Influence of storage conditions and sample processing on viral particle recovery from untreated municipal wastewater

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Submitted in fulfilment of the requirements of the degree of Master of Applied Sciences: Biotechnology Faculty of Applied Sciences

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APPROVAL

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This 1st day of March 2024 at the Durban University of Technology.

DECLARATION

I hereby declare that this thesis entitled **"Influence of storage conditions and sample processing on viral particle recovery from untreated municipal wastewater"** submitted for the degree of Master's in Applied Sciences: Biotechnology at the Durban University of Technology:

1. This is my original work and has not been submitted for a degree at any other university.
2. I further declare that a detailed reference list has been provided on all the sources cited or quoted

01/03/2024

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DEDICATION

To my loved ones that are no longer with us.

To my family, and every individual that has been instrumental in my academic journey.

Thank you for your love and support throughout this journey.

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I humbly acknowledge the divine presence of God, through whom all things are possible. My faith in you has been a source of comfort and resilience through this journey.

I thank my family and friends for their support and understanding. Mom, you have been my pillar of strength and my greatest motivation. I am eternally grateful for your love, patience, support, and the countless sacrifices you have made. Thank you for always believing in me. To my dad and grandmother, I am grateful for your love and encouragement. To my sister, Malisha Pyladh, thank you for always being by my side, and for being my shoulder to cry on. To my nieces and nephews, through this journey of academic pursuit, your presence and the hope for a brighter future have fuelled my determination.

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ABSTRACT

The emergence and re-emergence of several epidemic and pandemic related pathogens has highlighted the need for the development and implementation of rapid and sensitive disease surveillance tools. Wastewater-based epidemiology (WBE) is one such environmental surveillance tool that has been adapted to provide a near real-time reflection of a population's health dynamics through detecting and quantifying biomarkers from untreated wastewater. This information can be used to monitor disease progression, detect infection hotspots as well as emerging variants. The success of WBE, however, is determined by the accuracy of pathogen detection and quantification methods. Various factors, such as transportation, storage conditions, and wastewater characteristics can affect the quantification, leading to unreliable results. This study aimed at investigating the effects of storage temperatures on SARS-CoV-2 and influenza A degradation in raw wastewater samples and extracted nucleic acids. Additionally, different pre-treatment strategies for improving viral recovery from wastewater solids were also examined as part of the optimization process. Municipal wastewater influent was collected and stored at 25°C, 4°C, -20°C, and -80°C for 84 days. A control sample (spiked with attenuated viruses) was also stored under the same conditions. The viral RNA was extracted and quantified from both the samples (stored wastewater and control) every two weeks. Additionally, the physicochemical characteristics of the wastewater at different temperatures were evaluated for their possible impact on degradation.

Supernatant samples and solid particles were used separately for RNA extraction and quantification to assess viral attachment to solid particles. Degradation of influenza A was observed in all samples with the highest at ±25°C in the supernatant (88.89%) and pellet (83.47%) of the wastewater influent, ±25°C in the spiked supernatant (90.69%) and 4°C in the spiked pellet (92.64%). The highest degradation of SARS CoV-2 was observed at ±25°C in the supernatant (94.1%) and pellet (92.66%) of the wastewater influent, 4°C in the spiked

supernatant (95.57%) and -20°C in the spiked pellet (50.79%). The lowest degradation of SARS-CoV-2 in the spiked pellet was observed at ±25°C (44.82%) and 4°C (31.53%). This may be indicative of viral adhesion to wastewater solids. The assessment of the physicochemical characteristics indicated of changes in stored wastewater samples. Salinity, DO, pH, COD, TS, and TFS were correlated with viral degradation. pH was also found to be correlated with viral particles attaching to wastewater solids.

The effect of storage temperature on stored RNA was also studied using the extracted RNA for a period of 168-days. The degradation between -20°C and -80°C was significantly different for RNA storage. The lowest degradation of SARS-CoV-2 occurred at -80°C (32.51% - wastewater influent; 33.42% - viral controls). In the case of influenza A, the lowest degradation occurred at -80°C for wastewater influent (43.28%) and -20°C for viral controls (36.02%). Statistical analysis conducted comparing storage of wastewater influent to extracted RNA indicated that degradation was higher in the wastewater samples.

Additionally, to enhance viral recovery from wastewater solids, sodium pyrophosphate and ultrasonication were explored as pre-treatments strategies. SARS-CoV-2 concentrations in the supernatant were significantly increased between 3.30 - 35.65% by ultrasonication at frequencies ranging from 4 to 16 kHz, indicating that significant amounts of RNA may be attached to solid particles based on the contact time. These results highlight the importance of additional pre-treatment methods for maximizing RNA recovery from wastewater samples. The findings of this research may contribute significantly to the improvement of WBE detection methods for disease surveillance.

PREFACE

Research outputs

Sueyanka Subroyen, Leanne Pillay, Sheena Kumari, and Faizal Bux. 2024. Impact of storage conditions and enhancement strategies on viral biomarker recovery for WBE applications. *WISA 2024 Biennial Conference & Exhibition.* **(Submitted)**

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LIST OF ABBREVIATIONS

AT	-adenine-thymine
COVID-19	– corona virus disease 2019
DDPCR	– droplet digital polymerase chain reaction
DNA	– deoxyribonucleic acid
DO	– dissolved oxygen
dsDNA	– double stranded DNA
dsRNA	– double stranded RNA
E	– envelope protein
EC	– electrical conductivity
GC	-guanine-cytosine
H1N1	– influenza A
HA	– hemagglutinin
M	– membrane
M1	– matrix proteins 1
M2	– matrix proteins 2
MERS	– Middle-Eastern respiratory syndrome
N	– nucleocapsid

NA – neuraminidase

NP – nucleoprotein

NS – non-structural proteins

Nsp – non-structural proteins

ORF – open reading frame

PA – polymerase acidic

PB – polymerase basic

PCR – polymerase chain reaction

PEG – polyethylene glycol

qPCR – quantitative polymerase chain reaction

RNA – ribonucleic acid

S – spike protein

SARS-CoV-2 – severe acute respiratory coronavirus 2

ssRNA – single stranded RNA

TDS – total dissolved solids

TFS – total fixed solids

TS – total solids

WBE – wastewater-based epidemiology

WHO – World Health Organisation

CHAPTER 1 : INTRODUCTION

The unpredictable powers of nature driving the evolution of pathogenic microorganisms poses a significant threat to public health (WHO, 2018). Since the 1970s, more than 1500 new pathogens and more than 40 new infections have been identified (WHO, 2018; Sims and Kasprzyk-Hordern, 2020). In the early 2000s, humankind faced several disease outbreaks that resulted in severe epidemics, including Ebola, Zika, H1N1 (influenza A), SARS (severe acute respiratory syndrome), MERS (Middle-Eastern respiratory syndrome), and most recently COVID-19 (Mao *et al.*, 2020). Early detection and monitoring of pathogens through clinical testing is essential in preventing outbreaks (Hata and Hondo, 2020; Oran and Topol, 2020). However, in many parts of the world this is merely impossible due to the fact that only individuals exhibiting symptoms or those who are extremely ill seek medical attention resulting in the underreporting of disease prevalence (Zahedi *et al.*, 2021).

The recent global COVID-19 pandemic caused by the etiological agent SARS-CoV-2 was first reported in Wuhan, China (Wu *et al.*, 2020). In December 2019, there were several reports of a severe pneumonia which was later confirmed by the World Health Organization to be caused by a new variant of corona virus which they later named as SARS-CoV-2 (Zahid and Perna, 2021). Due to its highly infectious nature, by March 2020, it had spread to 6 continents infecting more than 10 000 individuals and resulting in more than 160 deaths (Wu *et al.*, 2020). As at 08 October 2023, there have been 6,961,014 deaths globally, as reported by WHO (WHO, 2023). Clinical manifestations ranged from asymptomatic or mild cases with cough, fever, and headache to extremely fatal cases with pneumonia, and organ failure (Aguiar-Oliveira *et al.*, 2020). Approximately 45% of SARS-CoV-2 infections were asymptomatic leading to inaccuracy in public health surveillance (Oran and Topol, 2020). This posed a challenge to infection control as an undetected route for the potential transmission of the virus (Al-Tawfiq,

2020; Hata and Hondo, 2020). This has also contributed to the evolutionary changes of the virus increasing its threat to public health (Tegally *et al.*, 2021).

The virus is shed in bodily excreta (e.g. saliva, sputum, and fecal matter) by both symptomatic and asymptomatic individuals which ultimately enters the sewage network (Hata and Hondo, 2020; Oran and Topol, 2020). Due to the presence of high viral RNA in fecal matter, much attention has been given to sewage surveillance for the epidemiological monitoring of SARS-CoV-2 to be used as a complementary approach to clinical surveillance as an early warning system for disease outbreaks (Mauro *et al.*, 2020; Pillay *et al.*, 2021; Zahedi *et al.*, 2021).

Wastewater based epidemiology (WBE) is a concept based on extraction, detection, and subsequent analysis and interpretation of biomarkers from wastewater that are either biological or chemical in nature. The identification and quantification of human biomarkers (of endogenous and exogenous origin) in wastewater has the potential to provide an indication of a populations health trends in real-time (Sims and Kasprzyk-Hordern, 2020). Furthermore, it has been proposed that the surveillance of SARS-CoV-2 through WBE for monitoring the emergence of variants of concern may be a more suitable approach than whole genome sequencing (Heijnen *et al.*, 2021; Izquierdo-Lara *et al.*, 2021). During the course of the pandemic, SARS-CoV-2 has constantly and consistently mutated resulting in several lineages of the virus. These variants poses a public health concern due to mutations that increase transmissibility, illness severity, ability to elude immune responses, or reduced susceptibility to vaccination (Walensky *et al.*, 2021).Variant surveillance is a key factor in understanding the dynamics of transmission within communities to implement suitable measures for public health protection (Heijnen *et al.*, 2021). Whilst the complexity of the matrix may pose a challenge, several studies have successfully implemented WBE in the monitoring of variants thereby supporting its application for the monitoring of the spatiotemporal changes in the presence of variants (Heijnen *et al.*, 2021; Izquierdo-Lara *et al.*, 2021; Boogaerts *et al.*, 2022).

Studies have shown that the structure of the virus may play a role in their persistence and infectivity in the environment (Corpuz *et al.*, 2020; Polo *et al.*, 2020). The enveloped viruses comprise of a nucleocapsid (nucleic acid and capsid) that is enclosed by a lipid layer which is extremely susceptible to environmental stress (Tsen and Tsen, 2016; Polo *et al.*, 2020). However, non-enveloped viruses comprising only of a nucleocapsid, have a high resistance to harsh environmental conditions such as high temperatures, low pH, and other oxidants, ensuring their survival for prolonged periods of time in environmental settings (Polo *et al.*, 2020).

Pre-COVID-19, wastewater surveillance studies were limited to human enteric viruses, with the onset of the pandemic, this scope was broadened to include respiratory viruses such as SARS-CoV-2 and influenza A (Farkas *et al.*, 2020; Waseem *et al.*, 2023). Influenza A, like SARS-CoV-2 is known for being a causative agent of global pandemics (Dumke *et al.*, 2022). During the COVID-19 pandemic, the concept of WBE has been extensively explored for its successful application as a disease surveillance tool (Ahmed *et al.*, 2020a; Pillay *et al.*, 2021). Several studies have focused on the optimization of sampling techniques, viral concentration methods (Ahmed *et al.*, 2020c), extraction and quantification methods (Pillay *et al.*, 2022) etc. There are however important considerations for the effective implementation of WBE as a disease surveillance tool. The need to store a large number of samples during COVID-19 was one of the major challenges due to the unavailability of reagents or due to the lockdown restrictions. There have been a few studies conducted on the impact of different storage conditions on the degradation of samples (Ahmed *et al*., 2020b; Hokajärvi *et al.*, 2021). A lack of understanding, however, exists with regard to the impact of sample matrix on the degradation process at different temperatures during long-term storage of wastewater samples. This study focused on understanding the influence of different storage conditions (temperature and time) on the degradation of enveloped viral particles (SARS-CoV-2 and influenza A) from

wastewater. The commonly employed storage temperatures (4°C, -20°C, and -80°C) and the time of storage (180 days) on viral nucleic acid degradation using polyethylene glycol 6000 (PEG) precipitation and subsequent droplet digital PCR quantification was evaluated. Additionally, since previous studies have found high concentrations of SARS-CoV-2 in wastewater solids (Hokajärvi *et al.*, 2021), mechanical (ultrasonication), and chemical (sodium pyrophosphate) pre-treatment methods were explored to enhance viral recovery from wastewater solids. In addition to this, the impact of storage temperature and time (168 days) on RNA degradation/quality was evaluated.

1.1. Aim

Investigation of the impact of storage and sample treatment on the recovery and degradation patterns of SARS-CoV-2 and influenza A RNA from wastewater influent for developing an efficient wastewater surveillance program

1.2. Objectives

- To determine the effect of storage temperature and incubation time on viral nucleic acid from wastewater influent samples
- To optimize different pre-treatment methods for maximum viral recovery from the solid fraction of the wastewater influent
- To determine the impact of storage conditions on the integrity of extracted viral RNA using droplet digital PCR

1.3. Thesis Structure

This thesis consists of five main chapters:

- **Chapter 1**: Introduction, aim, and research objectives.
- **Chapter 2:** Literature and background information on wastewater-based epidemiology, the presence and persistence of viruses in wastewater, the application of WBE as a

disease surveillance tool, the factors affecting viral stability and quantification in wastewater, and the impact of storage and sample processing on viral quantification using WBE.

- **Chapter 3:** In this chapter, the results from objectives 1 and 2 are combined into a technical chapter and focuses on assessing the impact of storage parameters and physicochemical characteristics of wastewater on viral particles degradation as well as the enhancing viral recovery using pre-treatment.
- C**hapter 4:** The results of objective 3 have been presented in this technical chapter, which examines the impact of storage conditions on the integrity of extracted viral RNA using droplet digital PCR
- **Chapter 5:** General conclusions and recommendations.

CHAPTER 2 : LITERATURE REVIEW

Wastewater is a complex matrix comprising of several colloidal, soluble, and suspended constituents such as metals, heavy metals, nutrients, pathogenic and non-pathogenic microorganisms, and various micro pollutants (McCall *et al.*, 2016). The quantitative and qualitative analysis of wastewater to detect the presence of excreted human metabolites as well as parent compounds, referred to as biomarkers, proves to be an inexpensive and non-intrusive method to provide near real-time reflection of a populations dynamics and health (Choi *et al.*, 2018; Sims and Kasprzyk-Hordern, 2020).

2.1.WASTEWATER-BASED EPIDEMIOLOGY

2.1.1. The concept and evolution of WBE

The concept of environmental surveillance of wastewater, known as WBE, was first introduced in 2001 by Daughton as a tool for monitoring drug residues in wastewater (Daughton, 2001). The use of WBE for the surveillance of chemical and biological compounds is based on the initial raw wastewater sample collection, and sample preparation which includes a centrifugation or filtration step for the removal of solid matter (Lorenzo and Picó, 2019). The identification process varies depending on the target (either chemical or biological markers). For the detection and quantification of chemical markers, instruments such as ultra-high performance liquid chromatography, liquid chromatography-mass spectrometry, and gas chromatography-mass spectrometry are being used (Lorenzo and Picó, 2019; Sims and Kasprzyk-Hordern, 2020; Picó and Barceló, 2021). The biological markers, mainly includes extraction of the nucleic acids from wastewater and subsequent detection or identification of the targets using molecular techniques such as polymerase chain reaction (PCR), quantitative PCR (qPCR), droplet digital PCR (ddPCR), or sequencing (Abdeldayem *et al.*, 2022).

2.1.2. Applications of WBE

The first use of WBE was to monitor the use of cocaine in a community by detecting concentrations of the parent compound i.e. cocaine and the metabolite i.e. benzoylecgonine (Zuccato *et al.*, 2005). Other illicit drug consumption that were monitored through WBE include heroin, cannabis, ketamine, new psychoactive substances (e.g. synthetic cannabinoids, psychedelics, cathinone's, etc.), and performance enhancing drugs (Choi *et al.*, 2018). Licit drugs (caffeine, alcohol, and tobacco), pharmaceutical (antimicrobials, antidepressants, opioids, etc.) and personal care products, and industrial chemicals (pesticides, flame retardants, etc.) have also been monitored through WBE (Choi *et al.*, 2018; Gracia-Lor *et al.*, 2018).

In recent years, WBE has been adapted as a public health surveillance tool that monitors both endogenous and exogenous human biomarkers related to various diseases such as diabetes and cancer as well as their specific treatments (Gracia-Lor *et al.*, 2017). The application of WBE was expanded to focus on the nucleic acid amplification for the monitoring of antibiotic resistance trends, metabolic disorders, and epidemiological studies for emerging pathogens (Gracia-Lor *et al.*, 2017; Choi *et al.*, 2018; Gracia-Lor *et al.*, 2018). In more recent years, WBE has gained widespread attention as a disease surveillance tool, especially during the most recent COVID-19 pandemic (Choi *et al.*, 2018; Lorenzo and Picó, 2019). WBE offers several advantages over clinical testing such as its capability to quickly detect disease outbreaks, monitor population infection trends in near-real time, estimation of cases, assess the effectiveness of infection control interventions, identifying emerging variants, and monitoring for variants of concern (Sims and Kasprzyk-Hordern, 2020; Ahmed *et al.*, 2021). Early research conducted using similar approaches to WBE, identified viral strains including human adenovirus, hepatitis A virus, hepatitis E virus, noroviruses, and rotavirus within communities supporting the application of WBE in epidemiological studies as a monitoring and early warning system for disease outbreaks (Kokkinos *et al.*, 2011; Hellmér *et al.*, 2014; Gracia-Lor

et al., 2017). A study by Kazama *et al.* (2017) had successfully associated clinically reported cases of gastroenteritis with norovirus concentrations measured in sewage samples by performing a cross-correlation analysis over a 3-year period. Additionally, the results of the study revealed variants which were detected in sewage 1 year prior to its identification in clinical cases, proving the importance of wastewater surveillance as an early warning system in monitoring for disease outbreaks (Kazama *et al.*, 2017).

