ENHANCED MOLECULAR METHODS FOR THE DETECTION AND QUANTIFICATION OF SARS-COV-2 IN WASTEWATER AND SANITATION SYSTEMS

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

Due to the economic and practical shortcomings of medical screening for SARS-CoV-2 and other infectious diseases, researchers are now turning to wastewater-based epidemiology (WBE) as an alternative method for disease surveillance in large communities. Using WBE for disease surveillance of communities is less expensive and quicker than clinical screening since it provides a snapshot of a community's health status without clinically diagnosing all infected individuals. Nevertheless, wastewater-based surveillance for SARS-CoV-2 is underutilized since there are no uniform methods for viral RNA concentration/enrichment, RNA extraction, selection of target genes, or molecular methods to detect or quantify viral RNA. In this study the first aim was to optimize protocols for the collection and extraction of SARS-CoV-2, Influenza A and Rotavirus RNA fragments from wastewater samples, including untreated wastewater, surface water, urine and faecal sludge, while the second aim focused on optimizing molecular methods (ddPCR and qPCR) for the detection and quantification of viral fragments from these viruses. For SARS-CoV-2, this involved evaluating the various gene targets (N2, E, ORF1ab) and selecting the most suitable target gene. For Influenza A and Rotavirus A, the goal was to optimize qPCR and ddPCR protocols for detection and quantification of the InfA and JVK genes, respectively. Final objective was to evaluate the newly developed methodologies on different environmental matrices (waste water, surface water, urine and faecal sludge) for three months to determine their effectiveness and applicability.

In combination with the Qiagen QiAMP Viral RNA Mini Kit, Centricon® Plus-70 ultrafiltration method provided the best results for viral concentrations and RNA extractions from the liquid samples (wastewater, urine, and surface water). In the case of faecal sludge samples, the commonly used commercial kits such as the Macherey-Nagel NucleoSpin RNA Plus Kit and the Qiagen QiAMP RNA MiniKit did not yield adequate results, despite following the manufacturer's recommendations for modifying the protocols. On the other hand, the TRIzolTM (Invitrogen) protocol (which requires a higher sludge biomass) produced better results for faecal sludge, although further optimization is necessary. Thus, it is crucial to develop a more effective protocol for the isolation of viruses from faecal sludge in addition to the modified TRIzolTM (Invitrogen) protocol developed in this study.

Comparing ddPCR to qPCR, ddPCR had a better detection limit, with 0,2, 2, and 3,1 gene copies/µl for the N2, E, and ORF1ab, while qPCR detected 13, 100, and 12 copies/µl for the

same gene targets. The limit of detection for Influenza A was determined to be 0.18 copies/µl by ddPCR, while qPCR could not detect concentrations below 7 copies/µl. In light of the lower limit of detection, the N2 gene was selected as the gene of choice for SARS-CoV-2 detection. The conclusion can be drawn that although qPCR is widely used for viral detection and quantification, ddPCR should be considered the gold standard method for applications requiring high sensitivity and high accuracy.

During the three-month monitoring period, optimized protocols developed for this project were able to accurately detect both N2 and InfA gene targets in raw wastewater from both treatment plants, while they were absent or below detectable thresholds in urine, faecal sludge, and surface water samples. The study has contributed significantly to the existing body of knowledge in wastewater-based epidemiology. The newly developed methods should provide a much more accurate estimation of viral loads in wastewater and other matrices, thereby enhancing the application of this tool to the decision-making process. Data from this study has also been shared with the South African Collaborative COVID-19 Environmental Surveillance System (SACCESS) network and other relevant stakeholders to support the national wastewater surveillance program. Furthermore, it is recommended that future research evaluate sampling strategies for different matrices so that representative samples can be obtained for the application of WBE techniques.

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LIST OF ACRONYMS

ALOD	:	Absolute Limit Of Detection
ddPCR	:	Digital Droplet Polymerase Chain Reaction
FS	:	Feacal Sludge
LOD	:	Limit Of Detection
NICD	:	National Institute Of Communicable Diseases
PBS	:	Phosphate Buffer Saline
PEG	:	Polyethylene Glycol
PPE	:	Personal Protective Equipment
qPCR	:	Quantitative Polymerase Chain Reaction
SACCESS	:	South African Collaborative COVID-19 Environmental Surveillance System
SW	:	Surface Water
WBE	:	Wastewater Based Epidemiology
WW	:	Wastewater
WWTP	:	Wastewater Treatment Plant

CHAPTER 1: INTRODUCTION

In the current COVID-19 pandemic, over 5,61 million deaths have been recorded, and over 355 million cases have been documented worldwide as of 26 January 2022 (WHO 2022). In South Africa, almost 3,58 million cases have been confirmed in the same period, with a mortality rate of 94 625 (JHU CSSE, 2021). Due to challenges with identification, isolation, and monitoring of infected individuals, this pandemic has put considerable strain on existing health systems.

With the practical and economic limitations of medical screening for SARS-CoV-2/COVID-19 becoming increasingly apparent worldwide, scientists and health authorities turned to wastewater-based epidemiology (WBE) as a potential tool for assessing and managing the pandemic – by detecting and quantifying the viral loads of SARS-CoV-2 in wastewater. Viruses are ubiquitous in raw and treated wastewater as well as in receiving water bodies such as surface waters (Fumian et al., 2010). One of the main sources of viruses in water is through human faecal matter shed by infected individuals. According to Gerba (2000), an infected person possibly sheds between 10^5 and 10^2 viral particles per gram of faeces – implying that the presence of viral fragment in wastewater bodies reflects the pattern of infection in the community. As such, adenovirus, rotavirus, hepatitis A, and Influenza A have all been previously detected in wastewater.

Regarding SARS-CoV-2, this approach is also based on the knowledge that some infected individuals shed viral particles and RNA in their urine and stool, regardless of whether they are symptomatic. Several studies have indicated that up to 25% of patients have gastrointestinal symptoms, such as diarrhoea, abdominal pain, or vomiting, although these symptoms are less frequent than respiratory symptoms (Yeo et al., 2020; Wang et al., 2020). These findings support the potential of stool, urine and wastewater analysis as an early warning system since viral RNA can be detected in faeces, and subsequently in wastewater, weeks before the onset of illness. Several studies have documented the detection of the SARS-CoV-2 virus in wastewater, which supported the adoption of WBE as a surveillance tool. In addition to serving as an early warning system, the detection of viral RNA in human excreta and wastewater has been referred to as a cost-effective method to identify infection hotspots. This is based on a calculation of the number of people infected within a catchment in response to viral loads found

in wastewater. Watzer et al. (2020) and Ahmed et al. (2020) reported a correlation between viral load in untreated wastewater and COVID-19 infections in the population served by wastewater treatment plants in Paris, France, and Queensland, Australia, respectively. Earlier studies in South Africa have also reported a similar correlation between viral loads in wastewater and reported clinically active cases in the eThekwini Municipality (Pillay et al., 2021). In spite of these positive results, it remains necessary to improve the sensitivity of the methods used for viral detection and quantification, as well as broaden the surveillance beyond wastewater to include other matrices.

1.1 Aim and Objectives

This project aimed to develop and optimize new advanced molecular methods for the detection and quantification of SARS-CoV-2 RNA from a variety of matrices, and had the following objectives:

1. To optimize RNA extraction methods for selected viruses in different sample matrices
2. To optimize quantification methods (ddPCR and qPCR) and their detection limits
3. To apply the optimized methods for detection and quantification of SARS-CoV-2, Rotavirus and Influenza A viral loads in wastewater environments

1.2 Report Structure

To address the objectives stated above, the report contains a general introductory chapter (this chapter) that presents the background and objectives of the study. A review of relevant literature is also provided to highlight the state of knowledge and the gaps that will be filled. The objectives stated above, then served as a guide in presenting the rest of the report. Each specific objective is covered by a technical chapter, which contains background information to highlight the rationale of the objective, a methodology and presentation of the results obtained. Chapter four (4) presents the optimization of viral concentration and RNA extraction methods, compared based on RNA quantity extracted (Objective 1). Chapter five (5) addresses Objective 2, which is the optimization of viral RNA quantification using ddPCR and qPCR, the assessment of recovery efficiency and determination of the limit of detection (LOD). The application of the optimized methods for the detection and quantification of the viral targets is presented in Chapter six, addressing the last objective of this study. A general conclusion and recommendation chapter completes this report.

CHAPTER 2: LITERATURE REVIEW

2.1 Wastewater Based Epidemiology

The concept of WBE for monitoring illicit drug consumption at the community level whilst preserving an individual's anonymity was first theorized in 2001 (Daughton, 2001). Based on systematic sampling and analysis of wastewater samples, it was possible to correlate the drug residues in wastewater to consumption in the community. The first practical implementation of WBE of cocaine consumption monitoring was achieved in 2005 (Mao et al., 2020; Zuccato et al., 2005; Tang et al., 2020a). Since then, WBE has undergone further improvements and was used for various studies including the presence of pharmaceutical or industrial waste (Baz-Lomba et al., 2016; He, 2020), viruses (Hou et al., 2020) and the potential emergence of antibiotic-resistant bacteria (Hutinel et al., 2019; Castrignanò et al., 2020). Substances including alcohol, amphetamines, counterfeit medicine, opiates and tobacco have also been successfully monitored at the community level via WBE (Hummel et al., 2006; Reid et al., 2011; Irvine et al., 2011; Lai et al., 2013; Venhuis et al., 2014; Castiglioni et al., 2015). More recently in 2017, WBE was applied for the first time to estimate human exposure to pesticides across several European cities by quantifying urinary biomarkers in their sewers (Rousis et al., 2017). Different authors have used it to complement other traditional disease surveillance methods successfully in monitoring Norovirus outbreaks in different countries including Australia, New Zealand and Brazil (Lun et al., 2018; Fioretti et al., 2018). In the Netherlands, WBE was applied to establish a timeline for the prevalence of Aichi virus infection in humans (Lodder et al., 2013). Currently, several countries have reported SARS-CoV-2 detection in wastewater, using it as a data source to determine the circulation of the virus in the community (Ahmed et al., 2020a, Lodder and de Roda Husman, 2020, Medema et al., 2020; Wu et al., 2020; Kumar et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; La Rosa et al., 2020; Wurtzer et al., 2020; Pillay et al., 2021). The WBE may provide a better resolution of the infection rate at the community level since all classes of COVID-19 patients (symptomatic, asymptomatic, and pre-symptomatic), may not be captured via clinical surveillance.

