DURBAN UNIVERSITY OF TECHNOLOGY
INYUVESI YASETHEKWINI YEZOBUCHWEPHESHE

Assessment of biomarkers for normalization of SARS-CoV-2 concentrations in wastewater

This work is submitted in complete fulfilment of the academic requirements for the degree of Master of Applied Sciences in the Department of Biotechnology and Food Sciences, Faculty of Applied Sciences at the Durban University of Technology, Durban, South Africa

By

Aaliyah Osman (21001341)

Supervisor: Prof. Sheena Kumari

Co-supervisor: Dr. Isaac Dennis Amoah

Co-supervisor: Prof. Faizal Bux

JUNE 2023

APPROVAL

I hereby approve the final submission of the following thesis.

Prof. Sheena Kumari Kuttan Pillai
Supervisor
PhD: Biosciences

Dr. Isaac Dennis Amoah
Co-supervisor
PhD: Health Science

Prof. Faizal Bux
Co-supervisor
D Tech: Biotechnology

DECLARATION

I hereby declare that the work reported in this dissertation titled "Assessment of biomarkers for normalization of SARS-CoV-2 concentrations in wastewater" and submitted to the Faculty of Applied Sciences, Department of Biotechnology and Food Sciences at the Durban University of Technology for a master's degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

11/08/2023

Aaliyah Osman (Student number: 21001341) Date
Student

DEDICATION

This thesis is dedicated to my beloved family (My Husband – Ahmed Raza and my Precious Kids – Muhammed Bilal and Khadija). To my Mum, thank you for believing in me, thank you for supporting me and most of all thank you mum, for being my pillar of strength.

ACKNOWLEDGEMENTS

I want to express my gratitude to Almighty Allah SWT for always watching over me. My friends and family for their support in helping me reach my goals. Even though this has been one of the most challenging goals, I am grateful to have made it this far.

Words will not be enough to convey my feelings to my mother. May Allah SWT bless you for your perseverance and sacrifices. I love you mom, for always going above and beyond for me.

I want to thank my supervisors for their unwavering support during this research and for allowing me to continue to grow. Thank you to Prof Sheena, Prof Bux, and Dr Isaac Dennis Amoah.

Special thanks to Durban University of Technology (DUT) for funding my study.

Prof. Sheena, through your endless love, support, and guidance, I have become a better version of myself. Thank you, Prof. Thank you for your consistent encouragement especially through the times when I felt like giving up. Most importantly thank you for believing in me.

Dr. Isaac Dennis Amoah, you are amazing. I will always be grateful that I was introduced to a co-supervisor like you. Despite your busy schedules, you never hesitated to assist me. May God Bless you always!

Mr. Mhlengi, thank you for always making the time to take me sampling, even on days when I had to reschedule.

Mr Kriveshin, Thank you for your assistance and guidance during my study.

I want to thank the IWWT team for all their love and support. I want to express my gratitude to my friends; Precious, Annette, Hlengiwe, Humeira, Nonsikelelo Precios, Riona, and Sphe.

My deepest gratitude goes out to my spouse Ahmed Raza, you've helped me grow into the better person I am today. I really believe that I could not have done this without you.

My children, Muhammad Bilal and Khadija: Remember that I will always love you. I am grateful for all the love, fun, and happiness you have brought into my life.

PREFACE

Osman A, Amoah ID, Kumari SK, Bux F.2023. Assessment of biomarkers for normalization of SARS-CoV-2 concentrations in wastewater.

Abstract – Accepted for TTW 6 – UK Conference

ABSTRACT

During the COVID-19 pandemic, the measurement of SARS-CoV-2 RNA levels in wastewater quickly emerged as an additional tool for monitoring and to provide an early warning system. This led to development of several regional, national and international projects aimed at applying this approach. The main principle is based on the detection of the viral signature in untreated wastewater to provide an indication of infection levels within connected populations. However, the concentration of the viral signature in wastewater can be impacted by dilution factors or population changes in the sewer shed, leading to misinterpretation of measurement results. Therefore, there is the need for normalization of wastewater to ensure accurate representation of infection numbers. The aim of this study was to evaluate different viral and bacterial markers in wastewater for their efficiency in normalizing SARS-CoV-2 WBE data, which will enhance the accuracy when interpreting the SARS-CoV-2 RNA concentrations in wastewater.

Weekly sampling was conducted from two wastewater treatment plants (WWTP A and WWTP B) within the eThekwini district over a period of three months (July-October 2022). Three biomarkers (*crAssphage*, *Bacteroides* (HF 183), and *Pepper Mild Motile Virus*) where chosen for this study to ascertain the most suitable for WBE data normalization. Biomarker and SARS-CoV-2 concentrations in the wastewater samples were determined using the droplet digital PCR (ddPCR). Physicochemical characteristics of the wastewater samples were also determined to identify the potential impact of these characteristics on the concentration of SARS-CoV-2 and the biomarkers. To determine the most suitable biomarker, correlation analysis and the Adaptive neuro fuzzy inference system (ANFIS) model was used.

Average concentrations of SARS-CoV-2 in the sampled WWTPs ranged from 0.28 copies/µL to 9.57 copies/µL. Among the three biomarkers studied, *crAssphage* recorded the highest concentration compared to *PMMoV* and *Bacteroides* HF183 in both the WWTPs. C*rAssphage* recorded the highest concentration of 7943 (±7.07) copies/µL for WWTP A and 8006 (±4.24) copies/µL for WWTP B. The *Bacteroides* HF183 highest concentrations were 10116 (±120.91) copies/µL for WWTP A and 2474 (±117.37) copies/µL for WWTP B. *PMMoV* had concentrations of 46 (±4.24) copies/µL for WWTP A and 84,1 (±5.48) copies/µL for WWTP B. *PMMoV* concentrations were observed to be the highest at Week 1. *CrAssphage* showed a greater association during the trend analysis with SARS-CoV-2 (0.499) than the other two biomarkers for WWTP A, (HF 183 and SARS-CoV-2 (-0.191) and *PMMoV* and SARS-CoV-2 (-0.562)).

Among the physicochemical factors studied, electrical conductivity and temperature had a significant correlation with SARS-CoV-2 and the *crAssphage* biomarker for both WWTPs. Using the ANFIS model, it was shown that the levels of the measured biomarker concentrations in wastewater had a significant association with chemical oxygen demand (COD), dissolved oxygen (DO), and volatile solids (VS). These results indicate a possible impact of these parameters on the concentration of these biomarkers in the wastewater. Furthermore, the viral RNA quantities of SARS-CoV-2 in wastewater were demonstrated to be influenced by other parameters such as electrical conductivity, pH and temperature. This indicates a difference in the physicochemical parameters that influence both biomarkers and SARS-CoV-2. However, when all physicochemical parameters, biomarkers and SARS-CoV-2 were combined, it was determined that the best biomarker was *crAssphage*, with potential impact from COD and the VS. The results of this study highlight the significance of including wastewater characteristic in WBE studies for reliable and accurate results. As shown in this study, *crAssphage* can serve

as a biomarker for efficient WBE for COVID-19 surveillance. In addition, it has been demonstrated that the detection and quantification of targets of concern, including SARS-CoV-2, may be enhanced when combined with wastewater characteristics, which may enhance the monitoring of COVID-19 infections.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ANFIS	Adaptive Neutral Fuzzy Interference System
ARG's	Antimicrobial resistant Genes
BCoV	Bovine Coronavirus
BLAST	Basic Local Alignment Search Tool
BRSV	Bovine respiratory syncytial virus
cDNA	Commentary deoxyribonucleic acid
COD	Chemical Oxygen Demand
COVID-19	Corona Virus Disease
CoVs	Coronaviruses
CRISPR	Clustered regularly interspaced short palindromic repeats
ddPCR	Droplet Digital PCR
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMV	Electronegative Membrane Vortex
FIB	Faecal Indicator Bacteria
FP	Forward Primer
Gc/GC	Gene Copies
HF 183	Human Forward 183
HIAA	Hydroxy indoleacetic acid
KHP	Potassium hydrogen phthalate
L	Litre
MgV	Mengovirus
mL	Millilitres
ML/d	Million Litres per day
MST	Microbial Source Tracking
NCBI	National Center for Biotechnology Information
nm	Nanometres
NPS	New Psychoactive Substances

OTC	Over the Counter
P.aeruginosa	*Pseudomonas aeruginosa*
PARA	Paraxanthine
PCR	Polymerase Chain Reaction
PCS	Primary Clarified Sludge
PEDV	Porcine Epidemic Diarrhea Virus
PEG	Polyethylene Glycol
PGS	Post grit solids
pH	Potential Hydrogen
PMMoV	*Pepper Mild Mottle Virus*
PPE	Personal Protective Equipment
rcf	Relative Centrifugal Force
RNA	Ribonucleic Acid
RP	Reverse Primer
rRNA	Ribosomal Ribonucleic Acid
RT-LAMP	Reverse Transcription Loop Mediated Isothermal Amplification
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus -2
TBE	Tris Borate Ethylenediaminetetraacetic acid
TDS	Total Dissolved Solid
TGEV	Transmissible Gastroenteritis Virus
TMA	Transcription Mediated Amplification
TS	Total Solid
µL	Microlitre
µM	Micromolar
UV	Ultraviolet
vs	verses
VS	Volatile Solids
WBE	Wastewater-based epidemiology data
WHO	World Health Organization
ZR	Zymo Research

CHAPTER ONE

1. Introduction

Wastewater-based epidemiology (WBE) refers to evaluation of wastewater to identify the presence of chemicals or biological markers for the purpose of monitoring public health (O'Keeffe, 2021, Aguiar-Oliveira *et al.*, 2020). Previously, WBE has been used extensively to detect the presence of pharmaceutical or industrial waste, drugs, viruses, and the potential emergence of antibiotic-resistant bacteria (Robins *et al.*, 2022). Wastewater monitoring allows for the timely detection of infection dynamics in residential facilities, which includes retirement homes, nursing homes, schools, hospitals, and other public or private facilities, in addition to data collecting at the wastewater treatment plants (Galani *et al.*, 2022, Costa *et al.*, 2021, Gettings *et al.*, 2022, Elbadawi *et al.*, 2021). The recent application of WBE have shown that wastewater monitoring offers an advantage over diagnostic testing of up to two weeks (Stadler *et al.*, 2020). As a result, wastewater monitoring can act as an early warning system (Bibby *et al.*, 2021). The analysis of wastewater for indicators of illicit drug use was one of the first proposed applications of WBE (Daughton, 2001). Thereafter this has also been applied to estimate public exposure to alcohol (Van Wel *et al.*, 2016, Reid *et al.*, 2011, Baz-Lomba *et al.*, 2016), counterfeit medicines (Van Nuijs *et al.*, 2009, Causanilles *et al.*, 2018, Venhuis *et al.*, 2014), and tobacco (Castiglioni *et al.*, 2015). In the recent COVID-19 outbreak, WBE has become a very important tool for monitoring infections in communities (Maida *et al.*, 2022, Islam *et al.*, 2022, O'Keeffe, 2021, Kumblathan *et al.*, 2021, Medema *et al.*, 2020b). Compared to clinical individual testing, the wastewater analysis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is found to be considerably faster, less expensive, and more effective in comprehensive anonymous testing of communities (Mousazadeh *et al.*, 2021). Worldwide, WBE has already been employed in numerous ways to track SARS-CoV-2 ribonucleic acid (RNA) in wastewater (Ahmed *et al.*, 2020b, Hillary *et al.*, 2020, Haramoto *et*

al., 2020, Kumar *et al.*, 2020, Randazzo *et al.*, 2020b, Wolfaardt *et al.*, 2021, Amoah *et al.*, 2021, Medema *et al.*, 2020b). While being largely a respiratory infection, SARS-CoV-2 RNA has also been discovered to be shed in feces at high concentrations (approximately 5.7^{3}-$10^{7.6}$ copies/mL) depending on the infection course (Foladori *et al.*, 2020, Peccia *et al.*, 2020, Hamouda *et al.*, 2021). Regardless of a person's health status (symptomatic, asymptomatic, pre-symptomatic, convalescent), these RNA fragments are present in their feces and can be found in wastewater (Wolfaardt *et al.*, 2021). Studies have shown that viral shedding in feces begins before clinical symptoms appear (Miura *et al.*, 2021, Cevik *et al.*, 2021). In spite of its simplicity, the approach still poses some challenges in public health monitoring. Matrix complexity and dilution rate due to stormwater incursion are some of the challenges associated with WBE (Choi *et al.*, 2018, Daughton, 2012). Additionally, several factors affect the stability of viral RNA in wastewater including inhibitory compounds and physicochemical parameters (eg: detergents, salts, pH, organic matter etc). There is also the possibility of considerable loss of viral RNA during transit times through the wastewater network due to decay and sorption, concentration and extraction methods (Ahmed *et al.*, 2020b, Corpuz *et al.*, 2020). Another difficulty is that population fluctuations and the resulting uncertainty about population size can cause issues with monitoring infectious diseases in wastewater because the presence of commuters or tourists in a catchment area may make it challenging to monitor infectious diseases in the local community (Ort *et al.*, 2014).

There are several technologies that can be used to reduce sources of uncertainty related to population size. This includes water chemistry parameters of established analytical techniques which can assist in estimating the population contributing to the catchment area of the wastewater collection points over a given time period (Been *et al.*, 2014, Van Nuijs *et al.*, 2011). However, the results are influenced by the wastewaters composition which creates additional uncertainties about these procedures, such as variability in sample collection and

analysis (Ort *et al.*, 2014, Castiglioni *et al.*, 2013, Ort *et al.*, 2010). Therefore, for COVID-19 analysis to be useful for public health and decision-making, a deeper understanding of the variations in SARS-CoV-2 concentrations in wastewater and how it corresponds to the actual incidence or prevalence of COVID-19 in the contributing population is required. The use of biomarkers in WBE gives data on population behaviour, illness prevalence, and health (Vitale *et al.*, 2021). The discipline of WBE has developed from the concept of using biomarkers selected from concentrations found in wastewater to provide demographic information. WBE is based on the analysis of specific human metabolic excretion products in feces known as 'biomarkers' in wastewater as indicators to address the variability within the wastewater population (Mao *et al.*, 2020). Several options exist that may allow estimation of relative human fecal load including genetic biomarkers for example human mitochondrial DNA or RNA (Tanvir Pasha *et al.*, 2020), cross-assembly phage (*crAssphage*) (García-Aljaro *et al.*, 2017, Reynolds *et al.*, 2022), *Pepper Mild Mottle Virus* (*PMMoV*) (Gyawali *et al.*, 2019, Dhakar and Geetanjali, 2022), organic or inorganic compounds (wet chemical data), such as ammoniacal nitrogen, urea and creatinine and contaminants, like pharmaceuticals (Lin *et al.*, 2019, Choi *et al.*, 2018). In order to compare or normalize measured levels of RNA to fecal load, it is useful to know how many people are contributing to the wastewater. This warrants the need for normalization of wastewater data to study the actual infection trend in an infection over a period of time within a connected community. For example, in cases where the number of people contributing to the sewer-shed is expected to change over the surveillance period due to factors such as: dilution, tourism, office hours, temporary workers etc. Thus, it is important to normalize SARS-CoV-2 concentrations by the population number served by the sewer system, especially if the number of people contributing to the wastewater in a particular location is expected to vary (Hsu *et al.*, 2022). This provides a better understanding of

fluctuations in viral RNA concentration, allowing for comparisons of wastewater samples over time and between locations together with normalization.

1.1 Rationale

The primary purpose of wastewater surveillance is to provide timely and reliable data to protect public health. This also requires the urgent understanding of the variability and limitations in the WBE data. The unaccountable variability in WBE data introduces a major challenge with the accuracy and application of WBE for disease surveillance. In such cases normalizing SARS-CoV-2 RNA concentrations by the amount of human feces in wastewater is key for calculating the actual SARS-CoV-2 RNA concentrations and comparing the trend over time. This variability can be better understood by normalizing the wastewater using specific human fecal biomarkers. Various biomarkers have been proposed with varying accuracy; however, an efficient and universal normalization method has not yet been developed. These biomarkers can be population specific or region specific which warrants the need for research in this area. Therefore, there is a need to better understand factors that influence observable levels of the SARS-CoV-2 RNA concentrations in wastewater to validate the approach for surveillance purposes. Therefore, this study aims at improving WBE data for COVID-19 surveillance.

1.2 Aim

To evaluate different viral and bacterial biomarkers in wastewater for their efficiency in normalizing SARS-CoV-2 WBE data

1.3 Objectives

•To detect and quantify biomarkers (*crAssphage*, *Pepper Mild Mottle Virus (PMMoV)*, and *Bacteroides* HF183) in untreated wastewater using conventional PCR and ddPCR

•To monitor changes of viral and bacterial biomarkers and determine the effect of physicochemical parameters of the biomarker concentration in wastewater using ddPCR and the Adaptive Neural Fuzzy Inference System (ANFIS) Modeling technique

•To evaluate the suitability of the biomarkers in normalizing SARS-CoV-2 WBE data using the Spearman's Rank Correlation analysis

1.4 Thesis Structure

The thesis is presented in Five (5) main chapters. **Chapter one** presents the background, rationale, aim and objectives of the study. The literature review is captured in **Chapter two**. **Chapter three** addresses objective 1, which focused on the detection and quantification of the biomarkers (*crAssphage, PMMoV*, and *Bacteroides* (HF183)) in untreated wastewater. It has a brief introduction and methodology, results, discussion and a conclusion. **Chapter four** focused on objective 2 and objective 3. Therefore, an introduction, methodology, results, discussion and conclusion is presented for the suitability of the biomarkers in normalizing SARS-CoV-2 WBE data. **Chapter Five**, draws conclusions, study limitations and recommendations from this study.

CHAPTER TWO

2.1 Literature Review

2.1.1 Wastewater-Based Epidemiology (WBE)

Public health can be assessed and improved by implementing tools that can track the appearance, spread, or reappearance of diseases, drug use, and other biological or chemical indicators (Tulchinsky and Varavikova, 2014). WBE has emerged as an efficient tool to present a snapshot of the overall health of a community based on what is being excreted in pooled sewage (O'Keeffe, 2021). There are many different uses for WBE, some of which are more developed technologically and in use than others. This includes estimation of drug or pharmaceutical consumption, sources of antimicrobial resistance, and estimation of infectious diseases. In the recent pandemic, WBE played a significant role in tracking the progress of SARS-CoV-2 infection within the community (Medema *et al.*, 2020b). The success of WBE relies on various steps including sampling, sample preparation, analysis, data processing and interpretation, as well as reporting (Kumblathan *et al.*, 2021). Figure 2.1 provides a representation of the various steps involved in WBE (Kumblathan *et al.*, 2021). Since the idea of WBE was initially introduced more than 20 years ago (O'Keeffe, 2021), it has been used in a variety of situations, from analysing drug usage (Zuccato *et al.*, 2008) to recent research, determining the presence of diseases such as COVID-19 in the community (Ahmed *et al.*, 2020b, Kitajima *et al.*, 2020, Navarro *et al.*, 2021, Chen and Li, 2020, Medema *et al.*, 2020b).

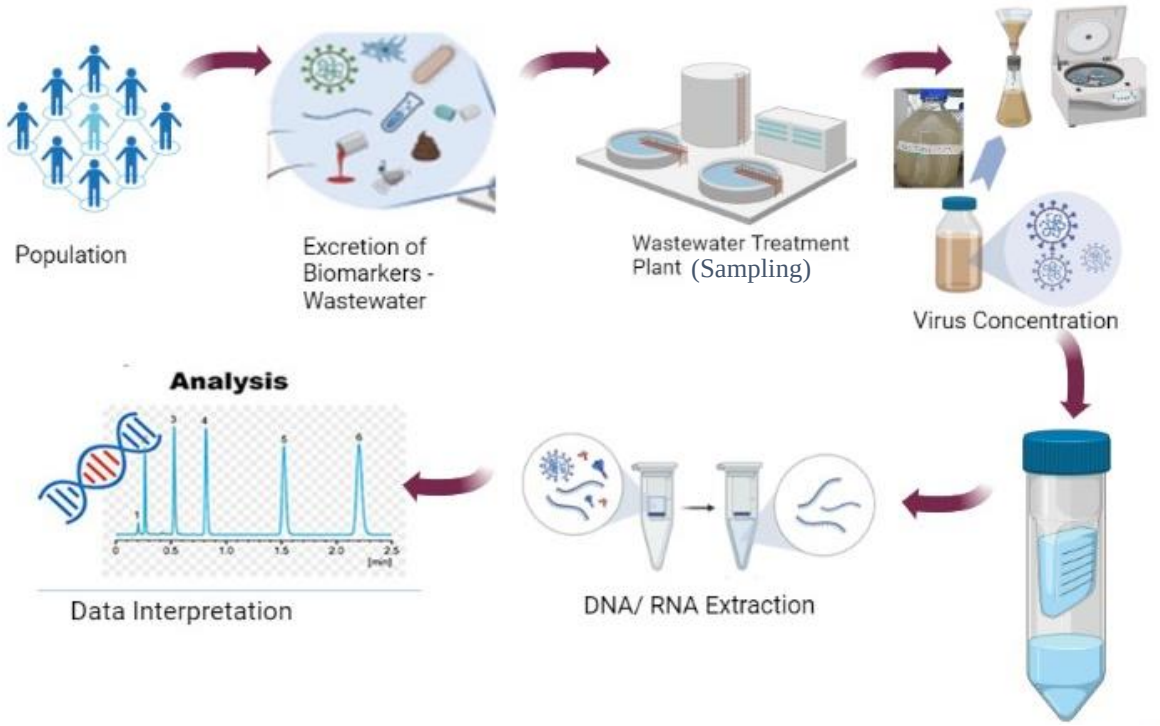


Figure 2.1: Representation of the various steps involved in monitoring of WBE. Adapted from (Kumblathan *et al*., 2021)

2.2 Applications of WBE

2.2.1 Estimating drug consumption

One of the first suggested uses for WBE was the analysis of wastewater for signs of drug usage (Daughton, 2001). By quantifying cocaine and its urinary metabolite benzoylecgonine in wastewater and surface waters of Italian cities, Zuccato *et al.*, (2005b), delivered one of the first proof of concept studies for estimating drug consumption in a community. The research showed that the usual methods for measuring cocaine-use from surveys, consumer interviews, medical records, and crime statistics, were probably underestimating the use. Some substances, such as cocaine, and 3,4-Methylenedioxymethamphetamine, can be reliably found in wastewater, but others are more challenging to detect (Keshaviah, 2017). This might be caused by specific biomarkers excreted in trace amounts (such as the metabolites of marijuana and heroin), analytical difficulties, or a lack of precision brought on by interference from other

sources (e.g., heroin metabolizes to morphine) (Choi *et al.*, 2018, Keshaviah, 2017, Werschler and Brennan, 2019). Additionally, wastewater contains a variety of chemical interferences, some of which may have chemical spectra and characteristics that are similar to those of the target chemicals, making extraction and analysis challenging. Despite the analytical difficulties, drug surveillance in wastewater has been utilized to identify potential targets for public health interventions or to assist law enforcement in locating drug production facilities (Boogaerts *et al.*, 2021a, Subedi and Burgard, 2019, Brandeburová *et al.*, 2020).

Comparison with conventional surveillance data and other complementary data has been used to refine estimates of consumption (e.g., comparison with prescribing or over the counter (OTC) sales data). The use of complementary data has also been studied to identify correlations such as the comparison of fentanyl concentrations with drug death data (Gushgari *et al.*, 2019) or by comparing cannabis biomarkers with sales data from legitimate sources to determine the percentage of the illegal market that legalization has replaced (Burgard *et al.*, 2019). Alcohol and nicotine are examples of legal addictive substances that have been estimated using WBE in order to determine patterns of geographical and temporal use (Boogaerts *et al.*, 2021b, Brandeburová *et al.*, 2020, Choi *et al.*, 2018, Driver *et al.*, 2020, Montes *et al.*, 2020). It is important to remember that WBE can help uncover relationships between licit and illicit drugs, indicating poly-drug consumption. Strong associations between the use of numerous pairings of illicit drugs, such as heroin and cocaine and methcathinone and ketamine, were found in a recent study conducted in China (Liu *et al.*, 2021). WBE has been used to research several aspects of lifestyle, such as alcohol intake (mentioned above) and smoking prevalence. The metabolite of ethanol, ethyl sulphate, has mostly been used in WBE for alcohol consumption (Baz-Lomba *et al.*, 2016, Mastroianni *et al.*, 2014). Conversely, smoking uses nicotine and its metabolites, cotinine and hydroxy cotinine in WBE (Castiglioni *et al.*, 2015). Anatabine and anabasine, two other biomarkers linked to tobacco use, have also been discovered in

wastewater (Tscharke *et al.*, 2016). Since then, WBE has been used for a variety of different indicators, including those related to alcohol, nicotine, infections, treatments, and antimicrobial resistance markers (AMR) (Boogaerts *et al.*, 2021a, Senta *et al.*, 2020, Sims and Kasprzyk-Hordern, 2020).

2.2.2 Estimating pharmaceutical consumption

There are many uses for measuring pharmaceutical amounts in wastewater. Indicators of consumption vs disposal practices include the ratio of parent chemicals to metabolites (Casas *et al.*, 2021b). Use and misuse patterns can be detected to focus prevention measures or properly allocate resources when utilized in conjunction with other complementing data sources (e.g., prescribing, OTC , or survey data) (Boogaerts *et al.*, 2021a). In addition to prescription or OTC sales data, WBE data may be used to detect the use of rogue pharmacies. Venhuis *et al.*, (2014b), linked pharmacy dispensing databases to the wastewater loads of the erectile dysfunction medication sildenafil in three Dutch cities. They discovered that dispensing statistics could not account for at least 60% of the sildenafil identified in sewage, indicating widespread usage of online or rogue pharmacies for this medication. The quantity of typical medicines in wastewater has been assessed in numerous studies (Ahmed *et al.*, 2020a, Ahmed *et al.*, 2021a) as a metric of illness prevalence. For instance, oxypurinol, an allopurinol urine metabolite, has been measured in wastewater to evaluate the prevalence and trends of gout (a gout treatment). By assessing the presence of metformin, a medication used to treat type II diabetes, in wastewater, a similar method has been used to determine the prevalence of the illness by Yan *et al.*, (2019). There are various drawbacks to using medicines as a stand-in for disease frequency. Some therapies are used to treat multiple diseases, and not all patients with the disease are diagnosed or treated in the same way. There are uncertainties in back-calculating precise population averages due to the variability of individual usage habits and excretion levels. A substitute wastewater indication of overall population ‘health’ has been

proposed using ubiquitous biomarkers of oxidative stress (such as isoprostanes), which may indicate systemic disorders such as cancer, diabetes, heart disease as well as psychological stress (Daughton, 2012).

2.2.3 Sources of Antimicrobial resistance

One of the top hazards to public health and medicine in the twenty-first century is antimicrobial resistance (AMR) (O'Neill, 2016). According to the World Health Organisation (WHO), AMR is when microorganisms such as bacteria, viruses, fungi, and parasites evolve with mutations that are resistant to drugs previously used to treat the diseases they cause (WHO, 2022). This process is a natural occurrence, but the improper use of medications speeds it up even more. Currently, a large amount of surveillance is dependent on prescription and clinical data. WBE offers a chance to accomplish a whole population strategy for combating AMR as a complementary technique. It has been suggested that monitoring antibiotics, their metabolites, infections, and antibiotic resistance genes (ARGs) in wastewater could serve as a proxy for regional AMR and show how changes can develop over time (Kwak *et al.*, 2015, Larsson *et al.*, 2018). Determining where and how much resistant bacteria are introduced into the environment by testing wastewater for the presence and diversity of antimicrobial resistant genes for various medications (Aarestrup and Woolhouse, 2020). Increased quantities of antibiotics in wastewater have been linked to higher levels of resistance (e.g., hospital sewage vs. municipal) (Hutinel *et al.*, 2019). Further research into these associations may help determine where to direct mitigation efforts. Finding suitable surrogates, using established protocols, using uniform units of measurement, and comprehending how to use the data to assist risk assessment or focused actions are some of the remaining issues (Nguyen *et al.*, 2021).

2.2.4 Estimation of infectious disease prevalence

The idea of using wastewater for disease surveillance is not new, and has received much attention in the literature since the corona virus (COVID-19) pandemic (McClary-Gutierrez *et al.*, 2021, Graham *et al.*, 2020, Foladori *et al.*, 2020). For instance, environmental polio surveillance in wastewater has been established (Hovi *et al.*, 2012, Roberts, 2013). Before COVID-19, in the 20th century, Poliomyelitis known as polio, was one of the most feared diseases worldwide, paralysing hundreds of thousands of children yearly (Tseha, 2021). Soon after the introduction of effective vaccines in the 1950s and 1960s, polio was brought under control and practically eliminated as a public health problem. WBE was utilized as a tool to assess polio circulation within populations and evaluate immunisation efficacy against poliovirus (Brouwer *et al.*, 2018). In Finland (Hovi *et al.*, 2012) and Israel (Roberts, 2013), environmental surveillance of poliovirus in wastewater has been carried out since the 1980s to measure the quantities of poliovirus circulating in the populations (Organization, 2003, Manor *et al.*, 1999, Shulman *et al.*, 2006, Lago *et al.*, 2003).

WBE has been used in the retrospective prediction of disease outbreaks for hepatitis A and norovirus-associated gastroenteritis (Sims and Kasprzyk-Hordern, 2020, Hellmér *et al.*, 2014). Researchers have accurately anticipated norovirus and hepatitis A outbreaks in wastewater in the past (Hellmér *et al.*, 2014) and wastewater samples have been shown to contain influenza (Heijnen and Medema, 2011). WBE has also been applied to monitor bacterial infections (Yan *et al.*, 2018). A study in Hawaii, Diemert and Yan, (2019), showed the prevalence of enteric *Salmonella* in a population, causing diarrhoea and illnesses. Additionally, direct measurements of microbial DNA/RNA for infectious disorders brought on by viruses and bacteria can show the presence of infection in a population, and the strength of the signal can show their prevalence. Following a poliovirus outbreak in Israel in 2013, the virus was identified in sewage, and a dose-dependent correlation between the viral content in sewage and the number

of positive cases was discovered (active shedders) (Berchenko *et al.*, 2017). The discovered virus, and molecular features allowed for the location and origin of infections to be determined (travel-related introductions) (O'Keeffe, 2021). WBE has been used to measure the population prevalence of a number of other human viruses, such as measles (Benschop *et al.*, 2017), enteroviruses (Brinkman *et al.*, 2017), hepatitis A (Hellmér *et al.*, 2014, McCall *et al.*, 2021), norovirus (Hellmér *et al.*, 2014, Lu *et al.*, 2021) and additionally, more recent substantial SARS-CoV-2 analysis (Ahmed *et al.*, 2020b). WBE has been employed in some of these research as an early warning sign of outbreaks (Hellmér *et al.*, 2014) or to indicate trends in the prevalence of different viruses (Brinkman *et al.*, 2017). WBE may also be used to understand the evolution of viruses and patterns of spread. Lu *et al*., (2021) used WBE in China to detect the introduction of novel norovirus strains in sewage and provided early warning of a new variation gaining dominance. Metagenomic sequencing was used in a study of the urban virome in sewage from several cities to demonstrate the capacity to identify various viruses and their seasonal and geographical variations (Nieuwenhuijse *et al.*, 2020). The difficulty of sequencing, the abundance of unclassified sequences, the absence of defined procedures, and difficulties in interpreting the results are only a few of the difficulties that still exist (Mao *et al.*, 2020). Additionally, WBE can more easily detect some viruses than others (e.g., enteric pathogens that may be more persistent compared with other pathogens that are more incidentally shed). The first study on the presence of SARS-CoV-2 in sewage in the Netherlands was conducted by Medema *et al*., (2020b). The findings indicated that water samples taken from two locations contained the SARS-CoV-2 virus. Notably, Medema *et al*., (2020b), and the team mentioned that the initial water sample contaminated with the virus was observed for four days following the initial positive SARS-CoV-2 coronavirus test. This was a significant and intriguing discovery which then lend to numerous studies conducted globally to track SARS-CoV-2 monitoring in community using WBE. Thereafter, there has been

significant interest in utilizing WBE to track the evolution and spread of the SARS-CoV-2 virus throughout the COVID-19 pandemic thus to gain information on various aspects of public health (Róka *et al.*, 2021, Ahmed *et al.*, 2020b). As a result, WBE activity has increased globally, and this has brought attention to the possibility for WBE to be employed more widely in public health surveillance.

