Hasan, 2022) As illustrated in **Figure 56**, the Delta variant was dominant in South Africa from May 2021 until October 2021, with the Omicron variant quickly becoming dominant in November 2021 resulting in the 4th wave of infections. The shift in dominance from the Delta variant to the Omicron variant was apparent in samples taken in November 2021 from the Jukskei Source and the Jukskei downstream of Alexandra.

It was therefore possible to generate sequence data from RNA extracted from environmental samples, with sufficient genome coverage to match BLAST hits to other SARS-CoV-2 sequence submissions by >99%. Variants could be assigned to 12 of the 28 successful libraries (43%). Tracking of variants in environmental samples is therefore a viable method for determining the incidence of these variance in the non-sewered communities impacting on these water sources.

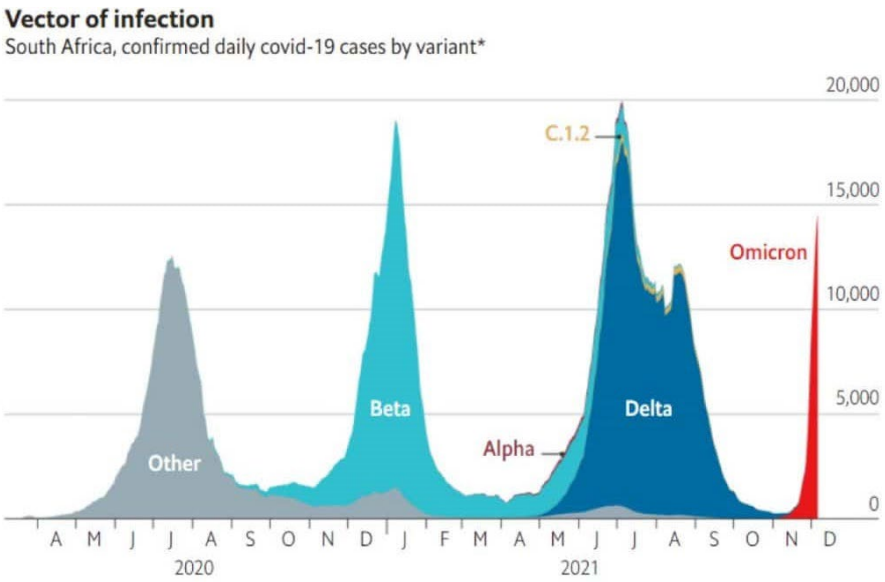


Figure 56: Confirmed daily COVID cases in South Africa by variant, based on sequences submitted to GISAID (Source: Wenseleers (2021) South Africa National Institute for Communicable Diseases; GSAID)

3.4 RESULTS OF SCREENING FOR OTHER ENTERIC VIRUSES OF CONCERN: NOROVIRUS AND HEPATITIS E

3.4.1 Norovirus in surface samples

A subset of 87 samples were screened for norovirus Genogroup I (GI) and Genogroup II (GII). Norovirus GI was detected in 48% (42/87) and norovirus GII in 53% (46/87) of samples. Both genogroups were present in 34% (30/87). The median Ct value for the GI target was 35.45, (range 22.9-39.8) and the median for the GII target was 33.8 (range 26.4-39.6).

Results for assay of norovirus GI and GII targets for a subset of samples from rivers and surface run-off from the different provinces are presented in the figures below. The results for the GI and GII assay internal positive controls are also indicated in blue and grey in each figure. Results from the Gauteng rivers, the Jukskei River downstream of Alexandra, the Kaalspruit downstream of Tembisa, and the Rietspruit at Thatchfield are presented in **Figure 57**.

Results for the Crocodile River in Mpumalanga downstream of Kanyamazane are presented in **Figure 58**, results for the KZN rivers, the Palmiet and Umhlangane rivers and surface run-off from the Quarry Road and Johanna Road informal settlements are presented in **Figure 59** and the results for the Western Cape for run-off into the Plankenbrug River and the Franshoek River downstream of the Langrug informal settlement are presented in **Figure 60**.

The assay successfully detected norovirus RNA in the river and surface samples, with both GI and GII targets being detected in most samples tested. The same method applied for the concentration of samples and extraction of nucleic acids for assay of SARS-CoV-2 in environmental samples can therefore be applied to Norovirus screening as well.

