

**Incidence and diversity of SARS-CoV-2 from wastewater samples recovered from
selected municipal wastewater treatment facilities in Buffalo City Metropolitan
Municipality, Eastern Cape Province, South Africa**



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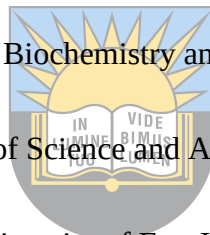
Okuhle Mayoyo (201406501)

A thesis submitted in fulfilment of the requirements for the degree of

Doctor Of Philosophy (Microbiology)

Department of Biochemistry and Microbiology

Faculty of Science and Agriculture



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Alice

5700

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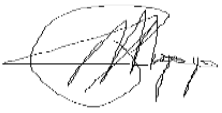
2024

DECLARATION

I, undersigned, thus declare that the Doctor of Philosophy thesis titled “Incidence and diversity of SARS-CoV-2 from wastewater samples recovered from selected municipal wastewater treatment facilities in Buffalo City Metropolitan Municipality, Eastern Cape Province, South Africa” submitted to the University of Fort Hare for the award of Doctor of Philosophy degree in Microbiology in the Faculty of Science and Agriculture is my original work except for the citations and has not been submitted to any other institution for consideration of any kind. I am aware of the University of Fort Hare’s Research Ethics Committee policy and have taken every precaution to comply with the regulation. The study was conducted under the ethics code OKO011SMAY01.



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DATE: 10th July 2024

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DEDICATION

This body of work and fruits to come are dedicated to my family: my uncles and aunts, especially my dearest mother, Nonzaliseko Mayoyo, my sister Kwakhanya Mayoyo and brother Linembasa Mayoyo. Your work and dedication to ensuring I complete what I have started is immeasurable. I will forever be grateful to you. May God bless you and expand all your territories.

Lastly ...

Xabiso Mayoyo



Ntombodumo Wasa Manto

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Mninawa Koti

Uthimna “My lollipop” Mpalo (It’s finally here my angel)

...your wishes were prayers to God’s ears. Continue resting in peace.

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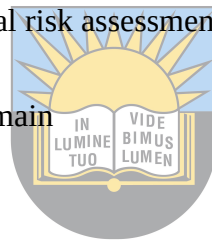
ABBREVIATIONS AND ACRONYMS

ACE2	– Angiotensin-converting enzyme 2
BCM	– Buffalo City Metropolitan
BCMM	– Buffalo City Metropolitan Municipality
cDNA	– complementary Deoxyribonucleic Acid
CDC	– Centre for Disease Control and Prevention
COVID-19	– Coronavirus Disease 2019
COV	– Coronavirus
C _T	– Cyclic threshold
ddPCR	– Droplet digital Polymerase Chain Reaction
DNA	– Deoxyribonucleic Acid
DWS	– Department of Water and Sanitation
ECP	– Eastern Cape Province
GC	– Genomic copies
HCOV	– Human Coronavirus
LSD	– Least Significant Difference
MERS	– Middle East Respiratory Syndrome
MERS-CoV-1	– Middle East Respiratory Syndrome Coronavirus type 1
NICD	– National Institute for Communicable Disease
NGS-SA	– Network for Genomics Surveillance in South Africa



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NSP	– Non-structural proteins
NTD	– N-terminal domain
OFR	– Open reading frames
PCR	– Polymerase Chain Reaction
PFU	– Plaque forming units
PHEIC	– Public Health Emergency of International Concern
$P_{(i)}$	– Probability of infection
PPE	– Personal protective equipment
QMRA	– Quantitative microbial risk assessment
RBD	– Receptor binding domain
RNA	– Ribonucleic Acid
RT-PCR	– Reverse Transcription Polymerase Chain Reaction
RT-qPCR	– Quantitative Reverse Transcription Polymerase Chain Reaction
SARS	– Severe Acute Respiratory Syndrome
SARS-CoV-1	– Severe Acute Respiratory Syndrome Coronavirus type 1
SARS-CoV-2	– Severe Acute Respiratory Syndrome Coronavirus type 2
SNP	– Single nucleotide polymorphism
UK	– United Kingdom
USA	– United States of America
UTR	– Untranslated region



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UV	– Ultraviolet
WBE	– Wastewater-Based Epidemiology
WHO	– World Health Organisation
WWTP	– Wastewater treatment plant
VBM	– Variants being monitored
VOC	– Variant of concern
VOI	– Variant of interest



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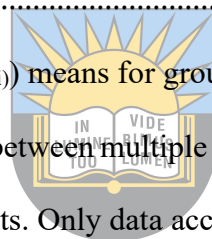
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General abstract

Wastewater surveillance has gained traction since the Severe Acute Respiratory Syndrome Coronavirus type 2 (SARS-CoV-2) induced global health crisis. SARS-CoV-2 is an enveloped *Betacoronavirus* that has evolved into numerous variants. The virus and its genomic variants contributed to COVID-19 transmission dynamics, disease severity, immune-response escape, and economic collapse in developing countries. This study elucidated the incidence and diversity of SARS-CoV-2 in municipal wastewaters in the Buffalo City Metropolitan Municipality (BCMM). Wastewater influent samples were aseptically collected monthly from ten wastewater treatment facilities in the municipality over a 12-month period from June 2021 to June 2022 to represent the four seasons of autumn, spring, summer and winter in South Africa. A total of 494 wastewater-influent samples were collected, and the total viral RNA was extracted from the samples following the standard operating protocols. The Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) was utilised to screen for the nucleocapsid phosphoprotein-inducing gene of SARS-CoV-2. Subsequently, the TaqMan SARS-CoV-2 mutation panel genotyping-molecular-specific RT-PCR assay, targeting the single nucleotide polymorphisms (SNPs) (*delH69V70*, *E484K*, *K417N*, *L452R*, *N501Y*, *P681H* and *P681R*), was used to rapidly detect SARS-CoV-2 variants with the panel related to the B.1617.2 and the B.1.1.529 lineages of SARS-CoV-2 variants. About 71% ($n = 351$) of the 494 samples were positive for the nucleocapsid gene, indicating SARS-CoV-2 presence. The total viral load retrieved from all study sites in winter (2021) was a total concentration of 22.08×10^4 GC/mL, spring (2021) 13.09×10^4 GC/mL, summer (2021-2022) 7.61×10^4 GC/mL and autumn (2022) 29.30×10^4 GC/mL. The circulation of variants of concern (VOC) was ascertained as 93% of SARS-CoV-2 samples ($n = 45$) with viral loads ≥ 1000 GC/mL were positive for the target SNPs. The predominant amino acid mutation was the *N501Y* (22%), shared between Alpha (B.1.17), Beta (B.1.33, B.1.351) and Omicron lineages. The geospatial

analysis revealed the EB WWTP, a combined sewer network, as the diverse SARS-CoV-2 genome hotspot. Nevertheless, the spike protein mutations linked to VOCs were circulating in other areas. Overall, a direct correlation was observed between SARS-CoV-2 signals in wastewater, VOC-linked SNPs and clinical cases in the region. A significant spike in all three factors was observed in September 2021, during the third epidemic. VOC Delta (B.1.617.2) was the predominant variant during this period. Furthermore, a more significant peak was in SARS-CoV-2 with a total viral load exceeding 20×10^4 GC/mL, *L452R*, *N501Y* and *P681H* were detected more frequently during this epidemic driven by BA.4 & BA.5. This finding emphasises the significance of wastewater-based epidemiology as a non-invasive population-based surveillance strategy to monitor circulating pathogens and diseases. Quantitative Microbial Risk Assessment (QMRA) model was carried out to evaluate the potential occupational health risks of exposure to SARS-CoV-2 amongst wastewater treatment plant workers at different exposure scenarios. As expected, the $P_{(i)}$ values for wastewater treatment plant (WWTP) operators working for 6 hours without personal protective equipment (PPE) and inadvertently exposed to up to 20 mL of contaminated bioaerosol, droplets and fomites were 35.9×10^{-2} (winter 2021), 5.1×10^{-2} (spring 2021), 6.8×10^{-2} (summer 2021-2022), and 18.2×10^{-2} (autumn 2022). There was no significant correlation between the seasons and the potential health risks ($p \geq 0.05$). Therefore, the potential health risks associated with the incidence of the SARS-CoV-2 genome in wastewater across the epidemiological seasons in the study WWTPs are low at a significance level of 95%. Nevertheless, the controversies in the literature regarding the viability of SARS-CoV-2 in the wastewater milieu accentuate the coronavirus as a public health concern and demand stringent precautionary measures in occupational settings. Irrevocably, the incidence of the SARS-CoV-2 genome in the municipal wastewaters of the BCCM is an indicator of the circulation of the virus in the metropolitan. The implication of occupational and environmental safety protocols is crucial to curb the

dissemination of the pathogen, and wastewater-based epidemiology coupled with QMRA are imperative approaches that could be continuously employed as complementary tools for clinical surveillance. This surveillance strategy for communicable diseases is imperative for reaching the 2030 Agenda for Sustainable Development.



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1. CHAPTER ONE

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GENERAL INTRODUCTION

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Introduction

As of March 2020, a viral agent's emergence profoundly impacted the global community and brought it to a standstill. The etiological agent-related complications immediately claimed a vast number of lives. Consequently, several countries, including ours, declared a national state of disaster as the World Health Organisation (WHO) announced the global coronavirus pandemic.

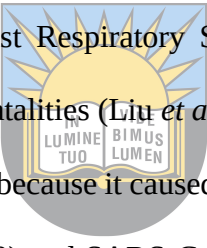
A call was made to find cost-effective and brisk measures to study and monitor the novel agent driving the biological catastrophe due to the severity of the then-emerging disease. Wastewater-based epidemiology fits the criteria and is excellent for tracking diseases via human excreta, thereby aiding as a proxy for disease trends within various communities (Corpuz *et al.*, 2020; Prado *et al.*, 2021). Wastewater-based epidemiology serves as a prospective predictive monitoring system for infectious disease epidemics. It can be applied as a complementary methodology for present infectious disease surveillance strategies (Sims and Kasprzyk-Hordern, 2020) through the evaluation of a broad spectrum of pathogens inhabiting wastewater matrices (Kumar *et al.*, 2020; Medina *et al.*, 2022).

Wastewater remains a viable reservoir for emerging pathogens of public health significance. Bacterial and viral pathogens are among the myriad microorganisms ubiquitous in wastewater (Corpuz *et al.*, 2020). The Severe Acute Respiratory Syndrome Coronavirus type 2 (SARS-CoV-2) is within the populace of pathogens in wastewater despite initially being a respiratory virus (Wu *et al.*, 2020; Xiao *et al.*, 2020; Xu *et al.*, 2020). The virions move through the gastrointestinal tract and are excreted with faecal matter, thus ending in the wastewater milieu (Hamouda *et al.*, 2021). Furthermore, studies have indicated the occurrence of SARS-CoV-2 genomic fragments in hospital wastewater (Tiacharoen *et al.*, 2022), raw wastewater,

wastewater samples after secondary treatment, and also in freshwater environments (Haramoto *et al.*, 2020; Randazzo *et al.*, 2020; Rimoldi *et al.*, 2020).

The documented reports trail preceding literature reports on the 2003 SARS-CoV-1-induced pandemic. The studies highlighted wastewater systems and potential pathways for distributing virions and viral fragments (Yu *et al.*, 2004; McKinney *et al.*, 2006). Essentially, wastewater is a probable exposure pathway that facilitates viral transmission via the faecal-oral route, airborne transmission (e.g. inhalation of bioaerosol) (Kang *et al.*, 2020; Tang *et al.*, 2020).

The eruption of SARS-CoV-2 marked the third and most devastating human pathogenic coronavirus outbreak within the 21st century. It trails the 2003 Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), which claimed over seven hundred lives and preceded the 2012 epidemic of the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), which resulted in over eight hundred fatalities (Liu *et al.*, 2020). MERS-CoV exhibited higher mortality and lower transmission rates because it caused more severe conditions than the novel Coronavirus Disease 2019 (COVID-19) and SARS-CoV (Feng *et al.*, 2020; Petrosillo *et al.*, 2020).



The logo of the University of Fort Hare is centered over the text. It features a shield with a sunburst at the top, a book in the center, and the motto 'LUMINE TUO BIMUS LUMEN' on a banner below the book. The text 'University of Fort Hare' and 'Together in Excellence' are written in a serif font below the shield.

Cui *et al.*, (2019) and Cao *et al.*, (2021) depict SARS-CoV-2 as a positive single-stranded RNA virus (+ssRNA) characterised by a “crown-like” morphology, attributed to the incidence of protrusions (spike glycoproteins) on the lipid bilayer envelope (Caldas *et al.*, 2020; Farkas and Haigh, 2021) when observed by electron microscopy. The enveloped virus has a large genome (29.9 kb) and belongs to the Coronaviridae family, order Nidovirales (V’kovski *et al.*, 2020; Brant *et al.*, 2021; Rubio, de Toro and Pérez-Pulido, 2024). According to Low *et al.*, (2021) and (Abdolmaleki *et al.*, 2022), among the genomic attributes differentiating SARS-CoV-1 and SARS-CoV-2 is the occurrence of a novel furin proteolytic site in the spike glycoprotein of

SARS-CoV-2 (Laue *et al.*, 2021; Zhao *et al.*, 2022). This feature might potentially affect the pathogenicity and the viral life cycle (Johnson *et al.*, 2021; Peacock *et al.*, 2021).

SARS-CoV-2 has since become a significant global health concern, as the virus is the pathogenic agent causing the Novel Coronavirus Disease 2019 (COVID-19) (Jernigan, 2020). The initial COVID-19 outbreak was primarily recognised in Wuhan, China, in December 2019 (Zhu, Wei and Niu, 2020). Subsequently, it became a global pandemic (WHO, 2020). The disease exhibited quite a range of symptoms, such as flu-like symptoms, conjunctivitis, diarrhoea, and more severe symptoms, including breathing complications and chest pains (Sheikhi *et al.*, 2020).

SARS-CoV-2 has instigated so much obliteration since the pandemic ensued. Accompanied by a proliferation of SARS-CoV-2 variants, a subsequent surge in COVID-19 cases was observed in the different waves of infection (WHO, 2021). In South Africa, local cases of variants of concern (VOC), such as Delta (first detected in India) and Alpha, were primarily reported in May 2021. The Delta variant drove the most dire epidemic with an extreme hospitalisation rate (Jassat *et al.*, 2022; Madhi *et al.*, 2022; Mahase, 2022; Yang and Shaman, 2022).

Much like any other virus, SARS-CoV-2 has been gradually evolving and mutating with time. The mutations, including the most prominent ones on the spike protein, have altered the rate at which SARS-CoV-2 is transmitted and disease intensity. Furthermore, the mutations have reduced the efficacy of some therapeutics, vaccines, and diagnostic tools (Feng *et al.*, 2020; Harvey *et al.*, 2021). SARS-CoV-2 variants can be attributed to detrimental modifications in the COVID-19 epidemiological landscape across the globe (Forster *et al.*, 2020; Chadha, Khullar and Mittal, 2022; Okoh *et al.*, 2022).

The Centre for Disease Control and Prevention, (2022) defines a variant as a viral genome that may comprise a single or multiple genomic modifications. Such modifications may

differentiate one variant from other SARS-CoV-2 variants. As anticipated, accounts of numerous SARS-CoV-2 variants have been observed globally throughout the pandemic (Korber *et al.*, 2020; Zhou *et al.*, 2021). A group of variants with similar genetic changes, such as lineages, may be classified as Variants of Concern (VOC), Variants of Interest (VOI), and Variants being Monitored (VBM). The mutual features observed in these genomic variations mandated public health response (Rambaut *et al.*, 2020; Konings *et al.*, 2021).

New mutated SARS-CoV-2 lineages with amino acid mutations *N501Y*, *K417N*, and *E484K* were detected after the first epidemic in South Africa by the Network for Genomics Surveillance in South Africa (NGS-SA). The strain was detected in the worst-affected metropolitan areas within the Eastern Cape Province among two other provinces in South Africa (Tegally *et al.*, 2020). The NGS-SA reported that the strain, not previously seen in South Africa, comprised a cluster of slightly different variants with ten to twenty mutations (NICD, 2021).



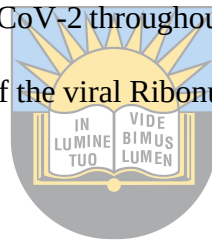
Multiple SARS-CoV-2 variants have been observed, with a specific mutation known as D614G. Variants with this mutation tend to spread at an accelerated rate. Some of the early detected variants of concern are represented in Table 1.1

Table 0.1: Early accounts of SARS-CoV-2 genomic variants.

Name	Alternative name	Country of first detection	Reference
B1.1.7	501Y.V1	United Kingdom	(WHO, 2020)
B1.1.7 + E484K			
B1.351	501.V2	South Africa	
B1.4.29	S:452R	United States of America	
P1	501YV3	Brazil/Japan	
B1.617.2	-	India	

The preliminary step towards managing SARS-CoV-2 infections is a diagnosis, wherein the causative virus is identified. Early and precise diagnosis of COVID-19 is crucial for epidemic surveillance, treatment and vaccine development (Farkas and Haigh, 2021). Samples for diagnostic evaluation can be obtained from clinical (e.g., through nasopharyngeal fluids) and environmental (e.g., wastewater) sources.

With a wide array of diagnosis evaluation tests (Rando *et al.*, 2022), molecular techniques and genome sequencing are employed to detect, quantify and characterise the viral genome obtained from municipal wastewater (Habibzadeh *et al.*, 2021; Heijnen *et al.*, 2021; Child *et al.*, 2023). SARS-CoV-2 genome sequencing contributes to the diagnosis of COVID-19 when the nature of the infection is unique or complicated (Hemarajata, 2020), as the technique has identified genetic variations in SARS-CoV-2 throughout its circulation (Pohl *et al.*, 2021). This is all on the condition that the purity of the viral Ribonucleic acid (RNA) is ensured during the extraction procedure.



Another essential tool in diagnosing COVID-19 is the amplification of target genes through Polymerase Chain Reaction (PCR). Primarily, the diagnosis of SARS-CoV-1 was based on epidemiologic and clinical criteria. However, many respiratory viral infections had similar clinical presentations (Subbarao and Mahanty, 2020; Vetter *et al.*, 2020); hence, Emery *et al.*, (2004) recommended more reliable diagnostic tests for SARS-CoV infections.

The real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) and the Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) bridge that gap by enabling the rapid and accurate detection of such infections and quantifying the viral genome, including SARS-CoV-2. Real-time RT-PCR remains the benchmark for detecting viral particles (Liu *et al.*, 2020; Oliveira *et al.*, 2023), as it detects target gene amplification in the exponential phase of the reaction. RT-PCR can detect SARS-CoV-2 genomic determinants in

upper respiratory secretions and wastewater samples (Medema *et al.*, 2020; Kevadiya *et al.*, 2021). To complement the technique's rapid detection and accuracy, Mo *et al.*, (2012) elucidated RT-PCR as a relatively uncomplicated and inexpensive technique extensively utilised in biomedical sciences for quantitative analysis.

The combination of such techniques, RT-qPCR and single nucleotide polymorphism (SNP) genotyping, in monitoring the genetic diversities and disease burden in specific communities is worth noting.

1.2 Statement of research problem

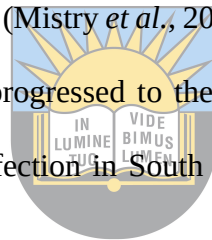
The most recent COVID-19 pandemic was the most devastating human pathogenic coronavirus outbreak (Liu *et al.*, 2020). The pandemic preceded the previous health crises caused by coronavirus, i.e., the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) epidemic, which impacted seventeen countries, resulting in seven hundred and forty-four deaths (Sampathkumar *et al.*, 2003), and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Prabhat and Suresh, 2020) which infested twenty-one countries and claimed approximately eight hundred and sixty lives (Al-Omari *et al.*, 2019; Rabaan *et al.*, 2021).

The exceedingly contagious SARS-CoV-2 primarily erupted in Wuhan, Hubei Province, China (Chakraborty *et al.*, 2021). The initial phase of the outbreak began when several individuals who had gone to the Huanan (Southern China) Seafood Wholesale Market presented “pneumonia of unknown aetiology.” By the time the etiological agent was identified, person-to-person transmission had occurred (Li *et al.*, 2020).

The second phase of the distribution of SARS-CoV-2 was through nosocomial infections and family transmission within the Hubei Province hospital setting (Chan *et al.*, 2020; Wang *et al.*, 2020). The epidemic spread to Beijing City and Guangdong Province (Kang *et al.*, 2020) and

Taiwan, where the infection index case was a Wuhan traveller (Y.-C. Liu *et al.*, 2020; Wilasang *et al.*, 2022). Consequently, SARS-CoV-2 infections rapidly spread to other regions of Asia and, subsequently, to different continents through international travel.

Since the unprecedented pandemic ensued, over 6.8 million fatalities, with approximately 753 million confirmed cases, have been documented worldwide (Koelle *et al.*, 2022; WHO, 2023). Due to the hasty transmission and pathogenicity, the World Health Organisation declared SARS-CoV-2 a Public Health Emergency of International Concern (PHEIC) in March 2020 (WHO, 2020). After the primary COVID-19 case in South Africa, several SARS-CoV-2 variants of concern (VOC) have dominated COVID-19 infections. These variants include the Alpha (B.1.1.7) variant, primarily identified in late September 2020 and rapidly becoming the United Kingdom's predominant strain (Mistry *et al.*, 2022). The Beta (B.1.351/501.V2) variant, initially observed in October 2020, progressed to the most prevalent strain and driving the second epidemic of SARS-CoV-2 infection in South Africa (Adepoju, 2021; Tegally *et al.*, 2021).



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The Delta (B.1.617.2) variant, on the other hand, was initially observed in December 2020 and was accountable for the immense escalation in cases, leading to the second epidemic in India and post-immunisation infections in the United States of America (Mistry *et al.*, 2022) with higher transmissibility. The Omicron (B.1.1.529) variant was initially detected through genomic surveillance laboratories in South Africa in November 2021 and has been circulating since discovery. Also, the BA.5 and sub-lineages were dominant in November (2022), December (2022) and January (2023), with BA.2.75 detected at low prevalence. BQ.1 and sub-variants have increased in prevalence since October 2022 and were the dominant Omicron lineages (January 2023); the XBB.1.5 was detected in December 2022 and circulating in January 2023.

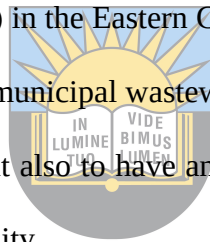
Sixteen months into the pandemic, the hospital environments in South Africa were completely overwhelmed, as there were concerns about insufficient beds and ventilators. This deterred the advancements towards the United Nations' 2030 Agenda for Sustainable Development by primarily affecting Sustainable Development Goals 3: Good health and well-being, among others (Clemente-Suárez *et al.*, 2022; Qadeer *et al.*, 2022). The highly contagious coronavirus threatened the health and well-being of people and universal health coverage. Throughout the abrupt COVID-19 epidemics, healthcare facilities were repressed while the national healthcare facilities faced shortages of ambulances and equipment such ventilator machines. Such resulted in delayed treatment and consequently increased mortality (Rapanyane, 2022; Sono-Setati *et al.*, 2022).

The commencement of the COVID-19 pandemic prompted an exceptional catastrophe. Not only has the global health system been shocked, but the quality of education as well. Students have suffered various attendance breaks as different cohorts were allowed back to school at different intervals due to restrictive measures and general fear. Moreover, the pandemic had substantial economic consequences, exceeding the impact of the 2008 financial crisis, leading to extensive unemployment and poverty on a global scale (Abdool Karim and Baxter, 2022; Bustamante-Sanchez *et al.*, 2022; Clemente-Suárez *et al.*, 2022; Qadeer *et al.*, 2022; Spulbar *et al.*, 2022).

Due to the drastic economic decline, wastewater monitoring became the most cost-effective and suitable medium to track COVID-19 indicators in various populations. The incidence of SARS-CoV-2 in wastewater samples is due to faecal excreta. The virus-infected mucus of COVID-19-positive individuals moves through the gastrointestinal system into biological waste. The problem with a viable virus in environmental samples is faecal aerosol transmission, consequently contributing to COVID-19 epidemics (Kang *et al.*, 2020; Tang *et al.*, 2020).

The Eastern Cape is one of the country's impoverished provinces with poor infrastructure and service delivery. It is not rare to find dilapidated wastewater treatment plants, blocked sewer systems, and open defecation, among other things, leaving biological waste and pathogens of medical importance exposed or disseminated into the environment. Consistent with numerous news reports, Buffalo City Metropolitan is among several municipalities found accountable for sewage spills to the environment (Sgqolana, 2022a; Sgqolana 2022b; Fray, 2023; Mndayi, 2023; Velaphi, 2024).

This alone may be a contributing factor to the resurgence of a variety of pathogens. The factors mentioned earlier are a significant concern of public and environmental health importance for Buffalo City Municipality, with the second largest population density of 317.8/km² (Buffalo City Metropolitan Municipality, 2019) in the Eastern Cape Province, and South Africa, which necessitates adequate surveillance of municipal wastewater, not only as an early warning sign of the occurrence of SARS-CoV-2 but also to have an understanding of the epidemiology of the prevalent variants in the municipality.



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Additionally, the incidence of SARS-CoV-2 and broad spectrum of variants of concern in municipal wastewater reflects the incidence and pervasiveness of the coronavirus in the municipal region. Often, clinical case data solely represents data from tested individuals and may not necessarily reflect the community status. Wastewater surveillance captures viral particles from asymptomatic and symptomatic individuals, and tested and non-tested individuals (McElfish *et al.*, 2021; Souch and Cossman, 2021; Nomali *et al.*, 2023).

There is a paucity of information concerning the incidence and persistence of SARS-CoV-2 in municipal wastewaters in the Buffalo City Municipality, Eastern Cape, South Africa. Hence, this present study sought to elucidate the incidence and diversity of SARS-CoV-2 in some wastewater milieus in Buffalo City Municipality, Eastern Cape, South Africa.

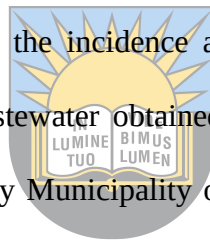
1.3 Hypothesis

This study was premised on the null hypothesis that there is no prominent difference in the molecular epidemiology of the SARS-CoV-2 genomes in the municipal wastewater obtained from the designated wastewater treatment facilities in the Buffalo City Municipality during the surveillance period (June 2021 – June 2022) amidst the COVID-19 global pandemic.

1.4 Aim and objectives

1.4.1 Research aim

This study was aimed at elucidating the incidence and the diversity of the SARS-CoV-2 genome attained from municipal wastewater obtained from selected municipal wastewater treatment facilities in the Buffalo City Municipality of the Eastern Cape Province in South Africa.



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1.4.2 Specific objectives

1. To collect wastewater influent samples from selected municipal wastewater treatment facilities within the Buffalo City Municipality for a surveillance period of 12 months and analyse the samples to evaluate the incidence and distribution of the SARS-CoV-2 genome.
2. To screen the influent samples for Single Nucleotide Polymorphism (SNP) to determine the relevant SARS-CoV-2 mutations circulating in the communities within the catchment areas, carryout a spatiotemporal analysis and correlate the findings with the

frequency of SARS-CoV-2 in the wastewater and clinical cases of COVID-19 reported in the Eastern Cape Province between June 2021 and June 2022.

3. To extrapolate the seasonal distribution of SARS-CoV-2 genomes across the target municipal wastewater treatment plants.
4. To carry out a Quantitative Microbial Risk Assessment (QMRA).



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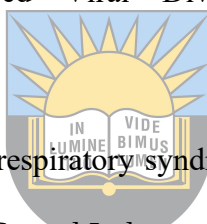
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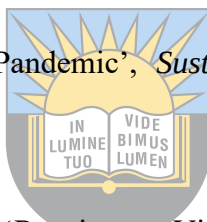
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2. CHAPTER TWO



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LITERATURE REVIEW
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LITERATURE REVIEW

Humanity has cohabitated the Earth with various organisms within the animal kingdom and a broad spectrum of microorganisms. In the documented existence of all organisms, humans have survived numerous disease outbreaks and magnitudes of pandemics, most of which have detrimental effects on major biological systems such as the nervous, cardiovascular, and respiratory systems, crucial for the basic functioning of the human body. Therefore, this review briefly explores some of the viral pandemics of the past two decades that mainly affected the human respiratory system. This review further explicates the causative agent driving the most recent coronavirus pandemic.

2.1 The Human Respiratory System



The respiratory system is an intricate organisation of organs and tissues responsible for gas exchange that facilitates breathing. The primary function is extracting atmospheric oxygen from the air to the blood, reaching the body's cells and, in reverse, releasing carbon dioxide carried in deoxygenated blood into the atmosphere. The human respiratory system is divided into the upper and lower respiratory tracts (Dominelli *et al.*, 2019; Jaeger *et al.*, 2019; Ahookhosh *et al.*, 2020).

The upper respiratory tract is anatomically associated with an interconnected cavity system composed of the nose, mouth, rhino-pharynx, and oral cavity. The Eustachian tube connects it to the mouth and middle ear cavity (Liu *et al.*, 2023). Each anatomical component has a specific characteristic, including oxygen concentration, epithelial cell type, temperature, and humidity. The lungs play a vital role in the primary function of the upper respiratory system as they facilitate the filtration, heating, and humidification of the passing air (Santacroce *et al.*, 2020).

On the other hand, the lower respiratory tract includes the airways that carry oxygen (trachea, bronchial, and bronchial) and the alveoli, where gas exchange occurs. The conducting airways are surrounded by respiratory epithelium similar to that of the upper respiratory tract, with epithelium cells gradually moving along the respiratory tree towards a cuboidal shape. The lungs' alveoli are lined with functionally different epithelial cells (Man *et al.*, 2017; Man *et al.*, 2019; Gaibani *et al.*, 2021).

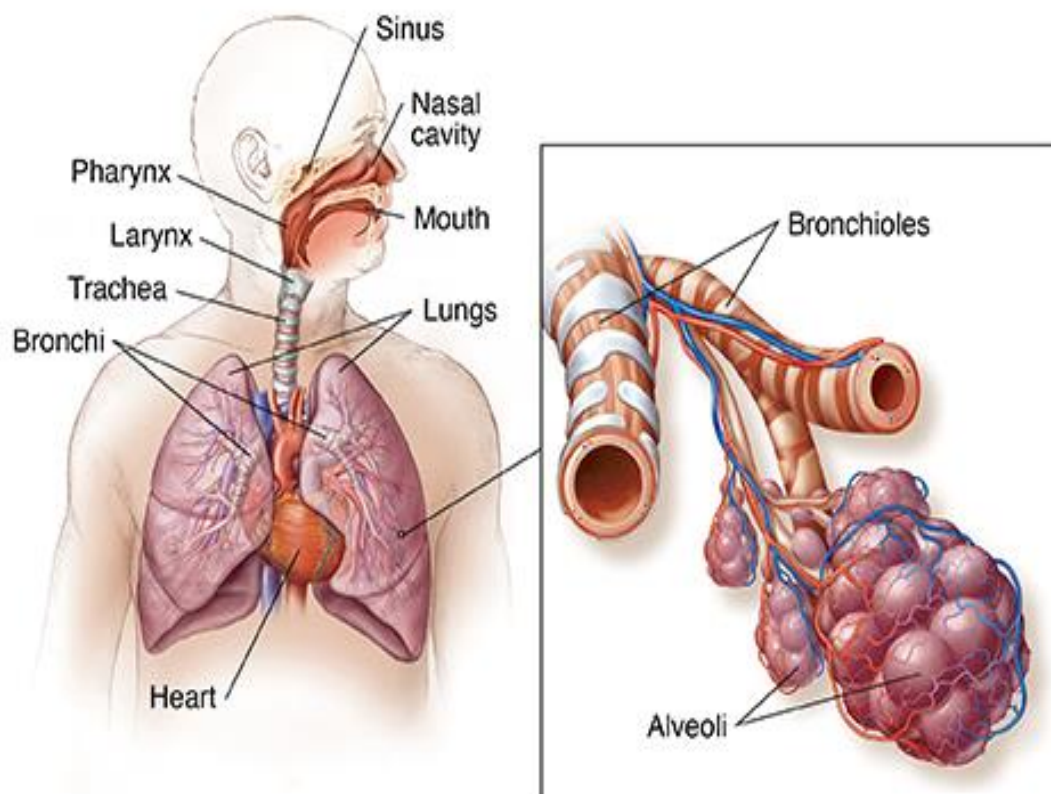


Figure 2.1: Pictorial depiction of the anatomy of the human respiratory system displaying the location of the sinus, nasal cavity, mouth, pharynx, larynx, trachea, lungs, bronchi, hearts and an enlarged display of the bronchioles and alveoli (Corwell Health Diabetes and Endocrinology, 2024) .

2.1.1 Microbiological perspective and associated pandemics

From a microbiological viewpoint, the respiratory system is continuously exposed to various microorganisms, including bacterial pathogens, fungi, and viruses. These microorganisms can potentially cause upper respiratory or lower respiratory infections and diseases such as pneumonia, sinusitis, bronchiolitis, tuberculosis, human respiratory syncytial virus disease and the novel coronavirus diseases 2019. Amidst the global pandemic caused by the latter, a tremendous death toll, distress and turmoil in everyday life have been witnessed (Burney *et al.*, 2017; Peltola *et al.*, 2020; Tao *et al.*, 2021; Volz *et al.*, 2021; Elkhatab *et al.*, 2022).

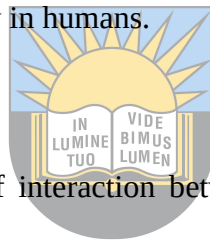
Pandemics are not a modern phenomenon; fatal pandemics have occurred throughout documented history. A pandemic relates to the incidence of a health condition related to its anticipated frequency and dissemination in a geographical area. Therefore, the term pandemic denotes the global dissemination of an outbreak/health condition. Several documented deadly pandemics are infamous for their detriment to the United Nations' 2030 Agenda for Sustainable Development (Shulla *et al.*, 2021).

They have had detrimental impacts on the status quo, including the destruction of political regimes, and have caused psychosocial and economic burdens. Generally, rising infectious diseases have initiated the deadliest and most disastrous pandemics, with the 1918 influenza pandemic being a great example (Huremovic, 2019; Piret & Boivin, 2021).

Most of these communicable disease pandemics, such as plaques, flu, SARS, and MERS, result from animal zoonosis to humans. The zoonotic pathogens are spread to humans by heightened interactions among humans and animals. Activities encouraging such transmissions include hunting, farming, and international trade (Belay *et al.*, 2017; Latif *et al.*, 2020).

Parrish *et al.*, (2008) and Li *et al.*, (2023) highlight how the progression of cross-species transmission occurs. According to Piret and Biovin, (2021), cross-species transmission involves five steps:

1. In the natural environment, pathogens transmit disease to animals only.
2. Evolution occurs, and the pathogen develops mechanisms that enable transmissibility to humans, exclusive of continuous transmission amongst humans.
3. The pathogen undertakes secondary transmission between humans at low frequencies.
4. The pathogen still infects animals, but continuous secondary transmission amongst humans transpires without contact with the animal.
5. The pathogen causes a disease which solely infects humans.
6. The disease occurs exclusively in humans.



The nature and rate of recurrence of interaction between humans and animals possessing pathogens may contribute to regulating the risk of cross-species transmission (Miller *et al.*, 2017; Piret and Biovin, 2021; Qian *et al.*, 2022).

Pathogenic microorganisms, including viruses, are the etiological agents driving major pandemics in history. Viruses are miniature infectious and obligate intracellular parasites. Encompassed in a protective protein coat is a segment of nucleic acids, i.e., DNA genome or RNA genome. The intracellular parasites are clustered according to their morphology (size, shape, chemical composition, genome structure) and replication mode.

Two morphology categories are distinct in viruses:

1. “Helical morphology: is observed in the nucleocapsid of filamentous and pleomorphic viruses. Helical nucleocapsids consist of a helical array of capsid proteins (protomers) wrapped around a helical filament of nucleic acids.

2. Icosahedral morphology is characteristic of the nucleocapsids of many” “spherical” viruses. The number and arrangement of the capsomeres (morphology subunits of the icosahedron) are helpful in the identification and classification. Many viruses have an outer envelope.”

(Louten, 2016; Twarock, 2019; Parvez, 2020; Robb, 2022; Voytekhevsky, 2023).

Pandemics that emerged in the last two decades are linked to the emergence of viruses, as Bhadoria *et al.*, (2021) reported. The pandemics frequently involved respiratory viruses such as the influenza viruses causing influenza A H1N1 Swine flu and coronaviruses, instigating three significant global pandemics, namely the Severe Acute Respiratory Syndrome Coronavirus 1, Middle East Respiratory Syndrome Coronavirus, and Severe Acute Respiratory Syndrome Coronavirus 2 pandemics. Table 2.1 denotes some of the virus-induced pandemics between 2002 and 2023.

Table 2.1: Virus-induced pandemics observed worldwide between 2002 and 2023. Four of the viral pandemics that arose in the past 20 years are associated with the invasion of the respiratory system.

Name	Etiological agent	Period	Country of Origin	Death Toll	References
SARS	SARS-CoV-1	2002 - 2003	China	774	(Peeri, 2020)
Swine Flu	H1N1 Influenza A virus	2009 - 2010	Mexico	284 400	(Aris-Brosou <i>et al.</i> , 2017)
MERS	MERS-CoV	2012	Saudi Arabia	858	(Peeri, 2020)
Ebola	Ebola virus	2013 - 2016	Guinea	11 310	(Faria <i>et al.</i> , 2016)
Zika virus	Zika virus	2015-2016	Brazil	-	(Kazmi <i>et al.</i> , 2020)
COVID-19	SARS-CoV-2	2019 - 2023	China	>6 000 000	(Wise, 2023)

Some of the significant viral pandemics from 2002-2023 include:

- **The 2009 Influenza A H1N1 Swine flu pandemic** (involves infection invasion of the human respiratory system): H1N1 influenza A is the etiological agent causing Swine flu. According to Scalera *et al.*, (2009), Influenza A group viruses had initiated two significant pandemics. The first documented pandemic emerged in 1918 and was labelled the 1918 Spanish flu. The second influenza A group virus pandemic was the Swine flu. Three-way re-assortment involving avian, swine and humans caused the virus's progression. The triple re-assortment brought about the term "swine flu", which is an inaccurate label as the virus uses humans as hosts (Bhadoria, Gupta and Agarwal, 2021).

The disease primarily emerged in Mexico (Echevarria-Zuno *et al.*, 2009) and was disseminated globally (i.e., 121 countries), consequently infecting a tenth of the global population. The global transmission route was through international travel and global trade. Persons infected by the virus display symptoms such as fever, cough, sore throat, myalgia, shortness of breath, vomiting and diarrhoea. Respiratory failure was the cause of death in disease cases. A study by Pfischer *et al.*, (2016) revealed that among 9555 cases of severe maternal morbidity, (5 %) had respiratory disease due to the H1N1 influenza. (Cao *et al.*, 2009; Mukhopadhyay *et al.*, 2010; Engels *et al.*, 2017).

- **The 2013-2016 Ebola pandemic:** Ebolaviruses, members of the Filoviridae family belonging to the order Mononegavirales, are the etiological agents causing the Ebola disease. The clinical manifestations of the disease include non-specific viral syndrome, elevated body temperature, muscle aches, fatigue, subsequent vomiting and diarrhoea, which lead to loss of extracellular fluids and dehydration. Individuals displaying severe

infection suffer gastrointestinal bleeding and mucosal haemorrhage (Holmes *et al.*, 2016; Aris-Brosou *et al.*, 2017).

Over 20000 people were infected by the Ebola virus. The haemorrhagic fever that killed 11000 individuals erupted in Guinea, West Africa, rapidly spreading to Liberia and Sierra Leone. One of the earliest reported cases in Guinea was that in December 2013 of an 18-month-old boy. The source of infection was direct contact with the body fluid of the infected bat. The main route of transmission was contaminated blood and body fluids (Chertow *et al.*, 2014; Chowell and Nishiura, 2014; Dixon and Schafer, 2014; Goeijenbier *et al.*, 2014).