2.2.THE PRESENCE AND PERSISTANCE OF VIRUSES IN WASTEWATER

2.2.1. Sources and types of viruses in wastewater

Since the discovery of the yellow fever virus in 1901, more than 219 species of viruses capable of infecting humans have been discovered, with the number increasing each year (Woolhouse *et al.*, 2012; Mirza *et al.*, 2021)., These pathogens are classified based on their morphology (i.e. enveloped or non-enveloped) and genomes (i.e. DNA or RNA) (Mirza *et al.*, 2021). Non-enveloped viruses, which comprise only of nucleic acid and a capsid (nucleocapsid), have a high resistance to harsh environmental conditions such as high temperatures, low pH, and other oxidants, ensuring their survival for prolonged periods of time in environmental settings (Polo *et al.*, 2020). Many non-enveloped viruses are waterborne enteric pathogens that are commonly detected in wastewater (Ibrahim *et al.*, 2021). In contrast, enveloped viruses consist of a nucleocapsid that is enclosed by a lipid layer that is extremely susceptible to damage by environmental stress (Tsen and Tsen, 2016; Polo *et al.*, 2020). Clinical data indicates that enveloped viruses are shed into wastewater systems by means of the excretion of bodily fluids of infected individuals, although, viral titers are low (Silverman and Boehm, 2021). The protein envelope makes these viruses extremely susceptible to degradation in wastewater due to the harsh nature of the matrix (Wigginton *et al.*, 2015; Tsen and Tsen, 2016; Polo *et al.*, 2020). RNA viruses are a major concern due to their ability to rapidly mutate with high error rates (Jiang *et al.*, 2023). Many of these viruses, such as rotavirus, hepatitis virus, and enterovirus

are waterborne pathogens. However, several viruses such as Zika virus and Ebola, that may be classified as zoonotic viruses, are non-waterborne pathogens but have been detected in wastewater (Xagoraraki and O'Brien, 2019; Bhatt *et al.*, 2020).

2.2.2. Pandemic-related pathogens in wastewater

Majority of the wastewater surveillance studies thus far has focused on human enteric viruses. However, since the recent COVID-19 pandemic, viral surveillance of wastewater has been broadened to include the surveillance of respiratory viruses such as SARS-CoV-2 (Farkas *et al.*, 2020). Another major pandemic that mankind encountered was the influenza A virus (H1N1) pandemic of 2009, both pandemics being caused by enveloped, respiratory viruses. Furthermore, coronaviruses were responsible for 3 pandemics in the last 2 decades (Jiang *et al.*, 2023), and influenza viruses were the causative agent of 3 pandemics in the last century (Monto and Fukuda, 2017). Wastewater surveillance of respiratory viruses were previously overlooked since the virus is spread by means of person-to person contact as opposed to transmission via the fecal-oral route, as in the case of enteric viruses. However, given the severity of the COVID-19 pandemic, it's important to explore wastewater surveillance as an early warning system for different virus types. Nonetheless, this shows the viability of WBE as an early warning system.

2.2.3. Structure of influenza A and SARS-CoV-2

Influenza A is a negative sense, single-stranded RNA virus, with a genome consisting of 8 RNA segments encoding 10 structural and non-structural proteins as shown in Figure 2.1.A (Piret and Boivin, 2021). These proteins include the envelope proteins, hemagglutinin (HA) and neuraminidase (NA), polymerase proteins, namely polymerase acidic (PA), polymerase basic 1 (PB1) and 2 (PB2), matrix proteins 1 (M1) and 2 (M2), nucleoprotein (NP), and the non-structural proteins 1 (NS1) and 2 (NS2) (Hao *et al.*, 2020).

SARS-CoV-2 is a positive sense, single-stranded RNA virus with a genome size of approximately 30 kb with around 29 900 nucleotides, making it the largest RNA virus (Wang *et al.*, 2020b; Szczesniak *et al.*, 2023). It comprises of fourteen open reading frames (ORF) making up two thirds of the viral genome, which are separated into two categories i.e. ORF1a and ORF1ab, responsible for the translation of two polyproteins (i.e. pp1a and pp1ab) (Yan *et al.*, 2022). These code for 2 proteases, namely papain-like proteases and main-protease, which are responsible for the production of sixteen non-structural proteins (Nsp), i.e. Nsp1 to Nsp16 (Arya *et al.*, 2021). The last third of the viral genome encodes for the structural proteins of the viral particles, i.e. spike (S), nucleocapsid (N), membrane (M), and envelope (E) as shown in Figure 2.1. B.

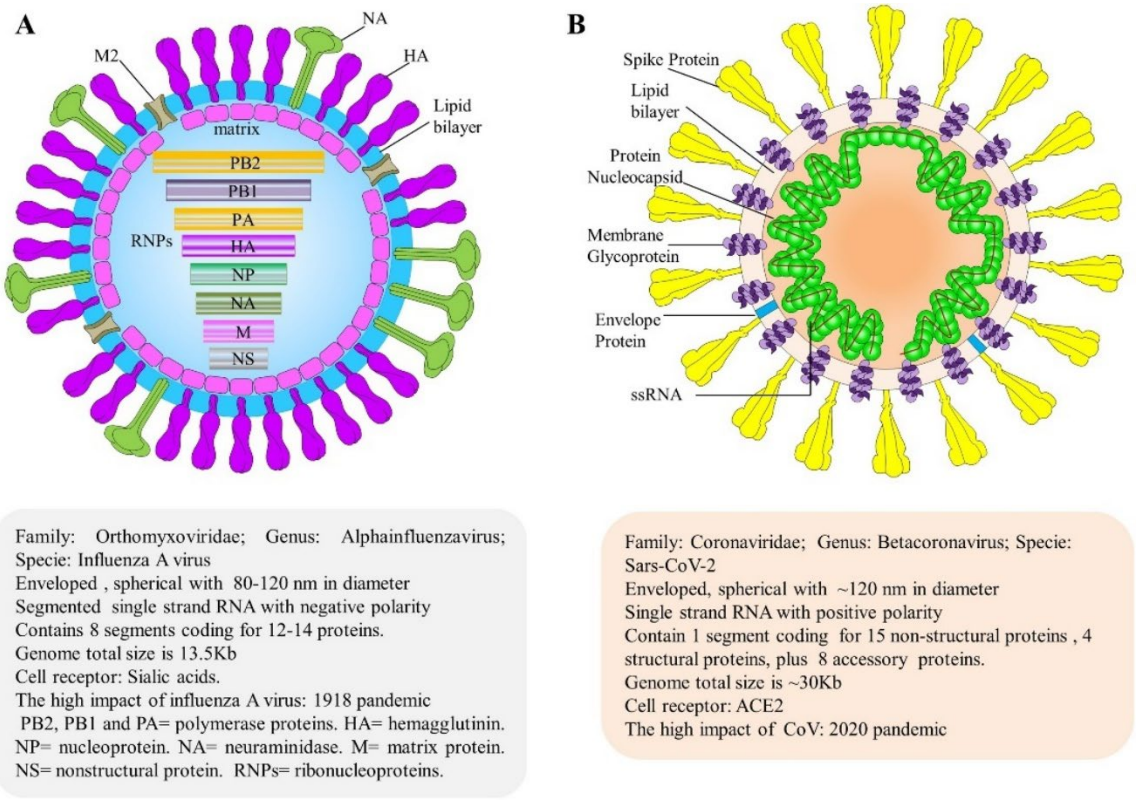

Figure 2.1: Viral structure and genome of (A) – influenza A and (B) – SARS-CoV-2 (Costa *et al.*, 2020)

2.3.WBE AS A DISEASE SURVEILLANCE TOOL

In wastewater, viruses are present in low concentrations which necessitates the addition of a primary concentration step. This will allow for large volumes of wastewater to be concentrated for a more accurate quantification (McMinn *et al.*, 2021). Primary concentration is usually conducted following a preconditioning method, which is generally centrifugation for the removal of larger solid particles and debris (Sapula *et al.*, 2021). Commonly used primary concentration techniques for viral particles in wastewater include aqueous polymer separation using polyethylene glycol (PEG), ultracentrifugation, electronegative membrane filtration, electropositive membrane filtration, and ultrafiltration (Lu *et al.*, 2020a). It is important to note that these techniques were initially used for the recovery of non-enveloped enteric viruses (Ye *et al.*, 2016; Lu *et al.*, 2020a). Ye *et al*. (2016) showed that PEG, ultrafiltration, and ultracentrifugation had a significantly lower recovery of enveloped viruses when compared to non-enveloped viruses. However, since the beginning of the COVID-19 pandemic, significant progress has been made in adapting these techniques for optimum enveloped virus recovery (Lu *et al.*, 2020a). A comparison of the concentration methods for different types of viruses is summarized in Table 2.1. Viral recovery is followed by nucleic acid extraction and subsequent detection as described for biological markers. Based on this, several studies have demonstrated the efficacy of WBE as both a predictive and surveillance tool for the monitoring of disease outbreaks (Kokkinos *et al.*, 2011; Hellmér *et al.*, 2014; Kazama *et al.*, 2017; Medema *et al.*, 2020; Polo *et al.*, 2020; Sims and Kasprzyk-Hordern, 2020; Ahmed *et al.*, 2021; Pillay *et al.*, 2021).

Table 2.1: Summary of concentration methods used for enveloped and non-enveloped pathogenic viruses detected in wastewater

Virus	Viral Structure	Genome	Fecal shedding	Concentration method	Concentration and/or persistence in wastewater	References
Influenza A	Enveloped	Negative sense, ssRNA	Yes	Ultrafiltration	-	(Heijnen and Medema, 2011; Hutchinson, 2018)
Ebola	Enveloped	Negative sense, ssRNA	Yes	-	1 day	(Elsamadony *et al.*, 2021)
Zika	Enveloped	Positive sense, ssRNA	Yes	-	-	(Musso and Gubler, 2016; Xagoraraki and O'Brien, 2019; Muirhead *et al.*, 2020)

SARS-CoV-1	Enveloped	Positive sense, ssRNA	Yes	Positive charged filter media PEG elution	2-3 days	(Gundy *et al.*, 2009; Elsamadony *et al.*, 2021)
SARS-CoV-2	enveloped	Positive sense, ssRNA	Yes	PEG elution Electronegative filtration Ultrafiltration Ultracentrifugation	Approximately 10^5 – $10^{6.5}$ copies per liter	(Lu *et al.*, 2020a; Elsamadony *et al.*, 2021)
Adenovirus	Non-enveloped	dsDNA	Yes	Ultrafiltration	Approximately 10^8 copies per liter	(Xagoraraki and O'Brien, 2019; Jahne *et al.*, 2020; Elsamadony *et al.*, 2021)

Hepatitus A virus	Non-enveloped	Positive sense, ssRNA	Yes			(Xagoraraki and O'Brien, 2019; Elsamadony *et al.*, 2021)
Rotavirus	Non-enveloped	dsRNA	Yes		Approximately 10^7 copies per liter	(Xagoraraki and O'Brien, 2019)
Enterovirus	Non-enveloped	Positive sense, ssRNA	Yes	PEG elution Glass wool filters		(Pennino *et al.*, 2018; Xagoraraki and O'Brien, 2019)
Norovirus	Non-enveloped	Positive sense, ssRNA	Yes	Ultrafiltration	Approximately 10^9 copies per liter	(Xagoraraki and O'Brien, 2019; Jahne *et al.*, 2020; Elsamadony *et al.*, 2021)

2.3.1. Adaption for WBE for the surveillance of enveloped virus

Early studies on disease surveillance using WBE looked at non-enveloped viruses. With the recent COVID-19 pandemic, there have been reports of SARS-CoV-2 RNA being detected several days before the first reported clinical case (Medema *et al.*, 2020; Ahmed *et al.*, 2021). Additionally, Wu *et al*. (2020) showed viral concentrations in wastewater indicated a higher number of infections than clinically confirmed cases (Wu *et al.*, 2020). This can be attributed to asymptomatic cases. Whilst an asymptomatic patient may not display symptoms, viral RNA is still shed in the excreta of the infected individual (Aguiar-Oliveira *et al.*, 2020; Gonçalves *et al.*, 2021). Hence, the presence of viral nucleic acids in wastewater proves to be a valuable tool for the surveillance of novel viruses and monitoring of disease outbreaks (Zahedi *et al.*, 2021).

Since the start of the COVID-19 pandemic, wastewater surveillance has gained much attention for the monitoring of SARS-CoV-2. Initial clinical testing involved molecular detection of SARS-CoV-2 isolated from nasopharyngeal swabs. However, several studies had later detected viable SARS-CoV-2 and SARS-CoV-2 RNA in the stool of infected individuals being disseminated into the wastewater systems (Medema *et al.*, 2020; Wang *et al.*, 2020a). Medema *et al*. (2020) were the first to detect SARS-CoV-2 in wastewater in the Netherlands and proposed sewage surveillance as an early warning detection tool to monitor increases in infection. Since then, several proof-of-concept studies were conducted to determine the applicability of wastewater surveillance for disease monitoring (Ahmed *et al.*, 2020a; Medema *et al.*, 2020; Pillay *et al.*, 2021). In addition to a surveillance tool, several authors proposed that WBE be used as a tool to estimate infection rates based on viral loads using mathematical models (Ahmed *et al.*, 2020a; Vallejo *et al.*, 2020). However, there are several limitations that may impact the accuracy of these models. Pillay *et al*. (2021) predicted a range of 95 000 to 2 300 000 infected individuals in a catchment when applying

the model proposed by Ahmed *et al*. (2020a). However, clinical data showed the estimated infection range to be between 21 000 to 377 000, which was significantly lower than the predicted infection rate (Pillay *et al.*, 2021).

In addition to surveillance for correlation with infection rates and analysis of trends, the data obtained from wastewater surveillance was used to monitor the efficacy of lockdown interventions. This was achieved by associating an increase in viral concentrations with the easing of lockdown regulations, and monitoring for the emergence or variants of concern (Ai *et al.*, 2021; Rios *et al.*, 2021; Weidhaas *et al.*, 2021). Ai *et al*. (2021) conducted whole genome sequencing on SARS-CoV-2 extracted from wastewater obtained from 8 sites which enabled the identification of several variants, including emerging variants of B.1.427/429 strains with variant allele frequencies ranging from 7 – 10%. Rios *et al*. (2021) had also successfully used wastewater surveillance to identify the emergence of a variant of the B.1.1.7 lineage in one catchment that quickly spread to other catchments within the study area. Comparison of this data with clinical samples verified the variant of SARS-CoV-2 thus proving the applicability of WBE as a valuable epidemiological tool (Rios *et al.*, 2021).

WBE has several advantages over clinical surveillance. Clinical testing is an expensive approach with a long turnaround time, and it is also limited by the underestimation of positive cases as only individuals displaying symptoms seek medical attention to determine the nature of their illness (Sims and Kasprzyk-Hordern, 2020; Amoah *et al.*, 2022). Whereas WBE allows for community wide analysis of all positive cases including asymptomatic and pre-symptomatic cases (Amoah *et al.*, 2022). However, there are -several limitations which are outlined below.

2.4. METHODS FOR DETECTION AND QUANTIFICATION OF VIRAL PARTICLES FROM WASTEWATER

2.4.1. Sample Collection

The most important and critical step in WBE is sample collection. Most underdeveloped or rural areas lack sewage collection systems and rely on latrines, septic tanks, or open spaces for waste disposal which complicates sample collection. However, urban areas have the advantage of wastewater treatment systems which offer a convenient sampling point representing a wastewater confluence of the service population (Xagoraraki and O'Brien, 2019).

For WBE, samples are usually collected at influent points of the wastewater treatment plants. However, the method of sample collection may vary. Composite wastewater sampling is the most commonly employed sampling technique with a sampling event taking place at increments, usually 24 hr (Lorenzo and Picó, 2019; Bibby *et al.*, 2021). Another sampling technique that may be employed is grab sampling i.e. the total volume of samples is collected at the same point in time (Bernard *et al.*, 2019; Betancourt *et al.*, 2021). Although grab sampling is a simpler technique, it is limited by its capacity to provide a temporal representation of concentrations within a sample (Bernard *et al.*, 2019). A study by Curtis *et al*. (2021) showed grab samples had lower viral loads than composite samples that were collected simultaneously from the same wastewater treatment plant. An important consideration is the in-sewer travel time, based on the configuration of the treatment plant, this may be as long as 35 hrs (Bibby *et al.*, 2021). Based on this, the time of sampling is a crucial factor to consider. Additionally, the high concentrations of compounds within the sample matrix can lead to viral RNA decay and continuous fluctuations in influent flow rates at wastewater treatment plants impact viral concentrations (Lorenzo and Picó, 2019; Curtis *et al.*,

2021). Once samples are collected, they are usually transported on ice to the laboratory, this can further influence RNA decay (Bibby *et al.*, 2021).

2.4.2. Viral Concentration and RNA Extraction

As previously mentioned, following a preconditioning step, the low concentration of viruses in wastewater necessitates a primary concentration step which include aqueous polymer separation using polyethylene glycol (PEG), ultracentrifugation, electronegative membrane filtration, electropositive membrane filtration, and ultrafiltration .(Lu *et al.*, 2020a). Since these methods were initially adapted to non-enveloped viruses, the recovery efficiencies of these methods may differ between enveloped and non-enveloped viruses owing to their structural differences (Ahmed *et al.*, 2020c).Ye *et al*. (2016) showed that PEG, ultrafiltration, and ultracentrifugation had a significantly lower recovery of enveloped viruses when compared to non-enveloped viruses. A study by Ahmed *et al*. (2020c) explored the recovery efficiencies of electronegative membranes (adsorption-extraction method), ultrafiltration, PEG precipitation, and ultracentrifugation on murine hepatitis virus (MHV), a surrogate virus for SARS-CoV-2. Two of the adsorption-extraction methods used had the highest mean MHV recovery, followed by ultrafiltration, PEG precipitation, and ultracentrifugation (Ahmed *et al.*, 2020c). Table 2.2 below summarizes some of the advantages and disadvantages associated with the above-mentioned methods for viral recovery. Whilst these steps are necessary to facilitate the detection of low viral titers, the processes involved may lead to sample loss which ultimately affects the accuracy and reproducibility of the technique (Gonzalez *et al.*, 2020). These methods sometimes require a pre-filtration or pre-centrifugation step whereby the larger particles are removed from the liquid fraction of the wastewater and analysis is only performed on the liquid fraction. This is problematic as studies have detected viral RNA in the particulate matter of wastewater suggesting adsorption of the virus onto the solids

(Hokajärvi *et al.*, 2021; Kitamura *et al.*, 2021). These findings necessitate the addition of a pre-treatment step to dislodge the viruses from the solid matter or the need to include both components of wastewater.

Following viral concentration, nucleic acid extraction is another key step in viral recovery from wastewater. The method of extraction chosen is based on the type of sample being used and needs to take into consideration the quality and quantity of the extracted RNA. Factors that may impact RNA integrity and purity include cross-contamination by other nucleic acids and the presence of other contaminants from the sample matrix or reagents used in the extraction process (Ahmed *et al.*, 2020d). Recent studies employing WBE have utilized a plethora of commercially available RNA kits, and more recently, automated RNA extraction methods (Ahmed *et al.*, 2020d; Marzinotto *et al.*, 2020; Pérez-Cataluña *et al.*, 2021). It is important to consider that the components, sample and elution volumes, and processes of different extraction methods will vary, and this will lead to variability in recovery efficiencies. Despite the variability, commercial kits have the advantage of shorter incubation periods, fewer steps, and the removal of inhibitors that may affect subsequent PCR identification (Ahmed *et al.*, 2020d).

