SARS-CoV-2 infectivity potential in municipal wastewater: Implications for public health & water treatment

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Abstract

The COVID-19 pandemic has emphasized the importance of wastewater surveillance to monitor the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Increased surveillance requires attention to municipal workers who collect and handle samples at treatment facilities. While SARS-CoV-2 is primarily transmitted through respiratory droplets, evidence suggests virus can be transmitted via fecal-oral route and contaminated environmental samples, including municipal wastewater. This project investigated SARS-CoV-2 infectivity in wastewater and assessed potential risks to municipal workers and all those in contact with wastewater. Vero E6 infectivity assays determined inactivation kinetics of SARS-CoV-2 in time and temperature-based assays, aerosolization assays, and UV disinfection studies. Stability in infectivity was observed in 23, 15 and 4°C spiked samples with longer viral survival observed at colder temperatures. SARS-CoV-2 in wastewater aerosols was infectious directly to Vero E6 cells and also maintained infectivity in aerosolized droplets settling on various materials such as plastic and stainless-steel. The log reduction kinetics were also defined using collimated-beam UV irradiations to determine the optimal UV dosage for viral inactivation. Understanding SARS-CoV-2 infectivity in the wastewater treatment process is essential to inform public health by providing crucial information on risks associated with handling wastewater to prevent community spread.

Keywords

Coronavirus disease 19 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), wastewater-based epidemiology (WBE), wastewater surveillance (WWS), infectivity, inactivation kinetics, aerosolization, ultraviolet (UV), collimated-beam.
Summary for Lay Audience

The coronavirus disease 19 (COVID-19) pandemic has brought to light the importance of monitoring wastewater for the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Wastewater surveillance is an effective way to track the virus in communities since it can detect traces of the virus in fecal matter even before individuals show symptoms. This can help public health officials identify outbreaks and implement measures to prevent further spread. However, this increased surveillance comes with its own set of challenges. Municipal workers who collect and handle samples at treatment facilities are at risk of exposure to the virus. While the primary mode of transmission of the virus is through respiratory droplets, evidence suggests that the virus can also be transmitted through the fecal-oral route and contaminated environmental samples, including wastewater.

To better understand the risks associated with handling wastewater, this project was conducted to investigate the infectivity of SARS-CoV-2 in wastewater. The project used Vero E6 cell infectivity assays to determine the inactivation kinetics of the virus under different conditions such as time and temperature-based studies, UV disinfection, and aerosolization assays. Results from these assays found that there were differences in the inactivation of the virus in samples spiked with SARS-CoV-2 held at different temperatures including 23, 15, and 4°C. The project found that infectious virus can be carried in wastewater aerosols in material experiments with plastic and stainless-steel, as well as direct sprays onto Vero E6 cells which means that workers who handle wastewater need to take extra precautions at the treatment facilities to prevent exposure. The log reduction kinetics were also defined using collimated-beam UV irradiations, where the amount of UV exposure required to inactivate the virus was identified.

Understanding SARS-CoV-2 infectivity in the wastewater treatment process is crucial to inform public health and provide information on the risks associated with handling wastewater. This information can help prevent community spread and ensure the safety of workers who are on the front lines of handling wastewater. It is essential to continue research in this area to further develop guidelines and protocols to minimize the risks associated with handling wastewater.
First, I would like to express my heartfelt gratitude to my supervisor, Dr. Eric Arts. Through my time in your lab as a research technician at the onset of the COVID-19 pandemic and throughout my masters, your continuous support and unwavering belief in my research have been invaluable. The seamless transition from HIV research to SARS-CoV-2 research was pivotal for public health, and it highlighted your impactful contributions that extend far beyond the confines of academic papers, leaving a lasting imprint on the community. Your collaborative approach, fostering connections between academia, industry, and government, has emphasized how collective support can yield amazing results. I am profoundly grateful for the numerous opportunities you have provided me, as they have played a pivotal role in shaping me into a better researcher.

I would also like to express my appreciation to my advisory committee members, Dr. Ryan Troyer and Dr. Stephen Barr, for their invaluable encouragement and insightful guidance in the field of SARS-CoV-2. The Troyer and Barr labs played crucial roles in Western's response to the COVID-19 pandemic, conducting research on SARS-CoV-2. Their expertise in virology greatly influenced the design of my research objectives, and I am grateful for their contribution to my work.

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<td>Angiotensin converting enzyme 2</td>
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<td>CPE</td>
<td>cytopathic effect</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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Chapter 1

1 Introduction

1.1 Research Focus

The COVID-19 pandemic has underscored the need to understand the transmission dynamics of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). While respiratory droplets are considered the primary mode of transmission, alternative routes, such as the fecal-oral route, are being investigated. This raises concerns about the presence of SARS-CoV-2 in municipal wastewater and its potential infectivity, necessitating further examination to safeguard public health and optimize water treatment practices.

Studying SARS-CoV-2 in municipal wastewater has gained significant attention due to its implications for public health. Wastewater can reflect the health status of a community by capturing the collective viral load shed by infected individuals. Monitoring SARS-CoV-2 in wastewater offers valuable epidemiological data and insights into the virus's spread within a population. Assessing the infectivity of SARS-CoV-2 in wastewater is vital for evaluating risks, especially for wastewater treatment workers and individuals near treatment facilities.

Apart from public health concerns, the presence of SARS-CoV-2 in municipal wastewater presents challenges for water treatment plants. Conventional treatment processes are designed to remove organic matter and pathogens, but the virus may survive in the presence of organic matter and biofilms. Concerns also arise from potential aerosolization of the virus during treatment, posing respiratory exposure risks. Given the unique characteristics of SARS-CoV-2, including its infectivity, stability, and environmental resistance, current water treatment strategies need re-evaluation. Effective removal or inactivation of SARS-CoV-2 during water treatment is essential to prevent potential transmission through treated wastewater intended for reuse or environmental discharge.
This thesis aims to contribute to the research on wastewater-based epidemiology (WBE) by examining the persistence and infectivity potential of SARS-CoV-2 in wastewater. It seeks to understand the implications for public health and water treatment, identify challenges, and propose mitigation strategies. By doing so, it provides guidance for future research and strategies to effectively manage the risks associated with SARS-CoV-2 and other novel viruses in municipal wastewater.

1.2 COVID-19 and SARS-CoV-2

1.2.1 COVID-19 Pandemic Overview

The emergence of the COVID-19 pandemic in late 2019 has resulted in an unprecedented public health crisis with the rapid spread of SARS-CoV-2, causing substantial morbidity and mortality worldwide\(^1\). COVID-19 is characterized by a wide range of symptoms, ranging from mild respiratory symptoms to severe pneumonia and multiorgan failure with the potential for long-term negative health consequences\(^2\). The disease has strained healthcare systems, disrupted economies, and profoundly affected daily life for individuals in every corner of the world\(^3\).

The pandemic's rapid progression can be attributed to the highly contagious nature of SARS-CoV-2 and its continuous evolution\(^4\). Human-to-human transmission occurs primarily through respiratory droplets expelled when an infected individual coughs, sneezes, talks, or breathes heavily\(^5\). These droplets can be inhaled by individuals in close proximity or deposited on surfaces, facilitating indirect transmission\(^6\). The virus can maintain stability on different surfaces depending on material, temperature, and time exposure which further contributes to its persistence and spread\(^7-9\).

Furthermore, the asymptomatic and presymptomatic transmission of SARS-CoV-2 has presented challenges in controlling its dissemination\(^10,11\). Infected individuals who show no symptoms or have not yet developed symptoms can unknowingly transmit the virus to others, making it difficult to identify and isolate all potential carriers\(^12\). With up to one third of infections estimated as being asymptomatic this feature has contributed to the
rapid community transmission observed, making effective containment strategies a considerable challenge\textsuperscript{25}.

Efforts to control the pandemic have focused on a combination of non-pharmaceutical interventions, such as social distancing, mask-wearing, hand hygiene, and vaccination campaigns\textsuperscript{1,26,27}. Additionally, extensive testing and contact tracing have been crucial in identifying and isolating infected individuals to prevent further transmission\textsuperscript{28}. The development and distribution of effective vaccines have provided an effective strategy in managing the pandemic, but ongoing research is necessary to understand the virus and its variants to ensure continued protection and effective response measures\textsuperscript{29,30}.

The COVID-19 pandemic has had a profound impact on global health, economies, and societies. Understanding the epidemiology, transmission dynamics, and characteristics of SARS-CoV-2 is crucial for formulating effective strategies to reduce its spread and minimize the impact of the disease.

### 1.2.2 SARS-CoV-2 pathophysiology

SARS-CoV-2 is a novel coronavirus that belongs to the *Coronaviridae* family, which includes other well-known human pathogens like SARS-CoV and MERS-CoV\textsuperscript{31}. The virus has a positive-sense single-stranded RNA genome and is characterized by its distinctive spike (S) protein which plays a crucial role in binding to host cell receptors, facilitating viral entry, and initiating infection\textsuperscript{32}.

The genetic sequence of SARS-CoV-2 has been extensively studied, with phylogenetic analysis revealing its close resemblance to bat coronaviruses, particularly SARS-related coronavirus (SARSr-CoV) RmYN02 found in Malayan horseshoe bats (*Rhinolophus malayanus*) sharing 93.3\% genetic identity to SARS-CoV-2\textsuperscript{33–35}. This similarity suggests a zoonotic origin, where SARSr-CoVs have been identified in potential intermediate animal host such as Chinese pangolins (*Manis pentadactyla*) facilitating transmission to humans\textsuperscript{36}. The high genetic similarity between SARS-CoV-2 and bat coronaviruses indicates that the virus likely originated from a natural spillover event\textsuperscript{37}.
SARS-CoV-2 primarily infects respiratory epithelial cells, targeting the angiotensin-converting enzyme 2 (ACE2) receptor on host cells\(^3\). The binding of the viral spike protein to ACE2 facilitates viral entry and subsequent replication within the host. The virus's ability to infect and replicate in the respiratory tract contributes to its efficient human-to-human transmission through respiratory droplets\(^3\) (Figure 1-1).

Figure 1-1-1. SARS-CoV-2 pathophysiology. (1) SARS-CoV-2 is a coronavirus with a crown-like structure created by surface (S) proteins. (2) Cell entry is dependent on viral S protein subunit 1 (s1) binding to ACE2 receptor on target cell surface and priming facilitated by serine protease TMPRSS2\(^3\). (3) SARS-CoV-2 infection of ACE2 expressing cells in the respiratory tract including the sinonasal epithelium and alveolar epithelium\(^4\). (4) Primary transmission of SARS-CoV-2 occurs via the oral-nasal route
when infected individual release respiratory droplets through coughing or sneezing\textsuperscript{1,39}. The clinical presentation of SARS-CoV-2 infections varies widely, with some individuals experiencing mild symptoms or remaining asymptomatic, while others develop severe respiratory distress and multiorgan failure\textsuperscript{24,41}. Risk factors for severe disease include advanced age, underlying health conditions, and compromised immune systems\textsuperscript{42,43}. The mechanisms underlying the varying clinical outcomes of SARS-CoV-2 infection are still being elucidated, and ongoing research is focused on understanding the host-virus interactions between new SARS-CoV-2 variants and immune responses\textsuperscript{44}.

Though a comprehensive understanding of the characteristics of SARS-CoV-2, including its genetic makeup, viral structure, and mechanisms of infection, insights can be made into the virus's behaviour and its potential to persist and spread within different environments. This knowledge is vital in assessing the presence and infectivity potential of SARS-CoV-2, contributing to the development of effective strategies for monitoring and controlling the spread of the virus.

### 1.2.3 SARS-CoV-2 transmission routes

The transmission routes of SARS-CoV-2 play a crucial role in understanding its spread and designing effective control measures. Respiratory droplets generated when an infected person coughs, sneezes, talks, or breathes heavily are considered the primary mode of transmission. These droplets, varying from 1 to 2000\(\mu\)m, carry viral particles and can be directly inhaled by individuals in close proximity, leading to infection\textsuperscript{45}. Respiratory droplets released from infected individuals can also result in the generation of aerosols containing SARS-CoV-2 as well as deposits of virus on contact materials\textsuperscript{46}. The virus can persist both in the air on inanimate surfaces without losing its infectivity depending on environmental conditions such as temperature\textsuperscript{47}. Water plays a vital role in the longevity of SARS-CoV-2 considering that a lipid enveloped virion such as SARS-CoV-2 is almost immediately inactivated in a few seconds in the absence of a water layer and dehydration\textsuperscript{48}. Regardless of the definition of
airborne virus, this mode of transmission highlights the implementation of social distancing measures and wearing masks when sick to mitigate the risk of spreading respiratory droplet transmission.

Mounting evidence suggests that SARS-CoV-2 transmission can also occur through other routes, including the fecal-oral route with studies showing the gastrointestinal (GI) tract can be an alternative route for SARS-CoV-2 infection in Rhesus macaques (Macaca mulatta)⁴⁹. Viral RNA can readily be detected in stool samples from infected individuals, indicating the potential for fecal shedding of the virus from the GI tract in patients with no association to the presence of GI symptoms or severity of illness⁵⁰. These findings also raise concerns regarding the possibility of transmission through the fecal-respiratory route, particularly in settings with poor ventilation, sanitation or inadequate hygiene practices⁵¹,⁵². The presence of viral RNA in wastewater further supports the notion that the fecal-oral route may contribute to the dissemination of the virus.

It is worth noting that the detection of viral RNA in stool samples does not necessarily imply the presence of infectious virus particles. The CDC reports that infectious SARS-CoV-2 can be isolated in 2 of 3 stool samples indicating infectious virus in feces may be common manifestation for COVID-19 patients⁵⁰. Viable clinical stool samples also have resulted in infection in ferrets after intranasal inoculation⁵³. While infective SARS-CoV-2 can be isolated for patient stool samples, further research is required to address the duration of viral infectivity in stool samples, and the viability of the virus as it enters the wastewater system⁵⁴.