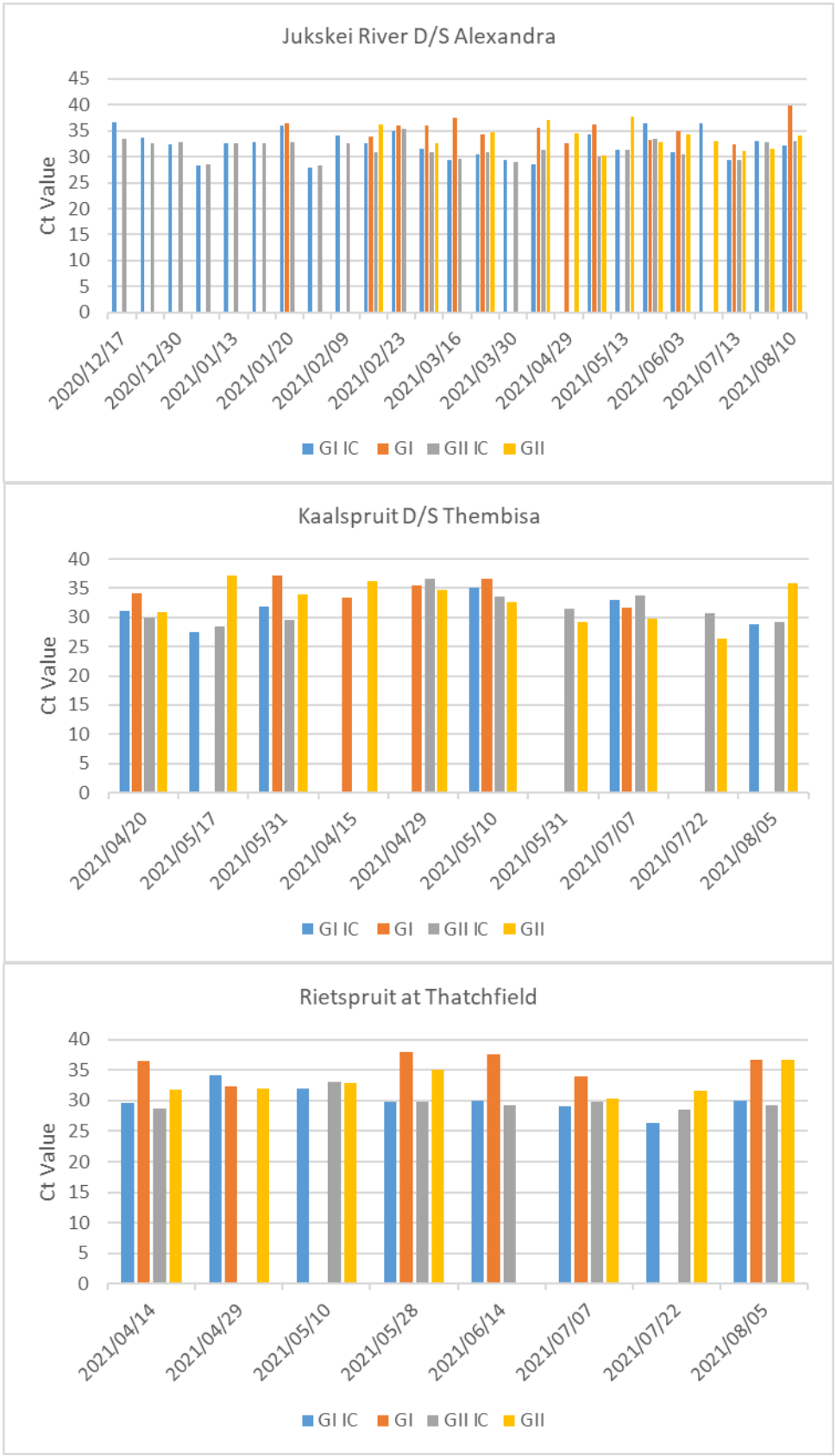


Figure 57: Norovirus RNA detection in Gauteng rivers

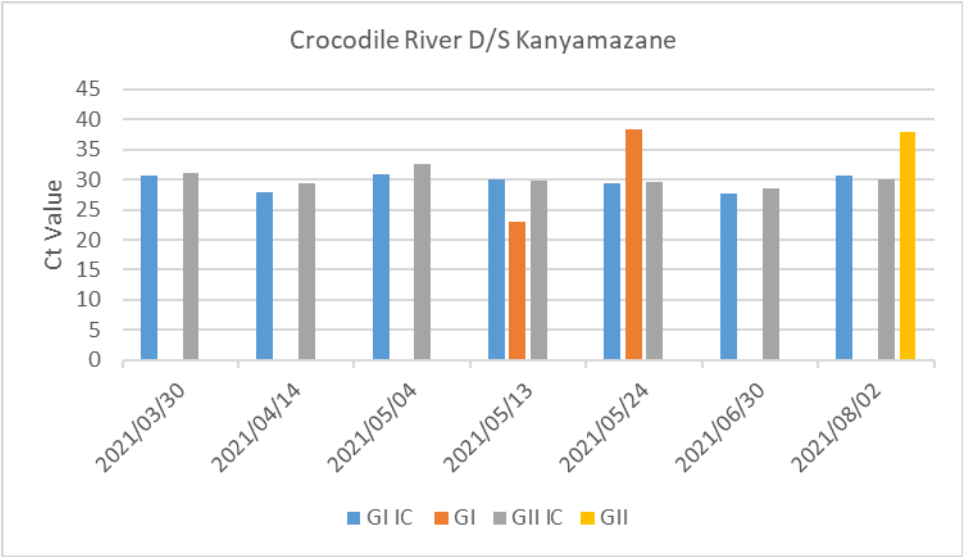


Figure 58: Norovirus RNA detection in Mpumalanga rivers

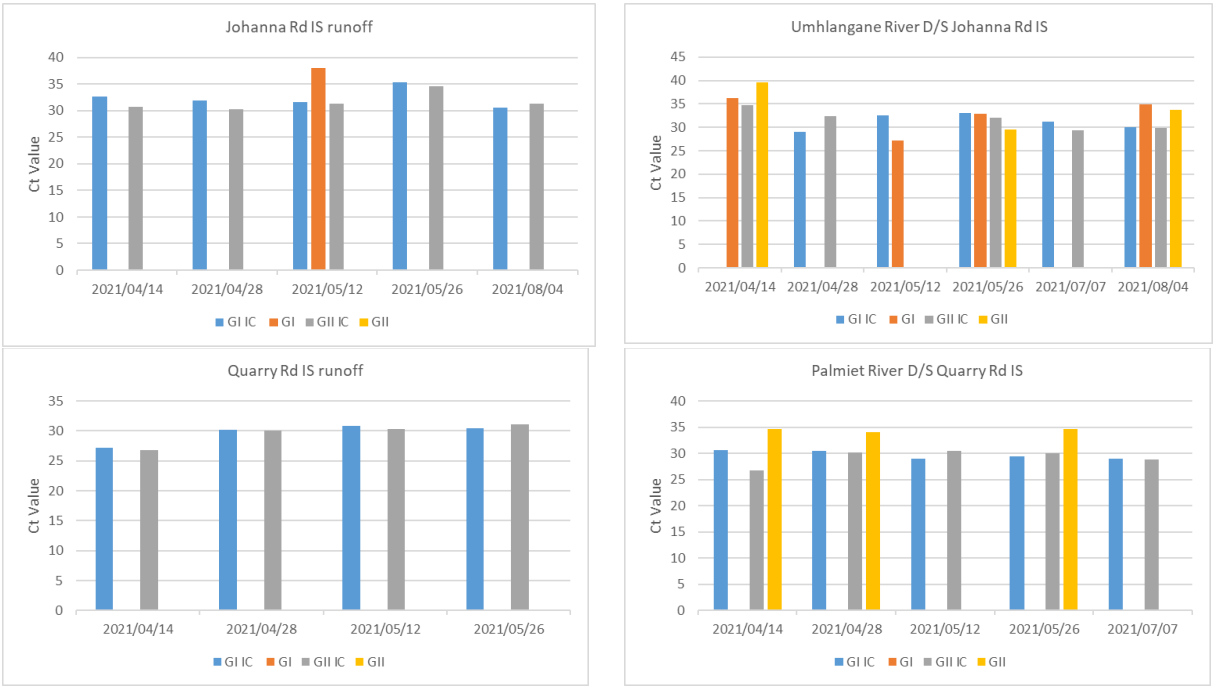


Figure 59: Norovirus RNA detection in KZN rivers and surface run-off

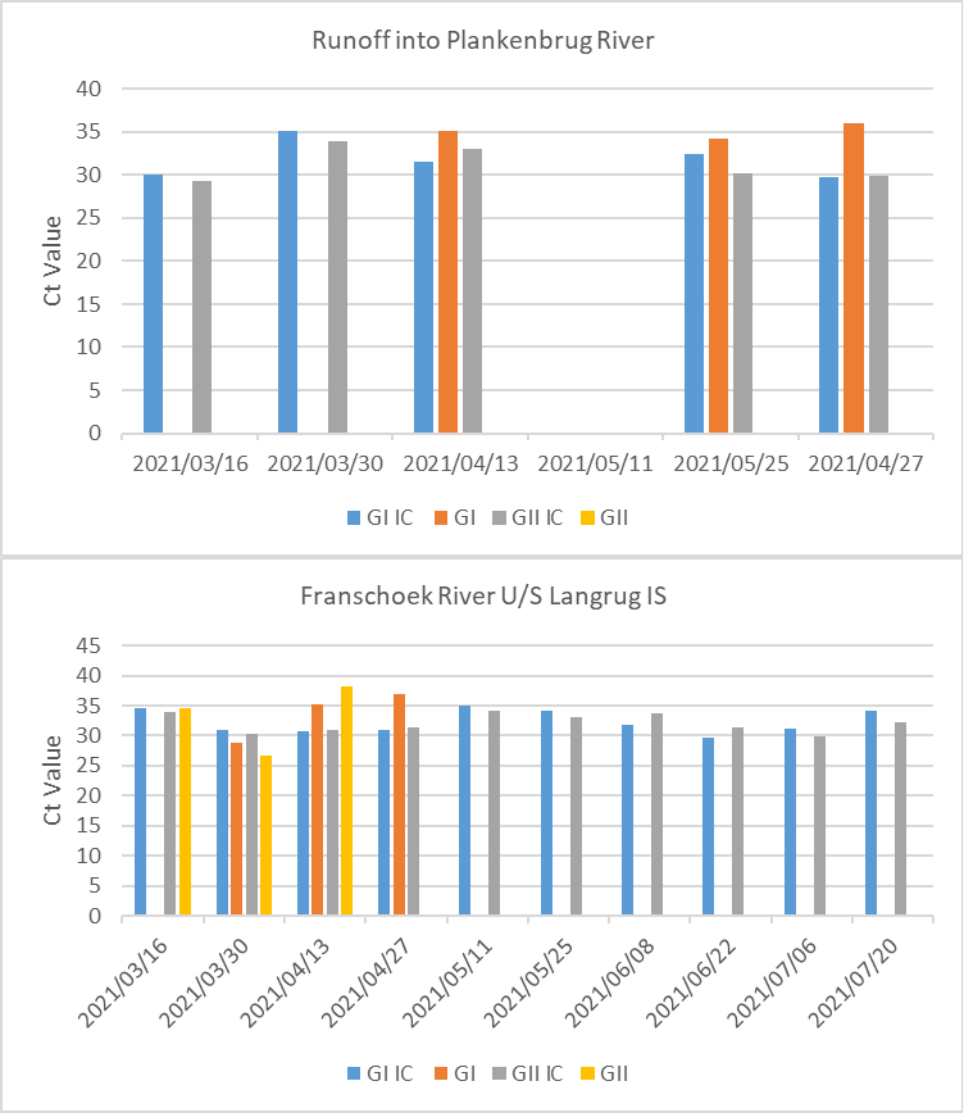


Figure 60: Norovirus RNA detection in Western Cape rivers

3.4.2 Hepatitis E virus screening

A subset of 44 samples were screened for Hepatitis E virus from the same rivers and surface water sample sites as were screened for norovirus.