Additional uncertainties in WBE for viral monitoring include normalizing results to population size, individual variation in shedding patterns for particular viral infections, the stability of the target indicators, and a lack of linkage with current public health and clinical data to verify results (Xagoraraki and O'Brien, 2020). Comparing the results from sampling at different locations could also be necessary to validate findings. Numerous research studies have used pathogen genetic material, such as DNA or RNA, to track the spread of an infection in a community (Chaudhary *et al.*, 2021). However, a more efficient way to measure the spread of disease at the community level has been demonstrated by combining the analysis of other pertinent biomarkers with the genetic makeup of the pathogen in the issue (Daughton, 2020b). The broad range of information gathered from wastewater opens the possibility of expanding the WBE to other human biomarkers providing clues about diet, health, diseases and exposure to contaminants (Gracia-Lor *et al.*, 2017, Arnold, 2016, Covacic *et al.*, 2016). For example, by linking exposure to the environment or food contaminants with health outcomes such as diabetes or cancer.

2.2.5 SARS-CoV-2 and WBE

Coronaviruses (CoVs) are a member of the *Coronaviridae* family. Their rounded shape and the spikes on their surface give them the name corona. In contrast to 'naked' viruses, which have no lipid membrane sheath around their surface, coronaviruses are enclosed. Coronaviruses are more delicate than other viruses due to their lipid envelope (Walls *et al.*, 2020) and hence is relevant to understanding their environmental persistence, transmission and susceptibility to

inactivation by disinfection. The lipid structure holds the membrane, envelope, and spike proteins together, with the spike protein protruding from the envelope as illustrated in Figure 2.2 (Won and Lee, 2020).

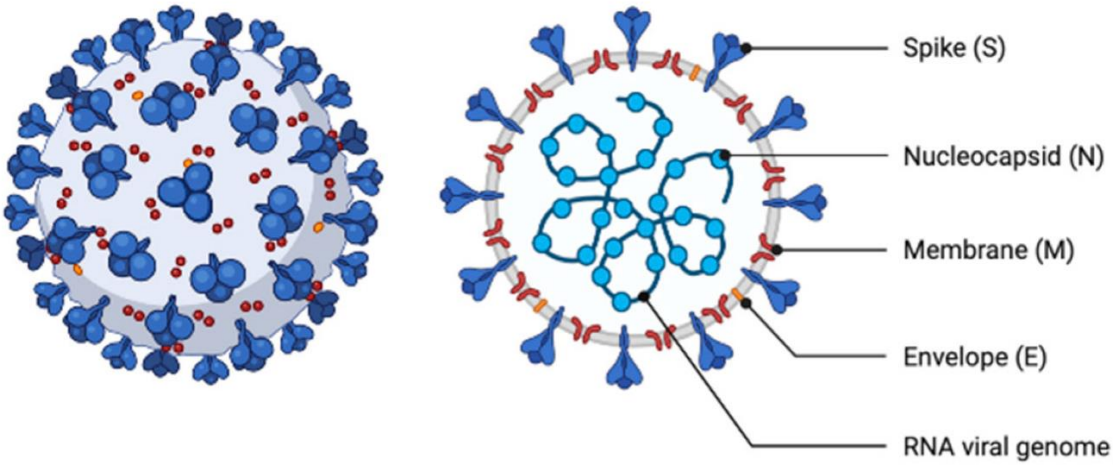


Figure 2.2: The Structure of human coronavirus: the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. (Won and Lee, 2020)

The coronaviruses are a broad family of viruses that includes the SARS-CoV-2 (Ortiz-Prado *et al.*, 2020, Chen *et al.*, 2023). Both people and some animals can contract these viruses. In 2019, SARS-CoV-2 was first identified as a human pathogen (Singhal, 2020, Wu *et al.*, 2020a). The virus is believed to be transmitted from person to person through droplets expelled during coughing, sneezing, or talking by an infected person (Wang *et al.*, 2020b). A less prevalent method of transmission is through mouth, nose, or eyes through contact surfaces (Pitol and Julian, 2021). Since the SARS-CoV-2 virus has been spreading globally, variants have emerged and been identified in many countries around the world. Some of which are referred to as Variants of Concern (VOC) and others as Variants of Interest (VOI). SARS-CoV-2 contains just one mutation (D614G mutation); however, the alpha version has a total of 23 mutations. Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) are the listed variants of concern (Sanyaolu *et al.*, 2021). Figure 2.3 represents the different

variants of concern and flags of the countries from which they were first detected. The variant alpha had been reported in 178 countries, beta in 123 countries, gamma in 75 countries and delta in 111 countries (Pillay *et al.*, 2021, Misra *et al.*, 2023, Organization, 2021).

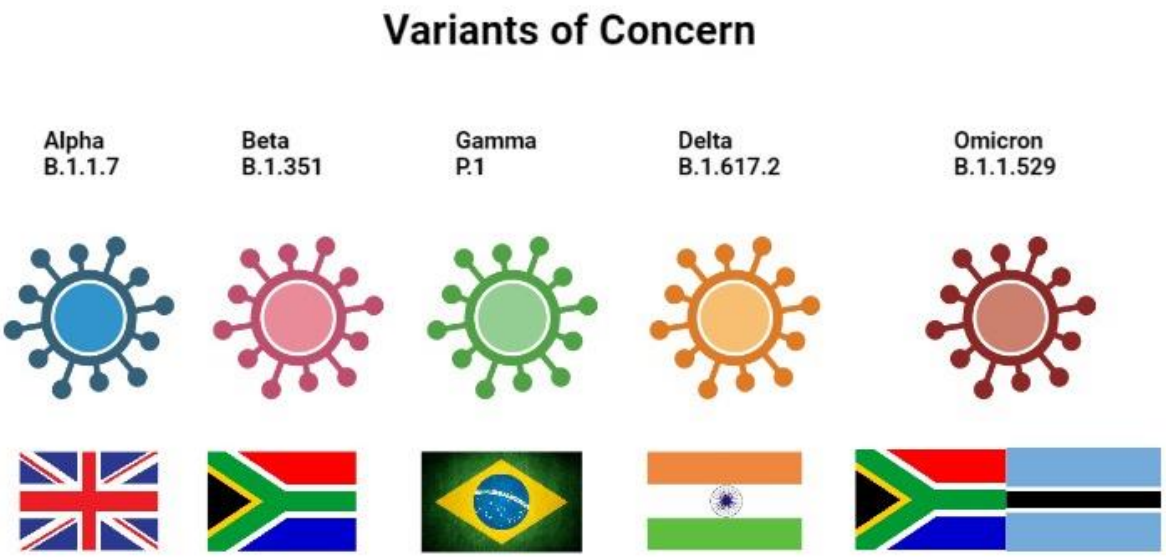


Figure 2.3: Variants of concern and flags of the countries from which they were first detected (Choi and Smith, 2021).

A study by Haramoto *et al*., (2020) detected the SARS-CoV-2 concentrations in wastewater to be 2.4 x 10^3 copies/L. The shedding rate of SARS-CoV-2 in feces is approximately 10^2 – 10^7 (gc/ml) (Jones *et al.*, 2020). The shedding duration of an infected person after the symptoms subside are to be between 14–28 days (Jones *et al.*, 2020).

Worldwide, numerous methods have been employed using WBE to detect SARS-CoV-2 RNA in wastewater (Ahmed *et al.*, 2020b, Hillary *et al.*, 2020, Haramoto *et al.*, 2020, Kumar *et al.*, 2020, Randazzo *et al.*, 2020b, Wolfaardt *et al.*, 2021, Amoah *et al.*, 2021, Medema *et al.*, 2020b). Medema *et al*., (2020b), conducted the initial study on the occurrence of SARS-CoV-2 in sewage in the Netherlands. Studies thereafter had found links between the rise in viral RNA and the number of COVID-19 cases that have been reported (Medema *et al.*, 2020b, Chen *et al.*, 2021, Yu *et al.*, 2022). Some studies have found SARS-CoV-2 RNA in wastewater prior

to community reports of cases or have noted an increasing trend prior to the observation of an increase in cases in clinical data (D'Aoust *et al.*, 2021a, Medema *et al.*, 2020b, Omori *et al.*, 2021). Table 2.1 presents studies that reported the detection of SARS-CoV-2 RNA in wastewater and sewage sludge prior to clinical reports. The main use of WBE for SARS-CoV-2 monitoring up to this point has been to corroborate patterns found in clinical tests and epidemiological data. WBE have also been found useful as an early warning indicator on a smaller scale (e.g., university campuses and residential structures) (Betancourt *et al.*, 2021, Colosi *et al.*, 2021, Wong *et al.*, 2021) as well as to detect unknown (asymptomatic) COVID-19 cases (Betancourt *et al.*, 2021). Similar study was also conducted in South Africa targeting university residents. Although there were few verified instances of COVID-19 reported, the study nonetheless showed the scope of potential infections at specific sites (Mangwana *et al.*, 2022). Similarly, using WBE to find SARS-CoV-2 in an apartment building in China led to the testing of further residents and the discovery of previously unreported positive cases (Wong *et al.*, 2021). Monitoring wastewater for new genetic variants have also been suggested for early detection and targeted mitigation strategies where genetic variants of concern are on the rise. There are numerous studies that have used sequencing methods for detecting VOCs. One such study by Ning *et al.*, (2022), has developed a rapid SARS-CoV-2 variant detection method that can be adapted for specific detection of future SARS-CoV-2 variants. Another study by Alhamlan *et al.*, (2023), has used SARS-CoV-2 spike gene Sanger sequencing methodology to identify the SARS-CoV-2 VOCs. It has been shown that it is possible to discover genetic variations and that they correspond with trends and clinical data (Ai *et al.*, 2021, Carcereny *et al.*, 2021, Lin *et al.*, 2021).

Table 2.1: Examples of Studies reporting the detection of SARS-CoV-2 RNA in wastewater and sewage sludge prior to clinical reports.

Time of detection prior to clinical reporting	Type of sample	References
3 Weeks	Raw wastewater 24-hour composite sample	(Ahmed *et al.*, 2021b)
48 Hours	Primary sewage sludge	(D'Aoust *et al.*, 2021a)
10-16 days	Raw wastewater grab sampling	(Randazzo *et al.*, 2020b)
6 days	Raw wastewater 24-hour composite sampling	(Medema *et al.*, 2020b)
1-4 days	Primary sewage sludge	(Peccia *et al.*, 2020)

2.2.6 Detection methods of SARS-CoV-2

Accurate and early identification of SARS-CoV-2 is crucial for lowering the risk of transmission by swiftly enabling isolation and contact tracking. The ability to quickly identify contaminated cases has the greatest impact on public health. Clinical symptoms and contact history with other potentially infected people are the main bases for clinical COVID-19 detection. Since the clinical indications and symptoms of infection (pneumonia, dyspnea, fever, cough, and respiratory symptoms) are not always clear (Filipić *et al.*, 2020) supporting diagnostic and serological tests are essential for the diagnosis of COVID-19. The type of test, the time it takes to get results, the testing precision, and the amount of testing resources required all have an impact on how effective diagnostic approaches are. In other words, the best strategy is to quickly identify suspect individuals in order to enable an effective response and prevent transmission. Different SARS-CoV-2 diagnostic assays have been developed using serological, molecular, and nanotechnology techniques. Several techniques are frequently used to identify viral nucleic acids, including high-throughput sequencing, reverse-transcription-polymerase

chain reaction (RT-PCR), RT-loop-mediated isothermal amplification (RT-LAMP) (Zhang *et al.*, 2020b, Khalilov *et al.*, 2020, Amoah *et al.*, 2021), and quantitative real-time PCR (qPCR). Molecular approaches used for detection of SARS-CoV-2 infection are Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Isothermal Nucleic Acid Amplification, Nucleic Acid Hybridization using Microarray, Amplicon-Based Metagenomic Sequencing. RT-PCR is the best test for coronavirus disease 2019 (COVID-19) laboratory diagnosis. It is also the most affordable, trustworthy, and regarded as the gold standard test (Carter *et al.*, 2020). It effectively amplifies relatively small amounts of viral genetic material in a mixture of other nucleic acid sequences. Recent studies indicate that RT-PCR-based detection approach has also been used with serum, eye, and stool samples (Mizumoto *et al.*, 2020, Gallo *et al.*, 2020). Reverse transcription quantitative PCR (RT-qPCR) and reverse transcription digital droplet PCR (RT-dPCR) are the most popular methods for virus quantitation (Huge *et al.*, 2022). In both cases, reverse transcriptase converts the RNA virus into a DNA strand that can be amplified by PCR. Amplification at a constant temperature is possible by adopting isothermal nucleic acid amplification. Information regarding the primers and probes reported for SARS-CoV-2 RT-PCR assays is summarized in table 2.2. A new technique called Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP), which combines LAMP and reverse transcription to increase test sensitivity, has been developed to identify SARS-CoV-2 quickly and inexpensively. RT-LAMP becomes a quick and accurate viral detection instrument when only heating and visual inspection procedures are required (Hong *et al.*, 2004, Amoah *et al.*, 2021). Digital PCR is based on the principles of limited dilution, end-point PCR, and Poisson statistics, with absolute quantification (Vogelstein and Kinzler, 1999, Marchini *et al.*, 2023). The sample is randomly divided into various partitions made up of thousands of droplets, some of which have no templates and others of which include one or more templates. Once the partitions have been amplified to their end point, a droplet reader counts the positive

partitions, and the concentration can then be roughly estimated using a Poisson distribution model. Low amplification efficiency and possible amplification inhibitors in samples hence have less of an effect on quantification. Sample partitioning effectively concentrates template molecules within the micro reactions, increasing analytical sensitivity for rare species by removing rivalry between different targets for amplification reagents in the reaction mixture (Morón-López *et al.*, 2023, Lucansky *et al.*, 2023, Marchini *et al.*, 2023). RT-qPCR measures the number of cycles in a polymerase chain reaction to generate a signal that exceeds a threshold, the Ct value. The Ct value is inversely exponentially related to the concentration of virus in the sample, dilute samples generate relatively large Ct values, and calibration is required to quantitate the virus concentration (Huge *et al.*, 2022, Yaniv *et al.*, 2021).

The other isothermal amplification method is transcription-mediated amplification (TMA) which can amplify specific DNA and RNA portions (Carter *et al.*, 2020). SARS-CoV-2 has been identified using CRISPR (Clustered regularly interspaced short palindromic repeats) technology. Techniques for CRISPR-based detection are made possible by the use of Cas nucleases (Cas12 and Cas13) (Gootenberg *et al.*, 2017, Li *et al.*, 2018). SARS-CoV nucleic acids have also been accurately and sensitively detected using microarray tests. The microarray assays start with the cDNA synthesis from viral RNA, which is then labelled with certain probes. Labelled cDNAs are placed onto microarray trays with solid-phased oligonucleotides attached. If the hybridization process takes place, it will be possible to see whether there is viral-specific nucleic acid (Chen *et al.*, 2010).

SARS-CoV-2 has been identified using amplicon-based metagenomic sequencing, which combines amplicon and metagenomic sequencing. Metagenomic sequencing was used to make the initial discovery of the related microbiota in infected individuals. Amplicon-based

sequencing assesses the potential for molecular epidemiology investigations, viral evolution research, and contact tracing (Eftekhari *et al.*, 2021).

Table 2.2: Information regarding the primers and probes reported for SARS-CoV-2 real-time reverse-transcription PCR assays.

Target genes	**Name**	**Sequence (5' → 3')**	**Reference sequence**	**Nucleotide position**	**Reference**
RdRp	Forward primer: Reverse primer:	CAAGTGGGGTAAGGCTAGACTTT ACTTAGGATAATCCCAACCCAT	--	14961-14983 15283-15304	(Chan *et al.*, 2020)
RdRp /nCoV IP2	Forward primer: Reverse primer: Probe	ATGAGCTTAGTCCTGTTG CTCCCTTTGTTGTGTTGT Hex-AGATGTCTTGTGCTGCCGGTA-BHQ-1	SARS-CoV, NC_004718	12621-12727	(Etievant *et al.*, 2020)
RdRp/nCoV IP4	Forward primer: Reverse primer: Probe	GGTAACTGGTATGATTTCG CTGGTCAAGGTTAATATAGG FAM-TCATACAAACCACGCCAGG-BHQ-1	SARS-CoV, NC_004718	14010-14116	(Etievant *et al.*, 2020)
RdRp gene	Forward primer; Probe 2; Probe 1; Reverse primer	GTGARATGGTCATGTGTGGCGG FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ CARATGTTAAASACACTATTAGCATA (W=A/T; R=A/G; M=A/C; S=G/C)	MN908947	15431-15452 15470-15494 15469-15494 15505-15530	(Corman *et al.*, 2020)
ORF1a	Forward primer: Reverse primer: Probe	AGAAGATTGGTTAGATGATGATAGT TTCCATCTCTAATTGAGGTTGAACC FAM-TCCTCACTGCCGTCTTGTTGACCA-BHQ1	Alignment of sequenced virus genomes	--	(Lu *et al.*, 2020)
ORF 1ab	Forward primer: Reverse primer: Probe	TGATGATACTCTCTGACGATGCTGT CTCAGTCCAACATTTTGCTTCAGA ROX-ATGCATCTCAAGGTCTAGTG-MGB	MN908947	15704-15728 15823-15846 15749-15768	(Wu *et al.*, 2020b)

Target genes	Name	Sequence (5' → 3')	Reference sequence	Nucleotide position	Reference
ORF 1ab	Forward primer: Reverse primer: Probe	CCCTGTGGGTTTTACACTTAA ACGATTGTGCATCAGCTGA FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ	MN908947	13342-13362 13442-13460 13377-13404	(Primers, 2020)
ORF1b-nsp14	Forward primer: Reverse primer: Probe	TGGGGYTTTACRGGTAACCT AACRCGCTTAACAAAGCACTC FAM/ZEN-TAGTTGTGATGCWATCATGACTAG-IBFQ (W=A/T; Y=C/T; R=A/G)	MN908947	18778-18797 18889-18909 18849-18872	(Chu *et al.*, 2020)
N gene	Forward primer: Reverse primer: Probe	GGGGAACTTCTCCTGCTAGAAT CAGACATTTTGCTCTCAAGCTG FAM-TTGCTGCTGCTTGACAGATT-TAMRA	MN908947	28881-28902 28958-28979 28934-28953	(Primers, 2020)
N gene	Forward primer, Reverse primer, Probe	TAATCAGACAAGGAACTGATTA CGAAGGTGTGACTTCCATG FAM/ZEN-GCAAATTGTGCAATTTGCGG-IBFQ	MN908947	29145-29166 29236-29254 29179-29198	(Chu *et al.*, 2020)
N1 gene	Forward primer: Reverse primer: Probe	GAC CCC AAA ATCAGCGAA AT TCTGGTTACTGCCAGTTGAATCTG FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1	MN908947	28287-28306 28335-28358 28309-28332	(Coronavirus, 2019)
N2 gene	Forward primer: Reverse primer: Probe	TTACAA ACATTGGCCGCA AA GCGCGACATTCCGAAGAA FAM-ACA ATTTGCCCCCAGCGTTAG-BHQ1	MN908947	29164-29183 29213-29230 29188-29210	(Coronavirus, 2019)
N3 gene	Forward primer: Reverse primer: Probe	GGGAGCCTTGAA TAC ACC AAA A TGTAGCACG ATTGCAGCATTG FAM-AYCACATTGGCACCCGCA ATCCTG-BHQ1	MN908947	28681-28702 28732-28752 28704-28727	(Coronavirus, 2019)
NIID_2019-nCOV_N_F2	Forward primer: Reverse primer: Probe	AAATTTTGGGGACCAGGAAC TGGCAGCTGTGTAGGTCAAC FAM-ATGTCGCGCATTGGCATGGA-BHQ1	MN908947	29125-29144 29263-29282 29222-29241	(Shirato *et al.*, 2020)

Target genes	**Name**	**Sequence (5' → 3')**	**Reference sequence**	**Nucleotide position**	**Reference**
N gene	Forward primer: Reverse primer: Probe	CGTTTGGTGGACCCTCAGAT CCCCACTGCGTTCTCCATT FAM-CAACTGGCAGTAACCA-BQH1	MN908947	28320-28339 28358-28376 28341-28356	(Corman *et al.*, 2020)
E gene	Forward primer: Reverse primer: Probe	ACAGGTACGTTAATAGTTAATAGCGT ATATTGCAGCAGTACGCACACA FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	MN908947	26269-26294 26360-26381 26332-26357	(Corman *et al.*, 2020)
Spike	Forward primer: Reverse primer:	CCTACTAAATTAAATGATCTCTGCTTTACT CAAGCTATAACGCAGCCTGTA	MN938384 MN975262	22712-22741 22849-22869	(Chan *et al.*, 2020)
RNase P gene (RP)	Forward primer: Reverse primer: Probe	AGATTTGGACCTGCGAGCG GAGCGGCTGTCTCCACAAGT FAM -TTCTGACCTGAAGGCTCTGCGCG-BHQ-1	CDC internal control	--	(Coronavirus, 2019)
GAPDH	Forward primer: Reverse primer: Probe	TCAAGAAGGTGGTGAAGCAGG CAGCGTCAAAGGTGGAGGAGT VIC-CCTCAAGGGCATCCTGGGCTACACT-BHQ1	internal control	--	(Lu *et al.*, 2020)

2.3 Challenges associated with WBE

2.3.1 Complexity of wastewater matrix

The complexity of the wastewater matrix is one of the foremost challenges for its successful application for disease monitoring. The wastewater matrix contains high concentrations of nutrients, heavy metals, and micropollutants, as well as dissolved and particulate matter (with a chemical oxygen demand of 250–1000 mg/L), microorganisms (up to 10^9 number/mL), and solids (total solids 350–1200 mg/L) (Warwick *et al.*, 2013). Uncertainty in comparing research is increased by varying methods for testing and normalizing data (Mao *et al.*, 2020). These constraints restrict how effectively WBE data may be used to inform policy. Wastewater complexity involved high concentrations of inhibitors, which could affect the survival of RNA (Corpuz *et al.*, 2020). The catchment area's size and consequent vulnerability to diurnal fluctuations in flow and/or virus detection rates need to be carefully taken into account (Cornman *et al*., 2018, Dong, 2015). In addition, the variety of disinfectants and detergents concurrently entering the sewage network may make it difficult to sample SARS-CoV-2 wastewater (Aguiar-Oliveira *et al.*, 2020). This also makes extraction of RNA difficult and higher rates of degradation (Aguiar-Oliveira *et al.*, 2020). Which leads to high levels of uncertainty for many target indicators due to variability in excretion rates, patterns of use, fluctuation in the contributing population, unknown sources of disposal, environmental factors, or degradation of the signal in the sewer network (Corpuz *et al.*, 2020). There are also many analytical challenges related to analysis of sewage, which is a complex matrix with many analytical interferences (Aguiar-Oliveira *et al.*, 2020). Viruses are susceptible to inactivation once released into the environment and several factors including temperature, pH, Ultraviolet (UV) light, inorganic matter and antagonistic microbial interaction can affect these viral particles (Auffret *et al.*, 2019). Many research utilizing various types of water have also assessed the effect of temperature on the survival of viruses in water (Casanova *et al.*, 2009,

Gundy *et al.*, 2009, Nazir *et al.*, 2010). Coronaviruses are less viable at high temperatures (over 30 °C), although they can survive for more than 28 days at low temperatures (4 °C) (Kampf *et al.*, 2020). According to Darnell *et al.*, (2004), pasteurization may be quite successful at inactivating the virus. Darnell *et al*., (2004), found that SARS-CoV-1 rapidly inactivated at progressively higher temperatures. Similarly, pH has also shown an impact on the viability of viruses in wastewater. A pH range between 5 and 7.4 is a stable range for coronaviruses (Casanova *et al.*, 2009). Over a testing period of 1 hour, a recent study has demonstrated that SARS-CoV-2 persists throughout a wide pH range (3–10) without any discernible loss, however it is still unclear how its stability changes over a longer period of time (Chin *et al.*, 2020). The presence of suspended solids in wastewater in addition affects the coronavirus survival by increasing the likelihood that the virus particles will attach to other particles present in the wastewater (Gundy *et al.*, 2009). It has been reported that the presence and concentration of disinfection chemicals such as residual chlorine is another aspect that affect the viral stability in wastewater (García-Ávila *et al.*, 2020). SARS-CoV-2 was found to be more effectively inactivated by free chlorine than by chlorine dioxide (Wang *et al.*, 2005).

2.3.1.1 Concentrations of the virus particles from wastewater

The difficulties associated with WBE for disease surveillance, include virus recovery due to dilution and matrix effect. There are numerous techniques that have been developed for their efficient recovery. Several studies determined the recovery efficiency using a variety of enveloped ((Phi 6 phage, porcine epidemic diarrhea virus (PEDV), bovine coronavirus (BCoV), bovine respiratory syncytial virus (BRSV), and transmissible gastroenteritis virus (TGEV) and non-enveloped viruses (F-specific RNA phage, mengovirus (MgV)) as indicated in table 2.3. These techniques combine filtration, ultracentrifugation, and Polyethylene glycol (PEG) precipitation with a variety of pH values, chemicals, filter types, centrifuge speeds and purifying processes (Polo *et al.*, 2020).

Table 2.3: Recovery Efficiency from wastewater using different methods.

Types of wastewater	Volume of wastewater concentrated (mL)	Virus concentration methods used	% recovery	RT-PCR assay/target gene used	Sequencing	Reference
Untreated wastewater	500 (mostly 24-h time or flow composite)	Direct flocculation	35.5 ± 13.0% using TGEV (whole process control)	EliGene® COVID19 BASIC A RT kit	NU	(Mlejnkova *et al.*, 2020)
Untreated wastewater	250 (24-h composite)	Two-phase (PEG-dextran method) separation	2.04 ± 0.70% using Alphacoronavirus HcoV-229E)	ORF1ab E gene RdRP	Direct sequence	(La Rosa *et al.*, 2021)
Influent **Secondary treated**	200 (grab) 5000 (grab)	Electronegative membrane-vortex (EMV) method Adsorption-extraction	71.6 ± 25.2% using MS2 for EMV 8.5 ± 3.7% using MS2 for the adsorption- extraction (RNA extraction and RT-qPCR)	N_Sarbeco NIID_2019-nCOV_N CDC N1 CDC N2	Direct sequence	(Haramoto *et al.*, 2020)
Influent **Secondary** **Tertiary effluent**	200 (grab, 7-12 am)	$Al(OH)_3$ adsorption-precipitation	10 ± 3.5% using PEDV (influent) 3.3 ± 1.6% using PEDV (effluent) 10 ± 2.1% using MgV (influent) 6.2 ± 1.0% (MgV effluent)	CDC N1, N2, N3 CDC N1, N2, N3 CDC N1, N2, N3	NU	(Randazzo *et al.*, 2020b)

Types of wastewater	Volume of wastewater concentrated (mL)	Virus concentration methods used	% recovery	RT-PCR assay/target gene used	Sequencing	Reference
Untreated wastewater	200 (grab, 10 am – 12 pm)	$Al(OH)_3$ adsorption-precipitation	2.56 - 18.8% using MgV	CDC N1 CDC N2	NU	(Randazzo *et al.*, 2020a)
Untreated wastewater	250 (24-h flow composite)	Ultrafiltration	73 ± 50% using F-specific RNA phages for purification and concentration) 30.4 ± 22.3% using F-specific RNA phages for RNA extraction and RT-qPCR)	CDC N1 CDC N2 CDC N3 E_Sarbeco	NU	(Medema *et al.*, 2020b)
Untreated wastewater **Secondary treated effluent** **Final effluent**	100-750 (grabs at 7 - 11am and 24-h composite)	Centrifugation and ultrafiltration Adsorption and elution with electronegative membrane	54-56% using Phi 6	CDC N1 CDC N2	NU	(Sherchan *et al.*, 2020)
Sewage	125 (24-h flow composite)	InnovaPrep Electronegative filtration	5.5 ± 2.1% using BCoV) 7.6 ± 3.0 using BRSV) 4.8 ± 2.8% using BCoV) 6.6 ± 3.8 using BRSV)	CDC N1 CDC N2 CDC N3	NU	(Gonzalez *et al.*, 2020)

NU: Not undertaken

2.3.2 Sampling method

The two most popular techniques for sampling wastewater are grab sampling and 24-hour composite automatic sampling (Augusto *et al.*, 2022). Grab sampling is practical and simple, and it may be carried out along the outflow tracks of most catchments since no additional equipment installation is necessary (Wilson *et al.*, 2022). Grab sampling, however, may miss viral shedding discharges to sewers and provide less representative surveillance since the contents of wastewater fluctuate greatly depending on the time of day and related human activities. To overcome this limitation, automatic samplers are installed to collect 24-hour composite samples. It is not always possible to use the automatic sampling method for small catchments, even though composite samples capture variations in wastewater over time. This is due to numerous factors, including the high cost of equipment and maintenance, the need for skilled personnel to install and power the sampler, a lack of available space for installation, difficulty in accessing the sampling site, difficulties in areas with sub-zero temperatures, and low wastewater flow. While autosamplers can be used to collect composite samples over a representative time, such as 24 hours, the cost of the refrigeration units required to stop viral deterioration makes them impractical (Wilson *et al.*, 2022). Alternative sampling techniques may therefore be advantageous for small catchments. The benefits of grab and automated sampling are combined in passive sampling techniques, which frequently take the shape of a modified ‘Moore swab’ (Sikorski and Levine, 2020). An abiotic device known as a passive sampler may include absorbent materials or membranes. It is positioned in a certain sewage catchment where it will capture viruses for a predetermined amount of time. Using passive samplers has numerous advantages. They can be utilized in any accessible sewage line because they are simple to deploy, gather from tiny catchments, and are powerless. Additionally, as passive samplers gather viruses throughout their whole stay, shedding events are unlikely to go unnoticed. In terms of detecting SARS-CoV-2 in wastewater from manholes in small towns,

Rafiee *et al.*, (2021) discovered that Moore swab passive sampling outperformed grab sampling and performed comparably to automatic composite sampling. Moore swabs and grab samples were examined by Liu *et al.*, (2020) from a hospital's sewage line, and they discovered that passive sampling was more sensitive than grab sampling for the identification of SARS-CoV-2. To check for COVID-19 cases at a university residence hall, Corchis-Scott *et al.*, (2021) utilized a tampon as a Moore swab. In order to stop the disease from spreading inside the apartment COVID-19 testing was initiated and contact tracing was used to prevent the spread of disease in the residence. Although Moore swab-style passive samplers for COVID-19 surveillance have yielded promising results, these passive samples are prone to disruption of the contact required between the swab and wastewater caused by particles in the wastewater (Schang *et al.*, 2021). Moore swabs may also be damaged or lost if they come into contact with solids flowing in the sewer pipe (Hayes *et al.*, 2021). There is limited information about passive sampler's effectiveness in detecting SARS-CoV-2 in wastewater, despite research demonstrating their potential for viral detection in wastewater. Therefore, there are no uniform method that currently can be considered as the most efficient and need to be optimized according to the needs and resource availability.