Understanding the various transmission routes of SARS-CoV-2 is crucial for implementing targeted interventions and control strategies. Efforts should be directed not only towards respiratory droplet protection, but also towards promoting proper sanitization of contact surfaces and hygiene practices to minimize the risk of other routes including fecal-oral transmission⁵⁵. Knowledge of the transmission routes helps public health authorities tailor their recommendations and guidelines to effectively reduce the spread of the virus in diverse settings, including households, healthcare facilities, and community spaces⁵⁶–⁵⁸.
1.2.4 SARS-CoV-2 viral shedding in feces and presence in wastewater

The detection of viral RNA in wastewater serves as a valuable indicator for monitoring the circulation and potential hotspots of the virus within a community\((59)\). WBE, coupled with clinical data, can provide valuable insights into the overall prevalence and transmission dynamics of SARS-CoV-2 infection within a population\((60)\).

The key factor contributing to the presence of SARS-CoV-2 in wastewater is viral shedding by infected individuals resulting in the release and excretion of viral particles\((61)\). In the case of SARS-CoV-2, viral shedding can occur through various body fluids, including saliva, urine and stool\((62)\). While normally shed through the respiratory tract, the multiorgan tropism of SARS-CoV-2 S protein allows it to infect and replicate in other bodily tissues and organs expressing the ACE2 receptor, such as the small intestine\((63)\) (Figure 1-2).
Figure 1-2. SARS-CoV-2 multiorgan tropism and replication in the intestine. (1) Although the main target of SARS-CoV-2 is the respiratory tract, there are many other ACE2-expressing cells in tissues and organs such as the brain, heart, liver, and vasculature which are permissive to infection\(^63\) as well as (2) the small intestine which also expresses ACE2 supporting SARS-CoV-2 infection that can result in observable GI symptoms. (3) SARS-CoV-2 infects ACE2-expressing cells in the intestinal epithelium. (4) SARS-CoV-2 replication in intestinal epithelium can release virions into the intestinal lumen, resulting in excretion of intact virions in feces\(^64\). Generated with BioRender.com

In infected individuals, viral shedding in the feces can be detected within the first week post infection\(^65\), even before symptoms manifest, making asymptomatic and presymptomatic individuals’ potential sources of viral transmission\(^66\). The duration and magnitude of viral shedding can vary among individuals and may depend on factors such as the severity of illness and immune response with RNA detected up to 7 months post diagnosis, suggesting prolonged GI infection is possible in severe cases\(^65\). Understanding the patterns and dynamics of viral shedding is crucial for assessing the potential presence of SARS-CoV-2 in wastewater.

Quantitative analysis and detection methods, such as reverse transcription-quantitative polymerase chain reaction (RT-qPCR), are employed to determine the viral load and RNA concentration of SARS-CoV-2 shed in wastewater samples\(^67\). These methods provide valuable insights into the relative abundance and trends of SARS-CoV-2 in wastewater, allowing for the estimation of community-wide viral prevalence\(^68\). However, it is important to note that these methods have limitations, including limits of detection, environmental context at sampling sites including rainfall and inlet flow rate, and challenges in quantifying the exact number of infectious viral particles present depending on the assay parameters set by different research groups\(^69–71\). It is important to note that infectious viral particles in wastewater is a small component of the SARS-CoV-2 content, including inactivated virus and viral remnants, measured by RT-qPCR.

The presence of SARS-CoV-2 shed in wastewater samples validates the need for further investigation into the infectivity potential of the virus. Wastewater monitoring can
provide valuable information on the circulation and trends of SARS-CoV-2 within communities, helping public health authorities make informed decisions and implement timely interventions to limit the spread of COVID-19.

1.3 Wastewater-based Epidemiology

1.3.1 WBE as a surveillance tool

WBE has emerged as a powerful and innovative tool for disease surveillance, offering the potential for early warning of infectious disease outbreaks and monitoring public health at a community level. By analyzing wastewater samples, insights can be gained into the overall health status of a population, regardless of whether individuals show symptoms or seek clinical testing. This approach enables the detection and monitoring of infectious diseases, including those with asymptomatic or mild cases that may go unnoticed in traditional surveillance methods. The municipal wastewater system acts as a repository of biological and chemical markers that reflect the health conditions and activities of the served population. The analysis of wastewater samples can provide valuable information about the presence and concentration of specific markers, such as viral genetic material, drug metabolites, or specific disease biomarkers.

WBE has several advantages over traditional surveillance methods. Firstly, it offers population-scale monitoring, providing a broader perspective on disease prevalence and trends within a community. Secondly, WBE offers a non-invasive and cost-effective approach to disease surveillance by regularly collecting from wastewater treatment plants (WWTPs) or strategic sampling points within the sewerage system. This non-invasive nature simplifies the data collection process and reduces the associated costs, making WBE a scalable and sustainable surveillance tool. Furthermore, WBE has the potential for early detection and monitoring of disease outbreaks. By analyzing wastewater samples at regular intervals, trends can be identified in changes in pathogen concentration, providing early warning signals for the emergence or re-emergence of infectious diseases within a community. This early detection can in turn facilitate
timely public health responses, including targeted testing, contact tracing, and the implementation of control measures to limit the spread of the disease.

WBE has been successfully applied in the surveillance of various infectious diseases, demonstrating its versatility and effectiveness in tracking and providing early warnings for potential outbreaks. WBE has been used to track the presence of the poliovirus in wastewater, providing valuable information to aid in the eradication of polio from the population. Similarly, WBE has been employed for monitoring the prevalence of norovirus, a common cause of gastrointestinal illness, by detecting viral RNA in wastewater samples. WBE is also regularly used for routine monitoring of community levels of hepatitis A which can inform local vaccination efforts.

WBE represents a valuable and complementary approach to traditional disease surveillance methods. By analyzing wastewater samples, it offers population-scale monitoring, non-invasive data collection, and the potential for early detection of disease outbreaks. As the field of WBE continues to advance, it holds great promise for enhancing our understanding of disease dynamics, improving public health responses, and ultimately safeguarding community well-being.

1.3.2 Advantages and challenges of WBE for SARS-CoV-2 surveillance

The application of WBE for wastewater surveillance (WWS) of SARS-CoV-2 has garnered considerable attention as a complementary approach to traditional testing. While WWS offers several advantages in monitoring the presence and trends of the virus within a community, it also presents certain challenges that need to be addressed for effective monitoring. One of the major advantages of using WWS of SARS-CoV-2 surveillance is its ability to detect trends in viral shedding earlier than clinical data. Wastewater trends can precede clinical cases by 4–10 days, offering advanced notice of disease transmission.

Another advantage is the ability to provide population-level data on the spread of new and emerging variants. WWS can capture both viral load and sequence information...
from a broad spectrum of individuals, enabling the monitoring of viral evolution in the population over time\textsuperscript{85,86}. This population-scale monitoring offers an all-inclusive understanding of the viral prevalence and transmission dynamics of SARS-CoV-2 variants within a community. It can help identify outbreaks of new variants of concern (VOC), including the rapid transition between Delta and Omicron waves observed here in Canada in January 2022\textsuperscript{87}. WBE surveillance efforts have more recently aided in tracking the evolution of Omicron sublineages including BQ.1, BQ1.1, BA2.75 and XBB.1 as these VOC overtook the previously dominant BA.4 and BA.5 lineages\textsuperscript{88}.

However, implementing WWS for SARS-CoV-2 comes with certain challenges. One significant challenge is the need for sensitive detection methods to accurately identify and quantify viral genetic material in wastewater\textsuperscript{89}. The presence of inhibitors and low viral concentrations in wastewater samples can affect the sensitivity and reliability of the detection process\textsuperscript{90}. Developing standardized methods for viral RNA extraction, amplification, and quantification is essential to ensure accurate and consistent reporting across different sampling locations\textsuperscript{69,91}.

Interpreting WBE data in the context of actual infection rates poses another challenge. While the detection of viral RNA in wastewater does correlate with the number of infected individuals, it gives no insight into the infectious potential of the virus and its current circulating VOC. While WWS data can act as an indicator of VOC virulence when compared to hospitalizations in limited clinical testing cases, factors, such as infectivity, transmissibility, and pathogenicity are difficult to obtain from wastewater\textsuperscript{92}.

Viral shedding patterns, decay rates, and dilution within the wastewater system can also influence the concentration of viral genetic material in samples\textsuperscript{93}. Therefore, it is important to establish correlations between the viral RNA levels in wastewater and the epidemiological data from clinical surveillance to estimate the true prevalence of SARS-CoV-2 infections accurately.
Our work on the wastewater surveillance initiative in London, Ontario, in collaboration with the Ministry of the Environment, Conservation and Parks' (MECP) WWS initiative, yielded highly promising outcomes for public health surveillance. The wastewater-based approach demonstrated its effectiveness in detecting and monitoring viral load values of SARS-CoV-2 in the community. Building on the success of this innovative approach, the ministry made a significant decision to transition from reporting COVID-19 case numbers to exclusively reporting viral load values in early 2022. This shift reflects the recognition of the robustness and reliability of wastewater surveillance data in providing real-time information about the prevalence of the virus in the community.

Figure 1-3. London, Ontario COVID-19 wastewater vs. case data. Viral load sampling result are compiled from all five wastewater treatment plants in London, Ontario three times per week. All samples are 24-hour composites of the raw wastewater entering the plants. The results shown are weighted averages from all plants, weighted by the population of the plant catchment areas. The viral load of SARS-CoV-2 is normalized by the viral load of pepper mild mottle virus (PMMoV), which is a fecal indicator that results from human consumption of peppers and pepper products. Case data is no longer being updated due to new testing guidelines severely underestimated true case counts. Figure used from 519covid.ca
WBE holds great promise as a surveillance tool for SARS-CoV-2. It offers population-level monitoring, non-invasive data collection, and the potential for early detection of outbreaks. However, addressing challenges related to sensitivity of detection methods, data interpretation, and standardization of protocols is crucial for realizing the full potential of WBE in monitoring SARS-CoV-2. Regarding the infectivity potential of the virus in wastewater, there are still many unknowns. Sample preparation from wastewater plays a large role in retaining potentially infectious virus from municipal samples. Pretreatment and concentration steps may result in turning SARS-CoV-2 non-viable meaning that if RNA is detected through WWS, infectivity cannot be ruled out.

1.3.3 Potential risks associated with SARS-CoV-2 transmission through wastewater

Understanding the potential risks associated with SARS-CoV-2 transmission through wastewater is of paramount importance for public health and effective disease control strategies. While the detection of viral RNA in wastewater does not necessarily indicate the presence of infectious virus particles, there is evidence to suggest that SARS-CoV-2 can remain viable in wastewater and river outlets. Therefore, it is crucial to investigate the infectivity potential of the virus in wastewater and the potential for transmission through environmental exposure pathways.

One potential risk is the accidental release of infectious SARS-CoV-2 particles from wastewater treatment facilities into the environment. Wastewater treatment processes are designed primarily to remove pollutants and pathogens through activated sludge, but they may not completely eliminate all infectious agents in downstream treatment stages. Monitoring treatment plants at their entry and exit points reveal that “treated” wastewater may still contain infectious human viruses including hepatitis A, rotavirus, and enterovirus which can represent a threat to public health. Inadequate treatment or mechanical failures in wastewater infrastructure could lead to the discharge of viable SARS-CoV-2 particles into surface waters or the contamination of soil, posing a risk to reverse zoonosis events like that observed in white-tailed deer (Odocoileus virginianus).
Additionally, the presence of SARS-CoV-2 in wastewater may facilitate the transmission of the virus to individuals who come into contact with contaminated water sources. This could occur through interaction with receptor water bodies, or the use of untreated wastewater for irrigation in agricultural settings where agricultural irrigation accounts for 70% of water use worldwide\textsuperscript{100}.

Another concern is the potential for indirect transmission of SARS-CoV-2 through exposure to contaminated aerosols generated during the handling of wastewater or sewage\textsuperscript{11,101}. Wastewater workers, including those involved in sample collection, treatment plant operation, and maintenance, may be at an increased risk of exposure to the virus\textsuperscript{101}. Adequate personal protective equipment (PPE), hygiene measures, and proper training are crucial to mitigate occupational risks and prevent the transmission of the virus to wastewater workers and the wider community\textsuperscript{102}.

Transmission directly from fecal material from toilets and bathrooms typically not factored as a route for infection by respiratory pathogens and is not well understood. As described earlier, infection with certain respiratory viruses can result in active replication in the GI tract and high viral loads in fecal material. Public bathrooms with poor ventilation can result in five times the normal levels of fecal microbes in areas including the toilet bowl, sinks, and walls related to aerosolized fecal material due to flushing, and contaminated hands\textsuperscript{103}. While this thesis provides analyses of the lowest frequency event for potential of SARS-CoV-2 transmission through diluted virus in wastewater future studies can explore the possibilities of transmission in bathrooms especially those accessed by the public. By addressing these risks and enhancing our knowledge of the infectivity potential of SARS-CoV-2 in wastewater, we can develop effective strategies to assess viral infectivity in different environments.

1.4 Persistence and survival of SARS-CoV-2 in wastewater

1.4.1 Environmental factors affecting SARS-CoV-2 in wastewater

Several factors can influence SARS-CoV-2 longevity in wastewater, highlighting the dynamic nature of viral behaviour in this environment. Temperature plays a critical role,
as studies have shown that higher temperatures can accelerate the decay of the virus in wastewater\textsuperscript{104}. SARS-CoV-2 exhibits reduced viability at elevated temperatures, suggesting that warmer wastewater systems may be less conducive to viral survival. Awareness of the impact of temperature on viral persistence can inform wastewater treatment strategies and the design of systems that maximize viral inactivation.

Another important factor is pH, which plays a role in the stability of SARS-CoV-2 and its molecular interactions\textsuperscript{105}. The virus is generally more stable at pH values ranging from 6 to 9, indicating that the conditions present in wastewater might enhance viral survival\textsuperscript{104,106}. This finding underscores the potential for viral persistence in wastewater with lower pH levels, necessitating the consideration of pH adjustment strategies during wastewater treatment processes\textsuperscript{106}.