- **The 2015 Zika Virus Pandemic:** 2015 Brazil was distressed by the re-emergence of a viral disease caused by the Zika virus. This disease was initially reported in Uganda in 1947 and was named Zika after the Zika forest of Uganda. The Zika Virus pandemic began in 2015 with relatively rare deaths, spreading to over 87 countries. The virus is a member of the family Flaviridae and is assigned to the genus Flavivirus. The virus typically infects monkeys and is circulated in the enzootic cycle of mosquito-monkey-mosquito transmission. The virus spreads through the bite of *Aedes* mosquitos, namely *Aedes aegypti* and *Aedes albapictus* (Nhan and Musso, 2015; Afolabi, Sani and Okunowo, 2016; Gutiérrez-Bugallo *et al.*, 2019).

Like other flaviviruses, human beings are accidental virus hosts (Cerbino-Neto *et al.*, 2016). The most crucial transmission of the Zika virus is vertical transmission at the time of delivery or during the antenatal period, causing neural abnormalities ranging from microcephaly to the development of neurocognitive disorders in adulthood. (Van der Linden *et al.*, 2016; Heukelbach *et al.*, 2016; Baud *et al.*, 2017; Schwartz, 2017; Bhadoria, Gupta and Agarwal, 2021).

A maximum of three pandemics that emerged in the last two decades were of coronavirus origin, affecting the respiratory system. The first was the emergence of the Severe Acute Respiratory Syndrome Coronavirus 1, which caused SARS and lasted for a year. The second case was a Saudi Arabian outbreak caused by the Middle East Respiratory Syndrome Coronavirus. The third and most recent coronavirus pandemic was caused by the Severe Acute Respiratory Syndrome Coronavirus 2.

2.1.2 Viral invasion of the human respiratory system

In cases of infection of the respiratory system, the primary course of entry for viruses is through the respiratory tract, which includes the nose, throat, and lungs, causing common colds, flu, and more severe respiratory illnesses like COVID-19.

The invasion of the human respiratory system, or rather the primary route of viral infection of the human respiratory system, constitutes three mechanisms: direct transmission, indirect transmission, and airborne transmission (Pöhlker *et al.*, 2023). Direct transmission of respiratory viruses among humans involves the conventional (physical) contact from an infected person to a susceptible person (e.g., shaking of contaminated hands) and, consequently, the self-inoculation of mucous membranes. The indirect transmission mode occurs through fomites, where viruses are transferred from contaminated inanimate objects (door handles, keys and cell phones) (Rabaan *et al.*, 2021).

The airborne transmission of respiratory viruses is through trajectories such as aerosols and droplets. Contaminated aerosols are produced during the respiration of an infector. Aerosols are small, have a long-time suspension rate and long-distance transmission, and have easy access to the deep respiratory tract, leading to a high infection risk and a significant threat to

public health. Conversely, droplets are produced through coughing, sneezing, and talking. They do not remain suspended in the air for long as they are relatively larger. Droplets can travel one metre before settling the mucosa of a susceptible contact (Jones and Brosseau, 2015; Otter *et al.*, 2016; Das *et al.*, 2020; Paton *et al.*, 2021). Understanding the mechanisms of respiratory viral transmission is a crucial preventative measure.

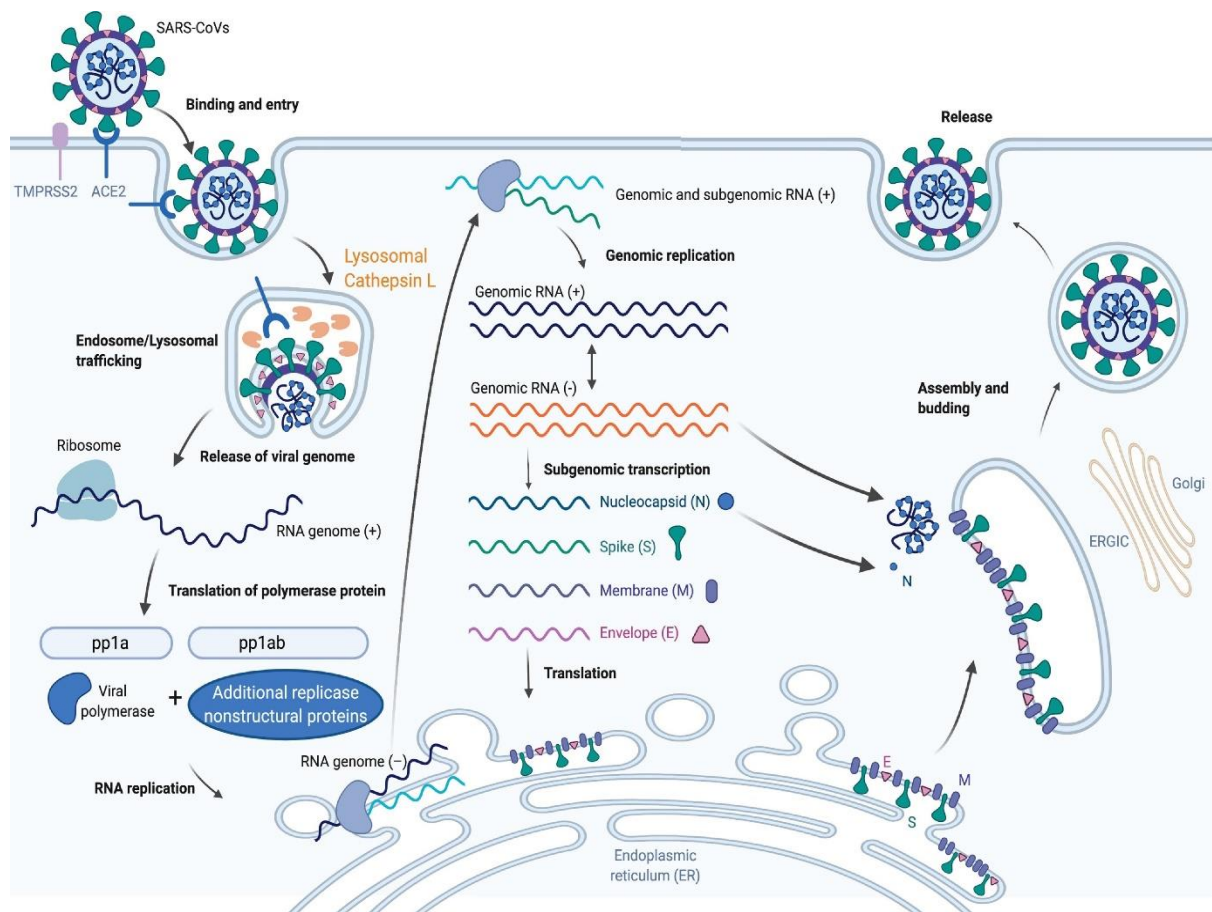
The general synopsis of how viruses invade the human respiratory system is as follows:

1. **Transmission:** Viruses can be disseminated from amongst individuals through various mechanisms, such as respiratory droplets (coughing, sneezing), contaminated aerosols, direct contact with infected individuals, or touching contaminated surfaces and then touching the face, especially the mouth, nose, or eyes.
2. **Attachment and Entry to Respiratory Epithelium:** After the virus is transmitted to a susceptible individual, it must attach and enter the pseudostratified columnar ciliated epithelium to initiate infection. The initial point of attachment is usually the cells with specific receptors on their surface that the virus can recognise and bind. Respiratory viruses possess specific surface proteins that enable them to attach to receptors on the respiratory epithelium. For example, the spike proteins of the SARS-CoV-2 bind to the angiotensin-converting enzyme 2 (ACE2) receptor in respiratory epithelial cells.
3. **Receptor Binding:** Different viruses have different target receptors. For example, the influenza virus targets cells with sialic acid receptors, and the virus liable for COVID-19 primarily targets cells with ACE2 receptors. The binding of the virus to its specific receptor facilitates viral entry into the host cell.
4. **Endocytosis or Fusion:** Once attached to the cell, viruses can enter the host cell through two main mechanisms:

- **Endocytosis:** Some viruses are engulfed by their host's cell membrane, forming a vesicle that transports the virus into the cell.
 - **Fusion:** Other viruses can directly fuse their viral envelope with the host cell membrane, releasing the viral genetic material into the cell.
5. **Replication and Spread:** Once inside the host cell, the virus takes over the cellular machinery to replicate its genetic material and produce new viral particles. These new viral particles can then spread to neighbouring cells in the respiratory tract and continue the infection, causing damage to the respiratory epithelium. This leads to inflammation and an immune response, resulting in respiratory symptoms.
6. **Immune Response:** The body's immune system detects the presence of the virus and mounts an immune response to control and eliminate the infection. The immune response can involve various components, such as antibodies, T cells, and other immune cells.



(Grove and Marsh, 2011; Crowe, 2014; Millet and Whittaker, 2014; Bellinghausen *et al.*, 2016; Sobhy, 2017; Battles and McLellan, 2019; Subbarao and Mahanty, 2020; Vimalajeewa *et al.*, 2022; Pöhlker *et al.*, 2023).



Trends in Immunology

Figure 2.2: SARS-CoV-2 lifecycle; entry and release from host cell as described by Harrison *et al.*, (2020).

Understanding how viruses enter the respiratory tract is essential for developing strategies to prevent and control respiratory infections. Measures such as vaccinations, good hygiene practices (e.g., handwashing, wearing masks), and maintaining respiratory health are crucial in plummeting the risk of viral respiratory infections, subsequently curbing the potential emergence of global outbreaks.

In the last two decades, three significant pandemics were caused by coronaviruses. The following section of this review introduces the coronavirus-induced pandemics and explicates the etiological agent of COVID-19.

2.2 Coronaviruses: Description, classification, and host range.

Coronaviruses are a set of viruses primarily identified in the 1960s. The group of viruses have emerged as a central public health concern in recent years, with the recent outbreak of COVID-19 being the most current and notable example. Coronaviruses are found in avian and mammalian species and resemble one another in morphology and structure. In addition, the viruses are renowned for their ability to mutate rapidly, alter tissue tropism, cross the species barrier, and adapt to different epidemiological situations (Woo *et al.*, 2012; Helmy *et al.*, 2020; Maganga *et al.*, 2020).

The coronaviruses are large enveloped, positive-sense RNA viruses with the largest genomes (26-32 kb) among all RNA virus families. The coronavirus genome encompasses 6–11 open reading frames (ORFs) encoding 9680 amino acid polypeptides (Guo *et al.*, 2020; Naqvi *et al.*, 2020). The first ORF comprises approximately 67% of the genome that encodes 16 non-structural proteins (nsps), whereas the remaining ORFs encode accessory and structural proteins (Chan *et al.*, 2020; Finkel *et al.*, 2020; Kim *et al.*, 2020). Each viral transcript has a 5'-cap structure and a 3' poly (A) tail, and a single virion's diameter ranges from 50 nm to 200 nm. *Coronavirus* stems from the viruses' appearance with large petal-shaped surface projections or crown-like spikes on a 120–160 nm pleomorphic body similar to a solar corona (Monto *et al.*, 2014; WHO, 2020).

2.2.1 Taxonomic classification

The International Committee of Taxonomy of Viruses (ICTV) classifies coronaviruses into the realm Riboviria, kingdom Orthornavirae, phylum Pisuviricota, class Pisoniviricetes, order Nidovirales and the Coronaviridae family (Mavrodiev *et al.*, 2020). The Coronaviridae family has over thirty viruses and has the largest reported genome of all viruses of 30 Kb. All members

of this family have helical nucleocapsids comprising various copies of a single primary phosphoprotein species (N). A number of protein species encompassed in the virion envelope are crucial for morphogenesis and host infectivity. Coronaviridae members' morphogenesis involves the virion assembly through the budding nucleocapsids at the intracellular membranes' early Golgi compartments (Artika *et al.*, 2020; Saraste and Prydz, 2021).

Coronaviridae is categorised into two subfamilies: Letovirinae and Orthovoronavirinae. Subfamily Letovirinae includes the *Alphaletovirus* genus, and subfamily *Orthocoronavirinae* is subdivided into four genera, as mentioned below. The phylogenetic analysis and genome structure of coronaviruses reveal four major genera: *Alphacoronavirus* (17 species), *Betacoronavirus* (12 species), *Gammacoronavirus* (2 species) and *Deltacoronavirus* (7 species) (Helmy *et al.*, 2020). Before 2019, only six human coronaviruses (HCoV) could be held accountable for respiratory diseases (Arshad Ali *et al.*, 2020; Ebomah *et al.*, 2022). The viruses of Coronaviridae, such as Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and related Middle East Respiratory Syndrome Coronavirus (MERS-CoV), are highly pathogenic.



2.2.2 Coronavirus host range

Coronaviruses infect a broad spectrum of wild and domestic animals. *Delta* and *Gammacoronaviruses* predominantly infect birds, whereas *Alpha* and *Betacoronaviruses* infect mammalian species, including humans. *Alphacoronaviruses* include animal pathogens such as feline coronaviruses, canine coronaviruses, and common human coronaviruses 229E and NL68 (Li *et al.*, 2022), which can also infect humans and cause mild respiratory illnesses. The distinguishing features of the genus include the distinct type of non-structural protein-1 and the presence of a mutual accessory for a dispensable multi-spanning *Alphacoronavirus* membrane

protein (α mp). At the same time, for some *Alphacoronaviruses*, α mp is the only accessory protein. Others may carry up to at least six accessory genes, according to the ICTV (V'kovski *et al.*, 2021).

On the other hand, *Betacoronaviruses* consist of separate lineages: lineage A (OC43, HKU1), lineage B (SARS-CoV and SARS-CoV-2) and lineage C (MERS-CoV) (Cimolai, 2020). The genus encompasses human pathogens such as SARS-CoV and SARS-CoV-2 and human coronaviruses OC43 and HKU1. The RNA genome and spike proteins on the surface characterise the two genera (Patarca and Haseltine, 2022).

Moreover, several coronaviruses have a broad host range, including wildlife, pets or livestock, such as in the case of bat coronaviruses (BatCoV, CDPHE15 and HKU10). The viruses also include Porcine Enteric Diarrhoea Coronavirus (PEDV), Transmissible Gastroenteritis Virus (TGEV), Feline Infectious Peritonitis Virus (FIPV), Bovine Coronavirus (BCov), sudden acute diarrhoea syndrome (SADS-CoV) and others (Woo *et al.*, 2012; Zhou, Qiu and Ge, 2021).

The ecological distribution of coronaviruses is a complex issue that requires a comprehensive understanding of the virus, its hosts, and the environment. Studies have shown that coronaviruses inhabit various hosts, including birds, bats, and mammals (Wu *et al.*, 2020). Until the beginning of this century, coronaviruses were considered minor pathogens for humans. However, CoVs have caused significant disease outbreaks in recent decades: the Severe Acute Respiratory Syndrome (SARS) in 2002–2003, the Middle East Respiratory Syndrome (MERS) in 2012, and the recent COVID-19 pandemic 2019-2023 (Cui, Li and Shi, 2019; Arshad Ali *et al.*, 2020; Foladori *et al.*, 2020; Peeri *et al.*, 2020; Wise, 2023).

2.3 Human Coronaviruses: Disease Outbreaks and Symptomatology

2.3.1 The Severe Acute Respiratory Syndrome Coronavirus outbreak

In November 2002, a *Betacoronavirus* (SARS-CoV) outbreak was primarily identified in the Guangdong Province in China (Peeri *et al.*, 2020). China reported over eight thousand cases of infection during the SARS epidemic, claiming seven hundred and seventy-four lives. *Paguma larvata* was considered the reservoir host of the disease, and the open markets were considered the foci of transmission to humans (Berger *et al.*, 2011; Mulabbi *et al.*, 2021; Liu *et al.*, 2022). Since 2004, no known cases have been reported.

Severe Acute Respiratory Syndrome (SARS) is a vastly infectious disease (Agarwal, 2023) with significant morbidity and case fatality. The SARS-CoV-1 transmission route among humans was via the deposition of contaminated droplets or aerosols on the respiratory epithelium. The transmission was infrequent during the first five days of illness, partly because of the low viral load in respiratory secretions during that phase (Boncristiani *et al.*, 2009).

Once transmitted to the respiratory epithelial tissue, the virus (SARS-CoV-1) permeates the intracellular space of the host cell after binding the angiotensin-converting enzyme 2 (ACE2) receptors. Following the intrusion of the airway epithelia and the alveolar spaces, SARS-CoV-1 is reported by Cavallieri *et al.*, (2022) to spread to the brain, resulting in the upregulation of pro-inflammatory cytokines and chemokines in both the lung and the brain.

The major clinical features of SARS include obstinate fever, chills, myalgia, malaise, dry cough, headache, and dyspnoea. Less common symptoms include sputum production, sore throat, coryza, dizziness, nausea, vomiting and diarrhoea (Hui, Wong and Wang, 2003).

2.3.2 The Middle East Respiratory Syndrome Coronavirus outbreak

In 2012, a second coronavirus disease succeeded the emergence of SARS. MERS first emerged in Saudi Arabia once a sixty-year-old man presented with severe pneumonia. It took two years for an outbreak of the virus to occur, resulting in a significant global health threat. Consequently, twenty-one countries spanning Europe, Asia, the Middle East, and North America were infected (Cho *et al.*, 2016; Abdelrahman, Li and Wang, 2020).

Since 2012, over two thousand laboratory-confirmed cases of MERS have been reported, and eight hundred and fifty-eight associated deaths have occurred (34.4 % case-fatality ratio) (Peeri *et al.*, 2020). MERS-CoV is the positive-sense enveloped single-stranded RNA virus causing MERS. The Betacoronavirus belongs to the *Merbecovirus* subgenus (Toosy and O’Sullivan, 2019). Habituated with zoonotic transmission, MERS has fatal effects on *Homo sapiens*. In humans, dipeptidyl peptidase 4 (DPP4), the cellular receptor of MERS-CoV, originates in the epithelium of the lower respiratory tract, which most likely causes disease severity in humans as compared to other mammals (Beltz, 2023).

MERS causes a variety of illnesses as well as asymptomatic infections. Most MERS patients develop severe influenza-like illness, typically presenting with fever, shortness of breath and cough (Saleri and Ryan, 2019). Other symptoms include malaise, myalgia, nausea, diarrhoea and more dire symptoms such as respiratory failure, organ failure and death (Zaki and Keating, 2018).

2.3.3 The Coronavirus Disease-2019 global pandemic/SARS-CoV-2 outbreak.

COVID-19 is an infectious novel coronavirus disease with an upsurge of unusual viral pneumonia. The respiratory disease was named the 2019 novel coronavirus until 11th February 2020, when the WHO officially named it COVID-19.

The 2019 viral outbreak occurred in Wuhan, Hubei Province, China. By the time Chinese authorities could contain the endemic, the virus had spread through international travel. COVID-19 was already disseminated to various countries on different continents, including Asia, Europe, and North America, when WHO declared a global pandemic in March 2020.

The first reported COVID-19 case in Africa was in Egypt. Subsequently, the virus spread to other African countries such as Nigeria (imported from Italy), Senegal (The first four cases were imported from France and Italy), and South Africa. The first confirmed case in South Africa was of a male citizen who recently travelled from Italy, the epicentre of COVID-19 at the time. The virus spread like wildfire as COVID-19 cases increased hastily. On 23rd March 2020, the Republic of South Africa was declared a state of disaster, and strict measures were implemented as the country went on a national lockdown. Furthermore, the country experienced several waves of infection as the virus was transmitted from person to person (Amzat *et al.*, 2020; Dia *et al.*, 2020; Tuite *et al.*, 2020; Giandhari *et al.*, 2021)

The virus causing COVID-19 underwent zoonotic transmission (Holmes, 2022) to infect humans and cause a viral outbreak, leading to a global pandemic (Banks *et al.*, 2022). The transmission dynamics of SARS-CoV-2 are relatively similar to other respiratory viruses (Aggarwal *et al.*, 2022). Studies by Jaimes *et al.*, (2020) and Wan *et al.*, (2020) reveal similarities in the cellular infection models between the viruses causing COVID-19 and SARS, as both viruses predominantly target the respiratory system, especially the lungs.

Following entry, the virus binding domains (spikes) attach to the host-cell angiotensin-converting enzyme 2 receptors in the lungs. Subsequently, a proteolytic cleavage occurs, mediating membrane fusion and viral infectivity (Belouzard *et al.*, 2009; Meyer *et al.*, 2021; Takeda, 2022). Essentially, the genomic material of the virus is released, polyproteins are then translated, and replication and transcription occur. Not only does this occur within the respiratory tract, but the active replication of the viral particles may occur in host enterocytes, subsequently shedding into human faecal matter. The shedding of infectious viral RNA into faeces poses another mode of transmission: faecal-oral transmission, as reported by Cuicchi *et al.*, (2021) and several researchers (Yeo, Kaushal and Yeo, 2020; Zaneti *et al.*, 2021; Cerrada-Romero *et al.*, 2022).

2.3.3.1 COVID-19 clinical manifestations

The clinical manifestations are broad and vary from entirely asymptomatic to severe disease and death. The plethora of COVID-19 indicators reported for symptomatic patients include cough, fever, dyspnoea, ageusia, anosmia, and rhinorrhoea, with the most common manifestations being cough, fever, and fatigue (Nehme *et al.*, 2021). Moreover, COVID-19 patients have presented gastrointestinal symptoms such as abdominal pain, vomiting and diarrhoea (Aguar-Oliveira *et al.*, 2020).

A spectrum of COVID-19 complications may result in severe illnesses such as pneumonia, liver injury, acute respiratory distress syndrome, cardiac injury and death (Merad *et al.*, 2022). Although considered a respiratory disease, Spudich and Nath, (2022) suggest that acute COVID-19 may manifest through innumerable neurologic difficulties, including confusion, stroke, and neuromuscular disorders (Jaago *et al.*, 2022), and is reported to have long-lasting manifestation, a phenomenon called long COVID (Nalbandian *et al.*, 2021; Sudre *et al.*, 2021).

Long COVID, i.e., heterogeneous disease, causes numerous organ and system distress. The unprecedented manifestations of prolonged COVID-19 stress the quality of life and pose a burden to the healthcare system. Individuals may exhibit persistent or relapsing long COVID manifestations, weeks or months further than the first inception of acute symptoms (Hastie *et al.*, 2023). Additionally, a narrative review by Sapkota *et al.*, (2021) highlights the mimicry of long COVID rheumatic diseases. This finding raises concerns about the challenges in diagnosis.



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2.4 SARS-CoV-2: Description and genomic variations

2.4.1 SARS-CoV-2: Description

The etiological agent driving the devastating COVID-19 global pandemic is SARS-CoV-2 (van Doremalen *et al.*, 2021). SARS-CoV-2 is the seventh known coronavirus to infect humans, cause respiratory disease and has a spectrum of clinical presentation ranging from asymptomatic, mild and severe (Corman *et al.*, 2020); four coronaviruses (229E, NL63, OC43, and HKU1) only cause minor common cold symptoms.

SARS-CoV-2 are large spherical particles (also pleomorphic and oval-shaped) with surface projections. The helical capsid containing nucleoprotein and the RNA genome is enclosed within the envelope. SARS-CoV-2 has a large genome, and its organisation is 89% identical to other CoVs. The SARS-CoV-2 genome is organised in two large open reading frames (ORF1a and ORF1b, located at the 5' end) coding for replicase polyproteins, followed, in the terminal one-third of the genome by a region encoding for the structural proteins (spike, envelope, membrane, and nucleocapsid protein) (La Rosa *et al.*, 2020; Naqvi *et al.*, 2020).

The genome lacks the hemagglutinin-esterase gene. However, it comprises two flanking untranslated regions (UTRs) at 5' end of 265 and 3' end of 358 nucleotides. The nsps include two viral cysteine proteases, including papain-like protease (nsp3), chymotrypsin-like, 3C-like, or main protease (nsp5), RNA-dependent RNA polymerase (nsp12), helicase (nsp13), and others likely to be involved in the transcription and replication (Kumar *et al.*, 2020).

The envelope enclosing the virion genomic material is embedded with protein molecules. The molecules are the lipid bilayer envelope (E), the membrane proteins (M), and the nucleocapsid phosphoprotein (N). These three proteins protect the virus outside the host cell, while the spike glycoprotein (S) protrusions give it its characteristic form. The 180 - 200 kDa spike protein is a significant factor in SARS-CoV-2 infection as it mediates viral entry into host cells through

receptor recognition, cell attachment, fusion, and endosomal escape (Watanabe *et al.*, 2020; Xia *et al.*, 2020).

The SARS-CoV-2 glycoprotein comprises of two subunits. The S1 subunit comprises the receptor binding domain (RBD) responsible for recognising and binding the ACE2 of the host. The second subunit, S2, forms a six-helical bundle via the two-heptad repeat domain to arbitrate viral cell membrane fusion (Huang *et al.*, 2020; Harvey *et al.*, 2021). Numerous mutations associated with variants of concern occur in the S protein, affecting viral detection, immune response, and SARS-CoV-2 epidemiology (Flores-Vega *et al.*, 2022; Rothenberger *et al.*, 2022).

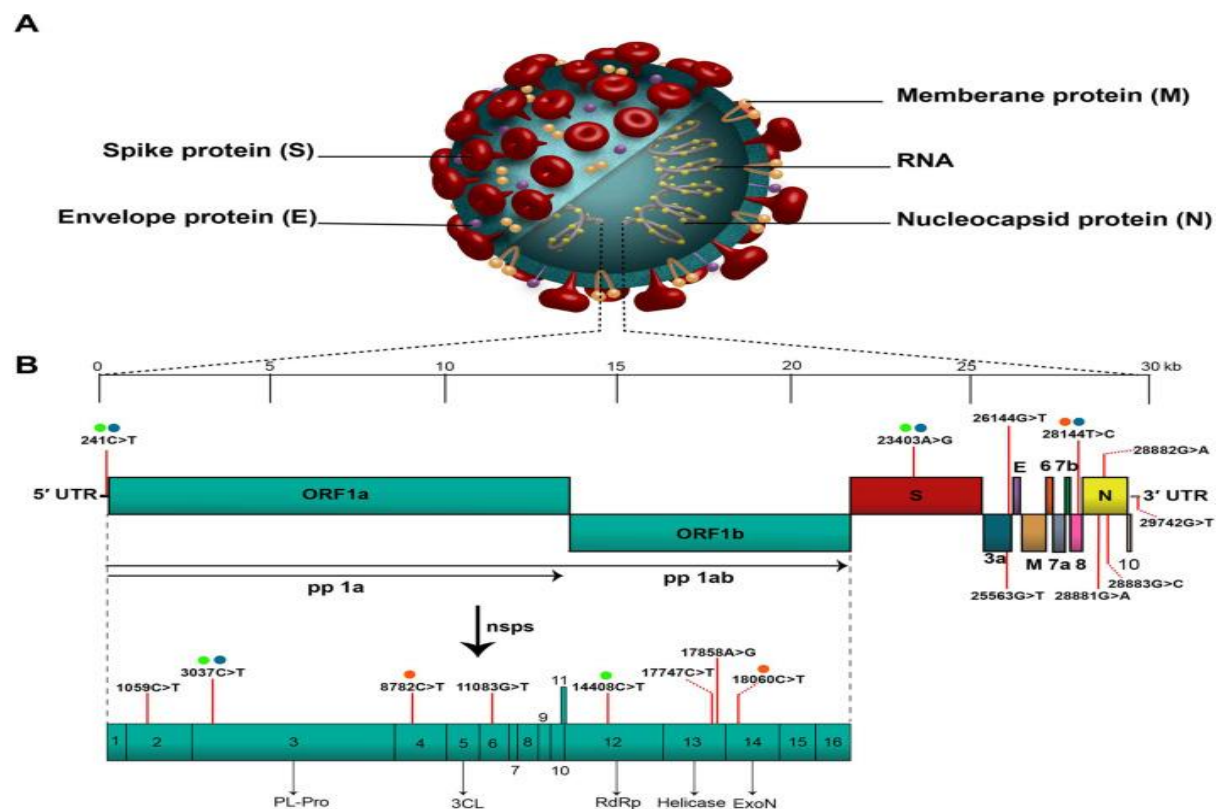


Figure 2.3: A graphical depiction of the structure and genome organisation of SARS-CoV-2 as described by Rahimi *et al.*, (2021). (A) An annotated SARS-CoV-2 virion with the spike, envelope, membrane, nucleocapsid protein and RNA highlighted. (B) A representation of the genome organisation.

2.4.2 SARS-CoV-2: Spike protein mutations - Key VOC-linked mutations

Viruses are continuously changing and evolving because of genetic selection. These viral variations result from nucleotide changes that occur in the genome during replication. The nucleotide changes in CoVs occur at a significantly lower rate than those of other RNA viruses. The variation is due to the incidence of an enzyme responsible for correcting some errors during replication (Korber *et al.*, 2020; Mistry *et al.*, 2022).

The frequent spread and evolution of SARS-CoV-2 have created numerous variants of interest and variants of concern with varied degrees of transmissibility, disease severity, diagnostics and the effectiveness of treatment and vaccines (Lusvarghi *et al.*, 2022). The WHO classifies SARS-CoV-2 variants into two categories, i.e., variants of concern (VOC) and variants of interest (VOI). The CDC describes a VOC as a variant accountable for increased virus transmissibility, increased virulence, resistance to some therapeutics such as vaccines, resistance to acquired immunity, and, therefore, has the aptitude to evade detection. Since the outbreak in the year 2019, VOCs have emerged from wild-type strains isolated in Wuhan (Ahmad *et al.*, 2021; Aksamentov *et al.*, 2021). Excellence

Mutations on the spike protein are mutual features of many SARS-CoV-2 variants. Receptor-binding-domain mutations enhance the structural stability and increase the human ACE2 receptor-binding capacity of the spike protein, which could be an essential characteristic that would explain the high virus spread and the severe infectivity (Magazine *et al.*, 2022). One of the initial S protein alterations detected was the D614G amino acid substitution within the receptor-binding motif, which rapidly dominated variants globally (Zhou *et al.*, 2021).

In a study conducted by Volz and colleagues, variants with this amino acid substitution were found to be more infectious than wild-type strains (Volz *et al.*, 2021). The D614G spike substitution is among a surfeit of variant-associated mutations/ alterations within the receptor-

binding domain, mainly in the ACE2-binding site (Harvey *et al.*, 2021), including, but not limited to, the following:

2.4.2.1 N501Y Substitution

The mutation is a substitution of one of six essential amino acids. These amino acids are involved in the binding of the ACE2 receptor. This substituted amino acid is asparagine (N) at position 501. Its substitution with tyrosine (Y) increases binding affinity to the host receptor (Tegally *et al.*, 2021).

Reports reveal that this amino acid substitution increases cell infectivity. The mutation has a role in viral penetration into host (human) cells. The N501Y substitution is mutual to the Alpha, Beta, Gamma, and Omicron variant strains. Additionally, mutation N501Y has possibly contributed to the increased transmissibility and higher viral burden in both the Alpha and Beta variants (Makowski *et al.*, 2021; Vega-Magaña *et al.*, 2021; Mistry *et al.*, 2022).

2.4.2.2 E484K/Q/A Substitutions

The E484K/Q/A are spike protein mutations in the Beta, Gamma, and Omicron lineages. E484K, a mutation in the receptor-binding domain of the spike protein (Rothenberger *et al.*, 2022), has a glutamate (E) to lysine (K) substitution at position 484 (Yang *et al.*, 2022). SARS-CoV-2 variants possessing the E484K mutation have reduced susceptibility to vaccines, monoclonal antibody treatment, and convalescent sera (Andreano *et al.*, 2021; Greaney *et al.*, 2021).

2.4.2.3 K417N/T Substitutions

The K417N/T spike protein mutations are amino acid substitutions in the Beta, Gamma, and Omicron strains. The substitutions of lysin (K) occur at position 417, where amino acids asparagine (N) or threonine (T) substitute lysin. SARS-CoV-2 variants possessing this

mutation are associated with reduced neutralisation and immune evasion, further disturbing the host immune response (Tegally *et al.*, 2021; Lusvarghi *et al.*, 2022).

2.4.2.4 L452R Substitution

The mutation occurs on the amino acid leucine at position 452 in the receptor-binding domain of the spike protein. This substitution is positioned near the ACE2 interaction interface associated with viral attachments and cell insertions. The L452R substitution is unique to the Beta (B.1.33, B.1.351) and Delta (B.1.617.0-1) VOCs. It is associated with immune evasion and enhanced transmission (Bittar *et al.*, 2021; Motozono *et al.*, 2021; Tchesnokova *et al.*, 2021).



2.4.2.5 DH69/ V70 Deletion

The DH69/ V70 deletion in the Alpha variant alters the spike protein. The deletion enhances viral infectivity in vitro and allows the virus to evade immune response. The failure of specific commercial kits to detect the S glycoprotein is associated with the mutation (Meng *et al.*, 2021; Rees-Spear *et al.*, 2021).

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2.4.3 SARS-CoV-2: Variants of Concern

2.4.3.1 Variant Alpha (B.1.1.7)

B.1.1.7 is a highly transmissible variant primarily detected as the predominant strain in the UK in late September 2020. The Alpha variant consists of 23 mutations in its genome, including spike mutations; N501Y substitution in the receptor-binding domain, P681H (Banks *et al.*, 2022) and N-terminal domain (NTD) deletions at positions 69–70 and 144. The 69–70 deletion

averts the amplification of three genomic segments in a commonly used diagnostic PCR assay, resulting in S-gene target failure.

The Alpha variant accounted for most infections in the USA and many European countries by the second quarter of 2021. Epidemiological studies suggest it was approximately 50% more transmissible than previously circulating UK variants (Davies *et al.*, 2020; Leung *et al.*, 2020). Tao *et al.*, (2021) note the association of the variant with threefold to eightfold higher upper-airway levels and an estimated 50% increased mortality.

2.4.3.2 Variant Beta (B.1.351)

Variant B.1.351 was identified initially in South Africa in December 2020 and became the dominant strain, resulting in the second wave in the country. The variant is associated with a substantial reduction in neutralising antibodies. The Beta variant escalated to one of the most predominant VOCs, harbouring the characteristic receptor-binding domain mutations; N501Y, K417N and E484K substitutions receptor-binding domain mutations involving K417N, E484K, and N501Y substitutions and five non-receptor-binding domain mutations, along with a deletion in the N-terminal domain (Lusvarghi *et al.*, 2022). The variant has since spread to over 60 countries and shares two common mutations (N501Y and E484K) with the P.1 Gamma variant (Johnson *et al.*, 2022).

2.4.3.3 Variant Gamma (P.1)

Similar to the Beta variant, the variant Gamma was detected in four Brazilians who were travelling to Japan in January 2021. The variant was responsible for the resurgence in Manaus, Brazil, despite elevated levels of previous infection (Tao *et al.*, 2021b). Akkız, (2022) indicates that the variant may pose resistance to naturally acquired immunity. Gamma encompasses 17

amino acid substitutions, including a similar trio in the S protein found in Beta. Studies by Zhang *et al.*, (2021); Lusvarghi *et al.*, (2022) established that the variant was more transmissible than the wild-type strain.

Since its detection, the Gamma variant has spread to more than thirty-seven countries with signature mutations, including N501Y, K417N, and E484K, which may aid the virus in evading immune response.

2.4.3.4 Variants Delta (B.1.617.2)

In December 2020, an upsurge in COVID-19 cases resulting in the second wave of infection in India (Tao *et al.*, 2021a) was attributed to variant Delta (B.1.617.2). The variant was also responsible for outbreak infections in several gatherings in the United States of America. Possessing two prominent mutations, E484Q and L452R (Johnson *et al.*, 2022), Variant Delta caused a devastating third wave in South Africa. It hastily displaced the Beta variant within three months of introduction into the country. The variant also replaced the Alpha variant in the UK.

2.4.3.5 Variant Omicron (B.1.1.529)

Omicron has 34 mutations (30 nonsynonymous mutations, three deletions, and one insertion) relative to the wild-type S protein, accounting for 2.5 % of all amino acids occurring within the protein. Variant B.1.1.529, a designated VOC by the WHO, was first detected in mid-November 2021 by world-class genomic surveillance laboratories in South Africa, as it comprised 76 % of the samples sequenced and was present in many countries around the world (Dyer, 2021). Lineage BA.1 and BA.2 became the predominant lineages and propelled the

fourth epidemic wave of infection in South Africa. In the first quarter of 2022, lineage BA.2 was displaced by the BA.4 and BA.5 lineages which further dominated the fifth epidemic wave of infection in the country (Jassat *et al.*, 2022).



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2.5 The occurrence of the SARS-CoV-2 genome in wastewater

Numerous studies have reported on the pervasiveness of SARS-CoV-2 (Sherchan *et al.*, 2020; Tran *et al.*, 2021) and SARS-CoV-2 variants (Pechlivanis *et al.*, 2022a) in the wastewater milieu (Johnson *et al.*, 2022). It is long established that the most common clinical indicators of COVID-19 are fever and respiratory symptoms. However, Benvari *et al.*, (2022) and several researchers reported gastrointestinal symptoms, suggesting the pathogenicity of SARS-CoV-2 in the gastrointestinal tract.

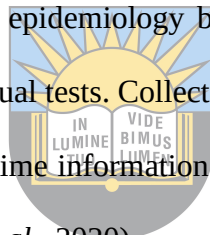
Nonetheless, both symptomatic and asymptomatic individuals shed SARS-CoV-2 RNA. Fascinatingly, viral shedding through faeces could continue even after negative respiratory tests as the virus migrates from the respiratory tract to the gastrointestinal tract and consequently to stool (Qian *et al.*, 2021; Cerrada-Romero *et al.*, 2022). SARS-CoV-2 RNA is introduced to wastewater (hospital and domestic) through several sources, such as handwashing, sputum, and vomit. Additionally, the viral RNA is excreted in infected individuals' faeces (reported extensively as the main route to wastewater), urine and saliva and can be detected in the sewage collected in wastewater treatment plants (Amman *et al.*, 2022).

Although only a few studies have isolated infective SARS-CoV-2 from stool and urine samples, many studies have detected SARS-CoV-2 RNA in stool and wastewater. Another probable route for SARS-CoV-2 RNA to the wastewater milieu is through contaminated surface runoffs and wastewater from healthcare facilities. For the latter, the virus is released into the wastewater system by facilities treating COVID-19 patients. This may also occur through the use of lavatories by patients and the discarding of patient samples (Bogler *et al.*, 2020).

A myriad of aspects, such as shedding-related factors, in-sewer factors, and population size, can influence RNA concentrations in wastewater. For example, documented reports by Tuite

et al., (2020) and Wu *et al.*, (2020). Wu *et al.*, (2021) and Wilder *et al.*, (2021) state the positive association between population size and SARS-CoV-2 concentrations in wastewater. However, there is a slight limitation to this deduction. People travel to and from various destinations for various purposes, thereby contributing to virus concentrations in those places at that time. Thus, Wade and colleagues explore the uncertainties and variabilities for wastewater monitoring and suggest factors such as the monitoring of instabilities of inhabitants within the catchment area to comprehend trends in RNA concentrations (Bertels *et al.*, 2022; Wade *et al.*, 2022).

In addition, wastewater samples can provide an overview of the community's circulating viral lineages by quantitative reverse transcription real-time PCR analysis (RT-qPCR) (Jahn *et al.*, 2022a) or genomic sequencing. The surveillance of SARS-CoV-2 by wastewater monitoring is highlighted as a powerful tool for epidemiology based on wastewater and public health interventions that complement individual tests. Collecting data from central sewerage systems and individual tests can provide real-time information on the distribution of SARS-CoV-2 in the communities concerned (Bogler *et al.*, 2020)



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Primary indicators of an outbreak can be identified as the development of SARS-CoV-2 concentration in wastewater, which precedes changes in confirmed COVID-19 cases by at least several days. Wastewater monitoring is instrumental in providing an early indication of the re-emergence of SARS-CoV-2 in communities that contained an initial outbreak and subsequently relaxed containment measures.

2.5.1 Detection and analysis methods for SARS-CoV-2 in wastewater

2.5.1.1 Molecular techniques

The isolation and detection of viruses are costly and laborious processes. Molecular technology advances have transformed emerging viruses' detection and characterisation (Payne, 2017). Molecular methods are effective in detecting viruses in environmental samples at low concentrations. This is due to the sensitivity and specificity of the technology used (Imwong *et al.*, 2014; Soares and Tasca, 2016).

Molecular methods are widely used today in diagnosing and studying pathogenic viruses. Rapid and precise detection of new viruses is essential for rapid response, optimal clinical care, and limiting the spread of these viruses. Ideally, diagnostic tests must be fast, cheap, accurate, and portable (Artika *et al.*, 2020; Mustafa *et al.*, 2020).