Twenty three percent (10/44) of these samples tested positive for Hepatitis E virus and the virus was detected in all four provinces. The median Ct value was 33.75. Results for each province are presented in Figure 61 to Figure 64. Results for the assay internal positive controls are included and are indicated in blue, with positive Hep E results indicated in orange.

While not as prevalent in the samples assayed as the norovirus, the presence of Hepatitis E in 23% of the limited number of samples screened across the four provinces indicates that incidence of the virus is widespread. As with the norovirus assay, the same methodology for processing of environmental samples for SARS-CoV-2 screening can be applied to Hepatitis E environmental surveillance.

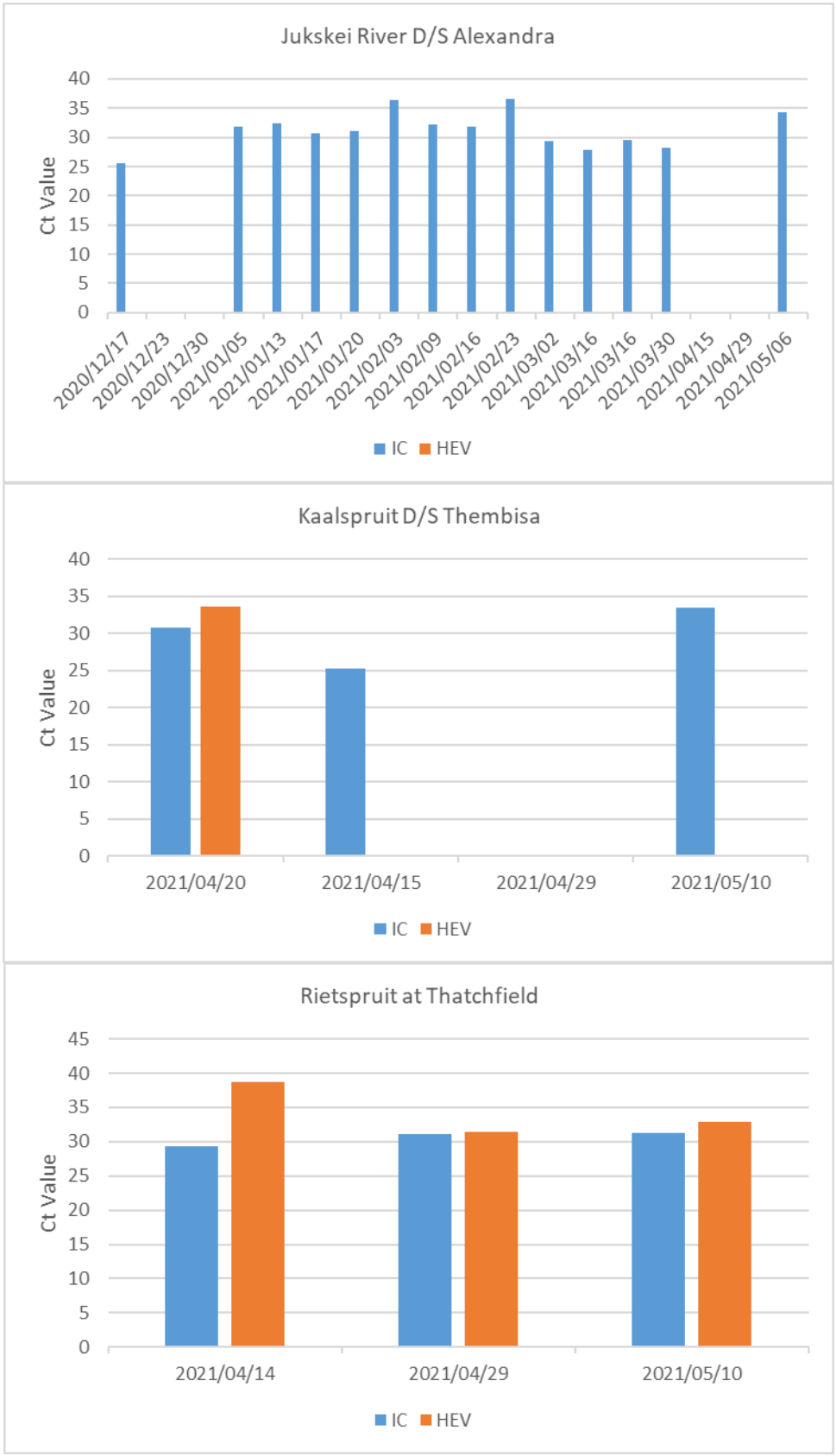


Figure 61: Hepatitis E RNA detection in Gauteng rivers

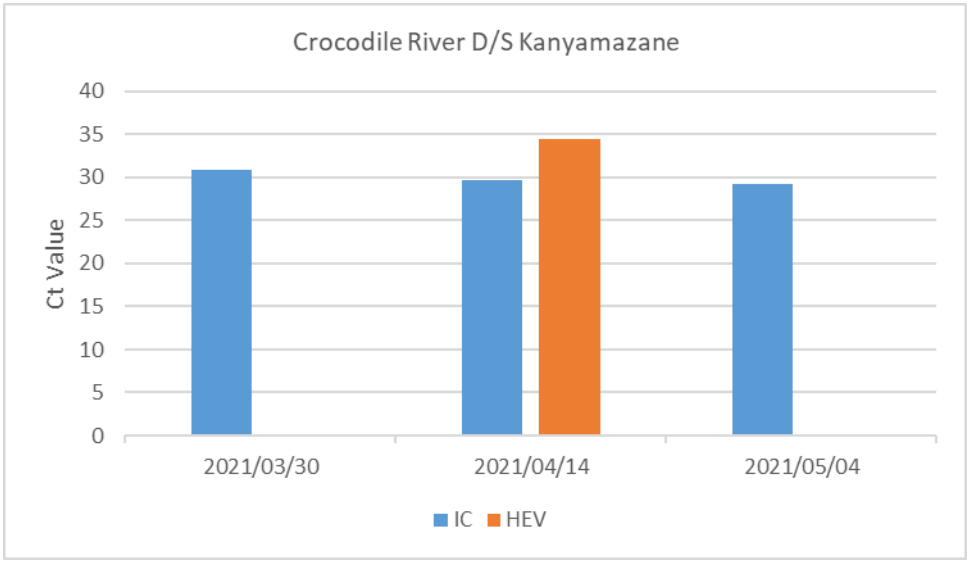