The interaction between SARS-CoV-2 and disinfectants or other chemical treatments such as chlorination in wastewater can impact the virus's viability\textsuperscript{107}. Disinfection processes employed during wastewater treatment can vary, and understanding their effectiveness in inactivating SARS-CoV-2 is crucial for ensuring safe handling and management of wastewater\textsuperscript{108}. The presence of microorganisms in wastewater can also influence the persistence of SARS-CoV-2. The ability of microbial activity to affect viral decay rates has been utilized in certain disinfection protocols, where micro-algae mediated approaches have been implemented to disinfect SARS-CoV-2\textsuperscript{109}.

Ultraviolet (UV) exposure is an additional factor that can affect viral persistence in wastewater\textsuperscript{108}. UV radiation present in sunlight has been shown to have a demobilizing effect on viruses, including SARS-CoV-2\textsuperscript{110}. Exposure to UV light can lead to the inactivation of SARS-CoV-2 in wastewater, reducing the risk of transmission. Incorporating appropriate UV disinfection systems or optimizing sunlight exposure during wastewater treatment processes can help mitigate the potential for viral persistence.

It is important to note that the interaction of these factors is complex, and their influence on viral persistence may vary depending on the specific environmental conditions\textsuperscript{111}. Therefore, comprehensive studies are needed to investigate the combined effects of
temperature, pH, microorganisms, disinfectants, and UV exposure to better understand and manage the persistence and survival of SARS-CoV-2 in wastewater systems. Such knowledge will contribute to the development of targeted strategies for wastewater treatment and disinfection to minimize the potential risks associated with the presence of the virus.

1.4.3 Infectious virus persistence in wastewater

The persistence of SARS-CoV-2 in wastewater has significant implications for the infectivity potential of the virus and the associated risks to those in close contact with municipal wastewater such as treatment facility workers. Research findings regarding sick leave among wastewater workers compared to other professions due to waterborne pathogen exposure are limited. While wastewater workers do face potential exposure to pathogens present in water, the extent to which this exposure directly leads to increased sick leave is not well defined. One study suggest that wastewater workers might experience higher rates of illness compared to some other occupations based on significantly higher sick leave taken by WWTP workers\textsuperscript{112}. While this increase may be attributed to waterborne pathogens and the nature of their work environment, the specific contribution of waterborne pathogens to sick leave is challenging to quantify accurately due to the presence of other potential factors, such as workplace safety practices, personal protective equipment usage, and overall health conditions.

SARS-CoV-2 can persist in wastewater for varying durations, with studies reporting viral RNA detection ranging from several days to several weeks. In one report the average log\textsubscript{10} reduction time (T90) for viral RNA ranged from 8.04 to 27.8 days in untreated wastewater, 5.71 to 43.2 days in autoclave treated wastewater, and 9.40 to 58.6 days in normal tap water dependent on temperature\textsuperscript{113}. Wastewater facilities have been noted as a repository for SARS-CoV-2, where viral RNA can persist for over 19 days post the end of recorded local outbreaks\textsuperscript{114}. While the detection of viral RNA in wastewater indicates the presence of the genetic material of the virus, it does not provide direct evidence of viral infectivity. However, studies have shown that the presence of viable, infectious
SARS-CoV-2 particles can be detected in spiked wastewater samples, suggesting the potential for viral transmission through environmental exposure pathways\textsuperscript{115}.

The path and residence time SARS-CoV-2 takes to travel to the wastewater plant can vary in time based on distance, flow rate and environmental conditions\textsuperscript{116}. As water travels through the municipal wastewater system, there are a few main stages that may contribute to the potential contact and spread of the virus. It is important to assess any sites of contact workers may have with SARS-CoV-2 and optimize its inactivation and removal from the system before release from the treatment facility (Figure 1-4).

Figure 1-4. Path of SARS-CoV-2 in the municipal wastewater system. (1) Viral shed through feces enters the system. (2) Virus exposed to different temperature and retention time conditions on path to treatment facility. (3) Aerosolization can occur during aeration stages of water treatment\textsuperscript{117}. (4) Effluent wastewater is then treated for viral inactivation, either chemically or through UV irradiation. (5) Treated water is released from plant. Generated with BioRender.com.
Although direct transmission of SARS-CoV-2 through wastewater is considered to be low, indirect transmission through exposure to contaminated water sources or aerosols generated during wastewater handling cannot be disregarded\textsuperscript{11,118}. The survival of SARS-CoV-2 observed in spiked wastewater and river water experiments increases the likelihood of potential transmission routes, particularly in situations where there is inadequate treatment, accidental release, or environmental exposure to untreated or inadequately treated wastewater\textsuperscript{115}.

Furthermore, the persistence of SARS-CoV-2 in wastewater poses challenges for wastewater workers who may come into direct contact with the virus during their work. Adequate safety measures, including PPE and proper training, are essential to moderate the occupational risks faced by wastewater workers and prevent the potential transmission of the virus while there have been issues with compliance to PPE guidelines\textsuperscript{119}. Studying the persistence of SARS-CoV-2 in wastewater and its implications for infectivity can guide the development of effective protocols to protect wastewater workers and ensure their safety.

The persistence and infectivity potential of SARS-CoV-2 must be further assessed to determine the risks associated with environmental exposure and implementing appropriate workplace and public health measures. The potential for indirect transmission and occupational risks to wastewater workers who play a role in WWS cannot be ignored\textsuperscript{120}. The aid WWS in early detection and response highlights the significance of investigating the infectivity potential of the virus in wastewater as a safe and reliable tool. By enhancing our knowledge of viral persistence and infectivity, we can develop effective strategies to manage wastewater systems, and protect wastewater workers who help in routine sampling\textsuperscript{121}.

1.4.4 Challenges and limitations of evaluating viral infectivity in wastewater samples

The evaluation of viral infectivity in wastewater samples presents several challenges and limitations that need to be considered in order to interpret the results accurately and
effectively. While assessing viral infectivity is crucial for understanding the potential risks associated with wastewater transmission of SARS-CoV-2, certain factors can affect the reliability and interpretation of the data obtained.

One of the key challenges lies in establishing a direct link between the detection of viral RNA or antigens in wastewater and the actual infectivity of the virus. While molecular methods and immunological assays can detect viral genetic material or proteins, respectively, they do not provide a definitive indication of whether the virus is still capable of causing an infection. Efforts to quantify intact SARS-CoV-2 have shown that full virions can be detected in wastewater using a modified RT-qPCR approach. In general, RNA is very labile within environmental samples and prone to rapid degradation. Thus, the detection of SARS-CoV-2 RNA in wastewater by RT-qPCR suggests that this RNA remains protected by the viral capsid. Although the presence of SARS-CoV-2 capsid integrity does not necessarily equate to the presence of infectious virus, these results could improve the interpretation of positive RT-qPCR results obtained from WWS and provide greater context to community spread.

The challenge of determining infectivity in SARS-CoV-2 in wastewater samples is compounded by the time it takes to collect the samples and bring them for analysis in a laboratory setting. The infectivity of the virus can rapidly diminish over time, especially in the presence of wastewater. As a result, delays in sample collection and transportation can potentially lead to inaccurate assessments of viral infectivity. Spiked SARS-CoV-2 experiments can be a valuable alternative when attempting to isolate infectious virus from wastewater samples. One major advantage is the ability to control and standardize the concentration of the virus introduced into the experiment, allowing for more precise measurements and comparisons. By spiking known quantities of the virus into a defined volume of non-infectious wastewater, a controlled environment can be created to evaluate the performance of detection methods and assess the stability and persistence of SARS-CoV-2 under specific conditions. This approach eliminates the potential variability associated with collecting and analyzing actual wastewater samples, including variations in viral load, sample preservation, and transportation and processing times.
1.5 Rational and hypothesis

In summary, this thesis aims to develop a comprehensive understanding of the infectivity potential of SARS-CoV-2 in municipal wastewater. By investigating the persistence and survival of the virus under different time and temperature conditions, aerosolization sprays, and UV irradiations, this study will contribute to existing knowledge regarding the risks associated with SARS-CoV-2 transmission through wastewater. It is hypothesized that infectious SARS-CoV-2 persists in wastewater fluids during the wastewater treatment process and can be inactivated using an ultraviolet light-emitting collimated-beam apparatus. The findings of this research will look to better inform public health measures and guide the development of effective strategies to protect wastewater workers and ensure the safe management of wastewater systems exposed to SARS-CoV-2 and the COVID-19 pandemic as well as potential future outbreaks.
1.6 References


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65. Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection. doi:10.1016/j.medj.2022.04.001


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### 1.7 Foreword for Chapters 2, 3, & 4

The following chapters 2, 3, and 4, have been thoughtfully separated in this thesis to ensure clarity and contextual understanding. The collective focus of these chapters
revolves around investigating the impact of SARS-CoV-2 on different stages of the wastewater treatment process. Each chapter sheds light on critical aspects of this pressing topic, providing valuable insights into the virus's behaviour within the wastewater system.

The research presented in these chapters covers an array of infectivity assays conducted using spiked SARS-CoV-2 samples to assess its infectivity potential in wastewater. These experiments have been thoughtfully compiled, and summary figures have been prepared to represent the multitude of infections performed. By exploring the virus's interactions and responses at various stages of wastewater treatment, we strive to unveil vital information that can shape our understanding of the virus's persistence and transmission dynamics throughout the entirety of the wastewater treatment process.

As we merge the information contained in these chapters into a single manuscript, we look forward to fostering further dialogue and collaborations among researchers, public health experts, and wastewater management professionals. Together, we endeavor to develop effective strategies to reduce viral transmission through wastewater and safeguard the well-being of communities.
Chapter 2

2 Time and temperature-based infectivity of SARS-CoV-2 spiked municipal wastewater samples

This chapter focuses on infectivity of SARS-CoV-2 spiked in wastewater over different time and temperature conditions. The objective was to determine the inactivation kinetics of the virus under varying conditions that the virus may be exposed to in the municipal wastewater system during its retention time in the water as it travels towards treatment facilities.

2.1 Abstract

Given that SARS-CoV-2 is shed into fecal material and can enter the sewer system, it is crucial to comprehend the inactivation kinetics of the virus within the municipal wastewater system. The primary objective of this study is to determine the decay and inactivation kinetics of SARS-CoV-2 in wastewater under varying time and temperature conditions. By simulating the retention time of the virus in the water as it travels towards treatment facilities, the study aims to shed light on the viability and infectivity of SARS-CoV-2 during its journey through the municipal wastewater system. To achieve this, wastewater samples were spiked with known concentrations of SARS-CoV-2, mimicking the presence of the virus in fecal material. These samples were then subjected to controlled time intervals and different temperature conditions to assess the decay and inactivation rates of the virus. By analyzing the time and temperature-based infectivity of SARS-CoV-2 in wastewater, this research contributes to our knowledge of the persistence and survival of the virus within the municipal wastewater system. The findings will help identify the factors that influence the infectivity of SARS-CoV-2 in wastewater, aiding in the development of strategies to mitigate the potential risks associated with its transmission.
2.2 Introduction

SARS-CoV-2 viral RNA can readily be detected in stool samples, indicating active viral replication in the GI tract\textsuperscript{123}. This sheds light on an additional potential transmission through the fecal-oral route and highlights the potential role of wastewater as a reservoir for the virus\textsuperscript{124}. The mechanisms of viral shedding and dissemination into the sewer system are multifaceted. Infected individuals can shed SARS-CoV-2 in their stool even in the absence of respiratory or gastrointestinal symptoms, making it difficult to identify cases solely based on clinical cases\textsuperscript{125,126}. The virus is believed to enter the sewer system primarily through human waste, as well as through wastewater from hospitals and healthcare facilities where infected patients are treated, with viral titres as high as $10^6$ copies per L\textsuperscript{127}. Additionally, surface runoff and stormwater can contribute to the rate at which the virus travels through the sewer system\textsuperscript{128}.

The retention time of wastewater in the sewage system is influenced by various factors that can impact the persistence and fate of contaminants like SARS-CoV-2. The primary factors affecting retention time include hydraulic loading, system design, flow rates, and the distance between the point of wastewater discharge and the treatment facility. Hydraulic loading refers to the volume of wastewater entering the system over a given period. High hydraulic loading can result in shorter retention times as the flow rates increase, allowing wastewater to move more quickly through the system\textsuperscript{129}. Conversely, low hydraulic loading can lead to longer retention times as the flow rates decrease, causing wastewater to dwell in the system for an extended period.

The design of the sewage system, including the configuration of pipes, pump stations, and treatment facilities, also plays a crucial role in determining retention time. System designs can vary significantly depending on factors such as population size, geographic location, and infrastructure availability\textsuperscript{130}. These design variations can result in differences in the travel distance and time of wastewater, affecting the overall retention time within the system by up to 37%\textsuperscript{130}. The retention time of wastewater in the sewage system has important implications for the persistence and infectivity of SARS-CoV-2. The virus, if present in the wastewater, can experience changes in its viability and concentration over time. Prolonged retention times may allow for natural decay.
processes, through interactions with Fe(II)/Fe(III) ions, pH alterations, irradiation and bacterial presence\(^{130}\). On the other hand, shorter retention times may limit the opportunity for these decay processes to take place, potentially increasing the likelihood of viral persistence and transmission.

The persistence and stability of SARS-CoV-2 in wastewater are critical factors in understanding the potential spread of the virus through the wastewater management system. Factors such as temperature, pH, and the presence of disinfectants or other organic matter in the wastewater matrix can influence the stability and persistence of the virus\(^{131}\). The wastewater matrix itself can play a crucial role in the stability and survival of SARS-CoV-2. The presence of organic matter, suspended solids, and chemicals in wastewater can affect the virus's ability to persist and remain infectious. Interactions between the virus and components of the wastewater matrix can impact viral adsorption, which could potentially shield the virus from the wastewater matrix\(^{132}\). Additionally, pH of wastewater can affect the stability of the virus, with a greater than 2 log\(_{10}\) drop in infectivity observed between pH 7.00 and 7.45 in chlorinated water\(^{133}\).

