Wastewater Surveillance of Influenza (A, B) and Respiratory Syncytial Virus (RSV) in Southern Ontario

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Wastewater-based surveillance (WBS) of SARS-CoV-2 has been highly effective at tracking trends of COVID-19 infections across the globe. This success led to the question of the possible applicability of WBS in monitoring other respiratory viruses that are also posing a concern for healthcare systems. Traditional influenza and RSV surveillance consists of monitoring hospital admissions, clinical testing data and outpatient visits. Even when clinical surveillance and hospitalization admissions are used to monitor the spread of these infections, they are often underestimated, with a lag between detection and community spread. In this study, wastewater surveillance of influenza A, influenza B and respiratory syncytial virus (RSV) was investigated, by quantifying the viral RNA of these viruses in wastewater and comparing these trends to clinical metrices.

The solid–liquid partitioning behaviors of influenza A, influenza B, RSV and SARS-CoV-2 in wastewater were examined to understand the behavior of the viruses in wastewater. Analyzing whether each virus is present in the liquid or solid fraction of wastewater may affect the data interpretation and inform further method development. The viral RNA in the liquid and solid fractions were separated and enriched by undergoing different centrifugation settings, overnight polyethylene glycol (PEG) precipitation followed by centrifugation, or ultrafiltration using a Centricon Plus-70 device. The influenza A, influenza B and RSV viral RNA concentrations were found to almost exclusively partition in the solids fraction of wastewater, which increased with an increase in centrifugal settings, unlike SARS-CoV-2 that had a more even split in signal between the solids and liquid fractions. The effectiveness of normalization using the endogenous pepper mild mottled virus (PMMoV) was also examined, by comparing the normalized influenza signal with the raw signal. Normalization using the PMMoV biomarker did not increase or hinder the correlation with clinical testing data relative to the raw influenza signal in wastewater.

Wastewater samples were collected once weekly from two wastewater treatment plants in the Region of Waterloo, Canada, from September 15, 2022, to June 21, 2023. Wastewater was treated overnight with polyethylene glycol (PEG), centrifuged (12,000g 1.5 h), then extracted (pellet) with Qiagen RNeasy PowerMicrobiome Kits and quantified using real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). The RNA concentrations of influenza A, influenza B and RSV in wastewater strongly correlated with the cases reported by episode date and hospitalizations, in

the cities of Kitchener and Waterloo (Spearman's Rank correlation coefficient rho ranging from 0.41-0.85). Wastewater surveillance can be a very effective additional surveillance tool to support public health officials in monitoring the trends of respiratory viruses in communities. Further development of WBS for respiratory virus monitoring will help public health to better prepare for these and other emerging pathogens in the future.

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Chapter 1
General Introduction

1.1 Wastewater-based surveillance

Wastewater-based Surveillance (WBS) emerged as an approach to community monitoring and was initially applied to measure pathogens (O'Keeffe, 2021), antimicrobial resistance (Hendriksen et al., 2019), and illicit drug use (Choi et al., 2018). Wastewater-based Surveillance is defined as the quantification and analysis of biological or chemical signals, in untreated wastewater, from sewer networks and wastewater treatment plants (WWTPs) (O'Keeffe, 2021). Even before the COVID-19 pandemic, scientists had indicated that WBS had promising potential in pathogen surveillance, due to its application in the global effort to eliminate poliovirus (O'Keeffe, 2021). Several polio environmental surveillance studies reported detecting polioviruses in wastewater, in the absence of clinical cases (Thompson & Duintjer Tebbens, 2017). WBS has the advantage of using passive techniques that capture input from entire communities without a participation burden (Hughes et al., 2022).