2.5.1.1.1 The Polymerase Chain Reaction

A widely used molecular method for detecting and identifying pathogens is the Polymerase Chain Reaction (PCR). PCR is a bio-enzymatic process that amplifies a single or a few DNA copies in various degrees, resulting in thousands to millions of copies of a particular DNA fragment. PCR has attracted considerable attention from researchers as it is a powerful technology that is fast, cost-effective, and easy to use. It is one of the most widely used in molecular biology (Artika, Wiyatno and Ma'roef, 2020; Cassedy *et al.*, 2021)

Among the promptest cases of coronavirus detection by PCR was the identification of SARS-CoV in a study conducted in 2003 by Ksiazek *et al.*, (2003) during the SARS pandemic (Emery *et al.*, 2004) over two decades ago. In the eruption of MERS, authors Corman, Eckerle and fellow researchers were amongst the first to identify the then-novel coronavirus driving the

pandemic (Corman *et al.*, 2012). However, other PCR techniques are more competent for detecting RNA viruses such as SARS-CoV-2.

- Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Works published by authors Drosten *et al.*, (2003) and Kuypers *et al.*, (2007) served as essential studies in signifying the importance of RT-PCR in the detection and characterisation of coronaviruses. Real-time RT-PCR is crucial for diagnosis in the field of virology due to its rapid and accurate identification fashion. The works by numerous researchers were fundamental for advances in diagnostic technology, as they describe the use and development of the technique in targeting specific gene markers, subsequently identifying coronaviruses (HCoV-NL63, SARS-CoV & MERS-CoV) in patient samples (Drosten *et al.*, 2003; Poon *et al.*, 2004; van der Hoek *et al.*, 2004; Zaki *et al.*, 2012).

RT-PCR is a typical end-point reaction that employs reverse transcription to transcribe a pure viral RNA template by reverse transcriptase to produce a complementary DNA (cDNA) template. The cDNA undergoes exponential amplification by PCR, and the amplicons are subsequently used for analysis. RT-PCR can be performed in two independent reactions or as a single reaction requiring more specific commercial enzymes. RT-PCR is predominantly used for the analysis of gene expression (Bachman, 2013)

- Quantitative Reverse Transcription - Polymerase Chain Reaction (RT-qPCR)

Quantitative PCR (qPCR) is employed for many applications for detecting and quantifying nucleic acids. In RT-qPCR, RNA transcriptions are often quantified by reverse transcription into cDNA, and consequently, qPCR is carried out. Comparable with standard PCR, DNA is amplified by three repeat steps: denaturation, annealing, and elongation. In qPCR, however, fluorescent labelling allows data to be collected as the reaction progresses. This technique has many advantages due to its variety of methods.

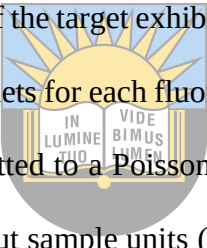
One of the primary reports about detecting SARS-CoV-2 using RT-qPCR was a study describing the identification and characterisation of the virus isolated from lower respiratory tract samples. The discovery of SARS-CoV-2 by real-time RT-qPCR propelled further research, which led to the initial discovery of SARS-CoV-2 RNA in wastewater by Medema in the year 2020 (Na Zhu *et al.*, 2020; Medema *et al.*, 2020). The technique accompanied by the emergence of wastewater-based epidemiology as a potential instrument to monitor and assess the phylogeny of SARS-CoV-2 isolated from wastewater (Ahmed *et al.*, 2020; Nemudryi *et al.*, 2020; 'et al., 2021).

The interpretation of the reaction's (RT-PCR, RT-qPCR) efficiency involves the analysis of metrical data produced during the reaction to ascertain the quality of the genome copies generated during the reaction. During the reactions, an amplification plot and standard curve are generated to indicate the amplification and efficiency of the reaction. Significant parameters to be evaluated include the amplification efficiency, the correlation value, and the cycle threshold value. Cycle threshold (C_T) refers to the number of cycles that fluorescent signals pass through a threshold, i.e., over background noise (Almeida *et al.*, 2013).

The semi-quantitative values are used to obtain more information about the amount of RNA and target DNA in specific samples. The C_T value broadly categorises the concentration of the viral genetic material. A low C_T value indicates higher concentrations of the target genetic material, typically associated with a high risk of infectivity, in the clinical scope (Wahab *et al.*, 2021). Higher C_T values entail a low target concentration, typically associated with a lower risk of infection. In other words, the C_T levels contradict the quantity of the targeted genetic material in each sample. The coherence of C_T values is used as a quality control measure during PCR when working with replicates and control samples (Fuller *et al.*, 2013; Rabaan *et al.*, 2021; Ebomah *et al.*, 2022).

- Droplet digital Polymerase Chain Reaction (ddPCR)

Another advancement of PCR is ddPCR. ddPCR is an exceedingly sensitive and precise PCR technology (Dobnik *et al.*, 2016; Demeke and Dobnik, 2018; Cocco *et al.*, 2020). It enables the absolute quantification of target nucleic acid molecules. According to Mao *et al.*, (2019), ddPCR generates micro-reaction units with water-in-oil and microfluid technology. The nucleic acids are randomly divided into water-oil drops that are individually subjected to PCR. Then, each droplet's signal is read with two-colour optical detection systems. Essentially, each partition acts as an individual PCR microreactor, and partitions containing amplified target sequences are detected by fluorescence.

In a ddPCR System, targets are amplified and detected using fluorescence-based probes. Droplets containing at least one copy of the target exhibit increased fluorescence. In the ddPCR System, the positive and negative droplets for each fluorophore are determined in each sample. The positive droplet fraction is then fitted to a Poisson distribution to determine the absolute starting copy number in copies/ μ L input sample units (Taylor *et al.*, 2017; García-Giménez *et al.*, 2019; Rinaldi *et al.*, 2022).  **University of Fort Hare**
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Preliminary research centring on PCR-based detection techniques laid the necessary groundwork for advanced ddPCR applications. The application has since attracted considerable attention from researchers following the comparative and validation studies by Taylor *et al.*, (2017), Moore *et al.*, (2020) and Li *et al.*, (2022) demonstrating the precision of ddPCR in detecting viral indicators at lower concentrations. Subsequently, the technique was employed to identify SARS-CoV-2 genetic markers and potential mutations (Heijnen *et al.*, 2021; Mills *et al.*, 2022). The technique was extensively applied in clinical surveillance studies (Chen *et al.*, 2020; Papadakis *et al.*, 2022; Moron-Lopez *et al.*, 2023).

2.5.1.1.2 Genome Sequencing

Sequencing is a secondary detection method. It is precisely employed for the validation and phylogenetic assessment of PCR amplicons. Sequencing is crucial in the molecular evaluation of PCR products retrieved from composite environmental samples such as wastewater, as contaminants may affect the reaction. Genome sequencing is an imperative technique used to monitor circulating viral genomes as it allows the analysis of exact nucleotide sequences (Alygizakis *et al.*, 2021). The basic principle of genome sequencing entails the process of determining the order of nucleotide bases of an organism/microorganism. Genome sequencing technologies include Sanger sequencing, Next-generation sequencing and Emerging third-generation sequencing, also known as long-read sequencing (Adawiah *et al.*, 2016; Heather and Chain, 2016).

Numerous federations have sequenced SARS-CoV-2 from wastewater, including the Netherlands, which has generated near-complete genome sequences from wastewater (Lara *et al.*, 2020). In the United States, the wastewater sequence provided similar results to clinical testing, including previously unknown mutation sequences before they appeared in clinical samples (Greenwald *et al.*, 2021). Sequencing the SARS-CoV-2 genome attained from wastewater samples is critical for monitoring community virus dissemination. Numerous bioinformatics tools are available for the analysis of genome sequences.

2.5.1.2 Wastewater-Based Epidemiology (WBE)

Various clinical assays have been developed to detect and characterise SARS-CoV-2, employing several techniques that are highly sensitive and precise (Reynolds *et al.*, 2022). These include RT-qPCR and ddPCR, which can be pragmatic as complementary tools in many scientific disciplines, such as environmental sciences. An emerging field of application of these

techniques is wastewater-based epidemiology (WBE). WBE was used as a crucial tool for monitoring drug utilisation, pharmaceuticals, pesticides, and many circulating chemicals that posed public health risks in various communities. WBE is now used to track biomarkers linked to infectious diseases in given communities.(Alygizakis *et al.*, 2021; Greenwald *et al.*, 2021; Medina *et al.*, 2022).

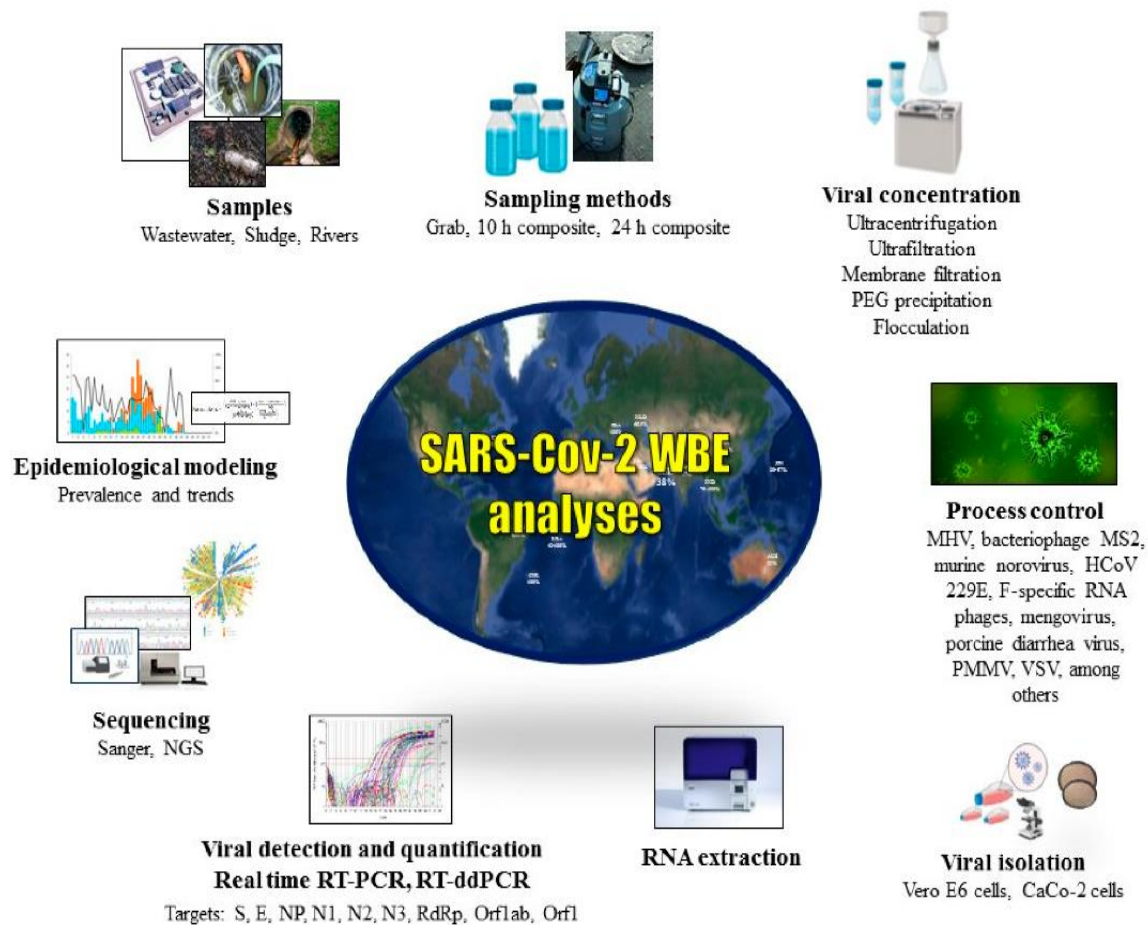


Figure 2.4: A summary of methods used in the wastewater-based epidemiology (WBE) analyses as described by Aguiar-Oliveira *et al.*, (2020).

The WBE protocol described in a review by Mousazadeh *et al.*, (2021) includes seven steps:

1. Sampling: Wastewater sampling is typically implemented by one of two techniques: composite or grab.

- a. Composite sampling technique: In this technique, a single sample is collected in aliquots over an extended period, usually 24 hours (Ahmed *et al.*, 2021).
 - b. Grab sampling technique: This is acquiring a volume of wastewater at once. It is like scooping a specific volume of sample from a wastewater body. This technique is preferred when evaluating the concentrations of a property, which may change rapidly and frequently, such as pH levels and SARS-CoV-2 RNA (Augusto *et al.*, 2022).
2. Storage: The storage and transportation of wastewater samples is vital for the survival of the virus. Wastewater samples are transported on ice and may be stored below 5 °C for same-day processing or between -20 °C and -80 °C for longer-term storage after the concentration step for further processing and analysis. SARS-CoV-2 RNA is stable at lower temperatures (Baldovin *et al.*, 2021; Beattie *et al.*, 2022).
3. Inactivation: Mousazadeh *et al.*, (2021) used a heating protocol to inactivate the virus from clinical samples as the procedure allows the detection and quantification of the viral RNA (D'Aoust *et al.*, 2021).
4. Preconditioning: This involves filtration/ centrifugation of wastewater samples at a minimum speed of 1200x g for a maximum period of 45 minutes.
5. Concentration: This step recovers SARS-CoV-2 RNA from the volume of water in the sample to maximise the overall performance of molecular assays. Ultrafiltration and Precipitation with polyethylene glycerol are the most employed techniques for the concentration of SARS-CoV-2 in wastewater samples (Kumar *et al.*, 2020; Jafferli *et al.*, 2021; Westhaus *et al.*, 2021).
6. Extraction: This is a fundamental phase in maintaining the integrity/quality of the viral RNA, which may generally influence the detection and quantification of the virus.

Commercial kits with specific instructions are available to extract SARS-CoV-2 from wastewater samples.

7. Detection and Quantification: This is the ultimate critical step in wastewater surveillance. This step comprises molecular techniques such as ddPCR and RT-qPCR, where different primers and probes are utilised to identify the SARS-CoV-2 genome in the given sample.



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2.6 Wastewater surveillance: Monitoring of communicable disease/ COVID-19

Infected individuals shed viral RNA through the faecal matter; hence, SARS-CoV-2 and variants can be detected in the influent collected in wastewater treatment plants (WWTP). Wastewater treatment plants are facilities where municipal or industrial wastewater is received, processed, and treated.

According to Warwick, Guerreiro and Soares, (2013) wastewater is an intricate medium exhibiting substantial concentrations of solids (total solids 350-1200mg/L) and particulate matter (chemical oxygen 250-1000mg/L). The composition of wastewater is 99.9% water. The 0.1% contains organic matter, inorganic compounds, and microorganisms (Wang *et al.*, 2017; Litvinov *et al.*, 2022). Viruses causing communicable diseases such as COVID-19 are among the spectrum of pathogens found in wastewater.

Studies have revealed that the frequency of SARS-CoV-2 variants in wastewater correlates with clinical statistics (Johnson *et al.*, 2022; Pechlivanis *et al.*, 2022b). Therefore, SARS-CoV-2 and SARS-CoV-2 variant surveillance in wastewater is an efficient and complementary approach to genomic epidemiology based on individual patient samples (Jahn *et al.*, 2022b; Atoui *et al.*, 2023). Wastewater surveillance is an epidemiology implementation with the potential to complement existing monitoring systems for infectious diseases (Yanaç *et al.*, 2022).

The initial objective of wastewater-based surveillance was to measure a population's exposure to certain chemicals and track illegal substance consumption in the community. The tool now serves as an early warning system for disease outbreaks within a community. Nevertheless, it cannot signal how much of the population is infected.

Researchers have used wastewater-based epidemiology as a surveillance tactic to monitor the prevalence and dissemination of SARS-CoV-2 in different communities. Wastewater sample

analysis further provides COVID-19 trends following the detection and quantification of the viral RNA. Dated disease surveillance approaches comprise diagnostic analysis, clinical samples, questionnaire surveys, and national census (Groseclose and Buckeridge, 2017; Murray and Cohen, 2017). These approaches have limitations. They are time-consuming, costly and involve extensive labour. Furthermore, the approaches cannot predict disease onset in real-time. Therefore, an approach capable of providing a real-time warning for disease outbreaks and surveillance is needed (Joseph-Duran *et al.*, 2022).

Wastewater-based epidemiology bridges that gap as it emerged as a potential strategy for disease monitoring and providing real-time data on the health status of a particular population. Complemented by molecular techniques, wastewater samples portray the circulating SARS-CoV-2 lineages and their diversity in the community through reverse transcription quantitative real-time PCR (RT-qPCR) analysis or genomic sequencing (Jahn *et al.*, 2022b). Moreover, wastewater-based epidemiology is utilised as a non-invasive surveillance approach to monitor SARS-CoV-2 concentrations within a given population (Zhang *et al.*, 2022).

The surveillance of COVID-19 through wastewater monitoring has attracted considerable attention from various countries. This method complements clinical testing systems and obtains qualitative and (semi)quantitative statistics on population infection levels (Bertels *et al.*, 2022; Kallem *et al.*, 2023).

Medema and fellow researchers' work has made a significant contribution to the field by researching the occurrence of COVID-19 indicators in wastewater. It was reported that their research was the primary study to identify SARS-CoV-2 RNA in wastewater in the Netherlands. They collected untreated wastewater samples from 6 wastewater treatment plants: Amsterdam, Amersfoort, Apeldoorn, Den Haag, Schiphol, Tilburg, and Utrecht. The findings confirmed the occurrence of SARS-CoV-2 in the wastewater from Amsterdam Schiphol

Airport in Tilburg and Kaatsheuvel, the wastewater treatment plants (Tran *et al.*, 2021). The study, in essence, provided evidence of the incidence and quantification of viral RNA in wastewater, although COVID-19 was relatively low. Therefore, it was possible to incorporate wastewater-based epidemiology into disease surveillance (Medema *et al.*, 2020).

In South Africa, the National Institute for Communicable Diseases (NICD), Centre for Vaccines and Immunology, and other research institutions adopted the monitoring system to track the SARS-CoV-2 genome in wastewater. The NICD collected wastewater-influent samples from 17 wastewater treatment facilities within five provinces in the country. Sampling was done every week. With each sampling period, SARS-CoV-2 levels in wastewater correlated with population infection levels, indicating the geographic distribution of COVID-19. Circulating VOC were identified in wastewater, and the findings corresponded with variant prevalence in the clinical sphere.

The monitoring of wastewater predicted the occurrence of the second wave in Spain 41 days before the first reported COVID-19 outbreak (Chavarria-Miró *et al.*, 2021), as SARS-CoV-2 was discovered in the wastewater. This discovery resulted in the implementation of deterrent measures to mitigate the rapid spread of the virus (Kumar *et al.*, 2020; Lastra *et al.*, 2022)

Literature affirms the diversity of SARS-CoV-2, the prevalence of SARS-CoV-2 genomic material within the wastewater setting, whether municipal, industrial or hospital and the implication to public health. Consequently, the occurrence of SARS-CoV-2 and SARS-CoV-2 variants in municipal wastewater influent from municipal WWTP in Buffalo City Metropolitan Municipality in the Eastern Cape Province was evaluated.

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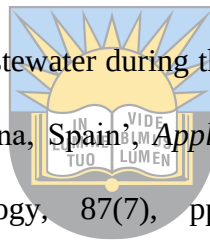
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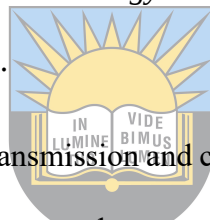
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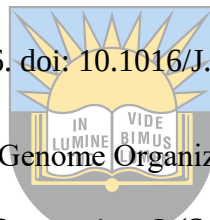
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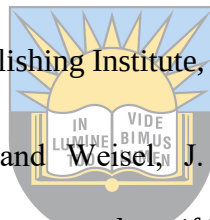
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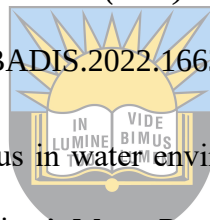
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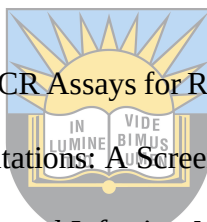
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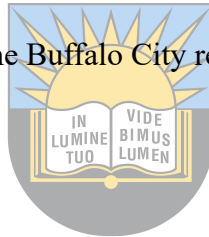
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3. CHAPTER THREE

Incidence and time-series analysis of SARS-CoV-2 in influent sourced from municipal
wastewater treatment plants in the Buffalo City region, Eastern Cape, South Africa



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**Incidence and time-series analysis of SARS-CoV-2 in influent sourced from
municipal wastewater treatment plants in the Buffalo City region, Eastern
Cape, South Africa**

Abstract

SARS-CoV-2 is the *Betacoronavirus* causing COVID-19. Since the unprecedented pandemic, over 6 million COVID-19-related fatalities have been observed worldwide, and the world economy has taken a toll. The virus is shed into the wastewater environment, posing significant environmental and public health concerns. Hence, this study sought to elucidate the incidence of SARS-CoV-2 in the municipal wastewater of Buffalo City Metropolitan Municipality. The viral RNA was extracted from a total of 494 wastewater-influent samples following the standard operating protocol. 351 (71%) tested positive by RT-qPCR for the nucleocapsid gene, indicative of SARS-CoV-2 in the municipal wastewater. A time series analysis revealed a relatively higher SARS-CoV-2 viral genome count in the autumn of 2022. The prevalence of SARS-CoV-2 in wastewater is a critical public health concern, offering a valuable indicator of community-level viral spread. The present study's findings substantiate our understanding of wastewater as a reservoir for pathogens of interest, emphasise the importance of wastewater surveillance and prompt the evaluation of the risk of exposure to the SARS-CoV-2 genome.

Keywords: Influent, RT-qPCR, SARS-CoV-2, viruses and wastewater

3.1 Introduction

Wastewater has been used as a non-invasive medium to monitor communities for chemicals, pharmaceutical drug usage, and circulating pathogens. Due to the recent pandemic, wastewater surveillance rapidly transitioned from being relegated to a conventional approach (Gracia-Lor *et al.*, 2017; Singer *et al.*, 2023). Municipal wastewater influents encompass a myriad of micropollutants, biological waste, and a broad spectrum of pathogens of medical importance.

Viruses and viral pathogens are within the populace of microorganisms inhabiting the wastewater habitat. Viruses are naturally ubiquitous, and their prevalence in wastewater is unsurprising. The primary source of viruses in wastewater is human excreta, predominantly from infected individuals. Additionally, infected individuals' virus-infected mucus/sputum moves through the gastrointestinal system into biological waste, reaching wastewater treatment facilities. Wastewater treatment facilities accumulate a variety of viruses excreted by both symptomatic and asymptomatic individuals (Corpuz *et al.*, 2020). Enteric and respiratory viruses are among the diverse categories found in wastewater influents (Fumian *et al.*, 2010; Ibrahim *et al.*, 2021; Ahmed *et al.*, 2023).

Detecting pathogenic viruses in wastewater has helped predict viral outbreaks, which has been the case for SARS-CoV-2 (Hellmér *et al.*, 2014; Bertsch *et al.*, 2020; Kumar *et al.*, 2020; Medema *et al.*, 2020). SARS-CoV-2 is the respiratory virus driving the most recent coronavirus pandemic. The Coronaviridae member primarily invades the respiratory system, and symptoms range from mild to severe disease and even death. The virus is accountable for over 6 million COVID-19-related fatalities globally (Rando *et al.*, 2021). The rapid detection and monitoring of this virus, a significant public health concern, is crucial in disease management practices. The pathogenic invasion by SARS-CoV-2 is concomitant with faecal shedding. Thus,

determining the quantity of the SARS-CoV-2 genome in wastewater can gauge the penetration and frequency of the virus in communities (La Rosa, Iaconelli, *et al.*, 2020).

Advancements in molecular techniques have facilitated the rapid identification of SARS-CoV-2 extracted from patient-derived samples and environmental sources. This study extensively used the Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) to detect and quantify the virus circulating in Buffalo City Metropolitan municipal wastewater samples. RT-qPCR is a molecular technique that transcribes RNA into complementary DNA, amplifying it into numerous copies. The technique is quick, highly sensitive and selective. The data obtained from the reaction can be used to deduce the amount of the target gene in the test samples.

The Eastern Cape Province is renowned among the most impoverished provinces of South Africa, with poor infrastructure and sanitation. It is not uncommon to find rivers of sewage caused by blocked sewer systems and dysfunctional, dilapidated, and vandalised wastewater treatment plants, exposing civilians to biological wastes infected with pathogenic viruses. Understanding and monitoring the circulating perilous pathogens such as SARS-CoV-2 is crucial. Such information can serve as a warning measure and is crucial for public health security.

Therefore, this study was conducted to explicate the incidence of SARS-CoV-2 in municipal wastewater influent attained from Buffalo City Metropolitan Municipality wastewater treatment facilities. Wastewater influent samples were selected to suit the surveillance nature of the study. This type of sample presents a more comprehensive representation of the pervasiveness of SARS-CoV-2 and any other pathogen, rather than effluent samples which reflect remnants of pathogens after all treatment processes. Moreover, the dilapidated treatment infrastructures in the resource-constrained Eastern Cape province further justifies the need of

wastewater influents monitoring as most of the WWT facilities discharged untreated wastewater due to consistent overflows, burst pipes and pump-malfunctions, among other things, which further exposes the public to potential risk of infection from microbial pathogens including SARS-CoV-2.

The data acquired from this study ought to serve as a cautionary tool to the possible public health safety predicament prompted by SARS-CoV-2 and other microbial pathogens as influent samples can assistance in recognising spikes in cases of infection permitting for rapid public health responses.



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3.2 Experimental design

3.2.1 Ethical clearance certificate and permits

Ethical clearance and permission to commence with the study were sought from and authorised by the University of Fort Hare Research Ethics Committee under the ethics code OKO011SMAY01.



Figure 3.1: Research methodology framework depicting the four stages of the work: sample collection, sample preparation for extraction and amplification, and analysis of amplification data. This was conducted at the National Wastewater Coronavirus Surveillance Laboratory at the University of Fort Hare.

3.2.2 Study area

The study was set at Buffalo City Metropolitan municipality, Eastern Cape Province, South Africa. Figure 3.2 depicts the study area.

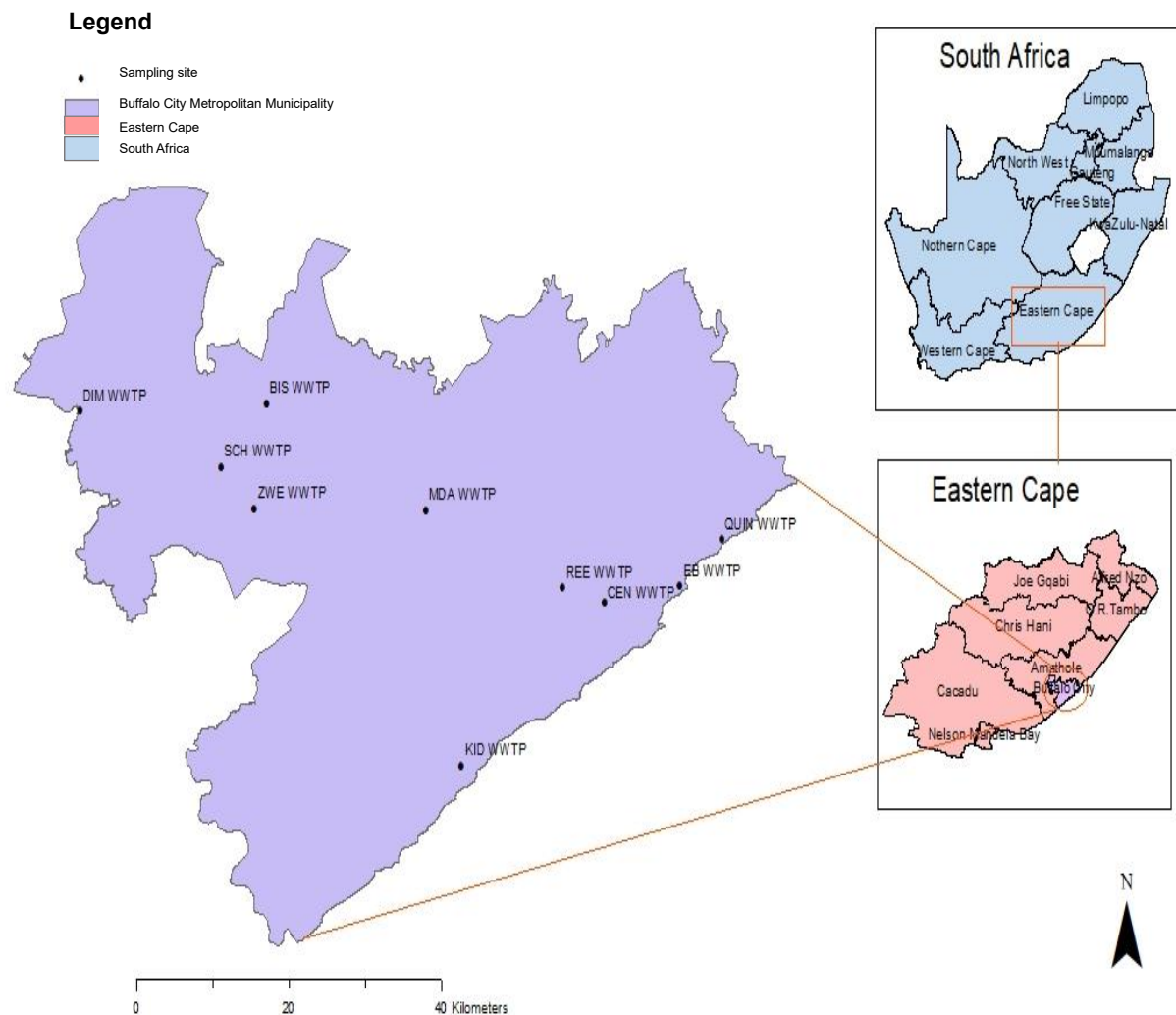


Figure 3.2: Map representing the study area depicted in purple. The selected wastewater treatment plants (WWTP): BIS WWTP, CEN WWTP, DIM WWTP, EB WWTP, KID WWTP, MDA WWTP, QUIN WWTP, REE WWTP, SCH WWTP and ZWE WWTP are represented by black pins on the map. ArcGIS 10.6.1 was used to construct all maps and the coordinates for the WWTPs were confirmed using Google Earth.

3.2.2.1 Study site description

The Eastern Cape Province falls within the scope of South Africa's nine provinces. Eastern Cape is one of the most impoverished provinces, mainly consisting of rural settlements with inadequate hygiene services and poor infrastructure. The province is divided into two metropolitan municipalities for local government purposes, one being Buffalo City Metropolitan Municipality.

Buffalo City Municipality is located on the eastern shore of the Eastern Cape and has a mild climate with an average of 850 mm of yearly rainfall. It accounts for approximately 10% of the overall populace in the Eastern Cape Province and was once declared as one of the COVID-19 hotspots in the country. Study samples were sourced from certain wastewater treatment plants situated within the Buffalo City Metropolitan Municipality of the Eastern Cape Province.

Sites and samples collected from the wastewater treatment facilities were represented in codes respective to the collection points. The EB WWTP and QUIN WWTP had two influent collection points EB-A and EB-B, GON-A and GON-B, respectively, and samples from each point were coded accordingly. The properties of each sample collection point are represented in Table 3.1. The influent reaching these facilities was composed of waste from household lavatories, sanitary sewage, wastewater from household sinks, laundry, food waste, rainwater run-offs in case of rainy days, Organic matter from run-offs, suspended solids, agricultural runoffs, microorganisms among myriads of contents.

Table 3.1: Characteristics of the Buffalo City Metropolitan Municipality wastewater treatment facilities (inquired during site visits and confirmed on the DWS Green Drop Report released in 2022 and the Department of Statistics South Africa, (2022). The EB WWRW and QUIN WWTW had two influent collection points, and the 494 samples retrieved from each point were encoded accordingly.

WWT Facility	Resource discharged into:	Wastewater technology used	System design capacity (ML/d)	Population served by facility
BIS WWTP	Yellowwoods River	Dose processing and oxidation ponds	2	150995
CEN WWTP	Buffalo River	Oxidation & Biofiltration	8	43100
DIM WWTP	Mdizeni Stream (Tributary of Keiskamma River)	Activated sludge system	7	18517
EB WWTP	Marine/sea discharge	Activated sludge system	40	141000
KID WWTP	Mcantsi River	Oxidation ponds	0.4	-
MDA WWTP	Unknown tributary of Buffalo	Activated sludge system	24	112900
QUIN WWTP	Sea discharge	Fine bubble aeration	18	30400
REE WWTP	Buffalo River	Vertical aeration blade method Biological odour control system	10	25400
SCH WWTP	Buffalo River	Activated sludge system Biofiltration, & Bioreactor system	7	35000
ZWE WWTP	Buffalo River	Activated sludge system	9	48900

3.2.3 Sample Collection and processing

Wastewater influent samples were sourced from the respective WWTPs weekly. Sampling was done for a 12-month sampling regime during the COVID-19 pandemic (June 2021 - June 2022). Sample collection was standardised by collecting influent between 07:00 am and 11:00 am. Wastewater influent samples were aseptically collected at a thoroughly mixed point using a Long handle sampler telescope and were aseptically transferred into sterile 500 mL Nalgene bottles through the grab-sampling technique and kept at 4°C to reduce changes in sample quality until analysis. The well-labelled samples were then transported to the National Wastewater Coronavirus Surveillance Laboratory at the University of Fort Hare for processing and analysis within six hours of sample collection.

Upon arrival at the lab, the facility was decontaminated with UV light. The wastewater samples were homogenised within a sterile Biosafety level 2 hood cabinet after decontamination after decontamination. After that, aliquots of the samples were dispensed into 50 mL capacity centrifuge tubes ($\times 4$ per sample, depending on the turbidity of the sample). The aliquots were then separated at 2500 rpm for 20 minutes (Johnson *et al.*, 2021; Peccia *et al.*, 2021; Street *et al.*, 2021). After centrifuging, the supernatant was discarded, and the concentrated pellet was used for nucleic acid extraction and subsequent RT-qPCR procedure. This method was validated in accordance with the standard operation protocol and manufacturer guidelines on the Qiagen RNeasy PowerSoil Total RNA extraction Kit.

3.2.4 Viral RNA Extraction and Quantification

Viral RNA extraction from the processed wastewater influent samples was conducted using the optimised protocol reported by Johnson *et al.*, (2021). Approximately 7.5 mL of the collected pellet (obtained in section 3.2.1) was used to extract total RNA using the QIAGEN RNeasy PowerSoil Total RNA Kit (QIAGEN, Germany) following the manufacturer's instructions as outlined in the extraction Kit information package.

Briefly, 7.5 mL of sample pellet was added into well-labelled 15 mL Bead tubes (x12). 2.5 mL of Bead solution, 0.5 mL of solution SR1 and 0.8 mL of solution IRS were pipetted onto the pellets. The mixtures were homogenised using a vortex in preparation for the addition of Phenol/chloroform/isoamyl alcohol (pH 6.5-8). The lights were switched off, and a volume of 2.5 mL of PCI was added to the 15 mL tubes using a pipette. The mixture was then vortexed until the biphasic layer disappeared. The bead tubes were placed on a vortex adapter at high speed for 15 min to allow the beads to lyse cells and organelles. Afterwards, the bead tubes were placed in a centrifuge, and the mixture was separated at 2500 rpm for 10 min.

Subsequently, three layers appeared following the separation process. The upper aqueous layer was transferred to a sterile 15 mL volumetric tube using disposable pipettes/droppers (ensuring not to disturb the interphase and lower phenol layer). The 15 mL tubes containing the remaining interphase and lower phenol layer were discarded. 1.5 mL of solution SR3 was pipetted into the aqueous phase, and the tubes were vortexed for homogenisation. The tubes were incubated at 2 - 8 °C for 10 min and then centrifuged at 2 500 rpm for 10 min at room temperature. While avoiding the pellet, the supernatant was transferred to new sterile 15 mL collection tubes, and the pellet was discarded. 5 mL of solution SR4 was added to the supernatant in the collection tubes. The collection tubes were inverted to mix, and the mixtures were incubated at room temperature for 30 min.

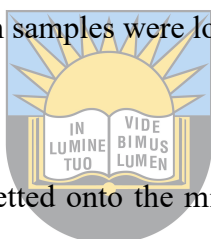
After incubation, the mixtures were separated at 2 500 rpm for 30 min. The surface of the Biosafety level 2 hood cabinet and paper towel were decontaminated using ethanol and RNA-away spray. The supernatant was discarded, and the 15 mL collection tubes were inverted onto sterile paper towels for 5 min/until dry. Solution SR5 was shaken, and 1 mL was pipetted into the pellets. The pellets were resuspended entirely by continuous pipetting and vortexing. When the pellet was not easily resuspended, the tubes were placed in a 45 °C for less than 10 min.

3.2.4.1 Preparation of mini-column for each RNA isolation sample.

The caps were removed from the 15 mL tubes, and the RNA capture columns were placed inside. The columns hung on the collection tubes. 2 mL of solution SR5 was pipetted onto the mini-columns. The solution was allowed to flow entirely through the column into the 15 mL collection tubes. Before RNA isolation samples were loaded, the columns were not allowed to dry out completely.

The RNA isolation samples were pipetted onto the mini-columns and were allowed to flow through the columns into the collection tubes. 1 mL of solution SR5 was added into the mini columns and allowed to flow by gravitation force through the columns into the collection tubes. Once the samples were fully collected into the 15 mL collection tubes, the mini-columns were transferred into new 15 mL collection tubes. Solution SR6 was shaken and mixed, and 1 mL of SR6 was added into the mini-columns to elute the bound RNA. The SR6 solution was allowed to flow through to the 15 mL collection tubes. The eluted RNA was transferred to 2.2 mL Eppendorf tubes. 1 mL of solution SR4 was pipetted into the RNA. The tubes were inverted to mix and were incubated at -15 to -30 °C for a minimum of 10 min.

The 2.2 mL Eppendorf tubes were centrifuged at 13 000 rpm for 15 min to pellet the RNA. After the RNA was separated, the supernatant was decanted, and the tubes were inverted onto a sterile paper towel in the lamina flow for 10 min to air dry the pellets. 50 µL solution SR7

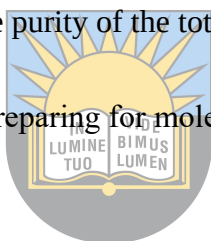


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was pipetted into the pellets to resuspend the RNA. The RNA was kept on ice while preparing for quantification.

The evaluation of the extracted RNA's purity and concentration were implemented through the utilisation of the Nanodrop™ One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA). 2 µL of solution SR7 was pipetted onto the pedestal to blank the spectrophotometer. After that, a sterile paper towel was used to wipe the pedestal. 1 µL of sample RNA was pipetted onto the spectrophotometer. The pedestal was sterilised each time a sample was loaded. Thereafter, the nanogram was exported in Microsoft Excel format for further calculations. For quality check, absorbance ratios at A260/A280 (1.8-2) and A260/A230 (1.8-2.1) wavelengths were measured to determine the concentration of the extracted RNA as well as to ensure the purity of the total RNA sample.

The RNA was stored at -80°C while preparing for molecular detection by RT-qPCR.



3.2.5 Detection of SARS-CoV-2 genome by Quantitative Reverse Transcription Polymerase Chain Reaction

iTaq Probe-Based qPCR assays (BIO-RAD, USA) were employed to determine and quantify the concentrations of SARS-CoV-2 virus in the extracted RNA samples using the QuantStudio 5 qPCR system (Applied Biosystems). The Nucleocapsid protein-encoding genes SARS-CoV-2_N1-P and SARS-CoV-2_N2-P (2019-nCoV, CDC, EUA) were targeted to amplify and quantify SARS-CoV-2 in the processed samples. The 2019-nCoV-N-Positive plasmid control (200 000 copies/µL) (Qauntabio, USA) was used as a positive control. Standards were prepared in 1:10 fold serial dilutions (for 200 000, 20 000, 2 000, 200 and 20 copies/µL standards) that were made with the 2019-nCoV-N-Positive plasmid control as a positive control and for viral copy number quantification (Johnson *et al.*, 2021; Peccia *et al.*, 2021; Street *et al.*, 2021). 1 uL

of each standard and the positive control (PTC) was used in every reaction. Table 3.2 describes the probes used to identify SARS-CoV-2 in the sample.