Temperature plays a crucial role in the wastewater treatment process and can have significant implications for the fate and behaviour of SARS-CoV-2. Different stages of wastewater treatment can encounter a range of temperatures depending on the specific processes involved. Studies focusing on SARS-CoV-1 have reported the detection of infectious virus in wastewater samples for extended periods ranging between 3 and 4 days at room temperature, and up to 14 days at 4°C suggesting its ability to remain viable for extended periods\(^{131}\). Other studies have observed a loss in infectivity after 2 days in 20°C wastewater, indicating more rapid loss of infectivity in higher temperatures\(^{134}\).

The influent wastewater entering treatment plants can exhibit temperature variations depending on external factors such as ambient temperature, seasonal changes, and the sources of the wastewater. Domestic wastewater typically reflects the temperature patterns of the local environment, while industrial wastewater may exhibit higher temperatures due to industrial processes. The temperature of the influent wastewater sets the initial conditions for subsequent treatment processes. Temperature has a profound
impact on microbial processes, including the inactivation and replication of viruses like SARS-CoV-2. Higher temperatures generally accelerate the rate of biological reactions, leading to increased microbial activity and faster decomposition of organic matter\textsuperscript{135}. Conversely, lower temperatures can slow down microbial activity and reduce the efficiency of biological processes\textsuperscript{135}.

Temperature profiles vary across different treatment processes within WWTPs. In primary treatment, which involves physical processes such as screening and sedimentation, temperature fluctuations may be minimal as the focus is primarily on solid-liquid separation. However, during secondary treatment, which involves biological processes such as activated sludge or biofilm systems, temperatures can reach 50 to 70°C, critically influencing the efficiency of microbial degradation and viral inactivation\textsuperscript{115}.

The disinfection stage of wastewater treatment is particularly important when considering the temperature-dependent inactivation kinetics of SARS-CoV-2. Disinfection processes, such as chlorination or UV disinfection, rely on specific temperature ranges to achieve effective viral inactivation. Higher temperatures can enhance the disinfection efficacy, while lower temperatures may require longer contact times or higher disinfectant concentrations to achieve comparable results\textsuperscript{21,136}.

Assessing temperature profiles throughout the different treatment processes is crucial for determining the inactivation kinetics of SARS-CoV-2 in wastewater. By characterizing temperature variations and their duration at each stage, we can gain insights into the potential impact on viral inactivation. This information is essential for optimizing disinfection strategies and ensuring the safe management of wastewater to minimize the risks of viral transmission.

Investigating the time and temperature-based infectivity of SARS-CoV-2 in wastewater is crucial for protecting the health and safety of wastewater workers. These individuals are at the forefront of handling and managing wastewater, potentially exposing them to infectious agents such as SARS-CoV-2. By understanding the infectivity dynamics of the virus under different time and temperature conditions, appropriate measures can be implemented to minimize the occupational risks faced by wastewater workers. This may
include enhanced PPE, improved hygiene protocols, and optimized disinfection strategies to ensure their well-being while carrying out their essential duties.

This study aims to identify the inactivation kinetics under varying time and temperature conditions. Through these analyses we can better understand the behaviour of the virus in wastewater and implement measures to mitigate the risks associated with SARS-CoV-2 transmission through wastewater pathways.

2.3 Materials and methods

2.3.1 SARS-CoV-2 viral RNA extraction

RNA extraction was performed on all samples using the QIAamp 96 Viral RNA Kit (Qiagen, Maryland, US) in accordance with the manufacturer's protocol. The centrifugation steps were conducted using an Allegra® X-14R Benchtop Centrifuge (Beckman Coulter, Brea, CA, US). To each sample, 560 µL of lysis buffer (AVL) containing carrier RNA was added, the mixture was thoroughly homogenized and incubated for 10 minutes at room temperature. Subsequently, 560 µL of 100% ethanol was added to each sample. The lysate was then transferred to the QIAamp 96-well plate and centrifuged at 4000 x g for 4 minutes. To ensure maximum RNA binding, a second round of centrifugation was performed using the remaining lysate. The samples were then washed with 500 µL of buffer AW1 and AW2, followed by a 10-minute drying spin. The RNA was finally eluted by adding 80 µL of buffer AVE. The RNA extracts were stored at -80°C until they were ready to be analyzed using reverse transcription-quantitative PCR (RT-qPCR).

2.3.2 SARS-CoV-2 RT-qPCR

All PCR reactions were performed on a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Waltham, MA, US) using TaqMan™ Fast Virus 1-Step reagents (Applied Biosystems, Waltham, MA, US) according to the manufacturer's protocol. The primer-probe pairs specific for the N gene detection are listed in Table 1 (IDT Coralville, IA, USA). Nuclease-free water was utilized as a negative control. A final reaction volume of 10 µL was prepared containing 2.5 µL of template and the following thermocycling
conditions were applied: cDNA synthesis at 50°C for 5 minutes; a hold step at 95°C for 20 seconds; 45 cycles of denaturation at 95°C for 15 seconds and annealing/elongation at 60°C for 40 seconds.

Table 2-1. Primer and probe pairing for N gene detection using RT-qPCR.

<table>
<thead>
<tr>
<th>Primer/probe name</th>
<th>Sequence (5' → 3')</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>nCOV_N1 Forward Primer</td>
<td>GAC CCC AAA ATC AGC GAA AT</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>nCOV_N1 Reverse Primer</td>
<td>TCT GGT TAC TGC CAG TTG AAT CTG</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>nCOV_N1 Probe</td>
<td>FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1</td>
<td>0.125 μM</td>
</tr>
</tbody>
</table>

2.3.3 SARS-CoV-2 strains and culture conditions

SARS-CoV-2 WA1 mNeonGreen (mNG) was chosen as the subject for experimentation due to its ability to express a yellow-green fluorescent protein akin to GFP. mNG exhibits an excitation maximum at 506 nm and an emission maximum at 517 nm, rendering it compatible with most GFP filters, while also possessing a brightness three times greater than GFP\textsuperscript{137}. To propagate the SARS-CoV-2 WA1 mNG virus, complete DMEM media was employed, and Vero E6 cells were utilized in a T150 flask. After 72 hours of infection, the supernatant was gathered and subsequently subjected to centrifugation in order to eliminate any cellular debris. 500 μL aliquots were then placed in 1.5 mL cryogenic tubes and stored at -80°C for future use.

2.3.4 Wastewater spiking conditions

24 h composite wastewater samples were spiked a SARS-CoV-2 mNG to make a working solution with 10\textsuperscript{7} viral copies per L calculated using RT-qPCR, with unspiked wastewater serving as a negative control. This concentration was selected for its
physiological relevance in being the upper threshold of observed titres through different wastewater monitoring programs.

2.3.5 Time and temperature-based inactivation assay

Prepared wastewater samples were placed on an Eppendorf Mixmate at 300 rpm for 72 h at set temperatures of 4, 15, and 23°C to simulate different environmental conditions in the sewer system. Timepoint samples were collected every hour ranging from the time of spike to 6 hours. Timepoint samples were also collected at 24, 48, and 72 hours. The samples were filtered through a 0.45 µm filter to eliminate any potential bacterial contamination before plating.

2.3.6 Vero E6 plate preparation

To prepare Vero E6 cells for infection in a 96-well plate format, cells were grown in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (Pen/Strep) at 37°C and 5% CO₂. Once the cells reached confluency, they were detached from the culture dish via trypsinization and counted. An inoculation density of 10⁴ cells per well in a final volume of 100 µL per well was determined and the cells were inoculated into the wells of the 96-well plate. The inoculated plates were then incubated for 24 hours at 37°C and 5% CO₂ to allow for the cells to reach 95% confluency prior to infection.

2.3.7 TCID₅₀ quantification assay

Filtered samples were added to a 96-well plate in triplicate for biological replication utilizing a 1:3 dilution series. To each well, 50 µL of sample was added to 100 µL of plated cells in a complete DMEM media. A total of 11 dilutions were employed on each plate, with the final row serving as an undiluted negative control. The plates were then incubated for 72 hours at 37°C and 5% CO₂, in order to allow adequate time for potential infection to occur and for the progression of any visible cytopathic effects (CPE) to be observed. Following the incubation period, the plates were scored for positive CPE using microscopy. All supernatants from each plate were saved for RT-qPCR to validate the
CPE scoring. TCID50 was then calculated based on scoring results to determine the infectious dosage of each sample.

### 2.4 Results

To determine the infectivity of SARS-CoV-2 in spiked wastewater samples, SARS-CoV-2 WA1 mNeon Green (mNG) was added at a concentration of $10^7$ copies/L (as calculated by RT-qPCR) to wastewater samples collected over a 24 h composite period from Greenway Wastewater Treatment Centre London, ON. Spiked wastewater samples were monitored over a 6-hour time frame with aliquots taken each hour, and a 72-hour time frame with aliquots taken every 24 hours for TCID$_{50}$ analysis. Post 72-hour incubation using neon green expression and CPE as positive indicators of infection.

A two phase decay non-linear regression analysis was conducted to examine the relationship between TCID$_{50}$ values derived from SARS-CoV-2 spiked wastewater treated under various temperature conditions and incubation times. The results of the regression analysis demonstrated strong correlations between the loss of TCID$_{50}$ at each condition over the sampling period sampling (**Figure 2-1 A-C**). For temperature condition (A) 23°C, the coefficient of determination $R^2 = 0.9427$, indicating that approximately 94.27% of the variability in TCID$_{50}$ could be explained by temperature alone. Similarly, the 15°C trial had a $R^2 = 0.9639$, suggesting that temperature accounted for approximately 96.39% of the observed variability in TCID50. In the 4°C condition, $R^2 = 0.8856$, indicating that temperature explained approximately 88.56% of the variation in TCID$_{50}$.

Regression analysis was also performed to evaluate the log reduction of infectious SARS-CoV-2 WA1 mNG titres in spiked wastewater under the different temperature and time conditions with the log reduction of the virus was quantified for each replicate over the course of the trial. The data presented in the results display the log TCID$_{50}$/mL at each time point, and three biological replicates were included in the analysis to ensure the reliability and consistency of the findings with CPE and mNG protein fluorescence imaging (**Figure 2-2**). The two phase decay non-linear regression analysis allowed for the examination of the relationship between time and the reduction in infectious viral titre.
(Figure 2-3) with $R^2$ values of 0.9056 for the 23°C trial, 0.9612 for the 15°C trial, and 0.7929 for the 4°C trial.
Figure 2-1. Time and temperature dependent effect on SARS-CoV-2 WA1 mNG infectivity in spiked wastewater. Two phase decay non-linear regression analysis of TCID$_{50}$ for SARS-CoV-2 WA1 mNG spiked wastewater sampled at different time and temperature conditions. Samples were assessed hourly over a 6 hour time frame as well as every 24 hours over a 72 hour period at temperature conditions (A) 23$^\circ$C; $R^2=0.9427$ (B) 15$^\circ$C; $R^2=0.9639$ (C) 4$^\circ$C; $R^2=0.8856$. 
A) T=0 -ve control WW, -ve CPE
B) T=0 -ve control WW, -ve mNG
C) T=0 $10^7$ spike WW, +ve CPE
D) T=0 $10^7$ spike WW, +ve mNG
E) T=48h $10^7$ spike WW, +ve mNG
F) T=24h $10^7$ spike WW, +ve mNG
G) T=72 $10^7$ spike WW, +ve mNG
Figure 2-2. Imaging of Vero E6 cells for TCID$_{50}$ calculation of time and temperature dependent SARS-CoV-2 WA1 mNG spiked wastewater inactivation. Imaging performed 72-hours post infection with cytopathic effect and neon green expression detected. (A) Negative control unspiked 15°C wastewater collected at T=0h with no signs of CPE. (B) Negative control unspiked 15°C wastewater collected at T=0h with no visual neon green expression. (C) Spiked 15°C wastewater collected at T=0 with positive CPE. (D) Spiked 15°C wastewater collected at T=0 with positive neon green protein fluorescence. (E-G) Spiked 15°C wastewater collected at T=24, 48 and 72h respectively with positive neon green protein fluorescence. All images are representative of first dilution well in TCID$_{50}$ assay.
Figure 2-3. Log reduction kinetics of the time and temperature dependent effect on SARS-CoV-2 WA1 mNG infectivity in spiked wastewater. Two phase decay non-linear regression analysis of log reduction of infectious SARS-CoV-2 WA1 mNG spiked wastewater sampled hourly over a 6 hour time frame as well as every 24 hours over a 72 hour period under temperature conditions 23°C; $R^2 = 0.9056$, 15°C; $R^2 = 0.9612$, and 4°C; $R^2 = 0.7929$. Data displayed as log TCID$_{50}$/mL at each time point of 3 biological replicates.
2.5 Discussion

These results demonstrate how the inactivation of SARS-CoV-2 in wastewater is highly dependent on both temperature and time. The virus is able to survive longer at colder temperature conditions such as 4°C and 15°C when compared to 23°C. The average temperature of water traveling through sewer systems can vary depending on various factors, including the location, climate, and time of year and typically range from 3.7 to 21°C\textsuperscript{138}. In general, sewer water tends to be influenced by the ambient temperature of its surroundings, which means it will typically be colder during the winter months and warmer during the summer months\textsuperscript{138}.

Employing a two-phase decay analysis to assess SARS-CoV-2 infectivity within spiked wastewater samples across varying temperatures offers a nuanced understanding of its behavior. The observed trend of an initial rapid decline in infectivity within the first hourly timepoints followed by a plateauing effect at the 24-hour timepoints underscores these complex dynamics. Colder temperatures might induce a higher rate of adsorption of the virus onto solid particles in the water over extended durations. This phenomenon could potentially act as a protective shield, hindering the virus's interaction with agents like detergents present in wastewater that possess virus-inactivating properties. By dissecting these patterns, a two-phase decay analysis not only unveils the temporal dynamics of SARS-CoV-2 infectivity but also offers insights into the potential influence of temperature has on its resilience in wastewater environments.