With the onset of the COVID-19 pandemic, WBS emerged as a potential technique to monitor the incidence and spread of the virus in communities, as a complementary public health surveillance tool (O'Keeffe, 2021). Viral SARS-CoV-2 RNA fragments were successfully detected in wastewater in several countries and were found to correlate with clinical cases (Ahmed et al., 2020; La Rosa et al., 2021; Medema et al., 2020). Viral shedding of SARS- CoV-2 was found to occur before the onset of symptoms and was detected in feces of asymptomatic patients as well (Wu et al., 2020). Wastewater surveillance has provided an advantage over clinical testing, as it can detect pre-symptomatic cases, asymptotic cases and individuals that do not get tested (Wu et al., 2020). It has also been able to monitor Variants of Concern (VOC) and detect their spread globally (Fuzzen et al., 2023; Graber et al., 2021). COVID-19 wastewater data has also served as an early detector of outbreaks in congregate high-risk settings, such as long-term homes, university residences and shelters, as viral shedding may occur before the onset of symptoms or reporting of clinical testing (Betancourt et al., 2021; Colosi et al., 2021; Haskell et al., 2024). With the current lack of clinical testing in the province of Ontario, wastewater surveillance has been a vital tool in monitoring the spread of COVID-19 in communities and the corresponding VOC (Dhiyebi, Cheng, et al., 2023).

Wastewater surveillance of COVID-19 has been demonstrated to be highly effective and a valuable tool for informing and supporting public health action. This led to the question of its applicability in monitoring other respiratory viruses of concern, that are also shed and detectable in stool, such as influenza (Arena et al., 2012), and respiratory syncytial virus (RSV) (von Linstow et al., 2006). The current testing program in Ontario, for influenza, and especially RSV may be severely underestimating the true number of cases (S. Drew, Region of Waterloo, personal communication). WBS may be a tool that is independent of clinical testing that could provide valuable information on the spread of these viruses in communities.

1.2 Influenza

Influenza, just like SARS-CoV-2, is a single stranded respiratory RNA virus (CDC, 2019). It is from the Orthomyxoviridae virus family composed of enveloped viruses with segmented negative-sense single strand RNA segments (Blut, 2009). Influenza is divided into four types A, B, C and D, however only influenza A and B are the viruses linked with endemic human seasonal influenza (CDC, 2019). More specifically, several subtypes of influenza A (H1N1 and H3N2) have been the cause of previous pandemics, with the most recent one occurring in 2009 when the novel influenza A (H1N1) virus emerged (CDC, 2019). Influenza pandemics occur when a novel influenza strain emerges that infects and spreads efficiently, with a lack of population immunity (CDC, 2019). The influenza A subtypes are differentiated depending on the type and combination of surface proteins (CDC, 2019). Influenza B on the other hand, is divided into two lineages B/Yamagata and B/Victoria and not subtypes, however just as with influenza A it is further classified into specific clades and sub-clades (CDC, 2019). Influenza A and B have eight RNA gene segments, with the surface proteins hemagglutinin (HA) and neuraminidase (NA) composing integral properties of the virus, as such the seasonal vaccine usually contains these two proteins (CDC, 2019).

In Canada, it is approximated that the seasonal flu is in the top 10 leading causes of death, resulting in approximately 12,200 hospitalizations and 3,500 annual deaths (IPAC, 2014). While worldwide it results in between 3 to 5 million severe annual infections and between 290,000 to 650,000 annual deaths (IPAC, 2014). These staggered numbers emphasize the need for additional surveillance systems to improve public health responses. Although clinical testing, hospital admissions and outpatient visits are used to monitor the spread of infections, they are not able to detect an onset or magnitude of a community outbreak quickly and accurately(Mercier et al., 2022).