Figure 62: Hepatitis E RNA detection in Mpumalanga rivers

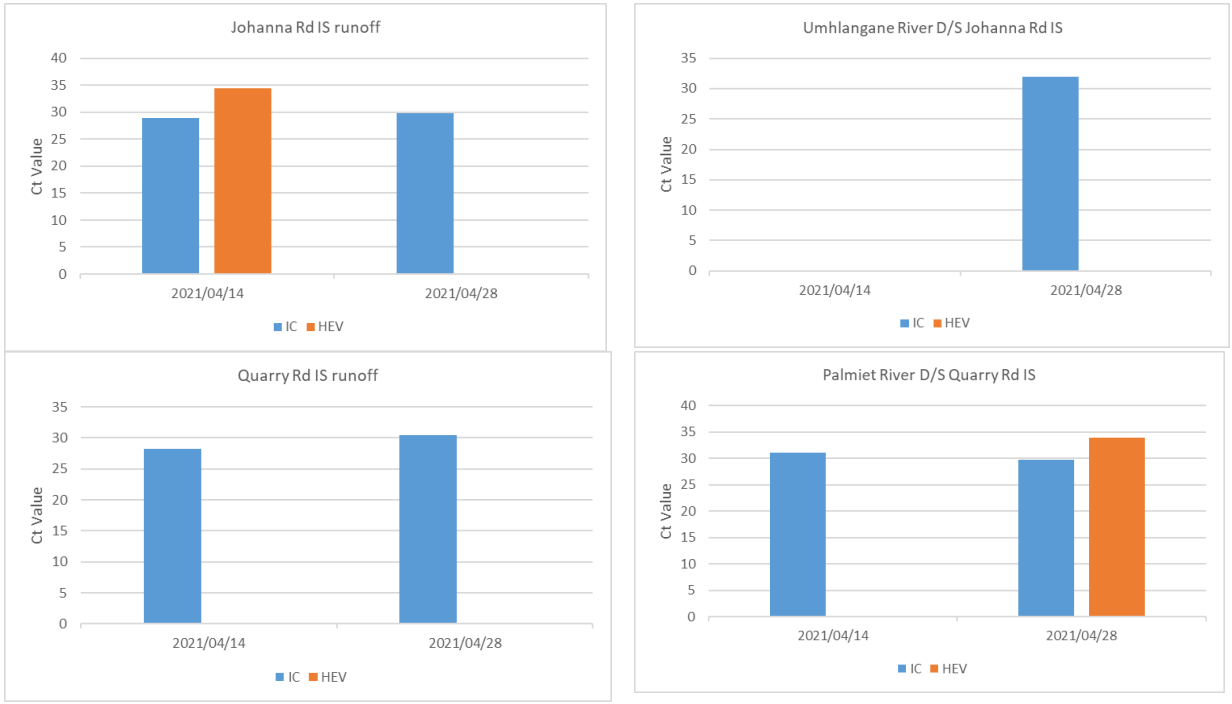


Figure 63: Hepatitis E RNA detection in KZN rivers and surface run-off

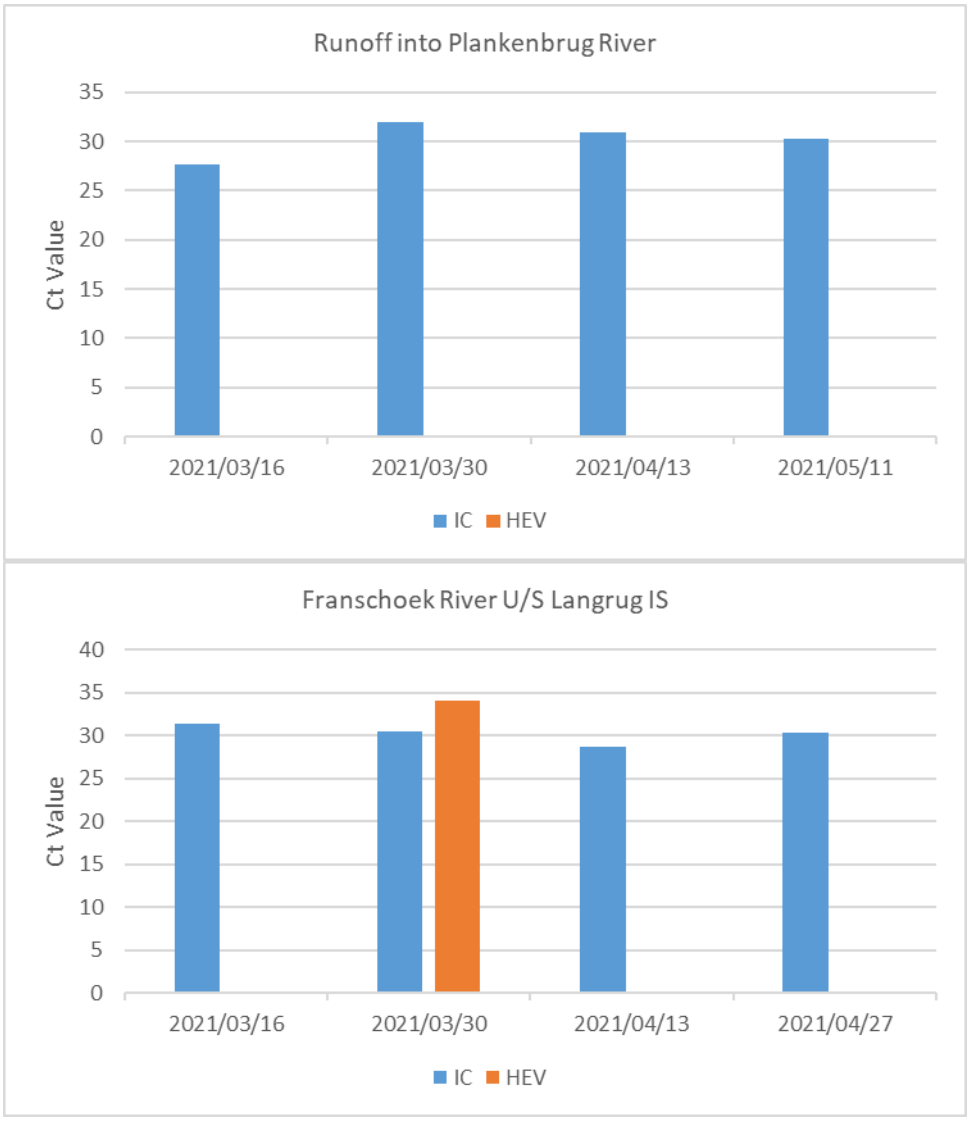


Figure 64: Hepatitis E RNA detection in Western Cape rivers

3.5 RESULTS OF CRASSPHAGE SCREENING AS FAECAL CONTAMINATION INDICATOR

A previously described crAssphage CPQ_064 specific PCR (Stachler et al. 2017) was used to quantify the crAssphage, a DNA-virus that is ubiquitously present in human intestinal tracts in high concentrations, in a subset of the river and surface run-off samples samples. Quantification was performed based on a standard curve generated with a dilution series of a synthetic quantified gBlock (obtained from IDT, Leuven, Belgium) containing the CPQ_064 gene fragment. The standard curve is presented in **Figure 65**.

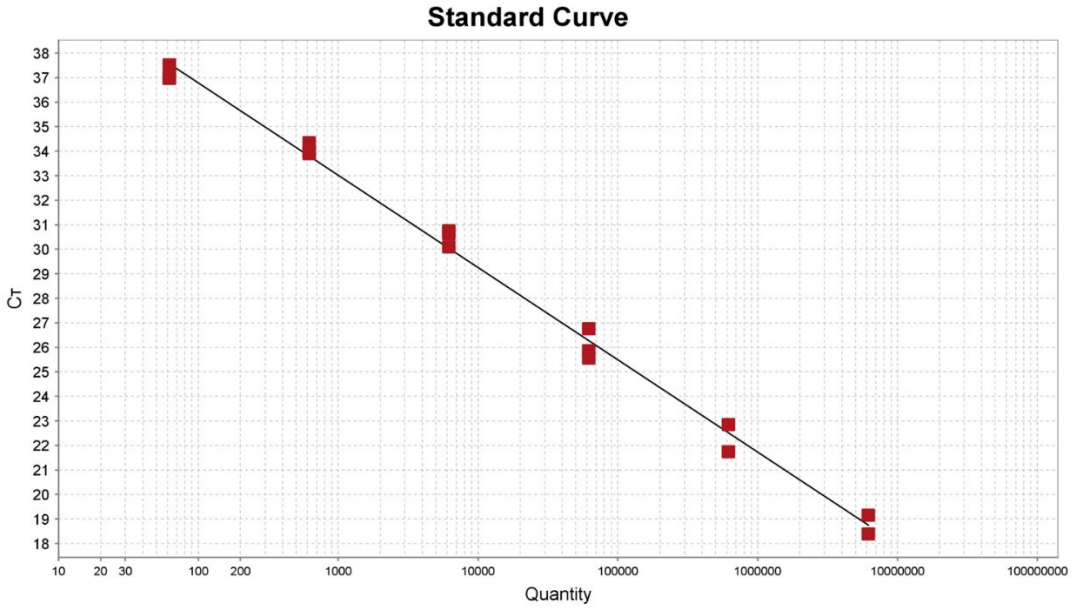
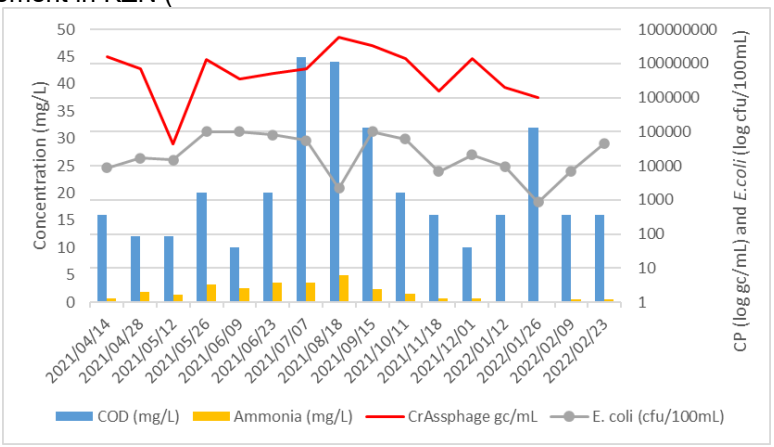