2.3.3 The shedding rate of the virus particles by the infected individuals

The viral particle shed from the infected person via the urine or feces, ends up in the sewage system (Graham *et al.*, 2020). Knowing shedding rates per individual is important to track the spread of the virus in the population. Many factors, such as viremia, the duration, severity, and stage of the illness, as well as age, may affect the shedding rate at which viruses are shed in the feces (Chen and Li, 2020). Viral loads in human feces or urine samples may be used to estimate levels in wastewater during an outbreak event in the absence of data on viral loads in wastewater (Sherchan *et al.*, 2020). There is a lack of information on whether the shedding rate could vary between the individuals and during the period of infection period. For example, a

person with diarrhoeal symptoms could excrete more virus particles than the one with no diarrhoeal symptoms. Similarly shedding rate between different aged groups could also vary. The shedding rate could vary from variant to variant. However, it is still unclear how long the virus will persist in symptomatic and asymptomatic patients and how often it will shed, though it is anticipated that these numbers will be significantly lower in wastewater (Wang *et al.*, 2020a, Xiao *et al.*, 2020, Tang *et al.*, 2020, Wu *et al.*, 2020c). By examining the presence of SARS-CoV-2 viral RNA in patient samples, Zhou *et al.*, (2020) calculated the average length of viral shedding. Although determining potential infectivity is a labour-intensive process, the mere presence of nucleic acid does not indicate whether a virus will shed or become infected.

2.4 Normalization of WBE data

The incorporation of population normalization to the WBE data provides the additional opportunity to determine patterns, trends, and possible comparisons across catchments with different population sizes. This ultimately helps identify infection hotspots identification (Medema *et al.*, 2020b). In WBE approach, normalizing analyte influent concentrations to per capita mass loads or consumption using the daily flow rate and catchment population provides population scale information on human activity within catchment boundaries (Choi *et al.*, 2018). This information can facilitate the assessment of the consumption, use, exposure, and release of chemicals among different populations. Sources of target signal variability in wastewater include inconsistencies in sample collection and laboratory processing (Ahmed *et al.*, 2020d, Ahmed *et al.*, 2020c, Feng *et al.*, 2021), nucleic acid degradation based on travel time and conditions in the sewer (Hart and Halden, 2020), and signal dilution due to rainfall and diurnal flow changes (Zahedi *et al.*, 2021).

Researchers have addressed some of these sources of variability through normalization using biomarkers or by increasing sampling frequency (D'Aoust *et al.*, 2021b, Feng *et al.*, 2021, Graham *et al.*, 2020, Nemudryi *et al.*, 2020, LaTurner *et al.*, 2021). Across WBE studies,

researchers have normalized wastewater concentrations to flow rate and population to calculate a per capita load (Zuccato *et al.*, 2008, Zuccato *et al.*, 2005a, Choi *et al.*, 2018, Chen *et al.*, 2014) or to a chemical parameter e.g. caffeine (Been *et al.*, 2014, Choi *et al.*, 2018). More recently biological markers have emerged as promising candidates to normalize SARS-CoV-2 RNA signal or fecal content.

Biomarkers can be classified based on their function as markers of exposure (compounds that give information about substances consumed or ingested) and markers of effect (indicators of measurable changes or alterations in an organism that can be associated with health problems or wellbeing) and based on biological nature (e.g., metabolites, hormones), or of the disease they can indicate (e.g., cardiovascular biomarkers, obesity biomarkers) (Pischon, 2009). Genetic biomarkers are crucial for determining disease incidence. A suited genetic marker must be stable, specific for a particular disease, consistent between distinct genders and ethnic groups, human-specific and excreted in urine or feces constantly, and not absorbable to particulate matter, such as, DNA/RNA, or antibiotic resistance genes (Mousazadeh *et al.*, 2021).

The fate of biomarkers in the sewer can also be predicted by using mathematical models to simulate physicochemical and microbial processes (McCall *et al.*, 2016, Bisceglia and Lippa, 2014, Ramin *et al.*, 2016). It is important to note that biomarker transformation pathways in the sewer might be different from human metabolic pathways (Mousazadeh *et al.*, 2021). For the biomarker to be detected, it must be discharged through urine or feces (Daughton, 2012).

Quantification of biomarkers would provide context to measure viral concentrations and ensure that differences in viral loads will not attribute to changes in population (Lorenzo and Picó, 2019). Various options exist which may allow estimation of relative human fecal load in different samples (Choi *et al.*, 2018). Most of these compare SARS-CoV-2 RNA concentrations with the concentration of a marker that is assumed to be generated at a set rate

per person. If the concentration of the marker decreases, the sample may be more dilute, e.g., due to rainwater ingress. Such markers may include biomarkers such as human mitochondrial DNA, human RNA, or *crAssphage* (a bacteriophage present in the human gut). A list of studies that utilized chemical and biological biomarkers for SARS-CoV-2 WBE normalization is presented in table 2.4. The calculation of the contributing population to wastewater samples, also known as population normalization, is a crucial step in the use of WBE (Daughton, 2012).

Population size uncertainties would further complicate the monitoring of infectious diseases in wastewater, as the presence of commuters or tourists in a catchment area might make it difficult to track the real spread of an infection (Daughton, 2020a). Even though these dynamics might not significantly affect the levels of biomarkers in big populations, they could increase uncertainty in smaller groups (Chen *et al.*, 2014, Ort *et al.*, 2014). In addition, due to the putative impact of weather events such as floods and storms on wastewater flow variations and viral detection rates remains to be determined. These events are common in tropical areas such as South America and Africa (Pereira *et al.*, 2013, Wolfaardt *et al.*, 2021, Olds *et al.*, 2018, Street *et al.*, 2020). A viral outbreak may be present when the observed virus concentrations are noticeably higher than the estimated population (O'Brien and Xagoraraki, 2019, Sims and Kasprzyk-Hordern, 2020).

Table 2.4: Chemical and Biological markers that have shown potential for SARS-CoV-2 normalization in WBE.

Name of Marker	Chemical/ Biological	References
PMMoV	Biological	(Hsu *et al.*, 2022, D'Aoust *et al.*, 2021b)
Caffeine	Chemical	(Hsu *et al.*, 2022)
Paraxanthine (PARA)	Chemical	(Hsu *et al.*, 2022)
crAssphage	Biological	(Langeveld *et al.*, 2023, Wilder *et al.*, 2021, Reynolds *et al.*, 2022)
HF 183	Biological	(D'Aoust *et al.*, 2021b, Reynolds *et al.*, 2022)
Human Mitochondrial gene (NADH dehydrogenase subunit 5)	Biological	(Hutchison *et al.*, 2022)
5-HIAA: 5 hydroxyindoleacetic acid	Chemical	(Hsu *et al.*, 2022)
Creatinine (Cre)	Chemical	(Hsu *et al.*, 2022)
Coprostanol	Chemical	(Reynolds *et al.*, 2022)
Biological Oxygen Demand	Chemical	(Hutchison *et al.*, 2022)
Chemical Oxygen Demand	Chemical	(Hrudey *et al.*, 2022)
Electrical Conductivity	Chemical	(Langeveld *et al.*, 2023, Hrudey *et al.*, 2022)
Ammonium	Chemical	(Hrudey *et al.*, 2022, Hutchison *et al.*, 2022)

Creatinine, cholesterol, coprostanol, nicotine, cortisol, androstenedione, as well as the serotonin metabolite 5-hydroxyindoleacetic acid have all been suggested as population biomarkers (5-HIAA) (Polo *et al.*, 2020). Nevertheless, all of these biomarkers need to be proficient in analytical chemistry. There is still uncertainty when applying these markers to various catchments because of varying consumption or disposal practices, stability, and sorption to particle matter (Rico *et al.*, 2017). Due to its low affinity for other species in

wastewater, stability, continual excretion by humans, and capability for quantification utilizing the same platforms and pipelines as the viral nucleic acid of interest, human nucleic acid has a significant potential to serve as a population biomarker. *Adenovirus, PMMoV,* and *crAssphage* are all very promising viral indicators of human activity (Tandukar *et al.*, 2020). In addition to normalizing for human feces, it's vital to collect data to account for other differences in wastewater caused by, for instance, changes in flow (due to heavy rain or a drought) and pH (which can affect the virus's stability).

2.5 Potential Viral and Bacterial Biomarkers

2.5.1 *Pepper Mild Mottle Virus*

PMMoV was recently discovered as the most abundant RNA virus in human feces (Kitajima *et al.*, 2018). *PMMoV* is rod shaped and not icosahedral like other human enteric viruses (Zhang *et al.*, 2016, Moriones and Navas-Castillo, 2010, Dhakar and Geetanjali, 2022). It is a plant virus belonging to the genus *Tobamovirus* in the family *Virgoviridae*. When detected in human feces (Zhang *et al.*, 2016, Dhakar and Geetanjali, 2022) it is through the consumption of peppers and their processed products such as hot sauces and spices (Gyawali *et al.*, 2019). Due to this *PMMoV* has been increasingly attracting research attention as a potential viral indicator for human faecal pollution in water treatment systems (D'Aoust *et al.*, 2021b, Burnet *et al.*, 2023, Li *et al.*, 2022, Kitajima *et al.*, 2018, Hamza *et al.*, 2011). The advantage of the use of *PMMoV* as an indicator organism is that it can be more consistently observed in quantifiable and higher concentrations without substantial seasonal fluctuations in environmental occurrence (Moriones and Navas-Castillo, 2010, Dhakar and Geetanjali, 2022). The unique feature of *PMMoV* in terms of environmental stability supports its use as a biomarker for WBE data normalization (Kitajima *et al.*, 2018). *PMMoV* has been detected in water samples, using the same methodologies for identifying enteric RNA viruses (Kitajima *et al.*, 2018). This is due to the fact that *PMMoV* possesses an RNA genome as with many human enteric viruses, such

as the SARS-CoV-2. *PMMoV* is deemed non-pathogenic for humans, which is an advantage as an indicator (Kitajima *et al.,* 2018).The major challenges associated with *PMMoV* is the morphology and inconsistent occurrence and behaviour with human viruses (Shirasaki *et al.*, 2017, Kitajima *et al.*, 2018). Under specific conditions, this may result in variations in environmental behaviour, removal/reduction rates during treatment processes, as well as recovery efficiency for virus concentration methods (Kitajima *et al.*, 2018). The exceptional environmental stability of *PMMoV*, according to Hamza *et al*., (2011) makes it potentially unsuitable for identifying fresh fecal pollution in water bodies, which is likely to be associated with pathogens (Hamza *et al.*, 2011).

2.5.2 *crAssphage*

Bacteriophages have long been considered as surrogates for modeling viruses in environmental systems and studies (Heffron *et al.*, 2019, Aranha-Creado and Brandwein, 1999). Bacteriophages are useful due to their similar size and morphology as pathogenic viruses, in addition to their high abundance in sewage (Dutilh *et al.*, 2014b, Bibby *et al.*, 2019). The genome sequence for *crAssphage* was assembled from an individual human gut metagenome and likely represents a *crAssphage* metapopulation (Dutilh *et al.*, 2014b). *CrAssphage* was found more abundant than all other known human gut phages combined and was mostly associated with human gut metagenomes. *CrAssphage* has been successfully identified in both sewage and environmental waters (Edwards *et al.*, 2019, Elyse and Kyle, 2014, Trefault *et al.*, 2019). This indicate that *CrAssphage* has the potential to be utilized as a highly sensitive and specific marker of human fecal pollution that could be used as a surrogate of viral-risk in environmental waters (Edwards *et al.*, 2019, Bibby *et al.*, 2019). It has a dsDNA (double stranded DNA), 97Kb genome (Dutilh *et al.*, 2014b). In addition, the initial evidence of high abundance and high human-specificity that *crAssphage* exhibits have shown that it could improve upon shortcomings witnessed with general fecal indicator bacteria (FIB) and other

developed Microbial source tracking (MST) assays (Bibby *et al.*, 2019) making *crAssphage* an ideal target for human-specific marker development and the normalization of WBE data. Being one of the most abundant phages of the human gut microbiome, there is a need to understand the use of *crAssphage* in wastewater and its contribution as a biomarker when correlating the concentration or abundance of *crAssphage* to other biomarkers that are present in wastewater (Stachler and Bibby, 2014, Elyse and Kyle, 2014). A recent study by Langeveld *et al*., (2023) used alternative normalization methods, where electrical conductivity and *crAssphage* have been studied and compared with the standard approach using flow measurements. Viral fecal indicators, including *crAssphage* and *PMMoV*, have been proposed as population biomarkers to normalize SARS-CoV-2 levels in wastewater and used in a study by Reynolds *et al.*, (2022). The study found that the *crAssphage* signal was found to be more stable than the *PMMoV* signal. Similar to this, levels of the SARS-CoV-2 virus that have used *crAssphage*-normalization have been reported, and relationships between these normalized levels and the number of clinical cases in New York have been recorded (Green *et al.*, 2020).

2.5.3 *Bacteroides* HF183 marker

The majority of human MST assays have been developed on bacterial targets, specifically the 16S rRNA (ribosomal RNA) gene of *Bacteroides* species. *Bacteroides* is one of the predominant genera in the human gut microflora, making it a potential target for the development of human-specific markers (Bernhard and Field, 2000). The most common target for human MST assay development to date has been the 16S rRNA gene of *Bacteroides* species. Due to their consistently high performance, the HF183 assays are widely considered to be the top-performing technology in human MST methods. A DNA fragment from the 16S rRNA gene of *Bacteroides* is primarily associated with human fecal material. It was named after the forward primer HF183 (e.g., ‘human’ ‘forward’ ‘183’) of the originally published PCR (Polymerase chain reaction) assay. *Bacteroides* HF183 is the most commonly used

sewage-associated marker gene that belongs to the genus *Bacteroides* (an obligate anaerobe) (Seurinck *et al.*, 2006, Unno *et al.*, 2018). The best studied human *Bacteroides* marker, the HF183 marker, is found in *Bacteroides dorei* and its closely related taxa and is located in the V2 hypervariable region of the 16S rRNA gene. This marker was first reported by Bernhard and Field (Bernhard and Field, 2000) as a PCR assay. The fecal anaerobic microorganism, *Bacteroides* has been utilized as a target for human fecal source detection since the early 2000s. *Bacteroides* HF183,(Bernhard and Field, (2000)) has been widely used to identify sewage contamination in environmental waters around the globe (Ahmed *et al.*, 2009, Seurinck *et al.*, 2006, Ahmed *et al.*, 2012, Jenkins *et al.*, 2009, Staley *et al.*, 2012, Chase *et al.*, 2012, Nshimyimana *et al.*, 2014). The high abundance of the HF183 marker gene in untreated sewage makes it easy to detect in source waters contaminated with low levels of sewage (Chase *et al.*, 2012, Hughes *et al.*, 2017). This marker has been widely used to identify human feces pollution in various environments but has shown little use as a population biomarker in SARS-CoV-2 wastewater surveillance studies (D'Aoust *et al.*, 2021b, Sala-Comorera *et al.*, 2021).

CHAPTER THREE

Detection and quantification of SARS-CoV-2 and biomarkers (*crAssphage, PMMoV* and *Bacteroides* HF 183) using PCR and droplet digital PCR in wastewater

3.1 Introduction

Wastewater-based epidemiology involves accurately detecting and quantifying pathogens in raw wastewater. However, the usability of WBE for disease surveillance is severely compromised by the unexplainable variability in WBE data caused by several factors, including the high dilution rate which decreases the virus load per volume (Ikner *et al*., 2012). The application of WBE therefore rely on efficient protocols for concentration, extraction and quantification of pathogens from raw wastewater. Nevertheless, these protocols vary depending on the type of pathogen being examined (Zhang *et al*., 2022). Grab sampling, composite sampling, and auto samplers are three common methods used for collecting wastewater samples (Larsen and Wigginton, 2020). A grab sample is taken at a specific time and location (Kmush *et al.*, 2022), while a composite sample is taken over a period of time and then combined to form a representative sample (Kmush *et al.*, 2022, Mendoza Grijalva *et al.*, 2022). This can be done either manually or using autosamplers (Vincent-Hubert *et al.*, 2022, Habtewold *et al.*, 2022). However, the methods may be adjusted depending on the type of wastewater, which is chemically and biologically complex and variable (Schang *et al*., 2021). Similarly, there have been several concentration procedures explored to identify and measure SARS-CoV-2 RNA in wastewater (Peinado *et al*., 2022, Kumblathan *et al*., 2023, Medema *et al.*, 2020b). This includes (a) aluminum hydroxide adsorption-precipitation (Randazzo *et al.*, 2020b, Yu *et al.*, 2022), (b) pretreatment with glycine buffer and PEG precipitation (La Rosa *et al.*, 2021, Torii *et al.*, 2022, Sapula *et al.*, 2021) and (c) ultrafiltration (Kevill *et al.*, 2022,

Medema *et al.*, 2020b) which have been extensively studies for their efficiency in detecting SARS-CoV-2 virus in wastewater.

The recovery efficiency of these methods though varied, have been reported to be satisfactory (Medema *et al.*, 2020b, Randazzo *et al.*, 2020b, Pillay *et al.*, 2021). Therefore, the choice of concentration methods can be influenced by a variety of factors, including the availability of equipment and cost. For the detection of pathogens in diluted environments such as wastewater, advanced detection methods such as quantitative real-time PCR (qRT-PCR), digital PCR (dPCR), and next-generation sequencing (NGS) have been investigated (Larsen and Wigginton, 2020). The most common method for the detection of SARS-CoV-2 in wastewater was qRT PCR (Farkas *et al.*, 2021, Yu *et al.*, 2022, Zhang *et al.*, 2020a, Ahmed *et al.*, 2020d, Ahmed *et al.*, 2020b, Ciesielski *et al.*, 2021), followed by digital PCR (Lou *et al.*, 2022, Ciesielski *et al.*, 2021, Amoah *et al.*, 2021). The choice of method however depends on factors such as the sensitivity and specificity required, the cost of the method, and the availability of equipment and expertise (Larsen and Wigginton, 2020). There has been an increase in studies and research involving the detection of SARS-CoV-2 RNA in wastewater since the initial study conducted by Medema *et al*. (2020b), however, methods for the concentration and subsequent quantification of SARS-CoV-2 viruses are not yet standardized. The variability in results may result from differences in methods, but it is unclear whether inter- and intra-laboratory variability will compromise the reliability of these methods for reliably assessing the degree of disease prevalence in a community and/or tracking temporal trends in SARS-CoV-2 occurrence. For example, a group of researches explored the patterns of SARS-CoV-2 quantification in wastewater influent solids (post-grit solids (PGS) and primary clarified sludge (PCS) from two municipal water resource recovery facilities (D'Aoust *et al.*, 2021b).

Additionally, they conducted a comparison of RT-qPCR and RT-droplet digital PCR (RT-ddPCR). PCS samples show signal inhibition using RT-ddPCR compared to RT-qPCR, with PGS samples showing similar quantifiable concentrations of RNA using both assays. However, another study by Ciesielski *et al.*, (2021) have also demonstrated that ddPCR is more accurate and sensitive than qPCR for detecting SARS-CoV-2 in WBE. Their study found that ddPCR displayed inhibitor resistance in a wastewater matrix and revealed great reproducibility between two processing groups. Additionally, in comparison to qPCR, which had a calculated LOD (Limit of detection) of 12.0 copies/L of template, ddPCR revealed superior analytical sensitivity with a lower LOD of 0.066 copies/L of template (Ciesielski *et al.*, 2021). In this study, the ddPCR technique has been used as a detection and quantification method for SARS-CoV-2 and the biomarkers in wastewater. This chapter therefore presented the detection and quantification of SARS-CoV-2 and the biomarkers (*crAssphage*, *PMMoV* and *Bacteroides* HF183) using PCR and droplet digital PCR in wastewater.

3.2 Material and Methods

3.2.1 Sample collection, concentration, and nucleic acid extraction

3.2.1.1 Bacterial biomarker

For biomarker detection, grab samples of approximately 2 litres of untreated wastewater (once off) were collected from a domestic WWTP in Durban, South Africa. The samples were transported to the laboratory in a cooler box. The wastewater samples of approximately 200 ml were homogenized upon arrival at the laboratory and then filtered through a 0.45 µm filter membrane (AXIVA membrane filters - cellulose nitrate). The membrane was then cut into small pieces and placed in a bead beating tube followed by bead beating at medium speed for 3 minutes. Bacterial DNA was extracted using the Zymo Research (ZR), ZymoBIOMICS™ DNA Miniprep Kit (D4300) following the manufactures instructions. The eluted DNA was stored at -20 °C and used for further analysis. The quality and quantity of the extracted DNA was determined using the Implen Nanophotometer®.

3.2.1.2 Virus Particles

Wastewater sampling was done as described in (Section 3.2.1.1). A sample volume 200 mL were equally divided into 50 mL centrifuge tubes and was centrifuged at 3400 rpm for 10 minutes at 4 °C. The supernatants from each tube were then pooled to a final volume of 60 mL. The method of ultrafiltration was used to concentrate the virus particles as previously described by Medema *et al*., (2020b). The concentrate was stored at 4 °C until further analysis.

Viral DNA and RNA was extracted using the Zymo Research quick DNA/RNA viral kit™ (D7020/D7021) according to the manufacturer's instructions. The eluted DNA was stored at -20 °C and the eluted RNA was stored at -80 °C until further analysis. The quality and quantity of the extracted DNA and RNA was determined using the Implen Nanophotometer®.

3.2.1.3 cDNA Synthesis

cDNA synthesis of the extracted viral RNA was performed using the Thermo Scientific™, Maxima H minus first strand cDNA synthesis kit following the manufacturer's instructions. The cDNA samples were stored at -80 °C until further analysis.

3.2.1.4 Determination of the recovery efficiency for SARS-CoV-2

The recovery efficiency was conducted and calculated as previously described by (Pillay *et al.*, 2021). Briefly, 400 mL of raw wastewater was spiked with 200 µL suspension of inactivated SARS-CoV-2 strain USA/WA1/2020 (Microbiologics, USA). Spiking was done in triplicate. The percentage of SARS-CoV-2 recovered was determined using Equation 3.1.

Equation 3.1:

$$Recovery\ \% = \frac{Csw - Cuw}{Csc} \times 100$$

The SARS-CoV-2 concentration in Milli-Q water or wastewater that has been spiked is represented by Csw, the concentration of SARS-CoV-2 in Milli-Q water or unspiked wastewater is represented by Cuw, and the concentration of SARS-CoV-2 in Milli-Q water or spiked wastewater is represented by Csc. Using the approach, the SARS-CoV-2 recovery from wastewater was recorded to be 62.90 (± 12.8) % effective. Due to the difficulty in obtaining positive controls for each of the biomarkers (*crAssphage, PMMoV* and *Bacteroides* HF 183) throughout this study, the recovery efficiency for the biomarkers could not be determined.

3.2.1.5 Polymerase chain reaction (PCR)

The extracted DNA/cDNA was used for screening of the various biomarkers for their detection using specific primers pairs listed in table 3.1. A working concentration of 50 ng (nanogram)/µL of the extracted DNA/ cDNA prepared using methods described in section 3.2.1 was used for the PCR analysis. A non-template control (NTC) that contained sterile

distilled water was used as a negative control. The PCR mixture (in the order listed in Table S3.1), for all targeted biomarkers contained 12.5 µL of high-throughput PCR: Emerald Amp GT PCR Master Mix, 4 µL of primer mix containing 2 µL of each primer set (final concentration of 0.2 µM), 2 µL of DNA template/cDNA, and 6.5 µL of molecular grade water in a final volume of 25 µL in a reaction tube. PCR amplification was performed in Veriti™ 96-Well Thermal Cycler. Thermal cycling conditions is captured in table 3.2. The PCR products were viewed on a 1% agarose gel (pre-stained with ethidium bromide). The PCR products were further verified using sequencing and analysis.

Table 3.1: Primer Sequences and References for biomarker

Primer Name	Primer Sequence	Expected Product Size	Reference
HF 183	ATC ATG AGT TCA CAT GTC CG	525bp	(Bernhard and Field, 2000)
BAC 708R	CAA TCG GAG TTC TTC GTG		(Bernhard and Field, 2000)
CPQ 056 F	CAG AAG TAC AAA CTC CTA AAA AAC GTA GAG	126bp	(Stachler *et al.*, 2017)
CPQ 056 R	GAT GAC CAA TAA ACA AGC CAT TAG		(Stachler *et al.*, 2017)
PMMoV CP-F	ATG GCT TAC ACA GTT TCC AGT	474bp	(Peng *et al.*, 2015)
PMMoV CP-R	CTA AGG AGT TGT AGC CCA GGT G		(Peng *et al.*, 2015)

Table 3.2: Thermal Cycling conditions for each biomarker

Target Organism	PCR Cycling Conditions						Results (Depicted Figure)
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Number of Cycles	
Bacteroides HF183 spp.	94 °C 2 minutes	94 °C 1 minute	63 °C 1 minute	72 °C 1 minute	72 °C 7 minutes	35	Figure 3.2
crAssphage (CPQ056f/CPQ056r),	95 °C 3 minutes	95 °C 1 minute	60 °C 1 minute	72 °C 1 minute	72 °C 7 minutes	35	Figure 3.3
PMMoV (PMMoV CP-f/PMMoV CP-r)	95 °C 2 minutes	95 °C 30 seconds	60 °C 30seconds	72 °C 30seconds	72 °C 7 minutes	40	Figure 3.4

3.3 Sequencing and Analysis

The PCR products were excised from the agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (#D4001, Zymo Research, South Africa) and sent for sequencing to Inqaba Biotech, Pretoria, South Africa. The obtained sequences were edited using Finch TV software (version 1.4.0). The partial sequences that were 99-100% were selected. The sequences obtained were compared to the National Centre for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) to determine the phylogenetic affiliations. The aligned sequences were exported into MEGA11 (version 2) where matrices of evolutionary distances were computed (Tamura *et al.*, 2013). Phylogenetic trees were then constructed and checked by bootstrap analysis (based on 1000 replicates) (Tamura *et al.*, 2011).

3.4 ddPCR Optimization for the detection of biomarkers from wastewater

A working concentration of 20 ng/µL of wastewater samples that showed positive identification of the biomarker's during PCR was further analysed using ddPCR. Using the optimized conventional PCR thermal cycling conditions listed in table 3.2, the concentrations of wastewater samples for each biomarker was determined using ddPCR analysis. The ddPCR was performed in a 22 µL reaction volume (in the order listed in Table S3.2), containing 10 µL of 2X QX200 ddPCR EvaGreen Supermix (Bio-Rad), 1 µL (20 ng/µL) of template DNA or cDNA, 1 µL each of the forward primers (FP) and reverse primers (RP), each at a final concentration of 0.2 µM and 9 µL of RNase/DNase free water.

Droplets were generated using an automated droplet generator [QX200TM AutoDG ddPCR system (Bio-Rad, USA)]. The plate containing the droplets (40 µL) was carefully removed from the automated droplet generator. The plate was then heat-sealed and loaded into a C1000 Touch^{TM} Thermal Cycler (Bio-Rad, USA) for amplification. The thermal cycling conditions,

as listed above table 3.2 were further optimized for each biomarker (*crAssphage*, *PMMoV*, and *Bacteroides* HF183). The optimized thermal cycling conditions for ddPCR is captured in section 3.4.1. After thermal cycling, the plates were loaded into a QX200^{TM} Droplet Reader (Bio-Rad, USA). The ddPCR analysis was conducted in triplicate and NTC were used. The QuantaSoft™ Analysis Pro software (version 1.0.596) (Bio-Rad, USA) was used for the analysis of the data. Thresholds were set manually for each sample using criteria defined for each assay. The quality of all droplets was analysed and rare outliers (e.g., doublets, triplets) were gated based on detector peak width. Analysis of the ddPCR data was performed with Quanta Soft analysis software (Bio-Rad) that accompanied the QX200 Droplet Reader. Figure 3.1 describes an overview on the workflow of the wastewater analysis using ddPCR for detection and quantification of the biomarkers.

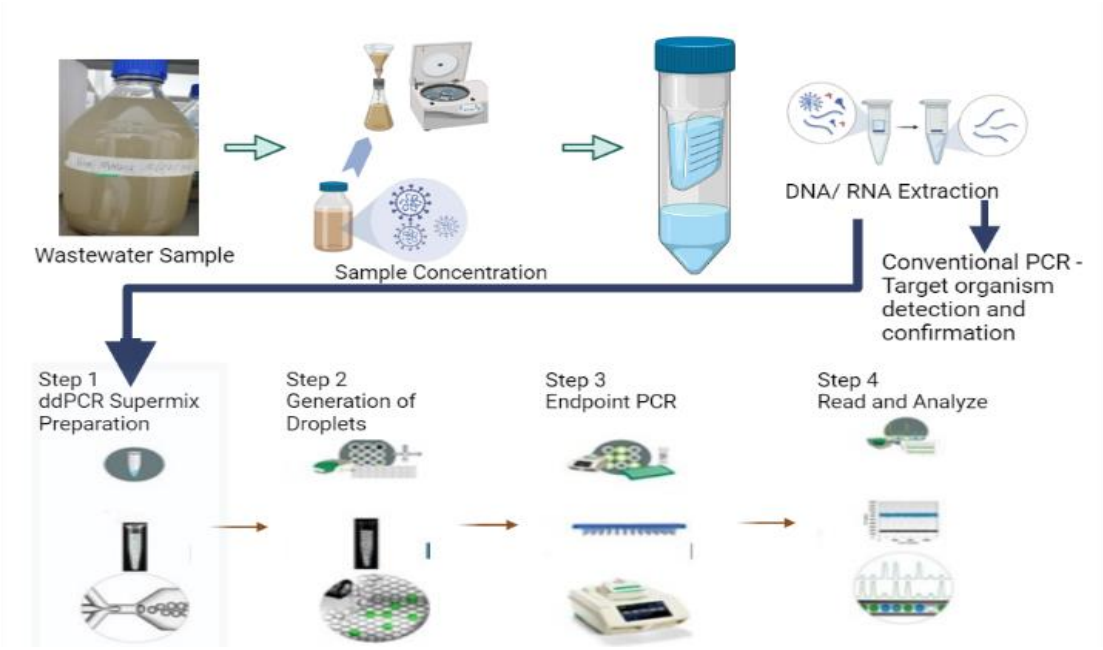


Figure 3.1: An overview on the workflow of wastewater analysis using ddPCR for detection of and quantification of viral and bacterial biomarkers.

3.4.1 ddPCR Optimization for biomarkers and SARS-CoV-2

HF183 *Bacteroides:* Optimized thermocycling condition included an initial denaturation step at 94 ℃ for 2 minutes and followed by 40 cycles of 94 ℃ for 1 minute. The annealing temperature of at 57 ℃ for 2 minutes, 72 ℃ for 1 minute (ramp rate 1 ℃/s), final incubation step was performed at 72 ℃ for 10 minutes and stabilization of the droplets at 4 ℃ for 30 minutes and held at 4℃ before reading the plate.

crAssphage: The PCR procedure for *crAssphage* began with an initial denaturation step at 95 ℃ for 10 minutes, followed by 35 cycles of denaturation at 95 ℃ for 1 minute. The annealing temperature of at 57 ℃ for 2 minutes, 72 ℃ for 1 minute (ramp rate 1 ℃/s), final incubation step was performed at 72 ℃ for 10 minutes and stabilization of the droplets at 4 ℃ for 30 minutes and held at 4℃ before reading the plate.

***PMMoV*:** For *PMMoV*, the PCR started with an initial denaturation step at 95 ℃ for 10 minutes, followed by 35 cycles of denaturation at 94 ℃ for 3 minutes. The annealing temperature was set at 57 ℃ for 30 seconds, 72 ℃ for 1 minute (ramp rate 1 ℃/s), final incubation step was performed at 72 ℃ for 10 minutes and stabilization of the droplets at 4 ℃ for 30 minutes and held at 4℃ before reading the plate.

SARS-CoV-2: The N2 region of the viral (SARS-CoV-2) genome was targeted using a primer and probes from the one-step RT-ddPCR advanced kit from Bio-Rad (USA). The 22 µL reaction mixture in which the ddPCR was performed contained: 4.49 µL nuclease-free water, 5 µL supermix, 1 µL dithiothreitol, 1.98 µL forward and reverse primers (10µM), 0.55 µL probe (10µM), 2 µL reverse transcriptase and 5 µL RNA template. According to (Giri *et al.*, 2021) and (Barra *et al.*, 2020) the forward primer sequence was 5' TTACAAACATTGGCCGCAPA3', the reverse primer sequence was 5' GCGCGACATTCCGAAGAA-3', and the probe was 5'- ACAATTTGC(ZEN)

CCCCAGCGCTTCAG-3' with 5' Modification with FAM and 3' Modification with Iowa Black@ FQ. The thermal cycling procedures included 40 cycles of denaturation at 94 °C for 30 seconds and annealing at 55 °C for 60 seconds, followed by 1 hour of reverse transcription at 50 °C and 10 minutes of enzyme activation at 95 °C. The droplets were stabilized at 4 °C for 30 minutes (ramp rate of 2 °C/s) after the enzymes were deactivated at 98 °C for 10 minutes (Giri *et al*., 2020; Barra *et al*., 2020).