In colder regions of Canada, such as northern provinces, the temperature of water in sewer systems may drop significantly during the winter, often reaching near-freezing temperatures. In contrast, in warmer regions or during the summer months, the water temperature in sewer systems can be relatively higher, approaching or even exceeding room temperature. It's important to note that these are general observations, and the actual average temperature of water in sewer systems can vary depending on the specific location and local conditions. Additionally, the temperature of wastewater can be
influenced by factors such as industrial discharges or mixing with groundwater, which can further affect its temperature profile\(^\text{139}\).

In a temperature inactivation experiment of infectious SARS-CoV-2, the results revealed that the rate of infectivity loss was higher in 23°C wastewater compared to 15°C and 4°C wastewater. This suggests that higher temperatures can contribute to a more rapid inactivation of the virus. At 23°C, the conditions were relatively warmer, resembling typical room temperature. In this environment, the infectious SARS-CoV-2 particles experienced a faster decline in their ability to remain infectious over time. This finding is consistent with the general understanding that higher temperatures can negatively impact the stability and viability of viruses. In contrast, the 15°C wastewater exhibited a slower rate of inactivation compared to 23°C. The lower temperature provided a more favorable environment for the virus to maintain its infectivity for a longer duration. Similarly, the 4°C wastewater, which represents colder conditions, showed the lowest slope in inactivation over time. Cold temperatures are known to have a preservative effect, potentially allowing the virus to persist and retain its infectivity for a longer period.

These results highlight the significance of temperature in determining the stability and infectivity of SARS-CoV-2 in wastewater. Understanding the effect of temperature on viral inactivation can aid in developing effective strategies for wastewater treatment and management. It emphasizes the importance of considering temperature factors when assessing the risk associated with the presence of the virus in wastewater systems and implementing appropriate measures to mitigate its transmission.

It was also observed that SARS-CoV-2 could persist for extended periods under certain conditions. Specifically, in the 15°C trial, the virus was found to remain viable for up to 48 hours, while in the 4°C trial, it persisted beyond 72 hours. These durations exceeded the typical residence time it takes for wastewater to travel from the sewage system to reach the treatment facility. These findings have important implications, particularly for workers in wastewater treatment facilities who may come into contact with samples that contain infectious virus. The prolonged persistence of the virus in colder temperatures
suggests that workers handling wastewater or involved in its treatment processes may be at risk of exposure to viable SARS-CoV-2 particles.

Considering the potential for workers to be exposed to and come into contact with samples containing infectious virus, it is crucial to prioritize their safety and implement robust measures to minimize the risk of transmission within wastewater treatment facilities. This may include strategies such as regular testing and surveillance, promoting physical distancing, and providing adequate training on infection prevention and control measures. By recognizing the potential for the persistence of SARS-CoV-2 in wastewater and its implications for worker safety, appropriate measures can be implemented to lower the risk of infection and protect the well-being of wastewater treatment facility workers.
2.6 References


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Chapter 3

3 Aerosolization infectivity assay of SARS-CoV-2 spiked wastewater.

There are multiple stages in the wastewater treatment process at municipal facilities which are aerated and exposed to the outside environment. This chapter focuses on the potential for aerosolized wastewater particles to transmit infectious SARS-CoV-2 particles to cell lines and different materials.

3.1 Abstract

The risk of SARS-CoV-2 to exposure to municipal wastewater workers through aerosols generated from routine operations is not well defined. These experiments focus on the behaviour of SARS-CoV-2 aerosols in wastewater systems. It explores the potential of aerosols to carry infectious virus, their deposition on different material surfaces, such as metal, plastic, Vero E6 cells and the implications for viral transmission. Spiked SARS-CoV-2 WA1 mNG wastewater samples were loaded into an aerosolization chamber, which vaporized the virus for collection on the different material conditions placed on the opposite side of the chamber. Results indicate that plastic surfaces have a higher viral recovery compared to metal surfaces, suggesting variations in the ability of different materials to support viral stability and adherence. Understanding the behaviour of SARS-CoV-2 aerosols and their interaction with various surfaces is crucial for implementing effective control measures to reduce the risk of viral transmission. These findings emphasize that wastewater aerosols do have the potential to carry pathogens that can be infectious and give importance to proper hygiene practices, targeted disinfection, and material selection in wastewater treatment facilities and other relevant environments to reduce the potential spread of SARS-CoV-2 through aerosols.
3.2 Introduction

Wastewater treatment plants (WWTPs) can serve as potential sources of aerosolized particles, which may pose a risk to both workers and those in the surrounding area with sampling able to detect viral presence at distances of up to 100 m\textsuperscript{140}. Aerosols can be generated during various stages of the wastewater treatment process, including aeration, agitation, and sludge handling\textsuperscript{141}. Bioaerosols can also be released from toilet flushing due to mechanical agitation\textsuperscript{142}. These aerosols can contain a wide range of microorganisms, including bacteria and viruses, which may serve as a potential route for the transmission of infectious agents\textsuperscript{140}. The generation, dispersion, and potential health risks associated with aerosols in WWTPs must be assessed for developing effective control strategies and ensuring the safety of workers and the general public.

Aerosols have been recognized as an important mode of disease transmission, particularly for respiratory infections\textsuperscript{143}. When inhaled, aerosolized particles can reach the respiratory tract, including the upper respiratory tract and deep lung regions, where they can deposit and potentially initiate infection\textsuperscript{143}. Compared to larger respiratory droplets, aerosols have the advantage of remaining suspended in the air for extended periods, allowing for long-range transport and the potential for inhalation by individuals at a distance from the emission source. The size distribution and composition of aerosols influence their behaviour, with smaller particles between 5 and 100 μm having the potential to travel longer distances and remain suspended for more than 5 seconds\textsuperscript{144}. Therefore, understanding the generation and fate of aerosols in the context of WWTPs is crucial for assessing the potential risk of disease transmission.

Aerosols in WWTPs can originate from various sources and processes. Aeration, which involves the introduction of air into wastewater to facilitate the growth of aerobic microorganisms for the breakdown of organic matter, is a significant source of aerosol generation\textsuperscript{145}. The vigorous mixing and agitation of wastewater during aeration can lead to the formation of fine droplets that become aerosolized. In addition, processes such as sludge handling, dewatering, and centrifugation can also generate aerosols due to the mechanical disruption of the sludge suspension\textsuperscript{146}. Moreover, the splashing and cascading of wastewater during its movement through different treatment units can contribute to the
release of aerosols. It is important to consider these various sources of aerosol generation in WWTPs to assess the potential risks and implement appropriate control measures.

The design and configuration of aeration systems, such as the type of diffusers and their placement within treatment tanks, can significantly impact the extent of aerosol generation\textsuperscript{147}. The characteristics of wastewater, such as its organic content, and total suspended solids (TSS) concentration, can also influence aerosol formation\textsuperscript{148}. Environmental factors such as temperature, humidity, and airflow patterns within the treatment plant can further affect the dispersion and transport of aerosols.

Several studies have demonstrated the detection of SARS-CoV-2 RNA in aerosols collected from WWTPs and other wastewater-related settings with titres as high as $10^4$ genome copies per L\textsuperscript{149}. The ability of these aerosols containing the virus to potentially transmit the infection is not well understood. Understanding the various routes of transmission, such as inhalation of aerosols or deposition on surfaces followed by subsequent hand-to-face contact, is crucial for implementing appropriate control measures. Additionally, assessing the risks associated with aerosolized SARS-CoV-2 transmission involves evaluating factors such as viral load, duration of exposure, and susceptibility of individuals\textsuperscript{150}. Investigating the transmission pathways and risks associated with aerosolized SARS-CoV-2 contributes to the development of effective mitigation strategies and safeguards the health and safety of individuals exposed to these aerosols.

Given the potential for aerosolization of the virus during various stages of wastewater treatment, it is crucial to understand the risks and implications for both wastewater workers and the general public. Assessing the infectious potential of aerosolized SARS-CoV-2 is crucial for understanding the risks associated with the transmission of the virus through these aerosols. Conducting research on aerosolized SARS-CoV-2 in wastewater presents several challenges, including the need for specialized sampling techniques, accurate measurement methods, and controlled laboratory settings that mimic real-world conditions. Obtaining representative aerosol samples and maintaining their viability for analysis can be technically challenging. These limitations and challenges need to be
carefully considered when interpreting and generalizing the findings from studies on aerosolization in wastewater. By conducting experiments that simulate real-world aerosolization scenarios, the viability and persistence of the virus in aerosols can be assessed. To address these challenges, an aerosolization chamber was designed and constructed in collaboration with the Western University Faculty of Engineering. This chamber serves as a controlled environment for generating aerosols from spiked wastewater samples. The chamber's ability to mimic real-world aerosolization conditions, such as temperature, humidity, and airflow patterns, is crucial for obtaining representative results.

By conducting systematic controlled experiments, this study aims to provide valuable insights into the aerosolization processes, including the factors influencing aerosol generation, transport, and deposition. These tests aim to explore multiple routes of infection through testing the infectivity of aerosols directly on Vero E6 cells to mimic an inhalation model, and determining the infectivity of SARS-CoV-2 on plastic and stainless-steel following aerosolization to model touch transfer. This study aims to quantify the viability, and infectivity of aerosolized SARS-CoV-2 in wastewater samples. The findings of this study will contribute to a better understanding of the transmission dynamics and risks associated with aerosolized SARS-CoV-2, thereby informing the development of effective control strategies.

### 3.3 Materials and methods

#### 3.3.1 SARS-CoV-2 strains and culture conditions

SARS-CoV-2 WA1 mNG was used as a fluorescent indicator of infection. Virus was propagated using complete DMEM media and Vero E6 cells in a T150 flask. After 72 hours of infection, the supernatant was gathered and subsequently subjected to centrifugation in order to eliminate any cellular debris. 500 μL aliquots were then placed in 1.5 mL cryogenic tubes and stored at -80°C for future use.
3.3.2 Wastewater spiking conditions

Greenway 24 h composite wastewater samples were spiked a SARS-CoV-2 mNG to make a working solution with $10^7$ viral copies per L calculated using RT-qPCR, with unspiked wastewater serving as a negative control.

3.3.3 Aerosolization chamber configuration

The chamber utilizes a syringe inlet on one side to inject viral samples into the system. Aligned with the syringe inlet is an air compressor that supplies pressurized air at a predetermined and adjustable psi. This controlled air supply plays a crucial role in aerosolizing the viral sample inside the chamber. By releasing controlled amounts of pressurized air, the sample is atomized into fine droplets, facilitating the generation of aerosols (Figure 4-1).

On the other side of the chamber, slots are strategically placed to accommodate trays or collection surfaces for capturing the aerosolized droplets. These slots can be sealed shut, ensuring that the aerosolized droplets are contained within the chamber during the collection process. To maintain a controlled environment within the chamber, ports are incorporated to allow for temperature and humidity modification and monitoring. This enables the conditions inside the chamber to be altered to simulate specific environmental conditions or replicate real-world scenarios accurately. By controlling temperature and humidity, the chamber ensures that the aerosolized droplets remain stable and representative of the desired experimental conditions.
**Figure 3-1. Aerosolization chamber CAD schematic.** (A) Back face of the chamber including tray slots with lock and sealing mechanisms. (B) Isometric opaque view highlighting chamber dimensions and temperature and humidity control. (C) Isometric transparent view showing tray locations on chamber interior.
3.3.4 Aerosolization chamber material spray procedure

All aerosolization sprays were performed within a sealed chamber located in a Biological Safety Cabinet (BSC) within a Containment Level 3 (CL3) facility. For each spray a 5 mL syringe was connected to the chamber, and 250 μL of SARS-CoV-2 WA1 mNG spiked wastewater maintained at 15°C was loaded into the 5 mL syringe using a pipette. The plugger was used to gently push the virus suspension down into the aerosolization tubing. The long tray of the chamber was then loaded at the back edge with plexiglass plates containing three plastic or stainless-steel chips. The air cylinder was then charged to 35 psi, and released allowing for the aerosolization of the spiked wastewater into the chamber, maintained at high humidity conditions (>60% relative humidity).

After 1 minute the seal on the tray was released, and the tray was carefully pulled out. The plexiglass plates were then removed from the tray, and the tray was pushed back and sealed. To ensure thorough cleaning, the air pressure was set to approximately 50 psi and a 5-second high-pressure cleaning spray was performed to remove any remaining liquid from the spray section, pushing it into the chamber. A waiting period of 5 minutes allowed the aerosols inside the chamber to settle.

Following aerosolization, the material chips were removed from the plates using forceps and subsequently submerged in a 6-well plate containing 3 mL of DMEM media and washed using a pipette. Immediate disinfection was carried out by wiping down the plexiglass plates, which contained the material and virus droplets, with 70% ethanol. These steps ensured controlled aerosolization within the chamber while maintaining safety precautions and meeting the necessary experimental requirements for subsequent analyses (Figure 3-2).

3.3.5 Aerosolization chamber direct Vero E6 cell interface procedure

To prepare Vero E6 cells for aerosolization sprays individual small format well plates (5.1 cm²), were seeded in triplicate and grown in complete DMEM containing 10% Fetal FBS and 1% Penicillin/Streptomycin (Pen/Strep) at 37°C and 5% CO₂. The inoculated
plates were then incubated for 24 hours at 37°C and 5% CO2 to allow for the cells to reach 95% confluency prior to infection.

Prior to aerosolization spray, the DMEM media was removed from the plates. A 1:4 dilution series of wastewater spiked with an initial concentration of $10^7$ viral copies per L spiked SARS-CoV-2 WA1 mNG was prepared to match the dilution series used in the TCID$_{50}$ analysis of the plastic and metal material spray. Each spiked wastewater dilution was then aerosolized directly onto separate sets of Vero E6 cells to allow for TCID$_{50}$ calculation. The aerosolization procedure then followed the protocol listed in Section 3.3.4.