Figure 65: Standard curve generated with a dilution series of a synthetic quantified gBlock containing the CPQ_064 gene fragment in serial ten-fold dilution range using the QuantiFast Pathogen PCR+IC kit. Slope -3.762, R^2= 0.994, Efficiency= 84.426% for the CPQ_064 gene target

CrAssphage assays were performed on grab samples from the Jukskei River downstream of Alexandra (**Figure 66**), on the grab (**Figure 67**) and passive (**Figure 68**) samples of the run-off from the Silvertown informal settlement in Alexandra and grab samples from the Kaalspruit downstream of Tembisa in Gauteng (**Figure 69**). These results were compared with the water quality parameters where available as well as with Ct values from the SARS-CoV-2 assays. Internal control results of the SARS-CoV-2 assays are not shown. Similarly, crAssphage assays were also conducted on samples from the Crocodile River downstream of Kanyamazane in Mpumalanga (**Figure 70**), the Palmiet River downstream of the Quarry Road informal settlement in KZN (



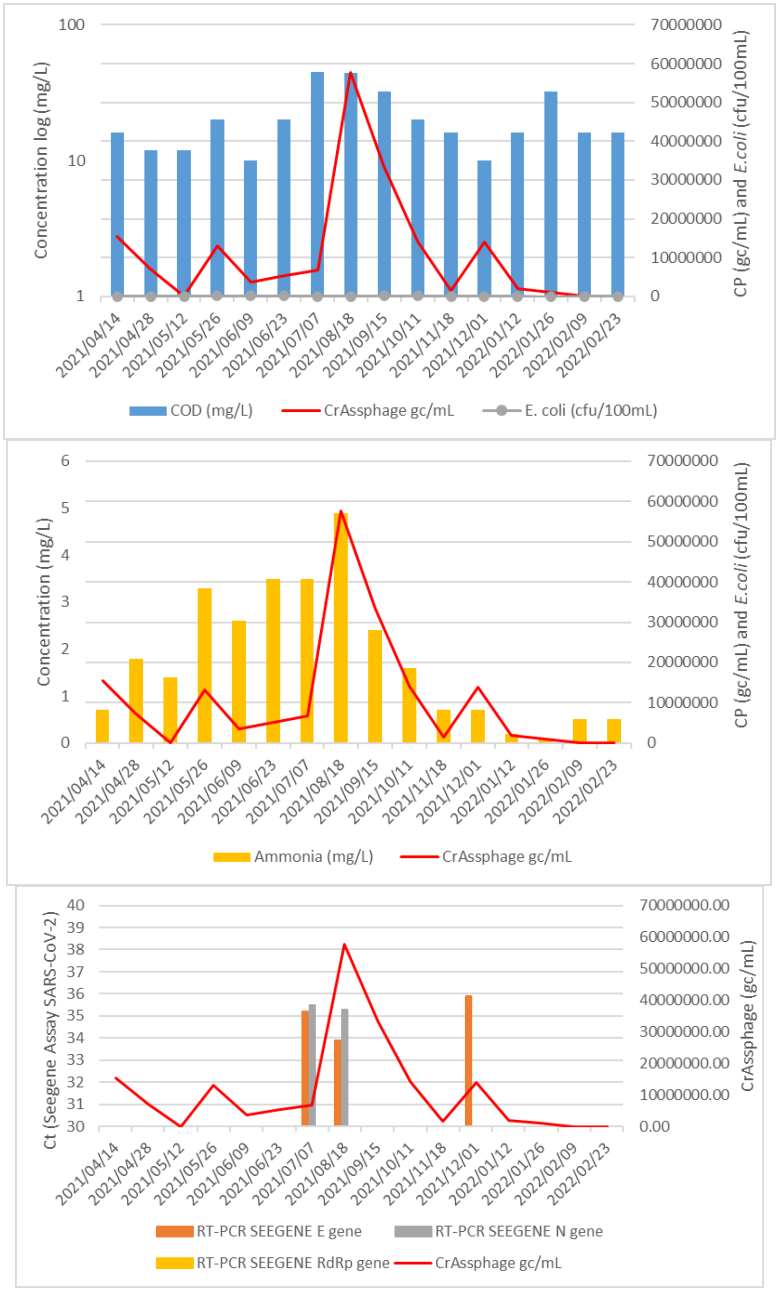


Figure 71) and the Plankenbrug River downstream of Kayamandi in the Western Cape (**Figure 72**).

The crAssphage copy number trend did not follow the exact trend of any of the water quality parameters. It should be noted that the maximum detection limit of the *E. coli* testing method used was 100 000 CFU/100 mL, and trends above this value could not be observed. The crAssphage copy number trend most closely followed that of the ammonia concentration, decreasing from June to September 2021, then increasing again from October 2021. This trend was more clearly observed in the less polluted rivers where the informal settlement had an obvious impact, such as the Palmiet River downstream of the Quarry Road Informal Settlement (**Figure 71**).

When comparing the crAssphage copy numbers with the SARS-CoV-2 assay, no clear trend was observed, but as they are not dependant parameters this is was expected. Ideally, crAssphage gene copies should be compared with SARS-CoV-2 gene copy numbers, but it can be seen even from the Ct values that caution should be taken not to misinterpret a strong positive SARS-CoV-2 result as indicating a higher case load when it may in fact be due to more concentrated sewage or a higher faecal load, perhaps due to dumping or a pollution event. For example, in the Palmiet River (**Figure 71**), positive assays for SARS-CoV-2 were observed in August and December 2021 which coincided with spikes in the crAssphage copy number, possibly due to spillage or run-off into the river.

As with the norovirus and hepatitis E assays, the same processing methods could be applied for the environmental samples for the crAssphage assay as for SARS-CoV-2 assay, giving this method an

advantage over water quality analysis which requires additional equipment. The crAssphage could also be detected in passive samples.

Figure 66: CrAssphage gene copies per L compared with water quality indicators (top) and SARS-CoV-2 gene assays (bottom) in the Jukskei River downstream of Alexandra in Gauteng