3.5 Results

3.5.1 Detection of biomarkers (*Bacteroides* HF183, *CrAssphage* and *PMMoV*) in untreated wastewater by conventional PCR

Conventional PCR was conducted for the initial screening of biomarkers in raw wastewater using specific primer pairs listed in Table 3.1. All samples were run in replicates. An expected product size of 525 bp (Figure 3.2), was obtained for *Bacteroides HF183* (HF183f and Bac708r), a product size of 126 bp indicated the presence of *crAssphage* as shown in Figure 3.3 and a 474 bp product for *PMMoV* (*PMMoV* CPf and *PMMoV* CPr) (Figure 3.4). Based on the detection of these biomarkers, indicates the presence in wastewater samples with varying intensities.

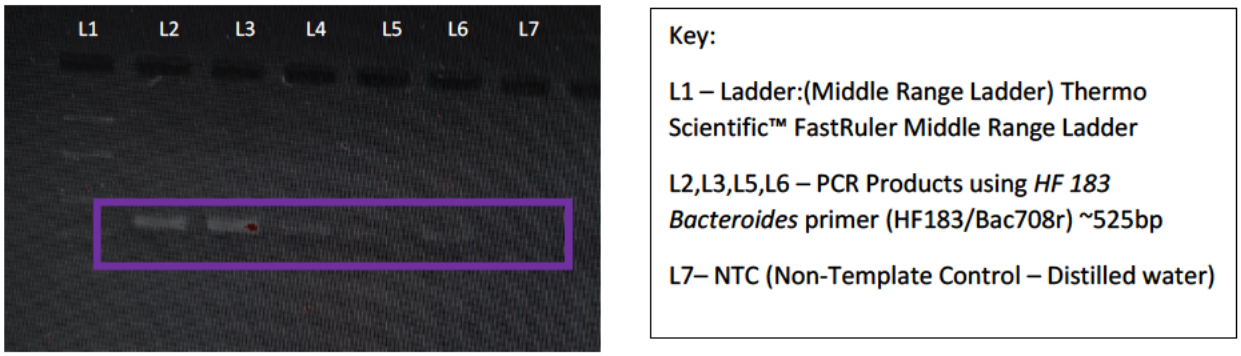
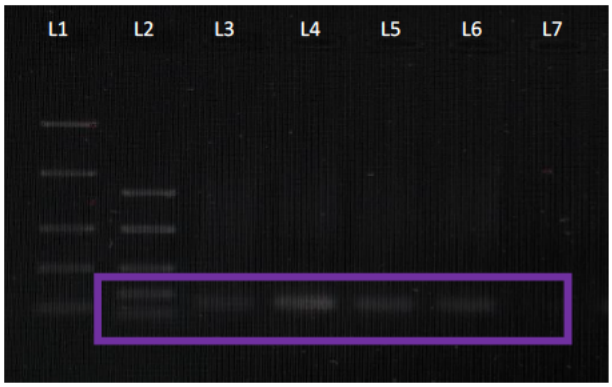


Figure 3.2: Agarose gel showing PCR products for *Bacteroides* HF183 (HF183f and Bac708r product size 525bp).



Key:

L1 – Ladder:(Middle Range Ladder) Thermo Scientific™ FastRuler Middle Range Ladder

L2 – Ladder:(Lower Range Ladder) Thermo Scientific™ FastRuler Lower Range Ladder

L3,L4,L5,L6 – PCR Products using *CrAssphage* primer (*CPQ056f and CPQ056r*) ~126bp

L7– NTC (Non-Template Control – Distilled water)

Figure 3.3: Agarose gel showing PCR products for *crAssphage* (*CPQ056*f and *CPQ056*r product size 126bp).

Key:

L1 – Ladder:(Lower Range Ladder) Thermo Scientific™ FastRuler Lower Range Ladder

L2 – Ladder:(Middle Range Ladder) Thermo Scientific™ FastRuler Middle Range Ladder

L4, L5,L7,L8,L10,L11 – PCR Products using *PMMoV* primer (*PMMoV* CPf and *PMMoV* CPr) ~474bp

Figure 3.4: Agarose gel showing PCR products for *PMMoV* (*PMMoV* CPf and *PMMoV* CPr) product size 474bp.

The PCR products were further verified using partial sequencing and analysis. The obtained sequences were aligned with the National Centre for Biotechnology (NCBI) GenBank to obtain the closely related sequences. Figure 3.5 A-C represents the phylogenetic tree of the biomarker sequences recovered from the WWTP which was used to construct a neighbor-joining tree from the resulting alignment with MEGA 11. Figure 3.5 A, indicates the relationship between the PCR product sequenced (Sample) to that of other *Bacteroides HF183* spp. Figure 3.5 B, represents the phylogenetic tree for *crAssphage*, where the sequenced PCR product (Sample) is shown against that of other *crAssphage* spp. The similarity of the sequences was chosen from those which had a similarity percentage of between 99-100% to that of the sequenced PCR products for each of the biomarkers. The *PMMoV*, as shown in Figure 3.5 C, was used to represent the relationship between the sequenced PCR product of *PMMoV* (Sample) to that of

other *PMMoV* spp. on the NCBI website. The results from the PCR and sequencing indicates the positive identification for each of the biomarkers in wastewater.

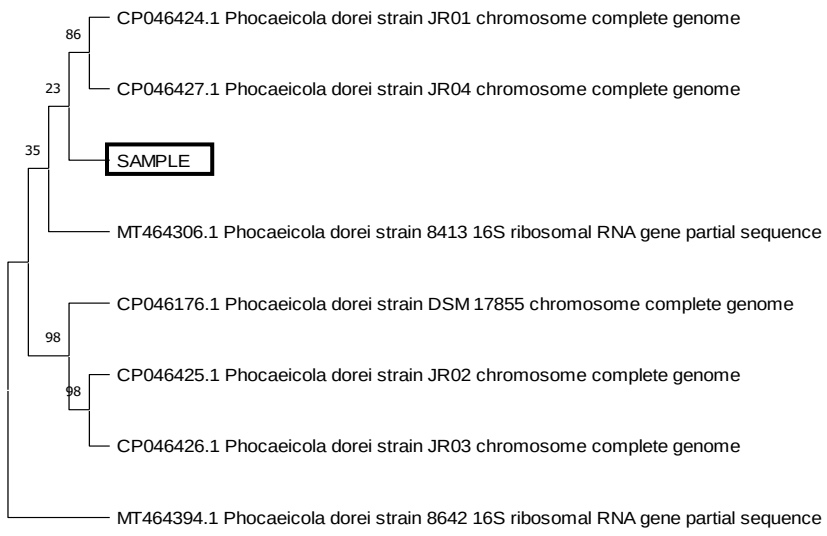


Figure 3.5 A: Phylogenetic tree of the bacterial *Bacteroides* HF183 sequences recovered from the WWTP was used to construct a neighbor-joining tree from the resulting alignment with MEGA 11 (Tamura *et al.*, 2011).

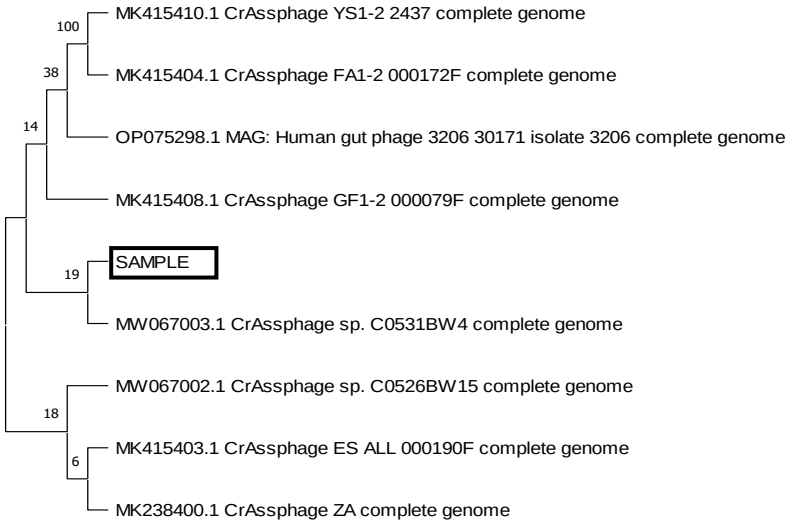


Figure 3.5 B: Phylogenetic tree of *crAssphage* sequences recovered from the WWTP was used to construct a neighbor-joining tree from the resulting alignment with MEGA 11 (Tamura *et al.*, 2011).

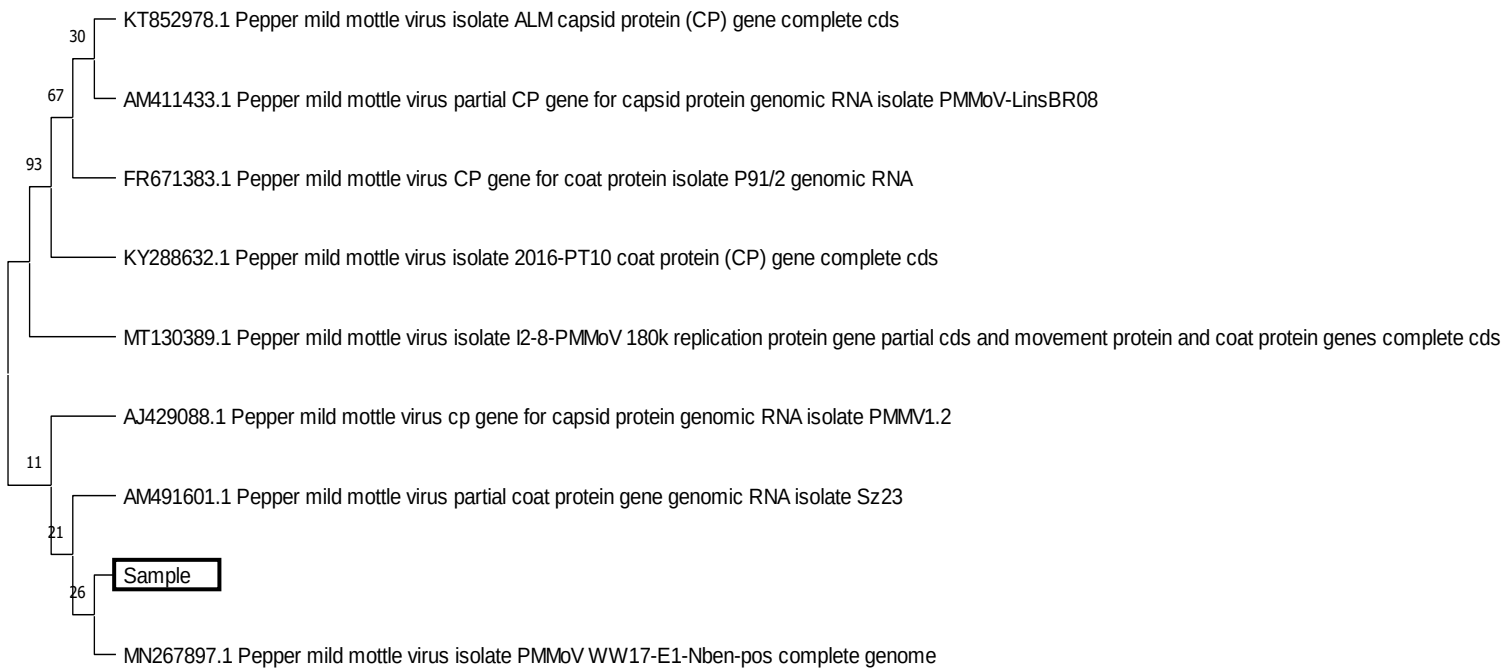


Figure 3.5 C: Phylogenetic tree of the viral *PMMoV* sequences recovered from the WWTP was used to construct a neighbor-joining tree from the resulting alignment with MEGA 11 (Tamura *et al*., 2011).

3.5.2 Optimization of ddPCR assays

The ddPCR method was used for the absolute quantification of the selected biomarkers in wastewater samples using ddPCR thermal cycling conditions listed in section 3.4.1. Thresholds were set manually for each sample using criteria defined for each assay. Figure 3.7 A-D represents the biomarkers detected using ddPCR. The ddPCR assay was applied for monitoring of the biomarkers and the SARS-CoV-2 concentrations in untreated wastewater samples during the study period. Figure 3.6 represents the copies/µL of each biomarker detected. Figure 3.7 (A-D) represents the manual threshold set for the biomarkers and the separation of positive and negative droplets. The concentration of biomarkers detected from the sample varied from biomarker to biomarker. The concentration of *crAssphage* in sample 1 (Figure 3.6) was 1185 (±91.92) copies/µL, the concentration of *PMMoV* is shown to be the lowest 57.6 (±15.34) copies/µL and *Bacteroides* HF 183 was 935.5 (±70.03) copies/µL. The concentration of sample 2 *crAssphage* (Figure 3.6) was 1280 (±98.99) copies/µL, the concentration of *PMMoV* is shown to be the lowest 30.4 (±10.49) copies/µL and *Bacteroides* HF 183 was 866 (±89.09) copies/µL.

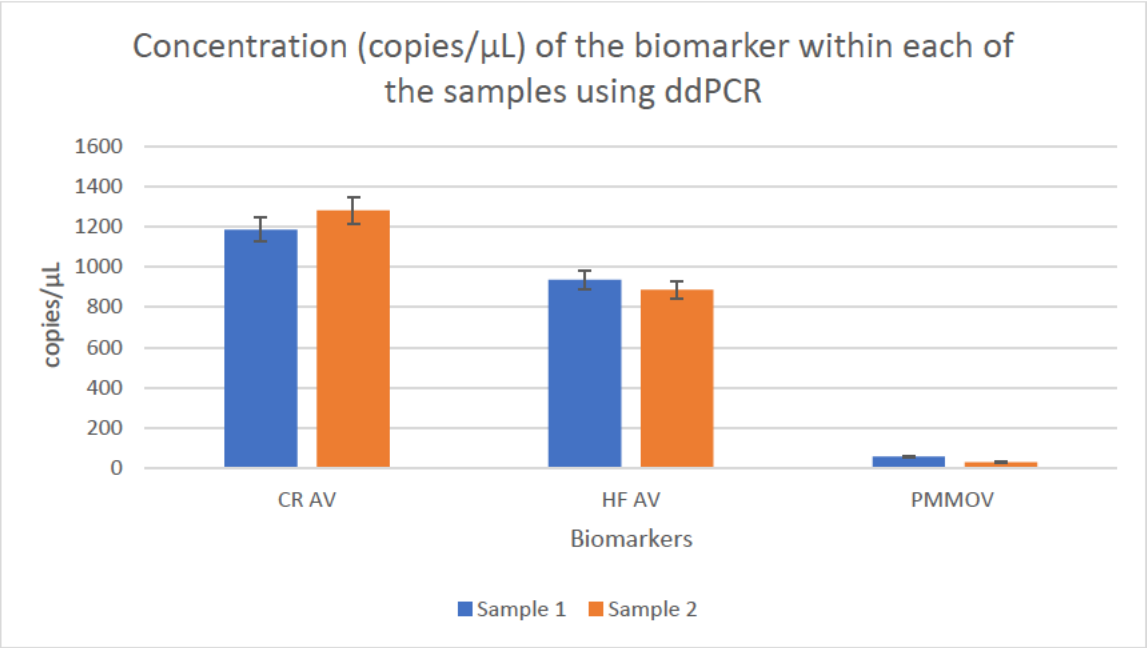


Figure 3.6: Concentration (copies/µL) of the biomarker detected in samples via ddPCR.

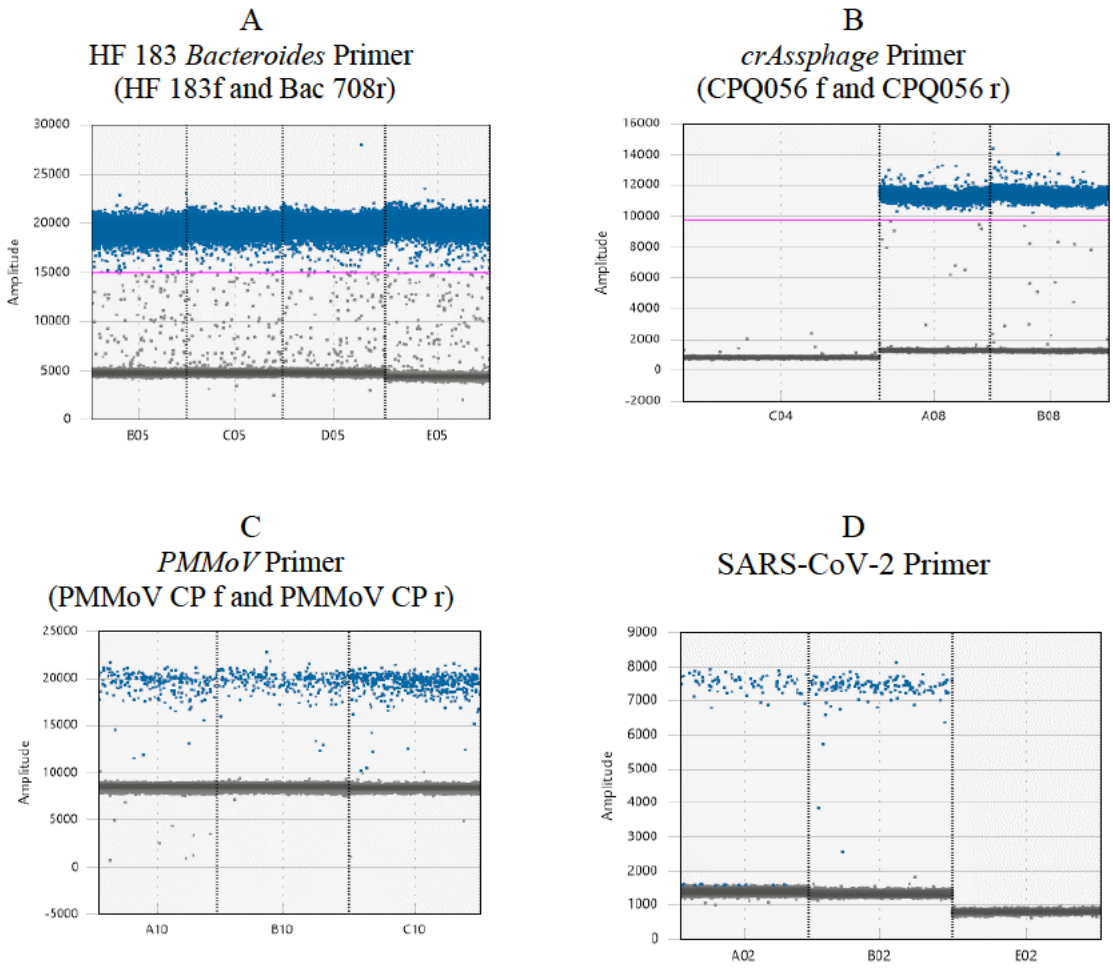


Figure 3.7 (A-D): ddPCR detection for the various biomarkers present in wastewater.

3.6 Discussion

Although the WBE approach is straightforward and offers a desirable advantage for public health monitoring, there are still some challenges to consider. The most popular technique identifying the presence of viral DNA is the PCR based techniques, but wastewater contains a variety of PCR inhibitors, such as fat, protein, and other compounds that could interfere with subsequent PCR testing (Suo *et al.*, 2020). Concentrating wastewater samples can improve detection of the biomarkers and SARS-CoV-2 RNA. Concentration may be more important for untreated wastewater samples than primary sludge samples. Concentrating the sample prior to viral detection from wastewater samples is important because it increases the concentration of the virus in the sample (Hjelmsø *et al.*, 2017). This makes it easier to detect the virus using molecular methods such as RT-PCR and ddPCR. A study conducted by (Hjelmsø *et al.*, 2017) showed that both the concentration of nucleic acids and the nature of the methods used for the extraction significantly influence the results of viral metagenomic analyses, particularly those of viral community composition, viral specificity, and viral pathogen detection.

Using ddPCR, the results showed that *crAssphage* biomarker had a greater dominance than the other two, *crAssphage* was detected wastewater samples using PCR. The concentration of *crAssphage* for sample 1 and 2 were noted to be 1185 (±91.92) copies/µL, and 1280 (±98.99) copies/µL respectively. The prevalence of *crAssphage* in wastewater was previously reported by various other authors (Sabar *et al.*, 2022, Wilder *et al.*, 2021, Tandukar *et al.*, 2020, Nam *et al.*, 2022). Given the high concentration of 4.26 to 8.25 log gene copies (GC)/ng in human feces, another study found that *crAssphage* is adequately prevalent in human feces (Nam *et al.*, 2022).

Pepper Mild Mottle Virus was detected using PCR in the wastewater sample; however, a lower concentration was observed via ddPCR. The *PMMoV* concentration observed was 57.6 (±15.34) copies/µL and 30.4 (±10.49) copies/µL for sample 1 and sample 2 respectively.

Similarly, (Bonanno Ferraro *et al.*, 2021), was the first to describe the presence and measurement of *PMMoV* in various Italian water habitats using the primer pairs (*PMMoV* CP-F/*PMMoV* CP-R). *PMMoV* was detected wastewater samples, however in significantly lower concentration compared to the other two markers. The fact that *PMMoV* is linked to a certain food intake (mainly peppers and their processed goods) may have contributed to the diet variance (Kitajima *et al.*, 2018). Another study found that *PMMoV* concentrations in domestic wastewater are consistently detected at 10^6 to 10^{10} gene copies per litre (Symonds *et al.*, 2019). *Bacteroides* HF 183 was detected the wastewater samples using PCR. Using ddPCR a higher dominance of this biomarker compared to *PMMoV* was observed. The concentrations of *Bacteroides* HF 183 for sample 1 and sample 2, were recorded as 935.5 (±70.03) copies/µL, and 866 (±89.09) copies/µL respectively. *Bacteroides* HF 183 used in a previous study which assessed the specificity and sensitivity evaluation of novel and existing *Bacteroidales* and *Bifidobacteria* specific PCR assays on feces and sewage samples and their application for fecal source tracking in Ireland. The study reported that the assay was 100% specific for *Bacteroides* (Dorai-Raj *et al.*, 2009). There are numerous studies that have utilized each of the aforementioned biomarkers individually or as a combination for normalization (Langeveld *et al.*, 2023, Hsu *et al.*, 2022, Vadde *et al.*, 2022, Duvallet *et al.*, 2022). However, there has been no study that has evaluated these biomarkers to determine which is the most suitable biomarker for normalization based on their concentration and frequency of detection. As a result, it is imperative to monitor the variability of different biomarkers across different regions, as their concentrations may vary from region to region based on human behaviour and dietary habits.

3.7 Conclusion

The focus of this chapter was to detect and quantify the biomarkers using PCR and droplet digital PCR in wastewater. The PCR analysis indicates the presence of the biomarkers in the wastewater samples. Using partial sequencing and analysis, the PCR products were further confirmed. The sequences were chosen based on their similarity to the sequenced PCR products for each of the biomarkers, which had a similarity percentage between 99 to 100%. The ddPCR was used to quantify these biomarkers, whereby *crAssphage* was observed to be the most dominant biomarker detected from the wastewater samples, followed by *Bacteroides* HF183 and lastly *PMMoV*. Thus, methods in this chapter were optimized to enhance the accuracy of the results which may be impacted by technical issues related to the WBE analysis.

CHAPTER FOUR

Assessment of biomarkers for normalization of SARS-CoV-2 concentrations in wastewater

4.1 Introduction

The incorporation of population normalization to the WBE data provides the additional opportunity for determination of patterns, trends, and possible comparison across catchments with different population sizes, ultimately helping in infection hotspot identification (Medema *et al.*, 2020a, Medema *et al.*, 2020b). It is advised to normalize SARS-CoV-2 concentrations in wastewater before calculating trends in order to account for variations in relative human waste input over time such as changes in population due to tourism, weekday commuters, and temporary workers, and variations in wastewater dilution (Covid and Team, 2021).

Molecular methods can be used to recover biomarkers from wastewater, and concentration measurements can subsequently be used to provide community-level health statistics and estimate the size of the shedding population (Xagoraraki and O'Brien, 2020). Biomarkers are intriguing since they can be quite specific for infectious diseases and, can be used for WBE (Daughton, 2012). Biomarkers include organisms or chemical compounds specific to human faeces that can be measured in wastewater to estimate the size of the population (Ballesté *et al.*, 2019, D'Aoust *et al.*, 2021b, Kitajima *et al.*, 2014, Medema *et al.*, 2020a). It is better to evaluate specific markers for normalization from different regions globally. For example, the biomarker could be linked to the diet of the population and can vary from region to region. Therefore, it is important to study their efficiency from different geographical regions. These biomarkers include but are not limited to viral or bacterial molecular targets *PMMoV,* a viral pathogen in *Capsicum sp*. commonly found in the human diet and persists in the feces (Jarret *et al.*, 2008, Zhang *et al.*, 2006, Hamza *et al.*, 2011), HF183 *Bacteroides* are widely used to

identify sewage pollution in environmental waters and is a reliable marker for detecting human fecal pollution (Ahmed *et al.*, 2008). A bacteriophage called *crAssphage* infects the commensal bacteria in the human stomach and is discharged in feces (Dutilh *et al.*, 2014a, Stachler and Bibby, 2014, Honap *et al.*, 2020). The goal of this study was to investigate the possible normalization biomarker for SARS-CoV-2 wastewater surveillance while considering the influence of wastewater's physicochemical properties. This has been overlooked during the study of biomarkers for SARS-CoV-2 monitoring thus far. To find the best universal biomarker that can be utilized for normalizing SARS-CoV-2. Wastewater surveillance monitoring can be challenging, and thus, it is crucial to include the specific wastewater physicochemical properties in biomarker assessment.

4.2 Methodology

4.2.1 Study Site and Sampling

Two WWTPs within eThekwini municipality were selected for this study, WWTP A (Figure 4.1) and WWTP B (Figure 4.2). WWTP A treats an average of 80 ML (million litres) per day (ML/d) and WWTP B 14 ML/d of wastewater respectively. WWTP A serves approximately 61 communities within the Durban Central region with types of wastewaters being domestic and industrial. WWTP B serves only on community and is mostly domestic wastewater. Grab sample of 2 litres untreated wastewater were collected from the respective plants, between 08:30 – 11:00 am on a weekly basis. The sampling was conducted for a period of three months (July – October 2022). Samples were stored during transit to the laboratory in a cooler box. Upon arrival to the laboratory, samples were analysed immediately (within 2 hours of sampling) for the physicochemical properties using a portable water-quality multiparameter instrument (YSI digital sampling system, YSI 556 MPS). Prior to analysis, samples were homogenized, and all analysis performed in triplicate.

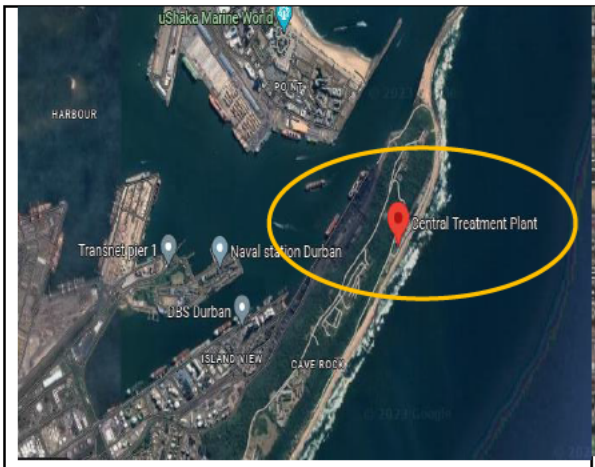


Figure 4.1: Sampling Site for Central WWTP (WWTP A)

Figure 4.2: Sampling Site for Isipingo WWTP (WWTP B)

4.2.2 Wastewater characteristics

The physicochemical properties analysed during this study using the portable water-quality multiparameter instrument (YSI digital sampling system, YSI 556 MPS) were temperature, Electrical conductivity (EC), Total dissolved solids (TDS), Salinity, Dissolved oxygen, and pH. Using methods outlined in Standard Methods (Rice *et al.*, 2012, Apha, 2012), wastewater parameters such as chemical oxygen demand (COD), total solids (TS) and volatile solids (VS), were additionally analysed.

4.2.2.1 Chemical Oxygen Demand (COD)

The COD concentrations in untreated wastewater were determined according to the standard method 5220D – Closed efflux colorimetric method using a microwave digester (APHA AWWA, 1998). Preparation of standards and reagents are listed in the appendices under appendix 1. Approximately 1.5 mL of digestion solution and to 3.5 mL of sulphuric acid were carefully added to the 2.5 mL sample. The reaction mixture was carefully mixed and tightly capped and thereafter digested at 150 °C for 2 hours in COD vials using the microwave digester (Milestone Start D, Sorisole, Italy). Standards and blank reagents were prepared and digested with the samples as controls. After cooling, the COD concentrations were measured using a

spectrophotometer (Jenway 7315) at 600 nm (Nanometres) and the results were recorded in mg/L.

4.2.2.2 Total Solids (TS) and Volatile Solids (VS)

The total solids and volatile solids were measured according to the standard methods (Apha, 2012). For the total solid measurement, 25 mL of untreated wastewater was added to a clean, pre-weighed ceramic crucible. The crucibles were heated at 105 °C overnight after which there were cooled in a desiccator containing silica gel. The crucibles were then weighed using a Mettler-Toledo ME204 analytical balance (Mettler-Toledo International Inc., USA) to gravimetrically determine the dry biomass. To determine the Volatile solids, the crucibles were subsequently incinerated at 550 °C for 1 hour. The crucibles were then cooled in a desiccator containing silica gel and thereafter weighed. The TS (Equation 4.1) and VS (Equation 4.2) were calculated using the equations below:

Equation 4.1:

$$\textbf{Total Solid}\ (\textbf{mg/L}) = \frac{(\text{Crucible} + \text{biomass}) - \text{mass (Crucible)}}{\text{Sample Volume (mL)}} \times 1000$$

Equation 4.2:

$$\textbf{Volatile Solid}\ (\textbf{mg/L}) =$$

$$\frac{(\text{Crucible} + \text{biomass}) - \text{mass (Crucible after incineration @ 550 °C)}}{\text{Sample Volume (mL)}} \times 1000$$

4.2.3 Droplet Digital PCR (ddPCR)

Using the droplet digital PCR (ddPCR) (Biorad - C1000 Touch Thermal cycler) platform the amplification of the targets was performed using the optimized conditions (From section 3.4.1) with primer pairs listed in Table 3.1. Samples were prepared and concentrated and extracted using methods described in section 3.2.1, for bacterial DNA and viral DNA/RNA. For the viral

RNA samples, cDNA synthesis was conducted as described in section 3.2.1.3 prior to the ddPCR analysis. Thereafter, ddPCR was conducted for the detection and quantification of the selected biomarkers and SARS-CoV-2 concentrations using the extracted DNA and prepared cDNA. Using the ddPCR platform the amplification of the targets the results after thermal cycling were read in the QX200 Droplet Reader, using the QuantaSoft 1.7 software (Biorad, USA). Droplet counts and amplitudes were exported to and analysed with the QuantaSoft™ analysis Pro software (Bio-Rad) and the appropriate assay information was entered into the analysis software package provided (QuantaSoft, Bio-Rad). All droplet qualities were examined, and based on detector peak width, rare outliers were gated (e.g., doublets, triplets).