Table 3.2: 2019 nCoV N1 (Cat number RV202001) and 2019 nCoV N2 (Cat number RV202002) oligonucleotide probes used in RT-qPCR to detect the SARS-CoV-2 genome in the municipal wastewater samples.

Name	Description	Oligonucleotide sequence (5' - 3')	References
SARS-CoV-2_N1-P	2019 nCoV_N1 Probe	FAM-ACC CCG CAT TAC GTT TGG ACC-NFQ-MGB	(CDC, 2020; Johnson <i>et al.</i> , 2021; Street <i>et al.</i> , 2021)
SARS-CoV-2_N2-P	2019 nCoV_N2 Probe	FAM-ACA ATT TGC CCC CAG CGC TTC AG-NFQ-MGB	

The qPCR was done in a 96-well 0.2 mL block reaction plate (Applied biosystems®) by aliquoting 1 uL of the total RNA extracts to 9 uL of RT-qPCR reaction mixture consisting of 5 uL iTaq universal probes reaction mix, 0.25 uL of iScript RT advanced reverse transcriptase (BIORAD, USA), 0.5 uL of each SARS-CoV-2_N1-P and SARS-CoV-2_N2-P (Table 3.2), and 3.25 uL of sterile Nuclease free water (BIO-RAD, USA), making up a final volume of 10 uL per reaction. The same procedure was followed for the negative control (where RNA was substituted with nuclease free water) the standards, positive control and 1 uL of each was aliquoted into 9 uL of RT-qPCR reaction mixture.

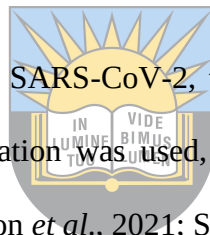
The reaction plate was carefully sealed with adhesive covers. All qPCR procedures ran for 40 cycles (Table 3.3) in the QuantStudio 5 qPCR system (Applied biosystems), followed by analysis using the Design & Analysis 2.4 RT PCR Software (Applied biosystems®). Analysis to obtain the quantitative data was done on each well, where positive samples were determined by the average Cycle Threshold (C_T) values ≤ 35 cycles, while the detection threshold limit

was determined at 0.02 per reaction. Tables 3.2 and 3.3 outline the probes and the PCR cycling conditions used to detect and quantify SARS-CoV-2 genomes.

Table 3.3: Thermal cycling conditions programmed for the amplification of target genes using specific N primer/probes which target the nucleocapsid region of the coronavirus.

AMPLIFICATION						
Real-Time PCR System	Setting	Reverse Transcription Reaction	Polymerase Activation and DNA Denaturation	Denaturation at 95 °C	Annealing/ Extension +Plate Read at 60 °C	Cycles
Quantstudio 5 PCR machine	Fast/Standard	10 min at 95 °C	3 min at 95 °C	15 sec at 95 °C	1 min at 60 °C	40

To evaluate the overall viral load of SARS-CoV-2, the data deduced (average quantity of genome copies (GC)) from amplification was used, and the values were substituted into Equation 3.1 and Equation 3.2 (Johnson *et al.*, 2021; Street *et al.*, 2021; Ngqwala *et al.*, 2024; Qongwe *et al.*, 2024).



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Equation 3.1 Average quantity × dilution factor (10) = GC/ µL

Equation 3.2 GC/ mL = (GC/ µL × 1000) / volume of the extracted sample

3.3 Results and Discussion

Samples were collected over a period of 46 surveillance weeks. Therefore, a sum of 552 samples was expected, i.e., 46 samples per site. However, there were times when sites were utterly inaccessible for sample collection. Sampling point EB-A was only discovered 9 weeks into sample collection. Ribonucleic acid concentrations detected from the influent wastewater samples retrieved from selected Buffalo City Municipality wastewater treatment works

In sum, 494 samples were retrieved from the wastewater treatment facilities. Viral RNA was extracted from the samples, and the quantity and quality of the RNA was ensured through nanodrop spectrophotometry. On average, influent samples collected from site EB-A yielded the highest average viral RNA concentration of 1.36×10^3 ng/ μ L, and the lowest concentration was observed in wastewater influent samples retrieved from site REE. Analysis of all sites revealed a median concentration of 1.08×10^3 ng/ μ L, corroborating Wu *et al.*, (2020) and Foladori *et al.*, (2020) about quantified viral titre of SARS-CoV-2 in wastewater. The weekly data was pulled together and the average concentration of RNA retrieved from each study site per month was determined and represented in Figure 3.3. This data showcases the overall biological material harbouring RNA present in the municipal wastewater. The figure depicts a constant indicator of the integrity of the samples as they passed quality validation (Nanodrop spectrophotometry) thereby ensuring the reliability of overall SARS-CoV-2 findings. Figure 3.3 illustrates samples that are devoid of impurities and contaminants and affirms the reliability and accuracy of the processing and extraction protocols.

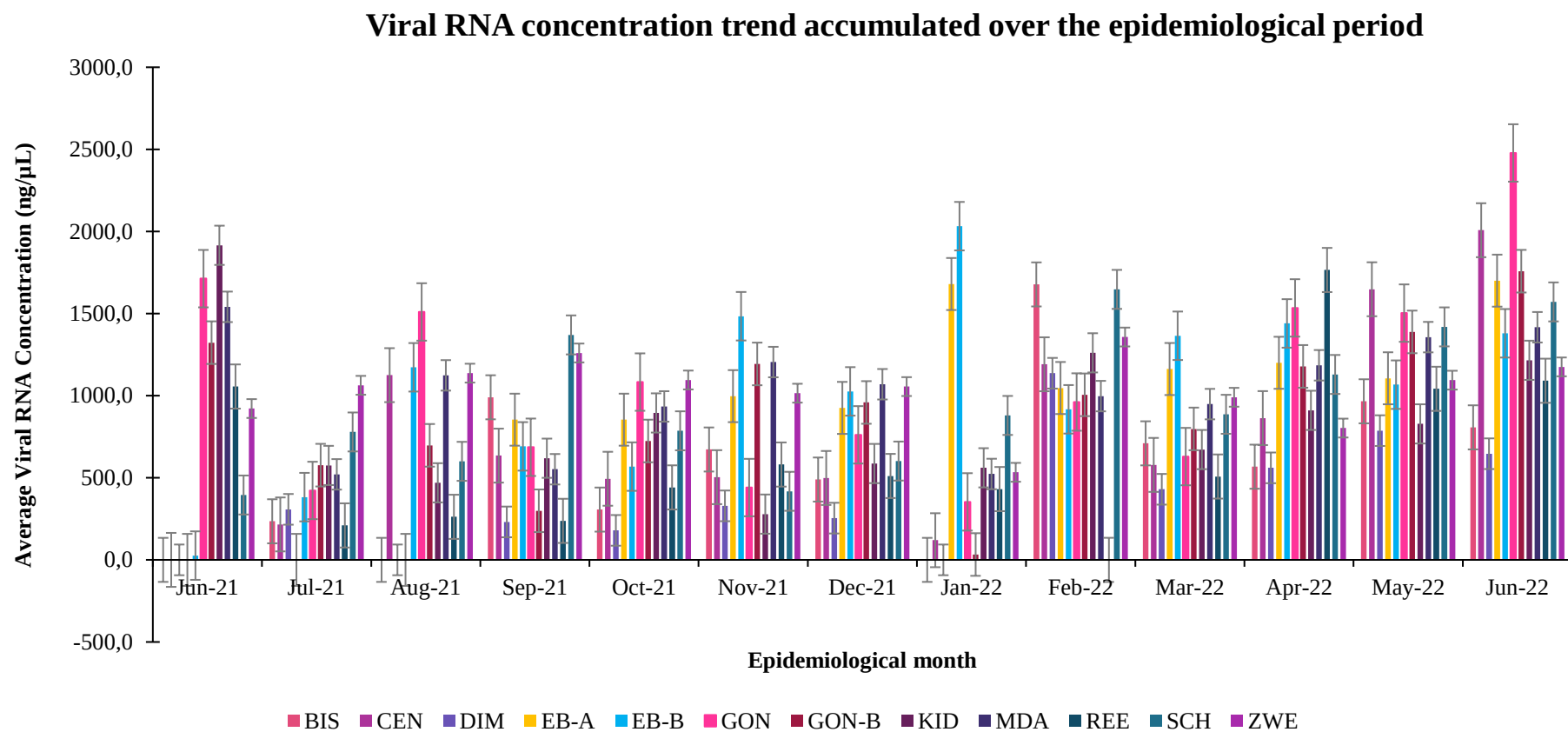


Figure 3.3: Time series of Nucleic acids (RNA) extracted from designated BCMM WWTPs. Variations in nucleic acid concentrations in municipal wastewater sourced from the WWTPs in BCMM were observed throughout the epidemiological period.

3.3.1 SARS-CoV-2 viral load attained from the influent wastewater samples retrieved from selected Buffalo City Metropolitan Municipality wastewater treatment works

Of 494 samples from municipal wastewater, 351 (71%) samples tested positive for the nucleocapsid genes N1 and N2, a SARS-CoV-2 indicator. Table 3.4 provides an overview of the proportional allocation of SARS-CoV-2 RNA in the wastewater samples recovered from each site.

Table 3.4: Percentage distribution of SARS-CoV-2 viral load in wastewater attained from the selected wastewater treatment facilities.

Sampling site (WWTP)	CT average		Number of samples retrieved from the sites	Samples positive for SARS-CoV-2 genome (%)
N1	N2			
BIS	31.9	30.1	28	86
CEN	35.4	30.9	39	72
DIM	33.8	31.7	28	75
EB-A	34.9	31.2	35	69
EB-B	36.6	32.1	45	76
GON-A	32.2	30.9	45	80
GON-B	33.9	30.7	45	67
KID	34.9	32.4	45	56
MDA	33.8	31.2	46	59
REE	34.4	30.9	38	71
SCH	34.6	34.1	46	72
ZWE	34.4	34.3	46	74

Equations 3.1 and 3.2 were used to determine the average N1 and N2 copy numbers/mL in the samples. To get the N1 and N2 average concentration per millilitre (mL) of wastewater sample, the average quantity of the target gene (N1 or N2) obtained at the 0.02 limit of detection threshold at average Ct values ≤ 35 cycles is multiplied by the dilution factor (10) to get target gene copy numbers in microlitres (μL). Moreover, the copy numbers (μL) are multiplied by

10^3 to convert the units to millilitres (mL). Once the units are converted, the target gene copy number is divided by the volume from which the template was extracted resulting in the concentration of the target gene in copy numbers/mL i.e. the SARS-CoV-2 viral load. Sample KID-40_i retrieved in epidemiological week 14 possessed the highest viral load which was approximately 26.91×10^3 GC/mL and was extracted from the KID wastewater treatment works. On average, the sampling site EB-B possessed the highest SARS-CoV-2 viral load, and SCH had the lowest. However, most of the samples retrieved from BIS wastewater treatment works possessed the nucleocapsid protein, indicative of the SARS-CoV-2 virus. On observation, influent wastewater samples collected from the BIS ranged from clear to slightly turbid throughout the study. The average viral load detected in the wastewater from this facility is 1.47×10^3 GC/mL.

On observation, wastewater influent samples collected from the EB wastewater treatment works were extremely turbid throughout the study. The influent coming into this site is composed of sewage, agricultural wastes, organic matter, stormwater and household wastes from four suburbs. The average viral load detected in the wastewater from this facility is 1.83×10^3 GC/mL for EB-A and 19.84×10^3 GC/mL for EB-B. Figure 3.5 represents the temporal data for the sites.

Essentially, Figure 3.4 depicts the distribution of the SARS-CoV-2 genome across the study area. The figure below demonstrates the distribution of SARS-CoV-2 viral particles, it indicates the presence of SARS-CoV-2 signals (GC/ml) in the influents of the communities being served by these WWTP which indicates the circulation of this virus in the community.

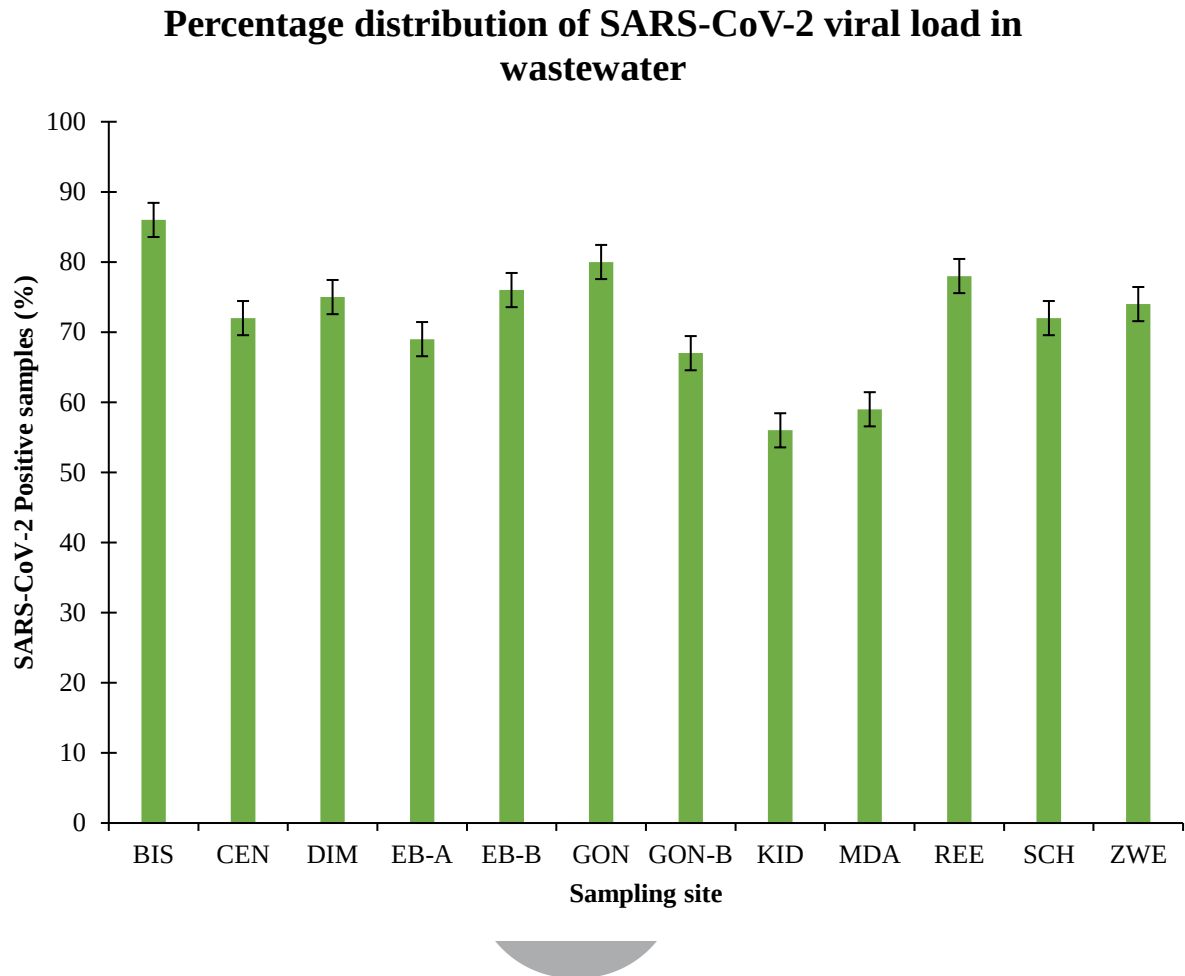


Figure 3.4: An overview of the percentage distribution of SARS-CoV-2 viral load yielded by the wastewater samples recovered from each sampling site between June 2021 and June 2022. The proportion of influent samples positive for the SARS-CoV-2 genome is relatively higher (86%) in samples retrieved from the BIS wastewater treatment works, and a lower percentage (56%) is observed from samples collected from KID.

A time series analysis was constructed to deduce the temporal fluctuations of the SARS-CoV-2 viral load and compare them to the clinical case data (extracted from the NICD surveillance reports) across the Buffalo City municipal region over 46 weeks.

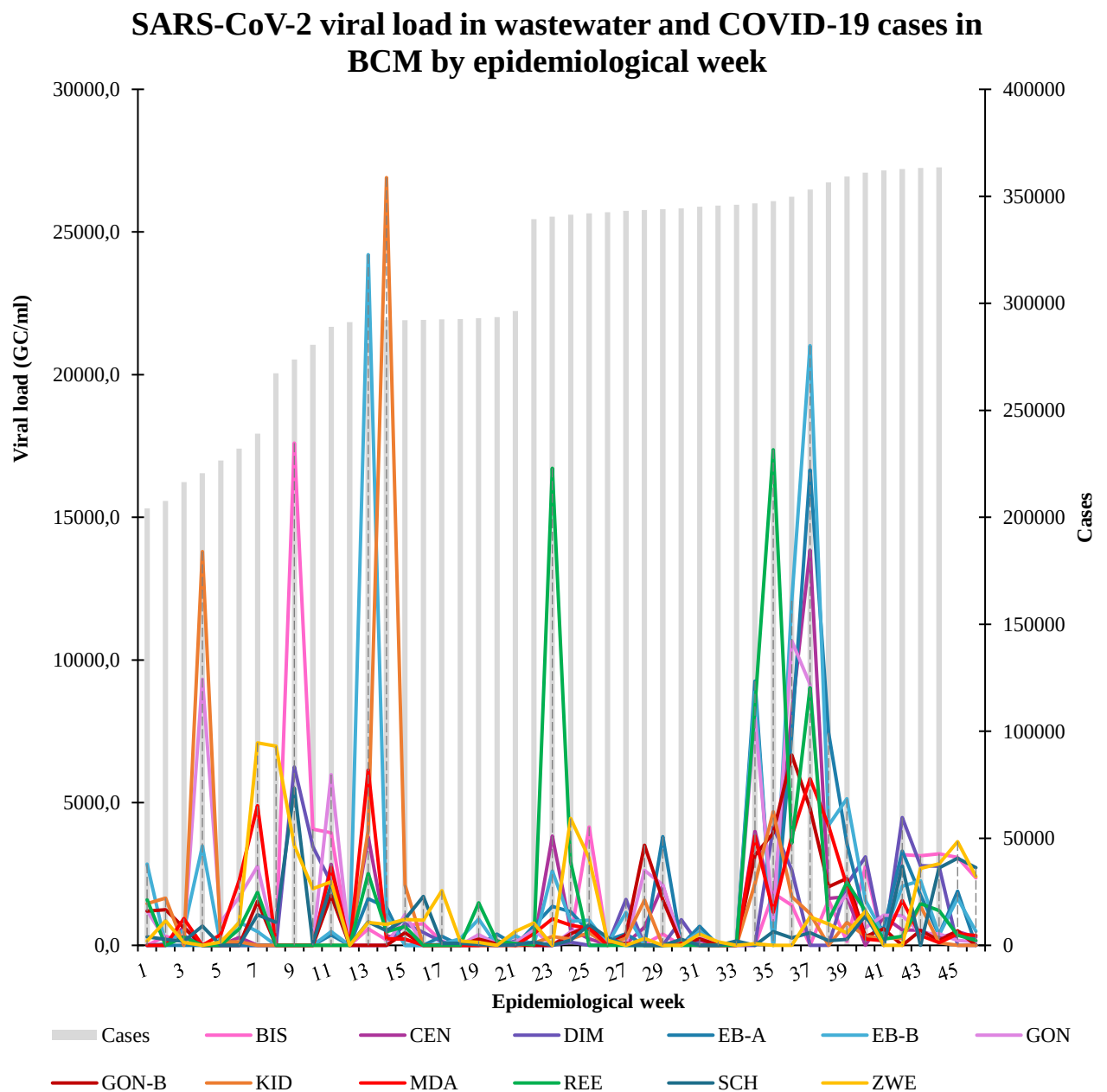
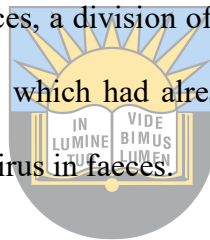


Figure 3.5: Temporal trends of the SARS-CoV-2 genome across the municipal wastewater treatment facilities. The light grey bars on the secondary axis represent the lab-confirmed clinical cases reported in the municipal region (NICD, 2021,2022). Fewer COVID-19 cases were reported from epidemiological week 25 to epidemiological week 46. In contrast, significant spikes in the SARS-CoV-2 genome in the wastewater are observed within that period (EPI week 34 to EPI week 38).

Fluctuations in SARS-CoV-2 levels in the municipal wastewater were observed throughout the study period. Figure 3.5 reveals the time series data of the SARS-CoV-2 RNA signals detected in the wastewater samples.

The plateau observed between epidemiological weeks 25 - 33 represents the period where there were no samples due to damage sustained by the pump station by the storms. However, the upward trendline is evidence of an increase in SARS-CoV-2 viral load. The National Institute for Communicable Diseases of South Africa (NICD) also reported a sudden spike observed in epidemiological week 23 (December 2021) in their WBE for SARS-CoV-2 surveillance report released on 11th December 2021 (NICD, 2021). This follows a spike in clinical cases reported in the municipal region as well as the Eastern Cape Province a week before (EPI week 22) by the National Health Laboratory Services, a division of the NICD. This suggests that the viral load in wastewater reflects infections which had already occurred in the region as infected individuals typically shed the coronavirus in faeces.



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3.3.2 Seasonal distribution of SARS-CoV-2 across the BCMM municipal wastewater

The temporal analysis reveals higher concentrations in winter, early spring, and mid-autumn (2022). The virus is persistent at low temperatures and low humidity. Figure 3.6 illustrates the different SARS-CoV-2 RNA concentration trends throughout the seasons. In essence, relatively high quantities of SARS-CoV-2 fragments were observed in the winter and autumn. The virus is more persistent at low temperatures and low humidity, conditions often observed in the two seasons.

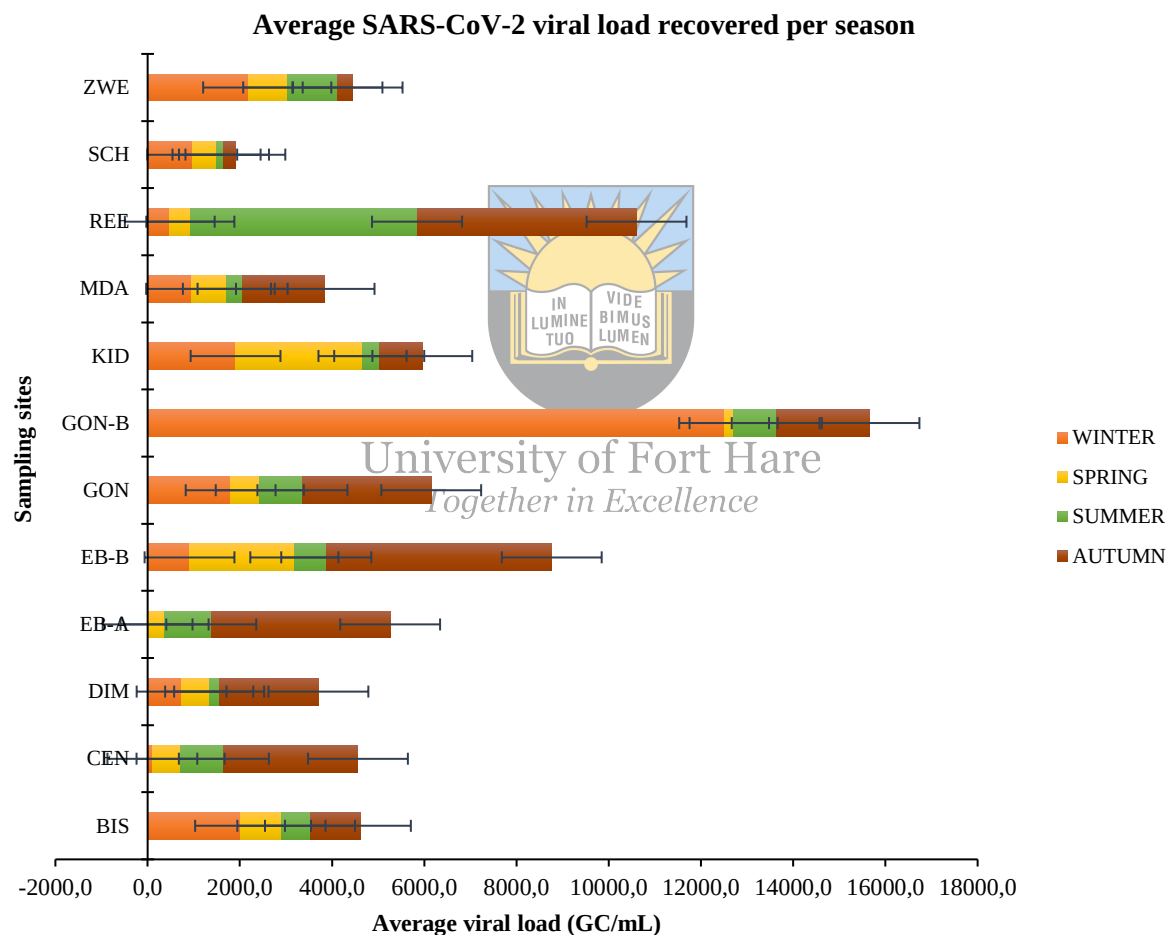


Figure 3.6: Seasonal distribution of SARS-CoV-2 in the wastewater of the BCM. The average concentration data reveals relatively high SARS-CoV-2 fragments in the winter and autumn epidemiological weeks. Winter (2021) is represented by orange bars, spring (2021) is

represented by yellow bars, summer (2021 - 2022) is represented by light green bars and autumn (2022) is represented by brown bars. The total viral load recovered across the study sites in winter was approximately 22.08×10^4 GC/mL, spring 13.09×10^4 GC/mL, summer 7.61×10^4 GC/mL and autumn 29.30×10^4 GC/mL. When assessing the viral load accumulated when evaluating seasonal data, higher concentrations were extracted from wastewater samples attained from the QUIN WWTP throughout the epidemiological period.

Wastewater is an intricate medium comprising organic and inorganic substances, an array of microorganisms, fragments of genetic material and viruses (Chahal *et al.*, 2016; Koul *et al.*, 2022). Viruses are ubiquitous and persistent in raw and treated wastewater and the receiving water bodies (Chigor and Okoh, 2012; Corpuz *et al.*, 2020). Numerous investigations have reported the incidence of viruses in municipal wastewater, and their findings ascertain the evidence of viral genome in the wastewater milieu (Adefisoye *et al.*, 2016; Gcilitshana *et al.*, 2017; Ibrahim *et al.*, 2021).

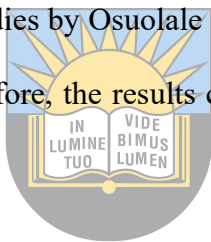


Nonetheless, there is diminutive data on the incidence and temporal trends of SARS-CoV-2 in influent sourced from wastewater treatment facilities in Buffalo City Metropolitan Municipality in the Eastern Cape Province. Hence, the present study explicates the incidence of the SARS-CoV-2 genome from selected municipal wastewater treatment facilities in the Buffalo City Region. Wastewater influent samples were collected from ten selected wastewater treatment plants using the grab sample technique. Subsequently, total viral RNA was extracted from the wastewater samples by means of commercially available extraction kits comprising three characteristic phases.

The primary phase is sample preparation involving microbeads and bead solution and the subsequent lysis of cells. The second phase is the organic extraction/phase separation, where RNA is separated from other compounds in the wastewater samples. The last phase is the

purification of nucleic acids. Nanodrop spectrophotometry further confirmed the quality of the extracted RNA. The spectrophotometer generally uses the principle of ultraviolet-visible spectrum absorbance to reflect the purity of ribonucleic acids from contaminants at the 260/280 and 260/230 absorbance ratios.

Wastewater influent samples from Buffalo City Metropolitan wastewater treatment facilities yielded average total RNA concentrations ranging between 0.8×10^3 - 1.5×10^3 ng/ μ L. The EB-A sampling site yielded an overall higher average total RNA concentration of 1.36×10^3 ng/ μ L. Samples from this site were highly turbid with a moderate flow rate throughout the study period, and the EB drainage reticulation served four suburbs with a combined population shown in Table 3.1. The incidence of ribonucleic acids in the wastewater samples corroborates the findings from the documented studies by Osuolale and Okoh, (2017), Wang *et al.*, (2020) and by Rothman *et al.*, (2021). Therefore, the results confirm the current viral indications in the wastewater environment.



Pure RNA extracts utilized for the molecular screening of the nucleocapsid gene. The nucleocapsid protein is fundamental for encapsulating the viral genome into the ribonucleotide complex and virion formation. It is the predominant protein in virions and is a potential target for most therapeutic practices (Dutta, Mazumdar and Gordy, 2020; Wu *et al.*, 2023). It is a significant diagnostic indicator for COVID-19 as the nucleocapsid-inducing gene is exclusive to SARS-CoV-2 and indicates the occurrence of the virus in test samples (Supekar *et al.*, 2021).

Quantitative Reverse-Transcription Polymerase Chain Reaction was employed to investigate the prevalence of SARS-CoV-2 in the municipal wastewater retrieved from the Buffalo City Metropolitan Municipality wastewater treatment facilities. The occurrence of viral load in wastewater is associated with the disease prevalence in the community. SARS-CoV-2 is shed in faecal waste from infected individuals. The virions pass through the gut and subsequently

reach wastewater facilities through household sewage (Duker *et al.*, 2024; Michie, 2024). Overtime SARS-CoV-2 underwent genomic mutations which altered COVID-19 severity and the transmissibility of the coronavirus. Throughout the surveillance period of the study, there was emergence of new variants which also could have contributed to the viral load in wastewater.

The Buffalo City region was declared a COVID-19 hotspot between June 2020 and December 2020, exceeding Nelson Mandela Bay Metropolitan by 25% (which is relatively larger in population). The overall trends reveal fluctuations in the SARS-CoV-2 signals in municipal wastewater. Higher concentrations (>5000 genomic copies/mL) can be observed from the fourth week (21st July 2021 to the 30th September 2021) of sample collection. This ensued during the third major epidemic of clinical cases. The Delta variant (B.1.617.2) predominated the third wave, which presented higher transmissibility than variant Beta (B.1.351, predominant in the second wave) (Bingham *et al.*, 2022).

Samples were collected and analysed during adjusted alert level 4, and sudden peaks were observed in epidemiological week 4, approximated 4 days (21st July 2021) before the transition from adjusted alert level 4 to 3. SARS-CoV-2 viral load in wastewater rose again in weeks 7 to 11 during the transition from adjusted alert levels 3 to 2. A significant rise was observed in epidemiological week 13 (the day alert level 2 was lifted), and a spike in the BIS WWTP was detected in epidemiological week 14 on 7th October 2021 (during the adjusted alert level 1).

From epidemiological weeks 14 to 22, the viral load stabilised. However, concentrations increased again for sites CEN, EB, MDA and REE in epidemiological week 23 (9th December 2021), approximately a week after the announcement of adjusted alert level 1, which was set in place on 1st December 2021, leading to the fourth wave of infections in the country

predominated by variant Omicron (B.1.1.529). The circulating variant enhanced disease transmissibility (WHO, 2021; Wolter *et al.*, 2022), hence the raised virus levels in wastewater.

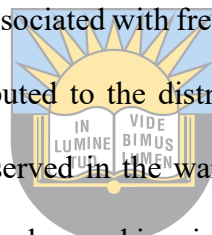
The viral load remained elevated until epidemiological week 25. The rise in SARS-CoV-2 concentrations in wastewater occurred during the local municipality elections, with an influx of people from different regions, and the country was at adjusted alert level one, where several restriction measures to prevent the transmission of the virus were lifted. A significant rise was observed in the REE WWTP with an average of 16.72×10^3 GC/mL because of the influx in into the area for voting purposes. `

The plateau observed in REE between epidemiology weeks 25 - 33 represents the period when sample collection was utterly impossible due to damage sustained by the pump station from the heavy storms that had occurred in that period. However, the upward trend line is evidence of an increase in SARS-CoV-2 viral load. The NICD also reported the sudden spike observed in epidemiological week 23 in their WBE for the SARS-CoV-2 surveillance report released on 11th December 2021. A variant of concern was discovered in South Africa during this period, and the World Health Organization further classified it as variant Omicron (B.1.1.529). Omicron is highly transmissible, resists the efficacy of therapeutics, and partially escapes acquired immunity.

The chronological patterns of SARS-CoV-2 epidemiology in wastewater are indicative of the fluctuations in the rate of infection, as seen in reported clinical cases of infection as well as the prevalence of variants in the municipal wastewaters. During the course of the study, spikes in SARS-CoV-2 signals in wastewater were observed as the number of reported cases of infection increased in the Eastern Cape Province. Prominent spikes in viral load were observed during the 3rd and 5th epidemic (wave) in the country. Cases of infection during the 3rd epidemic were predominated by VOC Delta (B.1.617.2). The proliferation of this variant was linked to the

increased immune evasion (Planas *et al.*, 2021), hence the elevated viral load during this period. July 2021 was reported as the peak period of the epidemic however the most significant rise in SARS-CoV-2 markers in wastewater was in September 2021 (Epidemiological week 9 to week 13). Since the inception of Omicron lineages, reported cases of infection in the country elevated. The 5th epidemic (wave) was driven by BA.4 and BA.5 which were highly transmissible and rendered lesser clinical severity than its predecessors (Jung *et al.*, 2022; Wolter *et al.*, 2022). A momentous increase in SARS-CoV-2 viral load was observed during this period in correlation to clinical cases and the emergence and circulation of variants.

The temporal distribution of SARS-CoV-2 viral load in wastewater depicts correlations between the increase in virus signals and the lifting of restriction measures. This can be attributed to basic human behaviour associated with freedom of movement. Environmental and climatic conditions may have contributed to the distribution of the virus in the wastewater milieu. Lower concentrations are observed in the warmer seasons, spring and summer (the warmest), whereas the opposite can be observed in winter and autumn. The latter are drier and characterised by lower temperatures.



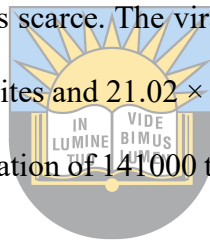
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Matsuki *et al.*, (2023) noted that winter conditions heightened the dissemination of viruses as the reduced temperatures are ideal for virion survival, hence high viral loads were observed in the winter. A review by Bisht and Velthuis, (2022) demonstrates that reduced humidity and temperatures facilitate the stability and spread of respiratory viruses. According to a study conducted by Peng *et al.*, (2023) the rate of decay in SARS-CoV-2 pseudovirus was significantly lower at reduced temperatures (< 25 °C).

Essentially, low environmental temperatures such as those observed in South African winter reduce the degradability of the coronavirus and preserve RNA integrity where the genome is generally stable as enzymatic activities are reduced. Moreover, extremely low temperatures

can damage the SARS-CoV-2 lipid envelope (ice crystals) and damage host-cell fusion and reduce replication (Bailey *et al.*, 2022; Ravi *et al.*, 2022; Peng *et al.*, 2023; Zhang *et al.*, 2024). According to Sharma *et al.*, (2020) an elevation in temperatures often observed in summer significantly disrupts stabilities in viral structure. A study conducted in Canada highlighted that coronaviruses were among the numerous respiratory viruses that are accountable for seasonal outbreaks, particularly in winter (Lagace-Wiens *et al.*, 2021).

Seasonal changes influence biological ecosystems via environmental relations and alter the epidemiology of infectious diseases. Climate change modulates host susceptibility and pathogen virulence. Several studies report increased mortality and morbidity in winter and autumn (Meo *et al.*, 2022; Lui *et al.*, 2023). Elevated viral loads were noted in mid-autumn as temperatures declined and rainfall was scarce. The viral load in this season ranged from $3.80 \times 10^3 - 17.37 \times 10^3$ GC/mL for other sites and $21.02 \times 10^3 - 810.99 \times 10^3$ GC/mL from the EB WWTP. This site serves a larger population of 141000 than other sites and is a combined sewer network.



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Many factors that need to be studied, such as shedding-related factors, in-sewer factors, and population size, can influence SARS-CoV-2 concentrations in influent wastewater (Bertels *et al.*, 2022). For example, documented reports by Wu *et al.*, (2021) and Wilder *et al.*, (2021) reveal the positive relationship between population size and SARS-CoV-2 concentrations in wastewater (Tuite *et al.*, 2020; Wu *et al.*, 2020). However, the population size within the catchment area cannot be assumed to be stationary as people commute from one place to another for different reasons. Hence, Wade and colleagues explore the uncertainties and variabilities for wastewater monitoring and suggest factors such as the monitoring of instabilities of inhabitants within the catchment area to comprehend trends in RNA concentrations (Bertels *et al.*, 2022; Wade *et al.*, 2022).

These findings confirm those of earlier studies, such as reviews and reports on the occurrences of the SARS-CoV-2 RNA in the wastewater environment by authors such as Foladori *et al.*, (2020), La Rosa *et al.*, (2020) and Medema *et al.*, (2020).



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3.4 Conclusion

These results emphasise the incidence of the ubiquitous nature of viruses in raw wastewater. The primary basis of SARS-CoV-2 genetic material in wastewater is human excrement of symptomatic and asymptomatic carriers. The molecular technique RT-qPCR facilitated the study's ability to give accurate data in real time. Upon reviewing the data, the findings elucidate the incidence of SARS-CoV-2 in the Buffalo City Metropolitan municipal wastewater. Elevated levels of the SARS-CoV-2 genome were observed in the colder months (winter and autumn), indicating the influence of colder climatic conditions on the dissemination of the virus. This finding emphasises further research beyond this study period to explore the influence of season on the distribution of SARS-CoV-2. This can assist in preparing health care systems and the public to better comprehend disease severity and circulation dynamics. A hasty public health response is imperative to avert further morbidity and mortality, as the pandemic has burdened healthcare systems in South Africa. Therefore, a regular wastewater surveillance study is highly recommended beyond this study period to predict the potential resurgence of SARS-CoV-2 and other disease outbreaks. For instance, pathogens such as the *Listeria monocytogenes* and *Vibrio cholerae* have caused in epidemics in the past in South Africa. Recently, Hammanskraal suffered the resurgence of a devastating cholera outbreak. To avoid a similar instance, it is quite imperative that the country has a proactive approach in monitoring communities for pathogens of medical importance and monitor their genetic composition to curb fatal outbreaks that may be caused through the evolution of virulent factors.

3.5 List of references

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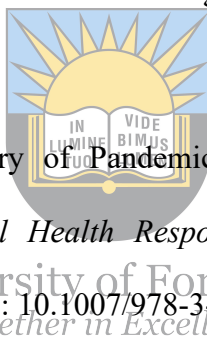
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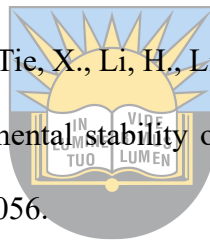
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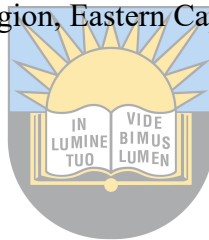
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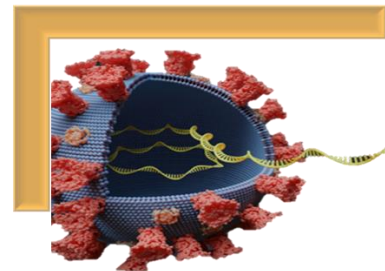
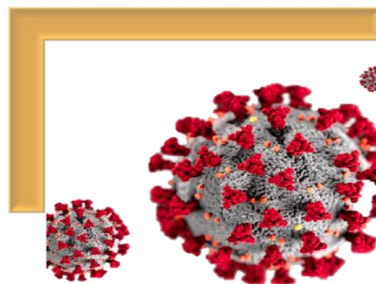
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4. CHAPTER FOUR

Real-Time PCR Targeting the Single Nucleotide Polymorphism (SNP) for the genotypic analysis of SARS-CoV-2 variants in selected wastewater Treatment facilities within the Buffalo City region, Eastern Cape, South Africa



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Real-Time PCR Targeting the Single Nucleotide Polymorphism (SNP) for the genotypic analysis of SARS-CoV-2 variants in selected wastewater Treatment facilities within the Buffalo City region, Eastern Cape, South Africa

Abstract

The inception of SARS-CoV-2 variants with remarkable transmissibility and infectivity has caused a great deal of global health calamity. The evolution of the coronavirus resulted in momentous surges in COVID-19 cases that brought about various epidemics in South Africa dominated by variants of concern. Thus, this study was aimed at the genotypic profiling of the spike-protein gene mutations signature to SARS-CoV-2 variants in samples from wastewater treatment plants in the Buffalo City region, South Africa. SARS-CoV-2 RNA fragments extracted from municipal wastewater influent samples were screened for the variant of concern signature mutations. The TaqMan SARS-CoV-2 mutation panel genotyping-molecular-specific RT-PCR assay, targeting the single nucleotide polymorphisms (SNP), was used to detect SARS-CoV-2 variants rapidly—the panel of mutations used related to the B.1617.2 and the B.1.1.529 lineages of SARS-CoV-2 variants. The present study revealed that the predominant amino acid mutation was the *N501Y*, shared between Alpha (B.1.17), Beta (B.1.33, B1.351) and Omicron. This result affirms the circulation of variants of concern in the specific municipal regions of Buffalo City, such findings underscore the essence of population-based approaches such the wastewater-based epidemiology.