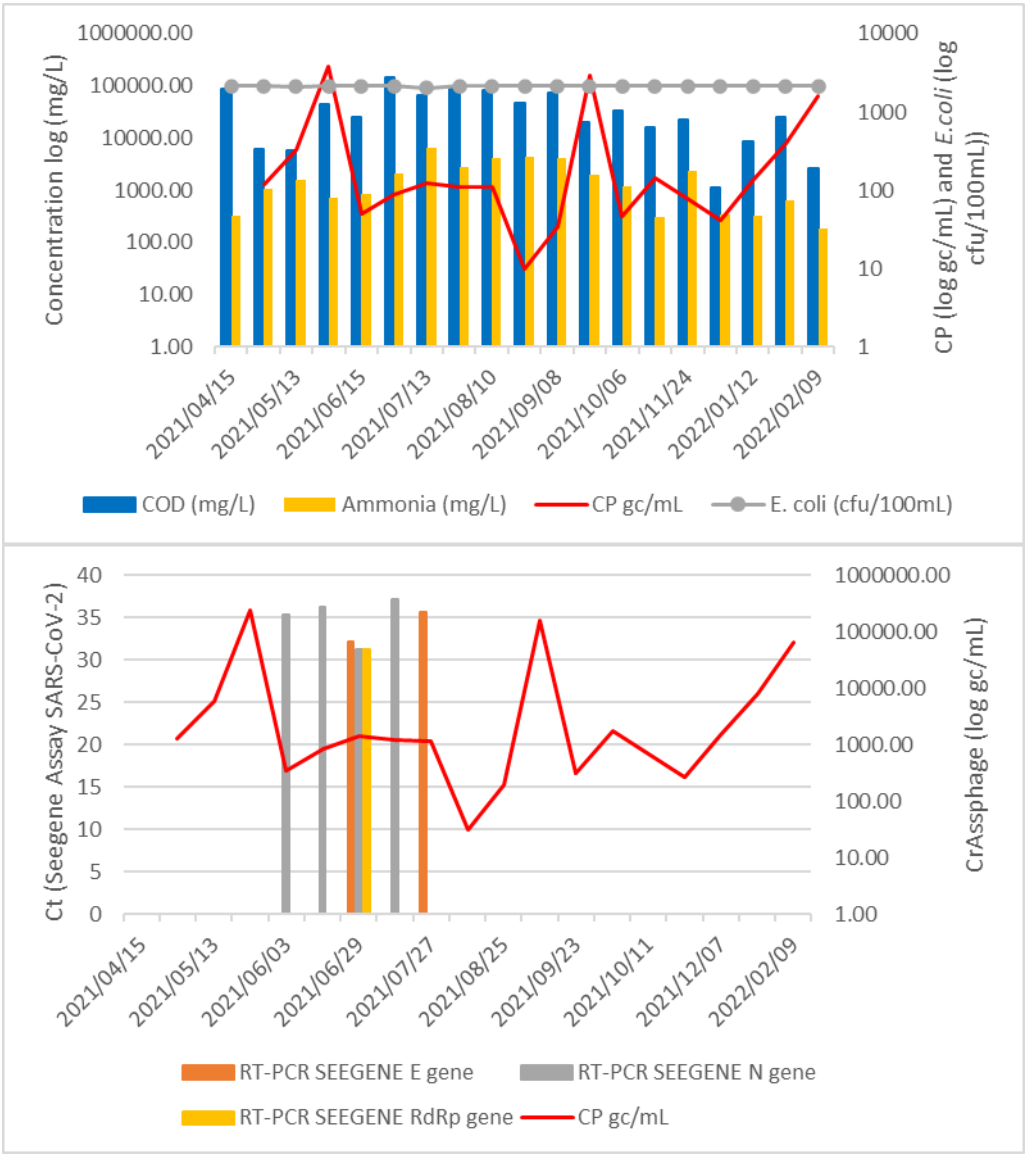


Figure 67: CrAssphage gene copies per L compared with water quality indicators (top) and SARS-CoV-2 gene assays (bottom) from grab samples taken from the Silvertown Informal Settlement in Alexandra in Gauteng

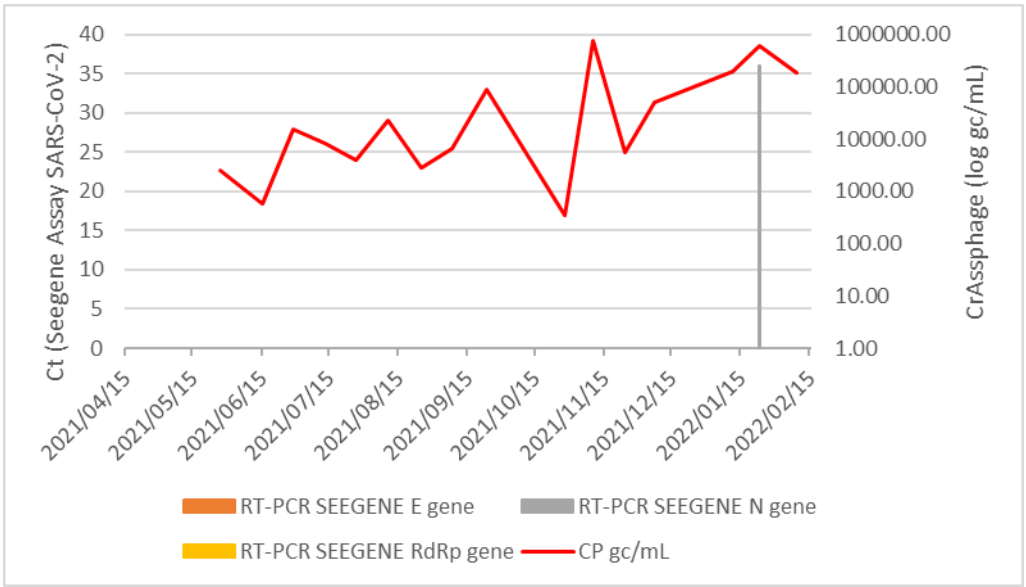


Figure 68: CrAssphage gene copies per L compared SARS-CoV-2 gene assays from passive samples taken from the Silvertown Informal Settlement in Alexandra in Gauteng

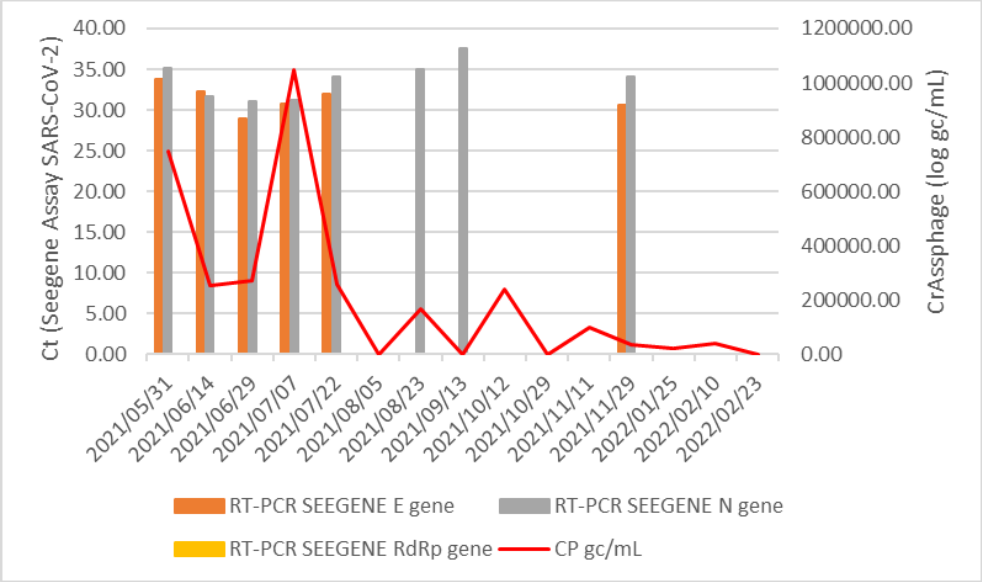


Figure 69: CrAssphage gene copies per L compared with SARS-CoV-2 gene assays from grab samples taken from the Kaalspruit River downstream of Tembisa in Gauteng