WBS is a promising surveillance candidate to fill this gap, as although influenza primarily targets the respiratory tract, gastrointestinal (GI) symptoms such as diarrhea, vomiting and abdominal pain also occur. Therefore, influenza is not only shed from nasal and oropharyngeal secretions but also through (GI) secretions (Al Khatib et al., 2021; Heijnen & Medema, 2011). There remains a significant knowledge gap on respiratory virus concentrations and shedding rates from gastrointestinal (GI) tract excretions, as not very much is known about the shedding rates at different stages of the infection. Nonetheless several studies have successfully detected and correlated influenza A in wastewater with clinical metrics (Dumke et al., 2022; Mercier et al., 2022; Nadeau et al., 2023; Vo et al., 2023; Wolfe et al., 2022). Just as with the wastewater surveillance of COVID-19, influenza A detected in congregate high-risk setting on a university campus was strongly correlated with clinical testing data (Wolfe et al., 2022). Subtyping of influenza A was also effective from wastewater surveillance, reflecting the possibility of predicting which subtype is circulating within communities and adjusting public health responses accordingly (Mercier et al., 2022). Measures put in place to limit the transmission of COVID-19, resulted in the decline of influenza over the first two years of the pandemic (Wolfe et al., 2022), but has rebounded in the spring and fall of 2022 (Mercier et al., 2022). Many jurisdictions, including Ontario, are now investigating whether wastewater surveillance of influenza can be effective and feasible.

1.3 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is also a single-stranded RNA, enveloped, negative sense virus, that infects the respiratory tract (Ruckwardt et al., 2018). It is classified into two subtypes, A and B, that typically alternate in different epidemic seasons, but can also simultaneously circulate (Ciarlitto et al., 2019). There are no apparent differences in severity between the two strains of RSV, however RSV A is more widespread, so it has resulted in a majority of the severe cases (Ciarlitto et al., 2019). RSV is the leading cause of severe pediatric respiratory disease and mortality in the elderly and the immunocompromised (Ruckwardt et al., 2018). It is a major global contributor to child morbidity and mortality, especially in the first 6 months of life, where one in every 28 deaths in children between 28 days to 6 months is due to RSV (Y. Li et al., 2022). Ninety-nine percent of mortality in children younger than five years old that are due to RSV occur in low and middle income countries (Carbonell-Estrany et al., 2023). However, even in upper middle and high income countries RSV remains the leading cause of infant morbidity and hospitalization (Carbonell-Estrany et al., 2023). Even with its severity, RSV is not a

reportable disease in the province of Ontario, and as most infected individuals do not require or seek care, cases go unreported.

Although RSV was identified approximately 65 years ago, there is still a lack of preventive strategy available for this virus (Carbonell-Estrany et al., 2023). Wastewater surveillance was vital during the Global Polio Eradication Initiative, particularly in the final stages of the program (Ivanova et al., 2019). Environmental surveillance, predominantly wastewater monitoring, aided in identifying the circulation and/or introduction of wild and vaccine-derived polioviruses in the absence of clinical cases in several countries (Benschop et al., 2017; Blomqvist et al., 2012; Esteves-Jaramillo et al., 2014; Ivanova et al., 2019). WBS is therefore a promising surveillance candidate to fill the gap currently present in RSV monitoring, as the current testing programs are biased towards the most severe (hospitalized) cases, and cases related to confirmed outbreaks. As with influenza there remains a knowledge gap on the concentration of RSV in the stool of infected patients, and the concentration of the virus at different stages of the infection. von Linstow et al., (2006) detected RSV RNA in stool samples of pediatric patients at different time intervals for three weeks, but did not report on the incidence rate, or a comparison of the concentrations at different stages of the infection. Akbari et al., (2017) also detected RSV from the stool of a pediatric patient, however just as with the previous study they did not provide the RSV RNA concentrations in the stool samples. Several studies have detected RSV (A&B) in wastewater and it was correlated with clinical trends of RSV (Ahmed et al., 2023; Hughes et al., 2022; Mercier et al., 2023; Rector et al., 2022). This approach can therefore provide valuable data that may strengthen health emergency response systems, to better prepare for hospital crises such as the one that occurred in the (2022/23) influenza and RSV season in Ontario (Zven et al., 2023)

1.4 Normalization with fecal indicators

Fecal indicators are a group of chemicals or organisms that have traditionally been used to indicate the presence of fecal contamination in recreational and drinking waters (Korajkic et al., 2020). Fecal indicator bacteria such as *Escherichia coli* are typically used when testing for contamination of surface waters, however they tend to exhibit poor correlation with virus fate in the environment (Tandukar et al., 2020). More recently in wastewater surveillance programs, fecal indicators have been used to normalize the targets of interest for quantitative detection, such as SARS-CoV-2 (Dhiyebi, Abu Farah, et al., 2023; Kakhki et al., 2020). They are used in a similar way to housekeeping genes in qPCR, acting as internal controls for quantitative detection (Ho & Patrizi, 2021; Turabelidze et al., 2010). Housekeeping genes are

cellular maintenance genes that regulate basic and ubiquitous cellular functions to maintain stable gene expression levels, therefore they are often used as controls (Ho & Patrizi, 2021; Turabelidze et al., 2010).