Keywords: SARS-CoV-2, variant, mutations, SNP, frequency, wastewater

4.1 Introduction

From the time when the COVID-19 global catastrophe ensued, SARS-CoV-2 has rapidly progressed by acquiring nucleotide changes attributed to elevated mutation frequencies, estimated between 10^{-5} and 10^{-3} , altering viral protein structures, function, and antigenic properties (Lauring and Hodcroft, 202; Abavisani *et al.*, 2022). The high rate of genomic alterations in the SARS-CoV-2 genome and on the viral spike trimeric surface glycoprotein leads to the emergence of variants (Rothenberger *et al.*, 2022). Since the first detection of variants, increased transmissibility, immune evasion, and disease severity have been observed.

The World Health Organization classified the variants that can escape immune response and increase transmissibility and the propensity to infect and increase disease severity as variants of concern (VOC). Throughout the global pandemic, SARS-CoV-2 VOCs rapidly proliferated in different regions globally. Various spike protein mutations and deletions are some of the morphological changes detected in the VOCs (Aksamentov *et al.*, 2021; Tao *et al.*, 2021). The variants display mutual alterations on the spike protein predominantly within the receptor-binding domain. These mutations are associated with enhanced structural stability and increased ACE2 receptor-binding capacity of the spike protein (Zhang *et al.*, 2021; Flores-Vega *et al.*, 2022). This attribute may be paramount in the heightened transmissibility and infectivity observed in the virus (Magazine *et al.*, 2022).

In May 2020, *D614G* amino acid substitution was detected in the receptor-binding motif of the S protein of SARS-CoV-2. The mutation was detected in 78% of the clinical samples worldwide and rapidly dominated variants globally (Zhou *et al.*, 2021). Amino acid mutations *N501Y*, *DelH69V70*, and *P681H* subsequently emerged. The mutations conferred increased transmissibility and infectivity. The strains possessing these alterations were subsequently

categorised as the Alpha (B.1.1.7) variant, which originated in September 2020 at the United Kingdom (Gobeil *et al.*, 2021).

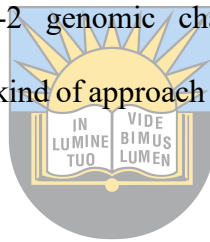
By December 2020, the Network for Genomics Surveillance in South Africa (NGS-SA) detected new SARS-CoV-2 strains possessing mutations *N501Y*, *K417N*, and *E484K*. These mutations are signature spike alterations of VOC Beta (B.1.351), which appeared following the first epidemic in South Africa. In that period, the virus had scourged two provinces, and among such was a metropolitan in the Eastern Cape Province where the VOC emerged (Tegally *et al.*, 2020; Johnson *et al.*, 2022). The VOC dominated COVID-19 cases of infections and accounted for 80% of SARS-CoV-2 genomes circulating in South Africa (Tambe *et al.*, 2023).

In January 2021, another variant, Gamma (P.1), was detected in Brazil, which was further detected in Japan in samples obtained from travellers from Brazil. Four months later, India was ravaged by a more infectious strain classified as the Delta (B.1.617.2) variant, which disseminated throughout the globe. In South Africa, the Delta-driven epidemic was the most severe wave, with excessive hospital admissions and a proliferated mortality rate (Amman *et al.*, 2022; Jassat, Abdool Karim, *et al.*, 2022). Moreover, in November 2021, samples from an immunocompromised patient possessed the Omicron variant (Akkız, 2022; Reincke *et al.*, 2022).

Omicron became the predominant variant just a month after its first detection (Jassat, Karim, *et al.*, 2022; Madhi *et al.*, 2022; Mahase, 2022; Yang and Shaman, 2022). Since its first discovery, Omicron has dominated COVID-19 cases. Clinical specimens from immunocompromised COVID-19 patients worldwide were continuously dominated by subvariants BA.1, BA.2, BA.3, BA.4, BA.5, XBB, and EG.5 (Fang *et al.*, 2023; Zhakparov *et al.*, 2023). These variations are attributable to shared traits and characteristics requiring public health interventions (Rambaut *et al.*, 2020; Konings *et al.*, 2021).

One of the interventions used to track the SARS-CoV-2 genome and infection trends in communities is wastewater surveillance. However, limited studies have utilised the approach to routinely screen for VOCs, as most researchers have utilised next-generation sequencing (NGS) to evaluate the genomic variations of SARS-CoV-2. NGS may be useful for identifying novel variants. However, the regular screening of wastewater by NSG for detecting and characterising known VOC mutations is expensive (Johnson *et al.*, 2022; Mills *et al.*, 2022).

This investigation sought to monitor wastewater influent through a rapid SNP genotyping assay utilising the TaqMan SARS-CoV-2 mutation panel to investigate the SARS-CoV-2 spike protein mutations associated with perilous VOCs circulating at the communities served by ten WWTPs in the Buffalo City region, South Africa. Genomic surveillance is one primary approach employed in SARS-CoV-2 genomic characterisation for detecting variants. Nevertheless, the disadvantage of this kind of approach is that it utilises data captured in clinical facilities.



The data represented in such databases is that of immunocompromised individuals examined in the facilities. Therefore, the genomic variations in SARS-CoV-2 from asymptomatic individuals and those not tested in the centres are not accounted for, thus not reflecting the viral genetic diversity in the population in question (Aguiar-Oliveira *et al.*, 2020; Tambe *et al.*, 2023).

4.2 Methodology

4.2.1 Ethical clearance certificate and permits

For ethical clearance and permission to commence with the study please refer to Section 3.2.1, page 100.

4.2.2 Sample collection, concentration and viral RNA extraction

Wastewater sample concentration, total RNA extraction and quantification were done according to the modified protocol as described by Johnson *et al.*, 2021. For a detailed protocol please refer to Section 3.2.3, page 104 and Section 3.2.4, pages 105-107.

4.2.3 Profiling of SARS-CoV-2 genomes by Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

The extracted RNA samples were subsequently profiled for the presence of SARS-CoV-2 genomes. The iTaq Probe-Based qPCR assay (BIO-RAD, USA) was used to determine and quantify the SARS-CoV-2 genomes targeting the nucleocapsid protein-encoding genes SARS-CoV-2_N1-P and SARS-CoV-2_N2-P (2019-nCoV, CDC, EUA) and the real-time polymerase chain reaction procedure for the detection and quantification of SARS-CoV-2 genomes from wastewater influents was performed based on the protocol as described by Street *et al.* (2021). Chapter 3, section 3.2.5 outlines in detail the RT-qPCR protocol, probes and thermal cycling conditions that were used in the study for profiling the incidence of SARS-CoV-2 in the wastewater.

4.2.4 Detection of the relevant SARS-CoV-2 variants by Single Nucleotide Polymorphism (SNP) genotyping.

TaqMan SARS-CoV-2 mutation panel genotyping-molecular-specific RT-PCR assay, targeting the single nucleotide polymorphisms (SNP), was used to detect the SARS-CoV-2 signature mutations. SARS-CoV-2 RNA samples with a viral load ≥ 1000 genome copies per mL were screened for the S-gene mutations using the TaqPath mutation panel (*L452R*, *P681H*, *N501Y*, *delH69V70*, *E484K*, *K417N*, *P681R*) was used. Tables 4.1, 4.2 and 4.3 represent the RT-PCR constituents, TaqPath mutation probes and the thermocycling parameters used for screening SARS-CoV-2 signature mutations.

Table 4.1: The RT-PCR reaction mixture constituents

Reaction components	X1 (μL)
TaqPath RT-PCR Master mix	5
TaqPath Mutation panel	0.5
Nuclease free water	9.5
RNA (2 μg RNA)	5
Total reaction volume	20

The reaction constituents depicted in Table 4.2 were aliquoted into a 96-well 0.2 mL block reaction plate (Applied biosystems®). Essentially, 5 uL of the total RNA pure extracts were aliquoted to 5 uL of TaqPath™ 1-Step RT-qPCR Master mix (Applied biosystems, Thermo Fischer Scientific), 0.5 uL of each TaqPath™ SARS-CoV-2 Mutation panel (Table 4.1), and 9.5 uL of sterile Nuclease free water (BIO-RAD, USA), yielding a total reaction volume of 20 uL per reaction. Adhesive covers were used to cover the reaction plate. All RT-PCR procedures (Table 4.3) were programmed in the Quant-Studio 5 RT-qPCR system (Applied biosystems), followed by analysis using the Design & Analysis 2.4 RT-PCR Software (Applied biosystems®).

Table 4.2: Spike protein primers/probes for RT-qPCR and SNP genotyping

Target	TaqPath SARS-CoV-2 mutational panel	Reference
Spike protein	S.delH69V70	(Johnson <i>et al.</i> , 2022)
	S.K417N.AAG.AAT	
	S.E484K.GAA.AAA	
	S.P681H.CCT.CAT	
	S.P681R.CCT.CGT	
	S.L452R.CTG.CGG	
	S.N501Y.AAT.TAT	

Table 4.3: Thermal cycling conditions programmed to amplify the TaqPath® mutation panel.

Step	Temperature (°C)	Time	No. cycles
Pre-read	60	30 seconds	1
Reverse transcription	50	10 minutes	1
Fast DNA polymerase activation	95	2 minutes	1
Denaturation	95	3 seconds	45
Annealing/Extension	60	30 seconds	
Post-read	60	30 seconds	1

Amplification data was analysed to evaluate the genotypes, where positive samples were determined by the amplification of target genes and evaluation of the allelic discrimination plot, which indicates precise discriminations between the wild-type samples (x-axis), no template control (NTC; bottom left corner of the plot) and the mutation samples (y-axis). The mutated samples are detected by fluorescein (FAM).

4.3 Results and Discussion

SARS-CoV-2 RNA samples ($n = 45$) with a viral load ≥ 1000 genome copies per mL (Table 4.4) were screened for the S-gene mutations

Table 4.4: SARS-CoV-2 samples screened for the spike protein mutations

Sample	Nucleic acid concentration (ng/uL)	SARS-CoV-2 viral load (GC/mL)
<i>BIS-27i</i>	1644	1667
<i>BIS-35i</i>	1779	17602
<i>BIS-36i</i>	1645	4072
<i>BIS-37i</i>	1365	3941
<i>BIS-67i</i>	2619	2304
<i>CEN-39i</i>	1412	3802
<i>CEN-62i</i>	1984	8119
<i>CEN-64i</i>	1267	1650
<i>DIM-56i</i>	2493	895
<i>EB-B-30i</i>	1635	3478
<i>EB-A-39i</i>	1352	1638
<i>EB-B-39i</i>	1821	24206
<i>EB-B-45i</i>	1979	2922
<i>EB-B-53i</i>	1056	1155
<i>EB-A-60i</i>	2635	9261
<i>EB-B-60i</i>	2323	8878
<i>EB-A-64i</i>	1698	7451
<i>EB-B-64i</i>	1617	4211
<i>GON-30i</i>	1526	9327
<i>GON-B-30i</i>	1785	107925
<i>GON-41i</i>	1058	1085
<i>GON-60i</i>	2635	7975
<i>GON-B-62</i>	1467	6667
<i>GON-64i</i>	1339	1948
<i>GON-B-64i</i>	1264	2052
<i>KID-40i</i>	1394	26905
<i>KID-41i</i>	1672	2109
<i>KID-60i</i>	1161	2169
<i>MDA-33i</i>	1306	4896
<i>MDA-37i</i>	1983	2735
<i>MDA-60i</i>	1580	3824
<i>MDA-61i</i>	1897	1157
<i>MDA-62i</i>	1076	3826
<i>MDA-64i</i>	1539	4186
<i>REE-60i</i>	1668	8367
<i>REE-61i</i>	2086	17368
<i>REE-62i</i>	1437	3581
<i>REE-64i</i>	1245	1869
<i>SCH-37i</i>	1995	2133

<i>SCH-41i</i>	1764	1922
<i>SCH-50i</i>	1458	2927
<i>ZWE-36i</i>	1337	1981
<i>ZWE-41i</i>	2348	1906
<i>ZWE-50i</i>	1474	4445
<i>ZWE-53i</i>	1003	1450

Samples with target gene amplifications were analysed for the incidence of SARS-CoV-2 mutations. An allele discrimination plot was generated after the RT-PCR reaction. On the cluster plot, samples with any of the mutations are clustered along the y-axis, and all the samples with wild-type or reference sequences are clustered along the x-axis of the cluster plot.

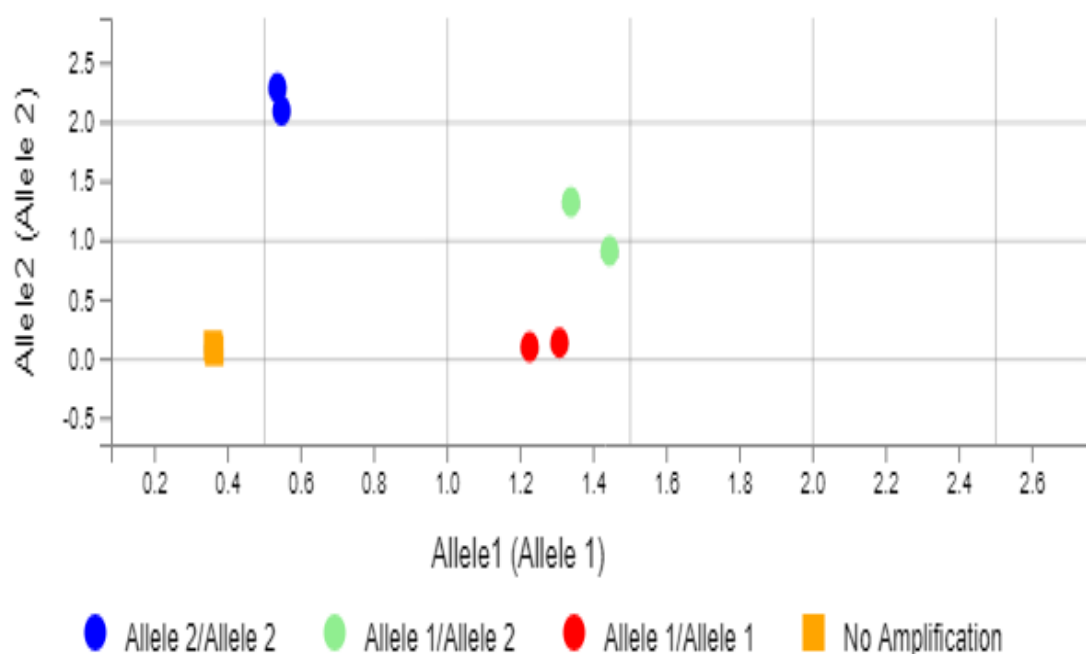
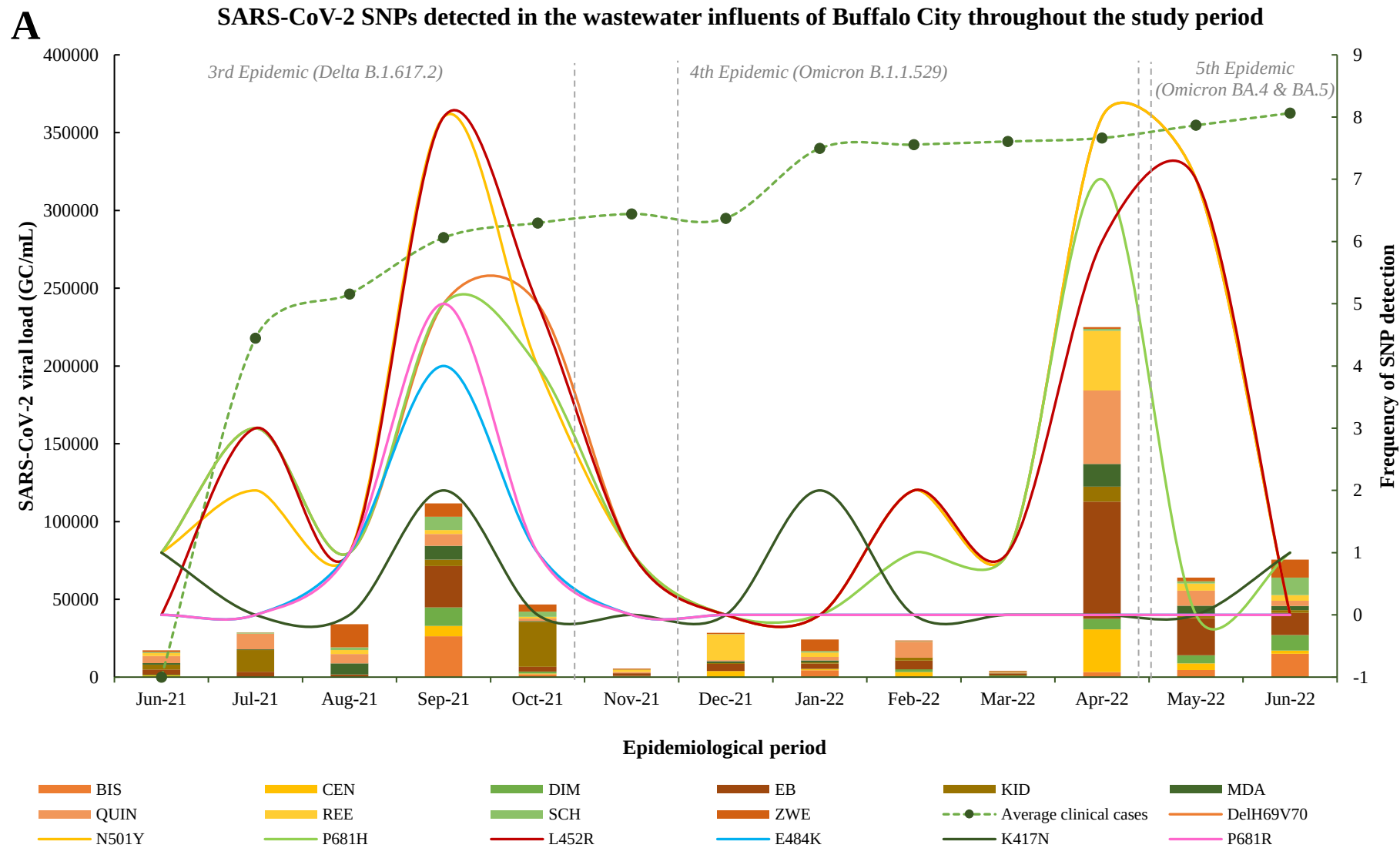


Figure 4.1: An allelic discrimination plot generated by the QuantStudio 5 RT-qPCR system and analysed through the Design & Analysis 2.4 RT-PCR Software. In this assay, allele 1 was determined by the allele reporter VIC, and allele reporter FAM determined allele 2. Allele 1/allele 2 represents samples with a heterogeneous genotype, i.e., a mixed mutation condition.

Table 4.5: A summary of the proportions (%) and genotypic characterisation of the mutation panel.

Spike protein mutation	No. samples tested	Not amplified (%)	Genotype		
			Heterozygous (%)	Homozygous (%)	
				Allele 1	Allele 2
<i>DelH69V70</i>	45	11	7	40	27
<i>N501Y</i>	45	11	11	29	44
<i>P681H</i>	36	11	11	6	64
<i>L452R</i>	42	10	12	2	69
<i>E484K</i>	6	0	0	100	0
<i>K417N</i>	8	25	0	75	0
<i>P681R</i>	16	50	0	0	50

The relevant SARS-CoV-2 mutations were determined, and the proportions and spatial analysis reveal that the *N501Y* signature mutation (associated with Alpha, Beta, and Omicron lineages) was the most predominant in the test samples within 84% of the SARS-CoV-2 genome retrieved in the municipal wastewater. 93% of SARS-CoV-2 RNA samples ($n = 45$) from the municipal influents collected from selected wastewater treatment sites with ≥ 1000 GC/mL possessed the target mutations. **Figures 4.2 and 4.3** illustrate the temporal and geospatial distribution of the SARS-CoV-2 genome across the Buffalo City region.



B

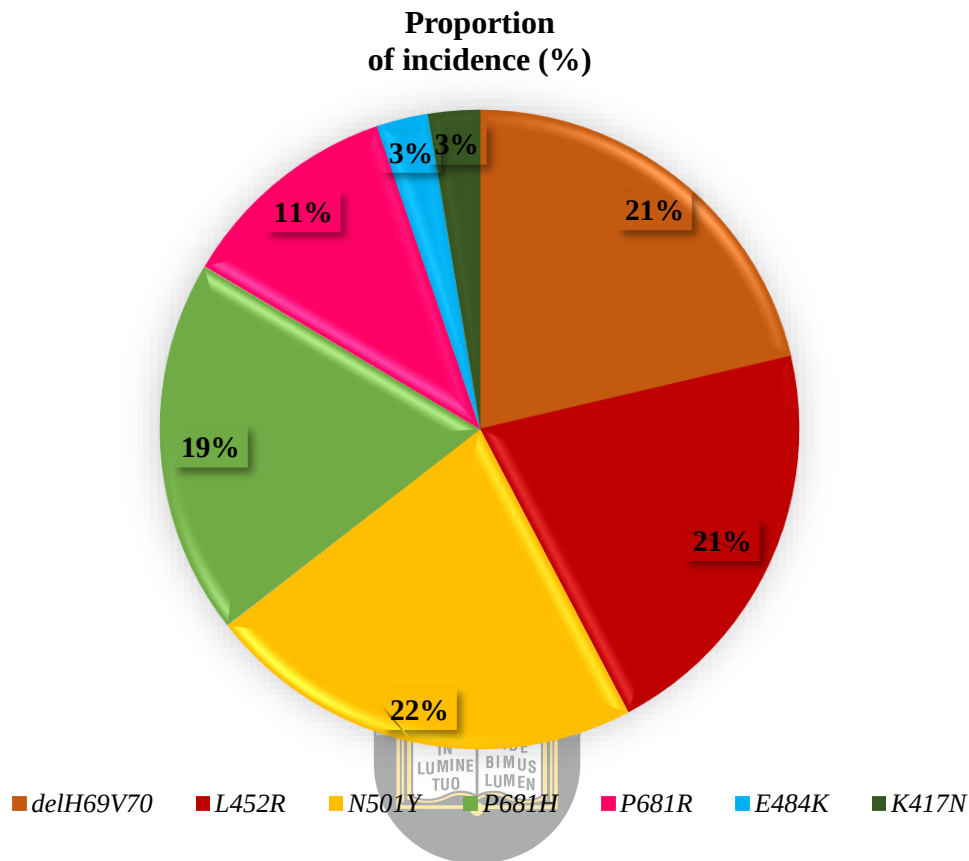


Figure 4.2: The temporal distribution and detection proportions of the SARS-CoV-2 spike protein mutations. Mutation *delH69V70* is presented in orange, *N501Y* presented in yellow, *P681H* presented in light green, *L452R* presented in dark red, *E484K* presented in blue, *K417N* presented in dark green and *P681R* presented in pink. **(A)** Frequency of detection of the signature mutations targeting the single nucleotide polymorphisms in the spike proteins of SARS-CoV-2 in relation to SARS-CoV-2 viral load in the wastewater throughout the surveillance period. The bars represent the SARS-CoV-2 viral load retrieved from the study sites; the line graph on the secondary axis represents SNP detected and the dotted line represents the average clinical cases within the epidemiological months recorded for the Eastern Cape Province on the NICD COVID-19 daily reports (June 2021- June 2022). Two prominent spikes are evident in September 2021 and in April 2021. **(B)** Proportions of the S-gene signature mutation panel. About 22% of SARS-CoV-2 RNA samples evaluated for SNPs possessed the *N501Y* mutation, while only 3% possessed the *E484K* and *K417N* spike protein mutations.

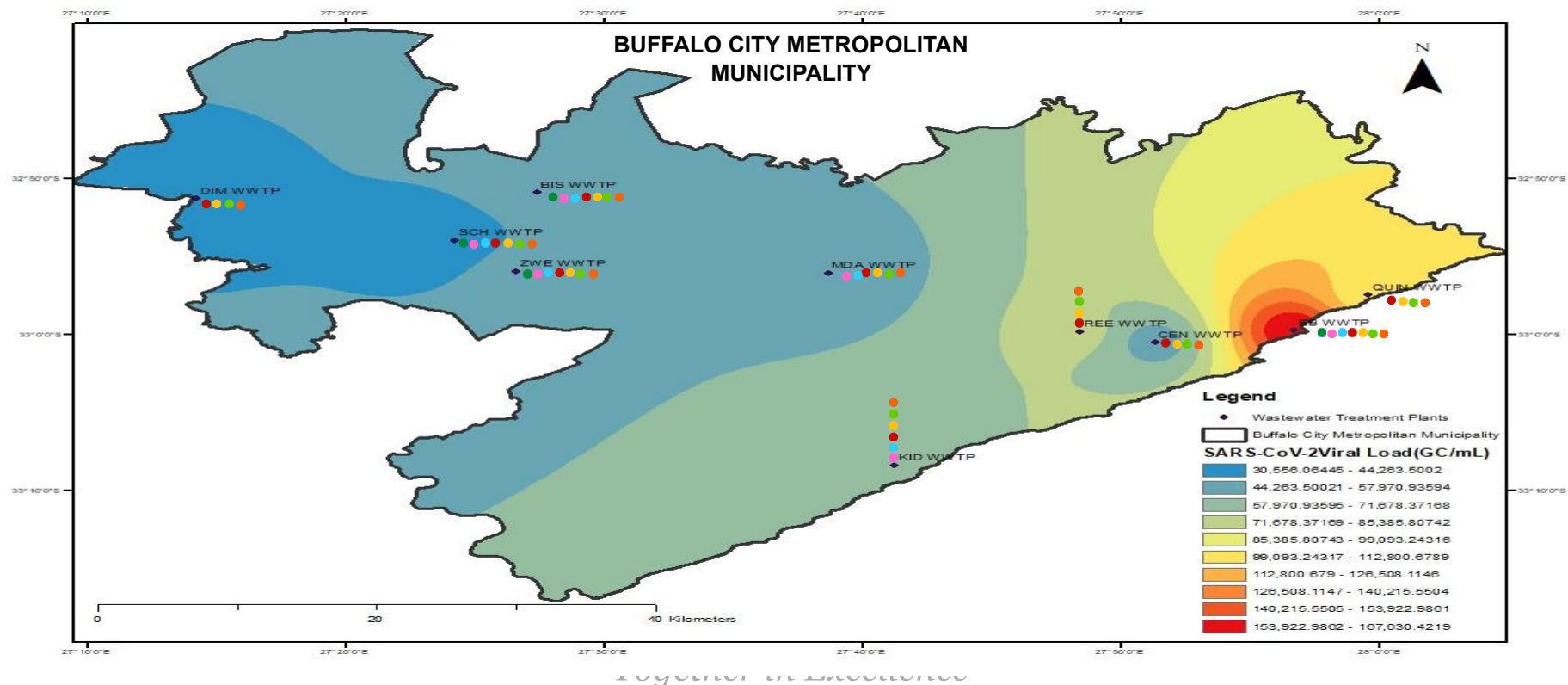


Figure 4.3: Geospatial distribution of SARS-CoV-2 and S-protein mutations linked with the variants of concern. Black spots on the map pinpoint the study sites. Sites BIS and EB equally had the most samples possessing the spike protein mutations, and site DIM had the least number of samples possessing the mutations. In terms of the viral load retrieved from the WWTPs site EB was the apparent hotspot represented in red. Wastewater samples from this site harboured all mutations screened for in this study. Coloured spots represent the spike protein mutations detected in the different locations: Orange represents delH69V70, yellow represents N501Y, light green represents P681H, dark red represents L452R, blue represents E484K, dark green represents K417N, and pink represents P681R.

The positive SARS-CoV-2 RNA samples recovered from the WWTPs throughout the study period tested positive for the spike protein mutations linked to VOCs: Alpha (*N501Y*, *P681H*, and *delH69V70*), Beta (*N501Y*, *E484K*, and *K417N*), Delta (*L452R*, and *P681R*), and the Omicron lineages (*delH69V70*, *N501Y*, *P681H* and *K417N*). Figures 4.2 and 4.3 underscore the frequency and distribution of SNP detected in wastewater sourced from the designated Buffalo City Metropolitan Municipal wastewater treatment facilities.

Approximately 7% of the SARS-CoV-2 RNA examined for the SNP of the spike proteins harboured at least six signature mutations (*delH69V70*, *N501Y*, *P681H*, *L452R*, *E484K* and *P681R*) from the mutation panel used. These samples were collected at different time intervals from three geographically distinct WWTPs. One sample was retrieved on the 12th August 2021 from the MDA WWTP, the second one was collected in epidemiologic week 37 (20th September 2021) from the SCH WWTP and the third one was retrieved from KID WWTP on 07th October 2021).



These were retrieved during the Delta-driven epidemic in the country. Table 4.6 depicts the epidemics and variants involved, where the total viral load was retrieved from the preliminary investigation on the incidence of SARS-CoV-2 in municipal wastewater of various populations within Buffalo City Metropolitan Municipality (Chapter Three).

Table 4.6: A summary of COVID-19 spikes/epidemics experienced in the Eastern Cape Province, associated variants, and the SARS-CoV-2 viral load. A higher viral load in municipal wastewater is observed during the Omicron-driven 4th COVID-19 epidemic—the number of clinical cases sourced from the NICD COVID-19 surveillance database made available for the public (*SARS-CoV-2 GENOMIC SURVEILLANCE UPDATE - NICD, 2020 - 2022*). The Eastern Cape Province was the first to usher the country into the second epidemic wave.

Epidemic/wave	Duration	Estimated number of cases in EC (NICD, 2022)	Total SARS-CoV-2 viral load (GC/mL)	Predominant lineage/variant
1st	Mar 2020 – Nov 2020	50 572	*	1.1.54, B.1.1.56 & C.1
2nd	Dec 2020 – Apr 2021	72 763	*	Beta (B.1.351)
3rd	May 2021 – Oct 2021	6 196	238 245.1	Delta (B.1.617.2)
4th	Dec 2021 – Apr 2022	332 120	305 146.3	Omicron (B.1.1529)
5th	May 2022 – Jul 2022	363 529	139 461.3	Omicron (BA.4 & BA.5)

* not within the study period

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Several studies have documented the occurrence of the SARS-CoV-2 genomes in the wastewater milieu (Sherchan *et al.*, 2020; Tran *et al.*, 2021; Johnson *et al.*, 2022; Pechlivanis *et al.*, 2022a). It has long been established that respiratory symptoms are the most common clinical indicators of COVID-19. Nevertheless, Benvari *et al.* (2022) and several researchers reported gastrointestinal symptoms as additional manifestations of the SARS-CoV-2 infection, suggesting the pathogenicity of the virus in the gastrointestinal tract. These reports explicate the incidence of the viral genome in wastewater, as it can be shed in faecal excreta (Jones *et al.*, 2020; Natarajan *et al.*, 2022).

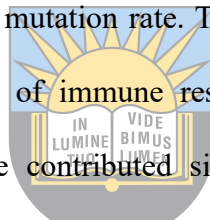
Wastewater comprises an assortment of non-infective SARS-CoV-2 particles shed by symptomatic and asymptomatic individuals (Tran *et al.*, 2021b; Johnson *et al.*, 2022). The viral fragments are extracted and identified to profile the presence and dissemination of variants among a population within a specific epidemiological region (Zhakparov *et al.*, 2023). This can be done through genotyping, which requires less effort than clinical surveillance (Harper *et al.*, 2021). Genotyping is crucial as SARS-CoV-2 lineages frequently evolve, affecting disease severity and transmissibility and impacting the efficacy of some therapeutics and some diagnostic tools (Neopane *et al.*, 2021; Greninger *et al.*, 2022).

Since wastewater harbours a myriad of SARS-CoV-2 particles from a broad range of individuals in a community, integrating wastewater-based epidemiology with SARS-CoV-2 surveillance strategies is beneficial for characterising the diverse genome of the virus and its dispersal patterns (Amman *et al.*, 2022; Karthikeyan *et al.*, 2022; Tambe *et al.*, 2023). This study uncovered the molecular epidemiology and the diverse genome of SARS-CoV-2 retrieved from wastewater samples in Buffalo City Metropolitan Municipality, South Africa.

A preliminary investigation was conducted, where the incidence of SARS-CoV-2 was determined in wastewater sourced from the Buffalo City municipal wastewater treatment

plants. Subsequently, the TaqMan SARS-CoV-2 mutation panel genotyping-molecular-specific RT-PCR assay, targeting the single nucleotide polymorphisms (SNP) was conducted. The selection of samples was restricted to those with a viral load > 1000 GC/mL, i.e. samples with the N1 and N2 average viral load greater or equal to 1000 GC/mL. These samples were subjected to rapid detection of SARS-CoV-2 S-protein mutations associated with VOCs. SNP genotyping was done as it is a cost-effective and rapid approach for variant surveillance. This study utilised mutations associated with the spike-protein gene to distinguish the incidence of VOCs Alpha, Beta, Delta, and Omicron in the municipal wastewater influent from the study sites.

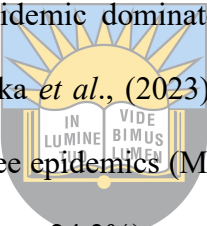
Since the onset of SARS-CoV-2 in South Africa in March 2020, an array of variants has been observed because of the coronavirus' mutation rate. These variants are attributed to reduced efficacy to pharmaceuticals, evasion of immune response, increased transmissibility and disease severity. These aspects have contributed significantly to the advancement of 5 sequential epidemics (waves) in South Africa. This maiden study was conducted during the 3rd epidemic to the 5th epidemic.



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During this period, key spike protein mutations associated with VOCs were observed in the municipal wastewaters of Buffalo City. In the course of the Delta (B.1.617.2) driven epidemic (3rd wave), there were fluctuations in clinical cases, SARS-CoV-2 fragments in wastewater and SNP. This finding demonstrates the correlation between the occurrence of SNPs, SARS-CoV-2 fragments and the number of COVID-19 cases. Spikes in the three aspects were observed at the peak of the epidemic (July 2021). During this period, variant delta had dominated approximately 66 % of the specimen collected in the country while variants alpha and beta circulated at low frequencies according to the Network for Genomic Surveillance South Africa (NGS-SA), (2021).

Throughout the third epidemic, vaccine administration (Pfizer-BioNTech (Comirnaty) and the Johnson & Johnson) was further extended to the general population in South Africa. This may have contributed to the stabilisation in the number of clinical cases and declines in the detection of SARS-CoV-2 and SNPs in municipal wastewater in the study area as the Pfizer-BioNTech COVID-19 vaccine was among the vaccines showing $\geq 90\%$ efficacy according to the WHO, (2020). Other factors contributing to this may include safety precautions and nonpharmaceutical measures. However, this was short lived as a significant spike was observed in September 2021 towards the end of the 3rd epidemic.

By the end of the 3rd epidemic the number of cases, SNPs and SARS-CoV-2 markers in wastewater had stabilised through to the 4th epidemic. A prominent spike was observed again following the inception of the 5th epidemic dominated by BA.4 and BA.5 Omicron sub-lineages. A study conducted by Mabuka *et al.*, (2023) demonstrated that rate of COVID-19 transmission was higher in the last three epidemics (Mean daily cases: 3rd epidemic = 15.7%, 4th epidemic = 18.4%, and 5th epidemic = 24.3%) compared to the first two epidemics which were similar in transmissibility.  University of Fort Hare
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This leads to the deduction that the rate of transmissibility inferred by VOCs delta and omicron lineages progressively increased with time. Several spike protein mutations detected in detected in these variants/lineages have an improved affinity for the ACE 2 receptors (Cele *et al.*, 2021; Davies *et al.*, 2021), hence, the elevation in SARS-CoV-2 transmission during these epidemics. The spike protein mutations (*L452R*, *P681H*, *N501Y*, *delH69V70*, *E484K*, *K417N*, *P681R*) were distributed across the municipal wastewater throughout the surveillance period.

The findings elucidate the incidence of SARS-CoV-2 lineages with numerous S protein signature mutations. The mutations are reportedly responsible for precluding possible infections

and decreasing vaccine efficacy. Annexure D: Table 7.1 reveals that VOCs share specific spike protein mutations, with *N501Y* being the most prevalent mutation.

The SARS-CoV-2 genome obtained from the municipal wastewater influent samples portrayed a random assortment of spike protein mutations. However, two distinct assortments were observed. 27% of the samples screened for the SNP assay mutation panel possessed four mutations: *delH69V70*, *N501Y*, *P681H*, and *L452R*. These samples were collected at different time intervals. Furthermore, samples possessing mutations (*N501Y*, *E484K*, and *K417N*) that can presumptively belong to VOC Beta were attained between epidemiologic weeks 27 and 37 (28th June 2021 – 13th September 2021). According to Tegally *et al.*, (2021), the Beta-driven wave subsided in April 2021. However, the variant circulated at low frequencies (Al Hasan *et al.*, 2022).

47% of the SARS-CoV-2 genome recovered from the Buffalo City Metropolitan municipal WWTP possessed mutations (*N501Y*, *delH69V70*, and *P681H*) which can be linked to VOC Alpha (B.1.17). These mutations were observed throughout the study period. Variant Alpha was primarily discovered in the United Kingdom in September 2020. These findings suggest that the VOC may have circulated in Buffalo City Metropolitan two years after its first detection.

Remarkably, samples with signature mutations (*N501Y*, *P681H*, *delH69V70*, *K417N*, *E484K*), which can also be linked to Omicron and its subvariants, were collected between 13th September 2021 and 07th April 2022. These findings indicate two possible deductions: either Omicron (B.1.1.529) was circulating the wastewater of Buffalo City two months before its discovery in South Africa (Viana *et al.*, 2022) or supporting literature on the commonality of key spike mutations in the variant spectrum (Magazine *et al.*, 2022; Islam *et al.*, 2023). Either



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way, this indicates the necessity for whole-genome sequencing strategies to complement SNP assay genotyping.

The Omicron variant dominated samples obtained in the fourth epidemic wave in South Africa (Mahase, 2022). According to WHO (2022), South Africa had recorded 1362 cases of the Omicron subvariant BA.2, 703 cases of BA.4, and 222 cases of BA.5 since the beginning of April 2022. The Omicron-dominant wave was less severe than previous epidemic waves, with more recorded cases but fewer hospital admissions (Dyer, 2021; Bager *et al.*, 2022; Jassat, Abdool Karim, *et al.*, 2022).

In the temporal distribution of mutations associated with VOCs, about 93% of the SARS-CoV-2 samples possessed the amino acid mutations of the spike-protein gene. The target mutations were detected in the epidemiological month of July 2021 (adjusted alert level 4 between 28th June and 25th July 2021) during the Delta-driven epidemic wave (May 2021 - October 2021) with an estimated 220568 COVID-19 reported cases of infections in the Eastern Cape Province. Throughout the third epidemic wave, SARS-CoV-2 samples from the WWTP possessed up to four VOC mutations, the *P681H*, *delH69V70*, *N501Y*, and *L452R*, that were observed consistently.

From June 2021, an increase in the frequency of the detection of the mutations was observed in July 2021. In July 2021, the country suffered civil unrest rooted in KwaZulu Natal and Gauteng province, leading to increased COVID-19 cases of infection and a subsequent rise of SARS-CoV-2 mutations. During this period interprovincial travels were permitted, one of the contributing factors to peaks and interprovincial circulation of SARS-CoV-2. This corroborates with findings documented by (Bingham *et al.*, 2022; Pulliam *et al.*, 2022). Nonetheless, the country eventually transitioned from adjusted alert level 4 down to adjusted alert level 1 between 28th June 2021 and 01st October 2021 (adjusted alert level 1 restrictions were lifted on

4th April 2022). During this period, stringent restrictions on travel and social gatherings were gradually relaxed.