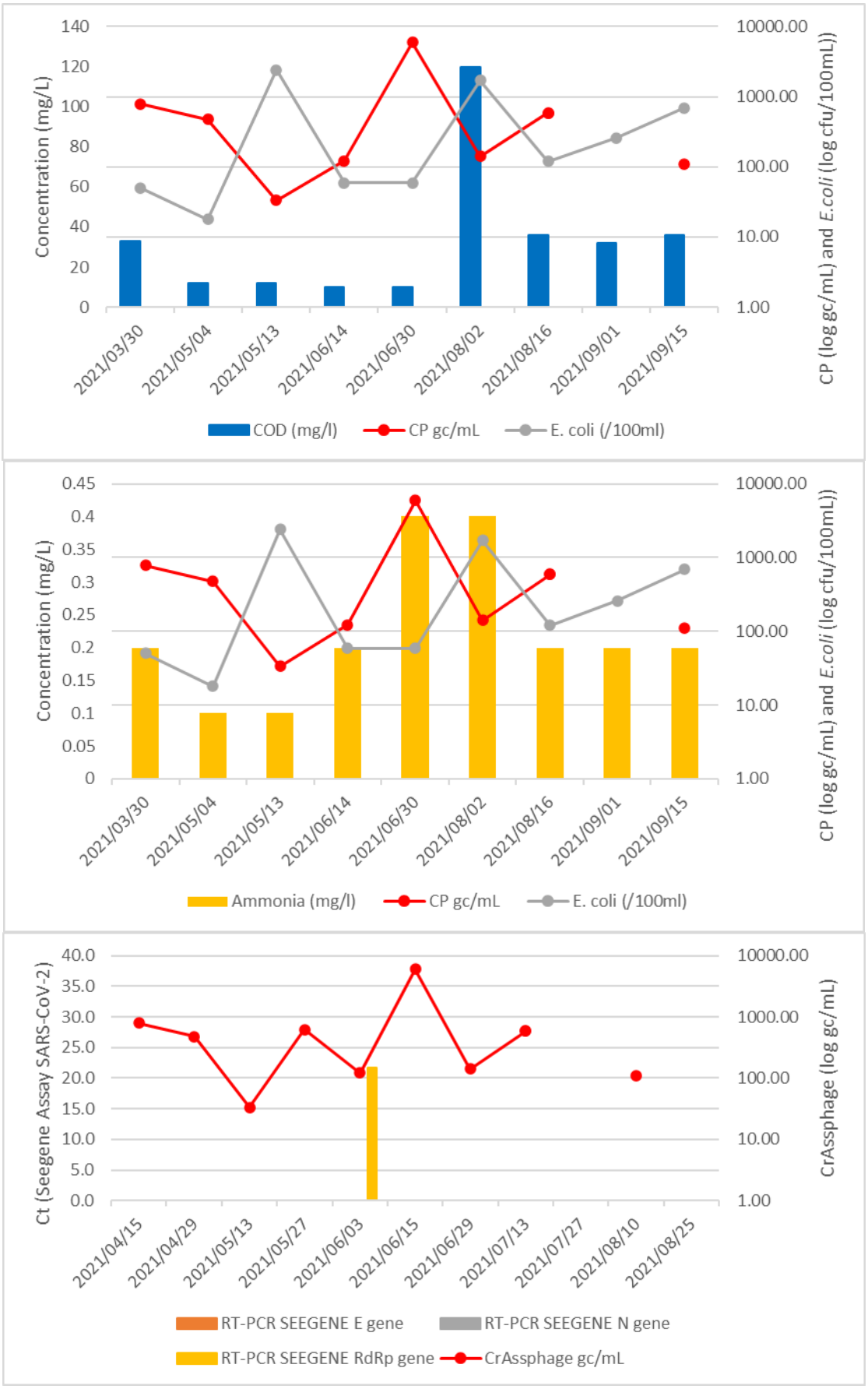


Figure 70: CrAssphage gene copies per L compared with water quality indicators (top and middle) and SARS-CoV-2 gene assays (bottom) from grab samples taken from the Crocodile River downstream of Kanyamazane in Mpumalanga

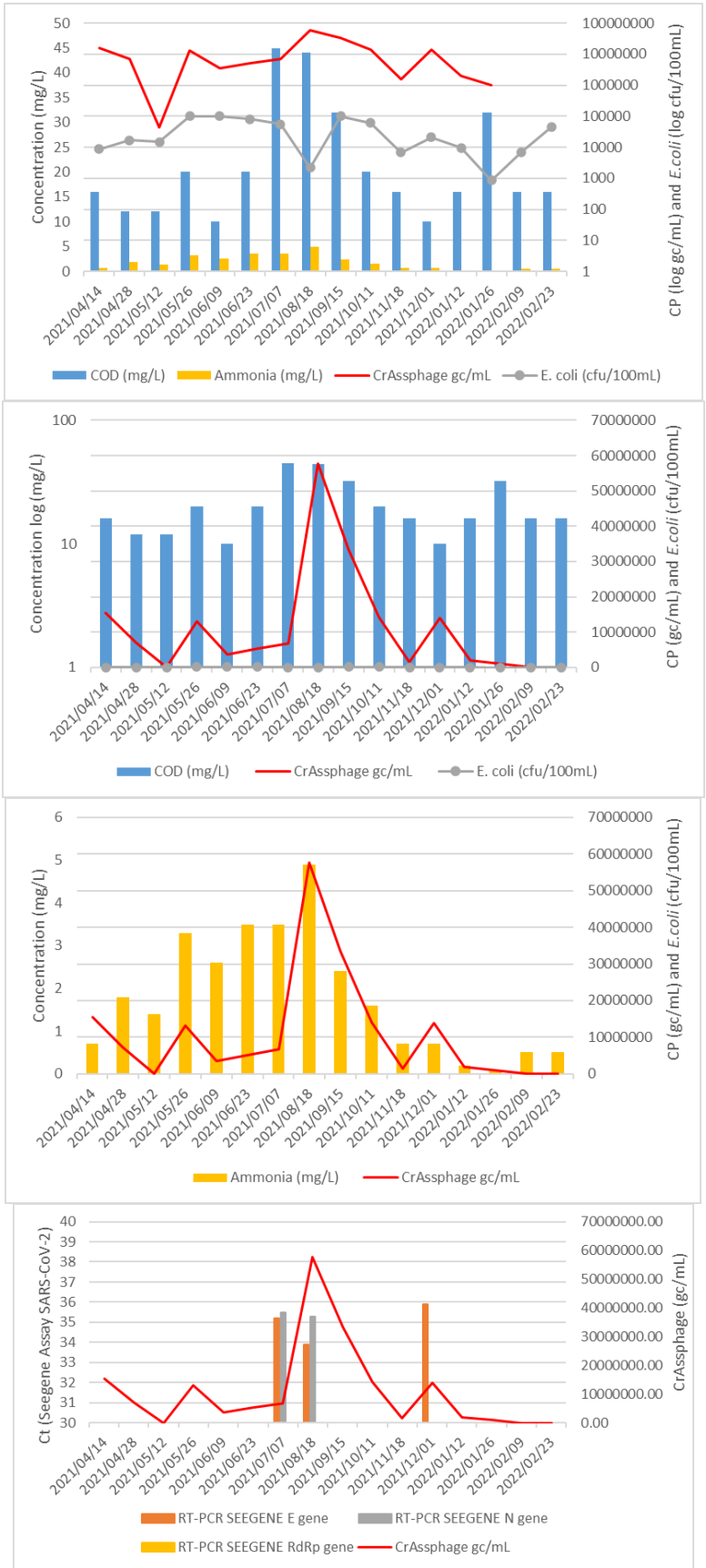


Figure 71: CrAssphage gene copies per L compared with water quality indicators (top) and SARS-CoV-2 gene assays (bottom) from grab samples taken from the Palmiet River downstream of the Quarry Road Informal Settlement in KZN