The fecal indicator chosen needs to be constantly released into the wastewater, be stable in the sewer network and have a similar fate to the target of interest. An ideal normalization biomarker would have minimal spatial variation per capita shedding rates and minimal temporal differences in wastewater loads in stable flow rates (Greenwald et al., 2021). It can be used to assess changes that occur in the sewer that are not related to the original fecal/viral load released, by normalizing the viral signal (i.e., SARS-CoV-2) and accounting for factors such as changes in flow rates or dilution (Dhiyebi, Abu Farah, et al., 2023). Viral based fecal indicators may be the more appropriate choice when performing viral wastewater surveillance, as they have higher specificities and greater similarities to enteric viruses (Korajkic et al., 2020).

Pepper mild mottle virus (PMMoV) is a plant enteric virus that belongs to the Virgoviridae family (Kitajima et al., 2018). It is one of the most abundant RNA viruses in human feces, with a dietary origin from peppers and their products, such as hot sauce (Kitajima et al., 2018). It is excreted mainly from healthy human populations, and rarely found in animal feces, unlike bacterial fecal indicators that are found in the gastrointestinal tract of many animals and have dissimilar fates in the sewer networks than pathogenic viruses. (Kitajima et al., 2018). Several studies have shown that PMMoV is found in greater amounts than human enteric viruses in sewage and are not highly affected by seasonal variations (Kitajima et al., 2018; Tandukar et al., 2020). PMMoV is the most used endogenous fecal indicator in SARS-CoV-2 surveillance, it often used to account for potential dilution effects, sewer shed conditions and sample variations that could alter the quantification of SARS-CoV-2 (Chik et al., 2021; D'Aoust et al., 2022). Normalization is done by dividing the viral signal (i.e., SARS-CoV-2) gene copies by the PMMoV gene copies on the same sample (Chik et al., 2021). Other biomarkers have also been used as endogenous controls to normalize the signal, including cross-assembly phage (crAssphage), tobacco mosaic virus (TMV) (Dhiyebi, Abu Farah, et al., 2023; Tandukar et al., 2020), human Bacteroides HF183 (Feng et al., 2021), and even caffeine and its metabolites (Hsu et al., 2022; Oloye et al., 2023). The normalized SARS-CoV-2 signal has not always improved trend and correlation analysis relative to the raw signal (Dhiyebi, Abu Farah, et al., 2023; Feng et al., 2021) When there is minimal variability in sewer flow, normalization has been shown to reduce the utility of normalization for the SARS-CoV-2 signal (Dhiyebi, Abu Farah, et al., 2023). As a result, the use of normalization needs to be considered carefully across the different sites.

1.5 Partitioning behaviours of influenza (A, B) and Respiratory Syncytial Virus (RSV)

The partitioning behaviours of influenza and RSV viral fragments (RNA) in the liquid and solid fractions of wastewater needs to be thoroughly investigated, to optimize the concentration and extraction protocols for the enrichment of influenza and RSV. Partitioning in wastewater is often operationally defined as the relative amount of virus in different phases, e.g., the solid fraction, colloidal fraction, the liquid, or dissolved fraction. Centrifugation-based methods are the most prevalent approaches, where the pellet formed represents the solid fraction, and the supernatant represents what is often referred to as the liquid fraction. The supernatant will always contain some non-settled solids and colloids, as factors such as particle size and density will affect how solids are settled out of solution under different conditions. The RNA in the solution is also often concentrated through ultrafiltration across a membrane under pressure after removal of the large particles (that may clog membranes). There is a range of ultrafiltration devices available, each with different characteristics and membrane sizes, allowing for different applications. Different flocculants such as polyethylene glycol (PEG) can be added to further settle the solids out of solution (Mullins et al., 2024; Zheng et al., 2023). PEG precipitation is one of the most conventional methods for virus concentration from environmental samples (Torii et al., 2022). The polymer PEG, traps solvent while excluding proteins, which allows for the proteins to concentrate and settle (Atha & Ingham, 1981). All these methods while varied are all aimed at concentrating the virus from wastewater to extract the RNA.