2.2 Methods used in the detection of SARS-CoV-2 in wastewater

The application of WBE for monitoring COVID-19 infections relies on the successful detection of SARS-CoV-2 viral RNA in untreated wastewater. Discussed below is the current state of knowledge on the methodological approaches published in the literature.

2.2.1 Sampling, Viral concentration and RNA extraction methods

Routine sampling of selective areas such as the WWTP influent, upstream pumping station, biological tanks, final wastewater effluent and/or collection at rivers or latrines are necessary for a widespread environmental surveillance and for early warning (WHO, 2020c; Reilly *et al*., 2020). To reduce areas requiring active public health investigations, more reliable sampling methodologies (especially for areas with low reticulated sewerage coverage), require further investigations (WHO, 2020c).

Sampling can range from simple batch methodologies (i.e. collection of individual samples), to time integrated sampling and continuous monitoring which closely represent "real-time" monitoring. Grab samples and automated sampling techniques such as conventional submersible *in-situ* high frequency auto samplers and conventional refrigerated auto-samplers are currently in use for wastewater sample collection. Most WWTPs are already undergoing automation, primarily for remote control. Further development extends to a wide range of sensors for characterisation of wastewater parameters. This could establish an important platform to automate the monitoring in WWTPs and remote reporting by the integration of WBE sensors (Daughton et al., 2020). The wastewater samples are subsequently transported on ice to the laboratory for analysis. Further processing of wastewater samples in laboratories should follow existing protocols and biosafety standards for proper handling of SARS-CoV2, i.e. BSL-2 (WHO, 2020d). Sampling personnel are required to use standard personal protective equipment (PPE) such as respirators, gowns, safety glasses, gloves, steel-capped boots and hard hats, to protect mucous membranes, skin, airways and clothing from infectious contaminants.

To increase the sensitivity of viral RNA detection, most studies have reported on viral concentration steps. Reliable concentration methods are fundamental for the detection of SARS-Cov-2 in wastewater, which is currently a key research need for WBE (Kitajuma et al., 2020; Ahmed et al., 2020; Farkas et al., 2020). All the reports of SARS-CoV-2 in wastewater has been based on the use of viral concentration methods (Ahmed et al., 2020a; Medema et al., 2020; Wu et al., 2020; Nemudryi et al., 2020; Wurtzer et al., 2020; Zhang et al., 2020; Bar-Or et al., 2020; Kocamemi et al., 2020; Randazzo et al., 2020; La Rosa et al., 2020).

The wastewater samples generally go through centrifugation and/or filtration to eliminate debris (Farkas et al., 2020). The current methods for concentration of the virus to recover SARS-CoV-2 RNA from wastewater includes ultrafiltration (Medema et al., 2020; Wurtzer et

al., 2020; Ahmed et al., 2020a), ultracentrifugation (Green et al., 2020; Ahmed et al., 2020b), polyethylene glycol (PEG) precipitation (Wu et al., 2020; Balboa et al., 2020) and skimmed-milk flocculation (Guerrero-Latorre et al., 2020). Globally, electropositive and electronegative membranes based on the electrostatic interaction between the viruses and filters have also been used to concentrate enteric viruses in wastewater samples (Ahmed et al., 2020a; Katajima et al., 2020). For this method, the viruses which are negatively charged, adsorb onto the filters via salt-bridging. The major disadvantage of these methods is the concentration of organic compounds, which may interfere with downstream processing for further detection and quantitative analysis (Farkas et al., 2020).

SARS-CoV-2 viral RNA is commonly extracted from the concentrated viral particles using commercial RNA extraction kits viz. RNeasy PowerMicrobiome Kit (Medema et al., 2020; Ahmed et al., 2020a; Ahmed et al., 2020b), Biomerieux Nuclisens kit (Medema et al., 2020), PowerFecal Pro kit (Wurtzer et al., 2020) and RNeasy PowerWater Kit and RNeasy (Ahmed et al., 2020a). Furthermore, a list of RNA extraction kits, that may be used for the assessment of SARS-CoV-2 in wastewater samples, was published in the Centres for Disease Control and Prevention of the US (CDC, 2020). For the successful application of WBE for early warning and COVID-19 surveillance, further improvements to efficient, rapid and cost-effective concentration and RNA extraction methods are pivotal for monitoring of SARS-CoV-2 and its nucleic acids in wastewater.

2.2.2 Methods for the detection and quantification of the viral RNA

2.2.2.1 Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The most common method used for amplification of the extracted viral RNA is the Real Time Quantitative Polymerase Chain Reaction (RT-qPCR). RT-qPCR is a type of PCR, which is considered quantitative in determining the number of copies of a target gene in a sample. A typical RT-qPCR reaction measures the amount of fluorescence in a sample during each cycle by employing the use of either SYBR Green or TaqMan probe-based chemistries (La Rosa and Muscillo, 2013). The standard curve set the threshold for the reaction, and during the reaction, the fluorescence of each sample is measured (Kuang et al., 2018). If the fluorescence exceeds that of the threshold, then it is scored as “positive”. The number of cycles a sample takes to reach this threshold level is referred to as the cycle threshold or “ct” value. A RT-qPCR reaction can be multiplexed to screen for multiple gene targets within a single sample with ease using specific internal probes that bind to different fluorochromes. To date, RT-qPCR is accepted as

the gold-standard for the detection of SARS-CoV-2 (Tahamtan and Ardebili, 2020). This has been applied to wastewater in various studies with the first being carried out by Ahmed et al. (2020) who used RT-qPCR together with sequencing for the detection of SARS-CoV-2 from pump stations and WWTPs in Australia. This was followed by Wang et al. (2020b) who screened for SARS-CoV-2 in untreated and partially treated wastewater while Nemudryi et al. (2020) used RT-qPCR to monitor raw sewage in municipal WWTPs in the USA. Since the publication of these initial uses of RT-qPCR several other studies (and still counting) have used this approach (Wu et al.,2020a; Wurtzer et al., 2020; Lodder & de Roda Husman, 2020; Randazzo et al., 2020; Green et al., 2020; Or et al., 2020; Balboa et al., 2020; Peccia et al., 2020; Kumar et al., 2020; Rimoldi et al., 2020).

2.2.2.2 Droplet Digital Polymerase Chain Reaction (ddPCR)

Droplet digital PCR is a relatively new surfactant-based platform which allows the user to quantify nucleic acids in a sample with greater precision than qPCR and without the need for a standard calibration curve. In a typical ddPCR reaction, the mastermix is mixed with oil and partitioned into approximately 20 000 nano-sized droplets (using an automated or manual droplet generator) in such a way that each droplet contains little to no copies of the template (either DNA or RNA) (Pinheiro et al., 2012; Quan et al., 2018). A normal PCR amplification step is carried out thereafter using fluorescently labelled TaqMan probes and amplification then proceeds within each encapsulated droplet. The droplets are then passed through an automated reader (similar to a cytometer) which measures the number of positive droplets within each sample well. Poisson statistical analysis is then applied to calculate the absolute concentration of template DNA within the sample and the designated software expresses it in copies per microliter (Roberts et al., 2013). Because the sensitivity of the reaction increases as the partitions increase, the ddPCR platform offers a significant advantage over qPCR in terms of precision and is quite useful in settings that require more robust and accurate quantification of target genes. ddPCR can also be used for gene expression analysis and rare mutation detection (Jamuar et al., 2016). While there is a dearth of information regarding the use of ddPCR for the detection of SARS-CoV-2 in wastewater, a clinical study by Suo et al. (2020) confirmed that ddPCR is superior to qPCR as it has a lower limit of detection (LOD) and is more specific than qPCR when used to screen for SARS-CoV-2 in clinical settings. This method has been used successfully for WBE during the current pandemic (Pillay et al., 2021; Gonzalez et al., 2021; Ciesielski et al., 2021).