Following aerosolization, the wells were removed from the trays 1 mL of complete DMEM media was added back to the well before sealing the plate. Immediate disinfection was carried out by wiping down the plexiglass plates, which contained the material and virus droplets, with 70% ethanol.

Figure 3-2. Workflow diagram for SARS-CoV-2 WA1 mNG spiked wastewater aerosolization trials. (1) SARS-CoV-2 mNG stock is spiked into 15°C wastewater sample. (2) Spiked sample is loaded into 5 mL syringe and loaded into the chamber. (3) Chamber tray are loaded with plexiglass plates holding either plastic, metal, or Vero E6 cell chips. Trays are sealed then 35 psi compressed air aerosolizes samples into the chamber. (4) After 1 minute in the chamber, trays are collected and material is rinsed with DMEM media. Samples are serially diluted for TCID$_{50}$ analysis 72 hours post infection. Generated with Biorender.com
3.3.6  TCID<sub>50</sub> quantification assay

Filtered samples were added to a 96-well plate in triplicate for biological replication utilizing a 1:3 dilution series. To each well, 50 µL of sample was added to 100 µL of plated cells in a complete DMEM media. A total of 11 dilutions were employed on each plate, with the final row serving as an undiluted negative control. The plates were then incubated for 72 hours at 37°C and 5% CO<sub>2</sub>, in order to allow adequate time for potential infection to occur and for the progression of any visible cytopathic effects (CPE) to be observed. Following the incubation period, the plates were scored for positive CPE using microscopy. All supernatants from each plate were saved for RT-qPCR to validate the CPE scoring. TCID50 was then calculated based on scoring results to determine the infectious dosage of each sample.

3.4  Results

3.4.1  Infectious SARS-CoV-2 on materials following spiked WW aerosolization and potential transmission from touch transfer

The possibility of transmission through touch transfer of infectious SARS-CoV-2 from contaminated materials was explored through aerosolization sprays onto plastic and stainless-steel material chips. In WWTPs, there are many exposed surfaces, mostly metal and plastic, on which SARS-CoV-2-containing droplets can settle following aerosolization from the wastewater, happening throughout processing. Thus, we have established a model system to aerosolize spiked wastewater, collecting droplets on various materials, and testing for infectivity through our TCID<sub>50</sub> measurements.

The SARS-CoV-2 WA1 mNG spiked wastewater was subjected to aerosolization, and the infectious viral titres were determined by quantifying the log TCID<sub>50</sub>/mL (Figure 3-3). Following 72 hours of incubation of the Vero E6 with the droplets washed from the material, the plates were imaged for presence of SARS-CoV-2 infection based on the detection of visual CPE and neon green protein fluorescence. The infectious viral titres were then determined from the different conditions and presented as the log TCID<sub>50</sub>/mL in Figure 3-3, with microscopy used TCID<sub>50</sub> calculation displayed in Figure 3-4. Aerosolization sprays were conducted in five biological replicates to ensure reliability of
the results as low recovery of infectious virus was expected to approach the limit of detection.

One-way ANOVA with Tukey's multiple comparisons test revealed statistically significant higher levels of virus titres obtained from aerosolized spike wastewater droplets settling on the plastic versus stainless-steel materials (p=0.0473).

3.4.2 Model for direct inhalation and infection of aerosolized SARS-CoV-2 spiked wastewater

Throughout the pandemic, our research team has been developing experimental models to study transmission of SARS-CoV-2 from direct inhalation following aerosolization of the virus or from materials harbouring SARS-CoV-2 droplets deposited by aerosolization. As described below, our experimental model to study inhalation transmission from aerosolized wastewater involves spraying spiked wastewater onto Vero E6 cells in the aerosolization chamber. This initial model will be improved with subsequent experiments using lung epithelial cell lines (CaLu) followed by the use of primary human nasal tissue.

In these preliminary experiments, SARS-CoV-2 WA1 mNG was added to wastewater with concentrations ranging from $10^7$ copies per L to $10^3$ copies per L in a 1:4 serial dilution series. Starting with the lowest concentrated samples, the Vero E6 wells were loaded into the chamber in triplicate and a syringe was loaded with 200 µL of the sample which was injected into the aerosolization nozzle of the chamber. Using 35 psi blast of compressed air the spiked wastewater was blown into the chamber, dispersing the virus aerosolized in wastewater droplets of various size, some of which fell onto the Vero E6 wells.

1 It should be noted that a manuscript is being prepared by Yiying Zhang, PhD candidate, describing all of the background data on aerosolization of SARS-CoV-2 in the chamber. This manuscript and data will be provided as appendix to the thesis following the lifting of the publication embargo of this thesis. For now, a slide presentation of the background aerosolization work with the chamber is provided as an addendum to this thesis.
Following 72 hours of incubation the Vero E6 wells were imaged for presence of SARS-CoV-2 infection based on the detection of visual CPE and neon green protein fluorescence. Aerosolization of spike wastewater directly onto Vero E6 cells resulted in significantly higher levels of infectious virus transfer than recovery of infectious virus from droplets settling on stainless steel and between stainless-steel and Vero E6 cells ($p=0.0161$). However, no significant difference was observed in viral titres between plastic and Vero E6 cells (Figure 3-3).

**Figure 3-3.** Infectivity of SARS-CoV-2 WA1 mNG spiked wastewater aerosols on different material conditions. Infectious viral titres reported in log TCID$_{50}$/mL. Aerosolization sprays performed in n=5 biological replicates. One-way ANOVA with Tukey's multiple comparisons tests yielded significance differences in viral titre between stainless-steel and plastic ($p=0.0473$) and stainless-steel and Vero E6 ($p=0.0161$), with no significant difference observed between plastic and Vero E6 cells.
A) Vero E6 $10^7$ spike WW, -ve CPE
B) Vero E6 $10^7$ spike WW, -ve mNG
C) Vero E6 $10^7$ spike WW, +ve CPE
D) Vero E6 $10^7$ spike WW, +ve mNG
E) plastic $10^7$ spike WW, +ve CPE
F) plastic $10^7$ spike WW, +ve mNG
G) metal $10^7$ spike WW, +ve CPE
H) metal $10^7$ spike WW, +ve mNG
Figure 3-4. Imaging of Vero E6 cells for TCID$_{50}$ calculation of aerosolized SARS-CoV-2 WA1 mNG spiked wastewater infectivity on different material conditions. Imaging performed 72-hours post infection with cytopathic effect and neon green expression from different materials. (A) Vero E6 cells material spray negative control with no CPE observed. (B) Vero E6 cells material spray negative control with no neon green expression. (C) Vero E6 cells material spray CPE observed. (D) Vero E6 cells material spray neon green expression observed. (E) Plastic material spray CPE detected accompanied by neon green protein fluorescence (F). (G) Stainless-steel material spray with positive CPE, along with neon green protein fluorescence (H). All images are representative of first dilution well in TCID$_{50}$ assay.

3.5 Discussion

At WWTPs, aerosols generated during various treatment processes have the potential to deposit virus-containing droplets on different surfaces throughout the facility. These surfaces include pipes, conduits, tanks, reactors, equipment, walkways, catwalks, control panels, and instrumentation systems. These surfaces can be made of plastic or metal materials commonly found in wastewater treatment infrastructure. In these aerosolization trials it was observed that wastewater samples spiked with SARS-CoV-2 WA1 mNG at a concentration of $10^7$ copies per L could be aerosolized to carry infectious virus, and that virus could directly infect Vero E6 cells. Infectious virus can also settle as droplets on material surfaces at relatively low titres of 1 to 2 log$_{10}$ scale. Notably, the results indicated that plastic surfaces had a higher viral recovery compared to metal surfaces which reflects other observations of the environmental stability of SARS-CoV-2 on different surfaces$^{151}$.

The ability of the spiked wastewater samples to carry infectious virus suggests that the virus can persist and remain viable even in wastewater aerosol environments. This finding raises concerns about the potential for viral transmission through contact with contaminated surfaces in wastewater treatment facilities or other settings where wastewater is handled. The differential viral recovery between plastic and metal surfaces is an interesting observation. It suggests that the material properties of the surfaces can influence the viability and adherence of the virus. Plastic surfaces may provide a more
favorable environment for viral stability and attachment, facilitating higher viral recovery rates. On the other hand, metal surfaces may have characteristics that make it less conducive for viral survival and adherence, resulting in lower viral recovery rates.

Plastic and metal surfaces can be conducive to the persistence and survival of viruses for extended periods. SARS-CoV-2 can survive longer on impermeable surfaces when compared to porous surfaces, such as paper, as these materials allow for prolonged viability of the virus suspended in droplets. These droplets provide a protection from evaporation and surface interactions with the material which can result in rapid inactivation\textsuperscript{152}. For these experiments, we utilized a polypropylene plastic similar to the plastics in WWTPs but we are currently comparing the potential of touch transfer transmission using various different plastic polymers, as well as organic surfaces (papers and cardboards), and different types of metals, considering that stainless steel is only one form metal used in WWTPs.

If these surfaces become contaminated with infectious viral particles, they can serve as a source of infection if they come into contact with susceptible individuals\textsuperscript{153}. This can occur when individuals touch the contaminated surfaces and then touch their face, mouth, or eyes, allowing the virus to enter their body\textsuperscript{153,154}. The virus can remain viable on these surfaces for hours to days, depending on various factors such as temperature, humidity, and the specific characteristics of the virus\textsuperscript{155}. Proper hygiene practices, including regular cleaning, and disinfection protocols are important in minimizing the risk of transmission from contaminated surfaces.

While the viral recovery from material surfaces in wastewater aerosols may be relatively low, it is important to consider the potential risk of prolonged exposure to these aerosols. Even at low titres, continuous or repeated exposure to SARS-CoV-2 in wastewater aerosols over time could potentially lead to infections. Prolonged exposure increases the likelihood of inhalation or ingestion of infectious particles present in the aerosols\textsuperscript{156}. It is worth noting that the infectious dose required to establish an infection is not fully understood, with estimates ranging from $10^2$ PFU to $10^6$ PFU can vary among individuals, and even a small number of viable viral particles can potentially cause
infection, especially in individuals with weakened immune systems or pre-existing health conditions\textsuperscript{156,157}. Other factors such as the concentration and persistence of the virus in the aerosols, as well as environmental conditions like temperature, humidity, and airflow patterns, can influence the actual risk of infection. It is important to consider that SARS-CoV-2 is known to remain viable in aerosols for variable periods of time dependent on atmospheric conditions, and sufficient exposure to these aerosols can potentially result in the transmission of the virus.

To reduce the risk of infection, it is crucial to prioritize the implementation of effective engineering controls, such as adequate ventilation systems and air filtration, to minimize the generation, dispersion, and inhalation of aerosols in wastewater treatment facilities and other relevant settings. Additionally, proper personal protective equipment (PPE) and adherence to hygiene practices, such as regular handwashing, can further reduce the potential for transmission. These results underscore the importance of surface disinfection and hygiene practices in settings where there is a potential for contact with wastewater or contaminated surfaces. Proper cleaning protocols and targeted disinfection of both plastic and metal surfaces can help limit the risk of transmission from these materials.
3.6 References


Chapter 4

4 Inactivation kinetics of SARS-CoV-2 spiked wastewater using UV disinfection

UV irradiations are a common form of treatment employed to remove pathogens from effluent wastewater samples before release from the treatment facility. This chapter focuses on UV experiments using a collimated-beam apparatus to determine the potential of UV treatment as a means of inactivation and determine the log reduction kinetics of SARS-CoV-2 with UV dosage.

4.1 Abstract

UV treatment is widely employed in WWTPs as an effective disinfection method used to return save purified wastewater to environment water sources. This purified wastewater constitutes part of the fish and aquatic habitat, provides source of water for terrestrial flora and fauna, is used for irrigation of crops, and drinking water for humans and livestock. WW with SARS-CoV-2 could pose a possible risk to employees in WWTPs to possible infections suggesting the need for WW treatment at the earliest point in wastewater treatment process. This study investigated the efficacy of collimated beam UV experiments on spiked SARS-CoV-2 WA1 wastewater samples. A dose series ranging from 0 to 5 mJ/cm² was applied to evaluate the reduction in infectious titres of SARS-CoV-2. Irradiated samples were plated on Vero E6 cells and assessed for presence of CPE 72 hours post infection and TCID\(_{50}\) calculation. The results revealed a substantial decrease in infectious titres by over 4 log scales, highlighting the exceptional effectiveness of collimated beam UV irradiation in inactivating the virus. The findings contribute to the growing body of knowledge on the application of UV treatment in wastewater systems for mitigating viral transmission. This research underscores the potential of UV disinfection as a valuable tool in wastewater treatment facilities to enhance public health protection by reducing the viability of SARS-CoV-2 and other pathogens in wastewater. The insights gained from these irradiation experiments can guide the implementation of UV disinfection strategies to ensure the safety and quality of treated wastewater before its release into the environment.
4.2 Introduction

UV disinfection is a widely employed technology in WWTPs for the inactivation of pathogenic microorganisms, including viruses. UV disinfection utilizes ultraviolet light, typically in the wavelength range of 200 to 300 nm, to destroy or inactivate the genetic material through pyrimidine dimers, rendering them unable to replicate. UV disinfection has proven to be an effective and environmentally friendly method for the treatment of wastewater, offering advantages such as rapid disinfection, no chemical residuals, and a broad spectrum of efficacy against a variety of microorganisms such as E. coli. UV also provides efficient inactivation of organisms that can be chemically resistant including chlorine-resistant Cryptosporidium and Giardia.