The partitioning behaviour of viruses in wastewater has not been thoroughly investigated, even with the widespread use of WBS for the surveillance of SARS-CoV-2, various studies are still targeting different fractions of wastewater (Chik et al., 2021; La Rosa et al., 2020; Pecson et al., 2021). Breadner et al., (2023) demonstrated that the apparent partitioning of SARS-CoV-2 showed an approximate even split between the supernatant and pellet fractions, under strong and long centrifugal conditions (i.e., 12,000g, 1.5 h). Therefore, various extraction and concentration methods that target different wastewater fractions can quantify SARS-CoV-2, as it is present in both fractions (Breadner et al., 2023). Many studies are therefore also operating under the assumption that influenza A, B and RSV will partition in the wastewater similarly to SARS-CoV-2 or to viral surrogates. However, Mercier et al., (2022) found that influenza A almost exclusively partitions in the solids fraction when using primary sludge and municipal wastewater. Yet, no study to date has thoroughly investigated the partitioning of influenza A, B, and RSV in wastewaters.

1.6 Research Goals

At the University of Waterloo, Professor Mark Servos, and his team, have been running a wastewater surveillance program that measures COVID-19 and its variants in municipal wastewater across Ontario, as part of the province's Wastewater Surveillance Initiative. The Wastewater Surveillance Initiative was established in 2020, and has been very successful since at tracking the trends of SARS-COV-2 (Dhiyebi, Abu Farah, et al., 2023; Dhiyebi, Cheng, et al., 2023). It also identified the possibility of monitoring influenza A, influenza B and RSV. These respiratory viruses have posed a potential concern for public health and better surveillance would lead to more informed public health action. RSV surveillance is of particular interest because it is a non-reportable disease in the province. At the time of initiating this study there was minimal information about the behaviour of these viral targets in wastewater.

The main objective was to conduct a case study on expanding the wastewater surveillance program beyond SARS-CoV-2. This was done to determine if wastewater surveillance can be applied to influenza and RSV to inform public health action and response, similarly to what was achieved with SARS-CoV-2. This case study intended to address the re-emergence of influenza and RSV in the 2022-2023 season. This was achieved by (1) optimizing and validating the methodology currently applied in the surveillance program to the detection, quantification, and enrichment of influenza (A, B) and RSV, (2) applying the method to wastewater from two treatment plants in Waterloo Region, Ontario, from September 2022 to January 2024 and (3) evaluating the relationship between wastewater data and available clinical cases and/or hospitalizations. The results were generated in near-real time and reported weekly, directly to the Waterloo Region Public Health Unit.

Chapter 2

Applying wastewater techniques to respiratory viruses beyond SARS-CoV-2 in Waterloo Region, Canada