2.3 Challenges associated with detection of SARS-CoV-2 in wastewater associated with primer specificity and stability of viral genome in wastewater

The selection and design of effective primers that are specific and sensitive enough for the diagnosis and tracking of SARS-CoV-2 are of immense importance, however, it comes with several challenges. Primers are the pivotal components of molecular based assays, but it is only effective if mutation and recombination do not occur within the viral genome (Li et al., 2020). The SARS-CoV-2 genome is approximately 30 kb and is arranged in the order of a 5′ untranslated untranslated region (UTR)-replicase complex (open reading frame [ORF] 1ab)-structural protein [Spike(S)-Envelope(E)-Membrane (M)-Nucleocapsid (N)]-3′ UTR and non-structural ORFs (Kailany et al., 2020). Proposed gene targets include N (nucleocapsid), E (envelope), ORF 1ab (open reading frame (or RdRP) and S (spike protein genes). The E gene is the most conserved while the N and RdRP genes are recommended for confirmatory purposes (Chan et al., 2020). Detection of a single target only is said to be prone to error therefore the sensitivity of the assay can be increased by screening for multiple targets (Green et al., 2020a). Although primers and probe designs are evaluated via in-silico analysis, experimental confirmation is of utmost importance. The WHO and the CDC have both published primers and probes for the detection of SARS-CoV-2. The concentration of these primers and probes, reaction conditions, melting temperatures and reverse transcription conditions all need to be optimized for maximum accuracy of the result. Using a positive control, it is important that each primer set be evaluated on the platform it is to be used on, and the absolute limit of detection (ALOD) be determined (Dobnik et al., 2016). Corman et al. (2020) reported ALOD values of 3.8, 5.2 and 8.3 copies per reaction for the detection of RdRp, E and N genes respectively while Shirato et al. (2020) reported that only assays for the N gene worked well. To date, screening for N gene seems to be the most widely used as it produces the most consistent results and has the lowest ALOD.

CHAPTER 3: OPTIMIZATION OF VIRAL CONCENTRATION AND RNA EXTRACTION METHODS

3.1 BACKGROUND

In recent publications, methods for recovering SARS-CoV-2 from wastewater have varied substantially, with consequences for cost, reagent availability, and scalability. Furthermore, previous research has shown that several common viral concentration techniques are ineffective in recovering wrapped viruses from ambient water samples (Kitajima et al., 2020). Various concentration methods have been used, including polyethylene glycol (PEG) precipitation (Wu et al., 2020), $AlCl_3$ precipitation (Randazzo et al., 2020), ultrafiltration with centrifugal filters (Medema et al., 2020), ultracentrifugation (Wurtzer et al., 2020), and membrane adsorption (Wurtzer et al., 2020). (Haramoto et al., 2020). RNA extraction has generally been performed using commercial kits from a variety of suppliers. The most common RNA extraction kits used are RNeasy PowerMicrobiome Kit (Medema et al., 2020; Ahmed et al., 2020), Biomerieux Nuclisens kit (Medema et al., 2020), PowerFecal Pro kit (Wurtzer et al., 2020) and RNeasy PowerWater Kit and RNeasy (Ahmed et al., 2020). In addition to these RNA extraction kits, the Centres for Disease Control and Prevention has published a list of RNA extraction kits that can be used for SARS-CoV-2, and potentially Rotavirus and Influenza A virus as well (CDC, 2020). The effectiveness of these different kits in extracting RNA from different wastewater needs to be confirmed in order to establish an effective method for RNA extraction which is a critical step in the use of sanitation systems as an approach for COVID-19 and other viral infection surveillance. This objective aimed at evaluating the different methods for the concentration of viruses and RNA extraction from wastewater, surface water, urine and faecal sludge.

3.2 METHODOLOGY

3.2.1 Sample collection: Wastewater and Surface Water

For the purpose of method optimization, untreated wastewater (WW) was collected from the Central WWTP in Durban, KwaZulu-Natal, once a week for a period of three weeks, taking into consideration the flow rate of the plant and its peak flow period. Samples were collected using sterile 1L sampling bottles previously disinfected with 70% ethanol and transported on ice to the laboratory. In addition, surface water (SW) samples were collected from the Isipingo River using sterile 1L bottles. Briefly, the sample bottle was inverted into the water body against the flow of the current to permit the movement of water into the bottle. The Isipingo

river was selected based on the reported anthropogenic activity near the river as well as receiving discharge from nearby wastewater treatment plants.

3.2.2 Sample collection: Urine and Faecal Sludge

Faecal sludge (FS) and urine samples were collected by WASH R&D Centre (University of Kwa-Zulu Natal). For faecal sludge collection, samples were collected from the pits of three UDDTs within the eThekwini municipality region using a long-handled spade and put into the sample containers. This was repeated three times from each household to achieve the desired volume and representativeness. The samples were then mixed into a composite sample and transferred into a 1L sample container. The containers with the samples were sealed tightly, tied in plastic packets, disinfected, and then packed into the 1-25L containers for transporting back to the lab. For the collection of urine, 1L sample containers were provided to each household from which faecal sludge was collected. This was done every week for a period of 3 weeks. After 2-3 days, samples were then collected from the households and transported to the laboratory for analysi3.2.3 Viral concentration for Wastewater, Surface Water and Urine.

Larger particles (debris, bacteria, etc.) was removed from the samples by centrifugation at 3500 x g for 30 min. The resulting supernatants of the WW, SW and Urine samples was then used for further analysis. Two methods of viral particle concentration were evaluated namely ultrafiltration and polyethylene glycol precipitation (PEG 8000). The ultrafiltration was done using Centricon® Plus-70 centrifugal ultrafilter (10 kDa) according to Medema et al. (2020). For the PEG 8000 concentration, PEG 8000/NaCl was prepared by dissolving 100g of PEG (Merck, Germany) and 17.5g of Sodium Chloride (Merck, Germany) in 200 ml of distilled water. The pH of each solution was adjusted to 7.0-7.2, and the resulting solution was filtered with a 0.2 µm filter. Supernatant from each sample was then mixed with PEG 8000/NaCl (10% w/v) and incubated overnight at 4ºC at 60 rpm. Following incubation, each sample was centrifuged at 5700 x g for 2 h. The resulting supernatant was discarded while the pellet was resuspended in 140 µl phosphate buffer saline (PBS) and stored at -80ºC (Wu et al., 2020). The faecal sludge samples did not undergo viral concentration.

3.2.3 Evaluation of RNA extraction methods

3.2.3.1 Wastewater, Surface Water and Urine

For WW, SW and Urine samples, the Qiagen QiAMP Viral RNA MiniKit (filter-based extraction method) required 140 µl of the viral concentrate from each of the concentration methods, while the Macherey-Nagel NucleoSpin® RNA Plus kit (magnetic-based extraction) required 200 µl of concentrate as a startup. Both commercial kits were used in triplicate according to the manufacturer's instructions. The extracted RNA was compared in terms of quality and yields using the NP80 ® Implen NanoPhotometer (Germany).

3.2.3.2 Faecal Sludge

RNA was extracted from the faecal sludge following TRIzol™ (Invitrogen) manufacturer's protocol after an initially modifying the protocol to use 0.1g of biomass. The faecal sludge was subjected to mechanical rupture at medium speed for 1 min 30 sec using the Bead Ruptor 12 (Omni International, Inc, Georgia). The RNA extraction was then completed following the Invitrogen™ TRIzol™ User Guide (Pub. No. MAN0001271 Rev. B.0). The extracted RNA's quality and quantity were assessed using the NP80 ® Implen NanoPhotometer (Germany). Modified methods for the extraction of viral RNA as prescribed by the manufacturers of the Macherey-Nagel NucleoSpin® RNA Plus Kit and Qiagen QiAMP Viral RNA MiniKit were initially evaluated however, the resulting RNA was of poor quality and did not yield any positive results.

3.2.4 Screening for SARS-CoV-2 and Influenza A

Wastewater, SW and Urine samples were screened for SARS-CoV-2 and Influenza A gene targets. If a sample did not contain any of the viral targets, they were spiked with inactivated forms of SARS-CoV-2 and/or Influenza A. Spiking was done by adding 60µl of inactivated SARS-CoV-2 strain USA/WA1/2020 and/or Influenza A strain H1N1 (Microbiologics, USA) to 60 ml of WW, SW or urine sample. Samples (spiked or unspiked) were then concentrated, and RNA extracted using a combination of either the PEG 8000 or Centricon® Plus-70 centrifugal ultrafilters for viral concentration together with either the Macherey-Nagel NucleoSpin® RNA Plus kit or the Qiagen QiAMP Viral RNA MiniKit for RNA extraction (methodology described above). The RNA concentrations obtained using each of the abovementioned methods were then analysed in respect of quantity and quality and compared.

3.2.5 Statistical analysis

Microsoft Excel (Microsoft Corporation, USA) was used to capture all the data, calculation of means and data visualization. Comparison of RNA quantity achieved by the two viral concentration methods and the two RNA extraction methods was performed using the Mann-Whitney test at a 95% confidence interval with GraphPad prism (GraphPad software, USA

3.3 RESULTS

3.3.1 Comparison of viral concentration and RNA extraction methods – comparing RNA concentrations

Figure 3.1 below shows the mean concentrations of RNA obtained when using both the Qiagen QiAMP Viral RNA Mini Kit and Macherey-Nagel NucleoSpin® RNA Plus kit in combination with either the Centricon® Plus-70 centrifugal ultrafilter or PEG 8000 from wastewater samples, while Figure 3.2 shows the RNA concentrations achieved using the Macherey-Nagel NucleoSpin® RNA Plus vs Centricon® Plus-70 centrifugal ultrafilter and PEG 8000 for SW samples. Figure 3.3 shows the same for urine samples. Based on the results obtained, the Qiagen QiAMP Viral RNA Mini Kit combined with the Centricon® Plus-70 centrifugal ultrafilter consistently yielded a higher concentration of RNA – making this combination the method of choice for viral particle concentration and extraction. This trend was consistent regardless of the sample matrix.