2.4.3. Detection and quantification methods

There are several molecular workflows employed for the detection and quantification of viral RNA including RT-PCR, RT-qPCR, and RT-ddPCR. In most WBE studies for the detection of SARS-CoV-2, the most commonly used platforms are RT-qPCR and RT-ddPCR (Ciesielski *et al.*, 2021). The platforms, protocols, reagents, and reporting methods of these techniques vary resulting in methodological inconsistencies indicating a lack of standardization which may hinder the wide-scale application of WBE as a surveillance tool (Ahmed *et al.*, 2020d; Ciesielski *et al.*, 2021).

Table 2.2: Advantages and limitations of viral concentration methods

Concentration Method	Advantages	Limitations	Reference
Electronegative membrane filtration	• Processing of large sample volumes (depending on the turbidity of the sample and the filter size) • Faster processing time (approximately 40 min) • Able to concentrate viruses from both the solid and liquid fractions of wastewater • Samples can be processed in the field	• May require a preconditioning step • Filters are easily clogged due to suspended solids • Filtration units are expensive (90 mm) • Sensitivity to organic matter • Recommended RNA extraction kits are expensive	(Cashdollar and Wymer, 2013; Ahmed *et al.*, 2020c; Lu *et al.*, 2020a)
Ultrafiltration	• Short processing times depending on sample turbidity (1 hr) • Viral recovery rates are high and consistent	• Suitable for viral recovery from liquid fraction of wastewater only • Filtration units are expensive • Turbid samples easily clog device • Centrifuge is expensive • Potentially concentrates PCR inhibitors	(Ahmed *et al.*, 2020c; Lu *et al.*, 2020a)

		• Adsorption of viruses to the filter may affect viral recovery • Samples cannot be processes in field
PEG precipitation	• Able to concentrate viruses from various water quality matrices • Useful in viral recovery in both solid and liquid phases of wastewater • High viral recovery rate • Processing of large sample volumes • A relatively inexpensive technique	• Long processing times • Loss of sample as only a portion of concentrate is used for analysis • Potential inhibition of viral activity • Samples cannot be processes in field • Variability in data
Ultracentrifugation	• Useful in viral recovery in both solid and liquid phases of wastewater • Separation of various viral types • Relatively low cost	• Long processing times • Smaller volumes and fewer samples can be processed at a time • Requires an ultracentrifuge which is expensive and not available in routine laboratories • Equipment can only be operated by trained individuals • Samples cannot be processes in field

For the detection and quantification of viruses in most WBE studies, RT-qPCR has been utilized. The technique uses fluorescence signal intensity to amplify the targeted product and is quantified using a reaction cycle threshold and standard curve (Mao *et al.*, 2019; Cervantes-Avilés *et al.*, 2021). Whilst RT-qPCR has several advantages including low cost, wide application, analysis of larger number of samples, etc. it is limited by its susceptibility to PCR inhibitors which impacts its amplification efficiency (Cervantes-Avilés *et al.*, 2021). A more sensitive approach is RT-ddPCR. This technique employs the generation of droplets to create micro-reactions of water-in-oil droplets that undergo PCR (Mao *et al.*, 2019; Cervantes-Avilés *et al.*, 2021). Although this technique is expensive and has a narrow dynamic range, it has several advantages such as the absence of external calibration curves, is less susceptible to PCR inhibitors, and offers greater precision in quantification (Cervantes-Avilés *et al.*, 2021). A study by Ciesielski *et al*. (2021) showed a lower limit of detection for SARS-CoV-2 from wastewater influent when using RT-ddPCR (0.066 copies/μl) when compared to RT-qPCR (12.0 copies/μl). This is attributed to the increased sensitivity and accuracy of the RT-ddPCR platform (Ciesielski *et al.*, 2021; Pillay *et al.*, 2021).

2.5.FACTORS AFFECTING VIRAL QUANTIFICATION IN WASTEWATER

2.5.1. Wastewater treatment plant infrastructure

An important consideration in the development of suitable WBE setup is the physical layout of the wastewater treatment plant infrastructure. Sewage networks are designed for the transport of wastewater and not for public health surveillance (Harris-Lovett *et al.*, 2021). The configuration and size of wastewater treatment infrastructure differs between developed and developing countries and the areas within these countries (Xagoraraki and O'Brien, 2019). These factors will impact viral degradation within the wastewater matrix. Viruses do not replicate outside of host cells so the viral titers of sample is a representation of the viral particles excreted by infected individuals (Xagoraraki and O'Brien, 2019). Factors such as hydraulic

retention times, and flow rates impact viral titers in the wastewater matrix (Ciesielski *et al.*, 2021; Pillay *et al.*, 2021). Another important consideration is the type of areas being serviced by the plants. Industrial and hospital influent will have high concentrations of chemicals and disinfectants that may influence viral decay. Furthermore, viral titers in wastewater are easily influenced by occurrences such as greywater input, floods, intensive runoffs, stormwater incursions, and septic tank discharge as the occurrences lead to additional dilutions to the sample matrix (Bernard *et al.*, 2019).

2.5.2. Population dynamics and viral shedding rate

There are several factors that need to be considered when looking at the assessment of RNA in wastewater to monitor disease outbreaks. WBE can provide information that can ultimately lead to the determination of hotspots by identifying trends and patterns in communities (Amoah *et al.*, 2022). Factors such tourism and commuting makes the estimation of population sizes difficult (Mao *et al.*, 2020; Sims and Kasprzyk-Hordern, 2020). Furthermore, there is no clear correlation between the concentration of viral particles shed and the mass of fecal matter excreted by the infected individuals. Hence, there is variation in the concentration of viral particles shed per gram of fecal matter (Amoah *et al.*, 2020). It is also important to note that an individual may continue to excrete viral RNA for several weeks after the infection has passed (Al-Sadeq and Nasrallah, 2020). This makes determination of the number of infected individuals in a community difficult. However, if used in conjunction with clinical surveillance, it can be used as a predictive tool to determine outbreaks and potential hotspots (Amoah *et al.*, 2022).

2.5.3. Wastewater matrix

Although wastewater proves to be a useful source of information, the complexity of this matrix can be pose to be a challenge in the detection of pathogenic microorganisms (Mao *et al.*, 2020). Wastewater is received from different sources and may be categorized into municipal

wastewater, hospital wastewater, treated and untreated wastewater (Tran *et al.*, 2021). The chemical and biological characteristics of different matrices will differ. These characteristics, categorized into micro-pollutants and macro-pollutants, have an impact on the stability of the virus (Achak *et al.*, 2021). Since WBE is to be used to monitor the health dynamics of a selected community, the selection of the WWTP is an important consideration. Based on the location, a WWTP may receive domestic, hospital, or industrial wastewater (Achak *et al.*, 2021; Ahmed *et al.*, 2022). The chemical and biological characterization of hospital and domestic wastewater is similar, although, the concentrations may vary (Achak *et al.*, 2021). Hospital wastewater may contain a higher microbial load, pharmaceuticals, and disinfectants whereas industrial wastewater will contain a higher concentration of chemical contaminants (Carraturo *et al.*, 2020). Therefore, it is important to choose a WWTP that receives domestic wastewater rather than one that receives domestic and hospital or industrial wastewater (Ahmed *et al.*, 2022).

In addition to the type of wastewater, another important consideration is the point of sampling in a WWTP. Depending on the type of wastewater being received, the treatment processes and disinfection concentrations may not be sufficient to inactivate the virus. Currently, some of the disinfection processes involve oxidants such as hydrogen peroxide, peracetic acid, and sodium hypochlorite. A study by Hasan *et al*. (2021) detected SARS-CoV-2 in municipal wastewater influent but not in treated effluents collected from the same WWTP's from the UAE (Hasan *et al.*, 2021). Additionally, a study by Sherchan *et al*. (2020) detected SARS-CoV-2 in 2 of the 15 untreated wastewater samples. However, secondary treated and final effluents tested negative for the virus. Thus, indicating the efficacy of the disinfection process in the reduction of the virus (Sherchan *et al.*, 2020).

The components of wastewater can affect the identification of viruses due the presence of PCR inhibitors (Mao *et al.*, 2020). To overcome this, more sensitive techniques such as ddPCR are

used. several studies have highlighted that temperature, time, the presence of other microorganisms and biological compounds (e.g. enzymes), pH, ammonia, level of organic matter and total solids that have the potential to affect viral RNA concentrations (Hart and Halden, 2020; Amoah *et al.*, 2022).

Temperature: Temperature impacts viral degradation. It is also indicated that viral structure also impacts viral susceptibility to degradation. (Kataki *et al.*, 2021). The increased temperature facilitates protein degradation and stimulates extracellular enzymatic activity contributing to the rapid decline of viral titers in wastewater (John and Rose, 2005; Gundy *et al.*, 2009). This may be attributed to the degradation of enveloped viruses that contain a protein envelope, compared to non-enveloped viruses that lack the outer protein layer (Kataki *et al.*, 2021). An early comparative study by Gundy *et al*. (2009) showed that viral titers of coronaviruses (feline infectious peritonitis virus (FIPV) and human coronavirus 229E (HCoV)) in unfiltered wastewater decreased by 99% (T_{99}) at 23°C in 1.71 (FIPV) and 2.36 (HCoV) days. However, poliovirus 1 LSc-2ab (PV-1) took 7.27 days to decrease by 99% under the same conditions (Gundy *et al.*, 2009). A study by Ahmed *et al* (2020b) showed higher and faster decay of SARS-CoV-2 and murine hepatitis virus (MHV) at 25°C and 37° in wastewater when compared to storage at lower temperatures, i.e. 4°C and 15°C which showed no significant decay. The duration for a 90% reduction in viral titers for SARS-CoV-2 and MHV respectively were 27.8 and 56.6 days at 4°C, 20.4 and 28.5 days at 15°C, 12.6 and 17.3 days at 25°C, and 8.04 and 7.44 days at 37°C (Ahmed *et al.*, 2020b). Based on these findings, it can be established that seasonal changes will impact virus persistence in the environment (Oliveira *et al.*, 2021).

pH: The pH of the matrix also has an influence on the survival of the virus. Depending on the species of the virus, the optimum pH that enables its survival will vary. Several in vitro studies conducted supported this. Studies show that a pH range of 5 – 8 at 33°C is considered to the stable range for HCoV, 5 – 7.4 at 37°C for MHV, 5 – 7 at 37°C for transmissible gastroenteritis

virus (TGEV), and 3 – 10 for SARS-CoV-2 (Amoah *et al.*, 2020; Bhatt *et al.*, 2020; Hamouda *et al.*, 2021). Since the pH of a solution influences melting temperature of RNA oligonucleotides, it is likely that pH fluctuations stimulate cycles of RNA separation and reannealing resulting in undesirable degradation (Mariani *et al.*, 2018; Amoah *et al.*, 2020). In addition to its influence of viral stability, the pH of a matrix impacts aggregation and adsorption properties of viruses by changing its surface charge (Mariani *et al.*, 2018; Amoah *et al.*, 2020; Corpuz *et al.*, 2020; Hamouda *et al.*, 2021).

Total solids: The presence of solids (dissolved solids, suspended solids, and settleable solids) in wastewater can affect viruses in wastewater in one of two ways, i.e. it may influence viral degradation due to the high concentration of organic and inorganic contaminants or lead to viral attachment to the solids (Bernard *et al.*, 2019; Oliveira *et al.*, 2021; Amoah *et al.*, 2022). Oliveira *et al.* (2021) conducted a study that looked at the persistence of SARS-CoV-2 in filtered and unfiltered river and wastewater samples. It was found that SARS-CoV-2 persisted longer in river water than in wastewater. Furthermore, SARS-CoV-2 survived for longer periods in filtered samples compared to unfiltered samples. This supports reports of viruses being more readily inactivated in complex matrices than simpler matrices (Bertrand *et al.*, 2012; Oliveira *et al.*, 2021). However, another study observed increased SARS-CoV-2 RNA recovery from samples with a higher total solids content. This was attributed to the affinity of the viral particles to the solid material due to decreasing pH of the sample (Amoah *et al.*, 2020; Forés *et al.*, 2021; Amoah *et al.*, 2022).

Microorganisms: Along with viruses, several other microorganisms can be found in wastewater. Biotic interactions between these microbial communities and viruses may influence their decay. Studies have shown predation of several bacteriophages and non-enveloped viruses by protozoa (Ahmed *et al.*, 2020b). Although, little is known about the predation of enveloped viruses, it is still a possibility.

2.5.4. Impact of storage and sample processing on viral quantification using WBE

The implementation of lockdown regulations contributed to delays in analysis of samples as this limits the movement of individuals limiting access to research facilities, and preventing the timeous delivery of reagents (Hokajärvi *et al.*, 2021). This results in prolonged storages of samples in the available facilities until the analysis is done. Furthermore, in order to handle the large number of samples received during COVID-19, the research facilities had to store the samples at non-desirable temperatures, such as 4°C, for an extended period of time. It is possible that all of these factors can affect the viral count, resulting in unreliable results. There are a few studies conducted on the impact of prolonged storage on sample matrices when considering WBE for disease surveillance.

Majority of these research conducted focused on the decay rates of SARS-CoV-2 at temperatures above 4°C to understand the survival and persistence under environmental conditions (Gundy *et al.*, 2009; Ahmed *et al.*, 2020b; Bivins *et al.*, 2020). Higher temperatures result in increased microbial metabolic processes, predation, and degradative enzyme activity. Therefore, in order to maintain viral stability for a prolonged period of time, temperatures would have to be lowered (Olson *et al.*, 2004). Recommendations made by standard microbiology guides suggests storage of environmental samples at 4°C for 48h and storage at -80°C for prolonged periods (APHA, 2002; Olson *et al.*, 2004). An early study by Olson *et al*. (2004) analyzed the decay of MS2, a non-enveloped bacteriophage, at different storage temperatures. Wastewater samples containing MS2 bacteriophage were stored at 4°C, -20°C, and -80°C, with the greatest decay at -20°C which was attributed to the formation of ice crystals that potentially caused viral damage (Olson *et al.*, 2004). It was also found that viruses began to degrade after 1 week of storage at 4°C, and viral titers stored at 4°C were significantly lower than samples that were stored at -80°C. In addition to this, results showed a nearly 80% reduction of viral titers in PBS compared to a 50% reduction in septic tank effluent when stored

at -80°C indicating the quality of aqueous environment impacts viral survivability (Olson *et al.*, 2004). Recently, a study by Hokajärvi *et al.* (2021) showed that SARS-CoV-2 and SARS betacoronavirus remained stable at 4°C, -20°C, and -80°C for up to 84 days. However, norovirus GII concentrations reduced by 1-log10 under the same storage conditions between 29 and 84 days of storage. Furthermore, it was also found that RNA concentrations increased in the particulate matter of wastewater. This is an important observation because most studies discard the solid fraction of wastewater and only focus on the liquid component.

As previously mentioned, the adsorption properties of viruses are influence by pH changes that influence the surface charge increasing their affinity to particulate matter (Mariani *et al.*, 2018; Amoah *et al.*, 2020; Corpuz *et al.*, 2020). The study by Hokajärvi *et al.* (2021) did not identify the reason for adsorption. There have been a few studies that have explored chemical (sodium pyrophosphate) and mechanical (ultrasonication) pre-treatment for viral enhancement, however, this has not been applied to SARS-CoV-2 and influenza A. Danovaro, *et al*. (2001) used a 10mM sodium pyrophosphate solution and sonication at 47kHz at different time intervals and found that sodium pyrophosphate had no impact whilst sonication increased viral concentrations. Brown, *et al*. (2015) used four dispersants (i.e. surfactants - polyoxyethylene-sorbitan monooleate and Triton X-100, and the ionic dispersants – sodium pyrophosphate and sodium cholate) and ultrasound treatment to dislodge floc bound viruses. They found that 5% Tween 80 and 10mM sodium pyrophosphate increased viral concentrations, whilst ultrasonication had no significant impact.

CHAPTER 3 : ASSESSING THE IMPACT OF STORAGE PARAMETERS AND ENHANCEMENT STRATEGIES ON VIRAL PARTICLE RECOVERY FROM WASTEWATER INFLUENT

3.1. INTRODUCTION

Since its first postulation in the late 1990's by Daughton and Ternes for the monitoring of chemical pollutants, the research field surrounding wastewater-based epidemiology (WBE) has gained remarkable momentum, evolving into a disease surveillance tool (Daughton and Ternes, 1999; Mackuľak *et al.*, 2021). More recently, during the COVID-19 pandemic, WBE was adapted to monitor infection trends to help ease the strain placed on the global healthcare systems by the alarming number of coronavirus infections (Pillay *et al.*, 2022). Whilst considerable progress has been made in the area of WBE, there is still a lack of comprehensive knowledge surrounding sample storage and its effect on the data generated for WBE (Mauro *et al.*, 2020; Zahedi *et al.*, 2021). Whilst temperature is a crucial factor driving microbial degradation during storage, there may be several other variables that influence microbial behavior and the stability of biomarkers in wastewater. Majority of the research conducted thus far focused on the degradation rates of SARS-CoV-2 biomarkers at temperatures above 4°C to understand the survival and persistence under environmental conditions (Gundy *et al.*, 2009; Ahmed *et al.*, 2020b; Bivins *et al.*, 2020). A few studies have evaluated the impact of storage temperatures on SARS-CoV-2, but the results of these studies have shown substantial inconsistency. The study by Weidhaas *et al*. (2021) showed a reduction in SARS-CoV-2 biomarkers after incubation at -80°C for a week, whereas the study by Hokajärvi *et al*. (2021) found no degradation at -20°C or -75°C after 84 days.

Considering the above, it is important to understand that wastewater is a complex environment containing numerous biological and chemical constituents that impacts the physicochemical

characteristics of the matrix. In addition to temperature, these properties may further impact viral persistence within the matrix (Amoah *et al.*, 2022). There is evidence that suggests factors such as pH and total solids may also impact viral persistence in a sample matrix (Amoah *et al.*, 2020; Bhatt *et al.*, 2020; Forés *et al.*, 2021; Hamouda *et al.*, 2021; Amoah *et al.*, 2022). A comparative study by Oliveira *et al*. (2021) looked at viral persistence in several matrices including river water, tap water, unfiltered wastewater, and filtered wastewater. The results from these studies show that viruses may persist for longer periods of time in less complex matrices (in this case river water) (Bertrand *et al.*, 2012; Oliveira *et al.*, 2021).

Additionally, Hokajärvi *et al*. (2021) reported high concentrations of viruses within the solid matter present in wastewater samples. This necessitates the addition of a pre-treatment step to dislodge these particles. Additionally, studies have observed increased adsorption of enveloped viruses to wastewater solids when compared to non-enveloped viruses owing to their structural differences (Ye *et al.*, 2016). Earlier studies have successfully applied ultrasonication and sodium pyrophosphate for the dislodgement of viral particles from solids (Danovaro *et al.*, 2001; Brown *et al.*, 2015). However, this has not been applied to enhance the recovery of SARS-CoV-2 and influenza A particles from wastewater samples. Herein this study focuses on investigating the combined effects of storage parameters (i.e. temperature and time) and the physicochemical characteristics of wastewater (i.e. pH, dissolved oxygen, electrical conductivity, total solids, total fixed solids, and chemical oxygen demand) on the degradation patterns of SARS-CoV-2 and influenza A. Additionally, different pre-treatment methods (ultrasonication and sodium pyrophosphate) for the enhancement of viral recovery from wastewater solids are explored to improve the efficiency of WBE as a disease surveillance system.