4.3 Statistical Analysis

4.3.1 ANFIS model illustration of the association between the biomarker and the wastewater characteristic

The Adaptive Neuro-Fuzzy Inference System (ANFIS) model was used to model the relationship or association between the wastewater parameters and the biomarker concentrations that are present in wastewater. ANFIS uses the Takagi-Sugeno (TS) fuzzy technique as its foundation. Fuzzy logic (FL) is implemented by the ANFIS within the confines of an artificial neural network (Abunama *et al.*, 2021). The most important inputs that correlate to a desired output are identified during the creation of ANFIS models (Shah *et al.*, 2021). The ideal criteria for selecting the ANFIS model structure with the fewest modelling mistakes, including the best kinds, numbers, and rules for related membership functions (MFs) (Abunama *et al.*, 2018). Amoah *et al*., (2022), employed the ANFIS model to determine how certain physicochemical factors affected the level of SARS-CoV-2 that was found in untreated, raw wastewater. In the current study, the targeted biomarker concentrations, which were established via ddPCR, are related to the wastewater characteristics, which are employed as input variables.

4.3.2 Evaluation of biomarkers for normalization of WBE data

To find the most stable biomarker in the wastewater from the two WWTPs and to ascertain any statistical relationships, the concentrations of the three biomarkers over the course of three months were compared. Furthermore, the relationship between each of these three markers and the concentration of SARS-CoV-2 was determined via the Spearman rank correlation analysis. The most suited biomarker for normalizing WBE data in connection to COVID-19 monitoring was determined using the correlation coefficients and the outputs from the ANFIS modeling as described above. The following equation (Equation 4.3) was used to calculate the Spearman rank correlation:

Equation 4.3: (Gauthier, 2001)

$$p = 1 - \frac{6 \sum d_i^2}{n(n^2-1)}$$

p = Spearman's rank correlation coefficient

d_i = difference between the two ranks of each observation

n = number of observations

4.4 Results

4.4.1 Trend Analysis of the biomarker concentrations and the SARS-CoV-2 concentrations over a 12-week period from the two WWTPs

The concentration of biomarkers differed greatly between the two WWTPs (Figures 4.3 and 4.4). The highest copies/µL of the biomarker *crAssphage* were observed in WWTP A followed by WWTP B at 7943 (±7.07) and 8006 (±4.24) respectively. The highest SARS-CoV-2 concentration was also observed from WWTP A, with an average of 9.57 (±0.65) copies/µL.

SARS-CoV-2 concentrations in WWTP A remained stable from the 2nd week of sampling to the 5th week with a concentration ranging from approximately 1,18 (±0.10) copies/µL to 1,51 (±0.11) copies/µL, recording an increase in concentrations (2,48 ±0.28 copies/µL) during the 6th week. Interestingly, fluctuating concentrations in the biomarkers were observed during this same period, with an increase in both *crAssphage* and *PMMoV* concentrations during week 3, reducing to 5200,01 (±70.71) and 3,47 (±1.76) copies/µL respectively during week 5 and increasing together with the SARS-CoV-2 concentrations during week 6 (Figure 4.3). A significant increase in SARS-CoV-2 concentrations occurred from week 10 onwards, which also coincided with a rise in *crAssphage* and *Bacteroides* HF183 concentrations.

At the WWTP B, HF183 *Bacteroides* and *crAssphage* concentrations exhibited a similar trend. For instance, an increase in the concentration of these two biomarkers was observed in week 2 (see Figure 4.4). A drop in concentration of these biomarkers was recorded in week 3 and an increase in week 4. This fluctuation in concentrations was observed mainly for these biomarkers. However, it is worth noting that despite a relatively low and stable concentration of SARS-CoV-2 over the course of the study, a slight increase in concentration was observed during week 4 being 1,49 (±0.01) copies/µL, corresponding to the increase in *Bacteroides* HF183 and *crAssphage*. Furthermore, during the 11th week of sampling, an increase in SARS-CoV-2 was observed to be 2,95 (±0.20) copies/µL, which corresponded with an increase in *crAssphage* concentration to 6708 (±16.97) copies/µL. The highest concentration for *PMMoV* (84,1 (±5.48) copies/µL) was at week 1 during the trend analysis (Figure 4.4). Week 5 recorded the 2nd highest rise in *PMMoV* concentration 19,9 (±1.34) copies/µL, and week 8 *PMMoV* concentration 17,4 (±1.27) copies/µL from WWTP B.

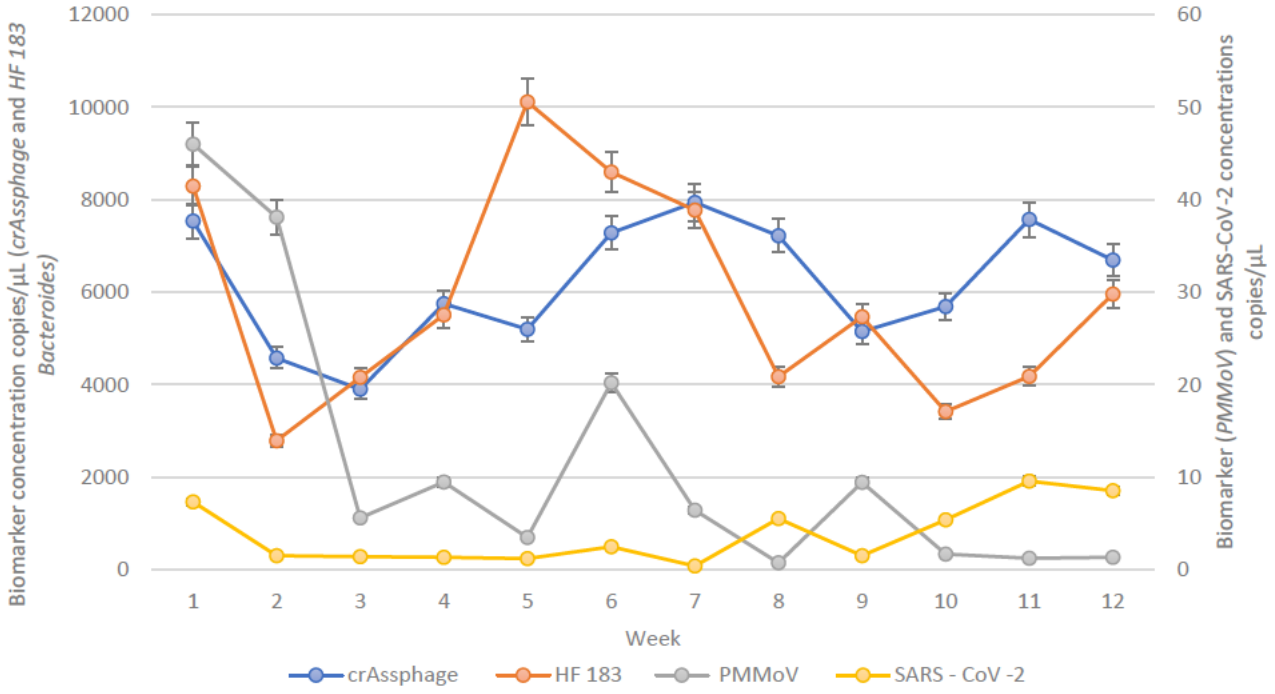


Figure 4.3: The trend of the biomarker and the SARS-CoV-2 concentration (copies/µl) for WWTP A over a 12-week period.

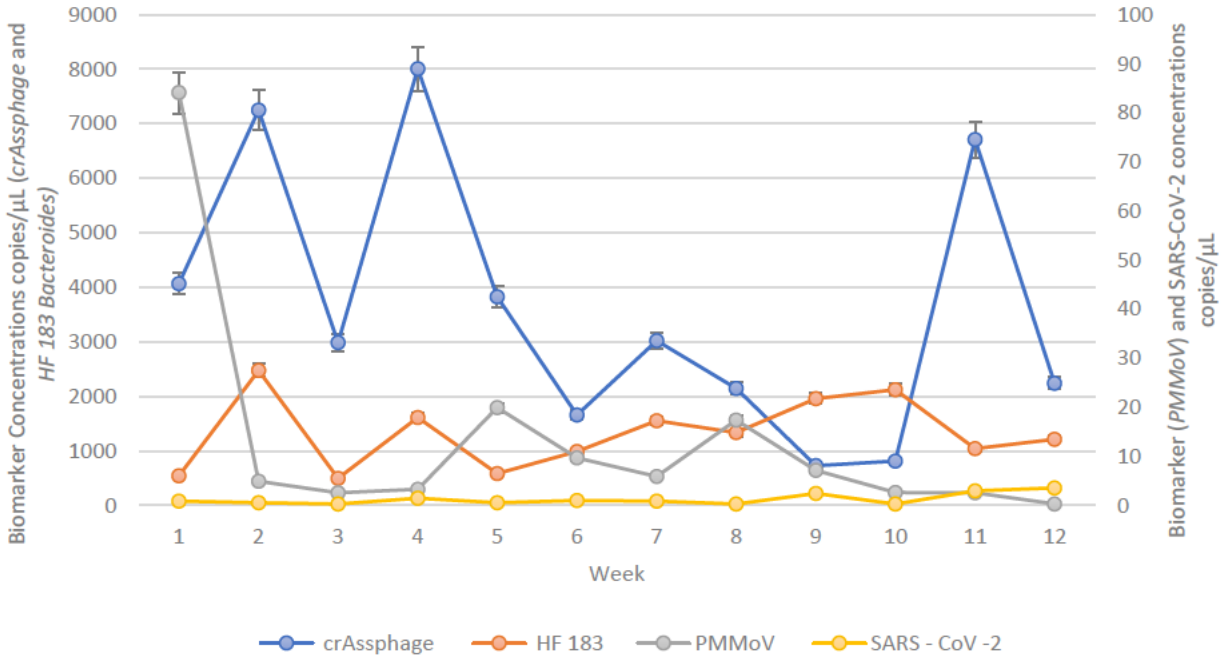


Figure 4.4: The trend analysis of the biomarkers and the SARS-CoV-2 copies/µl for WWTP B over a 12-week period.

4.4.2 Comparative analysis of the biomarker (*crAssphage, Bacteroides* HF183 and the *PMMoV*) concentration and the SARS-CoV-2 concentration varying associations

CrAssphage exhibited the highest positive correlation with SARS-CoV-2, with correlation co-efficient of 0.49 and 0.07 for WWTP A and B respectively, indicating a moderate association, as shown in Table 4.1. *PMMoV* concentrations exhibited a consistently negative correlation with SARS-CoV-2 concentrations. For instance, in WWTP A, a co-efficient of -0.56 was recorded and -0.21 in WWTP B (Table 4.1). Comparison of the various biomarkers among themselves also showed a variation in association. *crAssphage* and SARS-CoV-2 compared to the other two biomarkers (*Bacteroides* HF183 and *PMMoV*) exhibited a positive correlation for both WWTPs. *Bacteroides* HF183 and *PMMoV* showed a poor or negative correlation when compared to SARS-CoV-2 for both treatment plants.

Table 4.1: Correlation co-efficient of measured biomarker concentrations to the SARS-CoV-2 concentrations in untreated wastewater samples from two WWTPs.

Correlation co-efficient between the biomarkers and SARS-CoV-2	Correlation Coefficient WWTP A	Correlation Coefficient WWTP B
crAssphage and SARS-CoV-2	0.499	0.07
HF 183 and SARS-CoV-2	-0.191	0.01
PMMoV and SARS-CoV-2	-0.562	-0.215
crAssphage and *PMMoV*	0.013	0.005
crAssphage and HF 183	0.352	0.066
PMMoV and *crAssphage*	0.02	0.005
PMMoV and HF 183	0.135	-0.44

4.4.3 Physicochemical quality of wastewater samples

Physicochemical characteristics of the untreated wastewater at the different WWTPs varied considerably over the study period. The pH for WWTP A ranged from pH 6.9 – 7.5 and WWTP B from pH 6.5-7.5 (Supplementary Table S4.1 and S4.2). For both WWTP's the COD concentrations recorded were the highest at week 1 ranging from between 500mg/L -530mg/L. COD concentrations observed thereafter for WWTP A ranged from (120mg/L – 283mg/L) during the study period. For WWTP B, COD ranged from 175mg/L – 285mg/L (Supplementary Table S4.1 and S4.2). The electrical conductivity observed during the study period was higher for WWTP A. WWTP B, had relatively lower readings during the study period ranging between 620 - 740µS/cm. Only week 9 observed a lower electrical conductivity of 492µS/cm. For WWTP A, electrical conductivity ranged from 1000µS/cm – 2000µS/cm (Supplementary Table S4.1). Thus, WWTP A recorded the highest electrical conductivity values from between the two plants. This also indicates that the higher the electrical conductivity value the greater the presence of total dissolved solid (TDS) in the sample (Elsaidy *et al.*, 2022). This is further supported by WWTP A recording a higher TDS ranging from 0.5mg/L – 1.300mg/L (Supplementary Table S4.1), whereas WWTP B, TDS recording ranged from 0.3mg/L-0.480mg/L (Supplementary Table S4.2). A fluctuation in the DO concentrations were noted for both WWTPs. Weeks 1-3 recorded higher DO concentrations for both WWTPs.

4.4.4 Association of physicochemical quality of wastewater with biomarker and SARS-CoV-2 concentrations

A pH value above pH 7, were observed to be associated with high SARS-CoV-2 concentrations, for instance, a pH of 7.12 (±0.03), was associated with the highest SARS-CoV-2 concentration of 9.57 (±0.49) copies/µL at the WWTP A (Supplementary table S4.1). Similar results were obtained at the WWTP B, where a pH of 7.17 (±0.02), was associated with a high SARS-CoV-2 concentration of 3,57 (±0.65) copies/µL (Supplementary table S4.2). A high

COD value were also associated with a lower SARS-CoV-2 concentration at the WWTP B (Supplementary Table S4.2). However, in contrast, at the WWTP A, high COD of 507,14 (±0.70) mg/L was associated with a high SARS-CoV-2 concentration of 7,32 (±0.26) copies/µL (Supplementary Table S4.1). This shows site specific association of COD with SARS-CoV-2 concentrations, WWTP A with types of wastewaters being domestic and industrial as compared to WWTP B type of wastewater being solely domestic. WWTP A recorded the highest COD reading which may have resulted from a higher industrial composition. However, it is worth noting that a the WWTP A, the respective biomarkers and the SARS-CoV-2 concentrations showed a positive correlation to the COD content of the wastewater (Supplementary table: Table S4.3). Biomarker (*crAssphage*) also showed a positive correlation with pH (Supplementary table: Table S4.3). *CrAssphage* biomarker showed a correlation to majority of the physicochemical parameters analysed for both WWTPs, however a negative correlation was shown for *crAssphage* and DO% for WWTP A. *PMMoV* showed a correlation to only COD and DO% for WWTP A, and for WWTP B, a correlation for pH and COD. Thus, a positive correlation for *PMMoV* to COD was noted for both WWTPs (Supplementary table: Table 4.3). HF 183 *Bacteroides*, resulted in a positive correlation to pH and COD for WWTP A (Supplementary table: Table S4.3).

4.4.5 ANFIS Model prediction of the impact of the selected physicochemical parameters and on biomarkers and SARS-CoV-2 concentrations

The ANFIS model outputs shown the impact of physicochemical characteristics on the detection of the various biomarkers and SARS-CoV-2 between the WWTPs. For example, *crAssphage* exhibited a higher concentration for both WWTPs at a COD concentration, between 200-300 mg/L. As shown in Figure 4.5A and 4.5B, high *crAssphage* concentrations were also determined to be associated with high VS content (0.3mg/L -0.45mg/L). An increase in COD concentration does not coincide with their increase in their concentration. Similarly,

Bacteroides HF183 were observed to be high with COD values between 200-400mg/L, and VS values between 0.5-1 mg/L for WWTP A. Similar trend was observed at the WWTP B in respect to the COD associated with *Bacteroides* HF183. However, at the WWTP B an association with DO was also determined for HF 183 (Figure 4.6A and Figure 4.6B). A similar trend was also observed for *PMMoV* association with COD and DO, where high *PMMoV* concentrations were associated with high COD concentrations, such as >500 mg/L (Figure 4.7A and Figure 4.7B). However, DO concentrations varied between the WWTPs, but irrespective of the WWTP, high DO was predicted to result in high *PMMoV* concentrations (Figure 4.7A and 4.7B).

Figure 4.8A and Figure 4.8B, indicates the detection of SARS-CoV-2 concentrations for both WWTPs which were associated with electrical conductivity and temperature. A temperature of 19.8 °C at an electrical conductivity of 1154 (±1.41) µS/cm for central WWTP yielded a SARS-CoV-2 concentration (copies/µL) of 9.57 (±0.49) at a pH 7.12. For WWTP B, a concentration of SARS-CoV-2 3,57 (±0.65) copies/µL was detected at a temperature of 24.2 °C and an electrical conductivity of 646 (±2.12) µS/cm at a pH 7,17.

A B

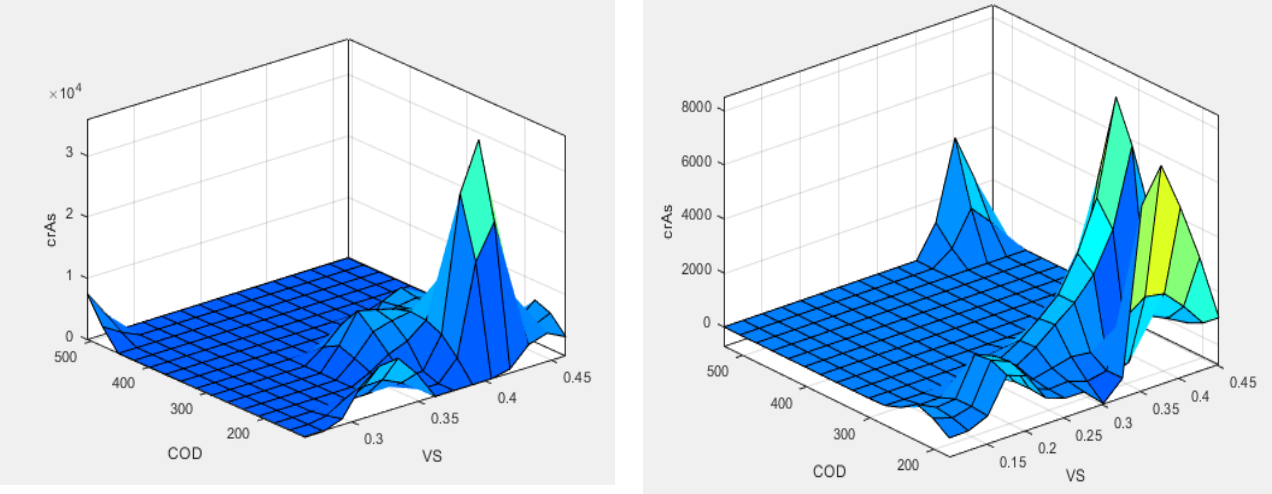


Figure 4.5: Modeled impact of COD and VS on the detection of the *crAssphage* in wastewater from WWTP A and WWTP B.

A B

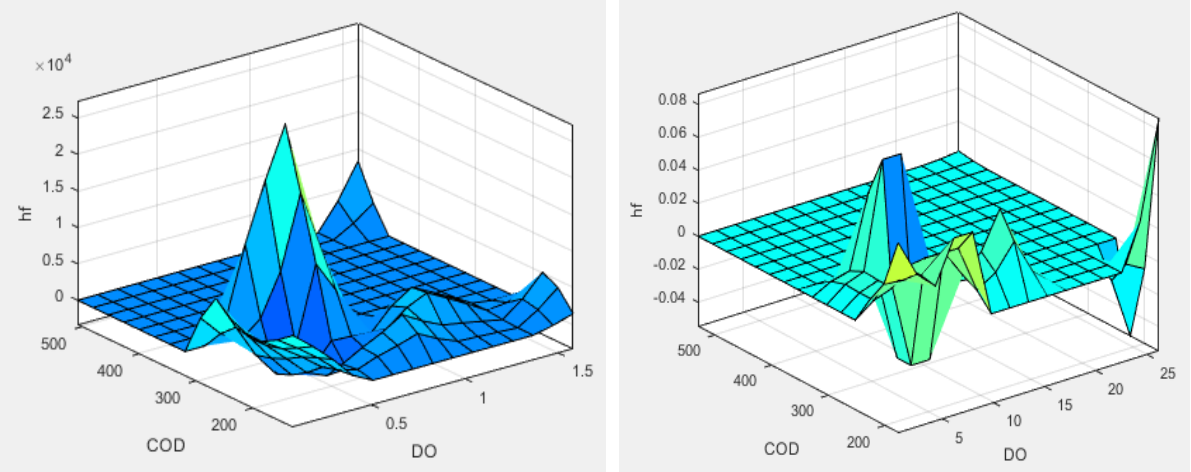


Figure 4.6: Modeled impact of DO rate and COD on the detection of the *Bacteroides* HF183 in wastewater from WWTP A and WWTP B.

A B

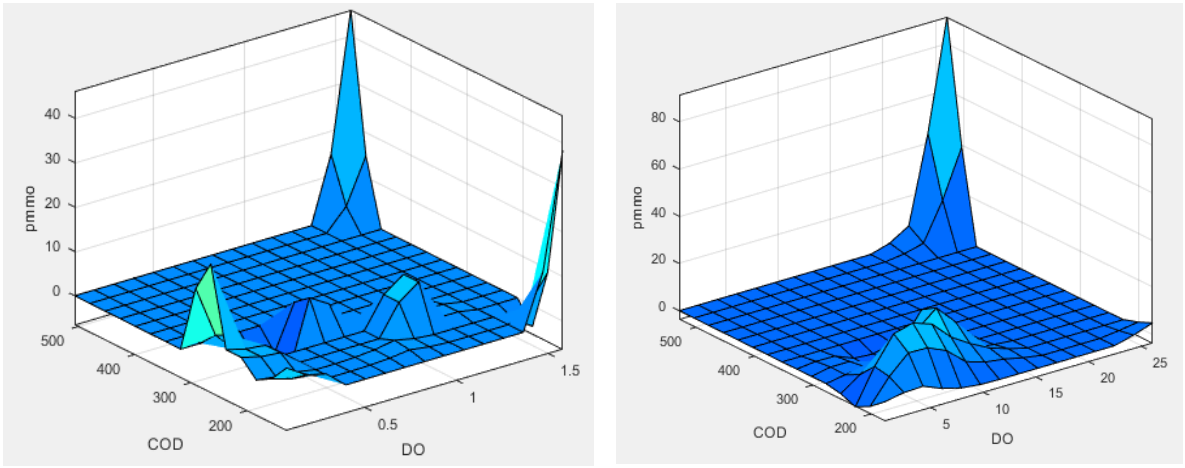


Figure 4.7: Modeled impact of DO rate and COD on the detection of the *PMMoV* in wastewater from WWTP A and WWTP B.

A B

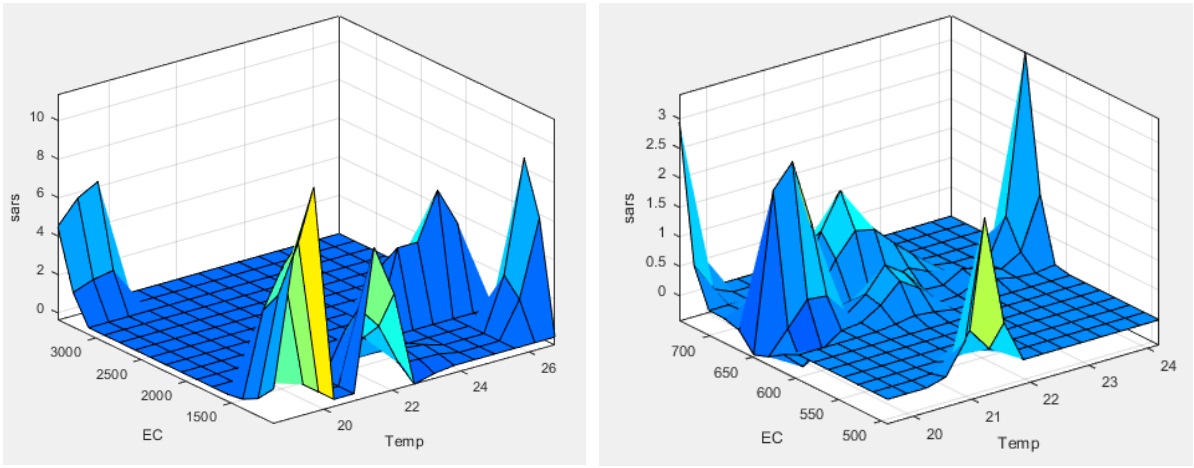


Figure 4.8. Modeled impact of electrical conductivity and Temperature on the detection of SARS-CoV-2 in wastewater from WWTP A and WWTP B.

4.5. Discussion

The concentration of SARS-CoV-2 RNA in wastewater can be affected by mixing household wastewater with water from other sources, such as stormwater incursion, and/or industrial wastewater (Langeveld *et al.*, 2023). Due to the diverse contributions of industrial effluent and rainfall, for example, dilution rates may vary over time and between catchment areas

(Langeveld *et al.*, 2023). To account for this unpredictable dilution, sewage surveillance and WBE, routinely normalize observed values, expanding on techniques and protocols created over the past decade primarily for drug use monitoring (Castiglioni *et al.*, 2014). In this study, three biomarkers were evaluated together with SARS-CoV-2 to find the suitable marker for wastewater surveillance in the selected WWTPs. *CrAssphage* had the highest abundance or detected copies/µL amongst the three selected biomarkers used in this study. *CrAssphage* is exclusively found in human feces, and is reported world-wide (Edwards *et al.*, 2019). Domestic wastewater is known to have high levels of *crAssphage*, and loads are said to not vary significantly with the seasons (Ballesté *et al.*, 2019), making them a potentially helpful indicator for the wastewater's human feces component. For WWTP A, the biomarker concentration of *crAssphage* was relatively stable during the study period, with a major decrease in its concentration at week 3. WWTP B, recorded fluctuations in *crAssphage* concentrations from weeks 1-5 and then again from week 10-12 for the study period. The difference in *crAssphage* between the two WWTPs during this study could be attributed from the capacity of WWTPs, the composition of the wastewater was different one purely domestic and other (WWTP A) was domestic and industrial, dilution factors and the difference in the population sizes. The influent composition, catchment size and consequent vulnerability to diurnal fluctuations in flow and/or virus detection rates need to be carefully taken into account (Cornman *et al.*, 2018, Dong, 2015). *PMMoV* was the least detected biomarker during the trend analysis. This however could result from the lower intake of pepper and pepper processed foods eg: spices and hot sauces. Healthy human populations excrete a substantial amount of this virus (Kitajima *et al.*, 2018). Findings from the literature show that *PMMoV* is present in a variety of water sources all over the world with minimum seasonal variation (Bivins *et al.*, 2020). However, during this study, there was an inconsistency in the biomarker *PMMoV* observed for

both WWTPs. A higher variation in concentrations were shown for WWTP A. A relatively lower concentration range (0.2 – 4.0 copies/µL) was observed for WWTP B.

The ANFIS model used in this study indicated a positive correlation between the biomarkers, SARS-CoV-2 and the measured physicochemical parameters of the wastewater. Electrical conductivity was more correlated with SARS-CoV-2 concentrations. Electrical conductivity is a recognized way to account for dilution in wastewater (Launay *et al.*, 2016). Electrical conductivity is easy to precisely measure and does not change as it travels through the wastewater system. Therefore, an increase in electrical conductivity is usually associated with increasing loads (de Sousa *et al.*, 2014). The association between electrical conductivity and the SARS-CoV-2 concentrations therefore shows the potential of using electrical conductivity to determine dilution. A study recently conducted by Langeveld *et al*., (2023), Normalization of SARS-CoV-2 concentrations in wastewater shows the potential use of electrical conductivity when considering dilutions (Langeveld *et al.*, 2023). The study concluded that an alternative for normalization in absence of flow data, *crAssphage* and electrical conductivity are suitable. The COD values showed relative stability during this study period except for week one, which peaked the highest COD recording values for both WWTPs. This could be due to higher human or industrial waste runoff from the increase in rainfall experienced over the study period. The COD in both WWTPs may have originated from different sources as the influent wastewater composition was different, being domestic and industrial. The difference in the physicochemical properties of the wastewater has shown to have an impact on the detection and quantification of the biomarkers and the SARS-CoV-2 RNA in different WWTPs. With the aid of the ANFIS model, weekly monitoring revealed significant relationships between the biomarker and SARS-CoV-2 concentrations, thus proving the importance of use of the physicochemical parameters for COVID-19 wastewater surveillance.

However, virus recovery and concentration methods that were previously developed for non-enveloped viruses, such as enteric viruses, can also affect the results of infectivity assays and overestimate the infectivity rates of these viruses present in wastewater (Corpuz *et al.*, 2020). Temperature, pH, matrix composition, or the presence of other microbes can all affect how long viruses can survive (Bosch *et al.*, 2006). Based on the results obtained from the two WWTPs, WWTP A yielded a higher biomarker and SARS-CoV-2 concentration as compared to WWTP B. This could imply that the population that the WWTP served have a potential influence on the concentrations of the biomarkers. The volume of a wastewater treatment plant can potentially influence biomarker concentrations. WWTP B is located near a rural suburb. Thus, making sampling in these areas can be more complex and challenging due to the lack of sewage collection systems. During the study period, an increase in rainfall was recorded which caused major damages to infrastructure and even resulted in malfunctioning of the WWTPs to date, thus leading to increase in dilutions (by incursions) being a contributor to detection and quantification of these target microbial communities within the WWTP. Additionally, the pandemic has increased water use due to greater hygiene concerns, which further dilutes virus concentrations in wastewater. Whereby it's worth noting that WWTP B, was greatly affected by heavy rainfall and infrastructure damages compared to WWTP A. The results for COD and EC for WWTP B, were recorded to be lower than that of WWTP A, thus, potentially resulting from the impact of heavy rainfall experienced causing dilution (due to stormwater incursion or other related issues). The dilution trend observed for the treatment plants showed a better correlation between the SARS-CoV-2 and *crAssphage* for both treatment plants, however the WWTP B correlation indicated a much poorer positive correlation as compared to the larger population served (WWTP A). *Bacteroides* HF183 correlation to the SARS-CoV-2 concentrations indicated a negative correlation for WWTP A and a poor positive correlation for WWTP B. *CrAssphage* and *Bacteroides* HF183 correlation between the biomarkers showed

a positive higher correlation as compared to the *PMMoV* biomarker. Thus, the results observed indicates that based on trend analysis the biomarker best suited with the highest positive correlation, *crAssphage* and SARS-CoV-2. It is noteworthy that with a pH value of ~7.0 – 7.3 (the highest) SARS-CoV-2 concentrations were observed from both WWTPs. The detected *crAssphage* concentration in wastewater was relatively comparable between the WWTPs and over time. This shows that, the population shedding is fairly stable in the population sizes. Given the wide variation in *crAssphage* concentration in the study's wastewater samples, it stands to reason that *crAssphage* would be less effective as a normalizer in smaller populations when in comparison to literature. Langeveld *et al.*, (2021), reported that *crAssphage* is better reported for a larger population as compared to a small population. The biomarker correlation co-efficient yields low or negative correlation in smaller populated areas thus making the biomarker less suited as a method of normalization for smaller population sizes. When *CrAssphage* is used in a larger population a more reliable correlation co-efficient is observed. It is worth noting that the correlation if *crAssphage* to electrical conductivity showed a positive correlation for both WWTPs. Based on the correlation coefficient data obtained *crAssphage* indicates to be a better biomarker when compared to SARS-CoV-2 concentration for use as a normalization biomarker for wastewater surveillance, However, more research to study the infection trend using *crAssphage* as a normalization biomarker is required. The physicochemical data in addition showed positive correlation of *crAssphage* to electrical conductivity for both WWTPs during the study period. A recent study concluded that an alternative for normalization in absence of flow data, (*crAssphage* and electrical conductivity) are suitable (Langeveld *et al.*, 2023), this further supports the current study highlighting the potential use of *crAssphage* as a normalization marker for SARS-CoV-2 concentrations. The physiochemical parameter, COD and *crAssphage* concentrations need to determine in the untreated wastewater as these have been shown to influence the SARS-CoV-2 concentrations.