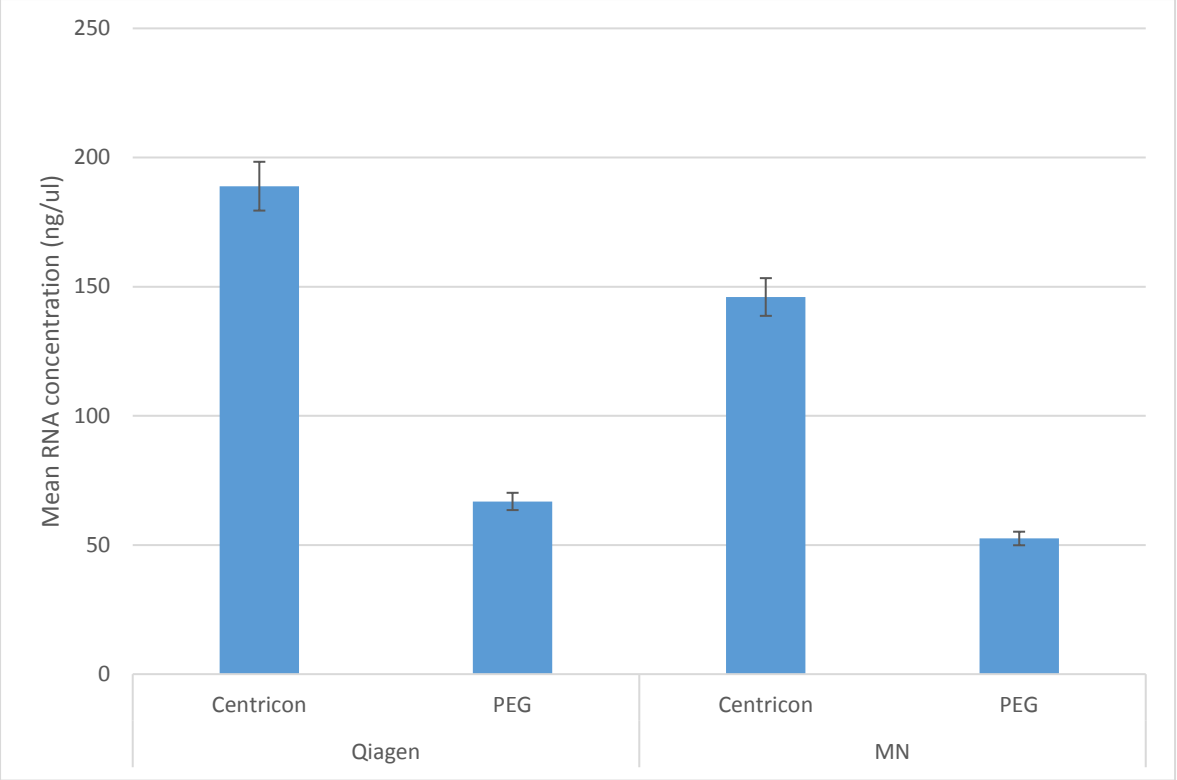

Figure 3.1: RNA concentrations obtained from wastewater using Qiagen QiAMP and Macherey-Nagel NucleoSpin® (MN) viral RNA extraction kits in combination with Centricon and PEG viral concentration methods

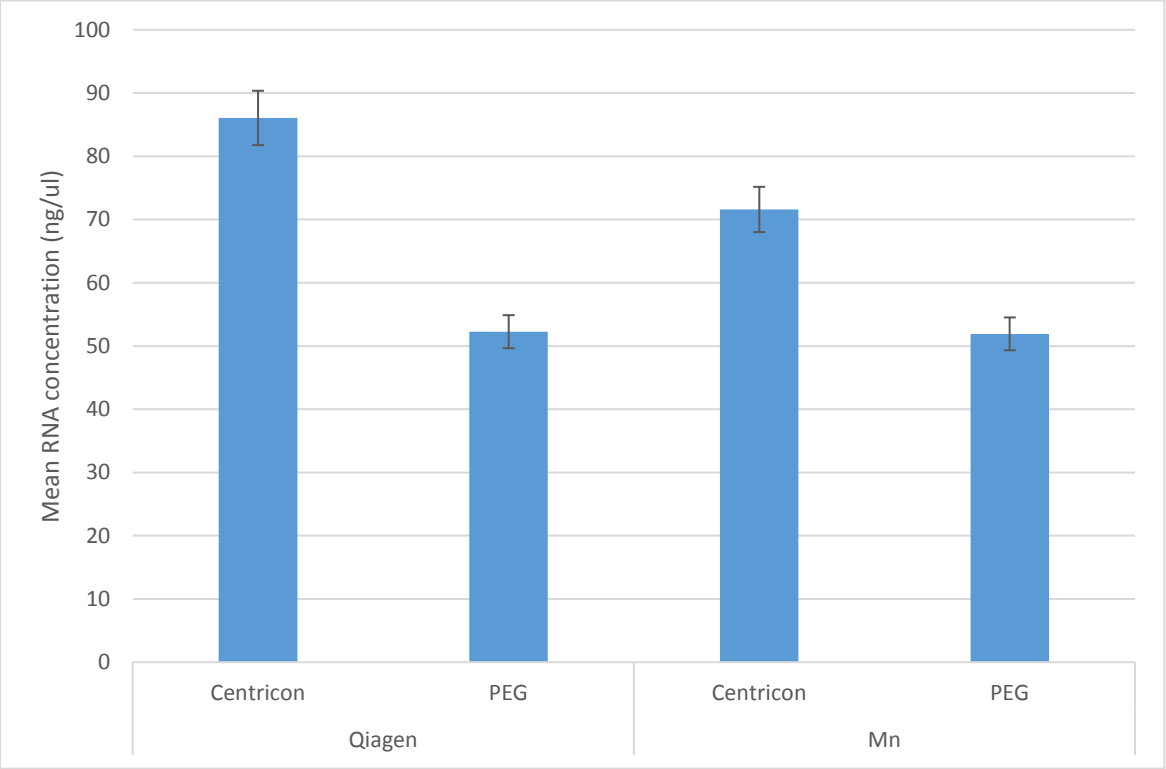

Figure 3.2: RNA concentrations obtained from surface water using Qiagen QiAMP and Macherey-Nagel NucleoSpin® (MN) viral RNA extraction kits in combination with Centricon and PEG viral concentration methods

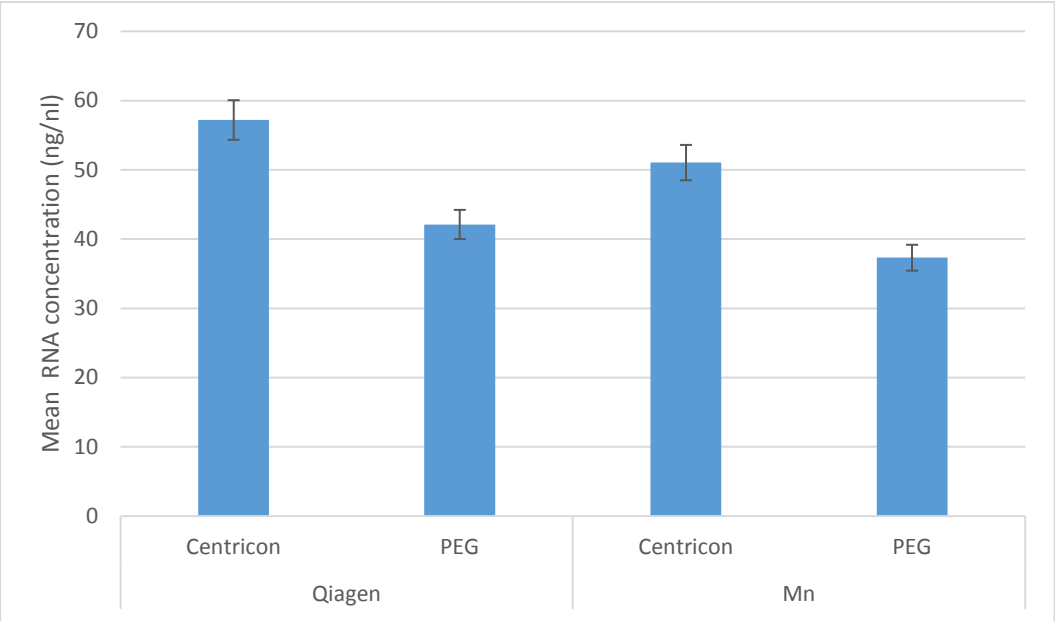

Figure 3.3: RNA concentrations obtained from urine using Qiagen QiAMP and Macherey-Nagel NucleoSpin® (MN) viral RNA extraction kits in combination with Centricon and PEG viral concentration method

CHAPTER 4: OPTIMIZATION OF QUANTIFICATION METHODS FOR SARS-COV-2, INFLUENZA A AND ROTAVIRUS

4.1 BACKGROUND

Reverse transcription polymerase chain reaction (RT-PCR) is recognized as a standard and commonly used technology for the characterization and quantification of diverse pathogenic RNA in laboratories and clinical diagnostics due to its great sensitivity in exponentially amplifying RNA molecules (Zhao et al., 2020). It has been used to identify SARS-CoV, Middle East respiratory syndrome coronavirus (MERSCoV), and other viral diseases such as Zika virus (ZIKV), Influenza A virus, and Dengue virus, among others (DENV) (Ng et al., 2020; Xu et al., 2016). Following the COVID-19 epidemic, many RT-PCR-based techniques and kits for detecting SARS-CoV-2 genomic RNA have been described (Corman et al., 2020; Chu et al., 2020). While RT-PCR-based approaches have been widely utilized to diagnose COVID-19, their applicability in accurate viral infection detection and epidemic control is limited by the tedious and time-consuming sample processing.