As the restrictions were relaxed, an upsurge in COVID-19 clinical cases and mutation frequency was observed in the epidemiological month of September 2021. Ramesh *et al.*, (2021) also noted a momentous increase in COVID-19 cases as the emergence of variants led to approximately 230 million cases globally in September. In this period, four SARS-CoV-2 lineages were categorised as the VOCs: Alpha, Beta, Gamma, and Delta variants. Variants in this category exhibit elevated transmission rates and can potentially increase disease severity (Choi and Smith, 2021; Harvey *et al.*, 2021; Patarca and Haseltine, 2022).

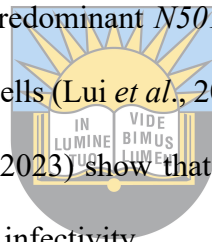
On the other hand, a spike in the frequency of occurrence was observed in April 2022 for three consecutive epidemiological weeks (07th April 2022 – 21st April 2022). Four mutations (*P681H*, *delH69V70*, *N501Y*, and *L452R*) were detected during this period following the re-emergence in February 2022. The mutations re-emerged in the influent samples from all WWTPs except for SCH WWTP on 10th February 2022 and were more frequent in April 2022 during the fifth epidemic spike predominated by Omicron subvariants (BA.2, BA.4, BA.5) (Mills *et al.*, 2022; Wolter *et al.*, 2022).

The spike in the epidemiological month of April 2022 arose two days after the National State of Disaster was lifted (05th April 2022). A similar spike was observed in the Western Cape Province, where the Delta variant dominated wastewater samples retrieved from Rawsonville WWTP, where four Omicron subvariants (BA.1, BA.2, BA.3, and BA.5), which had also spread globally, were confirmed (Johnson, Mangwana, *et al.*, 2022; Kumar, Karuppanan and Subramaniam, 2022).

All mutations on the panel were observed in samples obtained from BIS WWTP. 11% of the samples screened for the spike protein mutations were retrieved from this site. The range of

mutations observed in this site is consistent with at least four VOCs, namely Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.0-2), and Omicron (B.1.1.529). The WWTP serves a population of 150 995 (Department of Statistics South Africa, 2022) within its reticulation area and is located in the capital of the Eastern Cape Province. The plant is the second smallest in capacity design (2ML/d) but serves the most considerable population compared to other WWTPs within the study area. Apart from the inhabitants, many civilians converge into the areas the plant serves for work-related purposes, as several departmental headquarters and a university campus are in the catchment area. Hence, a diversity in the SARS-CoV-2 genome is observed from samples sourced from this site.

Notably, four mutations (*N501Y*, *L452R*, *P681H*, and *delH69V70*) are distributed among the samples in almost equal parts—the predominant *N501Y* deleterious mutation improves viral replication in primary human airway cells (Lui *et al.*, 2022). Research findings documented by Martin *et al.* (2021) and Aoki *et al.* (2023) show that spike protein mutation was evident in several variants as it is crucial in viral infectivity.



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The second most common mutation in the samples was the leucine substitution at position 452 in the spike protein's receptor-binding domain (*L452R*). *L452R* is linked to immune evasion and enhanced transmission (Bittar *et al.*, 2021; Motozono *et al.*, 2021; Tchesnokova *et al.*, 2021), hence the frequency throughout the surveillance period. The mutation is positioned near the angiotensin-converting enzyme 2 (ACE2) interface associated with viral attachments and cell insertions (Sun *et al.*, 2022). The mutation is unique to VOCs Beta (B.1.33, B1.351) and Delta (B.1.617.0 – 2). VOC Beta led the second epidemic (wave) in South Africa and is attributed to a reduction in neutralising antibodies (Tao *et al.*, 2021; Jassat, Abdool Karim, *et al.*, 2022). At the same time, the VOC Delta predominated the destructive third epidemic spike in the country.

The TaqMan SARS-CoV-2 mutation panel genotyping-molecular-specific RT-PCR assay technique is utilised to assess wastewater for amino acid mutations associated with SARS-CoV-2 variants circulation in communities (Nypaver, 2021; Ashford *et al.*, 2022). Additionally, the findings depict the time series inclinations, geospatial distribution of VOC signature mutations, and the frequency of incidence. Understanding the intricacies of a vigorously infectious strain in circulation will enable prompt responses and well-informed decision-making to manage and curb the dissemination of the virus. Similarly, recent studies have emphasised the significance of monitoring wastewater to track the prevalence of diseases in a general population (Street *et al.*, 2020; Amman *et al.*, 2022; Zhang *et al.*, 2022; Ahmed *et al.*, 2023). Additionally, this technique has proven valuable in monitoring signature VOC mutations.

SNP assay genotyping is a swift and cost-effective technique used to identify mutations. However, the technique has some limitations. SNP assay genotyping has restricted mutation coverage as it can only identify mutations in its design. The SARS-CoV-2 genome exhibits mutational diversity, i.e. specific variants share several mutations. Therefore, it is challenging to pinpoint novel variants and specific variants circulating at a certain point in time. For instance, in September 2021, mutations associated with VOCs alpha and Omicron were circulating in the municipal wastewater two months before the discovery of the Omicron variant.

Moreover, the assay's success depends on the RNA's quality and quantity. The RNA used to determine the incidence of SARS-CoV-2 in test samples, and duplicates is not enough to detect all mutations in duplicates in the SNP assay mutation panel. Therefore, a complementary technique such as sequencing is recommended to enhance the specificity of the method in determining SARS-CoV-2 variants.

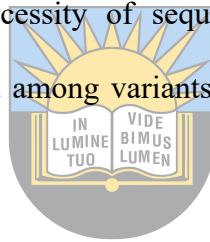
Conspicuously, this study is among a limited few conducted in the Buffalo City Metropolitan region of the Eastern Cape Province, South Africa, tracking the diverse genome of SARS-CoV-2 in influent municipal wastewater samples sourced from municipal wastewater treatment facilities using rapid and timeous SNP assay.



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4.4 Conclusion

In conclusion, this study has shown that VOC signature mutations were circulating in the different catchment areas of Buffalo City Metropolitan Municipality beyond the lifting of the National State of Disaster, suggesting the incidence of Alpha, Beta, Delta, and Omicron variants of concern. The detection frequency of mutations was directly proportional to the Eastern Cape COVID-19 cases, indicating an increase in SARS-CoV-2 transmissibility influenced by the incidence of variants. All mutations in the SNP assay mutation panel were present in wastewater samples sourced from BIS WWTP located in the capital of the Eastern Cape Province, suggesting an influx and circulation of an array of variants in the work district. The incidence of spike protein mutations linked to Alpha (B.1.17) and Omicron (B.1.1.529) in September 2021 emphasises the necessity of sequencing strategies to complement the methodology as mutations are shared among variants. Through this, a seamless tracking of novel variants can be achieved.



Furthermore, the surveillance of SARS-CoV-2 single nucleotide polymorphisms by wastewater monitoring is a potent instrument for wastewater-based epidemiology. The genomic surveillance technique used in this study validates that population-based strategies are more beneficial than individual-specific strategies, which may undermine data from asymptomatic individuals. Unceasing molecular epidemiology employing ultra-selective and specific techniques such as next-generation sequencing is essential in tracing mutations, improving diagnostics, and vaccine development.

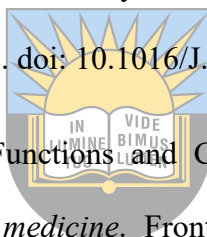
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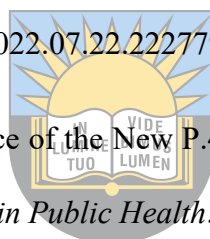
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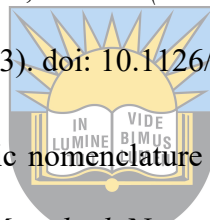
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5. CHAPTER FIVE

Using the Quantitative Microbial Risk Assessment (QMRA) model to understand the potential occupational health risks associated with WWTP workers' exposure to SARS-CoV-



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Using the Quantitative Microbial Risk Assessment (QMRA) model to understand the potential occupational health risks associated with WWTP workers' exposure to SARS-CoV-2

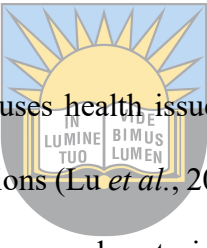
Abstract

Faecal-oral ingestion of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) through self-inoculation and the ingestion of contaminated droplets in the occupational setting are yet to be validated. Nevertheless, due to the pervasiveness of the virus, it is imperative to investigate potential health risks faced by wastewater treatment plant workers to SARS-CoV-2 across the four seasons. Quantitative Microbial Risk Assessment (QMRA) model was employed to understand the potential occupational health risks of exposure to SARS-CoV-2, which wastewater treatment plant workers are exposed to. The virus behind the most recent global health and socioeconomic catastrophe was detected in the municipal wastewater of Buffalo City Metropolitan through RT-qPCR. The total viral load retrieved from all study sites in winter was a total concentration of 22.08×10^4 GC/mL, spring 13.09×10^4 GC/mL, summer 7.61×10^4 GC/mL and autumn 29.30×10^4 GC/mL. In the aggressive (20 mL) scenario, $P_{(i)}$ values were 35.9×10^{-2} , 5.1×10^{-2} , 6.8×10^{-2} , and 18.2×10^{-2} in winter, spring, summer, and autumn, respectively. As expected, elevated $P_{(i)}$ values were observed for the considered exposure scenario. The findings indicate no significant correlation between the seasons and the potential health risks ($p \geq 0.05$) at a significance level of 0.05. Any observable variance in $P_{(i)}$ at varying exposure levels is more likely due to random chance than the direct effect of season, state of the facilities and the in-sewer factors. These findings accentuate the significance of interventions such as the QMRA in promoting occupational health strategies and curbing the potential transmission of SARS-CoV-2 genomes from wastewater treatment facilities to the workers and the populace within the reticulation area.

Keywords: SARS-CoV-2, Wastewater, QMRA, Contamination, Exposure

5.1 Introduction

Studies have identified a plethora of respiratory viruses in faeces (Al Khatib *et al.*, 2021; Lowry, Wolfe and Boehm, 2023), including respiratory syncytial virus, SARS coronavirus, adenovirus, and bocavirus (Arena *et al.*, 2012). Respiratory viruses like SARS-CoV-2 can disseminate via three mechanisms: direct transmission, indirect transmission, and airborne transmission (Pöhlker *et al.*, 2023). Direct transmission involves close interaction with an infected individual and, consequently, the self-inoculation of mucous membranes. Indirect transmission involves fomites, where viruses are transferred from contaminated inanimate objects (e.g. door handles and equipment) and airborne transmission, which involves the spread of viral particles through droplets and aerosols (bioaerosol) (Chen *et al.*, 2021; Rabaan *et al.*, 2021).



Exposure to contaminated surfaces causes health issues in occupational settings, and health complications such as epidermal irritations (Lu *et al.*, 2020) and “sewage worker’s syndrome,” presented by headaches, dizziness, fatigue, and gastrointestinal and pulmonary complications, can be prevalent in wastewater treatment operators (Karri *et al.*, 2021, Oza *et al.*, 2022). Several studies link these symptoms to toxins and excessive bioaerosols discharged throughout the typical wastewater treatment processes (Niazi *et al.*, 2015; Kowalski *et al.*, 2017; Chen *et al.*, 2021; Yan *et al.*, 2024).

According to Carducci *et al.*, (2018), QMRA is an essential approach for assessing microbiological risks based on evaluating principal pathogens in environmental matrices and their dose-response relationships. The QMRA evaluation is divided into four fundamental stages: hazard identification, exposure assessment, dose-response assessment, and risk characterisation. Work-related health risk evaluation for WWTP operators is challenging because of the distinct exposure scenarios, the indeterminate infectivity pattern, the broad

spectrum of pathogens in wastewater, the vulnerability spectrums of workers, and insufficient epidemiological research data. Furthermore, the probability of infection in such environments is contingent mainly upon the pathogen's virulence and pathogenicity, concentration, and duration of exposure (Carducci *et al.*, 2016, 2018).

Zaneti *et al.*, (2021) identified numerous studies that warrant urgent research on the persistence of viable SARS-CoV-2 virions in wastewater and the potential consequences for public health. These studies demonstrate a gap in QMRA for potential faecal-oral transmission routes, mainly in the developing world in contexts where employees in WWTPs encounter increased occupational risks mainly due to the less rigorous enforcement of personal and collective protective equipment (PPE and CPE) measures in contrast to those observed in the developed countries (Collivignarelli *et al.*, 2020; Heller, Mota and Greco, 2020; Gwenzi, 2021).

Assessing the potential health risks of exposure to contaminated surfaces, droplets, and bioaerosols using QMRA is vital to protecting workers in WWTPs, and several studies have employed QMRA to evaluate the potential risks of exposure to bioaerosols and pathogens in different environmental matrices (Allende *et al.*, 2018; Carducci *et al.*, 2018; Pasalari *et al.*, 2019; de Matos Nascimento *et al.*, 2020). The most recent global coronavirus pandemic has promoted the use of QMRA to evaluate the possibility of detrimental consequences of SARS-CoV-2 relating to wastewater exposure based on previous studies of other human coronaviruses (Haramoto, 2020; Silverman and Boehm *et al.*, 2020). There is a paucity of data on the potential microbial exposure risks faced by wastewater treatment plant workers in South Africa. Therefore, this study employs QMRA to assess and estimate the wastewater treatment plant workers' probability of exposure to the SARS-CoV-2 genomes harboured in municipal wastewater influent from the Buffalo City region across the seasons.

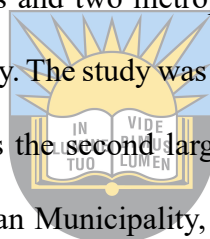
5.2 Experimental design

5.2.1 Ethical clearance certificate and permits

For ethical clearance and permission to commence with the study please refer to Section 3.2.1, page 100

5.2.2 Study site description

The study is based in the Eastern Cape Province, which is 170000 km² of a diverse landscape. It is one of South Africa's most impoverished provinces and was the first to enter the country's second epidemic wave. The Eastern Cape's population constitutes 13% of South Africa, with a population of approximately 6.6 million (EC Provincial Government, 2023). The province is divided into six district municipalities and two metropolitan municipalities, one of which is Buffalo City Metropolitan Municipality. The study was explicitly conducted in the Buffalo City Metropolitan Municipality, which has the second largest population density in the province (317.8/km²) (Buffalo City Metropolitan Municipality, 2019), making it easier to disseminate SARS-CoV-2.



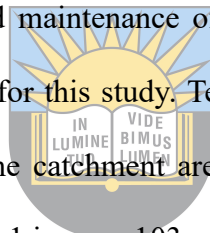
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The municipality is located on the eastern shoreline of the Eastern Cape and has a mild climate with an average of 850 mm yearly rainfall. Major industrial companies support the cosmopolitan area. Buffalo City is renowned for being an epicentre for leisure activities, fine establishments, nature and gaming reserves, shopping centres and malls, and is home to several educational institutions. People commute within and out of the municipal region for various reasons, such as work, education, and social activities, which consequently contribute to the spread of the coronavirus. The metropolitan nature of the municipality harbours a population from different socioeconomic standings.

Low-income individuals live in crowded and informal settlements located on the outskirts of Buffalo City, and some are part of essential services with increased risks of exposure to SARS-

CoV-2. Some of these low-income communities are exposed to raw sewage. According to several news reports, numerous parties have threatened to, and some have taken the municipality to court due to sewage spills (Sgqolana, 2022a; Sgqolana 2022b; Fray, 2023; Mndayi, 2023; Velaphi, 2024). During the sudden surges of COVID-19, the healthcare facilities in the municipality were utterly overwhelmed, leading to the rapid spread of the SARS-CoV-2 genome, delayed treatment, and increased mortality.

Buffalo City Metropolitan represents approximately 10% of the general population in the Eastern Cape Province and was once declared one of the COVID-19 hotspots in the country (Buffalo City Metropolitan Municipality, 2020). Figure 5.1 depicts the geographical setting of the study. The municipal region is a combination of rural, peri-urban and urban settlements. This is evident in the conditions and maintenance of the infrastructure observed from the wastewater treatment plants selected for this study. Ten wastewater treatment facilities were selected with the characteristics of the catchment area and populations served by the sites represented in Section 3.2.2.1, Table 3.1 in page 103.



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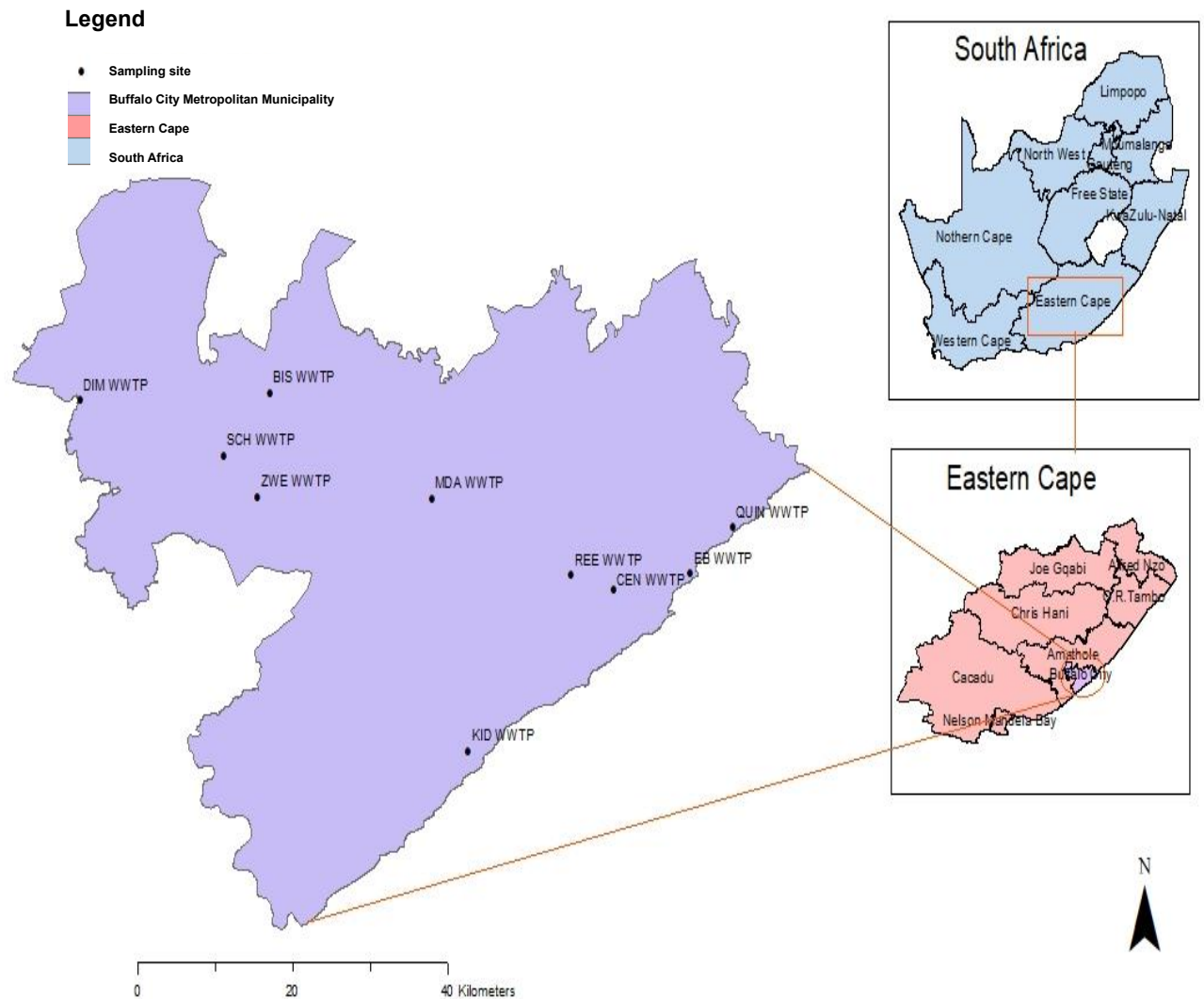


Figure 5.1: Geographical positioning of the wastewater treatment facilities in Buffalo City Metropolitan (depicted in purple). The Eastern Cape Province is depicted in pink, while the map of South Africa is depicted in blue. The selected wastewater treatment plants (WWTP): BIS WWTP, CEN WWTP, DIM WWTP, EB WWTP, KID WWTP, MDA WWTP, QUIN WWTP, REE WWTP, SCH WWTP and ZWE WWTP are represented by black pins on the map. ArcGIS 10.6.1 was used to construct all maps, and the WWTP coordinates were confirmed using Google Earth.

5.2.3 Rationale

The present study was premised in Buffalo City Metropolitan Municipality. It is not unusual to find vandalised, over-capacitated, non-functional wastewater treatment plants or blocked sewer systems exposing biological waste and pathogens of medical importance to the environment. Thus, wastewater treatment plant workers and civilians are exposed to a high risk of potential pathogen infections.



Figure 5.2: A pictorial representation of the state of some of the WWTPs observed and the working conditions WWTP workers were exposed to throughout the study period. In five

wastewater treatment plants, some workers have been observed conducting routine activities without complete PPE (A) A WWTP operating beyond its design capacity overflows with sewage. (B) A vandalised WWTP (situated 10 m away from civilian homesteads) that has not received maintenance and has been unkept, subsequently releasing effluent of poor quality. (C) Collection of influent wastewater samples (by researcher) from the over-capacitated and over-flooded WWTP for study purposes. (D) Sewage exposed to the environment, WWTP workers and civilians due to a burst pipe.

5.2.4 Sample Collection and Processing

Wastewater influent samples were collected from the designated facilities and the standard operating protocol was followed for a detailed protocol please refer to Section 3.2.3, page 104 and Section 3.2.4, pages 105-107.

5.2.5 Viral RNA Extraction and Quantification

Wastewater sample concentration, total RNA extraction and quantification were done according to the modified protocol as described by Johnson *et al.*, 2021. For a detailed protocol please refer to Section 3.2.4 page 105 and Section 3.2.4, pages 105-107.

5.2.6 Detection of SARS-CoV-2 genome by Quantitative Reverse Transcription Polymerase Chain Reaction

Please kindly refer to Chapter 3, section 3.2.5, pages 107-109 which outlines in detail the RT-qPCR protocol, probes and thermal cycling conditions that were used in the study for profiling the incidence of SARS-CoV-2 in the wastewater.



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5.2.7 Quantitative Microbial Risk Assessment model for the potential occupational health risks associated with WWTP workers' exposure to SARS-CoV-2 genomes.

The QMRA framework used in this study was sectioned into four primary stages: hazard identification, where SARS-CoV-2 was identified and quantified (through RT-qPCR) as a potential hazard for wastewater treatment plant workers; exposure assessment where an inspection of potential exposure pathways was assessed; dose-response assessment and risk characterisation using the exponential model $k = 4.1 \times 10^2$ (Watanabe *et al.*, 2010) and statistical analysis to characterise the potential risk of exposure. The framework was premised on the assumption that wastewater treatment plant workers without personal protective equipment (PPE) were exposed to viable aerosolised SARS-CoV-2 particles for at least 6 hours at varying volumetric intake. The potential exposure routes considered in this framework include the aerosol pathway and the accidental ingestion pathway.

5.2.7.1 Hazard identification and exposure assessment

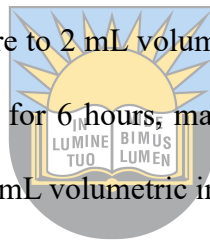
SARS-CoV-2 is a vastly infectious coronavirus that has become a significant public health concern. SARS-CoV-2 consequently caused a global health catastrophe that has adverse health implications and significant economic implications, surpassing the 2008 financial recess and causing unemployment and poverty globally. Previous research findings on the SARS-CoV-1 outbreak throughout the 2003 pandemic indicated that the viral particles could be dispersed through wastewater systems (Yu *et al.*, 2004; McKinney *et al.*, 2006). This suggests wastewater is a possible vehicle of viral particle transmission through the faecal-oral route, the respiration of aerosols and, consequently, airborne transmission (Kang *et al.*, 2020; Tang *et al.*, 2020).

The QMRA in this document took a conservative approach to protect the health of WWTP workers and assumed that the SARS-CoV-2 particles in raw wastewater are infectious (viable) to estimate the potential risk of infection. The framework of this study used the assumption

documented by Dada and Gyawali, (2021), entailing the correlation wherein a single plaque-forming unit (PFU) equates to 10^3 genomic copies (GC) (Equation 5.1).

$$\text{Equation 5.1} \quad PFU = (\text{volumetric intake} \times \text{viral load}) / 1000$$

Alarming, during wastewater sample collection, some WWTP operators were observed performing their routine duties such as manual coarse screening, grit removal and sampling, without wearing any PPE. This exposes them at a potentially high risk of exposure to contaminated bioaerosol and accidental ingestion of viral particles through poor hygiene/unsanitary practices. Therefore, the dose-response model, as described by Zaneti *et al.*, (2021), was adopted with some modifications. It was assumed that the operators worked for 6 hours per day. Therefore, three scenarios were fashioned to assess the probability of exposure (6 hours): minimum exposure to 2 mL volumetric intake of bioaerosols, wastewater droplets or spills during the agitation for 6 hours, maximum exposure to 10 mL volumetric intake, and aggressive exposure to 20 mL volumetric intake.



5.2.7.2 Dose-response assessment and risk characterisation.

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A dose-response model previously utilised for SARS-CoV-1 (Watanabe *et al.*, 2010) was implemented in this study due to the absence of an established dose-response model for SARS-CoV-2. The dose-response model previously described (Equation 5.3) by Zaneti *et al.*, (2021), Dada and Gyawali, (2021) was applied in the present study, and the dose was calculated using Equation 5.4.

$$\text{Equation 5.2} \quad P_{(i)} = 1 - \exp\left(-d/k\right)$$

$$\text{Equation 5.3} \quad d = i \times VC$$

Variables used in the equations:

P = probability of infection after a single exposure at the dose d

d = dose, as number of organisms ingested (PFU)

k = dose-response model (4.1×10^2)

i = Volumetric intake

VC = Viral concentration/viral load (GC/mL)

5.2.7.3 Statistical analysis

IBM SPSS Statistics 27 was employed to conduct ANOVA, and the significance level was 0.05.



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5.3 Results and Discussion

5.3.1 Detection and quantification of SARS-CoV-2 RNA in wastewater samples

494 samples were screened for the nucleocapsid protein-encoding genes SARS-CoV-2_N1-P and SARS-CoV-2_N2-P, and 351 (71%) were identified as SARS-CoV-2.

The SARS-CoV-2 viral loads (GC/mL) detected in the municipal wastewater retrieved from the Buffalo City region were quantified and are presented in Table 5.1 and Figure 5.3. Positive samples were screened against the reference standards prepared from the 2019-nCoV-N-Positive plasmid control and determined at the detection threshold limit of 0.02 per reaction by the average C_T values ≤ 35 cycles.

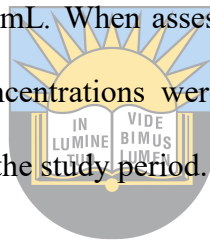
Table 5.1 demonstrates the seasonal trends of SARS-CoV-2 in municipal wastewater and potentially correlates it with the average number of clinical cases recorded in the epidemiological period in the Eastern Cape Province.

Table 5.1: SARS-CoV-2 average viral load (GC/mL) yielded by the municipal wastewater across the epidemiological period and the average clinical cases reported in EC in the study period.

WWTP	WINTER (2021)	SPRING (2021)	SUMMER (2021-2022)	AUTUMN (2022)
BIS	146,5	2578,4	826,9	1104,7
CEN	231,7	565,0	938,4	2907,6
DIM	190,1	1033,1	576,9	2152,4
EB	1022,0	1320,5	852,6	4385,0
KID	2145,7	2541,4	361,8	934,9
MDA	1060,1	717,8	339,4	1778,5
QUIN	1341,0	411,3	930,3	2402,0
REE	540,2	411,4	6555,7	4760,5
SCH	400,3	910,2	160,9	247,2
ZWE	2013,4	1052,9	1088,4	329,5
Average number of clinical cases	226748,3	290100,8	342080,6	351471,3

The clinical data was retrieved from the National Institute for Communicable Diseases Of South Africa (NICD) repository. This data was made available to the public through daily reports (NICD, 2021; 2022).

A prominent elevation in SARS-CoV-2 viral load was observed in the autumn epidemiological period of 2022 in at least 70 % of the sampling locations. Nevertheless, spikes were observed in the summer epidemiological period (2021 - 2022). A noteworthy spike was observed in the wastewater samples sourced from REE, EB, DIM and CEN WWTPs. Figure 5.3 demonstrates the SARS-CoV-2 viral load (GC/mL) accumulated from the study sites with each epidemiological season highlighted. The total viral load recovered across the study sites in winter was approximately 22.08×10^4 GC/mL, spring 13.09×10^4 GC/mL, summer 7.61×10^4 GC/mL and autumn 29.30×10^4 GC/mL. When assessing the viral load accumulated when evaluating seasonal data, higher concentrations were extracted from wastewater samples attained from the QUIN WWTP over the study period.



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SARS-CoV-2 viral load accumulated across the four seasons

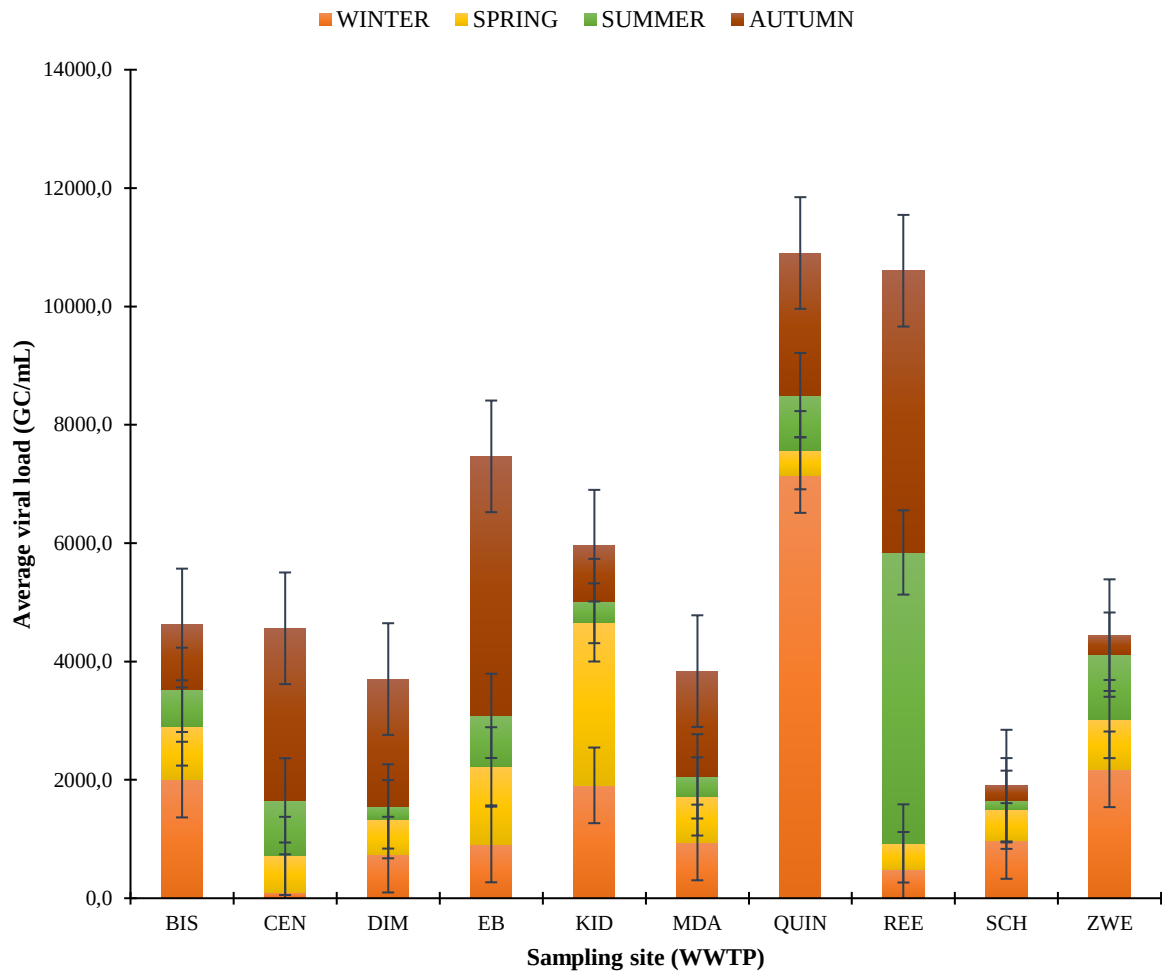


Figure 5.3: The average SARS-CoV-2 viral load (GC/mL) accumulated from wastewater from selected wastewater treatment facilities across the year's four seasons throughout the study period. Orange bars represent winter (2021), yellow bars represent spring (2021), green bars represent summer (2021 - 2022), and brown bars represent autumn (2022). The seasonal data analysis reveals relatively high SARS-CoV-2 genetic material in the winter and autumn seasons. Outstanding SARS-CoV-2 viral concentrations were observed in the wastewater sourced from the REE site in the summer season (49.17×10^2 GC/mL) and 47.61×10^2 GC/mL in the autumn season, followed by a viral load of 43.85×10^2 GC/mL retrieved from EB site in the autumn season and an average concentration of 71.51×10^2 GC/mL yielded by the wastewater from the QUIN site during the winter season.

Table 5.2 presents the total potential risk profiles observed for occupational exposure to SARS-CoV-2 virions through accidental ingestion/inhalation of wastewater droplets/bioaerosol or self-inoculation (faecal-oral ingestion) through contaminated hands and poor hygiene practices based on the virus concentration in the wastewater influents based on the minimum (2 mL), maximum (10 mL) and aggressive (20 mL) potential volumetric intake scenarios.

Table 5.2: Potential risk of SARS-CoV-2 infection ($P_{(i)}$) for WWTP operators at different WWTPs.

Type of exposure	Total Probability of Infection ($P_{(i)}$) per season			
	Winter	Spring	Summer	Autumn
	(2021)	(2021)	(2021-2022)	(2022)
Minimum (2 mL)	0,062	0,003	0,052	0,037
Maximum (10 mL)	0,056	0,027	0,036	0,126
Aggressive (20 mL)	0,359	0,051	0,068	0,182

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Higher levels are apparent for the aggressive exposure scenario. The highest $P_{(i)}$ value of 3.59 x 10⁻¹ is observed in winter for the aggressive exposure scenario, where the highest value for the maximum exposure scenario is observed in autumn.

The breakdown and site contributions of the potential seasonal risk profiles per facility are represented in Figure 5.4. No apparent pattern or influence of season on the probability of infection is observed. Nonetheless, elevated values are observed in all sites' aggressive (20 mL) exposure scenarios.

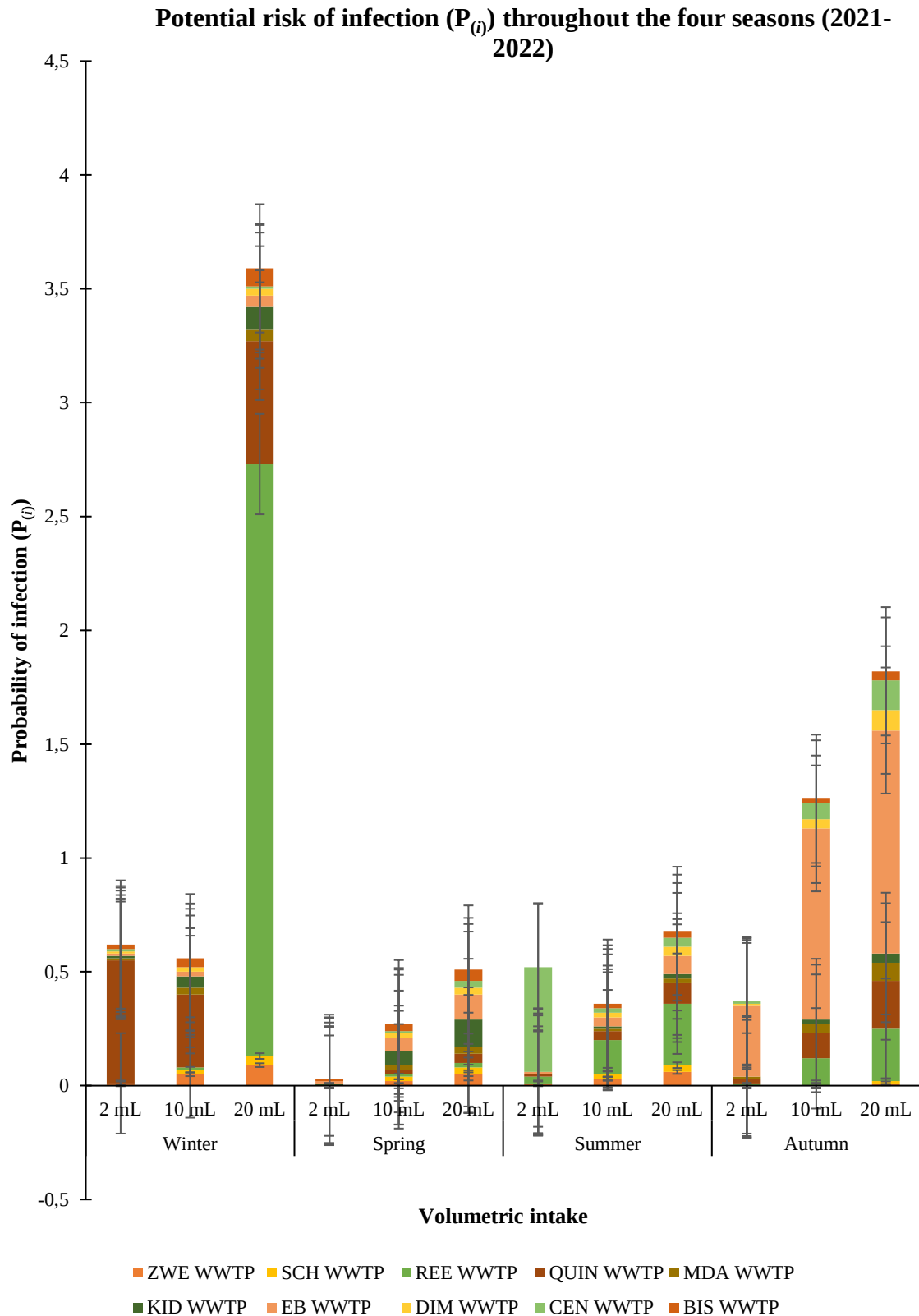
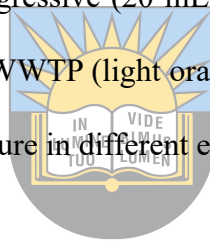


Figure 5.4: The probability of infection ($P_{(i)}$) to six hours of exposure to different volumetric intakes across the four seasons. Elevated probability of infection ($P_{(i)}$) values are evident as

the assumed volumetric intake was increased, i.e. as the volumetric intake increased. A linear trend is observed from the ZWE, SCH, MDA, KID, EB, DIM, CEN, and BIS WWTPs in winter (2021), where higher $P(i)$ values equal to 0.8 for the aggressive exposure scenario are observed in BIS WWTP. However, two sites stand out: QUIN WWTP, represented by the brown bars, and REE WWTP, represented by the lime-green bars. Elevated ($P(i)$) values are observed for the minimum (2 mL), maximum (10 mL) and aggressive (20 mL) exposure scenarios equivalent to 0.04, 0.32 and 0.54 ($P(i)$ units), respectively. A sudden spike at aggressive exposure to $P(i) = 2.6$ is observed from the REE WWTP. An unprecedented decrease in the probability of infection was observed in summer (2021-2022) for the maximum (10 mL) exposure scenario for the CEN WWTP, represented by light green bars, followed by a sudden spike of ($P(i)$) value of 0.46 at the aggressive (20 mL) exposure scenario. In autumn (2022) WWTP operators working at the EB WWTP (light orange bars) face elevated potential health risks due to SARS-CoV-2 RNA exposure in different exposure scenarios.



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5.3.2 Statistical assessment of the risk levels associated with SARS-CoV-2 exposure

Results obtained from the dose-response to the framework scenarios were subjected to statistical analysis, and the significance level was 0.05. The standard error was calculated to measure the accuracy and distribution of the mean probability of infection (POI). The observed standard error value was 0.08, indicating the assessment's reliability.