The effectiveness of UV disinfection is dependent on several key principles. Firstly, the intensity of the UV light, measured as energy per unit area, is crucial in determining the extent of microbial inactivation. Secondly, the wavelength of the UV light affects the penetration depth into the microbial cells and the absorption characteristics of their genetic material. Germicidal UV (UV-C) light in the range of 254 nanometers is commonly used due to its high bactericidal efficacy. Additionally, the exposure time of microorganisms to UV-C influences the degree of inactivation. Longer exposure times allow for increased absorption of UV energy and subsequent damage to the genetic material. Understanding these principles is essential for optimizing the design and operation of UV disinfection systems in WWTPs.

UV disinfection has been extensively utilized in wastewater treatment processes worldwide. It is commonly applied as a treatment step following primary and secondary treatment processes to ensure the removal of pathogenic microorganisms and reduce the risk of waterborne infections. However, UV light penetration can be challenging due to the presence of solids and turbidity in the samples. After primary and secondary treatment processes, a substantial number of viruses and other pathogens are removed in the TSS, resulting in a reduction in turbidity. This reduction in turbidity allows UV light to penetrate the water more effectively, facilitating the inactivation of any remaining pathogens. By utilizing UV treatment in the tertiary stage, the water undergoes an additional level of pathogen removal, enhancing the overall safety and quality of the
treated wastewater. Its application in wastewater treatment has proven successful in meeting stringent effluent quality standards, protecting public health, and safeguarding the environment. The use of UV disinfection is especially critical in areas where treated effluent is discharged into water bodies used for recreational activities, irrigation, or as a source for drinking water production\textsuperscript{167,168}.

The efficacy of UV disinfection in the inactivation of various viruses in wastewater has been well explored in the past. These studies have explored different parameters, such as UV dose, contact time, water quality, and the specific virus of interest. Research findings have demonstrated the effectiveness of UV disinfection in reducing viral concentrations and the risk of viral transmission. However, the efficacy of UV disinfection can vary depending on factors such as virus type, initial viral load, and wastewater characteristics. Certain viruses such as adenovirus show high resistance to UV disinfection compared to other common water-borne virus, such as enterovirus and norovirus, which are more UV sensitive\textsuperscript{169}. While RNA viruses are typically more susceptible to UV damage, certain RNA viruses such as aphthovirus exhibit high resistance to UV-C, highlighting the need for the UV-C kinetics of SARS-CoV-2 should be fully explored\textsuperscript{169}.

Determining the inactivation kinetics of SARS-CoV-2 in wastewater is crucial for implementing effective control measures and optimizing wastewater treatment processes. By investigating how SARS-CoV-2 behaves in wastewater and its susceptibility to UV disinfection, we can develop strategies to minimize the viral load and reduce the risk of transmission to wastewater workers, as well as protect the environment and downstream communities.

### 4.3 Materials and methods

#### 4.3.1 SARS-CoV-2 strains and culture conditions

SARS-CoV-2 WA1 was selected for spiking both wastewater and control samples. Virus was propagated using complete DMEM media and Vero E6 cells in a T150 flask. After 72 hours of infection, the supernatant was gathered and subsequently subjected to centrifugation in order to eliminate any cellular debris. 500 μL aliquots were then placed in 1.5 mL cryogenic tubes and stored at -80°C for future use.
4.3.2 Wastewater spiking conditions

To simulate the presence of SARS-CoV-2 in wastewater at the UV treatment stage, secondary effluent wastewater samples were spiked with a working solution containing \(10^4\) TCID\(_{50}\)/mL of SARS-CoV-2 WA1. This spike concentration was carefully chosen to ensure an adequate representation of the virus within the wastewater samples. To establish a reliable baseline for comparison, unspiked wastewater was also included as a negative control in the experiment. A DMEM spiked solution was also prepared as a control for inactivation in its normal suspension. 1:10 PBS to DMEM samples were prepared to allow for the desired transmittance of UV light through the sample spiked with a working concentration containing \(10^4\) TCID\(_{50}\)/mL of SARS-CoV-2 WA1.

4.3.3 Collimated beam apparatus configuration

The wastewater samples were subjected to irradiations using a bench-scale collimated beam apparatus (Figure 4-1). This apparatus consists of a low-pressure mercury lamp that emits a monochromatic spectrum centered at a wavelength of 254 nm. To ensure nearly parallel rays of light, a collimating tube was employed. The samples were placed in a small dish positioned on a magnetic stir plate within the apparatus. This setup allowed for controlled and uniform exposure of the samples to the irradiation source, facilitating the investigation of the effects of the specified wavelength on the samples. The use of the bench-scale collimated beam apparatus enabled precise manipulation of the irradiation parameters, providing valuable insights into the behaviour and response of the wastewater samples under controlled experimental conditions.
4.3.4 Collimated beam apparatus calibration

Prior to commencing experimental irradiations, the UV lamp is activated and allowed to undergo a warm-up period of no less than one hour to ensure the stability of the lamp output. Subsequently, the irradiance incident on the sample is quantified using a calibrated radiometer, with the UV sensor positioned at the same height as the liquid level within the sample dish. To obtain the average irradiance considering the sample volume, appropriate adjustments are applied to account for several factors. These factors encompass the reflection occurring at the air-liquid interface, the non-uniformity of the beam across the sample's surface, the absorption encountered as the beam traverses the sample, and the divergence attributable to less than perfect collimation. The experimental methodology employed, incorporating all these correction factors, closely adheres to the standard protocol established by Bolton and Linden (2003).
Table 4-1. Summary of standard parameters for collimated beam experiments.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of liquid sample</td>
<td>5 mL</td>
</tr>
<tr>
<td>Internal diameter of Petri dish</td>
<td>2.4 cm</td>
</tr>
<tr>
<td>Distance from lamp to sample surface ( (L) )</td>
<td>30.2 cm</td>
</tr>
<tr>
<td>Stir bar rotation speed</td>
<td>50 rpm</td>
</tr>
</tbody>
</table>

The reflection factor, denoted as \( F_{\text{ref}} \), accounts for the radiation that is reflected from the surface of the sample, which arises due to the difference in refractive index as the beam passes from the air medium to the liquid sample. By considering the refractive index of air as 1.000 and assuming the refractive index of the sample to be 1.375 (assuming it is equivalent to that of pure water), the reflection factor is determined to be \( F_{\text{ref}} = 0.975 \). This factor quantifies the proportion of radiation that undergoes reflection at the air-sample interface and is a crucial parameter in accurately estimating the effective irradiance received by the sample during the experimental procedures.

The Petri factor, denoted as \( F_{\text{Petri}} \), addresses the spatial variation of irradiance across the sample surface. It quantifies the relationship between the average incident irradiance over the sample surface and the central irradiance measured at the centroid of the sample dish. To determine the Petri factor, a grid-based approach was employed, where irradiance measurements were taken at 5 mm intervals on a grid using a radiometer positioned at the same level as the sample surface. In the case of the collimated beam apparatus utilized in this study, the measured Petri factor was determined to be \( F_{\text{Petri}} = 0.96 \). This value surpasses the recommended threshold of at least 0.9, as suggested by Bolton and Linden (2003).
The *divergence factor*, denoted as $F_{\text{div}}$, accounts for the divergence of the beam. It is calculated using the distance from the lamp to the surface of the sample, $L$, and the depth of the sample, $\ell$, as:

$$ F_{\text{div}} = \frac{L}{L + \ell} $$

Based on the geometry of the collimated beam configuration performed in these irradiations, the divergence factor for this experiment is $F_{\text{div}} = 0.964$.

The water factor, $F_{\text{water}}$, assesses the impact of water on irradiance measurements. It is determined by evaluating the ratio between the depth-weighted average irradiance and the central irradiance, $E_0$. The water factor is derived from the Beer-Lambert law and is expressed as:

$$ F_{\text{water}} = \frac{1 - 10^{-a\ell}}{a\ell \ln(10)} $$

where $a$ is the absorption coefficient (cm$^{-1}$). UV transmittance (%UVT) at 254 nm is measured for each sample and converted to the absorption coefficient (Table 2).

### Table 4-2. Summary of sample conditions for collimated beam experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>UV Transmittance (%)</th>
<th>Water Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media/Buffer</td>
<td>54.2</td>
<td>0.724</td>
</tr>
<tr>
<td>Secondary effluent wastewater</td>
<td>73.1</td>
<td>0.843</td>
</tr>
<tr>
<td>(WW1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary effluent wastewater</td>
<td>68.5</td>
<td>0.815</td>
</tr>
<tr>
<td>(WW2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Finally, the UV dose, \( D \), is calculated using the average irradiance, \( E_{avg} \), and the exposure time, \( t \), as:

\[
D = E_{avg} \cdot t
\]

where:

\[
E_{avg} = E_0 \cdot F_{ref} \cdot F_{Petri} \cdot F_{div} \cdot F_{water}
\]

### 4.3.5 Collimated beam irradiation procedure

A TrojanUV\textsuperscript{TM} collimated beam apparatus was used to calculate a series of UV doses spanning 0, 0.5, 1, 2, 3, 4, 5 and 8 mJ/cm\(^2\), which were corrected for Petri and reflection factor through calibration with a radiometer. 5 mL aliquots of working solution were added to a 10 mL glass beaker with a micro stir bar. The samples were placed on a stir plate set to low rpm speeds so as not to disturb the surface of the sample. The samples were exposed to the UV doses in duplicate while centered under the collimated beam. The samples were filtered through a 0.45 µm filter to eliminate any potential bacterial contamination post-irradiation before plating.

### 4.3.6 TCID\textsubscript{50} quantification assay

Filtered samples were added to a 96-well plate in triplicate for biological replication utilizing a 1:3 dilution series. To each well, 50 µL of sample was added to 100 µL of plated cells in a complete DMEM media. A total of 11 dilutions were employed on each plate, with the final row serving as an undiluted negative control. The plates were then incubated for 72 hours at 37°C and 5% CO\(_2\), in order to allow adequate time for potential infection to occur and for the progression of any visible cytopathic effects (CPE) to be observed. Following the incubation period, the plates were scored for positive CPE using microscopy. All supernatants from each plate were saved for RT-qPCR to validate the CPE scoring. TCID\textsubscript{50} was then calculated based on scoring results to determine the infectious dosage of each sample.
4.4 Results

The SARS-CoV-2 WA1 spiked wastewater samples and a DMEM media control were subjected to UV irradiations, and the infectious viral titres were determined by quantifying the log TCID$_{50}$/mL (Figure 4-2). 72 hours post infection with the plates were imaged for presence of visual CPE for TCID$_{50}$ calculation (Figure 4-3). UV irradiations were performed in biological duplicates with TCID$_{50}$ results measured as technical triplicates to determine the log reduction kinetics of SARS-CoV-2 caused by UV inactivation.

Simple linear regression analysis was conducted to examine the relationship between TCID$_{50}$ for SARS-CoV-2 spiked wastewater and various UV doses. The results of the regression analysis demonstrated strong correlations between TCID$_{50}$ and dose for each condition over the observed dose series (Figure 4-4). For the media control (A), the coefficient of determination $R^2 = 0.9730$ indicating a strong correlation with a significantly non-zero slope of -0.9333 ($p < 0.0001$). Similarly, the WW1 trial had a $R^2 = 0.9428$, suggesting that UV dose was strongly correlated to TCID$_{50}$ with a significantly non-zero slope of -0.7896. For the WW2 trial, $R^2 = 0.9531$, with a significant non-zero slope of -0.8240. The loss of infectivity due to increasing UV dose was significant by Pearson product-moment correlation (PPMC) for WW1 ($r = 0.9723$, $p = 0.0002$), WW2 ($r = 0.9846$, $p < 0.0001$), and media trials ($r = 0.9970$, $p < 0.0001$).

Regression analysis was also performed to evaluate the log reduction of infectious SARS-CoV-2 WA1 titres in spiked wastewater in response to UV irradiations. The log reduction of the virus was quantified for each replicate over the course of the trial, and 95% confidence intervals were calculated to assess the precision of the estimates.

The data presented in the results display the log TCID$_{50}$/mL at each UV dose. The results of the t-tests indicate statistically significant non-zero slopes ($p < 0.0001$) for both the media and two wastewater trials. These findings provide compelling evidence that UV dose can exert a critical influence on the inactivation kinetics of SARS-CoV-2 in wastewater.
Figure 4-2. UV dose dependent effect on SARS-CoV-2 WA1 infectivity in spiked wastewater. Regression analysis of TCID$_{50}$ for SARS-CoV-2 WA1 spiked samples with 95% confidence intervals sampled at different UV irradiation in dose series 0 to 5 mJ/cm$^2$. (A) DMEM media spiked control (B) Wastewater sample 1 (C) Wastewater sample 2. Data displayed as log TCID$_{50}$/mL at each time point of 2 biological replicates.
Figure 4-3. Imaging of Vero E6 cells for TCID50 calculation of SARS-CoV-2 WA1 spiked wastewater infectivity treated with low-pressure UV irradiation. Imaging of Vero E6 cells 72 hours post infection for TCID50 calculation of SARS-CoV-2 WA1 spiked UV inactivation. (A) Spiked DMEM control treated with UV dose = 5 mJ/cm² with no signs of CPE. (B) Spiked DMEM positive control (0 mJ/cm²) positive CPE. (C) Spiked wastewater sample 1 treated with UV dose = 5 mJ/cm² negative CPE. (D) Spiked wastewater sample 1 positive control (0 mJ/cm²) positive CPE. All images are representative of first dilution well in TCID50 assay.
Figure 4-4. Log reduction kinetics of UV dose dependent effect on SARS-CoV-2
WA1 infectivity in spiked wastewater. Regression analysis of log reduction of infectious SARS-CoV-2 WA1 spiked samples with 95% confidence intervals sampled at different UV irradiation in dose series 0 to 5 mJ/cm². Data displayed as log TCID₅₀/mL at UV dose of 2 biological replicates. The loss of infectivity caused by UV dose was significant PPMC for WW1 (r = 0.9723, p = 0.0002), WW2 (r = 0.9846, p < 0.0001), and media trials (r = 0.9970, p < 0.0001). t-test results show significantly non-zero slope for each trial (p<0.0001).
4.5 Discussion

The significance observed for the log reduction of SARS-CoV-2 by increasing UV dose in wastewater samples indicate a substantial effectiveness of UV treatment in reducing the viral load. These slope values for infectivity against UV dosage determined for each wastewater trial and DMEM media control, are relatively high when compared to slopes reported for other viruses in wastewater, indicating that SARS-CoV-2 has similar sensitivity to UV treatment as other RNA viruses such as rotavirus\textsuperscript{170}. The higher slopes suggest that SARS-CoV-2 is easily inactivated by UV irradiation compared to DNA viruses commonly found in wastewater such as adenovirus which can require UV doses higher that 150 mJ/cm\textsuperscript{2} to obtain the same log reduction observed in these experiments\textsuperscript{171}.