Additionally, this method has been seen to have lower efficiency and has resulted in some cases false negative or inconclusive reports. The use of the digital droplet PCR may help address this challenge. This has shown to be have an improved lower limit of detection, more sensitive and accurate compared to RT-qPCR (Lu et al., 2020; Dong et al., 2020). The use of dPCR may therefore aid in reducing false negatives and positives, especially in samples with low viral titer, like wastewater and surface water. This chapter therefore presents optimization and comparison of qPCR and ddPCR for the quantification of SARS-CoV-2, and Influenza A in different environmental matrices.

4.2 METHODOLOGY

4.2.1 Optimization of quantitative PCR and droplet digital PCR using positive controls

Optimization of ddPCR and qPCR conditions for the detection and quantification of viral gene targets was first done using positive controls. The following previously established protocols were first evaluated: SARS-CoV-2 (CDC, 2020; Pillay et al., 2021), Influenza A (Malhotra et al., 2016) and Rotavirus (Jothikumar et al., 2009). For rotavirus, additional evaluation was done using the method described by Li et al., 2011 and Zeng et al., 2008. For SARS-CoV-2, positive controls from Exact Diagnostics (Bio-rad, USA), which contains synthetic transcripts of the N2, E, ORF1ab and RdRp genes, was used. For Influenza A and Rotavirus, RNA was extracted

from inactivated strains of Influenza A and Rotavirus purchased from Microbiologics (USA) and used as a positive control in the PCR reactions. Despite trying 3 protocols as described above using different sets of primers, positive amplification for Rotavirus was unsuccessful for the positive control. There is a possibility that the problem is related to the positive control, either the RNA is degraded or contaminated. Attempts to purchase alternate control strains from other suppliers such as ATCC required import permits which could not be obtained in time due to delays related to COVID-19 during the study period. For Influenza A and SARS-CoV-2, previously published protocols were modified in terms of the concentration of the template RNA, number of PCR cycles as well as annealing temperatures to obtain the working protocols as stated in Table 4.1. A detailed description of the ddPCR protocol can be found in Appendix 1.

4.2.2 Determination of the recovery percentage of the viral targets from the different matrices

Once the qPCR and ddPCR protocols were optimized using the positive controls, it was applied to real samples to evaluate the recovery efficiency of the different concentration and extraction methods considered under this study. To determine the recovery efficiency, 60 ml each of WW, SW, and urine were each spiked with 60 µl of inactivated SARS-CoV-2 strain USA/WA1/2020 and Influenza A strain H1N1 (Microbiologics, USA). Since the presence of the N2 gene implies the presence of SARS-CoV-2, the recovery efficiency in spiked samples was only determined for the N2 gene (And not for ORF1ab and E). Based on previous in-house experimentation, every 60 ml of the spiked sample contained 504 and 780 copies of the N2 and InfA genes respectively. The ddPCR and qPCR assay to determine the concentrations of the gene targets were conducted on the same day as RNA extraction to avoid any losses in RNA integrity that may result from storing and/or freeze-thawing of the RNA. The primer and probe sequences, together with the optimized ddPCR and qPCR protocols for SARS-CoV-2 and Influenza A can be found in Table 4.1. Protocols for Rotavirus which were evaluated are attached in Appendix 1.

4.2.3 Determination of Limit of Detection

The limit of detection (LOD) for each of the gene targets was determined to avoid false positive and negative results as well as to reliably quantify the minimum amount of gene target copies that can be detected using each of the two PCR platforms. To determine the LOD for SARS-

CoV-2, the same SARS-CoV-2 standard (Exact Diagnostics), which contains synthetic RNA transcripts, was used (Medema et al., 2020; Suo et al., 2020). The standard was serially diluted, and a range of 10-fold serial dilutions was run through the QX200 droplet digital PCR system (Bio-rad) with an automated droplet generator to determine the limit of detection for each of the gene targets (N2, E, ORF1ab, InfA). To compare the quantitative detection limits of the two techniques, the same range of the 10-fold serially diluted positive standard (used for the ddPCR) was quantified using the RT-qPCR technique. The diluted positive standard was used as a template, mixed with One-step Prime Script RT-qPCR mastermix. The assay ran using the QuantStudio 3 Real-Time PCR System (Applied Biosystems) according to methods prescribed by Ahmed et al. (2020).

Table 4.1: Optimized ddPCR and qPCR protocols used in this study

Assay	Primer/Probe	Sequence (5'→3')	Cycling conditions qPCR	Cycling conditions ddPCR
N2	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	Reverse transcription at 55°C for 5 minutes, preheating at 95℃ for 10 seconds followed by 45 cycles of amplification at 95°C for 10 seconds and 55°C for 30 seconds.	Reverse transcription at 50°C for 1 h, enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 94°C for 30 s and annealing at 55°C for 60 s. Followed by: by enzyme deactivation at 98°C for 10 min and droplet stabilization at 4°C for 30 min with a ramp rate of 2°C/second
	2019-nCoV_N2-R	GCGCGACATTCCGAAGAA		
	2019-nCoV_N2-P	VIC-ACAATTTGCCCCCAGCGCTTCAG-ZEN/Iowa		
E	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT		
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA		
	E_Sarbeco_P	-FAM-ACACTAGCCATCCTTACTGCGCTTCG-ZEN/Iowa		
ORF1ab	ORF1ab-F	5′-CCCTGTGGGTTTTACACTTAA-3′		
	ORF1ab-R	5′-ACGATTGTGCATCAGCTGA-3′		
	Orf1ab-Probe	5′-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-ZEN/Iowa		
	JVKR	TCATTGTAATCATATTGAATACCCA		
	JVKP	(FAM-ACAACTGCAGCTTCAAAAGAAGWGT-Zen/Iowa		
InfA	InfA-F	GAC CRA TCC TGT CAC CTC TGA C	Reverse transcription at 50°C for 30 minutes, preheating at 95°C for 2 minutes followed by 45 cycles of amplification at 95°C for 15 seconds and 55°C for 30 seconds.	Reverse transcription at 50°C for 1 h, enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 94°C for 30 s and annealing at 62°C for 60 s. Followed by: by enzyme deactivation at 98°C for 10 min and droplet stabilization at 4°C for 30 min with a ramp rate of 2°C/second
	InfA-R	AGG GCA TTY TGG ACA AAK CGT CTA		
	InfA-P	FAM-TGC AGT CCT CGC TCA CTG GGC ACG-ZEN/Iowa		

4.3. Statistical Analysis

The recovery efficiency for wastewater (surface water and urine) was calculated based on the following equation:

$$\textbf{Recovery \%} = \frac{C_{SW} - C_{UW}}{C_{SC}} * \mathbf{100}$$

where C_{SW} *is* the concentration of SARS-CoV-2/Influenza A detected in spiked wastewater C_{UW} is the concentration of SARS-CoV-2/Influenza A detected in un-spiked wastewater C_{SC} is the concentration of SARS-CoV-2/Influenza A that was spiked into the wastewater. The Mann-Whitney test was used to compare the recovery percentage obtained by the two viral concentration and RNA extraction methods at a 95% confidence interval.

4.4 RESULTS AND DISCUSSION

4.4.1 Efficiency of the viral concentration and RNA extraction methods in detecting the viral targets from wastewater, surface water, urine and faecal sludge

Wastewater, surface water and urine samples were spiked with inactivated SARS-CoV-2 and Influenza A. The recovery percentage of the N2 and InfA genes was calculated taking into consideration that 60 ml of the spiked sample contained 504 and 780 copies of the N2 and InfA genes, respectively. Using the optimized PCR protocols, the recovery percentage of N2 and InfA in each sample matrix was then calculated. A recovery percentage of 80-120% was considered acceptable. Figures 4.1-4.4 show the recovery % of N2 and InfA in WW, SW and Urine samples – confirming that concentration of enveloped viruses in WW, SW, and Urine samples in this study was best achieved using ultrafiltration method as opposed to a precipitation-based method (PEG 8000)

In this study, RNA was most efficiently extracted from faecal sludge by using the TRIzol™ (Invitrogen) protocol when compared to the use of the Qiagen QiAMP Viral RNA Mini Kit or the Macherey-Nagel NucleoSpin® RNA Plus Kits. Using TRIzol™ method, the recovery efficiency of these viral targets from the faecal sludge varied from 25-35% for the InfA gene and 30-40% for the N2 gene of the SARS-CoV-2 (Figure 4.4). Based on the results, further optimization is needed for the extraction of RNA from faecal sludge in order to improve the recovery efficiency.