2.1 Introduction

Even before the COVID-19 pandemic, wastewater-based surveillance (WBS) had exhibited its capability of monitoring human pathogens. WBS has been applied to detect and track wild and vaccine-derived polioviruses since the early 2000s (O'Keeffe, 2021). During the COVID-19 pandemic WBS became an additional surveillance tool and was highly successful in tracking the virus and its corresponding variants in communities around the world. Prior to the emergence of the Omicron (BA.1) variant in December of 2021, wastewater surveillance had proven to be a reliable tool that tracked well with clinical cases in communities across Ontario (Dhiyebi, Cheng, et al., 2023). However, after the emergence of Omicron clinical PCR testing was overwhelmed and testing was greatly restricted in Ontario to hospitalized patients, healthcare workers, and residents and staff in congregate living settings (Dhiyebi, Cheng, et al., 2023). As a result of this policy wastewater surveillance of SARS-CoV-2 became a vital tool for monitoring COVID-19 as it was independent of clinical testing (Dhiyebi, Cheng, et al., 2023). In addition, even in the absence of PCR clinical testing the wastewater data exhibited strong relationships with the associated hospitalizations and had advanced detection times in comparison with hospital admissions (D'Aoust et al., 2021; Dhiyebi, Cheng, et al., 2023; Peng et al., 2023). Wastewater surveillance of COVID-19 has therefore been shown to be able to inform and support public health action in several studies around the world (Ahmed et al., 2020; La Rosa et al., 2021; Medema et al., 2020). The success of WBS application during the COVID-19 pandemic has led to the question of if wastewater surveillance could be expanded to other respiratory viruses that have the potential to also impact or even overwhelm healthcare systems. Viruses such as influenza (Arena et al., 2012) and respiratory syncytial virus (RSV) (von Linstow et al., 2006) are also detectable and quantifiable in human feces, which makes the application of WBS possible. The concentrations of influenza and RSV are expected to be low in wastewater (Mercier et al., 2022; Zulli et al., 2024), optimization of the extraction protocols for the enrichment of influenza and RSV are therefore needed. Mercier et al., (2022) suggested that influenza A partitions to the solids fraction in primary sludge and municipal wastewater samples and Roldan-

Hernandez & Boehm, (2023) had a similar conclusion for RSV. However, no studies to date have thoroughly investigated the partitioning of influenza A, B, and RSV in wastewaters.

Public health units rely on clinical surveillance to track and monitor influenza and RSV, but these methods can take several weeks to report trends in community transmission (Wolfe et al., 2022). The current clinical testing in Ontario, for influenza and RSV is limited to individuals that require or seek care, just as with COVID-19. Most cases as a result go unreported, and their prevalence in a community is underestimated. RSV incidence is particularly underestimated because it is not a reportable disease in the province, with testing restricted primarily to symptomatic children who present to the emergency room and require hospitalization. The current public health surveillance system for RSV relies on pediatric and adult hospitalization statistics. This surveillance system is a lagging indicator of infection prevalence in communities. As was demonstrated during the COVID-19 pandemic WBS is a tool independent of clinical testing, that can provide anonymous and aggregated data on the spread of respiratory viruses such as SARS-CoV-2. As these viruses may also be released to the sewer in feces, WBS may be a useful tool to fill the gaps in the surveillance of other respiratory viruses such as influenza and RSV.

Measures put in place to halt the transmission of COVID-19 early in the pandemic also severely limited the transmission of influenza and RSV (Ando et al., 2023; Groves et al., 2021; Zven et al., 2023). Once the mask mandate was lifted in the province of Ontario, in March of 2022, an unusual influenza A outbreak occurred in the spring (Mercier et al., 2022). In addition, the measures put in place during the first two years of the COVID-19 pandemic had the potential to impact the RSV hospitalization trends typically seen (Bourdeau et al., 2023). RSV hospitalizations are usually dominated by children 6 months and younger, however older children were not exposed to RSV and were also at risk of infection. With the return of society to pre-pandemic conditions (reduced masking mandates, social distancing, etc.) the transmission of these viruses was again expected.

Prior to the COVID-19 pandemic, influenza was among the top 10 leading causes of death in Canada (IPAC, 2014). Influenza A subtypes H1N1 and H3N2 are the two strains that typically circulate in the beginning of the fall flu season while influenza B typically peaks later. Influenza, like SARS-CoV-2, is a single stranded RNA virus, that is also shed from the GI tract and not exclusively through respiratory excretions (Al Khatib et al., 2021). Several researchers have demonstrated the capability of correlating the influenza A community incident rates with the levels in the wastewater and successfully applied it in communities and in congregate high-risk settings (Dumke et al., 2022; Kevill et al., 2022; Nadeau et al.,