3.2. MATERIALS AND METHODS

3.2.1. Sample collection, preparation, and storage

A composite raw influent sample of 8 L was collected from a municipal wastewater treatment plant located north of Durban, South Africa, and transported on ice to the laboratory. The physicochemical characteristics of the sample i.e., pH, temperature, dissolved oxygen (DO), salinity, electrical conductivity (EC), and total dissolved solids (TDS) was done using a YSI 556 MPS Handheld Multiparameter Instrument (YSI) upon arrival to the laboratory. The chemical oxygen demand (COD) was determined using the HACH high range (20 – 1500 mg/L) COD test kits and total solids and total fixed solids was determined as described in Standard Methods (APHA *et al.*, 2018). Samples were subsequently divided into two batches: the first batch was seeded with attenuated forms of SARS-CoV-2 strain USA/WA1/2020, and influenza A strain subtype H1N1 (Microbiologics, USA), of predetermined concentration of 10 copies/ µl and 2 copies/ µl respectively, and the second batch was unseeded wastewater influent sample. Both batches were homogenized using a magnetic stirrer at 1500 rpm for 10 min. Samples were then divided into 500 ml aliquots and stored in 1 L polypropylene sampling bottles at 4°C, -20°C, -80°C, and at room temperature (± 25°C) for a period of 84 days. Prior to analysis, the stored samples were thawed overnight at 4°C and allowed to adjust to ± 25°C. Thereafter, the physicochemical characteristics of the stored sample was assessed. The unseeded wastewater influent samples were analysed every 14 days throughout the 84-day period, and the seeded samples were analysed on days 0, 42, and 84. All experiments and analysis were conducted in triplicate.

3.2.2. Viral particle concentration

Viral particles were concentrated using polyethylene glycol (PEG) 6000 (Catalog number: P3750, Minema Chemicals, South Africa) together with NaCl (Catalog number: S3250, Minema Chemicals, South Africa) according to the methods described by Sapula *et al*. (2021).

Briefly, 100 ml of sample (2 batches of 50 ml) was clarified by an initial primary centrifugation (5810R, Eppendorf) at 5000 × g for 30 min at 4°C to remove large particles, and the resulting pellet was used for RNA extraction. To maximize viral recovery, the supernatant from the first round (primary) centrifugation was further precipitated overnight at 4°C with 15% PEG 6000 and 2% NaCl followed by centrifugation at 12 000 × g for 90 min at 4°C (Sapula *et al.*, 2021). An additional centrifugation (Hermle ZK 496, Lasec) step at 5000 × g for 5 min at 4°C was conducted in a swinging bucket rotor to ensure any residual particles that may have attached to the tube was dislodged. The final combined pellets were re-suspended in 140 µl of 1X phosphate buffered saline (PBS) (Catalog number: P5493-4L, Sigma Aldrich) for RNA extraction.

3.2.3. Viral RNA extraction, detection, and quantification using droplet digital PCR.

RNA was extracted from the pellets using the Qiagen QiAmp Viral RNA MiniKit (Hilden, Germany) following the manufacturers protocols using an Eins Sci (E-C15-24-2P) centrifuge with a AS24-2 rotor. Extracted RNA was eluted in 80 µl nuclease free water and stored at -20°C.

The purity (260 nm/ 280nm) and yield (260 nm) of the extracted RNA was assessed using the Implen Nanophotometer, NP80. Thereafter, the copy numbers of SARS-CoV-2 and influenza A was assessed by ddPCR using the One-Step RT-ddPCR Advanced Kit for Probes (Biorad, USA). The primer and probe sequences for each gene target can be found in Appendix Table A1. Each 22 µl ddPCR reaction contained 5 µl supermix, 2 µl reverse transcriptase, 1 µl dithiothreitol, 1.98 µl each of the forward and reverse primers (10 µM), 0.55 µl of 10 µM probe, 4.49 µl nuclease-free water, and 5 µl template RNA (1 ng).The droplets were generated using the QXD Automated Droplet Generator, read using the QX200 Droplet Reader together with the QuantaSoft 1.7 software, and analysed using the QuantaSoft Analysis Pro 1.0 software

(Biorad, USA). The limit of detection determined for this study for the N2 (SARS CoV-2) and INFA (Influenza A) genes were 0.2 and 0.18 copies/ µl.

3.2.4. Pre-treatment methods for enhancing viral recovery from wastewater samples

Mechanical (ultrasonication) and chemical (sodium pyrophosphate (Catalog number: GK0841, Glentham Life Sciences)) pre-treatment methods were evaluated for its efficiency in enhancing viral particle recovery from wastewater solids. The pellets obtained after primary centrifugation were weighed out (150 mg wet weight) and re-suspended in 100 µl nuclease free water and used for each pre-treatment. This was sonicated at 4 kHz, 8 kHz, 12 kHz, and 16 kHz using a 125 W sonicator (QSonica, Q125, USA) with probe attachment 422-A for 1 min and 3 min on ice and was interrupted after every minute to prevent overheating (Danovaro *et al.*, 2001). To test the effects of chemical pre-treatment, sodium pyrophosphate, an ionic dispersant, was added to the sample to a final concentration of 10 mM and was incubated at room temperature for 15 min, 30 min, 45 min, and 1 hr. The samples were centrifuged at 8000 $\times g$ for 1 min at 4°C from which 140 µl of the supernatant was removed for RNA extraction. The remaining pellet was re-suspended in 140 µl of 1X PBS and used for RNA extraction separately as previously described. Following this, viral particles were quantified using ddPCR.

3.2.5. Statistical analysis

Microsoft Excel was used for data entry and for determining mean values and standard deviations. Additionally, RNA copy numbers obtained from ddPCR was converted to $\log_{10}$ values prior to conducting any statistical analysis. The coefficient of correlation between storage temperature over a period of 84 days against physicochemical characteristics as well as viral RNA copy numbers and the physicochemical characteristics was determined by the Pearson's correlation test on R Studio with a significant correlation supported by a p value of <0.05. A correlation coefficient was used to determine positive, negative, or no correlation. Any significant differences between the means of RNA concentration stored at each

temperature was analysed by a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using GraphPad Prism, version 10 (USA) with a significant correlation supported by a *p* value of <0.05.

3.3. RESULTS

3.3.1. Impact of storage time at defined temperatures on wastewater characteristics

Visual inspection of the samples stored at 25°C after 7 days revealed a distinctive change in colour from brown to black as well as the odour of hydrogen sulphide along with the formation of a distinct layer at the top of the sample. At this point, adhesion of particles (biofilm-like) to the polypropylene storage bottles was observed. A similar trend was observed for samples stored at 4°C for 42 days. In contrast, no discernible differences were observed in samples stored at -20 and -80°C.

Figure 3.1: Pearson's correlation of storage time and changes in wastewater physicochemical characteristics

The study also examined the impact of storage time (up to 84 days) on wastewater physicochemical characteristics at different temperatures (Appendix Table A2). For samples stored at ± 25°C, the storage time positively correlated with EC, TDS, and TFS as shown in Figure 3.1. At 4°C, significant positive correlations were observed between EC and TDS and significant negative correlation was observed with TFS. At -20°C and -80°C, storage time had significant negative correlations with EC and TFS which are depicted in Figure 3.4 and Figure 3.5 for below were significance is represented as: * $p < 0.05$; ** $p < 0.002$; *** $p < 0.001$; blank – no significance; ? - no correlation.

3.3.2. Determining viral degradation in stored wastewater samples

Initial analysis of the wastewater influent samples revealed a higher concentration of SARS-CoV-2 (Appendix Table A3) RNA biomarkers in the supernatant (5.28±0.14 log copies/ 100 ml) when compared to the pellet (4.96±0.06 log copies/100 ml) while influenza A (Appendix Table A4) had a higher concentration in the pellet (4.99±0.12 log copies/100 ml) compared to the supernatant (4,87 ±0.04 log copies/100 ml). With the seeded samples, viral loads in the supernatant were higher when compared to the pellets for both SARS-CoV-2 (5.43 ±0.01 log copies/100 ml/ 4.48 ±0.01) log copies/100 ml) and influenza A (4.96±0.03 log copies/100 ml / 4.48±0.04 log copies/100 ml). SARS-CoV-2 concentrations in all the samples decreased over the course of the 84-day storage period, regardless of storage temperature (Figure 3.2).The highest degradation of SARS-CoV-2 occurred at ± 25°C (Figure 3.2A - supernatant: 94.1%, Figure 3.2B - pellet: 92.66%) and the lowest at -80°C (supernatant- 64.43%, pellet – 85.73%) in the wastewater influent sample. However, in the seeded sample the highest degradation in the supernatant (Figure 3.2C) occurred at 4°C (95.57%) and the lowest at -80°C (71.16%) and in the pellet (Figure 3.2D) the highest degradation occurred at -20°C (50.79%) and the lowest at 4°C (31.53%).

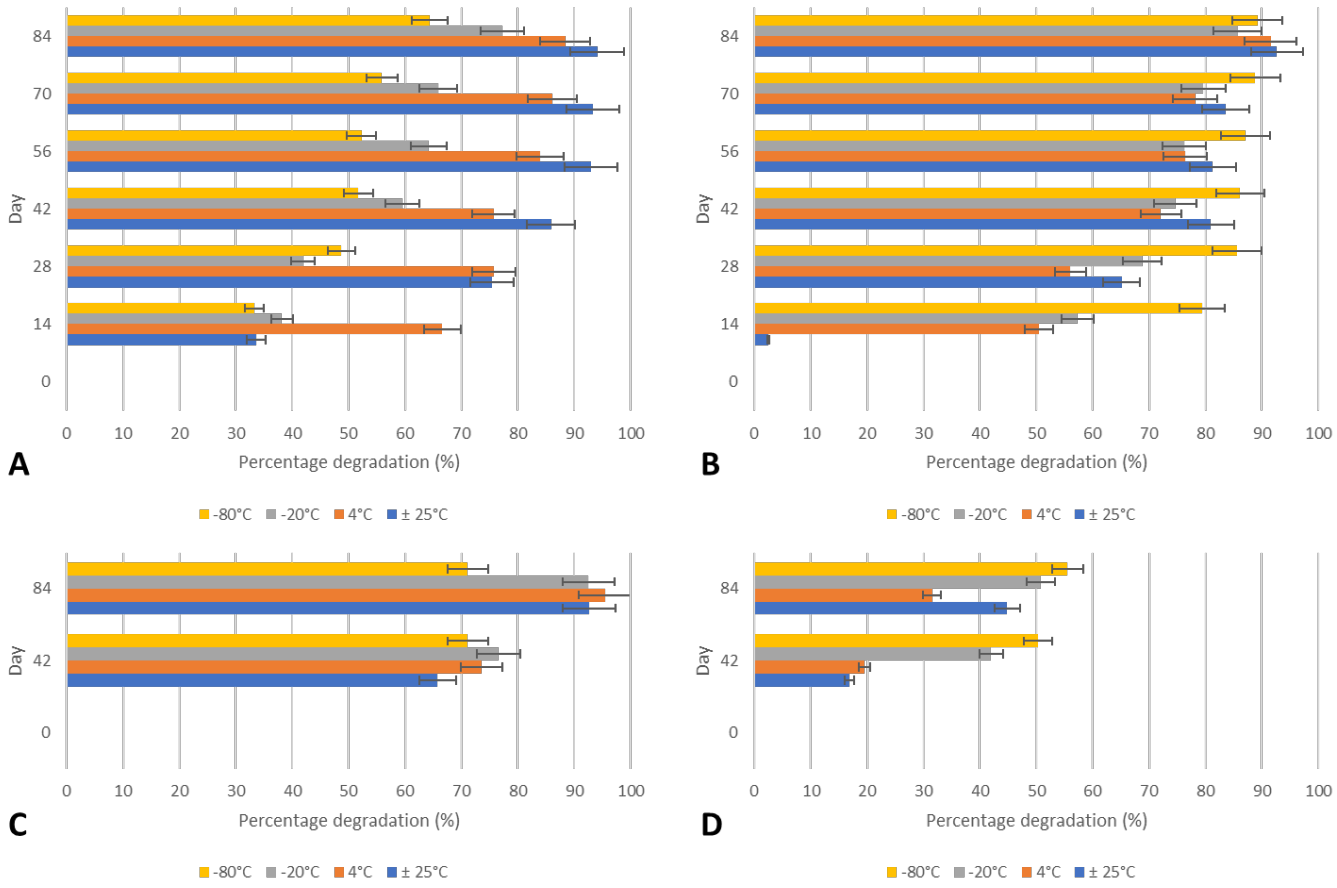

Figure 3.2: Percentage degradation of SARS-CoV-2 biomarkers in stored samples; A: wastewater influent – supernatant; B: wastewater influent – pellet; C: seeded wastewater – supernatant; D: seeded wastewater – pellet

A one-way ANOVA was conducted to determine the effect of storage temperature on SARS-CoV-2 degradation. A significant difference between SARS-CoV-2 degradation and at least three storage temperatures was observed (p <0.0001) (Appendix Table A5). Tukey's multiple comparisons test showed that SARS-CoV-2 degradation in the supernatants and pellets of the influent and seeded wastewater samples were significantly different between all storage temperatures (p <0.0001).

Whilst there was degradation in all stored samples, the degradation trend for influenza A differed from SARS-CoV-2 as shown in Figure 3.3. The highest degradation of influenza A in the influent samples occurred at ± 25°C in both the supernatant (Figure 3.3A - 88.89%) and the

pellet (Figure 3.3B 83.47%), and the lowest at -20°C in the supernatant (23.05%), and -80°C (49.46%) in the pellet. In the seeded samples, the highest degradation occurred at ± 25°C in the supernatant (Figure 3.3C - 90.69%) and 4°C in the pellet (Figure 3.3D - 92.64%) and the lowest at 4°C in the supernatant (47.05%) and -80°C in the pellet (69.15%) (Figure 3.3).

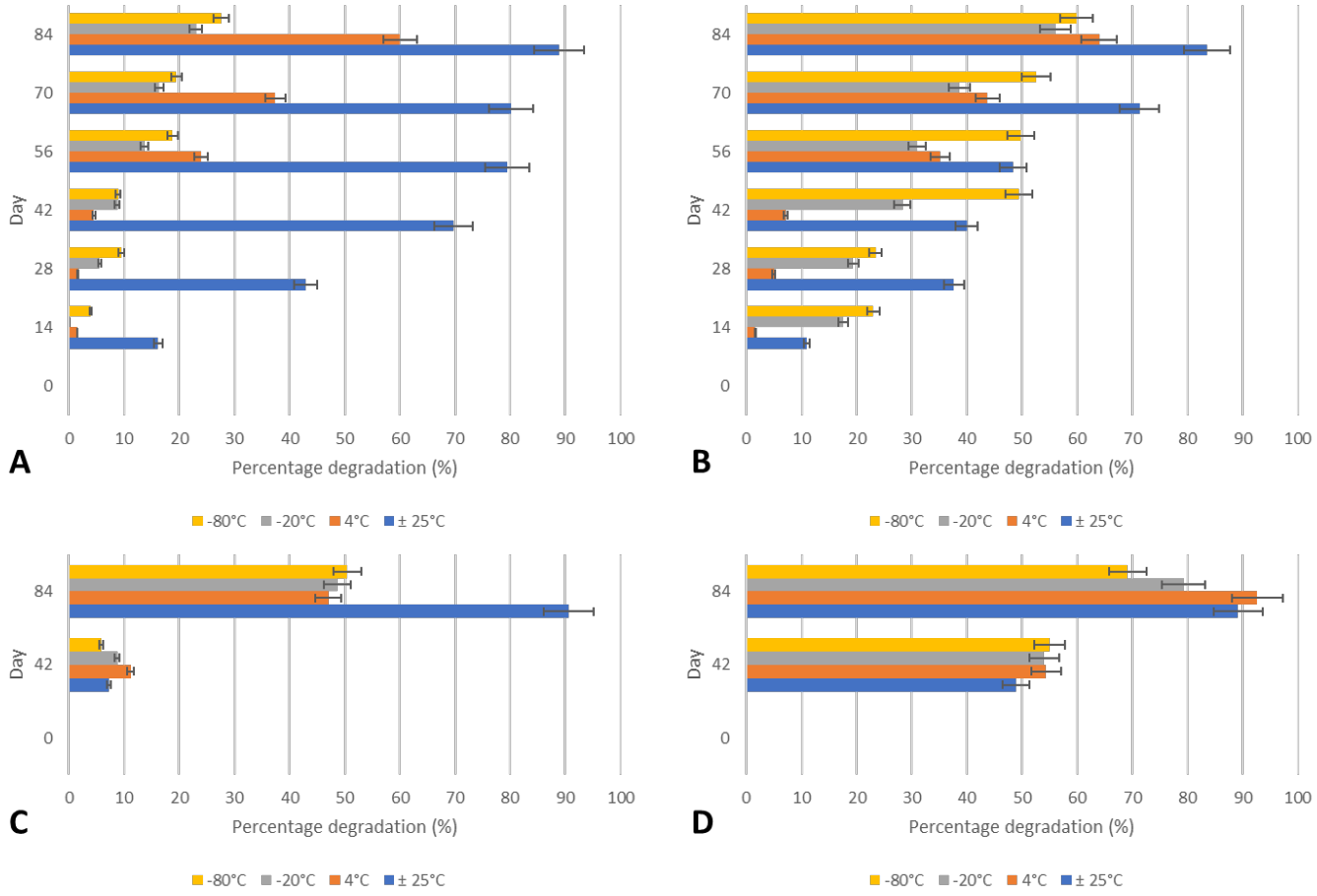

Figure 3.3: Percentage degradation of influenza A biomarkers in stored influent sample; A: wastewater influent – supernatant; B: wastewater influent – pellet; C: seeded wastewater – supernatant; D: seeded wastewater – pellet

A one-way ANOVA showed a significant difference between influenza A degradation between at least three storage temperatures (p <0.0001) (Appendix Table A6). Tukey's multiple comparisons test showed that influenza A degradation in the supernatants and pellets of the

influent and seeded wastewater was significantly different between all storage temperatures (p <0.0001).