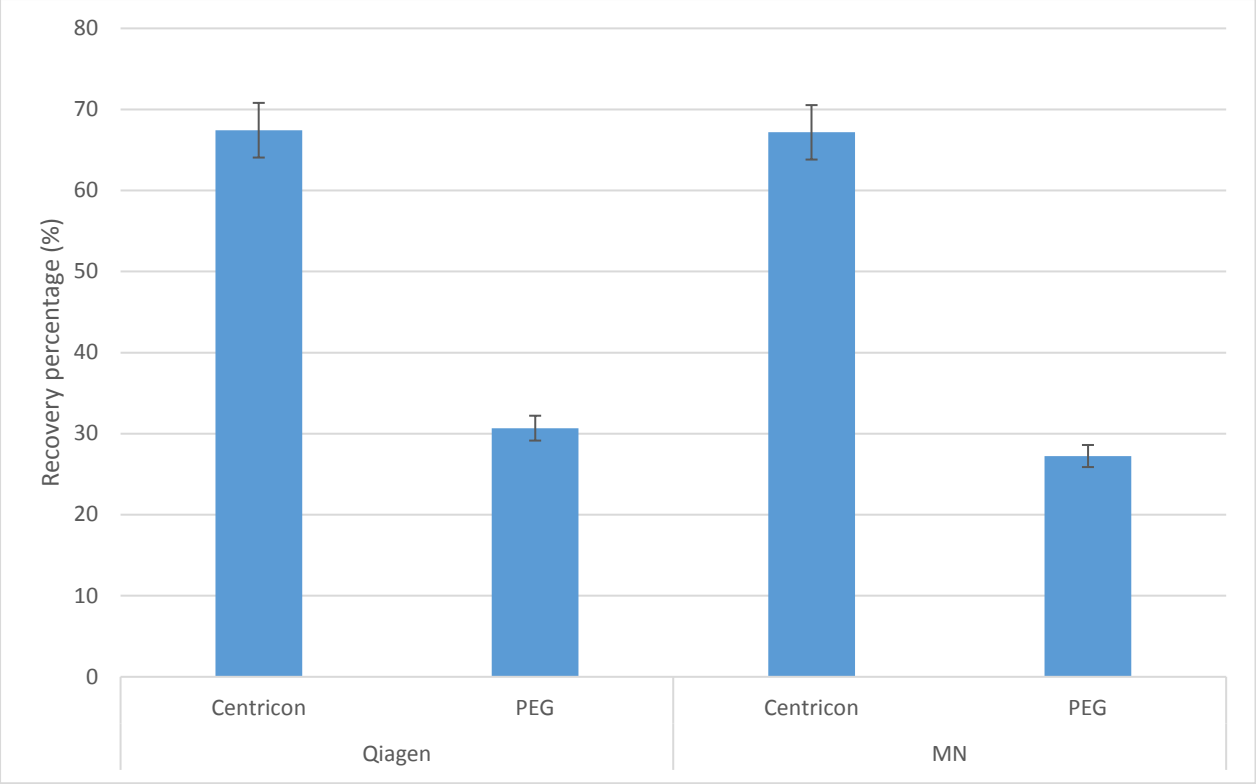

Figure 4.1: Recovery percentage (%) of InfA gene in wastewater

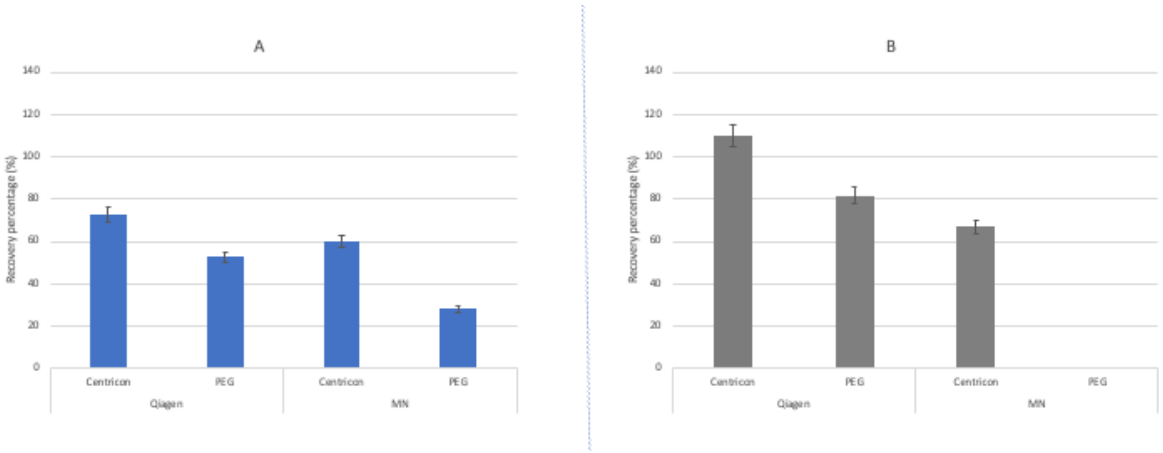

Figure 4.2: Recovery percentage (%) of InfA (A) and N2 genes (B) in surface water

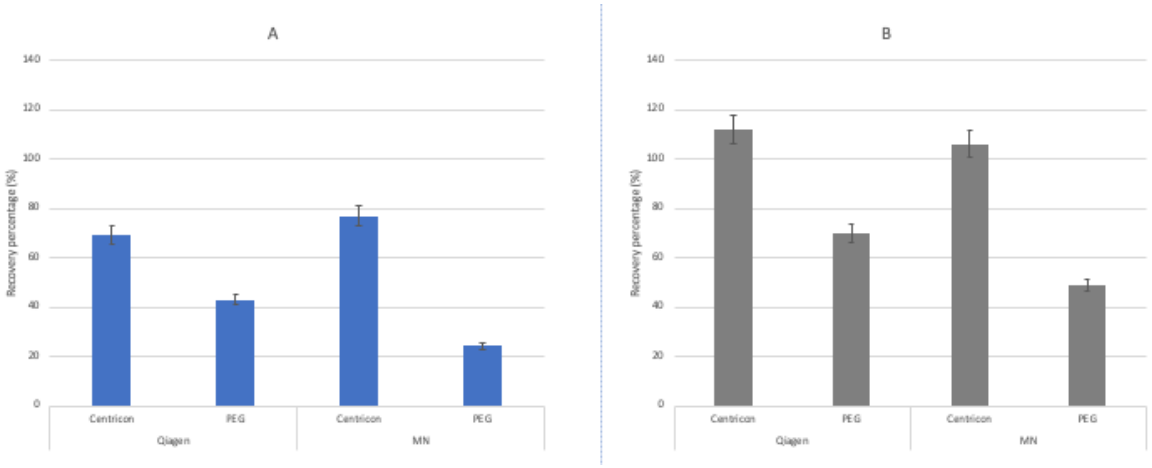

Figure 4.3: Recovery percentage (%) of InfA (A) and N2 genes (B) in urine

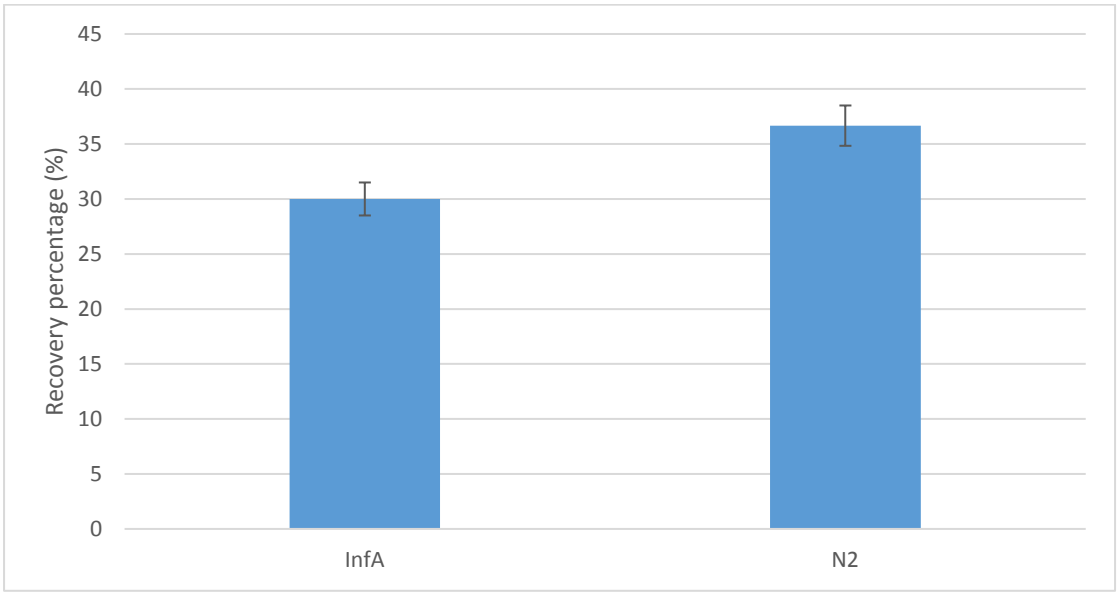

Figure 4.4: Recovery percentage (%) of InfA and N2 gene from faecal sludge

4.4.2 Determining the most suitable gene target for SARS-CoV-2 via LOD

The limit of detection for each of the gene targets of SARS-CoV-2 and Influenza A for ddPCR and qPCR are presented below in Tables 4.2 and 4.3, respectively. Based on these results, it can be deduced that ddPCR is a more sensitive detection method as it showed a lower limit of detection for all gene targets studied. For SARS-CoV-2, ddPCR was able to detect 0,2, 2, and 3,1 copies per µl of the N2, E and ORF1ab gene, respectively, while the LOD for qPCR was 13, 100, and 12 gene copies/µl for the same gene targets. For InfA, ddPCR has a LOD of 0,18 copies/µl, while qPCR failed to detect below seven copies/µl. Of the three genes for SARS-CoV-2, the N2 gene was detected most frequently and had the lowest LOD when compared to the E and ORF1ab genes – so N2 gene was chosen as the target of choice in further experiments.

The findings that the N2 gene is most abundant and most suitable for the detection of SARS-CoV-2 in samples are in accordance with other studies (Kim et al., 2020; Vogels et al., 2020).