4.6 Conclusion

For SARS-CoV-2 wastewater surveillance, *crAssphage* showed to be the most suitable biomarker. Additionally, the results point to the possibility of modifying WBE to normalize SARS-CoV-2 concentrations in larger populations. The ANFIS modeling technique, concluded that evaluation of wastewater characteristic is necessary for WBE COVID-19 surveillance purpose. The physicochemical properties, the biomarkers together with SARS-CoV-2 concentrations showed correlation for epidemiological data produced from wastewater, based on simple correlation analysis i.e., Spearman's Rank correlation co-efficient. During the trend analysis, *crAssphage* was shown to be the most abundant and stable biomarker in untreated wastewater. Furthermore, the weekly monitoring results showed a strong link between the *crAssphage* and SARS-CoV-2 suggesting the wastewater surveillance of COVID-19 is highly beneficial. Given that SARS-CoV-2 concentrations have been shown to be influenced by *crAssphage* and COD concentrations, this study was able to show the importance of determining these concentrations in untreated wastewater to have an impact on WBE for COVID-19 surveillance. These results further support the use of WBE as a new surveillance tool for tracking SARS-CoV-2 concentrations in communities.

CHAPTER FIVE

5. Conclusion, Limitations and Recommendations

5.1 General conclusion

This study has shown the potential of using both viral and bacterial assays for the normalization of SARS-CoV-2 WBE data from wastewater. The first objective was achieved via conventional PCR detection and sequencing analysis of the various biomarkers (*crAssphage*, *PMMoV*, and *Bacteroides* HF183) present in untreated wastewater. The detection of these biomarkers in the untreated wastewater from both WWTPs presents that these organisms are common in the catchment. The findings also show the potential for the adaptation of WBE for the normalization of SARS-CoV-2 concentrations in the served communities (larger populations). A highlight to the study was the potential application of the ANFIS modeling technique which determined the impact of the physicochemical parameters on the biomarker and the SARS-CoV-2 concentrations in WWTPs. The model indicated that since the physicochemical features show a correlation to the concentrations of the biomarker and the SARS-CoV-2 in wastewater, there is a need for assessing the wastewater characteristic for WBE COVID-19 surveillance purposes. Based on simple correlation analysis, Spearman's Rank correlation co-efficient, the findings provide additional evidence that *crAssphage* can be employed as a normalization marker for epidemiological data derived from wastewater. With its detection from WWTP being relatively consistent. Furthermore, the strong correlations observed between the *crAssphage* and SARS-CoV-2 from weekly monitoring indicates that the wastewater surveillance of COVID-19 is very valuable. Furthermore, these findings support the potential of WBE in providing an additional surveillance tool for monitoring SARS-CoV-2 concentrations in communities. Noting that SARS-CoV-2 concentrations have been shown to be influenced by *crAssphage* and COD concentrations, this study was able to show the

importance of determining these concentrations in untreated wastewater to have an impact on WBE for COVID-19 surveillance.

5.2 Study Limitations

- Limited Parameters: Environmental and operational parameters such as flow rates of the WWTP, population knowledge, and loading rates at the WWTP were inaccessible. It is known that these parameters could contribute to the respective SARS-CoV-2 and biomarker concentrations. Therefore, this limitation has been highlighted and should be addressed in future studies.
- Physicochemical Parameters: Physicochemical Parameters such as Nutrient Content (Phosphorous and Ammonia) should also be considered as these parameters contribute to the wastewater characteristics which could potentially have an association to the biomarker and SARS-CoV-2 concentrations. Therefore, it is important to draw attention to this constraint and take it into account for future research.
- Technical Limitation: Due to the lack of positive controls for the biomarkers, sequencing and analysis was conducted. (Unable to source positive controls).
- Virus Recovery Efficiency: The virus recovery efficiency during this study was overlooked for the selected biomarkers. During the study period we were unable to obtain positive controls for the biomarkers which the study co-currently ran during the COVID-pandemic. Thus, further research in this area will enhance the accuracy for WBE data during surveillance.

5.3 Recommendations

- During this study, *crAssphage* proved to be a promising candidate for normalization of SARS-CoV-2 concentrations when using wastewater samples. It is therefore recommended to quantify the biomarker *crAssphage* together with considering the physicochemical parameters such as COD, DO, pH, and VS. The detection and quantification of targets of concern, such as SARS-

CoV-2, can be improved by merging these metrics, resulting in more effective monitoring of COVID-19 infections by WBE.

- The advancement of WBE can benefit from the study's findings. Additionally, during the trend analysis, *crAssphage* was found to be the most frequent and stable biomarker in untreated wastewater. It is recommended that additional research using *crAssphage* as a biomarker for normalization should be conducted to supplement these findings.
- To strengthen and support the acceptability of using *crAssphage* as the best normalization biomarker for SARS-CoV-2 wastewater surveillance, there is a need to monitor more WWTPs for the chosen biomarkers that are part of this study from various sites in South Africa.
- The concentrations of the biomarker and SARS-CoV-2 were shown to be influenced by the physicochemical characteristics of the wastewater, proving that these characteristics can alter the usefulness of WBE during surveillance. For studies to enhance the accuracy for WBE data, a seasonal period study should be well captured.

6. References

Aarestrup, F. M. & Woolhouse, M. E. 2020. Using sewage for surveillance of antimicrobial resistance. *Science,* 367**,** 630-632.

Abunama, T., Ansari, M., Awolusi, O. O., Gani, K. M., Kumari, S. & Bux, F. 2021. Fuzzy inference optimization algorithms for enhancing the modelling accuracy of wastewater quality parameters. *Journal of environmental management,* 293**,** 112862.

Abunama, T., Othman, F. & Younes, M. K. 2018. Predicting sanitary landfill leachate generation in humid regions using ANFIS modeling. *Environmental monitoring and assessment,* 190**,** 1-15.

Aguiar-Oliveira, M. D. L., Campos, A., R. Matos, A., Rigotto, C., Sotero-Martins, A., Teixeira, P. F. & Siqueira, M. M. 2020. Wastewater-based epidemiology (WBE) and viral detection in polluted surface water: a valuable tool for COVID-19 surveillance—a brief review. *International journal of environmental research and public health,* 17**,** 9251.

Ahmed, F., Tscharke, B., O'brien, J., Thompson, J., Samanipour, S., Choi, P., Li, J., Mueller, J. F. & Thomas, K. 2020a. Wastewater-based estimation of the prevalence of gout in Australia. *Science of the Total Environment,* 715**,** 136925.

Ahmed, F., Tscharke, B., O'brien, J. W., Zheng, Q., Thompson, J., Mueller, J. F. & Thomas, K. V. 2021a. Wastewater-based prevalence trends of gout in an Australian community over a period of 8 years. *Science of the Total Environment,* 759**,** 143460.

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

Ahmed, W., Bertsch, P. M., Bibby, K., Haramoto, E., Hewitt, J., Huygens, F., Gyawali, P., Korajkic, A., Riddell, S. & Sherchan, S. P. 2020c. Decay of SARS-CoV-2 and surrogate

murine hepatitis virus RNA in untreated wastewater to inform application in wastewater-based epidemiology. *Environmental Research,* 191**,** 110092.

Ahmed, W., Bertsch, P. M., Bivins, A., Bibby, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., Mcminn, B. R. & Mueller, J. F. 2020d. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. *Science of The Total Environment,* 739**,** 139960.

Ahmed, W., Bivins, A., Bertsch, P. M., Bibby, K., Gyawali, P., Sherchan, S. P., Simpson, S. L., Thomas, K. V., Verhagen, R. & Kitajima, M. 2021b. Intraday variability of indicator and pathogenic viruses in 1-h and 24-h composite wastewater samples: implications for wastewater-based epidemiology. *Environmental Research,* 193**,** 110531.

Ahmed, W., Goonetilleke, A., Powell, D. & Gardner, T. 2009. Evaluation of multiple sewage-associated Bacteroides PCR markers for sewage pollution tracking. *water research,* 43**,** 4872-4877.

Ahmed, W., Masters, N. & Toze, S. 2012. Consistency in the host specificity and host sensitivity of the Bacteroides HF183 marker for sewage pollution tracking. *Letters in Applied Microbiology,* 55**,** 283-289.

Ahmed, W., Stewart, J., Powell, D. & Gardner, T. 2008. Evaluation of Bacteroides markers for the detection of human faecal pollution. *Letters in Applied Microbiology,* 46**,** 237-242.

Ai, Y., Davis, A., Jones, D., Lemeshow, S., Tu, H., He, F., Ru, P., Pan, X., Bohrerova, Z. & Lee, J. 2021. Wastewater-based epidemiology for tracking COVID-19 trend and variants of concern in Ohio, United States. *MedRxiv***,** 2021.06. 08.21258421.

Alhamlan, F. S., Bakheet, D. M., Bohol, M. F., Alsanea, M. S., Alahideb, B. M., Alhadeq, F. M., Alsuwairi, F. A., Al-Abdulkareem, M. A., Asiri, M. S. & Almaghrabi, R. S. 2023.

SARS-CoV-2 spike gene Sanger sequencing methodology to identify variants of concern. *Biotechniques,* 74**,** 69-75.

Amoah, I. D., Abunama, T., Awolusi, O. O., Pillay, L., Pillay, K., Kumari, S. & Bux, F. 2022. Effect of selected wastewater characteristics on estimation of SARS-CoV-2 viral load in wastewater. *Environmental Research,* 203**,** 111877.

Amoah, I. D., Mthethwa, N. P., Pillay, L., Deepnarain, N., Pillay, K., Awolusi, O. O., Kumari, S. & Bux, F. 2021. RT-LAMP: a cheaper, simpler and faster alternative for the detection of SARS-CoV-2 in wastewater. *Food and environmental virology,* 13**,** 447-456.

Apha, A. 2012. Wpcf.(2012) Standard methods for the examination of water and wastewater. *American Public Health Association, Washington*.

Apha Awwa, W. 1998. Standard methods for the examination of water and wastewater 20th edition. *American Public Health Association, American Water Work Association, Water Environment Federation, Washington, DC*.

Aranha-Creado, H. & Brandwein, H. 1999. Application of bacteriophages as surrogates for mammalian viruses: a case for use in filter validation based on precedents and current practices in medical and environmental virology. *PDA Journal of Pharmaceutical Science and Technology,* 53**,** 75-82.

Arnold, C. 2016. Pipe dreams: tapping into the health information in our sewers. National Institute of Environmental Health Sciences.

Auffret, M. D., Brassard, J., Jones, T. H., Gagnon, N., Gagné, M.-J., Muehlhauser, V., Masse, L., Topp, E. & Talbot, G. 2019. Impact of seasonal temperature transition, alkalinity and other abiotic factors on the persistence of viruses in swine and dairy manures. *Science of the total Environment,* 659**,** 640-648.

Augusto, M. R., Claro, I. C. M., Siqueira, A. K., Sousa, G. S., Caldereiro, C. R., Duran, A. F. A., De Miranda, T. B., Camillo, L. D. M. B., Cabral, A. D. & De Freitas Bueno, R.

2022. Sampling strategies for wastewater surveillance: Evaluating the variability of SARS-COV-2 RNA concentration in composite and grab samples. *Journal of Environmental Chemical Engineering,* 10**,** 107478.

Ballesté, E., Pascual-Benito, M., Martín-Díaz, J., Blanch, A., Lucena, F., Muniesa, M., Jofre, J. & García-Aljaro, C. 2019. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. *Water research,* 155**,** 233-244.

Barra, G. B., Santa Rita, T. H., Mesquita, P. G., Jácomo, R. H. & Nery, L. F. A. 2020. Analytical sensitivity and specificity of two RT-qPCR protocols for SARS-CoV-2 detection performed in an automated workflow. *Genes,* 11**,** 1183.

Baz-Lomba, J. A., Salvatore, S., Gracia-Lor, E., Bade, R., Castiglioni, S., Castrignanò, E., Causanilles, A., Hernandez, F., Kasprzyk-Hordern, B. & Kinyua, J. 2016. Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities. *BMC public health,* 16**,** 1-11.

Been, F., Rossi, L., Ort, C., Rudaz, S., Delémont, O. & Esseiva, P. 2014. Population normalization with ammonium in wastewater-based epidemiology: application to illicit drug monitoring. *Environmental science & technology,* 48**,** 8162-8169.

Benschop, K. S., Van Der Avoort, H. G., Jusic, E., Vennema, H., Van Binnendijk, R. & Duizer, E. 2017. Polio and measles down the drain: environmental enterovirus surveillance in the Netherlands, 2005 to 2015. *Applied and environmental microbiology,* 83**,** e00558-17.

Berchenko, Y., Manor, Y., Freedman, L. S., Kaliner, E., Grotto, I., Mendelson, E. & Huppert, A. 2017. Estimation of polio infection prevalence from environmental surveillance data. *Science translational medicine,* 9**,** eaaf6786.

Bernhard, A. E. & Field, K. G. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Applied and environmental microbiology,* 66**,** 4571-4574.

Betancourt, W. Q., Schmitz, B. W., Innes, G. K., Prasek, S. M., Brown, K. M. P., Stark, E. R., Foster, A. R., Sprissler, R. S., Harris, D. T. & Sherchan, S. P. 2021. COVID-19 containment on a college campus via wastewater-based epidemiology, targeted clinical testing and an intervention. *Science of The Total Environment,* 779**,** 146408.

Bibby, K., Bivins, A., Wu, Z. & North, D. 2021. Making waves: Plausible lead time for wastewater based epidemiology as an early warning system for COVID-19. *Water Research,* 202**,** 117438.

Bibby, K., Crank, K., Greaves, J., Li, X., Wu, Z., Hamza, I. A. & Stachler, E. 2019. Metagenomics and the development of viral water quality tools. *NPJ Clean Water,* 2**,** 9.

Bisceglia, K. J. & Lippa, K. A. 2014. Stability of cocaine and its metabolites in municipal wastewater–the case for using metabolite consolidation to monitor cocaine utilization. *Environmental Science and Pollution Research,* 21**,** 4453-4460.

Bivins, A., Crank, K., Greaves, J., North, D., Wu, Z. & Bibby, K. 2020. Cross-assembly phage and pepper mild mottle virus as viral water quality monitoring tools—potential, research gaps, and way forward. *Current Opinion in Environmental Science & Health,* 16**,** 54-61.

Bonanno Ferraro, G., Suffredini, E., Mancini, P., Veneri, C., Iaconelli, M., Bonadonna, L., Montagna, M., De Giglio, O. & La Rosa, G. 2021. Pepper mild mottle virus as indicator of pollution: Assessment of prevalence and concentration in different water environments in Italy. *Food and Environmental Virology,* 13**,** 117-125.

Boogaerts, T., Ahmed, F., Choi, P. M., Tscharke, B., O'brien, J., De Loof, H., Gao, J., Thai, P., Thomas, K. & Mueller, J. F. 2021a. Current and future perspectives for wastewater-based epidemiology as a monitoring tool for pharmaceutical use. *Science of the Total Environment,* 789**,** 148047.

Boogaerts, T., Jurgelaitiene, L., Dumitrascu, C., Kasprzyk-Hordern, B., Kannan, A., Been, F., Emke, E., De Voogt, P., Covaci, A. & Van Nuijs, A. L. 2021b. Application of wastewater-based epidemiology to investigate stimulant drug, alcohol and tobacco use in Lithuanian communities. *Science of the Total Environment,* 777**,** 145914.

Borecka, M., Białk-Bielińska, A., Siedlewicz, G., Stepnowski, P. & Pazdro, K. 2014. The influence of matrix effects on trace analysis of pharmaceutical residues in aqueous environmental samples. *Insights on Environmental Changes: Where the World is Heading***,** 1-16.

Bosch, A., Pintó, R. M. & Abad, F. X. 2006. Survival and transport of enteric viruses in the environment. *Viruses in foods***,** 151-187.

Brandeburová, P., Bodík, I., Horáková, I., Žabka, D., Castiglioni, S., Salgueiro-González, N., Zuccato, E., Špalková, V. & Mackuľak, T. 2020. Wastewater-based epidemiology to assess the occurrence of new psychoactive substances and alcohol consumption in Slovakia. *Ecotoxicology and Environmental Safety,* 200**,** 110762.

Brinkman, N. E., Fout, G. S. & Keely, S. P. 2017. Retrospective surveillance of wastewater to examine seasonal dynamics of enterovirus infections. *MSphere,* 2**,** e00099-17.

Brouwer, A. F., Eisenberg, J. N., Pomeroy, C. D., Shulman, L. M., Hindiyeh, M., Manor, Y., Grotto, I., Koopman, J. S. & Eisenberg, M. C. 2018. Epidemiology of the silent polio outbreak in Rahat, Israel, based on modeling of environmental surveillance data. *Proceedings of the National Academy of Sciences,* 115**,** E10625-E10633.

Burgard, D. A., Williams, J., Westerman, D., Rushing, R., Carpenter, R., Larock, A., Sadetsky, J., Clarke, J., Fryhle, H. & Pellman, M. 2019. Using wastewater-based analysis to monitor the effects of legalized retail sales on cannabis consumption in Washington State, USA. *Addiction,* 114**,** 1582-1590.

Burnet, J.-B., Cauchie, H.-M., Walczak, C., Goeders, N. & Ogorzaly, L. 2023. Persistence of endogenous RNA biomarkers of SARS-CoV-2 and PMMoV in raw wastewater: Impact of temperature and implications for wastewater-based epidemiology. *Science of the Total Environment,* 857**,** 159401.

Carcereny, A., Martínez-Velázquez, A., Bosch, A., Allende, A., Truchado, P., Cascales, J., Romalde, J. L., Lois, M., Polo, D. & Sánchez, G. 2021. Monitoring emergence of the SARS-CoV-2 B. 1.1. 7 variant through the Spanish national SARS-CoV-2 wastewater surveillance System (VATar COVID-19). *Environmental Science & Technology,* 55**,** 11756-11766.

Carter, L. J., Garner, L. V., Smoot, J. W., Li, Y., Zhou, Q., Saveson, C. J., Sasso, J. M., Gregg, A. C., Soares, D. J. & Beskid, T. R. 2020. Assay techniques and test development for COVID-19 diagnosis. ACS Publications.

Casanova, L., Rutala, W. A., Weber, D. J. & Sobsey, M. D. 2009. Survival of surrogate coronaviruses in water. *Water research,* 43**,** 1893-1898.

Casas, M. E., Schröter, N., Zammit, I., Castano-Trias, M., Rodriguez-Mozaz, S., Gago-Ferrero, P. & Corominas, L. 2021a. Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution. *Environment International,* 150**,** 106404.

Castiglioni, S., Bijlsma, L., Covaci, A., Emke, E., Hernández, F., Reid, M., Ort, C., Thomas, K. V., Van Nuijs, A. L. & De Voogt, P. 2013. Evaluation of uncertainties associated

with the determination of community drug use through the measurement of sewage drug biomarkers. *Environmental science & technology,* 47**,** 1452-1460.

Castiglioni, S., Senta, I., Borsotti, A., Davoli, E. & Zuccato, E. 2015. A novel approach for monitoring tobacco use in local communities by wastewater analysis. *Tobacco Control,* 24**,** 38-42.

Castiglioni, S., Thomas, K. V., Kasprzyk-Hordern, B., Vandam, L. & Griffiths, P. 2014. Testing wastewater to detect illicit drugs: state of the art, potential and research needs. *Science of the Total Environment,* 487**,** 613-620.

Causanilles, A., Cantillano, D. R., Emke, E., Bade, R., Baz-Lomba, J. A., Castiglioni, S., Castrignanò, E., Gracia-Lor, E., Hernández, F. & Kasprzyk-Hordern, B. 2018. Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater. *Environment international,* 115**,** 279-284.

Cevik, M., Tate, M., Lloyd, O., Maraolo, A. E., Schafers, J. & Ho, A. 2021. SARS-CoV-2, SARS-CoV, and MERS-CoV viral load dynamics, duration of viral shedding, and infectiousness: a systematic review and meta-analysis. *The lancet microbe,* 2**,** e13-e22.

Chan, J. F.-W., Yuan, S., Kok, K.-H., To, K. K.-W., Chu, H., Yang, J., Xing, F., Liu, J., Yip, C. C.-Y. & Poon, R. W.-S. 2020. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *The lancet,* 395**,** 514-523.

Chase, E., Hunting, J., Staley, C. & Harwood, V. 2012. Microbial source tracking to identify human and ruminant sources of faecal pollution in an ephemeral Florida river. *Journal of Applied Microbiology,* 113**,** 1396-1406.

Chaudhary, N., Weissman, D. & Whitehead, K. A. 2021. mRNA vaccines for infectious diseases: principles, delivery and clinical translation. *Nature reviews Drug discovery,* 20**,** 817-838.

Chen, C., Kostakis, C., Gerber, J. P., Tscharke, B. J., Irvine, R. J. & White, J. M. 2014. Towards finding a population biomarker for wastewater epidemiology studies. *Science of the Total Environment,* 487**,** 621-628.

Chen, Q., Li, J., Deng, Z., Xiong, W., Wang, Q. & Hu, Y.-Q. 2010. Comprehensive detection and identification of seven animal coronaviruses and human respiratory coronavirus 229E with a microarray hybridization assay. *Intervirology,* 53**,** 95-104.

Chen, W., Xiao, Q., Fang, Z., Lv, X., Yao, M. & Deng, M. 2021. Correlation analysis between the viral load and the progression of COVID-19. *Computational and mathematical methods in medicine,* 2021.

Chen, Y. & Li, L. 2020. SARS-CoV-2: virus dynamics and host response. *The Lancet Infectious Diseases,* 20**,** 515-516.

Chen, Y., Zhao, X., Zhou, H., Zhu, H., Jiang, S. & Wang, P. 2023. Broadly neutralizing antibodies to SARS-CoV-2 and other human coronaviruses. *Nature reviews Immunology,* 23**,** 189-199.

Chin, A. W., Chu, J. T., Perera, M. R., Hui, K. P., Yen, H.-L., Chan, M. C., Peiris, M. & Poon, L. L. 2020. Stability of SARS-CoV-2 in different environmental conditions. *The Lancet Microbe,* 1**,** e10.

Choi, J. Y. & Smith, D. M. 2021. SARS-CoV-2 variants of concern. *Yonsei medical journal,* 62**,** 961.

Choi, P. M., Tscharke, B. J., Donner, E., O'brien, J. W., Grant, S. C., Kaserzon, S. L., Mackie, R., O'malley, E., Crosbie, N. D. & Thomas, K. V. 2018. Wastewater-based epidemiology biomarkers: past, present and future. *TrAC Trends in Analytical Chemistry,* 105**,** 453-469.

Chu, D. K., Pan, Y., Cheng, S. M., Hui, K. P., Krishnan, P., Liu, Y., Ng, D. Y., Wan, C. K., Yang, P. & Wang, Q. 2020. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clinical chemistry,* 66**,** 549-555.

Ciesielski, M., Blackwood, D., Clerkin, T., Gonzalez, R., Thompson, H., Larson, A. & Noble, R. 2021. Assessing sensitivity and reproducibility of RT-ddPCR and RT-qPCR for the quantification of SARS-CoV-2 in wastewater. *Journal of Virological Methods,* 297**,** 114230.

Colosi, L. M., Barry, K. E., Kotay, S. M., Porter, M. D., Poulter, M. D., Ratliff, C., Simmons, W., Steinberg, L. I., Wilson, D. D. & Morse, R. 2021. Development of wastewater pooled surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from congregate living settings. *Applied and Environmental Microbiology,* 87**,** e00433-21.

Corchis-Scott, R., Geng, Q., Seth, R., Ray, R., Beg, M., Biswas, N., Charron, L., Drouillard, K. D., D'souza, R. & Heath, D. D. 2021. Averting an outbreak of SARS-CoV-2 in a university residence hall through wastewater surveillance. *Microbiology spectrum,* 9**,** e00792-21.

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., Bleicker, T., Brünink, S., Schneider, J. & Schmidt, M. L. 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance,* 25**,** 2000045.

Cornman, R. S., Mckenna Jr, J. E., Fike, J., Oyler-Mccance, S. J. & Johnson, R. 2018. An experimental comparison of composite and grab sampling of stream water for metagenetic analysis of environmental DNA. *PeerJ,* 6**,** e5871.

Coronavirus, N. 2019. Real-time rRT-PCR panel primers and probes. US Centers for Disease Control and Prevention, https://www. cdc. gov ….

Corpuz, M. V. A., Buonerba, A., Vigliotta, G., Zarra, T., Ballesteros Jr, F., Campiglia, P., Belgiorno, V., Korshin, G. & Naddeo, V. 2020. Viruses in wastewater: occurrence, abundance and detection methods. *Science of the Total Environment,* 745**,** 140910.

Costa, A. P., Manis, D. R., Jones, A., Stall, N. M., Brown, K. A., Boscart, V., Castellino, A., Heckman, G. A., Hillmer, M. P. & Ma, C. 2021. Risk factors for outbreaks of SARS-CoV-2 infection at retirement homes in Ontario, Canada: a population-level cohort study. *Cmaj,* 193**,** E672-E680.

Covacic, I. G.-M., Hapeshie, E., Kasprzyk-Hordernd, B., Kinyuac, J., Laic, F. Y., Letzelf, T., Lopardod, L., Meyerg, M. R., O'brienh, J. & Ramini, P. 2016. Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives 2. *perspectives,* 99**,** 131-150.

Covid, C. & Team, R. 2021. Sars-cov-2 b. 1.1. 529 (omicron) variant—united states, december 1–8, 2021. *Morbidity and Mortality Weekly Report,* 70**,** 1731.

D'aoust, P. M., Graber, T. E., Mercier, E., Montpetit, D., Alexandrov, I., Neault, N., Baig, A. T., Mayne, J., Zhang, X. & Alain, T. 2021a. Catching a resurgence: Increase in SARS-CoV-2 viral RNA identified in wastewater 48 h before COVID-19 clinical tests and 96 h before hospitalizations. *Science of The Total Environment,* 770**,** 145319.

D'aoust, P. M., Mercier, E., Montpetit, D., Jia, J.-J., Alexandrov, I., Neault, N., Baig, A. T., Mayne, J., Zhang, X. & Alain, T. 2021b. Quantitative analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19 incidence and prevalence. *Water research,* 188**,** 116560.

Darnell, M. E., Subbarao, K., Feinstone, S. M. & Taylor, D. R. 2004. Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV. *Journal of virological methods,* 121**,** 85-91.

Daughton, C. G. 2001. Illicit drugs in municipal sewage. *Pharmaceuticals and care products in the environment,* 791**,** 348-364.

Daughton, C. G. 2012. Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype. *Science of the total environment,* 424**,** 16-38.

Daughton, C. G. 2020a. Wastewater surveillance for population-wide Covid-19: The present and future. *Sci Total Environ,* 736**,** 139631.

De Sousa, D. N. R., Mozeto, A. A., Carneiro, R. L. & Fadini, P. S. 2014. Electrical conductivity and emerging contaminant as markers of surface freshwater contamination by wastewater. *Science of the total environment,* 484**,** 19-26.

Dhakar, V. & Geetanjali, A. S. 2022. Role of pepper mild mottle virus as a tracking tool for fecal pollution in aquatic environments. *Archives of Microbiology,* 204**,** 513.

Diemert, S. & Yan, T. 2019. Clinically unreported salmonellosis outbreak detected via comparative genomic analysis of municipal wastewater Salmonella isolates. *Applied and Environmental Microbiology,* 85**,** e00139-19.

Dong, G. Wastewater sampling and characterization–Raw sewage monitor-ing and results analysis. Proceedings of the 9th Annual WIOA NSW Water Industry Operations Conference. Orange, PCYC. Water Industry Operator's Association of Australia, 2015. 40-46.

Dorai-Raj, S., O'grady, J. & Colleran, E. 2009. Specificity and sensitivity evaluation of novel and existing Bacteroidales and Bifidobacteria-specific PCR assays on feces and sewage samples and their application for microbial source tracking in Ireland. *Water Research,* 43**,** 4980-4988.

Driver, E. M., Gushgari, A., Chen, J. & Halden, R. U. 2020. Alcohol, nicotine, and caffeine consumption on a public US university campus determined by wastewater-based epidemiology. *Science of the Total Environment,* 727**,** 138492.

Dutilh, B. E., Cassman, N., Mcnair, K., Sanchez, S. E., Silva, G. G., Boling, L., Barr, J. J., Speth, D. R., Seguritan, V. & Aziz, R. K. 2014a. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. *Nature communications,* 5**,** 1-11.

Duvallet, C., Wu, F., Mcelroy, K. A., Imakaev, M., Endo, N., Xiao, A., Zhang, J., Floyd-O'sullivan, R., Powell, M. M. & Mendola, S. 2022. Nationwide trends in COVID-19 cases and SARS-CoV-2 RNA wastewater concentrations in the United States. *ACS Es&t Water,* 2**,** 1899-1909.

Edwards, R. A., Vega, A. A., Norman, H. M., Ohaeri, M., Levi, K., Dinsdale, E. A., Cinek, O., Aziz, R. K., Mcnair, K. & Barr, J. J. 2019. Global phylogeography and ancient evolution of the widespread human gut virus crAssphage. *Nature microbiology,* 4**,** 1727-1736.

Eftekhari, A., Alipour, M., Chodari, L., Maleki Dizaj, S., Ardalan, M., Samiei, M., Sharifi, S., Zununi Vahed, S., Huseynova, I. & Khalilov, R. 2021. A comprehensive review of detection methods for SARS-CoV-2. *Microorganisms,* 9**,** 232.

Elbadawi, A., Elgendy, I. Y., Joseph, D., Eze-Nliam, C., Rampersad, P., Ouma, G., Bhandari, R., Kirksey, L., Chaudhury, P. & Chung, M. K. 2021. Racial Differences and In-Hospital Outcomes Among Hospitalized Patients with COVID-19. *Journal of Racial and Ethnic Health Disparities***,** 1-8.

Elsaidy, N. R., Elleboudy, N. S., Alkhedaide, A., Abouelenien, F. A., Abdelrahman, M. H., Soliman, M. M. & Shukry, M. 2022. Enhancement Effects of Water Magnetization and/or Disinfection by Sodium Hypochlorite on Secondary Slaughterhouse Wastewater Effluent Quality and Disinfection By-Products. *Processes,* 10**,** 1589.

Elyse, S. & Kyle, B. 2014. Metagenomic Evaluation of the Highly Abundant Human Gut Bacteriophage CrAssphage for Source Tracking of Human Fecal Pollution.

Etievant, S., Bal, A., Escuret, V., Brengel-Pesce, K., Bouscambert, M., Cheynet, V., Generenaz, L., Oriol, G., Destras, G. & Billaud, G. 2020. Performance assessment of SARS-CoV-2 PCR assays developed by WHO referral laboratories. *Journal of clinical medicine,* 9**,** 1871.

Farkas, K., Adriaenssens, E. M., Walker, D. I., Mcdonald, J. E., Malham, S. K. & Jones, D. L. 2019. Critical evaluation of CrAssphage as a molecular marker for human-derived wastewater contamination in the aquatic environment. *Food and Environmental Virology,* 11**,** 113-119.

Farkas, K., Hillary, L. S., Thorpe, J., Walker, D. I., Lowther, J. A., Mcdonald, J. E., Malham, S. K. & Jones, D. L. 2021. Concentration and quantification of SARS-CoV-2 RNA in wastewater using polyethylene glycol-based concentration and qRT-PCR. *Methods and protocols,* 4**,** 17.

Feng, S., Roguet, A., Mcclary-Gutierrez, J. S., Newton, R. J., Kloczko, N., Meiman, J. G. & Mclellan, S. L. 2021. Evaluation of sampling frequency and normalization of SARS-CoV-2 wastewater concentrations for capturing COVID-19 burdens in the community. *medrxiv*.

Filipić, A., Gutierrez-Aguirre, I., Primc, G., Mozetič, M. & Dobnik, D. 2020. Cold plasma, a new hope in the field of virus inactivation. *Trends in Biotechnology,* 38**,** 1278-1291.