3.3.3. Impact of physicochemical characteristics on viral degradation in stored samples

Storage time showed significant negative correlations with SARS-CoV-2 and influenza A degradation in the supernatant and pellets of the wastewater influent stored at all temperatures, and the supernatants of the seeded wastewater stored at ±25°C, 4°C, and -20°C. Changes in salinity showed a significant negative correlation with SARS-CoV-2 and influenza A degradation in the wastewater influent supernatant stored at ±25°C, and the pellets stored at ±25°C and 4°C. Changes in DO showed significant positive correlation with SARS-CoV-2 and influenza A degradation in the wastewater influent supernatants stored at ±25°C and pellet stored at ±25°C and 4°C. Additionally, a significant negative correlation was observed between salinity and SARS-CoV-2 and influenza A degradation of wastewater influent in the supernatant of samples stored at -20°C and -80°C and SARS-CoV-2 degradation in the wastewater influent pellet stored at -80°C. Changes in pH showed significant negative correlations with SARS-CoV-2 concentrations in seeded wastewater pellets stored at ±25°C and -20°C and significant positive correlations with SARS-CoV-2 concentrations in the wastewater influent pellet in samples stored at -80°C and seeded wastewater pellets stored at 4°C, and -80°C. Additionally, a significant positive correlation was observed between changes in pH and influenza A concentrations in seeded wastewater samples stored at all temperatures. Changes in COD showed significant positive correlation with viral degradation in the seeded wastewater supernatants at all temperatures. Changes in TS and TFS showed significant positive correlation with viral degradation in wastewater influent supernatants and pellets at all temperatures. These correlations are depicted in Figure 3.4 and Figure 3.5 for below were significance is represented as: * $p < 0.05$; ** $p < 0.002$; *** $p < 0.001$; blank – no significance;? - no correlation.

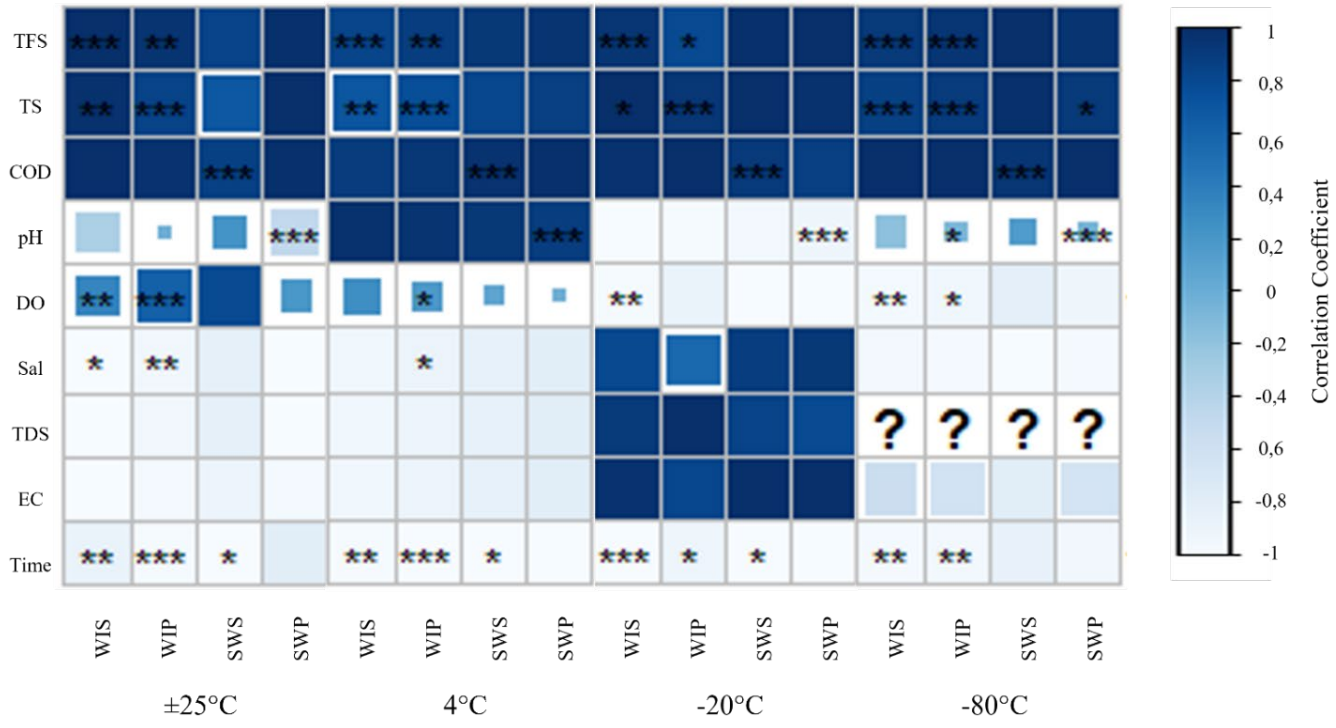

Figure 3.4: Pearson's correlation of SARS-CoV-2 degradation with storage time and changes in wastewater physicochemical characteristics. WIS – wastewater influent supernatant, WIP – wastewater influent pellet, SWS – seeded wastewater supernatant, SWP - seeded wastewater pellet

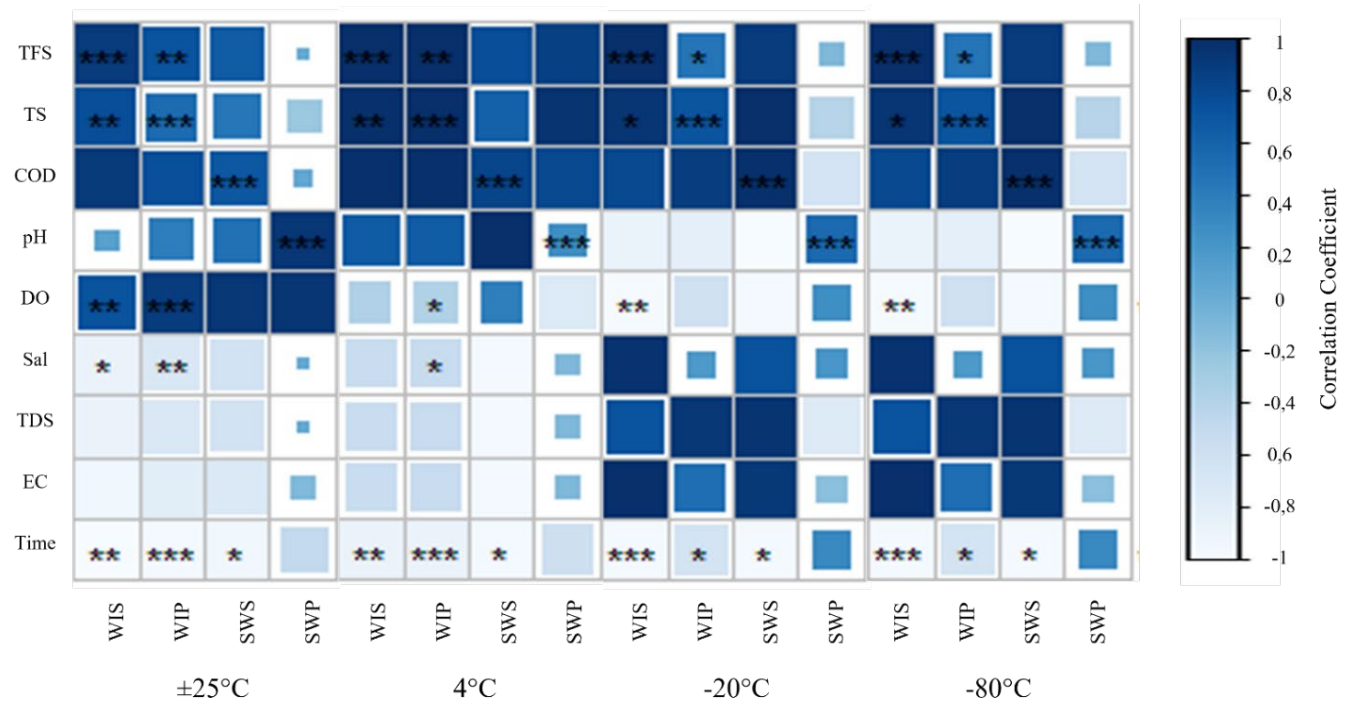

Figure 3.5: Pearson's correlation of influenza A degradation with storage time and changes in wastewater physicochemical characteristics. WIS – wastewater influent supernatant, WIP – wastewater influent pellet, SWS – seeded wastewater supernatant, SWP - seeded wastewater pellet

3.3.4. Effect of pre-treatment methods on enhancing viral recovery from wastewater influent solids

The ionic dispersant, sodium pyrophosphate did not enhance viral recovery, whilst sonication did effect the concentrations of viruses within the sample matrix. Chemical treatment using sodium pyrophosphate did not enhance SARS-CoV-2 and influenza A recovery from fresh wastewater samples. However, sonication at frequencies ranging from 4 – 16 kHz resulted in an increased SAR-CoV-2 abundance in the supernatant. In the untreated sample, there was an initial concentration of 4.52±0.18 log copies/ 100 ml SARS-CoV-2, after sonication there was an increase ranging from 3.30 – 35.65% as shown in Table 31. Sonication at 8 kHz for 3 min yielded the highest concentration increase of SARS CoV-2. In contrast, both pre-treatment

options negatively impacted influenza A copies in the supernatant samples. Before treatment, there were 4.38±0.04 log copies/100ml of influenza A in the supernatant, after pre-treatment, the highest increase observed was 0.28% after sonication for 1 min at 4 kHz. Thereafter, as the sonication frequency increased, influenza A copy numbers decreased.

Table 3.1: Percentage increase of SARS-CoV-2 and influenza A in wastewater supernatant after ultrasonication

Ultrasonication Frequency (kHz)	Treatment time (min)	SARS-CoV-2		Influenza A	
		Concentration (log copies/ 100 ml)	Percentage increase (%)	Concentration (log copies/ 100 ml)	Percentage increase (%)
0	-	4.52±0.18	-	4.38±0.04	-
4	1	6±1	29.29	4.49±0.18	0.28
	3	5.97±0.69	27.54	4.43±0.18	0.12
8	1	5.96±0.58	27.12	4.36±0.09	-
	3	6.08±0.65	35.65	4.2±0.12	-
12	1	6.07±0.44	34.68	4.14±0.06	-
	3	5.51±0.92	8.92	4.27±0.04	-
16	1	5.86 ±0.42	3.30	4.09±0.05	-

	3	5.58±1.02	10.62	3.85±0.01	-

3.4. DISCUSSION

In order to effectively use WBE for disease surveillance, it is necessary to understand the dynamics of the physical and biological changes of wastewater samples during storage (Parra-Arroyo *et al.*, 2023). Temperature is one of the most significant factors driving microbial processes and inactivation during storage (Kataki *et al.*, 2021; Parra-Arroyo *et al.*, 2023). In this study, SARS-CoV-2 and influenza A degradation was noted in samples stored at all temperatures, with the highest degradation observed in the samples stored at ± 25°C and the lowest at -20°C and -80°C. The difference in viral degradation at all temperatures were statistically significant.

SARS-CoV-2 and influenza A are enveloped viruses containing an outer protein layer that is susceptible to degradation in the external environment (Kataki *et al.*, 2021). Furthermore, the genomes of both viruses have a higher ratio of adenine and uracil bases (commonly known as the AT content) making them AT rich (Boussier *et al.*, 2020; Fumagalli *et al.*, 2023). A genome with a higher ratio of guanine and cytosine bases (commonly known as the GC content) tend to exhibit greater stability compared to genomes with a lower GC-content (Chan *et al.*, 2009). Exposure to high temperatures leads to the stimulation of extracellular enzymes capable of degrading viral particles or nucleic acid (Olson *et al.*, 2004; John and Rose, 2005; Gundy *et al.*, 2009). Furthermore, the observation of septic sludge formation after storing wastewater influent at 25°C for 14 days and at 4°C for 42 days may be associated with anaerobic microbial processes and chemical reactions, such as the reduction of sulphates to sulphides, as indicated by the smell of hydrogen sulphide (Muttamara, 1996). The chemical and biological reactions are further indicated by changes in the physicochemical characteristics. Certain changes i.e. salinity, DO, pH, COD, TS, and TFS may have potentially impacted viral concentrations as determined by statistical correlations. Factors such as the pH of a matrix impacts aggregation and adsorption properties of viruses by changing its surface charge (Mariani *et al.*, 2018;

Amoah *et al.*, 2020; Corpuz *et al.*, 2020; Hamouda *et al.*, 2021). This is further supported by the observation of biofilms in the samples stored at 25°C and 4°C which may have impacted viral concentrations in the samples (Li *et al.*, 2021) and by the lowest decrease of SARS-CoV-2 concentrations in the seeded wastewater pellets observed at 4°C. This may not be apparent in the other samples due to factors such as low viral concentrations or due to viral structure which may impact viral stability and adhesion properties. In a wastewater sample, enveloped viruses are extremely susceptible to degradation due to the high concentration of disinfectants and other surfactants (Bernard *et al.*, 2019). This degradation of the external viral structure coupled with the viral genetic material that has been shed through human excreta e.g. faecal matter, saliva, urine, etc. will result in a higher concentration of viral RNA when compared to whole virus (Mauro *et al.*, 2020; Zahedi *et al.*, 2021). In contrast, the seeded samples have been spiked with a pre-determined concentration of the attenuated viruses. As a result, the wastewater influent sample contained more viral genetic material than the seeded samples with attenuated whole virus, contributing to the difference between their storage properties. Whilst the structure of SARS-CoV-2 may differ from that of influenza A, the difference between the naturally occurring viruses and the spiked virus must also be accounted for. Influenza A contains a lipid envelope comprised of several surface glycoproteins which include hemagglutinin and neuraminidase surface glycoprotein, each having 16 and 9 known subtypes (Cheung and Poon, 2007). Similarly, SARS-CoV-2 has a nucleocapsid protein and an envelope made up of a spike protein, membrane protein, and envelope protein (Wang *et al.*, 2020b). These proteins have a surface charge, changes to this charge may influence viral aggregation and adsorption properties (Pielak and Chou, 2011; Amoah *et al.*, 2020). In addition to structural differences, the genomes of SARS-CoV-2 and influenza A have different polarities (Wang *et al.*, 2020b; Brázda *et al.*, 2021; Piret and Boivin, 2021; Szczesniak *et al.*, 2023), which may have further influenced viral adsorption and aggregation properties.

Most studies only focus on the liquid fraction of the wastewater which could lead to discrepancies in the data (Hokajärvi *et al.*, 2021). However, there have been reports on the presence of SARS-CoV-2 in the solids present in wastewater with some studies even showing a higher concentration of the virus in the settled solids when compared to influent wastewater (Graham *et al.*, 2020; Kitamura *et al.*, 2021; Simpson *et al.*, 2021).

Additional pre-treatments such as chemical (sodium pyrophosphate) and physical (sonication) methods have been found to be beneficial in dislodging the attached viral particles from the sludge and improving the efficiency of the recovery process (Danovaro *et al.*, 2001; Juel *et al.*, 2021). In this study, sonication led to an increase in the concentration of SARS-CoV-2 in the supernatant by 3.30 – 35.65%. Some of the results are contradictory. For instance, Juel *et al.* (2021) found that sonicating bovine coronavirus increased its concentration, but in their study, SARS-CoV-2 concentration decreased after sonication. It has been reported that a higher sonication frequency would result damage to the viral particles/genetic material (Wu and Liu, 2009), which was observed for influenza A. Despite this, the increase of SARS-CoV-2 after pre-treatment indicates that there will be discrepancies in results if only the supernatant is analysed as a substantial concentration of the virus may be lost to the pellet. This necessitates the addition of a pre-treatment step. However, an optimization step is crucial for wastewater samples due to the fact that each sample matrix and virus can differ considerably as seen in this study.

3.5. CONCLUSIONS

This study showed that storage temperature and time as well as changes to the physicochemical characteristics of the sample significantly impacted viral biomarker degradation. Based on the findings of this study, influenza A had the lowest degradation at -20°C and SARS-CoV-2 at -80°C. The difference in degradation between storage at -20°C and -80°C were significant.

It was further deduced that SARS-CoV-2 whole viral particles has an affinity for the solids in wastewater. This interaction between viral particles and wastewater solids may have been influenced by changes in the physicochemical characteristics of the sample. Based on the success of the pre-treatment methods in dislodging viruses from the wastewater solids in fresh samples, this can also be applied to stored wastewater samples.

It is evident that even at -80°C, viral degradation is high which will ultimately lead to discrepancies in data for stored samples. This necessitates the evaluation of wastewater preservation studies for WBE analysis. Since pH impacts viral aggregation, pH adjustment to prevent viral attachment to the solids in wastewater can also be explored. Additionally, pre-treatment methods for the dislodgement of viral particles should be implemented for wastewater sample. This would be especially useful for stored wastewater as we have observed viral affinity for wastewater solids.

CHAPTER 4 : DETERMINING THE IMPACT OF STORAGE CONDITIONS ON THE INTEGRITY OF EXTRACTED VIRAL RNA USING DROPLET DIGITAL PCR

4.1. INTRODUCTION

The diagnostic methods used for the detection of viruses has evolved significantly from traditional serological assays to more sensitive diagnostic tools such as PCR (Jartti *et al.*, 2013; Katsarou *et al.*, 2019). Wastewater-based epidemiology (WBE) a non-invasive diagnostic tool used for the screening of pathogens in sewage, is one of the more recent developments in disease surveillance (Choi *et al.*, 2018; Aguiar-Oliveira *et al.*, 2020; Islam *et al.*, 2023). Whilst there are several advantages to WBE, its effectiveness relies on nucleic acid integrity (Huang *et al.*, 2017; Islam *et al.*, 2023).

Nucleic acids, which are polymeric macromolecules containing the genetic information of biological organisms in the form of DNA or RNA (Khedkar *et al.*, 2016). Whilst both molecules are extremely susceptible to degradation, the structural difference between the two makes RNA more susceptible to degradation than DNA (Lehman, 2013). Therefore nucleic acid integrity during storage is an important consideration in disease surveillance. Since most viruses have an RNA genome, it is especially important to understand the factors that influence its stability. Factors including suboptimal sample handling and storage conditions compromises the stability of RNA molecules, influencing deterioration of the sample (Ahmed *et al.*, 2022). This directly influences the reliability and validity of downstream analyses leading to discrepancies in the data generated (Gessoni *et al.*, 2000; Gerbehaye *et al.*, 2002; Fabre *et al.*, 2014). Therefore, identifying optimal storage conditions for long-term storage is important should RNA be required for future analysis (Paoli, 2005).

Laboratories globally store RNA samples for many reasons including biobanking, sequencing, monitoring of variants, or backdating disease outbreaks (Paoli, 2005; Heijnen *et al.*, 2021; Simpson *et al.*, 2021). The most common way to minimize RNA degradation and maintain integrity is storage at -20°C or -80°C, however, RNA still remains reactive due to the presence of ribonucleases within the sample so this practice may not always be ideal (Huang *et al.*, 2017). Preservation techniques may induce additional damage to the nucleic acid, further compromising its integrity (Paoli, 2005). Most RNA extraction kits recommend that RNA remains stable up to a year when stored at −20°C. However, Hokajärvi *et al*. (2021) noted a decline of RNA stored at −20°C and −75°C by day 58 of analysis.