Table 4.2: Limit of detection for SARS-CoV-2 and Influenza A as determined by ddPCR (copies per µl)

Dilution factor	Concentration of INFA gene	Concentration of N2 gene	Concentration of E gene	Concentration of ORF1AB gene
Undiluted (Stock)	53,3	105	84	76
10^{-1}	53,1	87,5	72	53
10^{-2}	48,5	62,3	64	39
10^{-3}	44,7	54,9	43,5	22,4
10^{-4}	7,61	32	22,4	12,5
10^{-5}	7,59	12,5	8	6,2
10^{-6}	0,481	0,2	2	3,1
10^{-7}	0,18	0	0	0

*InfA is the Influenza A target gene

N2, E and ORF1ab are target genes of the SARS-CoV-2 virus

Table 4.3: Limit of detection for SARS-CoV-2 and Influenza A as determined by qPCR (copies per µl)

Dilution factor	Concentration of InfA gene	Concentration of N2 gene	Concentration of E gene	Concentration of ORF1AB gene
Undiluted (Stock)	47	92	152	65
10^{-1}	27	64	117	52
10^{-2}	16	35	105	32
10^{-3}	7	10	56	12
10^{-4}	0	0	15	0
10^{-5}	-	-	0	-

*InfA is the Influenza A target gene

N2, E and ORF1ab are target genes of the SARS-CoV-2 virus

CHAPTER 5: APPLICATION OF OPTIMIZED METHODS FOR DETECTION AND QUANTIFICATION OF SARS-COV-2, INFLUENZA A AND ROTAVIRUS

5.1 BACKGROUND

Sewage monitoring or wastewater-based epidemiology might be used to track the spread of an infection in the population, including both symptomatic and asymptomatic secretors, and therefore supplement existing clinical surveillance. Furthermore, wastewater-based epidemiology (WBE) might be beneficial as an early warning system for infection (re-) emergence, epidemic detection in the community, and pathogenic strain typing in the community. This method will detect all symptomatic, pre-symptomatic, and asymptomatic carriers in a given group, which clinical surveillance does not always do (Kumar et al., 2020; Bivins et al., 2020). Cocaine use (Zuccato et al., 2005; Mao et al., 2020; Tang et al., 2020), pharmaceutical consumption (Baz-Lomba et al., 2016; He, 2020), viral infections (Hou et al., 2020), and antibiotic-resistant bacteria infections have all been tracked using the WBE approach (Hutinel et al., 2019; Castrignan et al., 2020). Its application during the current COVID-19 pandemic has received a lot of global attention as an alternative to traditional clinical surveillance systems. This chapter therefore presents the use of the optimized methods presented in Chapters 3 and 4 for the surveillance of COVID-19 and Influenza A infections in the connected communities. The information presented here highlights the applicability of the methods optimized for tracking these infections via the detection of the target nucleic materials extracted from different environmental matrices. It is also bringing the fore the potential of achieving environmental surveillance via the use of matrices such as surface water, urine and faecal sludge, which expands the scope of WBE.

5.2 METHODOLOGY

5.2.1 Application of the optimized methods for a 3-month monitoring period

To apply the optimized methods, untreated wastewater samples were collected from the Central and Isipingo wastewater treatment plants using the methods described above while urine and faecal sludge samples were collected from the previously mentioned UDDT systems. The Central WWTP has a capacity of 80 Ml/d and treats both domestic and industrial influent while the Isipingo WWTP treats only domestic influent and is substantially smaller with a capacity of 14 Ml/d. Surface water samples were collected from the Isipingo River in Durban. This river was chosen due to the reported pollution form sources such as informal settlements, and wastewater treatment plants. Sample collection was done bimonthly for a period of 3 months

(October-December 2021). The extracted RNA was tested in duplicate for the N2 and InfA genes representative of SARS-CoV-2 and Influenza A respectively.

5.2.2 Statistical analysis

Viral concentrations were captured in Microsoft Excel (Microsoft Corporation, USA) and the mean concentrations calculated per sample. Variation in viral concentration over the 3 month study period was compared via the Kruskal-Wallis tests at a 95% confidence interval.

5.3 RESULTS AND DISCUSSION

For the application study, SARS-CoV-2 was detected in wastewater samples (Figure 5.1) from both treatment plants in accordance with 4th wave of COVID-19 pandemic, while it was not detected in any surface water, urine and faecal sludge samples analysed in this study. Failure to detect SARS-CoV-2 in surface water bodies may be due to possible dilution of the viral loads in such large bodies of water. The N2 gene was also not detected in urine and faecal sludge samples except for the spiked samples. This may be due to the fact that the samples were collected from 3 households with approx. 15-20 people contributing to the sample matrix. A person would have to be infected with COVID-19 at any time within the 3-month sample period for positive identification.

Unexpectedly, the InfA gene was detected during the 3-month monitoring period in untreated wastewater from both the Central and Isipingo WWTPs (Figure 5.2). This may be because Influenza cases have been on the rise since August 2021, with a sharp increase noted in November 2021 by the National Institute for Communicable Diseases (NICD). Influenza A (H1N1) has since been detected in all 5 provinces and was the second most detected subtype and lineage in South Africa. The increase in influenza cases during the summer period (atypical) is most likely due to the relaxation of non-pharmaceutical interventions to control COVID-19 combined with an immunity gap due to influenza not circulating for the last 2 years. Although many studies have utilized the same concentration and/or extraction methods employed in this study, it is important that these methods be evaluated in the context of South African wastewater systems.

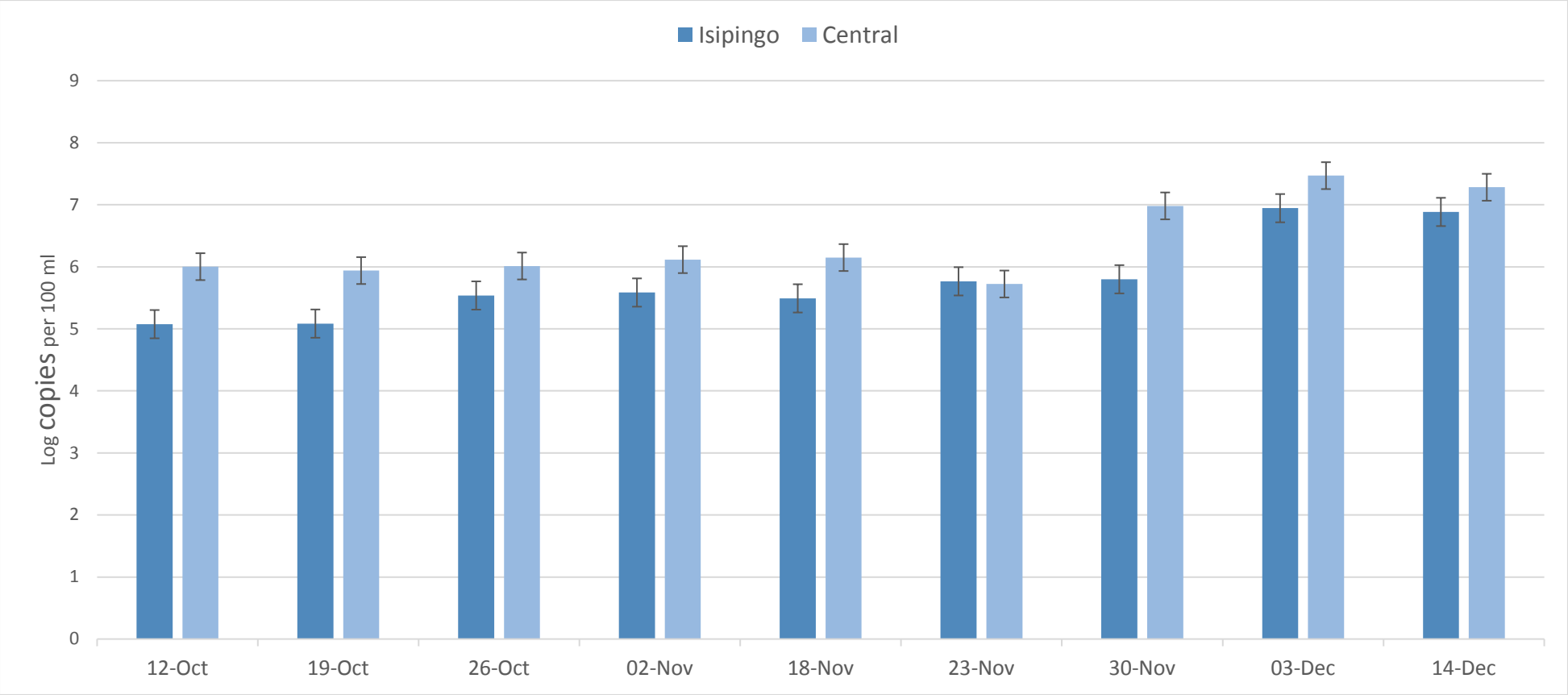

Figure 5.1: Quantification of SARS-CoV-2 (N2) in untreated wastewater over a 3-month period