Foladori, P., Cutrupi, F., Segata, N., Manara, S., Pinto, F., Malpei, F., Bruni, L. & La Rosa, G. 2020. SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review. *Science of the Total Environment,* 743**,** 140444.

Galani, A., Aalizadeh, R., Kostakis, M., Markou, A., Alygizakis, N., Lytras, T., Adamopoulos, P. G., Peccia, J., Thompson, D. C. & Kontou, A. 2022. SARS-CoV-2 wastewater surveillance data can predict hospitalizations and ICU admissions. *Science of The Total Environment,* 804**,** 150151.

Gallo, G., La Torre, M., Pietroletti, R., Bianco, F., Altomare, D., Pucciarelli, S., Gagliardi, G. & Perinotti, R. 2020. Italian society of colorectal surgery recommendations for good clinical practice in colorectal surgery during the novel coronavirus pandemic. Springer.

García-Ávila, F., Valdiviezo-Gonzales, L., Cadme-Galabay, M., Gutiérrez-Ortega, H., Altamirano-Cárdenas, L., Zhindón-Arévalo, C. & Del Pino, L. F. 2020. Considerations on water quality and the use of chlorine in times of SARS-CoV-2 (COVID-19) pandemic in the community. *Case Studies in Chemical and Environmental Engineering*, 2**,** 100049.

García-Aljaro, C., Ballesté, E., Muniesa, M. & Jofre, J. 2017. Determination of crAssphage in water samples and applicability for tracking human faecal pollution. *Microbial biotechnology,* 10**,** 1775-1780.

Gauthier, T. D. 2001. Detecting trends using Spearman's rank correlation coefficient. *Environmental forensics,* 2**,** 359-362.

Gettings, J. R., Gold, J. A., Kimball, A., Forsberg, K., Scott, C., Uehara, A., Tong, S., Hast, M., Swanson, M. R. & Morris, E. 2022. Severe Acute Respiratory Syndrome Coronavirus 2 Transmission in a Georgia School District—United States, December 2020–January 2021. *Clinical Infectious Diseases,* 74**,** 319-326.

Giri, B., Pandey, S., Shrestha, R., Pokharel, K., Ligler, F. S. & Neupane, B. B. 2021. Review of analytical performance of COVID-19 detection methods. *Analytical and bioanalytical chemistry,* 413**,** 35-48.

Gonzalez, R., Curtis, K., Bivins, A., Bibby, K., Weir, M. H., Yetka, K., Thompson, H., Keeling, D., Mitchell, J. & Gonzalez, D. 2020. COVID-19 surveillance in Southeastern Virginia using wastewater-based epidemiology. *Water research,* 186**,** 116296.

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., Verdine, V., Donghia, N., Daringer, N. M. & Freije, C. A. 2017. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science,* 356**,** 438-442.

Gracia-Lor, E., Castiglioni, S., Bade, R., Been, F., Castrignanò, E., Covaci, A., González-Mariño, I., Hapeshi, E., Kasprzyk-Hordern, B. & Kinyua, J. 2017. Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives. *Environment international,* 99**,** 131-150.

Graham, K. E., Loeb, S. K., Wolfe, M. K., Catoe, D., Sinnott-Armstrong, N., Kim, S., Yamahara, K. M., Sassoubre, L. M., Mendoza Grijalva, L. M. & Roldan-Hernandez, L. 2020. SARS-CoV-2 RNA in wastewater settled solids is associated with COVID-19 cases in a large urban sewershed. *Environmental science & technology,* 55**,** 488-498.

Green, H., Wilder, M., Collins, M., Fenty, A., Gentile, K., Kmush, B. L., Zeng, T., Middleton, F. A. & Larsen, D. A. 2020. Quantification of SARS-CoV-2 and cross-assembly phage (crAssphage) from wastewater to monitor coronavirus transmission within communities. *MedRxiv***,** 2020.05. 21.20109181.

Gundy, P. M., Gerba, C. P. & Pepper, I. L. 2009. Survival of coronaviruses in water and wastewater. *Food and Environmental Virology,* 1**,** 10-14.

Gushgari, A. J., Venkatesan, A. K., Chen, J., Steele, J. C. & Halden, R. U. 2019. Long-term tracking of opioid consumption in two United States cities using wastewater-based epidemiology approach. *Water research,* 161**,** 171-180.

Gyawali, P., Croucher, D., Ahmed, W., Devane, M. & Hewitt, J. 2019. Evaluation of pepper mild mottle virus as an indicator of human faecal pollution in shellfish and growing waters. *Water research,* 154**,** 370-376.

Hamouda, M., Mustafa, F., Maraqa, M., Rizvi, T. & Hassan, A. A. 2021. Wastewater surveillance for SARS-CoV-2: Lessons learnt from recent studies to define future applications. *Science of the Total Environment,* 759**,** 143493.

Hamza, I. A., Jurzik, L., Überla, K. & Wilhelm, M. 2011. Evaluation of pepper mild mottle virus, human picobirnavirus and Torque teno virus as indicators of fecal contamination in river water. *Water research,* 45**,** 1358-1368.

Haramoto, E., Kitajima, M., Hata, A., Torrey, J. R., Masago, Y., Sano, D. & Katayama, H. 2018. A review on recent progress in the detection methods and prevalence of human enteric viruses in water. *Water research,* 135**,** 168-186.

Haramoto, E., Malla, B., Thakali, O. & Kitajima, M. 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. *Science of the Total Environment,* 737**,** 140405.

Hart, O. E. & Halden, R. U. 2020. Modeling wastewater temperature and attenuation of sewage-borne biomarkers globally. *Water research,* 172**,** 115473.

Hayes, E., Sweeney, C., Anderson, L., Li, B., Erjavec, G., Gouthro, M., Krkosek, W., Stoddart, A. & Gagnon, G. 2021. A novel passive sampling approach for SARS-CoV-2 in wastewater in a Canadian province with low prevalence of COVID-19. *Environmental Science: Water Research & Technology,* 7**,** 1576-1586.

Heffron, J., Mcdermid, B., Maher, E., Mcnamara, P. J. & Mayer, B. K. 2019. Mechanisms of virus mitigation and suitability of bacteriophages as surrogates in drinking water treatment by iron electrocoagulation. *Water research,* 163**,** 114877.

Heijnen, L. & Medema, G. 2011. Surveillance of influenza A and the pandemic influenza A (H1N1) 2009 in sewage and surface water in the Netherlands. *Journal of water and health,* 9**,** 434-442.

Hellmér, M., Paxéus, N., Magnius, L., Enache, L., Arnholm, B., Johansson, A., Bergström, T. & Norder, H. 2014. Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks. *Applied and environmental microbiology,* 80**,** 6771-6781.

Hillary, L. S., Malham, S. K., Mcdonald, J. E. & Jones, D. L. 2020. Wastewater and public health: the potential of wastewater surveillance for monitoring COVID-19. *Current Opinion in Environmental Science & Health,* 17**,** 14-20.

Hjelmsø, M. H., Hellmér, M., Fernandez-Cassi, X., Timoneda, N., Lukjancenko, O., Seidel, M., Elsässer, D., Aarestrup, F. M., Löfström, C. & Bofill-Mas, S. 2017. Evaluation of methods for the concentration and extraction of viruses from sewage in the context of metagenomic sequencing. *PloS one,* 12**,** e0170199.

Honap, T. P., Sankaranarayanan, K., Schnorr, S. L., Ozga, A. T., Warinner, C. & Lewis Jr, C. M. 2020. Biogeographic study of human gut-associated crAssphage suggests impacts from industrialization and recent expansion. *PLoS One,* 15**,** e0226930.

Hong, T., Mai, Q., Cuong, D., Parida, M. & Minekawa, H. 2004. 318 Development and Evaluation of a Novel Loop-Mediated Isothermal 319 Amplification Method for Rapid Detection of Severe Acute Respiratory 320 Syndrome Coronavirus. *J Clin Microbiol,* 42**,** 1956-1961.

Hovi, T., Shulman, L., Van Der Avoort, H., Deshpande, J., Roivainen, M. & De Gourville, E. 2012. Role of environmental poliovirus surveillance in global polio eradication and beyond. *Epidemiology & Infection,* 140**,** 1-13.

Hrudey, S. E., Bischel, H. N., Charrois, J., Chik, A. H., Conant, B., Delatolla, R., Dorner, S., Graber, T. E., Hubert, C. & Isaac-Renton, J. 2022. Wastewater surveillance for SARS-CoV-2 RNA in Canada. Canadian Science Publishing 1840 Woodward Drive, Suite 1, Ottawa, ON K2C 0P7.

Hsu, S.-Y., Bayati, M., Li, C., Hsieh, H.-Y., Belenchia, A., Klutts, J., Zemmer, S. A., Reynolds, M., Semkiw, E. & Johnson, H.-Y. 2022. Biomarkers selection for population normalization in SARS-CoV-2 wastewater-based epidemiology. *Water Research,* 223**,** 118985.

Huge, B. J., North, D., Mousseau, C. B., Bibby, K., Dovichi, N. J. & Champion, M. M. 2022. Comparison of RT-dPCR and RT-qPCR and the effects of freeze–thaw cycle and glycine release buffer for wastewater SARS-CoV-2 analysis. *Scientific Reports,* 12**,** 20641.

Hughes, B., Beale, D., Dennis, P., Cook, S. & Ahmed, W. 2017. Cross-comparison of human wastewater-associated molecular markers in relation to fecal indicator bacteria and enteric viruses in recreational beach waters. *Applied and environmental microbiology,* 83**,** e00028-17.

Hutchison, J. M., Li, Z., Chang, C.-N., Hiripitiyage, Y., Wittman, M. & Sturm, B. S. 2022. Improving correlation of wastewater SARS-CoV-2 gene copy numbers with COVID-19 public health cases using readily available biomarkers. *FEMS microbes,* 3.

Hutinel, M., Huijbers, P. M. C., Fick, J., Åhrén, C., Larsson, D. G. J. & Flach, C.-F. 2019. Population-level surveillance of antibiotic resistance in Escherichia coli through sewage analysis. *Eurosurveillance,* 24**,** 1800497.

Islam, A., Hossen, F., Rahman, A., Sultana, K. F., Hasan, M. N., Haque, A., Sosa-Hernández, J. E., Oyervides-Muñoz, M. A., Parra-Saldívar, R. & Ahmed, T. 2022. An opinion on Wastewater-Based Epidemiological Monitoring (WBEM) with Clinical Diagnostic Test (CDT) for detecting high-prevalence areas of community COVID-19 Infections. *Current Opinion in Environmental Science & Health***,** 100396.

Jarret, R., Gillaspie, A., Barkley, N. & Pinnow, D. 2008. The occurrence and control of pepper mild mottle virus (PMMoV) in the USDA/ARS Capsicum germplasm collection. *Seed technology***,** 26-36.

Jenkins, M. W., Tiwari, S., Lorente, M., Gichaba, C. M. & Wuertz, S. 2009. Identifying human and livestock sources of fecal contamination in Kenya with host-specific Bacteroidales assays. *Water Research,* 43**,** 4956-4966.

Jones, D. L., Baluja, M. Q., Graham, D. W., Corbishley, A., Mcdonald, J. E., Malham, S. K., Hillary, L. S., Connor, T. R., Gaze, W. H. & Moura, I. B. 2020. Shedding of SARS-CoV-2 in feces and urine and its potential role in person-to-person transmission and the environment-based spread of COVID-19. *Science of the Total Environment,* 749**,** 141364.

Kampf, G., Brüggemann, Y., Kaba, H., Steinmann, J., Pfaender, S., Scheithauer, S. & Steinmann, E. 2020. Potential sources, modes of transmission and effectiveness of prevention measures against SARS-CoV-2. *Journal of Hospital Infection,* 106**,** 678-697.

Keshaviah, A. 2017. The potential of wastewater testing for public health and safety. *Washington: Mathematica Policy Research*.

Kevill, J. L., Pellett, C., Brown, M. R., Bassano, I., Denise, H., Mcdonald, J. E., Malham, S. K., Porter, J., Warren, J. & Evens, N. P. 2022. A comparison of precipitation and filtration-based SARS-CoV-2 recovery methods and the influence of temperature, turbidity, and surfactant load in urban wastewater. *Science of the Total Environment,* 808**,** 151916.

Khalilov, R., Hosainzadegan, M., Eftekhari, A., Hasanzadeh, A., Zadegan, H. & Nasibova, A. 2020. Necessity of different countries to deal with similar phenomena of COVID-19 coronavirus. *Adv. Biol. Earth Sci,* 5**,** 5-6.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C. P., Hamilton, K. A., Haramoto, E. & Rose, J. B. 2020. SARS-CoV-2 in wastewater: State of the knowledge and research needs. *Science of The Total Environment,* 739**,** 139076.

Kitajima, M., Iker, B. C., Pepper, I. L. & Gerba, C. P. 2014. Relative abundance and treatment reduction of viruses during wastewater treatment processes—identification of potential viral indicators. *Science of the Total Environment,* 488**,** 290-296.

Kitajima, M., Sassi, H. P. & Torrey, J. R. 2018. Pepper mild mottle virus as a water quality indicator. *NPJ Clean Water,* 1**,** 19.

Kumar, M., Patel, A. K., Shah, A. V., Raval, J., Rajpara, N., Joshi, M. & Joshi, C. G. 2020. First proof of the capability of wastewater surveillance for COVID-19 in India through detection of genetic material of SARS-CoV-2. *Science of The Total Environment,* 746**,** 141326.

Kumblathan, T., Liu, Y., Uppal, G. K., Hrudey, S. E. & Li, X.-F. 2021. Wastewater-based epidemiology for community monitoring of SARS-CoV-2: Progress and challenges. *ACS Environmental Au,* 1**,** 18-31.

Kwak, Y.-K., Colque, P., Byfors, S., Giske, C. G., Möllby, R. & Kühn, I. 2015. Surveillance of antimicrobial resistance among Escherichia coli in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society? *International journal of antimicrobial agents,* 45**,** 25-32.

La Rosa, G., Mancini, P., Ferraro, G. B., Veneri, C., Iaconelli, M., Bonadonna, L., Lucentini, L. & Suffredini, E. 2021. SARS-CoV-2 has been circulating in northern Italy since December 2019: Evidence from environmental monitoring. *Science of the total environment,* 750**,** 141711.

Lago, P. M., Gary Jr, H. E., Pérez, L. S., Cáceres, V., Olivera, J. B., Puentes, R. P., Corredor, M. B., Jímenez, P., Pallansch, M. A. & Cruz, R. G. 2003. Poliovirus detection in

wastewater and stools following an immunization campaign in Havana, Cuba. *International journal of epidemiology,* 32**,** 772-777.

Langeveld, J., Schilperoort, R., Heijnen, L., Elsinga, G., Schapendonk, C. E., Fanoy, E., De Schepper, E. I., Koopmans, M. P., De Graaf, M. & Medema, G. 2023. Normalisation of SARS-CoV-2 concentrations in wastewater: The use of flow, electrical conductivity and crAssphage. *Science of the Total Environment,* 865**,** 161196.

Larsson, D. J., Andremont, A., Bengtsson-Palme, J., Brandt, K. K., De Roda Husman, A. M., Fagerstedt, P., Fick, J., Flach, C.-F., Gaze, W. H. & Kuroda, M. 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. *Environment international,* 117**,** 132-138.

Laturner, Z. W., Zong, D. M., Kalvapalle, P., Gamas, K. R., Terwilliger, A., Crosby, T., Ali, P., Avadhanula, V., Santos, H. H. & Weesner, K. 2021. Evaluating recovery, cost, and throughput of different concentration methods for SARS-CoV-2 wastewater-based epidemiology. *Water research,* 197**,** 117043.

Launay, M. A., Dittmer, U. & Steinmetz, H. 2016. Organic micropollutants discharged by combined sewer overflows–characterisation of pollutant sources and stormwater-related processes. *Water Research,* 104**,** 82-92.

Li, C., Bayati, M., Hsu, S.-Y., Hsieh, H.-Y., Lindsi, W., Belenchia, A., Zemmer, S. A., Klutts, J., Samuelson, M. & Reynolds, M. 2022. Population Normalization in SARS-CoV-2 Wastewater-Based Epidemiology: Implications from Statewide Wastewater Monitoring in Missouri. *medRxiv***,** 2022.09. 08.22279459.

Li, S.-Y., Cheng, Q.-X., Wang, J.-M., Li, X.-Y., Zhang, Z.-L., Gao, S., Cao, R.-B., Zhao, G.-P. & Wang, J. 2018. CRISPR-Cas12a-assisted nucleic acid detection. *Cell discovery,* 4**,** 20.

Li, X., Zhang, S., Sherchan, S., Orive, G., Lertxundi, U., Haramoto, E., Honda, R., Kumar, M., Arora, S. & Kitajima, M. 2023. Correlation between SARS-CoV-2 RNA concentration in wastewater and COVID-19 cases in community: A systematic review and meta-analysis. *Journal of Hazardous Materials,* 441**,** 129848.

Lin, W., Zhang, X., Tan, Y., Li, P. & Ren, Y. 2019. Can water quality indicators and biomarkers be used to estimate real-time population? *Science of The Total Environment,* 660**,** 603-610.

Lin, X., Glier, M., Kuchinski, K., Ross-Van Mierlo, T., Mcvea, D., Tyson, J. R., Prystajecky, N. & Ziels, R. M. 2021. Assessing multiplex tiling PCR sequencing approaches for detecting genomic variants of SARS-CoV-2 in municipal wastewater. *Msystems,* 6**,** e01068-21.

Liu, P., Ibaraki, M., Vantassell, J., Geith, K., Cavallo, M., Kann, R. & Moe, C. 2020. A novel COVID-19 early warning tool: Moore swab method for wastewater surveillance at an institutional level. *MedRxiv***,** 2020.12. 01.20238006.

Liu, S.-Y., Yu, W.-J., Wang, Y.-R., Shao, X.-T. & Wang, D.-G. 2021. Tracing consumption patterns of stimulants, opioids, and ketamine in China by wastewater-based epidemiology. *Environmental Science and Pollution Research,* 28**,** 16754-16766.

Lorenzo, M. & Picó, Y. 2019. Wastewater-based epidemiology: current status and future prospects. *Current Opinion in Environmental Science & Health,* 9**,** 77-84.

Lou, E. G., Sapoval, N., Mccall, C., Bauhs, L., Carlson-Stadler, R., Kalvapalle, P., Lai, Y., Palmer, K., Penn, R. & Rich, W. 2022. Direct comparison of RT-ddPCR and targeted amplicon sequencing for SARS-CoV-2 mutation monitoring in wastewater. *Science of The Total Environment,* 833**,** 155059.

Lu, J., Peng, J., Fang, L., Zeng, L., Lin, H., Xiong, Q., Liu, Z., Jiang, H., Zhang, C. & Yi, L. 2021. Capturing noroviruses circulating in the population: sewage surveillance in Guangdong, China (2013–2018). *Water Research,* 196**,** 116990.

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B. & Zhu, N. 2020. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *The lancet,* 395**,** 565-574.

Lucansky, V., Samec, M., Burjanivova, T., Lukacova, E., Kolkova, Z., Holubekova, V., Turyova, E., Hornakova, A., Zaborsky, T. & Podlesniy, P. 2023. Comparison of the methods for isolation and detection of SARS-CoV-2 RNA in municipal wastewater. *Frontiers in Public Health,* 11**,** 740.

Maida, C. M., Amodio, E., Mazzucco, W., La Rosa, G., Lucentini, L., Suffredini, E., Palermo, M., Andolina, G., Iaia, F. R. & Merlo, F. 2022. Wastewater-based epidemiology for early warning of SARS-COV-2 circulation: A pilot study conducted in Sicily, Italy. *International Journal of Hygiene and Environmental Health,* 242**,** 113948.

Mangwana, N., Archer, E., Muller, C. J., Preiser, W., Wolfaardt, G., Kasprzyk-Hordern, B., Carstens, A., Brocker, L., Webster, C. & Mccarthy, D. 2022. Sewage surveillance of SARS-CoV-2 at student campus residences in the Western Cape, South Africa. *Science of The Total Environment,* 851**,** 158028.

Manor, Y., Handsher, R., Halmut, T., Neuman, M., Bobrov, A., Rudich, H., Vonsover, A., Shulman, L., Kew, O. & Mendelson, E. 1999. Detection of poliovirus circulation by environmental surveillance in the absence of clinical cases in Israel and the Palestinian authority. *Journal of clinical microbiology,* 37**,** 1670-1675.

Mao, K., Zhang, K., Du, W., Ali, W., Feng, X. & Zhang, H. 2020. The potential of wastewater-based epidemiology as surveillance and early warning of infectious disease outbreaks. *Current Opinion in Environmental Science & Health,* 17**,** 1-7.

Marchini, A., Petrillo, M., Parrish, A., Buttinger, G., Tavazzi, S., Querci, M., Betsou, F., Elsinga, G., Medema, G. & Abdelrahman, T. 2023. New RT-PCR Assay for the Detection of Current and Future SARS-CoV-2 Variants. *Viruses,* 15**,** 206.

Mastroianni, N., De Alda, M. L. & Barcelo, D. 2014. Analysis of ethyl sulfate in raw wastewater for estimation of alcohol consumption and its correlation with drugs of abuse in the city of Barcelona. *Journal of Chromatography A,* 1360**,** 93-99.

Mccall, A.-K., Bade, R., Kinyua, J., Lai, F. Y., Thai, P. K., Covaci, A., Bijlsma, L., Van Nuijs, A. L. & Ort, C. 2016. Critical review on the stability of illicit drugs in sewers and wastewater samples. *Water research,* 88**,** 933-947.

Mccall, C., Wu, H., O'brien, E. & Xagoraraki, I. 2021. Assessment of enteric viruses during a hepatitis outbreak in Detroit MI using wastewater surveillance and metagenomic analysis. *Journal of Applied Microbiology,* 131**,** 1539-1554.

Mcclary-Gutierrez, J. S., Mattioli, M. C., Marcenac, P., Silverman, A. I., Boehm, A. B., Bibby, K., Balliet, M., Francis Iii, L., Gerrity, D. & Griffith, J. F. 2021. SARS-CoV-2 wastewater surveillance for public health action. *Emerging infectious diseases,* 27.

Medema, G., Been, F., Heijnen, L. & Petterson, S. 2020a. Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges. *Current Opinion in Environmental Science & Health,* 17**,** 49-71.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. & Brouwer, A. 2020b. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environmental Science & Technology Letters,* 7**,** 511-516.

Misra, G., Hora, S., Ginwal, S., Singh, N. & Anvikar, A. 2023. SARS-CoV-2 Variants Impact on Key Signaling Pathways Metamorphoses into Severity. *Brazilian Archives of Biology and Technology,* 66**,** e23220261.

Miura, F., Kitajima, M. & Omori, R. 2021. Duration of SARS-CoV-2 viral shedding in faeces as a parameter for wastewater-based epidemiology: Re-analysis of patient data using a shedding dynamics model. *Science of The Total Environment,* 769**,** 144549.

Mizumoto, K., Kagaya, K., Zarebski, A. & Chowell, G. 2020. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Eurosurveillance,* 25**,** 2000180.

Mlejnkova, H., Sovova, K., Vasickova, P., Ocenaskova, V., Jasikova, L. & Juranova, E. 2020. Preliminary study of Sars-Cov-2 occurrence in wastewater in the Czech Republic. *International journal of environmental research and public health,* 17**,** 5508.

Montes, R., Rodil, R., Rico, A., Cela, R., González-Mariño, I., Hernández, F., Bijlsma, L., Celma, A., Picó, Y. & Andreu, V. 2020. First nation-wide estimation of tobacco consumption in Spain using wastewater-based epidemiology. *Science of The Total Environment,* 741**,** 140384.

Moriones, E. & Navas-Castillo, J. 2010. Tomato yellow leaf curl disease epidemics. *Bemisia: Bionomics and management of a global pest***,** 259-282.

Morón-López, S., Riveira-Muñoz, E., Urrea, V., Gutiérrez-Chamorro, L., Ávila-Nieto, C., Noguera-Julian, M., Carrillo, J., Mitjà, O., Mateu, L. & Massanella, M. 2023. Comparison of Reverse Transcription (RT)-Quantitative PCR and RT-Droplet Digital PCR for Detection of Genomic and Subgenomic SARS-CoV-2 RNA. *Microbiology Spectrum,* 11**,** e04159-22.

Mousazadeh, M., Ashoori, R., Paital, B., Kabdaşlı, I., Frontistis, Z., Hashemi, M., Sandoval, M. A., Sherchan, S., Das, K. & Emamjomeh, M. M. 2021. Wastewater based epidemiology perspective as a faster protocol for detecting coronavirus RNA in human populations: A review with specific reference to SARS-CoV-2 virus. *Pathogens,* 10**,** 1008.

Nam, S. J., Hu, W. S. & Koo, O. K. 2022. Evaluation of crAssphage as a human-specific microbial source-tracking marker in the Republic of Korea. *Environmental Monitoring and Assessment,* 194**,** 367.

Navarro, A., Gómez, L., Sanseverino, I., Niegowska, M., Roka, E., Pedraccini, R., Vargha, M. & Lettieri, T. 2021. SARS-CoV-2 detection in wastewater using multiplex quantitative PCR. *Science of the Total Environment,* 797**,** 148890.

Nazir, J., Haumacher, R., Ike, A., Stumpf, P., Böhm, R. & Marschang, R. E. 2010. Long-term study on tenacity of avian influenza viruses in water (distilled water, normal saline, and surface water) at different temperatures. *Avian diseases,* 54**,** 720-724.

Nemudryi, A., Nemudraia, A., Wiegand, T., Surya, K., Buyukyoruk, M., Cicha, C., Vanderwood, K. K., Wilkinson, R. & Wiedenheft, B. 2020. Temporal detection and phylogenetic assessment of SARS-CoV-2 in municipal wastewater. *Cell Reports Medicine,* 1**,** 100098.

Nguyen, A. Q., Vu, H. P., Nguyen, L. N., Wang, Q., Djordjevic, S. P., Donner, E., Yin, H. & Nghiem, L. D. 2021. Monitoring antibiotic resistance genes in wastewater treatment: Current strategies and future challenges. *Science of the Total Environment,* 783**,** 146964.

Nieuwenhuijse, D. F., Oude Munnink, B. B., Phan, M. V., Munk, P., Venkatakrishnan, S., Aarestrup, F. M., Cotten, M. & Koopmans, M. P. 2020. Setting a baseline for global urban virome surveillance in sewage. *Scientific Reports,* 10**,** 13748.

Ning, B., Youngquist, B. M., Li, D. D., Lyon, C. J., Zelazny, A., Maness, N. J., Tian, D. & Hu, T. Y. 2022. Rapid detection of multiple SARS-CoV-2 variants of concern by PAM-Targeting Mutations. *Cell Reports Methods,* 2**,** 100173.

Nshimyimana, J., Ekklesia, E., Shanahan, P., Chua, L. & Thompson, J. 2014. Distribution and abundance of human-specific Bacteroides and relation to traditional indicators in an urban tropical catchment. *Journal of applied microbiology,* 116**,** 1369-1383.

O'brien, E. & Xagoraraki, I. 2019. A water-focused one-health approach for early detection and prevention of viral outbreaks. *One Health,* 7**,** 100094.

O'neill, J. 2016. Tackling drug-resistant infections globally: final report and recommendations.

O'keeffe, J. 2021. Wastewater-based epidemiology: current uses and future opportunities as a public health surveillance tool. *Environmental Health Review,* 64**,** 44-52.

Olds, H. T., Corsi, S. R., Dila, D. K., Halmo, K. M., Bootsma, M. J. & Mclellan, S. L. 2018. High levels of sewage contamination released from urban areas after storm events: A quantitative survey with sewage specific bacterial indicators. *PLoS medicine,* 15**,** e1002614.

Omori, R., Miura, F. & Kitajima, M. 2021. Age-dependent association between SARS-CoV-2 cases reported by passive surveillance and viral load in wastewater. *Science of the Total Environment,* 792**,** 148442.

Organization, W. H. 2000. Antimicrobial resistance. *Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire,* 75**,** 336-336.

Organization, W. H. 2003. Guidelines for environmental surveillance of poliovirus circulation. World Health Organization.

Organization, W. H. 2021. Weekly epidemiological update. *Data as received by WHO from national authorities, as of,* 10.

Ort, C., Banta-Green, C. J., Bijlsma, L., Castiglioni, S., Emke, E., Gartner, C., Kasprzyk-Hordern, B., Reid, M. J., Rieckermann, J. & Van Nuijs, A. L. 2014. Sewage-based epidemiology requires a truly transdisciplinary approach. *GAIA-Ecological Perspectives for Science and Society,* 23**,** 266-268.

Ort, C., Lawrence, M. G., Rieckermann, J. & Joss, A. 2010. Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review. *Environmental science & technology,* 44**,** 6024-6035.

Ortiz-Prado, E., Simbaña-Rivera, K., Gómez-Barreno, L., Rubio-Neira, M., Guaman, L. P., Kyriakidis, N. C., Muslin, C., Jaramillo, A. M. G., Barba-Ostria, C. & Cevallos-Robalino, D. 2020. Clinical, molecular, and epidemiological characterization of the SARS-CoV-2 virus and the Coronavirus Disease 2019 (COVID-19), a comprehensive literature review. *Diagnostic microbiology and infectious disease,* 98**,** 115094.

Pandopulos, A. J., Gerber, C., Tscharke, B. J., O'brien, J., White, J. M. & Bade, R. 2020. A sensitive analytical method for the measurement of neurotransmitter metabolites as potential population biomarkers in wastewater. *Journal of Chromatography A,* 1612**,** 460623.

Peccia, J., Zulli, A., Brackney, D. E., Grubaugh, N. D., Kaplan, E. H., Casanovas-Massana, A., Ko, A. I., Malik, A. A., Wang, D. & Wang, M. 2020. Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics. *Nature biotechnology,* 38**,** 1164-1167.

Peng, J., Shi, B., Zheng, H., Lu, Y., Lin, L., Jiang, T., Chen, J. & Yan, F. 2015. Detection of pepper mild mottle virus in pepper sauce in China. *Archives of virology,* 160**,** 2079-2082.

Pereira, B. M. T., Morales, W., Cardoso, R. G., Fiorelli, R., Fraga, G. P. & Briggs, S. M. 2013. Lessons learned from a landslide catastrophe in Rio de Janeiro, Brazil. *American journal of disaster medicine,* 8**,** 253-258.

Pillay, L., Amoah, I. D., Deepnarain, N., Pillay, K., Awolusi, O. O., Kumari, S. & Bux, F. 2021. Monitoring changes in COVID-19 infection using wastewater-based

epidemiology: A South African perspective. *Science of The Total Environment,* 786**,** 147273.

Pischon, T. 2009. Use of obesity biomarkers in cardiovascular epidemiology. *Disease markers,* 26**,** 247-263.

Pitol, A. K. & Julian, T. R. 2021. Community transmission of SARS-CoV-2 by surfaces: risks and risk reduction strategies. *Environmental Science & Technology Letters,* 8**,** 263-269.

Polo, D., Quintela-Baluja, M., Corbishley, A., Jones, D. L., Singer, A. C., Graham, D. W. & Romalde, J. L. 2020. Making waves: Wastewater-based epidemiology for COVID-19–approaches and challenges for surveillance and prediction. *Water Research,* 186**,** 116404.

Primers, S. 2020. Probes for Detection 2019 Novel Coronavirus. *China National Institute For Viral Disease Control and Prevention: Beijing*.

Rafiee, M., Isazadeh, S., Mohseni-Bandpei, A., Mohebbi, S. R., Jahangiri-Rad, M., Eslami, A., Dabiri, H., Roostaei, K., Tanhaei, M. & Amereh, F. 2021. Moore swab performs equal to composite and outperforms grab sampling for SARS-CoV-2 monitoring in wastewater. *Science of The Total Environment,* 790**,** 148205.

Ramin, P., Libonati Brock, A., Polesel, F., Causanilles, A., Emke, E., De Voogt, P. & Plosz, B. G. 2016. Transformation and sorption of illicit drug biomarkers in sewer systems: understanding the role of suspended solids in raw wastewater. *Environmental science & technology*, 50**,** 13397-13408.