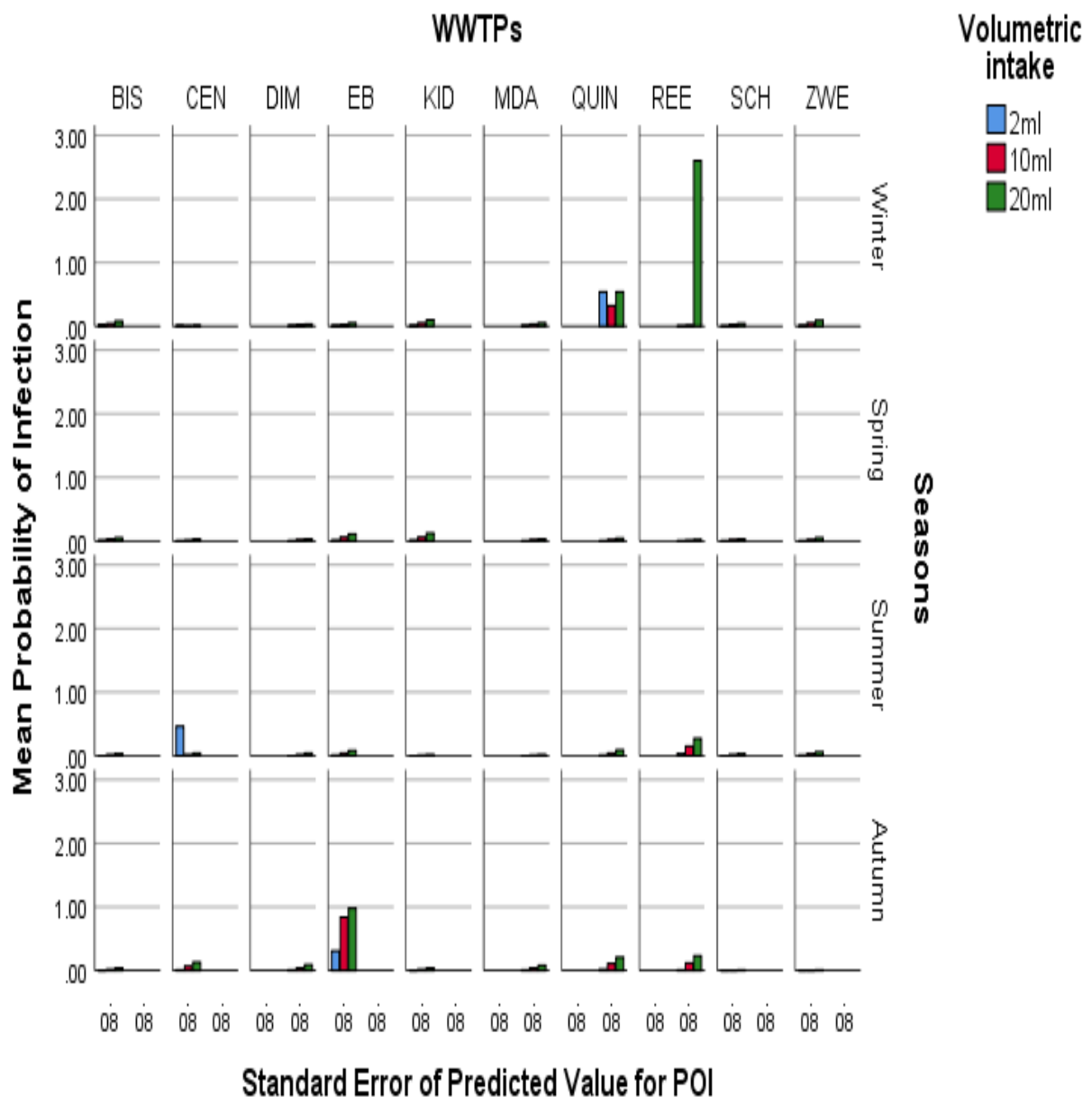


Figure 5.5: A graphic depiction of the distribution of the mean POI generated by OriginPro (version 2017, OriginLab Corporation, Northampton, MA, USA) with the highest value

observed at REE WWTP for the aggressive exposure scenario. According to the framework, WWTP operators working at EB WWTP were at a much higher potential risk of exposure during the autumn season. At the same time, the same was observed for WWTP operators working at the QUIN WWTP during the Winter season.

The Bayesian One-way ANOVA, General linear model (Univariate), and multiple comparison tests were carried out using IBM SPSS Statistics Data Editor. A total of 8 homogeneous subset tests and 12 multiple comparison tests were performed, and the significance values are represented in Tables 5.3 and 5.4, respectively.

Table 5.3: Probability of infection ($P_{(i)}$) means for groups in homogeneous subsets.

<i>Model</i>	WWTPs	N	Subset	
			1	2
<i>Student-Newman-Keuls^{a,b}</i>	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	
	BIS	12	.0283	
	KID	12	.0367	
	CEN	12	.0658	
	QUIN	12	.1617	
	EB	12	.2100	
	REE	12	.2875	
	Sig.		.278	
<i>Tukey HSD^{a,b}</i>	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	
	BIS	12	.0283	
	KID	12	.0367	
	CEN	12	.0658	
	QUIN	12	.1617	
	EB	12	.2100	
	REE	12	.2875	
	Sig.		.278	
<i>Tukey B^{a,b}</i>	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	

<i>Duncan^{a,b}</i>	BIS	12	.0283	
	KID	12	.0367	
	CEN	12	.0658	
	QUIN	12	.1617	
	EB	12	.2100	
	REE	12	.2875	
	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	
	BIS	12	.0283	
	KID	12	.0367	
<i>Scheffe^{a,b}</i>	CEN	12	.0658	.0658
	QUIN	12	.1617	.1617
	EB	12	.2100	.2100
	REE	12		.2875
	Sig.		.135	.065
	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	
	BIS	12	.0283	
	KID	12	.0367	
	CEN	12	.0658	
<i>Gabriel^{a,b}</i>	QUIN	12	.1617	
	EB	12	.2100	
	REE	12	.2875	
	Sig.		.711	
	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	
	BIS	12	.0283	
	KID	12	.0367	
	CEN	12	.0658	
	QUIN	12	.1617	
<i>Ryan-Einot-Gabriel-Welsch F^b</i>	EB	12	.2100	
	REE	12	.2875	
	Sig.		.442	
	SCH	12	.0142	
	MDA	12	.0250	
	DIM	12	.0258	
	ZWE	12	.0275	
	BIS	12	.0283	
	KID	12	.0367	
	CEN	12	.0658	
	QUIN	12	.1617	
	EB	12	.2100	

Ryan-Einot-Gabriel-Welsch Range^b

Hochberg^{a, b}

QUIN	12	.1617	
EB	12	.2100	
REE	12	.2875	
Sig.		.136	
SCH	12	.0142	
MDA	12	.0250	
DIM	12	.0258	
ZWE	12	.0275	
BIS	12	.0283	
KID	12	.0367	
CEN	12	.0658	
QUIN	12	.1617	
EB	12	.2100	
REE	12	.2875	
Sig.		.278	
SCH	12	.0142	
MDA	12	.0250	
DIM	12	.0258	
ZWE	12	.0275	
BIS	12	.0283	
KID	12	.0367	
CEN	12	.0658	
QUIN	12	.1617	
EB	12	.2100	
REE	12	.2875	
Sig.		.442	

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 0.071.

a. Uses Harmonic Mean Sample Size = 12

b. Alpha = 0.05

Table 5.4: Assessment of differences between multiple groups, in this case, $P_{(i)}$ mean difference of different wastewater treatment plants. Only data accumulated by test models such as Tukey HSD, LSD, and Dunnett's t-tests is represented.

Test Model	(I) WWTP	(J) WWTP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	BIS	CEN	-.0375	.10916	1.000	-.3900	.3150
		DIM	.0025	.10916	1.000	-.3500	.3550
		EB	-.1817	.10916	.813	-.5342	.1709
		KID	-.0083	.10916	1.000	-.3609	.3442
		MDA	.0033	.10916	1.000	-.3492	.3559
		QUIN	-.1333	.10916	.967	-.4859	.2192
		REE	-.2592	.10916	.351	-.6117	.0934
		SCH	.0142	.10916	1.000	-.3384	.3667
	CEN	ZWE	.0008	.10916	1.000	-.3517	.3534
		BIS	.0375	.10916	1.000	-.3150	.3900
		DIM	.0400	.10916	1.000	-.3125	.3925
		EB	-.1442	.10916	.947	-.4967	.2084
		KID	-.0292	.10916	1.000	-.3234	.3817
		MDA	.0408	.10916	1.000	-.3117	.3934
		QUIN	-.0958	.10916	.997	-.4484	.2567
		REE	-.2217	.10916	.580	-.5742	.1309
	DIM	SCH	-.0517	.10916	1.000	-.3009	.4042
		ZWE	.0383	.10916	1.000	-.3142	.3909
		BIS	-.0025	.10916	1.000	-.3550	.3500
		CEN	-.0400	.10916	1.000	-.3925	.3125
		EB	-.1842	.10916	.800	-.5367	.1684
		KID	-.0108	.10916	1.000	-.3634	.3417
		MDA	.0008	.10916	1.000	-.3517	.3534
		QUIN	-.1358	.10916	.963	-.4884	.2167
LSD	BIS	REE	-.2617	.10916	.338	-.6142	.0909
		SCH	.0117	.10916	1.000	-.3409	.3642
		ZWE	-.0017	.10916	1.000	-.3542	.3509
	CEN	BIS	.1817	.10916	.813	-.1709	.5342
		CEN	.1442	.10916	.947	-.2084	.4967
		DIM	.1842	.10916	.800	-.1684	.5367
		KID	.1733	.10916	.851	-.1792	.5259
		MDA	.1850	.10916	.796	-.1675	.5375
		QUIN	.0483	.10916	1.000	-.3042	.4009
	DIM	REE	-.0775	.10916	.999	-.4300	.2750
		SCH	.1958	.10916	.737	-.1567	.5484
		ZWE	.1825	.10916	.809	-.1700	.5350
	EB	BIS	.0083	.10916	1.000	-.3442	.3609

	CEN	-.0292	.10916	1.000	-.3817	.3234
	DIM	.0108	.10916	1.000	-.3417	.3634
	EB	-.1733	.10916	.851	-.5259	.1792
	MDA	.0117	.10916	1.000	-.3409	.3642
	QUIN	-.1250	.10916	.979	-.4775	.2275
	REE	-.2508	.10916	.399	-.6034	.1017
	SCH	.0225	.10916	1.000	-.3300	.3750
	ZWE	.0092	.10916	1.000	-.3434	.3617
MDA	BIS	-.0033	.10916	1.000	-.3559	.3492
	CEN	-.0408	.10916	1.000	-.3934	.3117
	DIM	-.0008	.10916	1.000	-.3534	.3517
	EB	-.1850	.10916	.796	-.5375	.1675
	KID	-.0117	.10916	1.000	-.3642	.3409
	QUIN	-.1367	.10916	.962	-.4892	.2159
	REE	-.2625	.10916	.333	-.6150	.0900
	SCH	.0108	.10916	1.000	-.3417	.3634
	ZWE	-.0025	.10916	1.000	-.3550	.3500
QUIN	BIS	.1333	.10916	.967	-.2192	.4859
	CEN	.0958	.10916	.997	-.2567	.4484
	DIM	.1358	.10916	.963	-.2167	.4884
	EB	-.0483	.10916	1.000	-.4009	.3042
	KID	.1250	.10916	.979	-.2275	.4775
	MDA	.1367	.10916	.962	-.2159	.4892
	REE	-.1258	.10916	.978	-.4784	.2267
	SCH	.1475	.10916	.939	-.2050	.5000
	ZWE	.1342	.10916	.966	-.2184	.4867
REE	BIS	.2592	.10916	.351	-.0934	.6117
	CEN	.2217	.10916	.580	-.1309	.5742
	DIM	.2617	.10916	.338	-.0909	.6142
	EB	.0775	.10916	.999	-.2750	.4300
	KID	.2508	.10916	.399	-.1017	.6034
	MDA	.2625	.10916	.333	-.0900	.6150
	QUIN	.1258	.10916	.978	-.2267	.4784
	SCH	.2733	.10916	.278	-.0792	.6259
	ZWE	.2600	.10916	.347	-.0925	.6125
SCH	BIS	-.0142	.10916	1.000	-.3667	.3384
	CEN	-.0517	.10916	1.000	-.4042	.3009
	DIM	-.0117	.10916	1.000	-.3642	.3409
	EB	-.1958	.10916	.737	-.5484	.1567
	KID	-.0225	.10916	1.000	-.3750	.3300
	MDA	-.0108	.10916	1.000	-.3634	.3417
	QUIN	-.1475	.10916	.939	-.5000	.2050
	REE	-.2733	.10916	.278	-.6259	.0792
	ZWE	-.0133	.10916	1.000	-.3659	.3392
ZWE	BIS	-.0008	.10916	1.000	-.3534	.3517
	CEN	-.0383	.10916	1.000	-.3909	.3142

		DIM	.0017	.10916	1.000	-.3509	.3542
		EB	-.1825	.10916	.809	-.5350	.1700
		KID	-.0092	.10916	1.000	-.3617	.3434
		MDA	.0025	.10916	1.000	-.3500	.3550
		QUIN	-.1342	.10916	.966	-.4867	.2184
		REE	-.2600	.10916	.347	-.6125	.0925
		SCH	.0133	.10916	1.000	-.3392	.3659
LSD	BIS	CEN	-.0375	.10916	.732	-.2538	.1788
		DIM	.0025	.10916	.982	-.2138	.2188
		EB	-.1817	.10916	.099	-.3980	.0347
		KID	-.0083	.10916	.939	-.2247	.2080
		MDA	.0033	.10916	.976	-.2130	.2197
		QUIN	-.1333	.10916	.225	-.3497	.0830
		REE	-.2592*	.10916	.019	-.4755	-.0428
		SCH	.0142	.10916	.897	-.2022	.2305
		ZWE	.0008	.10916	.994	-.2155	.2172
	CEN	BIS	.0375	.10916	.732	-.1788	.2538
		DIM	.0400	.10916	.715	-.1763	.2563
		EB	-.1442	.10916	.189	-.3605	.0722
		KID	.0292	.10916	.790	-.1872	.2455
		MDA	.0408	.10916	.709	-.1755	.2572
		QUIN	-.0958	.10916	.382	-.3122	.1205
		REE	-.2217*	.10916	.045	-.4380	-.0053
		SCH	.0517	.10916	.637	-.1647	.2680
		ZWE	.0383	.10916	.726	-.1780	.2547
	DIM	BIS	-.0025	.10916	.982	-.2188	.2138
		CEN	-.0400	.10916	.715	-.2563	.1763
		EB	-.1842	.10916	.094	-.4005	.0322
		KID	-.0108	.10916	.921	-.2272	.2055
		MDA	.0008	.10916	.994	-.2155	.2172
		QUIN	-.1358	.10916	.216	-.3522	.0805
		REE	-.2617*	.10916	.018	-.4780	-.0453
		SCH	.0117	.10916	.915	-.2047	.2280
		ZWE	-.0017	.10916	.988	-.2180	.2147
	EB	BIS	.1817	.10916	.099	-.0347	.3980
		CEN	.1442	.10916	.189	-.0722	.3605
		DIM	.1842	.10916	.094	-.0322	.4005
		KID	.1733	.10916	.115	-.0430	.3897
		MDA	.1850	.10916	.093	-.0313	.4013
		QUIN	.0483	.10916	.659	-.1680	.2647
		REE	-.0775	.10916	.479	-.2938	.1388
		SCH	.1958	.10916	.076	-.0205	.4122
		ZWE	.1825	.10916	.097	-.0338	.3988
	KID	BIS	.0083	.10916	.939	-.2080	.2247
		CEN	-.0292	.10916	.790	-.2455	.1872
		DIM	.0108	.10916	.921	-.2055	.2272

	EB	-.1733	.10916	.115	-.3897	.0430
	MDA	.0117	.10916	.915	-.2047	.2280
	QUIN	-.1250	.10916	.255	-.3413	.0913
	REE	-.2508*	.10916	.023	-.4672	-.0345
	SCH	.0225	.10916	.837	-.1938	.2388
	ZWE	.0092	.10916	.933	-.2072	.2255
MDA	BIS	-.0033	.10916	.976	-.2197	.2130
	CEN	-.0408	.10916	.709	-.2572	.1755
	DIM	-.0008	.10916	.994	-.2172	.2155
	EB	-.1850	.10916	.093	-.4013	.0313
	KID	-.0117	.10916	.915	-.2280	.2047
	QUIN	-.1367	.10916	.213	-.3530	.0797
	REE	-.2625*	.10916	.018	-.4788	-.0462
	SCH	.0108	.10916	.921	-.2055	.2272
	ZWE	-.0025	.10916	.982	-.2188	.2138
QUIN	BIS	.1333	.10916	.225	-.0830	.3497
	CEN	.0958	.10916	.382	-.1205	.3122
	DIM	.1358	.10916	.216	-.0805	.3522
	EB	-.0483	.10916	.659	-.2647	.1680
	KID	.1250	.10916	.255	-.0913	.3413
	MDA	.1367	.10916	.213	-.0797	.3530
	REE	-.1258	.10916	.251	-.3422	.0905
	SCH	.1475	.10916	.179	-.0688	.3638
	ZWE	.1342	.10916	.222	-.0822	.3505
REE	BIS	.2592*	.10916	.019	.0428	.4755
	CEN	.2217*	.10916	.045	.0053	.4380
	DIM	.2617*	.10916	.018	.0453	.4780
	EB	.0775	.10916	.479	-.1388	.2938
	KID	.2508*	.10916	.023	.0345	.4672
	MDA	.2625*	.10916	.018	.0462	.4788
	QUIN	.1258	.10916	.251	-.0905	.3422
	SCH	.2733*	.10916	.014	.0570	.4897
	ZWE	.2600*	.10916	.019	.0437	.4763
SCH	BIS	-.0142	.10916	.897	-.2305	.2022
	CEN	-.0517	.10916	.637	-.2680	.1647
	DIM	-.0117	.10916	.915	-.2280	.2047
	EB	-.1958	.10916	.076	-.4122	.0205
	KID	-.0225	.10916	.837	-.2388	.1938
	MDA	-.0108	.10916	.921	-.2272	.2055
	QUIN	-.1475	.10916	.179	-.3638	.0688
	REE	-.2733*	.10916	.014	-.4897	-.0570
	ZWE	-.0133	.10916	.903	-.2297	.2030
ZWE	BIS	-.0008	.10916	.994	-.2172	.2155
	CEN	-.0383	.10916	.726	-.2547	.1780
	DIM	.0017	.10916	.988	-.2147	.2180
	EB	-.1825	.10916	.097	-.3988	.0338

		KID	-.0092	.10916	.933	-.2255	.2072
		MDA	.0025	.10916	.982	-.2138	.2188
		QUIN	-.1342	.10916	.222	-.3505	.0822
		REE	-.2600*	.10916	.019	-.4763	-.0437
		SCH	.0133	.10916	.903	-.2030	.2297
Dunnett t (2-sided)^b	BIS	ZWE	.0008	.10916	1.000	-.2973	.2989
	CEN	ZWE	.0383	.10916	1.000	-.2598	.3364
	DIM	ZWE	-.0017	.10916	1.000	-.2998	.2964
	EB	ZWE	.1825	.10916	.450	-.1156	.4806
	KID	ZWE	.0092	.10916	1.000	-.2889	.3073
	MDA	ZWE	-.0025	.10916	1.000	-.3006	.2956
	QUIN	ZWE	.1342	.10916	.773	-.1639	.4323
	REE	ZWE	.2600	.10916	.117	-.0381	.5581
	SCH	ZWE	-.0133	.10916	1.000	-.3114	.2848

Based on observed means.

The error term is Mean Square (Error) = 0.071.

*. The mean difference is significant at the 0.05 level.

b. Dunnett t-tests treat one group as a control and compare all other groups against it.

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The present study was undertaken considering perturbations that some of the most vulnerable populations of the recent global SARS-CoV-2 pandemic may be exposed to raw wastewater. A handful of studies suggest the occurrence of viable SARS-CoV-2 in wastewater (Heller *et al.*, 2020; Xiao *et al.*, 2020; Oliveira *et al.*, 2021; Sherchan *et al.*, 2023).

In a report by Chattopadhyay and Taft, (2018), in the case where pathogens are not directly introduced into the wastewater system, the risk for conveyance is primarily due to the shedding of viable pathogens in bodily fluids, which maintain their infectious properties until further host exposure. The likely connection between the dissemination of diseases and pathogens in wastewater was acknowledged in the initial years of the 20th century, with the earliest publication documented in 1907, according to Johnson *et al.*, (2013). Sewage worker's syndrome has since propelled scholarly pursuits in work-related exposure to wastewater.

This study commenced on the assumption that wastewater treatment plant operators were exposed to viable SARS-CoV-2 virions from the respective municipal wastewater treatment facilities. While collecting wastewater-influent samples in the respective wastewater treatment plants, plant operators performed some of their manual tasks with limited protective gear. For example, some workers performed their duties while wearing overalls bottoms, a T-shirt, no gloves, and no splash masks, exposing themselves to likely contaminated droplets/bioaerosols and surfaces. Manual labour potentially exposes wastewater treatment plant operators to pathogens of medical importance. The laborious activities include repairing and maintaining infrastructure, manual handling of sludge and raw sewage, etcetera.

It is a known fact that infective SARS-CoV-2 can be tenacious on surfaces and inanimate objects for an extended time (several hours to a few days). The persistence and stability of SARS-CoV-2 on surfaces can vary depending on environmental conditions and the type of surface; for example, the virus can remain viable on plastic and surfaces for up to a week

(Doremalen *et al.*, 2020; Riddell *et al.*, 2020). According to observations by Minodier *et al.*, (2015), studies often focus on the molecular measures of enveloped viruses in the case of seasonal outbreaks and pandemics.

This is the maiden QMRA study conducted in the Buffalo City region in the Eastern Cape Province of South Africa to estimate the probable levels of occupational exposure of wastewater treatment plant operators to SARS-CoV-2 by accidental ingestion (self-inoculation) and inhalation of bioaerosols.

The accidental consumption of a substance occurs when it is transferred from the environment to the mouth, which can happen when contaminated hands or objects that have come into contact with the peri-oral area, causing the contamination to enter through (de Graaf *et al.*, 2017; Zaneti *et al.*, 2021). Fomites may exhibit a route from the hands to the mouth, nose, and eyes (Haas, 2020). A potential exposure pathway for SARS-CoV-2 transmission is the faecal route from carrier persons to the wastewater treatment system and the potential exposure pathways (i.e. spray or splashing, direct contact with contaminated surfaces, inhalation and ingestion) to wastewater treatment plant workers (Zaneti *et al.*, 2021).

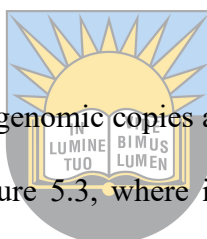
RT-qPCR was utilised in the present study to identify and quantify the SARS-CoV-2 RNA retrieved from the wastewater influent samples from the respective wastewater treatment plants throughout the study period. Table 5.1 depicts the average SARS-CoV-2 viral load (GC/mL) accumulated across the epidemiological seasons and the potential correlation to the clinical cases reported in the Eastern Cape Province. The peak in clinical cases was reported in summer (02 December 2021 – 24 February 2022). The correlation between the clinical cases and the viral load yielded by the wastewater samples was calculated to determine significant factors. REE, CEN, DIM, and EB contributed significantly to the epidemiological period peaks and positively correlated to the clinical cases, with correlation values 0.84, 0.75, 0.69 and 0.54,

respectively. These findings were observed during the Omicron (B.1.1529, BA.4 and BA.5) driven epidemic waves, which overlapped with the autumn epidemiological period.

The concentrations range yielded by the influent samples from all WWTPs in winter are approximately 2.04×10^4 GC/mL, spring 2.77×10^4 GC/mL, summer 1.18×10^4 GC/mL and autumn 10.06×10^4 GC/mL. According to Johnson *et al.*, (2013), the estimated viral load for faecal pathogens for common faecal-oral pathogens (e.g., norovirus) may ascend from 10^8 to 10^9 GC/g of faeces and at least 10^6 GC/mL of vomit. Chattopadhyay and Taft, (2018) have reported varying counts for common indicator pathogens complementary to the faecal-oral transmission pathways. The pathogen counts per gram of faeces are 10^6 number/gram faeces for enteroviruses and *Campylobacter* spp., and 10^5 number/gram faeces for *Vibrio cholerae* (World Health Organization, 2017).

The calculated average SARS-CoV-2 genomic copies accumulated at different seasons during the study period are provided in Figure 5.3, where it is apparent that the epidemiological seasons yielding high SARS-CoV-2 fragments were winter (2021) and autumn (2022), except for the case of REE with elevated concentrations in the summer season (2021). In summer, the average viral load attained from the REE facility was 4.92×10^3 GC/mL, and 4.76×10^3 GC/mL in autumn.

Elevated concentrations of 4.39×10^3 GC/mL in autumn were also observed in samples collected from EB, and QUIN yielded the highest concentrations (7.15×10^3 GC/mL) in winter. In South Africa, these seasons are categorised by strong winds and lower temperatures. Interestingly, the winter period had more rain than previous years. Moreover, outbreaks of respiratory infections and illnesses, such as the common cold caused by a panel of respiratory pathogens, are common during these seasons. This prompted using QMRA to understand the



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potential health risks of exposure of wastewater treatment plant workers to SARS-CoV-2 across the four seasons.

In the framework of this study, an assumption was made pertaining to the exposure scenario: wastewater treatment plant workers work for an average of six hours a day. During this period, they accidentally ingest and inhale up to 2 mL of wastewater harbouring SARS-CoV-2. The presumed volumetric intakes represent minimum (2 mL), maximum (10 mL) and aggressive (20 mL) case scenarios used to estimate the potential health risks.

The study findings reveal that the potential risk of infection is directly proportional to the accumulated SARS-CoV-2 viral load obtained from each study site, and the probability of infection values are provided in Table 5.4. As expected, higher probability of infection ($P_{(i)}$) values were observed for the aggressive exposure scenario (exposure to 20 mL for six hours a day). In the considered scenario, $P_{(i)}$ values were 35.9×10^{-2} , 5.1×10^{-2} , 6.8×10^{-2} , and 18.2×10^{-2} in winter, spring, summer, and autumn, respectively.

Elevated $P_{(i)}$ values were observed in Winter and Autumn. This result corresponds to the elevated SARS-CoV-2 viral load observed in the same study period. Coronaviruses are among various respiratory viruses accountable for seasonal outbreaks, particularly in winter (Lagacé-Wiens *et al.*, 2021). Site contributions to the seasonal values significantly influence the overall $P_{(i)}$ value variation. The variation in values can also be attributed to numerous factors, such as how much of the populace in the catchment area harbours SARS-CoV-2, varying influent flow rates, and the numerous subsets of the population contributing to these plants.

Data from the dose-response to the framework scenarios was subjected to statistical analysis. The standard error was calculated to measure the accuracy and distribution of the mean probability of infection (POI). The standard error of prediction was 0.8, indicating the acceptable reliability of the assessment. OriginPro (version 2017, OriginLab Corporation,

Northampton, MA, USA) was used to generate the plot illustrated in Figure 5.8, which shows the highest mean POI ($P_{(i)}$) values observed at the REE wastewater treatment plant for the aggressive exposure scenario.

According to the framework, wastewater treatment plant operators working at the EB wastewater treatment facility had a higher potential risk of exposure in the autumn season. At the same time, the same applies to wastewater treatment plant operators working at the QUIN WWTP in winter. Following the Bayesian One –way ANOVA and General linear model (Univariate) and multiple comparison tests on IBM SPSS Statistics Data Editor, Tables 5.3 and 5.4 display the statistical significance of the probability of infection ($P_{(i)}$) data attained from each study site at different dose levels.

The tests were successful as the probability of infection means were grouped into homogeneous subsets based on observed means. The observed error term was mean square (Error) equal to 0.07, *a.* used harmonic mean sample size equal to 12 and *b.* Alpha equaled to 0.05. Each model provided a significance value more significant than 0.05 ($p > 0.05$). This finding implies no statistical difference between the mean probability of infection ($P_{(i)}$) values for each site after the variance analysis. This conveys the impression that the potential health risks are not solely dependent on the facility where operators are employed. There is no apparent relationship between the facility and the worker's exposure to the coronavirus and the potential risk of infection.

Moreover, multiple comparison tests were employed using 12 models to evaluate the statistical significance of the variation relating to the probability of infection ($P_{(i)}$) to workers from the exposed facilities. The mean variation was statistically significant, with a p-value of 0.05. The error term was mean square (Error) equal to 0.071. The Dunnett t-tests treated one group as a control and assessed others against it. The $P_{(i)}$ values are not as statistically significant ($p \geq$

0.05) at a significance level of 0.05. This correlates with the homogenous subset data. The findings allude to the idea that the observed data is reasonably consistent with the assumption that the null hypothesis (H_0) is accurate specifically at the chosen significance level. Therefore, any observable difference in terms of $P_{(i)}$ at varying exposure levels, facilities conditions and in-site factors is more likely due to random chance than the direct effect of the factors as mentioned above. Interestingly, after the variation assessment, when compared to all other mean $P_{(i)}$ groups, REE $P_{(i)}$ are statistically significant at a significance level of 0.05 ($p \leq 0.05$) in 1 out of 12 test models; the Least Significant Difference (LSD). This particular finding indicates that there is about 8.3 % likelihood that the variables affecting SARS-CoV-2 concentrations in the wastewater collected from REE posit an effect on the potential occupational health risks.



In light of the preceding discussion, three sites yielding more SARS-CoV-2 particles stood out. The overall viral load accumulated from municipal wastewater samples from the EB, QUIN and REE wastewater treatment plants suggests that wastewater treatment plant operators working in these sites may potentially be exposed to higher SARS-CoV-2 concentrations and variants of concern than operators of other wastewater treatment plants. The sites were among the top five plants with a larger design capacity, and the geospatial distribution (Figure 4.3) in Section 4.2 presents EB as a potential hotspot for SARS-CoV-2 and its variants.

Essentially, the operators of the aforementioned facilities were potentially exposed to fragments of variants of concern. For instance, during the surveillance period, 7 (*P681H*, *P681R*, *L452R*, *N501Y*, *delH69V70*, *E484K* and *K417N*) SNP were detected in the municipal wastewater converging in the EB WWTP, while 4 (*L452R*, *N501Y*, *delH69V70* and *P681H*) were detected in the samples from QUIN and REE WWTPs. These mutations are associated with escape to acquired immunity as well as elevated transmissibility and are linked to the alpha, beta, gamma, delta and omicron variants.

Essentially, vulnerable wastewater treatment operators working in the designated wastewater treatment facilities in Buffalo City Municipality were potentially exposed to highly transmissible variants of concern especially during the 3rd epidemic (predominated by delta variants) and 5th epidemic (predominated by the omicron variants) as increased viral loads were observed during these periods when variants were circulating and contributing to viral load in wastewater.

Nonetheless, a myriad of factors influence the SARS-CoV-2 viral load levels collected in the respective wastewater treatment plants, hence the variations in viral load and, subsequently, the probability of infection values. These include factors such as the influent flow rate and how much of the facility's demographic is afflicted with SARS-CoV-2 (Tuite *et al.*, 2020; Wu *et al.*, 2020; Maida *et al.*, 2022).

The potential health risks outlined by QMRA relate exclusively to particular scenarios, such as when a wastewater treatment plant worker is exposed to untreated wastewater for six hours. That is a highly improbable situation in reality unless a wastewater treatment plant operator engages in sludge dehydration or manually collects and transports sludge for off-site treatment for an extended period (Bibby, Viau and Peccia, 2011; Bibby and Peccia, 2013; Carducci *et al.*, 2020; Singer and Wray, 2020).

Consequently, this study demonstrates potential health hazards for wastewater treatment plant workers involved in manual activities for extensive periods, corroborating Amoah *et al.*, (2022) and Dada and Gyawali (2021) but only during aggressive exposure scenarios.

5.4 Conclusion

The QMRA modelling of this study is an essential approach for measuring the potential health risks to which wastewater treatment plant operators are exposed. Incorporating the dose-response model allowed the QMRA framework to reveal the potential health risks in the wastewater treatment occupation. Despite the conservative approach adopted in the present study model, a low probability of infection risks was predicted during the minimum, maximum and aggressive exposure scenarios. The incidence of SARS-CoV-2 in wastewater suggests the viral shedding within the populace and a potential hazard to the well-being of wastewater treatment plant workers. The findings revealed a directly proportional relationship between the viral load and the potential risk of occupational exposure to SARS-CoV-2. Policies should be implemented in occupational settings to protect wastewater treatment plant workers and curb the rapid dissemination of SARS-CoV-2. This tool can be used as a complementary approach for epidemiological studies. Overall, some limitations to the model which prompt further research in this area were recognised. The assessment requires the incorporation of SARS-CoV-2 concentrations. Viral concentrations may vary for each plant depending on population dynamics and many in-sewer factors. This study advocates the significance of continued monitoring of potential viral incidences in wastewater milieu and an intensive multi-faceted risk assessment frameworks to alleviate the potential occupational health-hazards associated with the transmission of microbial pathogens in the environment.



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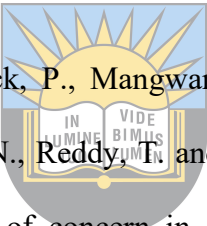
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6. CHAPTER SIX

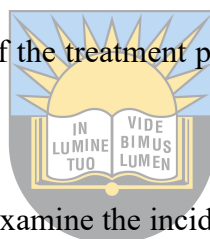


GENERAL DISCUSSIONS AND CONCLUSIONS
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GENERAL DISCUSSIONS AND CONCLUSIONS

6.1 Discussion

Wastewater-based epidemiology was employed for profiling the pervasiveness of SARS-CoV-2 genomes in wastewater and evaluating the probable health implications of occupational exposure associated with operating wastewater treatment plants amidst the COVID-19 pandemic across the four seasons in Buffalo City Metropolitan Municipality in the Eastern Cape Province, South Africa. This was accomplished through three principal experimental designs: quantification of the SARS-CoV-2 genomic markers extracted from municipal wastewater treatment plant influents, molecular assessment of the occurrence of the SARS-CoV-2 variants and the quantitative microbial risk assessment to evaluate the potential health implications related to the exposure of the treatment plant workers to SARS-CoV-2 genomes in wastewater matrices.



The fundamental procedural steps to examine the incidence and pervasiveness of SARS-CoV-2 genomes in the municipal wastewaters required a comprehensive analysis of the total viral ribonucleic acid (RNA) loads in the wastewater using RT-qPCR. Numerous studies have documented the incidences of an incredible array of pathogens, including respiratory viruses, in the wastewater milieu (Adefisoye *et al.*, 2016a; Gcilitshana *et al.*, 2017; Osuolale and Okoh, 2017; Stobnicka-Kupiec *et al.*, 2022; Lowry, Wolfe and Boehm, 2023).

The exploration of wastewater as a medium for the dissemination of SARS-CoV-2 was initiated in the course of the global pandemic. The eruption of a novel coronavirus disease (COVID-19) took the world by storm and caused a global health and socio-economic catastrophe. The disease primarily invades the respiratory system and has decimated approximately 7 million lives globally and over one hundred and two thousand lives in South Africa alone.

During disease circulation, the virus underwent genomic changes that yielded variants that altered the transmission and population-immunity dynamics. The rise in SARS-CoV-2 variants led to random surges in clinical cases with varying disease manifestations and subsequent devastations to healthcare and economic systems. Localised epidemics predominated by variants of concern (VOC) were observed in South Africa, with the Delta-driven epidemic dubbed as the worst epidemic due to excessive hospital admissions (Jassat *et al.*, 2022; Madhi *et al.*, 2022; Mahase, 2022; Yang and Shaman, 2022).

Studies in the Netherlands by Medema *et al.*, (2020), in Australia by Ahmed *et al.*, (2020) and in the United States of America by Peccia *et al.*, (2020) propelled the global race for the surveillance of SARS-CoV-2 in wastewater. The implementation of wastewater surveillance for tracking drugs and therapeutics circulating in specific communities and the fact that wastewater is a viable reservoir for emerging pathogens of communal health significance inspired the screening of wastewater for the incidence of potentially infectious pathogens (Corpuz *et al.*, 2020). This study used the wastewater-based epidemiology approach to monitor the incidence of SARS-CoV-2 viral concentrations in the Buffalo City Metropolitan Municipality in the Eastern Cape Province. This approach is an outstanding tool for monitoring emerging diseases via human excreta, thus assisting as a primary indicator of disease inclinations in respective communities (Corpuz *et al.*, 2020; Prado *et al.*, 2021).

The grab sampling technique was used to collect influent samples from the selected wastewater treatment facilities in the study community. A volume of 500 mL of wastewater influent was acquired from each sampling point for the study. This technique was of the utmost importance for this investigation as it provides preliminary data on the wastewater components at the specific sampling time, which is suitable for conducting a time series analysis. According to Augusto *et al.*, (2022), this technique is preferred when evaluating the concentrations of

substances, pathogens such as SARS-CoV-2, and pH levels, which may change rapidly and frequently.

Subsequently, the extraction of total ribonucleic acids from the collected samples was achieved using commercially available extraction kits comprising three characteristic phases. The primary phase was sample preparation involving microbeads and bead solution and the subsequent lysis of cells. The second phase was the organic extraction/phase separation, where RNA was separated from other compounds in the wastewater samples. The last phase was the purification of nucleic acids. Nanodrop spectrophotometry was used to determine the extracted RNA's concentration and quality. The spectrophotometer generally used the principle of ultraviolet-visible spectrum absorbance to reflect the purity of ribonucleic acids from contaminants at the 260/280 and 260/230 absorbance ratios.

RT-qPCR is widely utilised for the detection of RNA viruses, and it was also used in the present study to ascertain the viral load of SARS-CoV-2 genomes in wastewater. The reaction transcribes RNA into complementary DNA (cDNA) for subsequent amplification and analysis. The initial stages of this technique, the RT-PCR component, involved using iScript reverse transcriptase to reverse-transcribe the pure RNA extracts to cDNA at 95 °C for 10 minutes. Consequently, the reaction's quantitative component (qPCR) was carried out as cDNA's nucleocapsid gene (N1 and N2) region was amplified and quantified in 40 cycles.

The raw data file retrieved from the Design and Analysis Software was analysed to determine the efficiency of the reaction. A set of parameters determined the reaction's quality and the quantity of the SARS-CoV-2 nucleocapsid protein gene. For instance, the amplification efficiency ranged between 90% and 110%; the slope ranged from -3.3 to -3.8; the correlation value $r^2 \approx 1$; and the Y-intercept values ranged between 36 and 38. A parameter of utmost significance was the cycle threshold (C_T) value. C_T values reflect the cycles at which

fluorescent signals pass through a threshold, and this value broadly categorises the concentration of the nucleocapsid genes in the tested samples (Almeida *et al.*, 2013).

The circulating genomes were elucidated through the molecular detection method described and optimised by Johnson *et al.*, (2021) subsequent to the quantification of total RNA extracted from the study sites. The wastewater retrieved from the study sites yielded a median total RNA concentration of approximately 1×10^3 ng/ μ L, which corroborates the findings by Wu *et al.*, (2020) and Foladori *et al.*, (2020) about viral titre in wastewater. However, the same protocol used in total RNA extraction by Tomasino *et al.*, yielded a total RNA concentration of approximately 0.04×10^3 ng/ μ L. The appreciable difference in the RNA concentration could be attributed to numerous in-sewer dynamics such as wastewater velocity, the organic load and population dynamics (Bertels *et al.*, 2022; Boehm *et al.*, 2023). Essentially, the variations in nucleic acid concentrations in wastewater are inevitable.

The occurrence of RNA in the wastewater samples corroborates the findings from the documented studies by Osuolale and Okoh (2017), Wang *et al.*, (2020) and Rothman *et al.*, (2021). The findings ascertain evidence of viral particles in the wastewater milieu (Adefisoye *et al.*, 2016b; Gcilitshana *et al.*, 2017; Adriaenssens *et al.*, 2018; Dlamini *et al.*, 2024) and confirm the real-time viral signals in the wastewater milieu. The incidence of viral RNA in wastewater amidst the COVID-19 global crisis encouraged the molecular screening of the nucleocapsid protein gene, indicative of SARS-CoV-2. SARS-CoV-2 signals in the wastewater attained from the Buffalo City wastewater treatment facilities throughout the 12-month sampling regime are depicted in Figures 3.4 and 3.5.