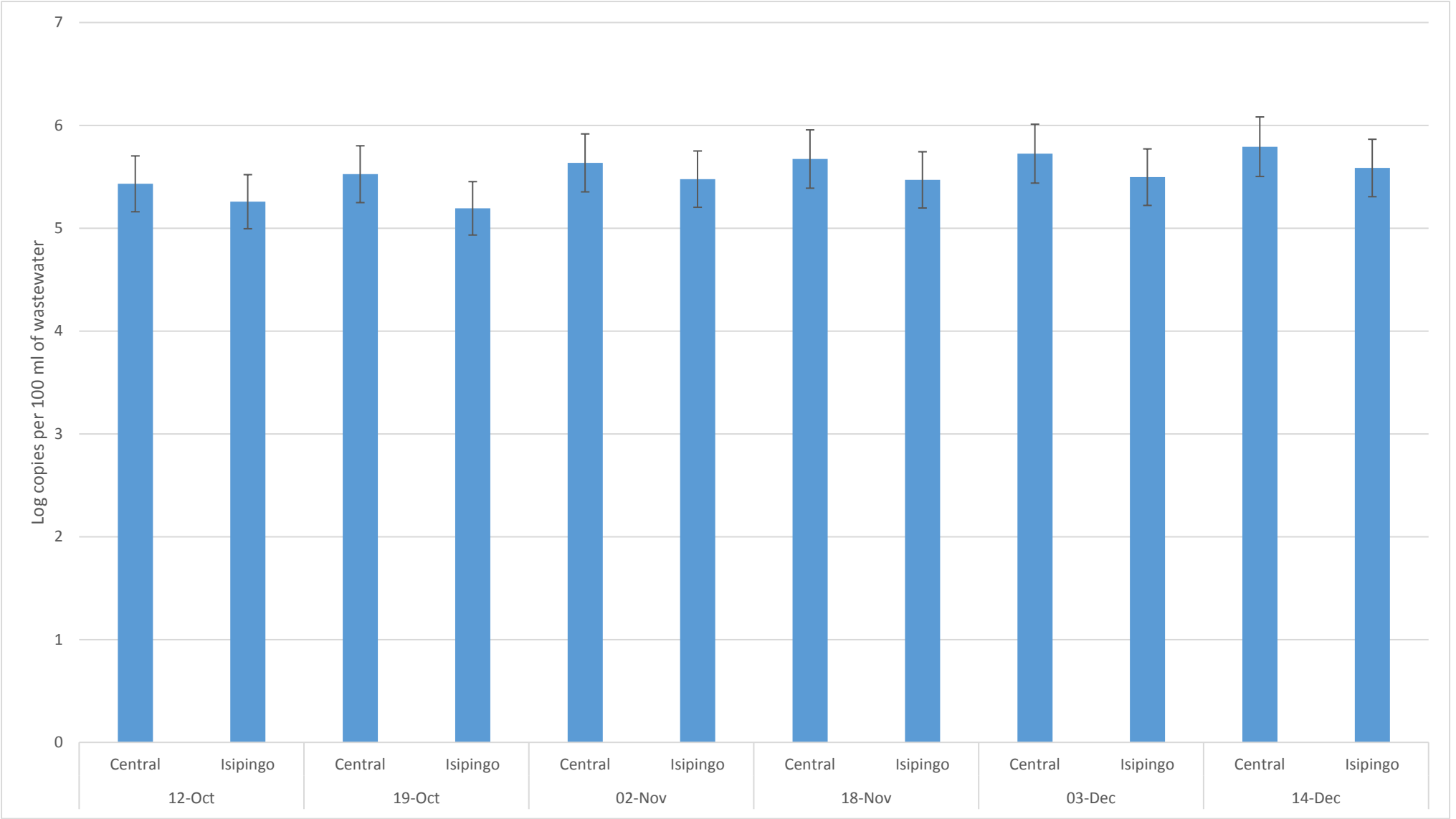

Figure 5.2: Quantification of Influenza virus (InfA) in untreated wastewater over a 3-month period

CHAPTER 6: CONCLUDING REMARKS AND RECOMMENDATIONS

The immense global burden of infectious disease outbreaks and the need to establish prediction and prevention systems have long been recognized by the World Health Organization. The central premise of the WBE approach is that community wastewater represents a snapshot of the status of public health. Wastewater analysis is equivalent to obtaining and analysing a community-based urine and faecal sample. Monitoring temporal changes in virus concentration and diversity excreted in community wastewater, in combination with monitoring metabolites and biomarkers for population adjustments could allow early detection of outbreaks (critical moments for the onset of an outbreak). However, effective disease surveillance via environmental analysis relies heavily on optimized methods for target concentration, nucleic acid extraction, detection, and quantification. This is especially critical when considering different matrices – and need to be addressed at the onset prior to considering the use of WBE for surveillance.

- This study was able to establish that the most effective viral concentration method for SARS-CoV-2 in liquid matrices, such as wastewater, surface water and urine is ultrafiltration while the best method for extraction of the viral RNA was achieved via a filter-based method (Qiagen QiAMP Viral RNA MiniKit), which was proven to outperform the precipitation-based method using PEG 8000.
- Furthermore, it was noted that for faecal sludge requires the use of alternate methods such as the TRIzol™ (Invitrogen) due to the little to no recovery of viable RNA from faecal sludge samples using commercial kits.
- Further development of RNA extraction and well as sampling methods for faecal sludge is therefore required if this avenue is explored for the purposes of WBE.
- The study was also able to demonstrate that droplet digital PCR performs better for both the detection and quantification of the viral targets in the various samples when compared to qPCR. This was based on a limit of detection of 0,2 obtained with ddPCR compared to 13 for qPCR when targeting the N2 gene of SARS-CoV-2. A similar trend was observed for the other genes of SARS-CoV-2 and the influenza A virus. However, when attempting to evaluate the effectiveness of the WBE approach, it is important that WBE data be supplemented with clinical data.

For WBE to be an effective and accurate surveillance tool, the following factors need to be taken into consideration for future research in the field:

1. **Normalization of WBE data:** Population normalization is a critical factor to consider when applying WBE to any setting. Quantification of biomarkers in wastewater would allow for an accurate estimation of the population serviced by the WWTPs via the use of statistical modelling. This in turn would provide context to the measured viral concentrations and give confirmation that changes in viral loads were not due to changes in populations. To date, creatinine, cholesterol and cortisol have been proposed as potential human biomarkers.
2. **Viral shedding and the impact of variants on viral loads:** The shedding rate is defined as the rate at which viruses are released from the body in excrement is an important factor to consider when using WBE for disease surveillance. The shedding rate depends upon the stage of infection at which a person is in as well as the severity of infection. It can also be speculated that different strains of viruses' illicit different symptoms in an individual and in turn affect the amount of viral particles released in excrement. It is therefore suggested that future studies focus on the average shedding rate of different variants of viruses.
3. **Correlation with public health records:** For a more accurate representation of the health status of a community, it is important that the relevant healthcare officials provide clinical case numbers at a district and sub-district level. Establishing correlations between wastewater data and reported clinical cases could serve as a validation for prediction models. Most jurisdictions do not have COVID-19 infection data down to the ward or street level, which means there is no data on the infection numbers within the specific catchment served by the wastewater treatment plant, which could impact the application of WBE for an effective early warning system

REFERENCES

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J. and Tscharke, B., 2020. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728, 138764.

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J.W., Choi, P.M., Kitajima, M., Simpson, S.L., Li, J. and Tscharke, B., 2020. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728, 138764.

Fumian, T.M., Leite, J.P.G., Castello, A.A., Gaggero, A., de Caillou, M.S.L, and Miagostovich, M.P., 2010. Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration. J. Virol. Methods, 170 (2010), pp 42-46.

Gerba, C. P., 2000. Assessment of enteric pathogen shedding by bathers during recreational activity and its impact on water quality. Quant. Microbiol. Pp. 55-68.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. and Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ. Sci. Technol. Lett., 7(7), 511-516.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. and Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. Environ. Sci. Technol. Lett., 7(7), 511-516.

Pillay, L., Amoah, I.D., Deepnarain, N., Pillay, K., Awolusi, O.O., Kumari, S., Bux, F., 2021. Monitoring changes in COVID-19 infection using wastewater-based epidemiology: A South African perspective. Sci. Total Environ., 786, 147273.

Suo, T., Liu, X., Feng, J., Guo, M., Hu, W., Guo, D., Ullah, H., Yang, Y., Zhang, Q., Wang, X. and Sajid, M., 2020. ddPCR: a more sensitive and accurate tool for SARS-CoV-2 detection in low viral load specimens. medRxiv https://doi.org/10.1101/2020.02.29.20029439

Vogels, C.B.F., Brito, A.F., Wyllie, A.L. et al., 2020. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT-PCR primer-probe sets. *Nat Microbiol* **5,** 1299-1305.

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., Wang, B., Xiang, H., Cheng, Z., Xiong, Y. and Zhao, Y., 2020. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *Jama*, *323*(11), pp.1061-1069.

WHO-World Health Organization, 2021. Middle East respiratory syndrome coronavirus (MERS-CoV). Available at https://www.who.int/emergencies/mers-cov/en/. Accessed on the 30th of April, 2021

Wurtzer, S., Marechal, V., Mouchel, J.M. and Moulin, L., 2020. Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlates with COVID-19 confirmed cases. *medRxiv*.

Yeo, C., Kaushal, S. and Yeo, D., 2020. Enteric involvement of coronaviruses: is faecal-oral transmission of SARS-CoV-2 possible?. *The Lancet Gastroenterology & Hepatology*, *5*(4), pp.335-337

Chu, D. K., Pan, Y., Cheng, S. M., Hui, K. P., Krishnan, P., Liu, Y., ... Poon, L. L. (2020). Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clinical chemistry*, *66*(4), 549-555.

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., ... Drosten, C. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance*, *25*(3), 2000045.

Xu, M. Y., Liu, S. Q., Deng, C. L., Zhang, Q. Y. & Zhang, B. (2016). Detection of Zika virus by SYBR green one-step real-time RT-PCR. *Journal of virological methods*, *236*, 93-97.

Zhao, Z., Cui, H., Song, W., Ru, X., Zhou, W. & Yu, X. (2020). A simple magnetic nanoparticles-based viral RNA extraction method for efficient detection of SARS-CoV-2. *BioRxiv*.

Ng, E. K., Hui, D. S., Chan, K. A., Hung, E. C., Chiu, R. W., Lee, N., ... Lo, Y. D. (2003). Quantitative analysis and prognostic implication of SARS coronavirus RNA in the plasma and serum of patients with severe acute respiratory syndrome. *Clinical Chemistry*, *49*(12), 1976-1980.