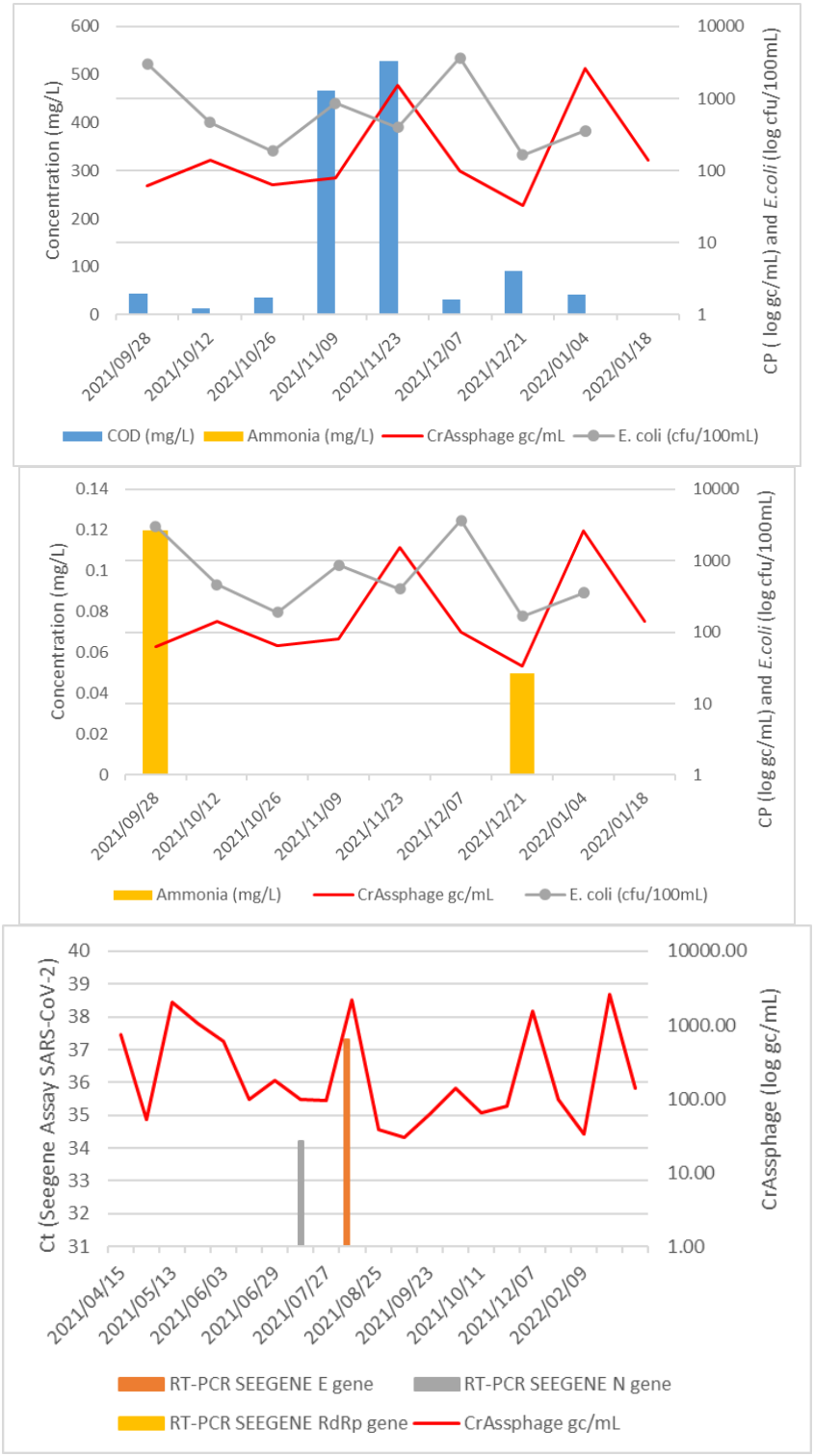


Figure 72: CrAssphage gene copies per L compared with water quality indicators (top and middle) and SARS-CoV-2 gene assays (bottom) from grab samples taken from the Plankenbrug River downstream of Kyamandi in the Western Cape

4 CONCLUSION AND WAY FORWARD

The data from this study indicated that COVID-19 could be identified in non-sewered community run-off, surface water and in the rivers which lie downstream of these communities. The incidence of COVID-19 in these communities was reflected in the Ct values obtained in the rivers and surface run-off samples. During the third wave the incidence of COVID-19 at detectable levels in these environmental samples increased with a corresponding increase in daily cases reported, and a similar increase was observed for the 4th wave in November and December 2021. In Gauteng province where the informal settlements are dense and the rivers are highly polluted by faecal matter from these communities, the trends were even more evident. It should be noted that the incidence of COVID-19 infections in the unsewered communities was very likely underreported as the cost of testing was prohibitive to these individuals and any free government testing would result in long queues and consequently time off work (if employed) would be required. Peaks in COVID-19 detection in the rivers were noted in March 2021, although these were not reflected in the clinical case load data, possibly due to unreported or untested infections.

Passive sampling of rivers has shown to be generally more sensitive that grab samples for the sites tested, with the passive samplers detecting SARS-CoV-2 earlier than the grab samples, and for longer into the inter-wave periods following the wave peaks. The use of passive samplers for detection of low viral loads will be particularly applicable during the rainy season when the dilution factor is high. Passive samplers were less effective when deployed in community run-off, most likely due to the concentration of inhibitors in the sample matrix.

Quantification of the human impact on a river is challenging as the number of individuals contributing to the viral load in the river is unknown. Therefore, it is necessary where possible to monitor indicators of human faecal contamination in these environmental samples. Use of the crAssphage as an indicator of faecal pollution has shown promise. screening of the Jukskei River downstream of Alexandra showed crAssphage trends in the river samples similar but not identical to that of the ammonia concentration and *E. coli* counts in the water quality. crAssphage screening on the Kaalspruit in Gauteng, as well as the run-off into the Plankenbrug River in the Western Cape, and the Palmiet River in KZN, showed that crAssphage trend more closely followed that of the ammonia concentration in the less polluted rivers where the informal settlement had an obvious impact on the downstream water quality, such as the Palmiet River downstream of the Quarry Road Informal Settlement. This study illustrated the potential application of crAssphage for normalisation of environmental sampling data to prevent misinterpretation of low Ct positive SARS-CoV-2 results. Low Cts indicating a higher case load may in fact be due to more concentrated sewage or a higher faecal load in the river or run-off water, perhaps due to dumping or a pollution event. CrAssphage may also serve as an indicator of stormwater dilution due to rainfall where river flow rates cannot be determined.

While this non-sewered surveillance programme focused on SARS-CoV-2, it was clearly illustrated that the same sample collection, recovery and extraction techniques used for SARS-CoV-2 screening could be successfully applied to collect contextual and public health information on other pathogens and indicators. Norovirus is an enteric virus that is very commonly present in South Africa's population and shed via stool, often used as reference viruses for sewage surveillance. Norovirus was found to be almost ubiquitously present in all four provinces (Gauteng, Mpumalanga, KZN and the Western Cape) across sites sampled, with norovirus GI detected in 48% and norovirus GII in 53% of 87 samples tested across eight sites. Both genogroups were present in 34% of samples. Similarly, twenty three percent of 44 samples tested positive for Hepatitis E, and positive samples were found in all four provinces tested. While not as prevalent in the samples assayed as the norovirus, the presence of Hepatitis E in 23% of the limited number of samples screened across the four provinces indicated that incidence of the virus is widespread. . CrAssphage as an indicator of sewage contamination was also successfully isolated using these techniques.

A subset of 29 positive samples with Ct values <34 for one of the gene targets representing each province over the duration of the study were selected for sequencing to confirm the SARS-CoV-2 assay and identify the specific variants present in those samples and potentially allow for tracking of variants over time. It was possible to generate successful sequencing libraries for 28 samples, with an average coverage of 92.9% when compared with the reference genome. 22 of these samples matched with other SARS-CoV-2 sequences in the BLAST database by >99%, confirming the positive assay results. Both grab samples and passive samples yielded successful libraries. Variant lineages were assigned to 12 of the 28 successful libraries (43%), and the shift in dominance from the Delta variant to the Omicron variant was apparent in samples taken in November 2021 from the Jukskei Source and the Jukskei downstream of Alexandra. Tracking of variants in environmental samples is therefore a viable method for determining the incidence of these variants in the non-sewered communities impacting on these water sources. More samples could be assigned to lineages in the Jukskei Source than other sites, indicating that the RNA may have been less fragmented and more like would be expected in a wastewater sample taken from a wastewater treatment

works inlet. The same was seen in tanker effluent which may be a useful source of variant tracking in non-sewered areas with conservancy tanks.

Sewage surveillance has proven to be a useful tool to monitor the circulation of SARS-CoV-2 in communities. However, this is most likely not going to be the only pandemic we will be facing in years to come. Urban water streams represent a rich and highly relevant source of information about exposure to pathogens as well as the opportunity to monitor emerging contaminants, lifestyle indicators, and antimicrobial resistance. This could be used to build a strategy and envision various scenarios about how this information can be used to prevent, mitigate, or even predict future outbreaks, as well as monitor human health on a broad scale, turning data into actionable insights for public health authorities and policy makers. It is important to consider how best to ethically and legally balance public health with civil liberties when handling this type of information (Gostin et al., 2020). One of the benefits of wastewater is that it has limited sociological bias with few if any ethical issues.

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