Randazzo, W., Cuevas-Ferrando, E., Sanjuán, R., Domingo-Calap, P. & Sánchez, G. 2020a. Metropolitan wastewater analysis for COVID-19 epidemiological surveillance. *International Journal of Hygiene and Environmental Health*, 230**,** 113621.

Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A. & Sánchez, G. 2020b. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low Prevalence Area. *Water Research,* 181**,** 115942.

Rashid, S. S., Harun, S. N., Hanafiah, M. M., Razman, K. K., Liu, Y.-Q. & Tholibon, D. A. 2023. Life Cycle Assessment and Its Application in Wastewater Treatment: A Brief Overview. *Processes,* 11**,** 208.

Reid, M. J., Langford, K. H., Mørland, J. & Thomas, K. V. 2011. Analysis and interpretation of specific ethanol metabolites, ethyl sulfate, and ethyl glucuronide in sewage effluent for the quantitative measurement of regional alcohol consumption. *Alcoholism: Clinical and Experimental Research,* 35**,** 1593-1599.

Reynolds, L. J., Sala-Comorera, L., Khan, M. F., Martin, N. A., Whitty, M., Stephens, J. H., Nolan, T. M., Joyce, E., Fletcher, N. F. & Murphy, C. D. 2022. Coprostanol as a population biomarker for SARS-CoV-2 wastewater surveillance studies. *Water,* 14**,** 225.

Rice, E. W., Bridgewater, L. & Association, A. P. H. 2012. *Standard methods for the examination of water and wastewater*, American public health association Washington, DC.

Rico, M., Andrés-Costa, M. J. & Picó, Y. 2017. Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study. *Journal of Hazardous materials,* 323**,** 156-165.

Roberts, L. 2013. Israel's silent polio epidemic breaks all the rules. American Association for the Advancement of Science.

Robins, K., Leonard, A. F., Farkas, K., Graham, D. W., Jones, D. L., Kasprzyk-Hordern, B., Bunce, J. T., Grimsley, J. M., Wade, M. J. & Zealand, A. M. 2022. Research needs for

optimising wastewater-based epidemiology monitoring for public health protection. *Journal of Water and Health,* 20**,** 1284-1313.

Róka, E., Khayer, B., Kis, Z., Kovács, L. B., Schuler, E., Magyar, N., Málnási, T., Oravecz, O., Pályi, B. & Pándics, T. 2021. Ahead of the second wave: Early warning for COVID-19 by wastewater surveillance in Hungary. *Science of the Total Environment,* 786**,** 147398.

Sabar, M. A., Honda, R. & Haramoto, E. 2022. CrAssphage as an indicator of human-fecal contamination in water environment and virus reduction in wastewater treatment. *Water Research***,** 118827.

Sala-Comorera, L., Reynolds, L. J., Martin, N. A., Pascual-Benito, M., Stephens, J. H., Nolan, T. M., Gitto, A., O'hare, G. M., O'sullivan, J. J. & García-Aljaro, C. 2021. crAssphage as a human molecular marker to evaluate temporal and spatial variability in faecal contamination of urban marine bathing waters. *Science of The Total Environment,* 789**,** 147828.

Sanyaolu, A., Okorie, C., Marinkovic, A., Haider, N., Abbasi, A. F., Jaferi, U., Prakash, S. & Balendra, V. 2021. The emerging SARS-CoV-2 variants of concern. *Therapeutic advances in infectious disease,* 8**,** 20499361211024372.

Sapula, S. A., Whittall, J. J., Pandopulos, A. J., Gerber, C. & Venter, H. 2021. An optimized and robust PEG precipitation method for detection of SARS-CoV-2 in wastewater. *Science of The Total Environment,* 785**,** 147270.

Schang, C., Crosbie, N. D., Nolan, M., Poon, R., Wang, M., Jex, A., John, N., Baker, L., Scales, P. & Schmidt, J. 2021. Passive sampling of SARS-CoV-2 for wastewater surveillance. *Environmental science & technology,* 55**,** 10432-10441.

Senta, I., Rodríguez-Mozaz, S., Corominas, L. & Petrovic, M. 2020. Wastewater-based epidemiology to assess human exposure to personal care and household products–a

review of biomarkers, analytical methods, and applications. *Trends in Environmental Analytical Chemistry,* 28**,** e00103.

Seurinck, S., Verdievel, M., Verstraete, W. & Siciliano, S. D. 2006. Identification of human fecal pollution sources in a coastal area: a case study at Oostende (Belgium). *Journal of water and health,* 4**,** 167-175.

Shah, M. I., Abunama, T., Javed, M. F., Bux, F., Aldrees, A., Tariq, M. A. U. R. & Mosavi, A. 2021. Modeling surface water quality using the adaptive neuro-fuzzy inference system aided by input optimization. *Sustainability,* 13**,** 4576.

Sherchan, S., Thakali, O., Ikner, L. A. & Gerba, C. P. 2023. Survival of SARS-CoV-2 in wastewater. *Science of The Total Environment,* 882**,** 163049.

Sherchan, S. P., Shahin, S., Ward, L. M., Tandukar, S., Aw, T. G., Schmitz, B., Ahmed, W. & Kitajima, M. 2020. First detection of SARS-CoV-2 RNA in wastewater in North America: a study in Louisiana, USA. *Science of The Total Environment,* 743**,** 140621.

Shirasaki, N., Matsushita, T., Matsui, Y. & Murai, K. 2017. Assessment of the efficacy of membrane filtration processes to remove human enteric viruses and the suitability of bacteriophages and a plant virus as surrogates for those viruses. *Water research,* 115**,** 29-39.

Shirato, K., Nao, N., Katano, H., Takayama, I., Saito, S., Kato, F., Katoh, H., Sakata, M., Nakatsu, Y. & Mori, Y. 2020. Development of genetic diagnostic methods for detection for novel coronavirus 2019 (nCoV-2019) in Japan. *Japanese journal of infectious diseases,* 73**,** 304-307.

Shulman, L. M., Manor, Y., Sofer, D., Handsher, R., Swartz, T., Delpeyroux, F. & Mendelson, E. 2006. Neurovirulent vaccine-derived polioviruses in sewage from highly immune populations. *PLoS One,* 1**,** e69.

Sikorski, M. J. & Levine, M. M. 2020. Reviving the “Moore swab”: a classic environmental surveillance tool involving filtration of flowing surface water and sewage water to recover typhoidal Salmonella bacteria. *Applied and Environmental Microbiology,* 86**,** e00060-20.

Sims, N. & Kasprzyk-Hordern, B. 2020. Future perspectives of wastewater-based epidemiology: monitoring infectious disease spread and resistance to the community level. *Environment international,* 139**,** 105689.

Singhal, T. 2020. A review of coronavirus disease-2019 (COVID-19). *The indian journal of pediatrics,* 87**,** 281-286.

Stachler, E. & Bibby, K. 2014. Metagenomic evaluation of the highly abundant human gut bacteriophage CrAssphage for source tracking of human fecal pollution. *Environmental Science & Technology Letters,* 1**,** 405-409.

Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K. & Shanks, O. C. 2017. Quantitative CrAssphage PCR assays for human fecal pollution measurement. *Environmental science & technology,* 51**,** 9146-9154.

Stadler, L. B., Ensor, K., Clark, J. R., Kalvapalle, P., Laturner, Z. W., Mojica, L., Terwilliger, A., Zhuo, Y., Ali, P. & Avadhanula, V. 2020. Wastewater analysis of SARS-CoV-2 as a predictive metric of positivity rate for a major metropolis. *MedRXiv***,** 2020.11. 04.20226191.

Staley, C., Gordon, K. V., Schoen, M. E. & Harwood, V. J. 2012. Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. *Applied and environmental microbiology,* 78**,** 7317-7326.

Street, R., Malema, S., Mahlangeni, N. & Mathee, A. 2020. Wastewater surveillance for Covid-19: an African perspective. *Science of The Total Environment,* 743**,** 140719.

Subedi, B. & Burgard, D. 2019. Wastewater-based epidemiology as a complementary approach to the conventional survey-based approach for the estimation of community consumption of drugs. *Wastewater-Based Epidemiology: Estimation of Community Consumption of Drugs and Diets.* ACS Publications.

Suo, T., Liu, X., Feng, J., Guo, M., Hu, W., Guo, D., Ullah, H., Yang, Y., Zhang, Q. & Wang, X. 2020. ddPCR: a more accurate tool for SARS-CoV-2 detection in low viral load specimens. *Emerging microbes & infections,* 9**,** 1259-1268.

Symonds, E. M., Rosario, K. & Breitbart, M. 2019. Pepper mild mottle virus: Agricultural menace turned effective tool for microbial water quality monitoring and assessing (waste) water treatment technologies. *PLoS pathogens,* 15**,** e1007639.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution,* 28**,** 2731-2739.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution,* 30**,** 2725-2729.

Tandukar, S., Sherchan, S. P. & Haramoto, E. 2020. Applicability of crAssphage, pepper mild mottle virus, and tobacco mosaic virus as indicators of reduction of enteric viruses during wastewater treatment. *Scientific reports,* 10**,** 3616.

Tang, A., Tong, Z.-D., Wang, H.-L., Dai, Y.-X., Li, K.-F., Liu, J.-N., Wu, W.-J., Yuan, C., Yu, M.-L. & Li, P. 2020. Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China. *Emerging infectious diseases,* 26**,** 1337.

Tanvir Pasha, A., Hinojosa, J., Phan, D., Lopez, A. & Kapoor, V. 2020. Detection of human fecal pollution in environmental waters using human mitochondrial DNA and

correlation with general and human-associated fecal genetic markers. *Journal of water and health,* 18**,** 8-18.

Torii, S., Oishi, W., Zhu, Y., Thakali, O., Malla, B., Yu, Z., Zhao, B., Arakawa, C., Kitajima, M. & Hata, A. 2022. Comparison of five polyethylene glycol precipitation procedures for the RT-qPCR based recovery of murine hepatitis virus, bacteriophage phi6, and pepper mild mottle virus as a surrogate for SARS-CoV-2 from wastewater. *Science of The Total Environment,* 807**,** 150722.

Trefault, N., Edwards, R. A., Vega, A. A., Norman, H. M., Ohaeri, M., Levi, K., Dinsdale, E. A., Cinek, O., Aziz, R. K. & Mcnair, K. 2019. Global phylogeography and ancient evolution of the widespread human gut virus crAssphage.

Tscharke, B. J., Chen, C., Gerber, J. P. & White, J. M. 2016. Temporal trends in drug use in Adelaide, South Australia by wastewater analysis. *Science of the Total Environment,* 565**,** 384-391.

Tseha, S. T. 2021. Polio: the disease that reemerged after six years in Ethiopia. *Ethiopian journal of health sciences,* 31.

Tulchinsky, T. H. & Varavikova, E. A. 2014. Measuring, monitoring, and evaluating the health of a population. *The New Public Health***,** 91.

Unno, T., Staley, C., Brown, C. M., Han, D., Sadowsky, M. J. & Hur, H. G. 2018. Fecal pollution: new trends and challenges in microbial source tracking using next-generation Sequencing. *Environmental Microbiology,* 20**,** 3132-3140.

Vadde, K. K., Al-Duroobi, H., Phan, D. C., Jafarzadeh, A., Moghadam, S. V., Matta, A. & Kapoor, V. 2022. Assessment of concentration, recovery, and normalization of SARS-CoV-2 RNA from two wastewater treatment plants in Texas and correlation with COVID-19 cases in the community. *Acs Es&T Water,* 2**,** 2060-2069.

Van Nuijs, A. L., Mougel, J.-F., Tarcomnicu, I., Bervoets, L., Blust, R., Jorens, P. G., Neels, H. & Covaci, A. 2011. Sewage epidemiology—a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium. *Environment international,* 37**,** 612-621.

Van Nuijs, A. L., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P. G., Bervoets, L., Blust, R., Meulemans, H. & Neels, H. 2009. Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium. *Addiction,* 104**,** 734-741.

Van Wel, J., Gracia-Lor, E., Van Nuijs, A., Kinyua, J., Salvatore, S., Castiglioni, S., Bramness, J. G., Covaci, A. & Van Hal, G. 2016. Investigation of agreement between wastewater-based epidemiology and survey data on alcohol and nicotine use in a community. *Drug and alcohol dependence,* 162**,** 170-175.

Venhuis, B., Zwaagstra, M., Keizers, P. & De Kaste, D. 2014. Dose-to-dose variations with single packages of counterfeit medicines and adulterated dietary supplements as a potential source of false negatives and inaccurate health risk assessments. *Journal of pharmaceutical and biomedical analysis,* 89**,** 158-165.

Vitale, D., Suárez-Varela, M. M. & Picó, Y. 2021. Wastewater-based epidemiology, a tool to bridge biomarkers of exposure, contaminants, and human health. *Current opinion in environmental science & health,* 20**,** 100229.

Vogelstein, B. & Kinzler, K. W. 1999. Digital pcr. *Proceedings of the National Academy of Sciences,* 96**,** 9236-9241.

Walls, A. C., Park, Y.-J., Tortorici, M. A., Wall, A., Mcguire, A. T. & Veesler, D. 2020. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell,* 181**,** 281-292. e6.

Wang, W., Xu, Y., Gao, R., Lu, R., Han, K., Wu, G. & Tan, W. 2020a. Detection of SARS-CoV-2 in different types of clinical specimens. *Jama,* 323**,** 1843-1844.

Wang, X., Li, J., Jin, M., Zhen, B., Kong, Q., Song, N., Xiao, W., Yin, J., Wei, W. & Wang, G. 2005. W.(2005). *Study on the resistance of severe acute respiratory syndrome-associated coronavirus. Journal of Virological Methods,* 126**,** 171-177.

Wang, Y., Xu, G. & Huang, Y.-W. 2020b. Modeling the load of SARS-CoV-2 virus in human expelled particles during coughing and speaking. *PLoS One,* 15**,** e0241539.

Warwick, C., Guerreiro, A. & Soares, A. 2013. Sensing and analysis of soluble phosphates in environmental samples: A review. *Biosensors and Bioelectronics,* 41**,** 1-11.

Werschler, T. & Brennan, A. 2019. Wastewater-based Estimates of cannabis and drug use in Canada: pilot test detailed results. Statistics Canada= Statistique Canada.

Wilder, M. L., Middleton, F., Larsen, D. A., Du, Q., Fenty, A., Zeng, T., Insaf, T., Kilaru, P., Collins, M. & Kmush, B. 2021. Co-quantification of crAssphage increases confidence in wastewater-based epidemiology for SARS-CoV-2 in low prevalence areas. *Water research X,* 11**,** 100100.

Wilson, M., Qiu, Y., Yu, J., Lee, B. E., Mccarthy, D. T. & Pang, X. 2022. Comparison of auto sampling and passive sampling methods for SARS-CoV-2 detection in wastewater. *Pathogens,* 11**,** 359.

Wolfaardt, G., Street, R., Mathee, A., Gray, G., Mdhluli, M., Gelderblom, H., Malema, S., Mutshembele, A., Van Der Walt, M. & Johnson, R. 2021. Qualitative and quantitative detection of SARS-CoV-2 RNA in untreated wastewater in Western Cape Province, South Africa. *South African Medical Journal,* 111**,** 198-202.

Won, J.-H. & Lee, H. 2020. The current status of drug repositioning and vaccine developments for the COVID-19 pandemic. *International Journal of Molecular Sciences,* 21**,** 9775.

Wong, J. C. C., Tan, J., Lim, Y. X., Arivalan, S., Hapuarachchi, H. C., Mailepessov, D., Griffiths, J., Jayarajah, P., Setoh, Y. X. & Tien, W. P. 2021. Non-intrusive wastewater surveillance for monitoring of a residential building for COVID-19 cases. *Science of the Total Environment,* 786**,** 147419.

Wu, D., Wu, T., Liu, Q. & Yang, Z. 2020a. The SARS-CoV-2 outbreak: what we know. *International journal of infectious diseases,* 94**,** 44-48.

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H. & Pei, Y.-Y. 2020b. A new coronavirus associated with human respiratory disease in China. *Nature,* 579**,** 265-269.

Wu, Y., Guo, C., Tang, L., Hong, Z., Zhou, J., Dong, X., Yin, H., Xiao, Q., Tang, Y. & Qu, X. 2020c. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *The lancet Gastroenterology & hepatology,* 5**,** 434-435.

Xagoraraki, I. & O'brien, E. 2020. Wastewater-based epidemiology for early detection of viral outbreaks. *Women in water quality: Investigations by prominent female engineers***,** 75-97.

Xiao, F., Tang, M., Zheng, X., Liu, Y., Li, X. & Shan, H. 2020. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology,* 158**,** 1831-1833. e3.

Xiao, K. & Zhang, L. 2023. Wastewater pathogen surveillance based on One Health approach. *The Lancet Microbe,* 4**,** e297.

Yan, M., Qi, H., Xia, T., Zhao, X., Wang, W., Wang, Z., Lu, C., Ning, Z., Chen, H. & Li, T. 2019. Metabolomics profiling of metformin-mediated metabolic reprogramming bypassing AMPKα. *Metabolism,* 91**,** 18-29.

Yan, T., O'brien, P., Shelton, J., Whelen, A. & Pagaling, E. 2018. Municipal wastewater as a microbial surveillance platform for enteric diseases: a case study for Salmonella and salmonellosis. *Environmental science & technology,* 52**,** 4869-4877.

Yaniv, K., Ozer, E., Lewis, Y. & Kushmaro, A. 2021. RT-qPCR assays for SARS-CoV-2 variants of concern in wastewater reveals compromised vaccination-induced immunity. *Water research,* 207**,** 117808.

Yu, L., Tian, Z., Joshi, D. R., Yuan, L., Tuladhar, R., Zhang, Y. & Yang, M. 2022. Detection of SARS-CoV-2 and Other Viruses in Wastewater: Optimization and Automation of an Aluminum Hydroxide Adsorption–Precipitation Method for Virus Concentration. *Acs Es&T Water,* 2**,** 2175-2184.

Zahedi, A., Monis, P., Deere, D. & Ryan, U. 2021. Wastewater-based epidemiology—surveillance and early detection of waterborne pathogens with a focus on SARS-CoV-2, Cryptosporidium and Giardia. *Parasitology research,* 120**,** 4167-4188.

Zhang, T., Breitbart, M., Lee, W. H., Run, J.-Q., Wei, C. L., Soh, S. W. L., Hibberd, M. L., Liu, E. T., Rohwer, F. & Ruan, Y. 2006. RNA viral community in human feces: prevalence of plant pathogenic viruses. *PLoS biology,* 4**,** e3.

Zhang, Y., Dai, C., Wang, H., Gao, Y., Li, T., Fang, Y., Shen, Z., Chen, L., Chen, Z. & Ma, X. 2020a. Analysis and validation of a highly sensitive one-step nested quantitative real-time polymerase chain reaction assay for specific detection of severe acute respiratory syndrome coronavirus 2. *Virology Journal,* 17**,** 1-13.

Zhang, Y., Odiwuor, N., Xiong, J., Sun, L., Nyaruaba, R. O., Wei, H. & Tanner, N. A. 2020b. Rapid molecular detection of SARS-CoV-2 (COVID-19) virus RNA using colorimetric LAMP. *MedRxiv***,** 2020.02. 26.20028373.

Zhang, Y., Sallach, J. B., Hodges, L., Snow, D. D., Bartelt-Hunt, S. L., Eskridge, K. M. & Li, X. 2016. Effects of soil texture and drought stress on the uptake of antibiotics and the internalization of Salmonella in lettuce following wastewater irrigation. *Environmental Pollution,* 208**,** 523-531.

Zhao, F., Heidrich, E. S., Curtis, T. P. & Dolfing, J. 2020. Understanding the complexity of wastewater: The combined impacts of carbohydrates and sulphate on the performance of bioelectrochemical systems. *Water Research,* 176**,** 115737.

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B. & Gu, X. 2020. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *The lancet,* 395**,** 1054-1062.

Zuccato, E., Chiabrando, C., Castiglioni, S., Bagnati, R. & Fanelli, R. 2008. Estimating community drug abuse by wastewater analysis. *Environmental health perspectives,* 116**,** 1027-1032.

Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S. & Fanelli, R. 2005a. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. *Environmental Health,* 4**,** 1-7.

Supplementary Table

Table S3.1: Indicates the PCR Reaction Components

A TAKARA PCR master mix was used.

PCR Components	Reaction Volume (µl)
Master mix	12.5
Distilled Water	6.5
Forward Primer (2µM)	2
Reverse Primer (2µM)	2
DNA or cDNA	2

Table S3.2: Indicates the ddPCR Reaction Components

QX200™ ddPCR™ EvaGreen Supermix was used.

PCR Components	Reaction Volume (µl)
Master mix	10.0
Forward Primer (2µM)	1
Reverse Primer (2µM)	1
Nuclease free water	9
DNA or cDNA	1

Supplementary Data

Table S4.1: Mean concentrations (±standard deviation) of the measured physicochemical characteristics, biomarker concentrations and SARS-CoV-2 concentrations in wastewater at the Central WWTP over a 12-week period.

Week	pH (@25 °C)	Temp °C	COD (mg/L)	TS (mg/L)	EC (µS/cm)	DO% (mg/L)	TDS (mg/L)	TS (mg/L)	VS (mg/L)	*crAssphage* copies/µl	*PMMoV* copies/µl	*HF183 Bacteroides* copies/µl	SARS-CoV-2 copies/µl
Week 1	6,95(±0.04)	21,37	507,14(±0.70)	0,74(±0.02)	1021(±2.12)	17,9(±1.06)	0,663(±0.00)	0,74	0,264	7536,2 (±5.65)	46(±4.24)	8290(±56.56)	7,32(±0.26)
Week 2	6,95(±0.03)	22,22	142,86(±1.96)	0,978(±0.02)	1026(±0.07)	17(±0.35)	0,565(±0.00)	0,978	0,438	4577(±21.21)	38,1(±2.68)	2790(±134.35)	1,51(±0.11)
Week 3	6,95(±0.05)	22,22	157,14(±3.53)	0,99(±0.04)	1026(±0.07)	17(±0.35)	0,565(±0.00)	0,99	0,438	3899,6(±13.22)	5,6(±0.84)	4156(±60.10)	1,41(±0.26)
Week 4	6,82(±0.02)	18,5	217,86(±2.12)	1,056(±0.00)	1120(±1.41)	11,6(±1.83)	0,728(±0.01)	1,056	0,432	5752(±293.44)	9,47(±0.74)	5511(±31.81)	1,32(±0.15)
Week 5	7,4(±0.15)	21,96	282,14(±0.70)	1,04(±0.06)	1313(±0.07)	6,4(±0.84)	0,854(±0.00)	1,04	0,414	5200,01(±70.71)	3,47(±1.76)	10116(±120.91)	1,18(±0.10)
Week 6	7,51(±0.03)	21,85	253,72(±0.70)	0,956(±0.00)	1313(±0.00)	0,8(±0.07)	0,854(±0.02)	0,956	0,378	7279,6(±98.99)	20,2(±0.98)	8595(±140.71)	2,48(±0.28)
Week 7	7,7(±0.02)	23,25	128,57(±1.41)	0,876(±0.00)	1250(±4.94)	1,7(±0.49)	0,812(±0.02)	0,876	0,326	7943(±74.24)	6,44(±0.07)	7770(±14.14)	0,393(±0.00)
Week 8	6,96(±0.04)	19,05	267,86(±0.35)	2,06(±0.01)	3410(±11.31)	6,9(±1.06)	2,217(±0.01)	2,06	0,452	7217,14(±17.67)	0,734(±0.06)	4175(±42.42)	5,51(±0.38)
Week 9	7,49(±0.01)	22,12	267,86(±0.35)	1,006(±0.00)	1291(±5.65)	1,9(±0.91)	0,839(±0.00)	1,006	0,408	5147,01(±137.88)	9,45(±0.61)	5470(±127.27)	1,51(±0.10)
Week 10	7,32(±0.01)	25,26	250(±0.00)	1,074(±0.01)	2006(±7.77)	2,2(±1.55)	1,3005(±0.01)	1,074	0,412	5687,02(±137.17)	1,69(±0.24)	3420(±21.21)	5,38(±0.26)
Week 11	7,12(±0.03)	19,8	178,57(±0.40)	0,932(±0.01)	1154(±1.41)	8,6(±8.20)	0,75(±0.00)	0,932	0,436	7571,33(±314.66)	1,22(±9.28)	4181(±100.40)	9,57(±0.49)
Week 12	7,2(±0.02)	26,76	164,29(±0.70)	1,686(±0.00)	1350(±0.07)	1,8(±0.07)	0.877(±0.01)	1,686	0,461	6694(±83.43)	1,35(±0.07)	5960(±98.99)	8,54(±0.67)

Table S4.2: Mean concentrations (±standard deviation) of the measured physicochemical characteristics, biomarker concentrations and SARS-CoV-2 concentrations in wastewater at the Isipingo WWTP over a 12-week period.

Week	pH (@25 °C)	Temp °C	COD (mg/L)	TS (mg/L)	EC (µS/cm)	DO% (mg/L)	TDS (mg/L)	TS (mg/L)	VS (mg/L)	*crAssphage* copies/µl	*PMMoV* copies/µl	*HF183 Bacteroides* copies/µl	SARS-CoV-2 copies/µl
Week 1	7,38(±0.02)	19,7	528,57(±0.30)	0,67(±0.00)	646(±8.49)	25,8(±0.85)	0,419(±0.00)	0,67	0,4	4068(±78.49)	84,1(±5.48)	543(±28.28)	0,906(±0.02)
Week 2	7,44(±0.06)	22,22	175(±0.32)	0,666(±0.02)	662(±4.95)	26,1(±0.21)	0,479(±0.01)	0,666	0,378	7248(±109.60)	4,91(±0.27)	2474(±117.37)	0,552(±0.06)
Week 3	7,44(±0.00)	19,7	196,43(±2.21)	0,666(±0.02)	662(±3.54)	26,1(±0.21)	0,479(±0.01)	0,666	0,378	2985(±140.00)	2,6(±0.50)	497(±12.02)	0,3(±0.14)
Week 4	7,29(±0.02)	21,5	278,57(±0.95)	0,728(±0.00)	682(±2.12)	7,2(±0.49)	0,443(±0.01)	0,728	0,392	8006(±26.70)	3,27(±0.55)	1609(±12.73)	1,49(±0.01)
Week 5	7,48(±0.24)	20,12	257,14(±1.73)	0,09(±0.00)	668(±3.54)	8,7(±2.89)	0,434(±0.00)	0,09	0,356	3826(±195.87)	19,9(±1.34)	586(±15.56)	0,527(±0.07)
Week 6	7,51(±0.00)	20,13	257,14(±0.04)	0,596(±0.01)	664(±0.70)	6,8(±0.49)	0,432(±0.00)	0,596	0,304	1656(±97.58)	9,68(±0.72)	989(±94.05)	1,01(±0.06)
Week 7	7,25(±0.01)	21,54	178,57(±1.13)	0,59(±0.04)	622(±1.41)	5,7(±0.35)	0,404(±0.00)	0,59	0,34	3021(±26.87)	5,88(±0.38)	1552(±132.94)	0,881(±0.01)
Week 8	6,58(±0.00)	19,89	200(±1.56)	0,438(±0.01)	632(±6.36)	5,4(±0.14)	0,41(±0.00)	0,438	0,18	2147(±34.65)	17,4(±1.27)	1334(±118.80)	0,302(±0.03)
Week 9	7,39(±0.03)	21,18	221,43(±0.92)	0,468(±0.01)	492(±1.41)	12,1(±1.77)	0,32(±0.00)	0,468	0,1	729(±39.60)	7,14(±1.05)	1956(±98.28)	2,45(±0.50)
Week 10	7,12(±0.02)	23,03	185,72(±0.04)	0,794(±0.01)	627(±6.36)	0,6(±0.64)	0,407(±0.00)	0,794	0,452	812(±9.19)	2,64(±0.12)	2121(±18.34)	0,285(±0.50)
Week 11	7,13(±0.01)	19,54	285,72(±0.70)	0,752(±0.02)	735(±0.00)	3,7(±1.34)	0,478(±0.02)	0,752	0,39	6708(±16.97)	2,55(±1.56)	1042(±5.65)	2,95(±0.20)
Week 12	7,17(±0.02)	24,2	200(±0.00)	0,742(±0.02)	646(±2.12)	0,7(±0.21)	0,42(±0.01)	0,742	0,382	2241(±32.53)	0,314(±0.34)	1214(±17.67)	3,57(±0.65)

Table S4.3: Correlation co-efficient of measured physicochemical characteristics and the various biomarker and SARS-CoV-2 concentrations from Central WWTP.

Biomarker	pH (@ 25 °C)	EC (µS/cm)	TDS (mg/L)	Salinity	COD (mg/L)	TS (mg/L)	DO% (mg/L)
crAssphage	0.2651	0.2320	0.2859	0.2334	0.2454	0.1184	-0.3591
PMMoV	-0.2719	-0.3924	-0.4077	-0.3890	0.5015	-0.4624	0.5844
HF 183	0.4849	-0.2525	-0.2021	-0.2531	0.4294	-0.2676	-0.2489
SARS-CoV-2	-0.2718	0.2046	0.2282	0.2085	0.2634	0.3081	-0.0167

Table S4.4: Correlation co-efficient of measured physicochemical characteristics and the various biomarker and SARS-CoV-2 concentrations from Isipingo WWTP.

Biomarker	pH (@ 25 °C)	EC (µS/cm)	TDS (mg/L)	Salinity	COD (mg/L)	TS (mg/L)	DO% (mg/L)
crAssphage	0.1481	0.6382	0.6596	0.5124	0.2120	0.1986	0.2727
PMMoV	0.0903	-0.0180	-0.1056	0.0480	0.8989	-0.1269	-0.4579
HF 183	-0.1473	-0.3745	-0.2644	-0.4273	-0.5110	0.2841	-0.1503
SARS-CoV-2	-0.0131	-0.0439	-0.1840	0.1002	0.0454	0.2786	-0.3824

Appendices

Appendix 1:

Preparation of chemical oxygen demand (COD) reagents

Digestion solution:

1.0216 g K_2CrO_7 (dried) in 50mL dH_2O

Added 16.7mL concentrated H_2SO_4

added 3.33g $HgSO_4$

Dissolved, cooled, and diluted with 100mL of dH_2O

Sulfuric acid - silver sulphate reagent

5.5 Ag2SO4→ 1 kg H2SO4

Fresh Potassium hydrogen phthalate (KHP) standard

Crushed, and dried (KHP), and placed in an oven set at 120 °C for 1 hour

Dissolved 425 mg KHP in dH_2O

diluted with 500 mL dH_2O

Now, the concentration is noted to be 1000 mg O_2/L

Appendix 2

TBE Buffer (TRIS BORATE EDTA) – Preparation
Tris Borate EDTA (TBE) is a common buffer used in nucleic acid electrophoresis.

Buffer Preparation 1 (BP1.1)
TBE Buffer 10x Stock Recipe
108 g tris base
55 g boric acid
900 ml double-distilled H2O
40 ml 0.5 M EDTA solution (pH 8.0)
Adjusted volume to 1 L.

1x TBE Preparation
Diluted 10x concentrated TBE buffer 10-fold with ultrapure water.

Making up a 1% Agarose Gel:
Measured 1 g of agarose.
Mixed agarose powder with 100 mL 1xTBE in a microwavable flask.
Microwaved for 1-3 min until the agarose was completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Poured onto the casting tray and allowed to set.

Agarose Gel Electrophoresis
For the purpose of visualisation and purification, the common laboratory process for separating DNA by size (for example, length in base pairs) is gel electrophoresis. Using an electrical field, negatively charged DNA moved through an agarose gel matrix and toward a positive electrode

during electrophoresis. DNA fragments that are shorter than those that are longer go through the gel more quickly. Hence, by running a DNA fragment alongside a DNA ladder on an agarose gel, you may roughly estimate the length of the fragment (a collection of DNA fragments of known lengths).