As previously highlighted, there are several challenges that may lead to analytical delays at different stages of the WBE workflow (Simpson *et al.*, 2021). Whilst the effect of storage temperature and time on RNA degradation has been extensively researched, there is little known about the stability of SARS-CoV-2 RNA during storage. The degradation can depend on nucleotide composition, therefore it is important to understand SARS-CoV-2 RNA stability under different temperatures. For the same reason, the degradation patterns of influenza A and SARS-CoV-2 can vary. Therefore, it is important to identify the impact of storage conditions on the unprocessed sample, as well as the impact of storage on RNA degradation. This chapter highlights the effect of temperature and long term storage on the integrity extracted viral RNA using ddPCR. Additionally, a comparison between the degradation patterns of extracted RNA and unprocessed samples were compared to determine the most appropriate storage conditions for long term storage of samples.

4.2. MATERIALS AND METHODS

4.2.1. Sample collection, preparation, and viral concentration

Wastewater influent was collected by retrieving and mixing several discrete samples to form a 4 L composite sample from a municipal wastewater treatment plant located in the north of Durban, South Africa and transported on ice to the laboratory. At the lab, the sample was homogenized using a magnetic stirrer for 10 min at1500 rpm. Viral particles were concentrated using polyethylene glycol (PEG) (Catalog number: P3750, Minema Chemicals, South Africa) together with NaCl (Catalog number: S3250, Minema Chemicals, South Africa) according to the methods described by Sapula *et al.* (2021). Briefly, 100 ml of sample (2 batches of 50 ml) was first clarified by centrifugation (5810R, Eppendorf) at 5000 $\times g$ for 30 min at 4°C to remove large particles. The pellet was discarded and the supernatant used for subsequent analysis. The recovery of viral particles was achieved by pelleting the sample at 12 000 $\times g$ for 90 min at 4°C (Sapula *et al.*, 2021). An additional centrifugation (Hermle ZK 496, Lasec) step at 5000 $\times g$ for 5 min at 4°C was conducted in a swinging bucket rotor to ensure any residual particles that may have attached to the tube was dislodged. The final pellet was re-suspended in 140 µl of 1X phosphate buffered saline (PBS) (Catalog number: P5493-4L, Sigma Aldrich) for RNA extraction.

4.2.2. Determining the effects of storage conditions on viral RNA integrity

RNA was extracted using the Qiagen QiAmp Viral RNA MiniKit (Hilden, Germany) following the manufacturers protocols using an Eins Sci (E-C15-24-2P) centrifuge with the AS24-2 rotor. Extracted RNA was eluted in 25 µl nuclease free water and stored at 4°C (temperature control), -20°C, and -80°C for a period of 168 days. Additionally, RNA was extracted from attenuated forms of SARS-CoV-2 strain USA/WA1/2020 and influenza A strain subtype H1N1 (Microbiologics, USA), eluted in 25 µl nuclease free water and stored under the same

conditions. During the 168-day period, samples were analysed weekly for the first 30 days, bi-monthly for the next 60 days, and once a month for the remainder of the storage period.

Viral copy number were determined by ddPCR using the One-Step RT-ddPCR Advanced Kit for Probes from Biorad (USA) with specific primers and probes for each viral target as shown in Supplementary Data Table S1. Each 22 µl reaction contained 5 µl supermix, 2 µl reverse transcriptase, 1 µl dithiothreitol, and 1.98 µl each of the forward and reverse primers (10 µM), 0.55 µl of 10 µM probe, 4.49 µl nuclease-free water, and 5 µl template RNA (1ng). Droplets were generated using the QXD Automated Droplet Generator, read using the QX200 Droplet Reader together with the QuantaSoft 1.7 software, and analysed using the QuantaSoft Analysis Pro 1.0 software (Biorad, USA). The limit of detection determined for this study for the N2 (SARS CoV-2) and INFA (Influenza A) genes were 0.2 and 0.18 copies/ µl.

4.2.3. Statistical analysis

Microsoft Excel was used for data entry and for determining mean values and standard deviations. Any significant differences between the means of RNA concentration stored at each temperature was analysed by a one-way analysis of variance (ANOVA) using GraphPad Prism, version 10 (USA). In addition to this, the differences in degradation of wastewater samples and extracted RNA samples were compared using an independent two sample t-test. Since the wastewater was only stored for a period of 84 days, only data for 84 days of RNA storage was used.

4.3. RESULTS

4.3.1. Determining RNA degradation in stored samples

Quantification of SARS-CoV-2 from the stored RNA samples showed degradation at all temperatures at the end of the 168-day period. With an initial concentration of 5.75± 0.29 log copies/ µl (Appendix Table A9) in the RNA extracted from wastewater influent, the sample

degraded by 92.46% (4°C), 44.96% (-20°C) and 32.51% (-80°C) by the end of the 168-day storage period, as shown in Figure 4.1. The concentration of SARS-CoV-2 extracted from viral controls was 5.99± 0.49 log copies/ µl (Appendix Table A9). After 168 days of storage, samples stored at cold temperatures, SARS-CoV-2 degraded by 95.24% (4°C), 36.34% (-20°C) and 33.42% (-80°C) as shown in Figure 4.2. The highest degradation was observed at 4°C and the lowest at -80°C for RNA samples extracted from wastewater and viral controls.

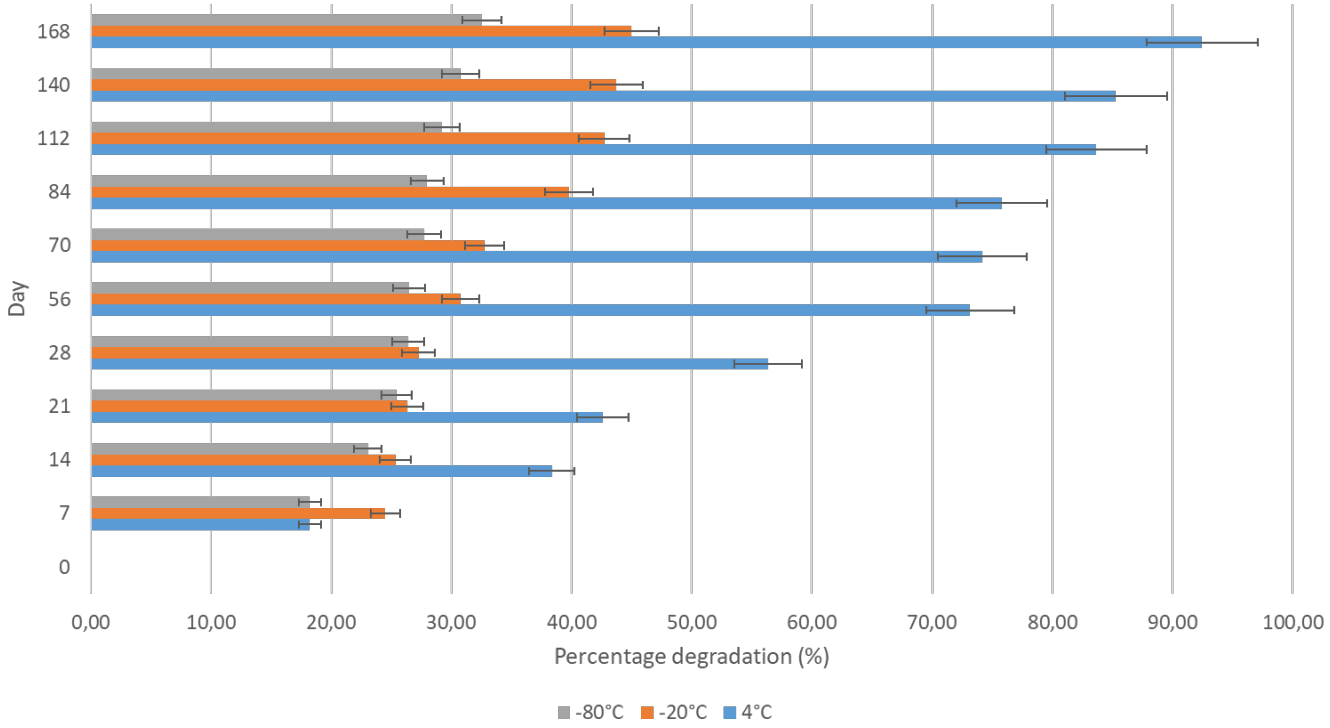

Figure 4.1: The percentage degradation of SARS-CoV-2 RNA from wastewater influent stored over a 168-day period at different temperatures

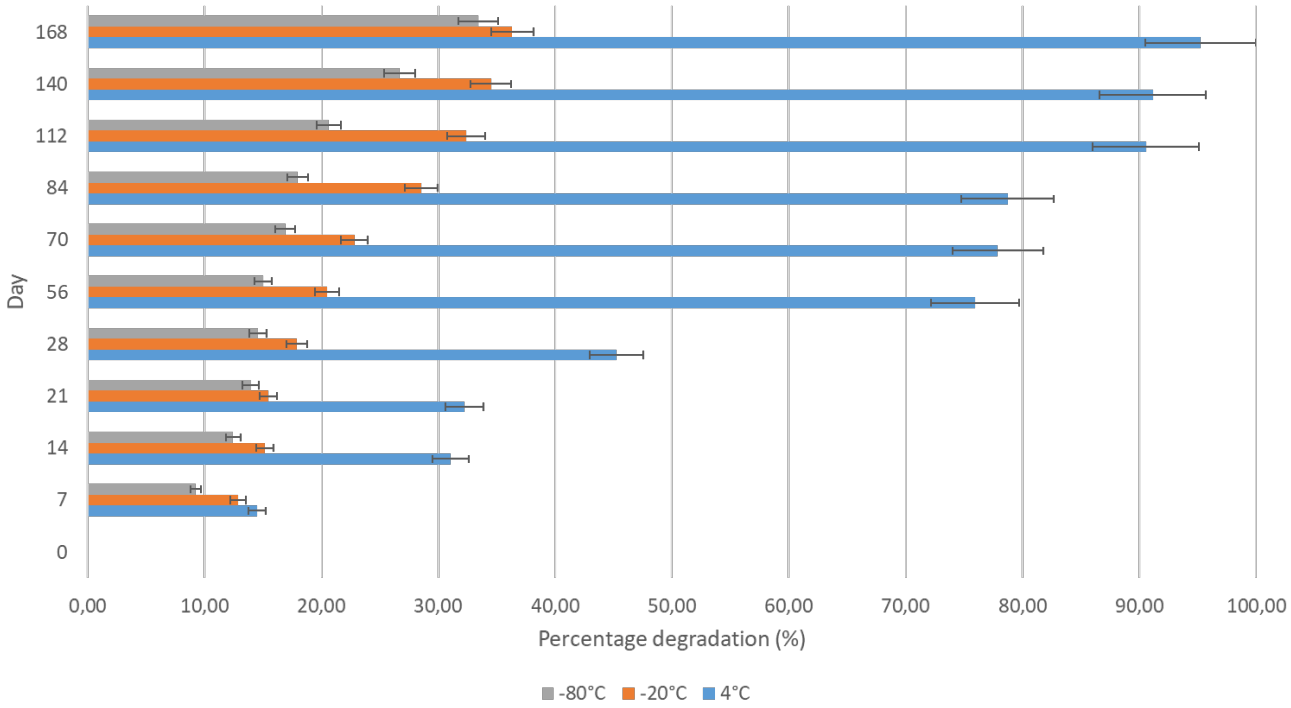

Figure 4.2: The percentage degradation of SARS-CoV-2 RNA from viral controls stored over a 168-day period at different temperatures

Quantification of influenza A from the stored RNA samples showed degradation at all temperatures at the end of the 168-day period. With an initial concentration of 5.13± 0.05 log copies/ µl (Appendix Table A9) in the RNA extracted from wastewater influent, there was 95.76% (4°C), 53.02% (-20°C) and 43.28% (-80°C) degradation by the end of the 168-day storage period, as shown in Figure 4.3. The concentration of influenza A extracted from viral controls was 5.42± 0.18 log copies/ µl (Appendix Table A9). By the end of the 168-day storage period there was 92.74% (4°C), 36.02% (-20°C) and 42.34% (-80°C) degradation, as shown in Figure 4.4. The highest degradation was observed at 4°C for RNA samples extracted from wastewater and viral controls. The lowest degradation was observed at -80°C for RNA samples extracted from wastewater and -20°C for RNA samples extracted from viral controls.

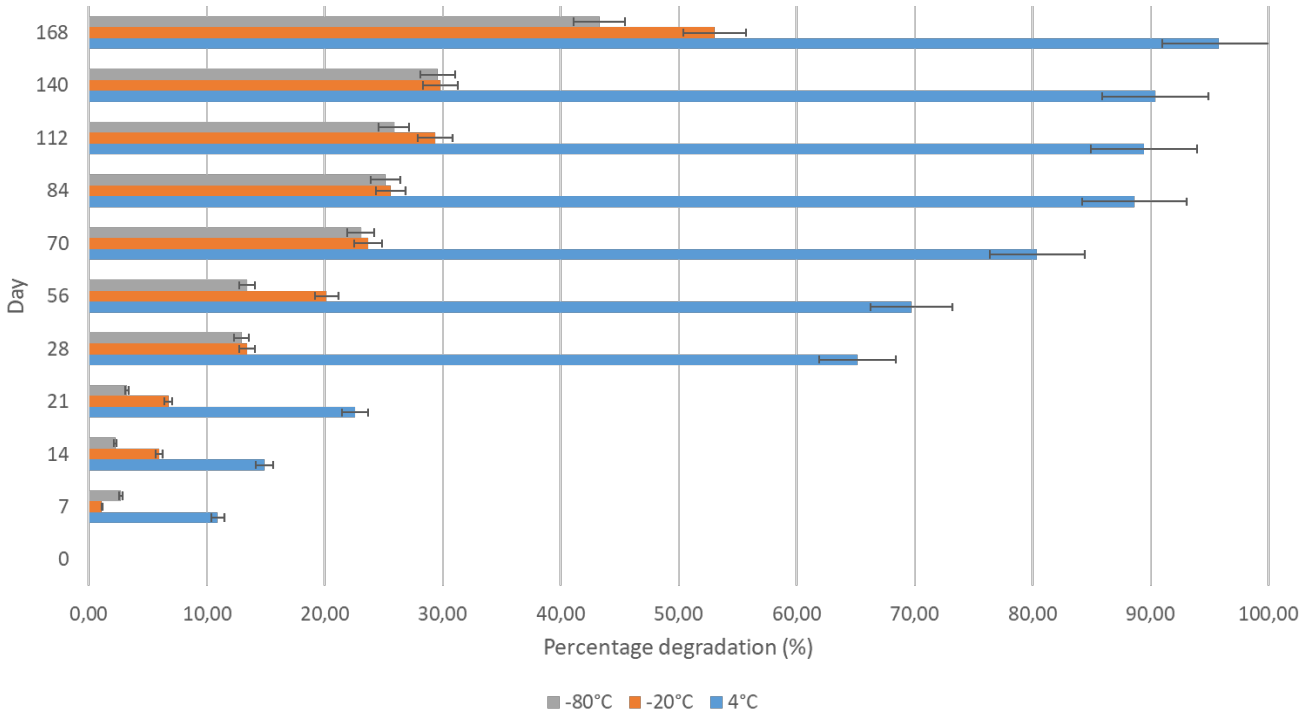

Figure 4.3: The percentage degradation of influenza A RNA from wastewater influent stored over a 168-day period at different temperatures

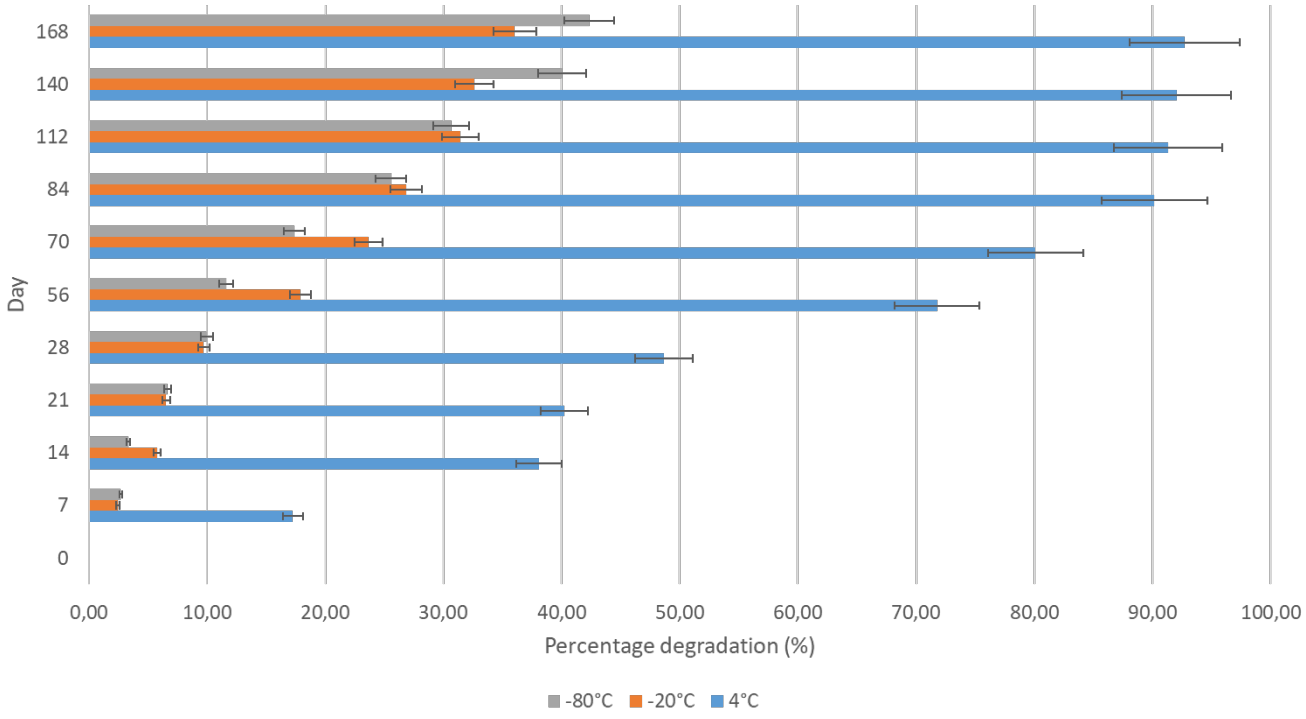

Figure 4.4: The percentage degradation of influenza A RNA from viral controls stored over a 168-day period at different temperatures

4.3.2. Effect of storage temperature on RNA degradation

A one way ANOVA was conducted to determine the effect of storage temperature on viral degradation in extracted RNA. A significant difference was noted between SARS-CoV-2 degradation and at least two groups in the case of RNA extracted from wastewater ($F(2, 3) = 15165567$) and RNA extracted from the control virus ($F(2, 3) = 17574978$). Tukey's multiple comparisons test showed that the difference in SARS-CoV-2 degradation in the RNA extracted from wastewater influent and viral controls were significant between all storage temperatures (Appendix Table A10). Degradation of SARS-CoV-2 RNA from wastewater influent samples and viral controls was significantly lower at -80°C when compared to all other storage temperatures.