The Pearson correlation coefficient was applied to evaluate the association between nucleic acid concentrations (ng/ μ L) and the SARS-CoV-2 viral load (GC/mL) retrieved from each sampling location. Nucleic acid concentrations (ng/ μ L) had a feeble correlation with SARS-

CoV-2 viral load (GC/mL). P-values < 0.05 indicated no significant correlation between the nucleic acid and SARS-CoV-2 viral load. It has been established that wastewater is a reservoir for numerous pathogens and RNA viruses. Therefore, to determine the direct correlation between the viral RNA concentration and SARS-CoV-2 viral load, a kit specifically designed to extract coronaviruses from wastewater would have to be used as the currently available kits are designed to extract the total nucleic acids in wastewater.

Fluctuations in SARS-CoV-2 signals were observed across the 46 week-epidemiological period. The study commenced during the adjusted alert level 4. This result depicted in Figure 3.5 was expected as COVID-19 levels cumulated nationally (NICD, 2021, 2022) and globally (WHO, 2021, 2022). The initial spikes in the SARS-CoV-2 viral load ($>5 \times 10^3$ GC/mL) were observed from epidemiological week 4 to epidemiological week 13 (21st July 2021 – 30th September 2021) in corroboration to inclinations recorded by the NICD, (2021) in the same period. This peak occurred throughout the third COVID-19 epidemic wave (May 2021 - October 2021) driven by variant delta (B.1.617.2) with an estimated 220568 COVID-19 reported cases of infections (NICD, 2021). These findings contradict the modelling projections Borchering *et al.*, (2021) made. Bekker and Ntusi, (2021) link the inclination of SARS-CoV-2 from July 2021 to the circulation of at least four variants of concern in correlation to a study undertaken by (Viana *et al.*, 2022).

Concurrently, SNP assay genotyping demonstrated the incidence of four target spike protein mutations in the epidemiological months of June and July 2021: *P681H*, *delH69V70*, *N501Y*, and *K417N* in June and *P681H*, *delH69V70*, *N501Y*, and *L452R* in July (adjusted alert level 4 between 28th June and 25th July 2021). In July 2021, the country suffered civil unrest rooted in KwaZulu Natal and Gauteng province, leading to increased COVID-19 cases of infection and a subsequent rise of SARS-CoV-2 mutations, Bingham *et al.* (2022) and Pulliam *et al.* (2022) documented similar observations. In the same period (July 2021), an upsurge in SARS-CoV-2

infections, even from vaccinated individuals, was observed in Massachusetts. This epidemic was attributed to the substantially transmittable Delta variant (B.1.617.2) dispersed through large social gatherings (Brown *et al.*, 2021; Gharpure *et al.*, 2022).

In August 2021, *P681H*, *delH69V70*, *N501Y*, *L452R* and *E484K* were ascertained in the wastewater samples possessing the SARS-CoV-2 nucleocapsid protein-encoding gene. All seven signature mutations were detected in September 2021 in more samples. These findings corroborated with the peaks in SARS-CoV-2 viral load (GC/mL) detected in epidemiological week 9 (9th September 2021) (highest signals observed in BIS WWTP with a concentration of 17.6×10^3 GC/mL) to week 13 on 30th September 2021 (highest signals observed in EB-B site with a concentration of over 24.2×10^3 GC/mL) followed by the spike in KID WWTP with approximately 26.9×10^3 GC/mL a week later from a sample collected on 7th October 2021.

Evidently, the upsurge of SARS-CoV-2 signals in wastewater and spike protein mutations interrelated with the upsurge in COVID-19-confirmed incidents over this period. Ramesh *et al.*, (2021) similarly highlighted a momentous increase in COVID-19 cases as the emergence of variants led to approximately 230 million cases globally in September 2021. In the same period, four SARS-CoV-2 lineages were categorised as the VOCs: Alpha, Beta, Gamma, and Delta. Variants of concern exhibit elevated transmission rates and potentially increased disease severity (Choi and Smith, 2021; Harvey *et al.*, 2021; Patarca and Haseltine, 2022). Apart from the wastewater sample sourced from KID WWTP yielding a vastly high viral load, the levels of SARS-CoV-2 in wastewater stabilised as the detection of S-protein mutations decreased and the intensity of the delta-driven wave subsided.

By December 2021, the alert levels in South Africa had regressed from adjusted level 4 to adjusted level 1 (confirmed on 1st December 2021). This implies that the rigorous limitations on travel, social gatherings and curfews were gradually relaxed. Nonetheless, a significant

spike in clinical cases was observed on 2nd December 2021, and a week later (09th December 2021), wastewater samples sourced from the BCCM region yielded more SARS-CoV-2 RNA fragments. REE WWTP significantly contributed to the weekly data as a viral load of approximately 16.7×10^3 GC/mL was observed from the site. A plateau had been observed in REE WWTP between epidemiological weeks 25 – 33. This represents the period where no samples were retrieved due to extensive damages sustained by the pump station caused by heavy storms.

An inconsistency in the functioning of the wastewater treatment facilities was observed in at least four wastewater treatment facilities. There were periods when some of these facilities would be left unattended and utterly inaccessible. This was mainly observed at the DIM and BIS WWTP. The one female staff member would leave the BIS WWTP unattended due to safety concerns as the facility was once broken into. The sightings at some of the facilities were absolutely revolting. Some of the plants would be left unattended and poorly maintained for weeks on end. For instance, the SCH WWTP was vandalised by cable and copper thieves. Since this incident, there has been an abhorrent buildup of waste due to poor maintenance; consequently, the plant released effluent of poor quality into the catchment river.

A similar sighting was observed at the MDA WWTP, where the influent flowed at a very slow rate due to blockages due to solid waste debris accumulation caused by zero maintenance. Another noteworthy observation was that the facility operated way over its design capacity at CEN WWTP, resulting in an overflow of influent, especially on rainy days. This placed the plant personnel at risk of exposure to hazardous substances and pathogens in the wastewater. An operator at the plant mentioned that there were plans in the near future to build pipelines to link the plant to another and have the influent coming in from some locations diverted to the REE WWTP. The damage to the pump station serving the REE WWTP meant no influent flowing to the facility for eight weeks. However, an upward trendline in SARS-CoV-2 signals

from this facility is an indication of a surge in SARS-CoV-2 viral load from the reticulation area.

In November 2021, the variant of concern primarily discovered in Botswana (a neighbouring country) was also detected in South Africa (Viana *et al.*, 2022) in the same period, and the World Health Organization further categorised it as variant Omicron (B.1.1.529). Omicron is highly transmissible, resists the efficacy of therapeutics, and partially escapes acquired immunity (Al Hasan *et al.*, 2022; Johnson *et al.*, 2022). Prior to and during the fourth epidemic wave (December 2021 – April 2022), driven by the Omicron variant, various spike protein mutation assortments were observed.

For instance, the assortment of spike protein mutations *N501Y*, *E484K*, and *K417N* are presumably associated with the Beta variant. This assortment was detected from samples attained between the epidemiological weeks 27 and 37 (28th June 2021 – 13th September 2021). According to a report by Tegally *et al.* (2021), the Beta-driven wave had abated in April 2021. However, Al Hasan *et al.*, (2022) reported that the VOC circulated at low frequencies beyond April 2021. Moreover, 47% of the SARS-CoV-2 genome possessed the signature mutations (*N501Y*, *delH69V70*, and *P681H*), which can be linked to the Alpha (B.1.17) variant that was initially detected in the UK in September 2020, indicating that the VOC was circulating the Buffalo City region two years post its initial discovery.

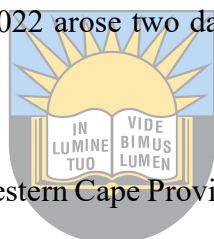
A noteworthy finding is the circulation of the spike protein mutation assortment *N501Y*, *P681H*, *delH69V70*, *K417N*, and *E484K* signature to the Omicron. These SNPs were detected in samples attained between 13th September 2021 and 07th April 2022. Additionally, these findings suggest two probable presumptions: either Omicron (B.1.1.529) disseminated within Buffalo City communities two months before its discovery in South Africa (Viana *et al.*, 2022) or supporting literature on the commonality of key spike protein mutations in the variant spectrum

(Magazine *et al.*, 2022; Islam *et al.*, 2023). Nonetheless, this indicates the obligation for whole-genome sequencing strategies to complement SNP assay genotyping and wastewater-based epidemiology. Madhi *et al.*, (2022) also documented prevalent underlying SARS-CoV-2 seropositivity before the Omicron-driven epidemic in the Gauteng Province.

Peaks in SARS-CoV-2 signals in wastewater occurred in the epidemiological months of April and May 2022 during the autumn season. This was accompanied by an increase in the detection frequency of spike protein mutations (*P681H*, *delH69V70*, *N501Y*, and *L452R*) occurring for three consecutive epidemiological weeks (07th April 2022 – 21st April 2022) followed by an upsurge in clinical cases (NICD, 2022) during the fifth epidemic wave predominated by Omicron subvariants (BA.2, BA.4, BA.5) (Mills *et al.*, 2022; Wolter *et al.*, 2022). The spike in the epidemiological month of April 2022 arose two days after the National State of Disaster was lifted (05th April 2022).

A similar spike was observed in the Western Cape Province, where the Delta variant dominated wastewater samples retrieved from Rawsonville WWTP, where four Omicron subvariants (BA.1, BA.2, BA.3, and BA.5), which had also spread globally, were confirmed (Johnson *et al.*, 2022; Kumar, Karuppanan and Subramaniam, 2022). Mahlangeni *et al.*, (2023) linked the spikes in SARS-CoV-2 signals to the Easter Holidays activities (15th to 18th April 2022).

A time series analysis revealed seasonal variations of SARS-CoV-2 signals in the wastewater of Buffalo City. The total viral load retrieved from all WWTPs in winter is 22.08×10^4 GC/mL, spring 13.09×10^4 GC/mL, summer 7.61×10^4 GC/mL and autumn 29.30×10^4 GC/mL. Overall, it is evident that elevated SARS-CoV-2 signals in wastewater were accumulated in the winter (2021) and autumn (2022) epidemiological periods. Site QUIN WWTP was the most significant contributor to the viral load (GC/mL) accumulated in winter, yielding an average



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viral load of 7.15×10^3 GC/mL. Autumn yielded the most SARS-CoV-2 signals, with REE WWTP contributing to the viral load (GC/mL), averaging 4.76×10^3 GC/mL.

In South Africa, autumn and winter are characterised by a temperature decline, high-velocity winds, and low humidity. Intriguingly, the winter of 2021 had more rainfall than previous years. Elevated SARS-CoV-2 signals were notable in mid-autumn as temperatures declined and rainfall was scarce. The viral load in this season ranged from 3.80×10^3 GC/mL – 17.37×10^3 GC/mL for other sites and 21.02×10^3 GC/mL – 810.99×10^3 GC/mL from the EB WWTP. This site serves a larger population of 141000 than other sites and is a combined sewer network. As a result, the geospatial distribution of spike protein mutations associated with VOCs, as represented in Figure 4.4, pinpoints EB WWTP as a VOC hotspot.

According to a review by Aboubakr *et al.*, (2021), coronaviruses are persistent in the environmental conditions characterised by reduced temperatures and humidity, climatic conditions often observed in winter and autumn. A study conducted in Canada highlighted that coronaviruses were among the numerous respiratory viruses that are accountable for seasonal outbreaks, particularly in winter (Moriyama, Hugentobler and Iwasaki, 2020; Lagacé-Wiens *et al.*, 2021; Meo *et al.*, 2022), while a report by Jenkins *et al.*, (2023) demonstrated that clinical cases in West and Southern Africa peaked at different seasons, particularly in monsoon and arid periods.

Conversely, significantly high SARS-CoV-2 concentrations of 49.17×10^2 GC/mL were observed in the wastewater sourced from site REE WWTP in the summer epidemiological period. This corroborates with findings by Sasikumar *et al.*, (2020), where approximately 72% of COVID-19 cases were clustered at temperatures as high as 31 °C in India. Studies have posited that in areas with temperate climates, numerous cases are attributed to dry and cold climatic conditions (McClymont and Hu, 2021) and that the seasonality of the virus spread is

presumed (Altamimi and Ahmed, 2020). However, seasonality cannot curb the dissemination of SARS-CoV-2 (Liu *et al.*, 2021). According to Adekunle *et al.*, (2020), the progression of COVID-19 in the continent of Africa had an inverse relationship with wind velocity and temperature.

Nonetheless, epidemics attributed to respiratory infections, such as the common flu induced by a panel of respiratory pathogens, are common in the colder seasons (Sooryanarain and Elankumaran, 2015). Seasonal changes influence biological ecosystems via environmental relations and alter infectious disease patterns. Climate change modulates host susceptibility and pathogen virulence. Several studies report increased mortality and morbidity in winter and autumn (D'Amato *et al.*, 2018; He *et al.*, 2018; Meo *et al.*, 2022; Madaniyazi *et al.*, 2024).

Many factors that need to be studied, including population size, in-sewer and viral shedding factors, can influence the concentration of SARS-CoV-2 in influent wastewater (Bertels *et al.*, 2022). For example, documented reports by Wu *et al.*, (2021) and Wilder *et al.*, (2021) reveal the positive association between population density and SARS-CoV-2 concentrations in wastewater (Tuite *et al.*, 2020; Wu *et al.*, 2020). However, the magnitude of the population served in the reticulation area should not be presumed as stationary as people commute from one place to another for different reasons. Hence, Wade and colleagues explore the uncertainties and variabilities for wastewater monitoring and suggest factors such as the monitoring of instabilities of inhabitants within the catchment area to comprehend trends in RNA concentrations (Bertels *et al.*, 2022; Wade *et al.*, 2022).

The temporal and geospatial distribution of the SARS-CoV-2 genome in wastewater depicts probable correlations between the increase in virus signals and the lifting of restriction measures within the country. This can be attributed to basic human behaviour associated with

freedom of movement. Environmental and climatic conditions may have contributed to the proliferation of the virus in the wastewater milieu.

The area where this study was conducted has the second largest population density in the province (317.8 km⁻²) (Buffalo City Metropolitan Municipality, 2019), making it easier to disseminate SARS-CoV-2. During the sudden surges of COVID-19, the healthcare facilities in the municipality became utterly overwhelmed, leading to the hasty distribution of the SARS-CoV-2 genomes, delayed treatment, and increased mortality. The municipal region combines rural, peri-urban and urban settlements. This is evident in the conditions and maintenance of the infrastructure observed from the wastewater treatment facilities selected for this study. Sewage spills and dilapidated infrastructure were usual sightings in the region. Consequently, wastewater treatment plant workers and civilians were exposed to untreated wastewater and a high risk of potential pathogen infections.

Wastewater treatment plant operators often present with health issues associated with exposure to contaminated surfaces and virion-infested bioaerosol. The most common health implications of such exposure are epidermal irritations and Sewage worker's syndrome, which is classified by the onset of headaches, dizziness, fatigue, and gastrointestinal and pulmonary complications (Kholi *et al.*, 2014; Sainath and Ahmed, 2014; Mokhtar *et al.*, 2022). The QMRA framework was employed in this study to evaluate the potential health risks and exposure profiles associated with working in wastewater treatment facilities in Buffalo City Metropolitan Municipality.

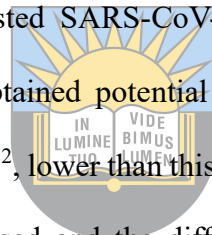
Joshi *et al.*, (2022) suggested no indications of viable virions in the wastewater milieus (Puhach, Meyer and Eckerle, 2022). While the virions are detectable in faecal matter, infectious SARS-CoV-2 has not yet been extracted from wastewater, indicating that the coronavirus may not be viable in this environment. However, studies have demonstrated the shedding of viable

SARS-CoV-2 in bodily excrements (Dergham *et al.*, 2021), including saliva (Chopoorian *et al.*, 2023), sputum and faecal matter (Jeong *et al.*, 2020), which are further discharged into wastewater (Kitajima *et al.*, 2020). Giacobbo *et al.*, (2021) are among the researchers stating that viable and infectious SARS-CoV-2 can be persistent in untreated wastewater for four to six days after being shed by infected individuals.

Despite the wastewater milieu harbouring potentially viable SARS-CoV-2, there is a paucity of standardised techniques for isolating and evaluating the viable SARS-CoV-2 genome in environmental samples (Robotto *et al.*, 2021). This shortfall has made it challenging to accurately monitor wastewater and other environmental matrices relating to the occurrence and pervasiveness of infectious SARS-CoV-2 (Zarza *et al.*, 2022). Such outcomes signify potential health issues associated with the occurrence of SARS-CoV-2 in wastewater systems. For the above reasons, the QMRA framework utilised in this study assumed that wastewater treatment plant operators were exposed to viable and infectious SARS-CoV-2 particles.

Some wastewater treatment plant operators performed routine tasks without appropriate protective gear. Essentially, WWTP operators were exposed to contaminated droplets, aerosol and surfaces. The framework was used to assess the probable occupational exposure levels of wastewater treatment plant operators to SARS-CoV-2 by accidental ingestion (self-inoculation) and inhalation of bioaerosols. This study used RT-qPCR to ascertain the incidence of SARS-CoV-2 signals in wastewater. Subsequently, the dose-response model previously developed by Watanabe *et al.*, (2010) for SARS-CoV-1 was applied as the exponential model as augmented by Dada and Gyawali, (2021) and Zaneti *et al.*, (2021) for SARS-CoV-2. Three exposure scenarios at various volumetric intakes yielded different probability of infection values ($P_{(i)}$), posing variable potential health risks to WWTP operators working without applying precautionary measures and PPEs.

As expected, elevated potential health risks ($P_{(i)}$) across the four seasons were observed in the aggressive scenario (20mL for six hours). In the considered scenario, $P_{(i)}$ values were 35.9×10^{-2} , 5.1×10^{-2} , 6.8×10^{-2} , and 18.2×10^{-2} in winter, spring, summer, and autumn, respectively. This result is consistent with the elevated SARS-CoV-2 signals observed in the same epidemiological timeframe. Coronaviruses are among various respiratory viruses accountable for seasonal epidemics, particularly in winter (Lagacé-Wiens *et al.*, 2021; Nichols *et al.*, 2021). This means that the probability of infection $P_{(i)}$ for an operator working without PPEs is a maximum of 35.9×10^{-2} in winter (2021) through inhalation self-inoculation (accidental ingestion). In comparison, the lowest $P_{(i)}$, approximately 3.00×10^{-3} , was observed in spring (2021) in the minimum exposure scenario (2 mL for six hours). The lowest reported $P_{(i)}$ is greater than the 4.6×10^{-7} of ingested SARS-CoV-2 reported by Ahmed *et al.*, (2020). Additionally, Tyagi *et al.*, (2022) obtained potential risk estimation for the lowest values ranging from 1.70×10^{-8} to 9.00×10^{-12} , lower than this study's findings. The variation in these reports is attributed to the models used and the differences in SARS-CoV-2 viral load in wastewater samples.



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In winter, higher $P_{(i)}$ for operators performing laborious tasks without PPEs are evident as the assumed volumetric intake was increased. An undeviating trend was observed in the ZWE, SCH, MDA, KID, EB, DIM, CEN, and BIS WWTPs, where higher $P_{(i)}$ values equal to 8×10^{-1} for the aggressive exposure scenario are observed in BIS WWTP. However, a different finding was observed for WWTP operators working at the QUIN and REE WWTPs. The potential health risks at QUIN WWTP, a combined sewer network, were higher in all exposure scenarios, with $P_{(i)}$ equivalent to 4.0×10^{-2} , 3.2×10^{-1} and 5.4×10^{-1} for the minimum (2 mL), maximum (10 mL) and aggressive (20 mL) volumetric intakes, respectively. The potential health risk suddenly increased for plant operators at REE WWTP to 0.26×10^1 in the aggressive exposure scenario (20 mL for six hours). As expected, in autumn (2022), all unprotected plant operators

working at the ten WWTPs had increased potential health risks attributable to the elevated SARS-CoV-2 signals in the wastewater. The breakdown and site contributions of the potential seasonal risk profiles per facility are represented in Figure 5.4. No distinct trend or evident influence of season on the potential health risks was observed. Nonetheless, precautionary measures must always be applied to safeguard occupational safety.

The QMRA was coupled with statistical analysis to characterise the potential exposure levels at $P = 0.05$. The standard error was calculated to evaluate the accuracy and distribution of the mean probability of infection (POI). The observed standard error value was 0.08, indicating the assessment's reliability. The Bayesian One-way ANOVA, General linear model (Univariate), and multiple comparison tests were carried out using IBM SPSS Statistics Data Editor. Eight homogeneous subset tests and twelve multiple comparison tests were performed, and the significance values are represented in Tables 5.4 and 5.5, respectively. The QMRA framework applied in this study indicated that wastewater treatment plant operators are at higher potential risks of exposure during periods when the SARS-CoV-2 signals are significantly elevated.



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The findings showed significance values of $p \geq 0.05$, suggesting that the potential health risks are not solely dependent on the facility where operators are employed. There is no apparent relationship between the facility and the worker's exposure to the coronavirus and the probable risk of acquiring an infection. Interestingly, after the variation assessment, when compared to all other mean $P_{(i)}$ groups, REE $P_{(i)}$ are statistically significant at the 0.05 ($p \leq 0.05$) threshold in one out of twelve test models, the Least Significant Difference (LSD). This finding indicates that there is about an 8.3 % likelihood that the variables affecting the SARS-CoV-2 viral load in the wastewater retrieved from REE WWTP posit an effect on the potential occupational health risks. Even if the potential health risks ($P_{(i)}$) are low, the QMRA approach should be

implemented in health and safety policies to ensure occupational health and curb the dissemination of pathogens of medical significance, such as SARS-CoV-2.

Notwithstanding limitations and disputes, QMRA is imperative to implement in managing and mitigating risks associated with SARS-CoV-2 and other infectious pathogens. The structured framework incorporating the quantification of SARS-CoV-2 and potential exposure pathways allows epidemiologists and relevant authorities to quantitatively evaluate the potential health risks and make informed decisions, as well as guidelines and regulations on public health. Continuous developments in molecular biology, environmental, and epidemiological studies further echo the importance of QMRA in protecting public health.

While an ongoing argument pertains to the incidence of viable and infectious SARS-CoV-2 in the wastewater environment, further research is crucial for developing standardised methods for detection and surveillance. Nonetheless, efforts to ensure wastewater effluent is efficiently disinfected are crucial for public health and curbing the potential propagation of SARS-CoV-2 into the environment (Gogoi *et al.*, 2024).

Overall, this study gives insights into the efficiency of wastewater-based epidemiology in addressing the community health status other than relying on clinical reports, which do not entirely reflect the status quo in the community as individuals are hesitant or ultimately decline to visit healthcare centres. The inability of residents within the remote areas/hinterlands/outskirts of the region to seek medical assistance is one factor that contributes to the misrepresentation of the community health status in which wastewater-based epidemiology comes to play a significant role in catering to such missing data.

6.2 Limitations

RT-qPCR genotyping has several restrictions in identifying variants relative to the benchmark NGS sequencing, which has the capability to detect previously established and novel and unidentified variants. This study used spike-protein mutations to determine the presumptive incidence of VOCs Alpha, Beta, Delta, and Omicron within the designated sampling locations. Literature reveals that all known variants excluding Delta share the *N501Y* mutation; Alpha and Omicron variants share mutations *delH69V70* and *P681H*; Beta and Omicron share mutation *K417N*, whereas Delta variant and Omicron subvariants BA.4 and BA.5 share the *L452R* spike protein mutation. This might result in assigning multiple variants to one sample, which might not accurately represent the prevalence of variants.

The QMRA model employed in this study SARS-CoV-2 depends on data derived from quantifying SARS-CoV-2 signals at a specific epidemiological period. Several aspects, such as population dynamics and environmental and in-sewer factors, influence the overall viral load. Moreover, complexities in viral dynamics, such as mutation or shedding rates, may thwart the accuracy of risk assessments. Furthermore, the frameworks employ assumptions which may not necessarily reflect literal scenarios.

Lastly, QMRA requires the amalgamation of multidisciplinary proficiency, the continuous collection of data and the corroboration of field observations. Nonetheless, QMRA is crucial for prioritising risk management policies and interventions that protect the health of wastewater treatment plant operators and the community.

6.3 Recommendations

Clinical surveillance strategies undermine overall community health/disease burden. Communities have a myriad of individuals. Some individuals are traditionalists who do not believe in modern medicine, and some fear the clinical setting. Other individuals show no symptoms and are unaware they are carriers of infectious pathogens, while some use home remedies for treatment. Capturing these individuals into the clinical surveillance system is unlikely. Therefore, the data on some databases may not truly reflect community health status. Wastewater-based epidemiology provides data encompassing such individuals and those who are immune-compromised. In some instances, peaks in SARS-CoV-2 signals have been observed before outbreaks. Therefore, wastewater-based epidemiology should be coupled with clinical surveillance to understand community disease trends.

The global health catastrophe caused by COVID-19 has reiterated the significance of community-based surveillance systems that are non-invasive and cost-effective to ensure global health and well-being as they are pivotal in reaching the United Nations' 2030 Agenda for Sustainable Development. Thus, continuous wastewater-based epidemiology is recommended to monitor circulating infectious pathogens and disease trends in communities before reaching the “epidemic” status.

RT-qPCR coupled with SNP assay genotyping are essential approaches for wastewater-based epidemiology as they provide real-time data on community health and are cost-efficient. However, as much as RT-qPCR is a gold standard in the molecular detection of organisms, RT-qPCR genotyping has various limitations; therefore, NGS sequencing is recommended as a complementary technique for detecting known and unknown variants.

The incidence of SARS-CoV-2 and VOCs in community influent should incite the attention of relevant authorities and policymakers. For instance, the Department of Water and Sanitation

(DWS) should investigate facilities and ensure that the plants are functional and do not release potentially infested effluent into the environment, as some civilians use water from catchment areas for primary and recreational purposes. Moreover, attention should be drawn to poorly maintained and dysfunctional facilities releasing untreated wastewater via burst sewer pipes and due to influent overflow.

A controversy concerning the viability of SARS-CoV-2 in the wastewater environment is circulating in the literature. Hence, the QMRA framework employed in this study used a conservative approach. A longitudinal examination of the pervasiveness of viable SARS-CoV-2 and exposure pathways at wastewater treatment facilities is recommended. The sample collection should include bioaerosol collection and swabbing surfaces and equipment. Furthermore, the QMRA framework should incorporate variants of concern and epidemics. This must be carried out to avert the exposure of plant personnel to viable SARS-CoV-2 and subsequent infection. Lastly, practical tactics for attenuating these risks must be developed.

Stringent safety and hygiene protocols should be applied in wastewater treatment facilities to reduce exposure to hazardous substances and infectious pathogens to alleviate potential health implications. All WWTP personnel are encouraged to practise good hygiene and wear protective gear such as safety boots, overalls, goggles, gloves, and face shields to protect them from micro-splashes, accidental ingestion (self-inoculation) and inhalation.

Furthermore, future research should develop methodologies for extracting viable SARS-CoV-2 from wastewater matrices as some facilities release effluent of poor quality, potentially disseminating the virions back into the communities. Henceforth, implementing effective disinfection and control efforts in wastewater management systems is vital for preserving public health by preventing the dissemination of the SARS-CoV-2 genome.

6.4 Conclusion

This study took the molecular route by utilising RT-qPCR and SNP assay genotyping to elucidate the incidence of the SARS-CoV-2 genome in the municipal wastewaters of BCMM. SARS-CoV-2 and the mutations linked to VOCs were detected in the municipal wastewaters of BCMM.

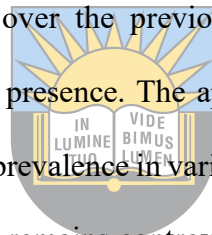
Prominent peaks appeared during the third epidemic (September 2021 yielding SARS CoV-2 viral load $> 8.5 \times 10^4$ GC/mL) driven by the highly transmissible Delta variant (B.1.617.2) and the fifth epidemic (April 2022 yielding a viral load $> 20 \times 10^4$ GC/mL) driven by Omicron lineages, BA.4 and BA.5. The spatiotemporal distributions of the diverse SARS-CoV-2 genome affirmed the correlation between COVID-19 cases of infection and SARS-CoV-2 markers in wastewater, indicating the influence of variants the temporal dynamics of SARS-CoV-2 infections. Additionally, in the course of the study, correlations were observed between the chronological dynamics of SARS-CoV-2 and its variants, and the lifting of National and Provincial restriction measures. This can be caused by various aspects such as basic human behavior associated with freedom of movement and a false sense of safety.

Furthermore, the occupational risk of potential exposure to SARS-CoV-2 was estimated using QMRA. The inception of variants and seasonal changes and are among numerous contributors to the resurgence of epidemics (due to host susceptibility, amplified transmission rate, the stability of the virus in winter etc) and the potential risks of infection in occupational settings. The overall findings of this study signify the essence of wastewater surveillance and the potential health risks in occupational settings as the aim of the study was achieved through the objectives.

The study, therefore, compels the execution of meticulous policies in occupational settings to ensure the safety of wastewater treatment plant workers and mitigate the rapid dissemination

of significantly infectious pathogens, such as SARS-CoV-2. This tool must be used as a complementary approach for epidemiological studies. Overall, some limitations to the model that prompted further research in this area were recognised. QMRA entails the incorporation of SARS-CoV-2 concentrations. The viral load may vary for each plant depending on population dynamics and many in-sewer factors. This study advocates the significance of continued monitoring of potential viral incidences in the wastewater milieu and an intensive multi-faceted risk assessment framework to alleviate the potential occupational health hazards associated with the transmission of microbial pathogens in the environment.

In a nutshell, wastewater surveillance is critical in monitoring the pervasiveness of SARS-CoV-2 and its variants in various communities. Reports have presented how wastewater-based epidemiology has hastily expanded over the previous years to its aptitude to provide a comprehensive measurement of viral presence. The approach is suitable for comprehending the dynamics of SARS-CoV-2 and its prevalence in various populations. Although the presence of viable SARS-CoV-2 in wastewater remains controversial, wastewater-based epidemiology is invaluable in tracking virus proliferation in communities.



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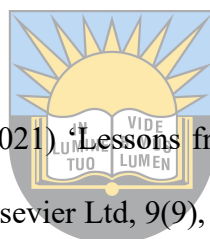
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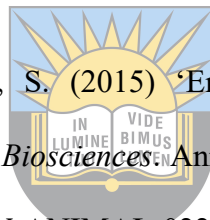
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7. Annextures to “Incidence and diversity of SARS-CoV-2 from wastewater samples recovered from selected municipal wastewater treatment facilities in Buffalo City Metropolitan Municipality, Eastern Cape Province, South Africa”

Annexture A: Amplification plot from the optimisation of SARS-CoV-2 detection by RT-qPCR

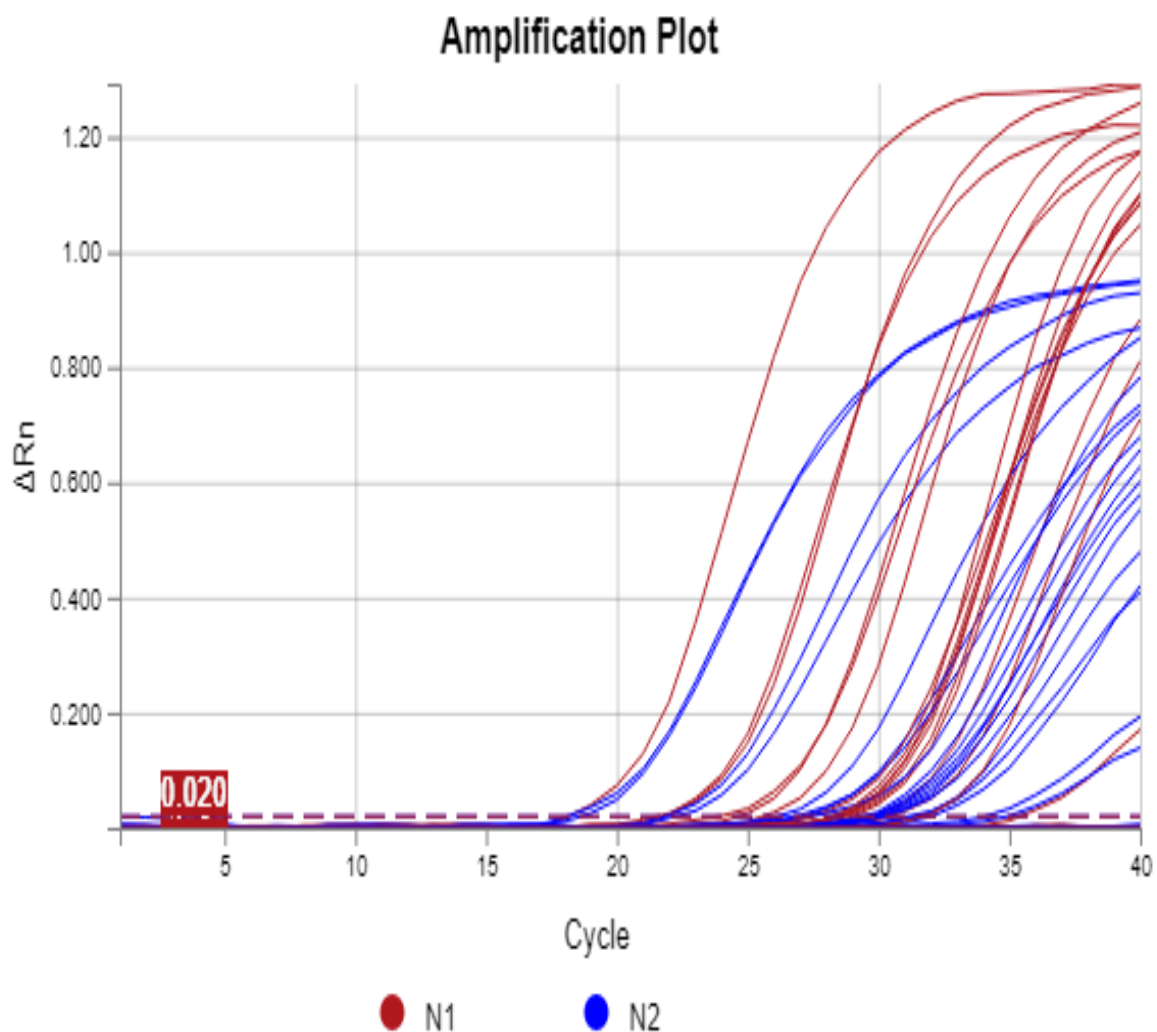
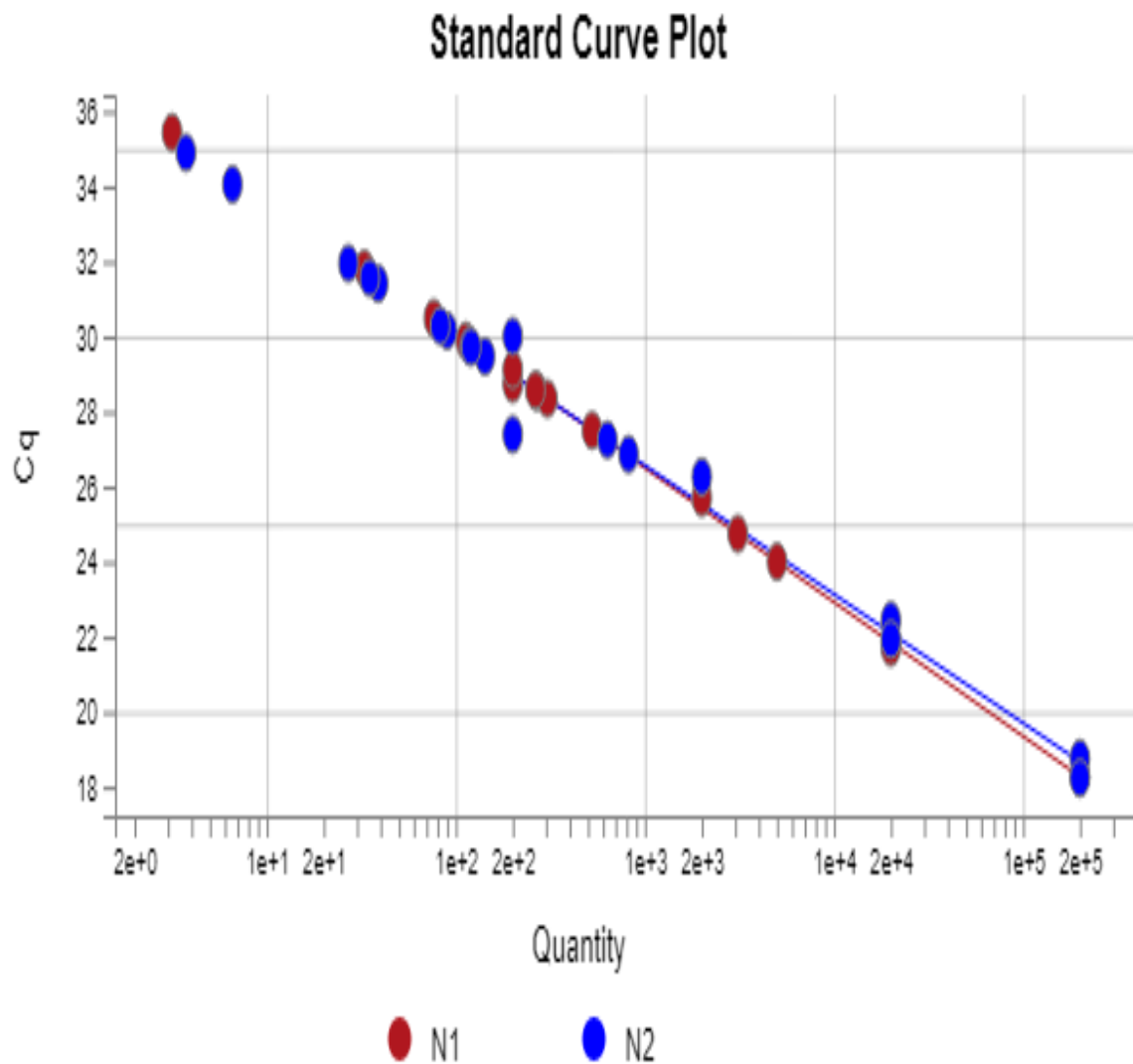


Figure 7.1: Amplification plot from the optimisation of SARS-CoV-2 detection by RT-qPCR

Annexture B: Standard curve generated from the optimisation of SARS-CoV-2 detection by RT-qPCR



Target: N1 Slope: -3.575 R^2 : 0.998 Y-Inter: 37.219 Eff%: 90.427 Error: 0.078

Target: N2 Slope: -3.432 R^2 : 0.963 Y-Inter: 36.858 Eff%: 95.608 Error: 0.302

Figure 7.2: Standard curve generated from the optimisation of SARS-CoV-2 detection by RT-qPCR

Annexure C:

Table 7.1: COVID-19 wave periods by weekly incidence and estimated number of cases observed in the Eastern Cape Province.

Wave	Peak date	Estimated number of cases	Weekly cases per 100,000 persons	Predominant lineage/variant	References
1st	June 2020	≈ 199,400	37.0 - 40.7	1.1.54, B.1.1.56 & C.1	(NICD, 2020, 2021; WHO, 2020,2021)
2nd	Dec 2020	≈ 170,687	31.1 - 41.5	Beta (B.1.351)	
3rd	July 2021	≈ 220,568	37.3 - 46.5	Delta (B.1.617.2)	

Annexure D:

Table 7.2: Tabular representation of the VOCs that emerged throughout the pandemic and their origins. The key spike protein mutations are shared among the variants, with *N501Y* being the most common amino acid alteration.

Variant of concern	Origin	Signature spike protein mutations
Alpha (B.1.1.7)	United Kingdom, Sep 2020	<i>N501Y, P681H, delH69V70</i>
Beta (B.1.33, B.1.351)	South Africa, Dec 2020	<i>N501Y, E484K, K417N, L452R</i>
Gamma (P.1)	Brazil, Jan 2021	<i>N501Y, E484K, K417N</i>
Delta (B.1.617.0-2)	India, May 2021	<i>L452R, P681R,</i>
Omicron (B.1.1.529) and subvariants	Botswana, Nov 2021	<i>delH69V70, N501Y, P681H, K417N</i>