The higher slope values suggest that increasing the UV dose leads to a more pronounced reduction in infectious SARS-CoV-2 levels. This indicates that UV treatment is particularly effective in inactivating and reducing the concentration of SARS-CoV-2 in wastewater samples. The observed effectiveness of UV treatment for SARS-CoV-2 in wastewater is crucial, as it highlights the use of UV disinfection as a reliable method to inactivate the virus in downstream treatment processes.\textsuperscript{170,171} The normal UV dose applied to wastewater at treatment facilities can vary depending on several factors, including the specific treatment objectives, the quality of the incoming wastewater, and the design of the UV disinfection system. UV dose is carefully calibrated to ensure effective disinfection of the wastewater while balancing the operational and cost considerations. A dose of 30 to 100 mJ/cm\textsuperscript{2} is typically to inactivate a significant portion of pathogens, including viruses, bacteria, and parasites, present in the wastewater\textsuperscript{170–172}. It's important to note that the UV dose required for effective disinfection may vary depending on the specific pathogens of concern, the required effluent quality standards, and regulatory requirements in different jurisdictions. Therefore, the actual UV dose applied at a wastewater treatment facility may be customized based on these factors to achieve the desired level of disinfection.

The results of the collimated beam irradiations on spiked wastewater samples demonstrated that even UV doses as low as 5 mJ/cm\textsuperscript{2} were able to significantly reduce the infectious titres of the virus by over 4 log scales. This indicates that UV treatment is
highly effective in inactivating the virus in wastewater. Considering that the normal UV doses applied at WWTPs typically fall within the range of 30 to 100 mJ/cm², these findings suggest that the UV application commonly used in wastewater treatment facilities is more than sufficient to inactivate any remaining virus that may be present in the water at this treatment stage. The UV dose used in actual practice far exceeds the minimum dose required to achieve substantial viral inactivation.

This level of UV treatment provides an additional layer of protection to ensure the safety of the treated wastewater before it is released from the facility's outlet. By employing the appropriate UV dosage, WWTPs can significantly reduce the risk of potential viral transmission and meet regulatory requirements for effluent quality standards. UV disinfection is a well-established and widely used technology in wastewater treatment due to its effectiveness against a broad spectrum of pathogens. The experiment's results affirm that UV treatment at the recommended doses effectively reduces the infectious titre of SARS-CoV-2 in wastewater, bolstering confidence in the ability of WWTPs to mitigate the transmission of the virus through treated effluent.
4.6 References


4.7 Acknowledgments

Special thanks go to Dr. Christopher DeGroot for his expertise in engineering and physics, which significantly contributed to this project. I would also like to express my gratitude to our collaborators at Trojan UV, Brian Petri, and Michelle Gabriel, for their guidance on the UV component of this project.
Chapter 5

5 General Discussion

In this study, we investigated key aspects related to the behaviour and management of SARS-CoV-2 in municipal wastewater. Chapter 1 provided an introduction to the potential risks associated with SARS-CoV-2 presence in wastewater and highlighted the importance of studying its infectivity and inactivation kinetics. Chapter 2 focused on examining the influence of time and temperature on the infectivity of SARS-CoV-2 spiked wastewater samples. Chapter 3 explored the aerosolization infectivity of SARS-CoV-2 in wastewater, shedding light on the potential risks to wastewater workers. Chapter 4 investigated the inactivation kinetics of SARS-CoV-2 in wastewater using UV disinfection, assessing its effectiveness as a potential mitigation strategy.

5.1 Implications of SARS-CoV-2 infectivity in municipal wastewater

The persistence of SARS-CoV-2 in wastewater raises significant concerns regarding the potential for viral transmission through wastewater pathways. While the findings of this thesis reveal intriguing insights into the infectivity potential of SARS-CoV-2 within wastewater, it becomes evident that SARS-CoV-2 demonstrates low signs of infectivity during the wastewater management process. This suggests that the risks of direct infection from SARS-CoV-2 via exposure to wastewater might be comparatively lower than initially speculated when compared to other pathogens present in the wastewater system.

Pathogens like norovirus, known for their robust survival capabilities in water environments, pose heightened risks due to their high transmissibility and capacity to cause gastroenteritis. Similarly, Salmonella, with its fecal origins and extended persistence in water, can induce severe symptoms of salmonellosis. Hepatitis A and E virus, capable of waterborne transmission, can lead to serious liver infections. Cryptosporidium, a chlorine-resistant parasite, can trigger cryptosporidiosis characterized by persistent diarrhea. Furthermore, enteric viruses such adenovirus and rotavirus and bacterial E. coli strains, and Shigella contribute to the spectrum of pathogens prevalent in
wastewater, each capable of causing various health issues, from gastrointestinal disturbances to severe diseases like meningitis, myocarditis and cancer\textsuperscript{177,178}. This underscores that the risk landscape extends beyond SARS-CoV-2, necessitating comprehensive strategies to manage a broader range of waterborne pathogens.

Detection of SARS-CoV-2 in wastewater provides valuable insights into the prevalence and circulation of the virus within a community. WWS serves as a complementary tool to clinical testing and provides a valuable perspective on virus infection levels in the population. However, the attention on WWS has not addressed the potential risks of infection and care must be taken when coming into contact with wastewater sources. By analyzing wastewater samples from different locations, patterns of high viral spread can be identified, indicating there may be a risk to infectious SARS-CoV-2 presence in the wastewater\textsuperscript{179}. WWS can assist in the allocation of resources and implementation of targeted interventions to limit the transmission of SARS-CoV-2\textsuperscript{180}. To ensure effective viral reduction and mitigate public health risks, wastewater treatment processes are crucial. Conventional WWTPs may require enhanced treatment strategies, including disinfection methods such as UV irradiation or chemical disinfection may be necessary in upstream processes to ensure effective inactivation of SARS-CoV-2 in wastewater prior to contact or handling at WWTPs.

Furthermore, the development and implementation of guidelines and regulations specific to SARS-CoV-2 in wastewater management are necessary. These guidelines should address the proper use of PPE when handling wastewater sampling and testing protocols, treatment requirements, and standards for safe discharge or reuse of treated wastewater\textsuperscript{181}. By adopting rigorous protocols and maintaining high treatment standards, the potential risks associated with SARS-CoV-2 in wastewater can be minimized, safeguarding workplace health and ensuring the protection of water resources.

Overall, understanding the implications of exposure to SARS-CoV-2 in wastewater is vital for effective disease surveillance, early detection of outbreaks, and implementation of appropriate interventions. By integrating WBE into public health strategies, we can enhance our ability to monitor viral transmission, protect workers, and prevent the
resurgence of community outbreaks. The effectiveness of disinfection strategies, such as UV disinfection, is of paramount importance in mitigating the risks associated with SARS-CoV-2 in wastewater. Therefore, optimizing UV disinfection systems and ensuring adequate UV-C and UV-A dosages and integration with other treatments, such as H₂O₂ and ozone, are crucial to achieving the desired level of viral inactivation and reducing the potential for viral transmission through wastewater. In addition to disinfection strategies, optimizing wastewater treatment processes is essential to ensure effective viral reduction and protect public health. The treatment should be designed and operated to target the removal or inactivation of viral pathogens, including SARS-CoV-2. This may involve implementing additional treatment steps or modifying existing processes to enhance the removal of viral particles. Advanced treatment technologies, such as membrane filtration or advanced oxidation processes, may be employed to further improve the removal efficiency and reduce the viral load in treated wastewater. Increased temperature requirements in upstream processes may be necessary even if more costly to rapidly inactivate any viral contamination. By continuously evaluating and optimizing treatment processes, wastewater management systems can effectively contribute to reducing the risks associated with SARS-CoV-2 in wastewater.

The presence of aerosolized SARS-CoV-2 in WWTPs can pose occupational hazards for wastewater workers. These frontline workers are at an increased risk of exposure to the virus due to their handling of contaminated wastewater and biosolids with the potential for aerosol generation during various stages of the treatment process. Inhalation of aerosols containing SARS-CoV-2 may lead to respiratory infections, thereby endangering the health and safety of wastewater workers. To mitigate these risks, it is crucial to ensure the use of appropriate PPE by wastewater workers. This includes respiratory protection such as N95 masks or respirators that can filter out airborne particles, as well as protective clothing, gloves, and eye protection. Adequate training and regular updates on the proper use and disposal of PPE are essential to maximize its effectiveness. Additionally, implementing enhanced hygiene protocols, such as frequent handwashing, sanitization of equipment, and maintaining proper personal hygiene practices, can further reduce the potential for viral transmission among wastewater workers.
Improving ventilation systems and implementing workplace safety measures are vital for safeguarding the health and well-being of wastewater workers. Adequate ventilation plays a crucial role in minimizing the concentration of aerosols in enclosed spaces, reducing the potential for viral transmission\textsuperscript{189}. Upgrading ventilation systems, ensuring proper air circulation, and considering the use of air purification technologies can help mitigate the occupational risks associated with aerosolized SARS-CoV-2 in WWTPs. Furthermore, implementing physical distancing measures, modifying work schedules to reduce crowding, and providing regular health monitoring and testing can contribute to maintaining a safe working environment for wastewater workers.

Furthermore, close collaboration between public health agencies, researchers, and wastewater treatment operators is crucial for addressing the wastewater management implications of SARS-CoV-2. Sharing knowledge, best practices, and experiences can contribute to the development of comprehensive guidelines and protocols that incorporate the latest scientific evidence and technological advancements. Additionally, fostering interdisciplinary collaborations among researchers, engineers, public health experts, and policymakers can facilitate the exchange of ideas and accelerate the implementation of innovative solutions to manage the risks associated with SARS-CoV-2 in wastewater.

By recognizing and addressing the occupational safety implications of SARS-CoV-2 in wastewater, we can ensure the well-being and protection of wastewater workers, who play a critical role in maintaining essential services and protecting public health. Collaborative efforts between wastewater management authorities, occupational health and safety experts, and workers are key to developing and implementing comprehensive occupational safety guidelines that consider the unique challenges posed by the presence of SARS-CoV-2 in WWTPs.

\section*{5.2 Future directions}

While our study has contributed valuable insights into the behaviour and management of SARS-CoV-2 in wastewater, there are still knowledge gaps that warrant further investigation. One such gap pertains to the long-term persistence of the virus in wastewater and its potential to act as a reservoir for transmission\textsuperscript{190}. Future studies could
focus on monitoring the presence and infectivity of SARS-CoV-2 in wastewater over short and long periods, considering seasonal variations and changes in community infection rates. Field testing methods for infectivity like those used for influenza A could be implemented at WWTPs to determine if infectious titres can be observed throughout the wastewater treatment process without relying on transferring and processing of samples in a laboratory environment.

Additionally, the role of different viral variants and their behaviour in wastewater systems requires further exploration. VOC such as Omicron and its subvariants, have raised concerns regarding their altered infectivity, vaccine breakthrough and transmissibility. Understanding their persistence, infectivity, and response to disinfection processes is crucial for adapting wastewater management strategies accordingly.

Future directions for experiments involving SARS-CoV-2 spiked wastewater could focus on testing in larger-scale environments to simulate more realistic scenarios. For instance, closed circuit pipe systems could be specifically designed to circulate water at controlled temperature conditions, simulating the conditions found in real wastewater systems. This setup would allow researchers to take regular aliquots from different points in the system for comprehensive analysis, providing a better understanding of viral persistence, behaviour, and transport within the wastewater infrastructure. Moreover, performing aerosolization experiments in larger facilities would enable the investigation of viral spread patterns and inactivation on various surfaces and materials. By simulating different environmental conditions, airflow patterns, and surface types, researchers can gain insights into the potential routes and dynamics of SARS-CoV-2 transmission via aerosols in different indoor settings. This information is crucial for developing effective mitigation strategies and engineering controls in wastewater treatment plants and other related facilities.

Additionally, observing the application of UV disinfection in tanks housing submerged UV disinfection units, as commonly seen in standard wastewater facilities, would provide valuable data on the inactivation efficiency of SARS-CoV-2. By monitoring the viral
load and assessing the effectiveness of UV treatment in these larger-scale setups, researchers can determine the optimal UV dosage and exposure time required for achieving desired levels of viral inactivation. By conducting larger-scale experiments in these more realistic scenarios, more insight can be gained into the infectivity potential of SARS-CoV-2 in wastewater and its transmission dynamics. This knowledge would contribute to improved risk assessment and the development of appropriate strategies to minimize the potential transmission of the virus through wastewater systems.

5.3 Concluding Statement

In conclusion, this study has contributed to the understanding of SARS-CoV-2 infectivity in municipal wastewater and its implications for public health and wastewater management. The findings underscore the importance of wastewater monitoring, inactivation, and aerosolization assessment of SARS-CoV-2 in wastewater systems. The knowledge gained from this study can inform risk assessment, guide the development of effective mitigation strategies, and ensure the protection of public health and the well-being of wastewater workers. Future research should continue to address the remaining knowledge gaps and further advance our understanding of the behaviour and management of SARS-CoV-2 in wastewater.
5.4 References


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# Curriculum Vitae

<table>
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<th>Name:</th>
<th>Justin Donovan</th>
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<tr>
<td><strong>Post-secondary Education and Degrees:</strong></td>
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</tr>
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<td>Western University</td>
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<td>2012-2016 B.Sc.</td>
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**Publications:**