In the case of influenza A, a significant difference was noted between influenza A degradation and at least three groups in the case of RNA extracted from wastewater ($F(2, 3) = 11244601$) and RNA extracted from viral controls ($F(2, 3) = 14745181$). Tukey's multiple comparisons test showed that the difference in influenza A degradation in the RNA extracted from both wastewater influent and viral controls were significant between samples stored at all temperatures ($p < 0.0001$; Appendix Table A10). Even though the difference in the degradation percentage between storage at -20°C and -80°C were minimal (i.e. <10%), the degradation of influenza A RNA was significantly lower at -80°C in the wastewater influent and at -20°C in the viral controls when compared to all other temperatures.

4.3.3. Comparison of degradation between stored wastewater influent samples and extracted RNA

A two sample t-test compared the difference in degradation between stored wastewater samples and stored extracted RNA. There was a significant difference in the degradation of SARS-CoV-2 and influenza A between wastewater influent samples and extracted RNA samples as shown in Table 4.1. In the case of SARS-CoV-2, RNA storage had a significantly lower degradation percentage at all temperatures when compared to wastewater influent storage. The same was observed for influenza A at 4°C. In contrast, wastewater influent storage had a significantly lower degradation percentage than RNA storage at -20°C and -80°C.

Table 4.1: t-test comparing difference in degradation between stored wastewater influent and stored extracted RNA

Sample Type	Storage Temperature	Percentage degradation (%) RNA / wastewater	t value	*p*-value
SARS-CoV-2 Extracted RNA vs wastewater influent	4°C	75.80 / 88.41	891,7(2)	<0.0001 (****)
	-20°C	39.75 / 77.21	2649(2)	<0.0001 (****)
	-80°C	27.97 / 64.43	2578(2)	<0.0001 (****)
Influenza A Extracted RNA vs wastewater influent	4°C	47.05 / 88.63	2940(2)	<0.0001 (****)
	-20°C	48.71 / 25.57	1636(2)	<0.0001 (****)
	-80°C	50.43 / 25.37	1772(2)	<0.0001 (****)

$p < 0.05$ - *; $p < 0.002$ - **; $p < 0.001$ - ***, $p < 0.0001$ - ****

4.4. DISCUSSION

The reliability of WBE as a disease surveillance tool depends on nucleic acid integrity. The guidelines included in most RNA extraction kits suggests that extracted RNA remains stable up to a year. RNA, being an extremely sensitive and unstable molecule, is known to be extremely susceptible to degradation (Ahmed *et al.*, 2022). It is therefore essential to understand the degradation patterns, especially if stored RNA is to be used for quantitative analysis, like in the case of WBE (Mao *et al.*, 2019; Cervantes-Avilés *et al.*, 2021).

In this study, degradation was observed at all storage temperature for both SARS-CoV-2 and influenza A. Samples stored at 4°C had degraded between 92.46% - 95.24% after 168 days of storage. Storage of RNA at higher temperatures are known to stimulate enzymatic and some non-enzymatic activity which leads to RNA cleavage (Kazakov *et al.*, 2006). This may occur through cleavage of the phosphodiester bonds, exposure to ozone, and exposure to chemicals and enzymes (Fabre *et al.*, 2014). A study by Fabre *et* al. (2014) showed that at room temperature, RNA degradation was strongly influenced by exposure to air. Contamination of the RNA by enzymes, namely RNAse's, due to improper laboratory practices, may also contribute to sample degradation (Tan and Yiap, 2009). Storage at ultra-low temperatures or in liquid nitrogen are known to inhibit these processes, but in some instances these processes may still continue or may even be accelerated (Kazakov *et al.*, 2006). Whilst the degradation was much lower, samples stored at -20°C (36.02% - 53.02%) and -80°C (32.51% - 43.28%) still degraded. The lowest degradation was observed at -80°C, with the exception of influenza A extracted from viral controls which had the lowest degradation at -20°C.

Whilst temperature is one of the main drivers of RNA degradation, it is important to consider other factors such as the GC-content. The GC-content greatly affects RNA stability (Chan *et al.*, 2009). Genomes with a higher GC-content are assumed to exhibit greater stability due to

an additional hydrogen bond that exists between the G-C pairs (when compared to A-T pairs). In this study, influenza A had a higher degree of degradation when compared to SARS-CoV-2 despite having a higher GC-content (43.37% vs 38%) (Brázda *et al.*, 2021). This finding may have been attributed to the fact that the GC-content of the target regions may differ from the GC-content of the whole genome. Furthermore, the concentration of influenza A was lower than SARS-CoV-2 on day 0 and may have contributed to the faster degradation. Additionally, RNA extraction reagents and processes may not have efficiently removed PCR inhibitors (Ahmed *et al.*, 2022). This would especially be problematic in samples obtained from wastewater as these matrices contain a mixture of chemicals such as detergents, pharmaceuticals, heavy metals, biological wastes, enzymes (such as RNAse's), and other substances (McCall *et al.*, 2016; Achak *et al.*, 2021). These contaminants may be particularly difficult to remove, even when using commercial extraction kits further facilitating the degradation of the extracted RNA (Ahmed *et al.*, 2022). As previously mentioned, the interaction of these contaminants leads to cleavage of the phosphodiester bonds in the RNA molecule (Fabre *et al.*, 2014).

The presence of chemical and biological contaminants are especially important to consider when comparing the storage of wastewater influent to the storage of extracted RNA. In an environmental setting, these contaminants are present at higher concentrations. This coupled with the presence of other microorganisms will accelerate the degradation process. Although present in the extracted RNA samples, these contaminants are residual so its influence on RNA degradation will not be as strong (Fabre *et al.*, 2014; McCall *et al.*, 2016; Achak *et al.*, 2021; Ahmed *et al.*, 2022). For SARS-CoV-2, degradation was significantly lower in the stored RNA sample when compared to the raw wastewater influent samples. In contrast, a significantly lower degradation was observed in the stored wastewater samples when compared to the extracted RNA of influenza A. There may be several reasons for that which were not

determined in this study. A limitation here maybe the presence of PCR inhibitors, which in addition to contributing to RNA degradation, may have resulted in false-positive or false-negative readings (Ahmed *et al.*, 2022).

4.5. CONCLUSIONS

Viral RNA stability is impacted by storage temperatures. Although, viral RNA was still detectable after 168 days of storage in nuclease free H_2O at ultra-low temperatures. For biobanking purposes, the extracted RNA should preferably be stored at -80°C. However, where resources are limited, RNA may be stored at -20°C. It can be concluded that RNA stored for extended periods of time should be used for detection and not quantification. The variables affecting the degradation of the extracted RNA are numerous and will therefore be difficult to determine. Should the RNA be required for quantification purposes, it should undergo additional preservation steps such as lyophilization.

Chapter 5 : CONCLUSIONS AND RECOMMENDATIONS

The primary focus of this study was to assess the stability of, and understand the degradation patterns of SARS-CoV-2 and influenza A under various storage conditions. The results have shown that the viral degradation was significantly influenced by storage temperature and time with a higher degradation observed at higher temperatures and longer incubation time. A notable finding was that the degradation patterns of influenza A and SARS-CoV-2 differed between ultra-low storage temperatures i.e. -20 °C and -80°. The lowest degradation of SARS-CoV-2 was at -80°C (64.43%) whereas influenza A had the lowest degradation at -20°C (23.05%). This showed that viral stability can vary depending on other factors such as virus structure, GC-content, and the complexity of the matrix. Whilst both viruses are enveloped viruses, they are structurally different which can account for the differences in results. Despite this significant difference in degradation between -20 °C and -80°, storage at -20 °C will be acceptable in resource constrained facilities.

Storage temperature and time had also influenced changes in the physicochemical characteristics of wastewater. It was further deduced that changes in Salinity, DO, pH, COD, TS, and TFS were found to impact viral degradation and influence viral interaction with wastewater solids during storage. pH in particular was correlated to viral concentrations in wastewater solids. Factors such as pH are known to influence viral aggregation properties which increases viral affinity for wastewater solids. This coupled with the unusual degradation patterns of SARS-CoV-2 suggests viral adsorption to the solid matter. Viral affinity to wastewater solids will affect downstream analysis leading to discrepancies in data. This necessitates the addition of wastewater solids in WBE analysis. Additionally, sonication effectively enhanced SARS-CoV-2 recovery up to 35.65% from wastewater samples, indicating pre-treatment should also be applied to stored wastewater samples.

In this study pre-treatment using sodium pyrophosphate was ineffective in enhancing viral recovery but sonication effectively enhanced SARS-CoV-2 concentration. In contrast, there was a decline in influenza A concentrations which suggests that the impact of pre-treatment will vary based on viral structure. Optimization will therefore be necessary depending on the target organism and sample type.

As with the storage of wastewater samples, storage of RNA samples degraded at all temperatures with higher degradation at higher temperatures. A comparison between wastewater influent and RNA storage revealed that SARS-CoV-2 degradation was significantly lower in the RNA sample (32.51%) than the wastewater influent (64.43%). In contrast, influenza A degraded faster in the extracted RNA form (43.28%) than in the wastewater influent (23.05%). This further supports the notion that viral structure and genome play a major role in stability and persistence. Additionally, stored samples should only be used for detection and not quantification. Since samples degraded significantly at all temperatures, the data generated will be inaccurate.

Whilst significant progress has been made in the field of WBE, the findings of this study highlight a few factors that makes the standardization of WBE a challenge. Based on these findings the following recommendations can be made:

- More emphasis needs to be placed on the importance of preservation techniques to maintain sample integrity for disease surveillance, especially in the case of biobanking.
- Studies to evaluate the addition of preservatives for maintaining RNA stability at higher temperatures should also be conducted.
- Physicochemical characteristics were found to impact viral concentration, based on this storage of post-centrifuged samples and concentrated samples should also be explored.

- pH adjustments for long term storage could potentially be explored to potentially reduce viral affinity for wastewater solids and reduce microbial processes.

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APPENDICES

Table A1: Target sequences and cycling conditions used in this study

Organism	Gene Target	Sequence	Cycling Conditions	Reference
SARS-CoV-2	N2-F	TTACAAACATTGGCCGCAAA	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	(Lu *et al.*, 2020b; Pillay *et al.*, 2021; Pillay *et al.*, 2022)
	N2-R	GCGCGACATTCCGAAGAA		
	N2-P	VIC-ACAATTTGCCCCCAGCGCTTCAG-ZEN/Iowa		
Influenza A	INFA-F	GACCRATCCTGTCACCTCTGAC	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	
	INFA-R	AGGGCATTYTGGACAAAKCGTCTA		

	INFA-P	FAM-TGC AGT CCT CGC TCA CTG GGC ACG-ZEN/Iowa	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	(Malhotra *et al.*, 2016; Pillay *et al.*, 2022)

Table A2: Physicochemical characteristics of wastewater

Parameter	Storage Temperature	Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
Temperature (ºC)	± 25°C	22.74 (±0.16)	21.15(±0.23)	20.04(±0.05)	21.98(±0.01)	23.39(±0.21)	21.47(±0.01)	20.65(±0.08)
	4°C		21.17(±0.05)	20.89(0.02)	21.36(±0.05)	23.32(±0.13)	21.63(±0)	20.50(±0.42)
	-20°C		21.45(±0.65)	20.15(±0.02)	20.65(±0.19)	23.81(±0.10)	20.24(±0.01)	20.01(±0.72)
	-80°C		21.31(±0.15)	19.87(±0.01)	19.32(±0.16)	22.61(±0.16)	20.09(±0.02)	19.13(±0.13)
	± 25°C	720(±2.08)	760(±8.39)	764(±2.89)	792(±1.73)	778(±0.58)	789(±0)	804(±2.08)

Electrical conductivity (µS/cm)	**4°C**		733(±3.46)	750(±2.52)	758(±0.58)	761(±0.58)	746(±6.03)	758(±6.81)
	-20°C		720(±12.42)	717(±0)	713(±0.58)	724(±0.58)	707(±6.11)	701(±1.53)
	-80°C		733(±2.52)	727(±0.58)	728(±1.15)	725(±3.79)	722(±0.58)	723(±0)
Total dissolve solids - TDS (g/L)	**± 25°C**	0.47(±0)	0.50(±0.01)	0.50(±0)	0.52(±0)	0.51(±0)	0.34(±0)	0.52(±0)
	4°C		0.48(±0)	0.49(±0)	0.49(±0)	0.49(±0)	0.42(±0.11)	0.49(±0)
	-20°C		0.47(±0.01)	0.47(±0)	0.46(±0)	0.47(±0)	0.45(±0)	0.46(±0)
	-80°C		0.47(±0.01)	0.47(±0)	0.47(±0)	0.47(±0)	0.47(±0)	0.47(±0)
Salinity (ppt)	**± 25°C**	0.35(±0)	0.37(±0)	0.37(±0.01)	0.39(±0)	0.38(±0)	0.36(±0)	0.39(±0.01)
	4°C		0.36(±0)	0.37(±0)	0.37(±0)	0.37(±0.01)	0.32(±0.08)	0.37(±0)
	-20°C		0.35(±0.01)	0.35(±0)	0.35(±0)	0.36(±0)	0.34(±0.01)	0.34(±0)
	-80°C		0.36(±0.01)	0.36(±0)	0.36(±0)	0.36(±0.01)	0.35(±0)	0.36(±0.01)

Dissolved oxygen (%)	**± 25°C**	13.37(±0.06)	10.8(±4.61)	24.77(±1.12)	19.17(±0.38)	34.5(±0.3)	31.9(±0)	5.87(±0.7)
	4°C		7.77(±0.85)	9.67(±0.35)	1.33(±0.31)	17.87(±2.64)	22.67(±7.97)	10.8(±1.71)
	-20°C		71.1(±0.20)	5.77(±3.30)	17.87(±0.15)	15.6(±2.09)	6.47(±0.32)	22.13(±1.74)
	-80°C		70.9(±1.71)	1.53(±0.35)	22.6(4.65)	15.3(±3.91)	1(±0.75)	29.47(±0.4)
Dissolved oxygen (mg/L)	**± 25°C**	1.43(±0.02)	0.96(±0.4)	2.24(±0.10)	1.67(±0.03)	3.59(±0.04)	2.93(±0)	0.53(±0.06)
	4°C		1.34(±0.8)	0.88(±0.03)	0.18(±0.11)	1.86(±0.27)	2.07(±0.73)	1.28(±0.14)
	-20°C		8.75(±0.09)	0.49(±0.29)	1.77(±0.02)	1.68(±0.22)	0.8(±0.38)	2.18(±0.2)
	-80°C		8.63(±0.4)	0.23(±0.18)	2.07(±0.43)	1.62(±0.41)	0.37(±0.24)	2.9(±0.03)
pH	**± 25°C**	6.74(±0.06)	6.28(±0.01)	6.81(±0.02)	7.2(±0.11)	6.79(±0.05)	7.11(±0)	6.65(±0.02)
	4°C		6.09(±0)	6.88(±0.02)	6.36(±0.04)	6.51(±0.02)	6.75(±0.04)	6.28(±0.12)
	-20°C		7.0(±0.01)	7.22(±0.01)	7.08(±0.01)	6.67(±0.01)	7.24(±0.02)	7.21(±0.11)

	-80°C		7.11(±0.02)	6.78(±0.01)	6.38(±0.01)	6.5(±0.01)	6.95(±0.03)	6.96(±0.01)
COD (mg/L)	**± 25°C**	1320(±5.51)	623(±16.5)	470(±47.95)	422(±2.49)	412(±8.73)	361(±15.28)	346(±33.32)
	4°C		1297(±18.48)	1266(±24.58)	1156(±14.3)	674(±30.35)	657(±34.51)	602(±20.55)
	-20°C		1251(±27.03)	961(±53.12)	837(±13.58)	831(±25.63)	808(±27.79)	754(±33.31)
	-80°C		1266(±24.58)	1035(±38.55)	953(±38.66)	857(±21.22)	849(±36.47)	787(±14)
Total solids (mg/L)	**± 25°C**	1.013(±0.07)	0.614(±0.009)	0.864(±0.03)	0.63(±0.02)	0.766(±0.009)	1.397(±0.007)	0.715(±0.004)
	4°C		0.884(±0.10)	1.027(±0.17)	1.043(±0.007)	1.007(±0.12)	0.869(±0.02)	0.782(±0.006)
	-20°C		0.534(±0.003)	1.114(±0.15)	0.793(±0.18)	0.727(±0.06)	0.699(±0.08)	0.635(±0.04)
	-80°C		0.641(±0.02)	0.855(±0.05)	0.677(±0.004)	0.826(±0.009)	0.735(±0.004)	0.75(±0.01)
Total fixed solids (mg/L)	**± 25°C**	0.481(±0.03)	0.175(±0.09)	0.225(±0.02)	0.202(±0.03)	0.267(±0.004)	0.696(±0.009)	0.191(±0.004)
	4°C		0.454(±0.02)	0.436(±0.09)	0.461(±0.07)	0.397(±0.01)	0.406(±0.02)	0.302(±0.006)

	-20°C	0.265(±0.007)	0.474(±0.07)	0.403(±0.15)	0.301(±0.04)	0.333(±0.04)	0.233(±0.02)
	-80°C	0.291(±0.01)	0.334(±0.02)	0.277(±0.004)	0.366(±0)	0.343(±0.001)	0.301(±0.007)

Table A3: Recovery of SARS-CoV-2 from stored wastewater samples

	Storage temperature	**Sample type**	**Log copies / 100 ml**						
			Day 0	**Day 14**	**Day 28**	**Day 42**	**Day 56**	**Day 70**	**Day 84**
Influent wastewater	**± 25°C**	**Supernatant**	5.28±0.14	5,11±0.08	4.59±0.37	4.11±0.09	4.42±0.16	4.06±0.07	4.08±0.29
	4°C			4..81±0.12	4.68±0.04	4.67±0.13	4.43±0.06	4.35±0.09	4.32±0.47
	-20°C			5.06±0.2	5.04±0.13	4.90±0.06	4.84±0.11	4.82±0.08	4.65±0.03
	-80°C			5.11±0.11	5±0.03	4.98±0.03	4.97±0	4.94±0.02	4.84±0.04
	± 25°C	**Pellet**	4.96±0.06	4.88±0.06	4.38±0.27	4.17±0.04	4.15±0.15	4.11±0.01	3.76±0.02

	4°C		4.58±0.09	4.53±0.08	4.32±0.15	4.25±0.15	4.22±0.15	3.82±0.08
	-20°C		4.17±0.22	4.04±0.05	4.01±0.24	3.94±0.29	3.93±0.02	3.92±0.17
	-80°C		4.52±0.04	4.39±0.06	4.27±0.08	4.27±0.21	4.19±0.13	4.05±0.02
Seeded wastewater	**± 25°C**	5.43±0.01	N/A	N/A	4.89±0.03	N/A	N/A	4.19±0.12
	4°C		N/A	N/A	4.74±0.06	N/A	N/A	3.96±0.12
	-20°C		N/A	N/A	4.77±0.31	N/A	N/A	4.2±0.02
	-80°C		N/A	N/A	4.78±0.05	N/A	N/A	4.79±0.12
	± 25°C	4.48±0.01	N/A	N/A	4.18±0.08	N/A	N/A	4.22±0.003
	4°C		N/A	N/A	4.43±0.1	N/A	N/A	4.35±0.09
	-20°C		N/A	N/A	4.30±0.05	N/A	N/A	4.02±0.03
	-80°C		N/A	N/A	4.18±0.07	N/A	N/A	4.10±0.002

Table A4: Recovery of influenza A from stored wastewater samples

	Storage temperature	Sample type	Log copies / 100 ml						
			Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
Influent wastewater (Low)	± 25°C	Supernatant	4,87 (±0.04)	4.78(±0.03)	4.62(±0.05)	4.35(±0.01)	4.17(±0.11)	4.11(±0.28)	3.90(±0.13)
	4°C			4.89(±0.04)	4.86(±0.07)	4.85(±0.01)	4.75(±0.02)	4.66(±0.05)	4.47(±0.04)
	-20°C			4.85(±0.02)	4.82(±0.04)	4.83(±0.02)	4.78(±0.01)	4.77(±0.04)	4.73(±0.04)
	-80°C			4.87(±0.01)	4.84(±0.04)	4.83(±0.02)	4.81(±0.01)	4.79(±0.05)	4.75(±0.03)
	± 25°C	Pellet	4.99(±0.12)	5.15(±0.14)	4.59(±0.21)	4.79(±0.06)	4.75(±0.05)	4.52(±0.02)	4.23(±0.06)
	4°C			5.12(0.17)	4.90(±0.08)	4.97(±0.04)	4.83(±0.02)	4.76(±0.02)	4.38(±0.19)
	-20°C			4.97(±0.07)	4.91(±0.07)	4.59(±0.13)	4.77(±0)	4.77(±0.02)	4.73(±0.04)
	-80°C			5.04(±0.07)	5.01(±0.04)	4.93(±0.05)	4.81(±0.1)	4.83(±0.02)	4.75(±0.14)

Seeded wastewater	**± 25°C**	**Supernatant**	4.96(±0.03)	N/A	N/A	4.8(±0.05)	N/A	N/A	4.01(±0.05)
	4°C			N/A	N/A	4.81(±0.13)	N/A	N/A	4.76(±0.001)
	-20°C			N/A	N/A	4.82(±0.13)	N/A	N/A	4.76(±0.01)
	-80°C			N/A	N/A	4.83(±0.15)	N/A	N/A	4.74(±0.04)
	± 25°C	**Pellet**	4.48(±0.04)	N/A	N/A	4.77(±0.14)	N/A	N/A	4.21(±0.22)
	4°C			N/A	N/A	4.72(±0.12)	N/A	N/A	4.12(±0.01)
	-20°C			N/A	N/A	4.73(±0.02)	N/A	N/A	4.56(±0.04)
	-80°C			N/A	N/A	4.72(±0.11)	N/A	N/A	4.74(±0.01)

Table A5: ANOVA and Kruskal-Wallis test showing variation between SARS-CoV-2 degradation in samples stored at different temperatures

Sample type	Storage Temperature	ANOVA (Tukeys multiple comparisons test)		Kruskal-Wallis (Dunn's multiple comparisons test)	
		Mean difference	*p* value	Mean rank difference	*p* value
Wastewater influent supernatant	**± 25°C vs 4°C**	-0.1671	>0.9999	0.1429	0.4108
	± 25°C vs -20°C	18.34	0.6510	7.000	0.1487
	± 25°C vs -80°C	24.16	0.4295	9.429	0.4413
	4°C vs -20°C	18.50	0.6445	6.857	>0.9999
	4°C vs -80°C	24.33	0.4236	9.286	>0.9999
	-20°C vs -80°C	5.826	0.9821	2.429	>0.9999

Wastewater influent pellet	**± 25°C vs 4°C**	-2,679	0,9987	2,714	>0,9999
	± 25°C vs -20°C	-5,196	0,9910	2,429	>0,9999
	± 25°C vs -80°C	-15,74	0,8112	-5,143	>0,9999
	4°C vs -20°C	-2,517	0,9990	-0,2857	>0,9999
	4°C vs -80°C	-13,06	0,8814	-7,857	0,4413
	-20°C vs -80°C	-10,55	0,9327	-7,571	0,5079
Seeded wastewater supernatant	**± 25°C vs 4°C**	-3,563	0,9997	-1,333	>0.9999
	± 25°C vs -20°C	-3,573	0,9997	-1,000	>0.9999
	± 25°C vs -80°C	5,367	0,9990	1,000	>0.9999
	4°C vs -20°C	-0,01000	>0,9999	0,3333	>0.9999
	4°C vs -80°C	8,930	0,9953	2,333	>0.9999

	-20°C vs -80°C	8,940	0,9952	2,000	>0.9999
Seeded wastewater pellet	**± 25°C vs 4°C**	3,530	0,9979	0,3333	>0.9999
	± 25°C vs -20°C	-10,36	0,9536	-1,667	>0.9999
	± 25°C vs -80°C	-14,72	0,8827	-2,667	>0.9999
	4°C vs -20°C	-13,89	0,8987	-2,000	>0.9999
	4°C vs -80°C	-18,25	0,8033	-3,000	>0.9999
	-20°C vs -80°C	-4,357	0,9962	-1,000	>0.9999

$p < 0.05$ - *

Table A6: ANOVA and Kruskal-Wallis test showing variation between influenza A degradation in samples stored at different temperatures

Sample type	**Storage Temperature**	**ANOVA (Tukeys multiple comparisons test)**	**Kruskal-Wallis (Dunn's multiple comparisons test)**

		Mean difference	*p* value	Mean rank difference	*p* value
Wastewater influent supernatant	**± 25°C vs 4°C**	35.47	0.0276 (*)	8.000	0.4108
	± 25°C vs -20°C	44.23	0.0047 (*)	9.857	0.1487
	± 25°C vs -80°C	41.29	0.0087 (*)	7.857	0.4413
	4°C vs -20°C	8.767	0.8761	1.857	>0.9999
	4°C vs -80°C	5.821	0.9589	-0.1429	>0.9999
	-20°C vs -80°C	-2.946	0.9943	-2.000	>0.9999
Wastewater influent pellet	**± 25°C vs 4°C**	19.32	0.4517	5.571	>0.9999
	± 25°C vs -20°C	14.43	0.6800	4.286	>0.9999
	± 25°C vs -80°C	4.810	0.9817	0.4286	>0.9999
	4°C vs -20°C	-4.894	0.9808	-1.286	>0.9999

	4°C vs -80°C	-14.51	0.6761	-5.143	>0.9999
	-20°C vs -80°C	-9.616	0.8767	-3.857	>0.9999
Seeded wastewater supernatant	**± 25°C vs 4°C**	13.33	0.9611	0.3333	>0.9999
	± 25°C vs -20°C	13.59	0.9589	0.3333	>0.9999
	± 25°C vs -80°C	13.98	0.9556	0.6667	>0.9999
	4°C vs -20°C	0.2600	>0.9999	0.000	>0.9999
	4°C vs -80°C	0.6500	>0.9999	0.3333	>0.9999
	-20°C vs -80°C	0.3900	>0.9999	0.3333	>0.9999
Seeded wastewater pellet	**± 25°C vs 4°C**	-2.997	0.9997	-1.000	>0.9999
	± 25°C vs -20°C	1.573	>0.9999	0.000	>0.9999
	± 25°C vs -80°C	4.607	0.9991	-0.333	>0.9999

	4°C vs -20°C	4.570	0.9991	1.000	>0.9999
	4°C vs -80°C	7.603	0.9959	0.6667	>0.9999
	-20°C vs -80°C	3.033	0.9997	-0.3333	>0.9999

$p < 0.05$ - *

Table A7: ANOVA and Kruskal-Wallis test showing variation between SARS-CoV-2 and influenza A degradation in the supernatant of stored samples

Storage Temperature	**Virus (wastewater matrix)**	**ANOVA (Tukeys multiple comparisons test)**		**Kruskal-Wallis (Dunn's multiple comparisons test)**	
		Mean difference	*p* value	Mean rank difference	*p* value
± 25°C	**Influenza A (influent vs seeded)**	21,16	0,8647	1,667	>0,9999

	Influenza A vs SARS-CoV-2 (influent)	-14,02	0,9095	-3,571	>0,9999
	Influenza A vs SARS-CoV-2 (influent vs seeded)	1,093	>0,9999	0,000	>0,9999
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-35,18	0,5830	-5,238	>0,9999
	Influenza A vs SARS-CoV-2 (seeded)	-20,07	0,9238	-1,667	>0,9999
	SARS-CoV-2 (influent vs seeded)	15,11	0,9443	3,571	>0,9999
4°C	**Influenza A (influent vs seeded)**	-0,9805	>0,9999	0,1905	>0,9999

	Influenza A vs SARS-CoV-2 (influent)	-49,66	0,0371 (*)	-7,000	0,1577
	Influenza A vs SARS-CoV-2 (influent vs seeded)	-37,94	0,3162	-4,810	>0,9999
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-48,68	0,1422	-7,190	0,4624
	Influenza A vs SARS-CoV-2 (seeded)	-36,96	0,4777	-5,000	>0,9999
	SARS-CoV-2 (influent vs seeded)	11,72	0,9449	2,190	>0,9999
-20°C	**Influenza A (influent vs seeded)**	-9,488	0,9499	-0,4762	>0,9999

	Influenza A vs SARS-CoV-2 (influent)	-39,92	0,0472 (*)	-6,143	0,3071
	Influenza A vs SARS-CoV-2 (influent vs seeded)	-46,71	0,0786	-6,143	0,7857
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-30,43	0,3522	-5,667	0,9812
	Influenza A vs SARS-CoV-2 (seeded)	-37,23	0,3249	-5,667	>0,9999
	SARS-CoV-2 (influent vs seeded)	-6,796	0,9805	0,000	>0,9999
-80°C	**Influenza A (influent vs seeded)**	-6,152	0,9787	0,000	>0,9999

Influenza A vs SARS-CoV-2 (influent)	-31,15	0,0863	-5,857	0,3774
Influenza A vs SARS-CoV-2 (influent vs seeded)	-34,83	0,1583	-6,333	0,7157
Influenza A vs SARS-CoV-2 (seeded vs influent)	-25,00	0,4075	-5,857	0,8981
Influenza A vs SARS-CoV-2 (seeded)	-28,68	0,4333	-6,333	>0,9999
SARS-CoV-2 (influent vs seeded)	-3,681	0,9952	-0,4762	>0,9999

$p < 0.05$ - *

Table A8: ANOVA and Kruskal-Wallis test showing variation between SARS-CoV-2 and influenza A degradation in the pellet of stored samples

Storage Temperature	Sample Type	ANOVA (Tukeys multiple comparisons test)		Kruskal-Wallis (Dunn's multiple comparisons test)	
		Mean difference	*p* value	Mean rank difference	*p* value
± 25°C	**Influenza A (influent vs seeded)**	-4,321	0,9979	-1,524	-1,524
	Influenza A vs SARS-CoV-2 (influent)	-16,33	0,8209	-3,143	-3,143
	Influenza A vs SARS-CoV-2 (influent vs seeded)	21,11	0,8204	3,143	3,143
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-12,01	0,9591	-1,619	-1,619
	Influenza A vs SARS-CoV-2	25,43	0,8125	4,667	4,667

	(seeded)				
	SARS-CoV-2 (influent vs seeded)	37,44	0,4372	6,286	6,286
4°C	**Influenza A (influent vs seeded)**	-26,64	0,5760	-3,762	>0,9999
	Influenza A vs SARS-CoV-2 (influent)	-38,33	0,1142	-6,000	0,3410
	Influenza A vs SARS-CoV-2 (influent vs seeded)	5,316	0,9936	1,571	>0,9999
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-11,69	0,9394	-2,238	>0,9999
	Influenza A vs SARS-CoV-2	31,95	0,5653	5,333	>0,9999

	(seeded)				
	SARS-CoV-2 (influent vs seeded)	43,64	0,1858	7,571	0,3759
-20°C	**Influenza A (influent vs seeded)**	-17,17	0,7958	-3,619	>0,9999
	Influenza A vs SARS-CoV-2 (influent)	-35,95	0,1015	-7,571	0,0975
	Influenza A vs SARS-CoV-2 (influent vs seeded)	-3,680	0,9972	-0,6190	>0,9999
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-18,78	0,7496	-3,952	>0,9999
	Influenza A vs SARS-CoV-2	13,49	0,9276	3,000	>0,9999

	(seeded)				
	SARS-CoV-2 (influent vs seeded)	32,27	0,3438	6,952	0,5242
-80°C	**Influenza A (influent vs seeded)**	-4,524	0,9959	-1,810	>0,9999
	Influenza A vs SARS-CoV-2 (influent)	-36,88	0,1279	-8,000	0,0666
	Influenza A vs SARS-CoV-2 (influent vs seeded)	1,579	0,9998	-0,4762	>0,9999
	Influenza A vs SARS-CoV-2 (seeded vs influent)	-32,36	0,4077	-6,190	0,7679
	Influenza A vs SARS-CoV-2	6,103	0,9940	1,333	>0,9999

	(seeded)				
	SARS-CoV-2 (influent vs seeded)	38,46	0,2675	7,524	0,3860

$p < 0.05$ - *

Table A9: Viral concentrations during the 168-day storage period

Day	**Storage Temperature**	**SARS-CoV-2**		**Influenza A**	
		Wastewater influent	**Viral controls**	**Wastewater influent**	**Viral controls**
0	**± 25°C**	5,73±0.79	5,99±0.49	5,13±0.05	5,42±0.18
7		5,38±0.4	5,78±0.1	4,82±0.06	5,29±0.23
14		4,99±0.11	5,42±0.36	4,62±0.04	4,93±0.17
21		4,49±0.06	5,17±0.55	4,59±0.21	4,73±0.12

28		4,01±0.04	4,78±0.06	4,40±0.01	4,43±0.07
56		3,79±0.01	4,57±0.23	4,09±0.02	4,28±0.09
70		2,95±0.01	3,76±0.01	4,04±0.01	4,27±0.01
84		2,64±0.01	3,61±0.02	3,76±0.01	3,78±0.02
112		-	-	3,33±0.04	3,59±0.03
140		-	-	-	-
168		-	-	-	-
0	**4°C**	5,73±0.79	5,99±0.49	5,13±0.05	5,42±0.18
7		5,67±0.33	5,92±0.26	5,08±0.38	5,34±0.1
14		5,54±0.49	5,82±0.64	5,06±0.33	5,21±0.55
21		5,51±0.14	5,82±0.04	5,02±0.07	5,19±0.38

28		5,39±0.21	5,72±0.1	4,67±0.03	5,13±0.14
56		5,18±0.07	5,37±0.36	4,61±0.22	4,87±0.21
70		5,17±0.16	5,33±0.1	4,42±0.08	4,72±0.09
84		5,14±0.23	5,31±0.07	4,18±0.02	4,41±0.06
112		4,97±0.04	4,96±0.06	4,15±0.04	4,36±0.01
140		4,92±0.06	4,93±0.05	4,11±0.01	4,32±0.08
168		4,63±0.12	4,66±0.02	3,75±0.09	4,28±0.02
0	**-20°C**	5,73±0.79	5,99±0.49	5,13±0.05	5,42±0.18
7		5,63±0.011	5,93±0.46	5,12±0.43	5,41±0.34
14		5,63±0.04	5,91±0.79	5,10±0.1	5,39±0.4
21		5,62±0.7	5,91±0.09	5,10±0.77	5,39±0.25

28		5,61±0.23	5,90±0.29	5,07±0.08	5,37±0.34
56		5,59±0.02	5,89±0.31	5,03±0.05	5,33±0.05
70		5,58±0.07	5,87±0.44	5,01±0.02	5,3±0.02
84		5,53±0.17	5,84±0.34	5±0.01	5,28±0.06
112		5,51±0.11	5,82±0.22	4,98±0.04	5,25±0.02
140		5,50±0.05	5,80±0.1	4,97±0.01	5,25±0.03
168		5,49±0.06	5,79±0.06	4,8±0.06	5,22±0.08
0	**-80°C**	5,73±0.79	5,99±0.49	5,13±0.05	5,42±0.18
7		5,67±0.21	5,94±0.88	5,12±0.29	5,41±0.45
14		5,64±0.42	5,93±0.88	5,12±0.23	5,40±0.91
21		5,63±0.1	5,92±0.02	5,11±0.02	5,39±0.33

28	5,62±0.15	5,92±0.07	5,07±0.01	5,37±0.29
56	5,62±0.39	5,92±0.12	5,07±0.09	5,36±0.2
70	5,61±0.06	5,91±0.04	5,01±0.12	5,33±0.13
84	5,61±0.07	5,90±0.09	5±0.03	5,29±0.03
112	5,60±0.03	5,89±0.35	5±0.07	5,26±0.2
140	5,59±0.06	5,85±0.42	4,98±0.01	5,2±0.04
168	5,58±0.16	5,81±0.33	4,88±0.04	5,18±0.06

Table A10: ANOVA showing variation between viral degradation in samples stored at different temperatures

Sample type	**Storage Temperature**	**ANOVA (Tukeys multiple comparisons test)**	
		Mean difference	*p* value

Influenza A - wastewater influent	**± 25°C vs 4°C**	4.25	<0.0001 (***)
	± 25°C vs -20°C	4699	<0.0001 (***)
	± 25°C vs -80°C	56.73	<0.0001 (***)
	4°C vs -20°C	42.74	<0.0001 (***)
	4°C vs -80°C	52.48	<0.0001 (***)
	-20°C vs -80°C	9.74	<0.0001 (***)
Influenza A – viral controls	**± 25°C vs 4°C**	7.27	<0.0001 (***)
	± 25°C vs -20°C	63.99	<0.0001 (***)
	± 25°C vs -80°C	57.67	<0.0001 (***)
	4°C vs -20°C	56.72	<0.0001 (***)
	4°C vs -80°C	50.4	<0.0001 (***)

	-20°C vs -80°C	-6.32	<0.0001 (***)
SARS-CoV-2 – wastewater influent	**± 25°C vs 4°C**	7.55	<0.0001 (***)
	± 25°C vs -20°C	55.05	<0.0001 (***)
	± 25°C vs -80°C	67.5	<0.0001 (***)
	4°C vs -20°C	47.5	<0.0001 (***)
	4°C vs -80°C	59.95	<0.0001 (***)
	-20°C vs -80°C	12.45	<0.0001 (***)
SARS-CoV-2 – viral controls	**± 25°C vs 4°C**	4.77	<0.0001 (***)
	± 25°C vs -20°C	63.7	<0.0001 (***)
	± 25°C vs -80°C	66.59	<0.0001 (***)
	4°C vs -20°C	58.93	<0.0001 (***)

	4°C vs -80°C	61.82	<0.0001 (***)
	-20°C vs -80°C	2.89	<0.0001 (***)

$p < 0.0001$ - ***