2023; Vo et al., 2023; Wolfe et al., 2022). RSV is also a single-stranded respiratory RNA virus, that disproportionately impacts pediatric, elderly, and immunocompromised patients (Ruckwardt et al., 2018). RSV A and B subtypes do not peak at different times in the season like influenza, but concurrently circulate (Ciarlitto et al., 2019). RSV (A&B) has also been detected in wastewater in several studies and successfully correlated with clinical metrices (Ahmed et al., 2023; Hughes et al., 2022; Mercier et al., 2023; Rector et al., 2022). WBS may provide public health officials with trends in the community spread of these important viruses, which could allow for improved public health responses, specifically with early detection of influenza and RSV.

The objective of this study was to expand the wastewater surveillance program to include the monitoring of additional respiratory viruses of concern. The concentration, extraction and detection methodologies used for wastewater surveillance of SARS-CoV-2 (Dhiyebi, Abu Farah, et al., 2023; Dhiyebi, Cheng, et al., 2023) were adapted for the enrichment of influenza A, B and RSV. The apparent partitioning of influenza A, B and RSV was determined and contrasted to SARS-CoV-2 on the same sample to inform and validate the method. Over a full respiratory year, from September 15, 2022, to June 21, 2023, weekly wastewater samples were collected from two treatment plants (Waterloo, Kitchener) in the Waterloo Region, Ontario as part of the SAR-CoV-2 surveillance program and analyzed in this case study. The influenza signal was also normalized with a fecal indicator Pepper mild mottle virus (PMMoV). PMMoV is a single stranded RNA virus with human dietary origins (Feng et al., 2021) that has been widely used to normalized the target virus with the intention to improve detection of trends (Dhiyebi, Abu Farah, et al., 2023). The wastewater viral signals, for influenza A, influenza B (unnormalized and normalized) and RSV were correlated with clinical metrices in each sewershed. In addition, the results were reported in near-real time to the Region of Waterloo Public Health and Emergency Services to help inform public health decisions.

2.2 Materials and Methods

2.2.1 Wastewater sampling

Wastewater samples were collected weekly from the headworks (influent) of the Region of Waterloo's Kitchener and Waterloo wastewater treatment plants (WWTPs), from September 15, 2022, to June 21, 2023. The WWTPs service 256,000 and 123,300 people respectively, or approximately 66% of the Region of Waterloo's population. Treatment plant operators collected the samples from a 24-h (4°C) composite, into sterile 250 mL HDPE bottles. Bottles were placed in coolers with icepacks and

transported to the nearby University of Waterloo. Upon arrival, sample bottles were wiped with 10% bleach, then with 70% ethanol, then placed in a biosafety cabinet for 30 minutes under ultraviolet light. Samples were held on ice or placed in a fridge (4°C) before concentration (normally within 2 days).

2.2.2 RNA concentration and extraction

The samples used in this study were inactivated, concentrated, and extracted using the same techniques used for SARS-CoV-2; as influenza and RSV are also single stranded RNA enveloped viruses (Dhiyebi, Abu Farah, et al., 2023). RNA was concentrated by centrifugation in polyethylene glycol and salt (PEG/NaCl), extracted and purified using RNeasy PowerMicrobiome Kits (QIAGEN), then quantified by RT-qPCR (Figure 1).

A 40 mL wastewater sample was added to a pre-weighed 50 mL Falcon tube with 4.0 g of PEG 8000 and 0.9 g of NaCl (Approx. 10% PEG, 2.25% NaCl). 229E (IC-1) was then spiked into the tube as a recovery surrogate. The sample was moderately shaken and vortexed at 2000 rpm for 30 sec, placed on ice and shaken for two hours at 150 rpm and then left overnight at 4°C. Samples were centrifuged at 12,000g at 4°C for 1.5 hours the following morning. The supernatant was discarded, and the tube centrifuged at 12,000g at 4°C for 5 minutes to firm up the pellet and remove any further supernatant. The pellet was weighed and sub-sampled if the weight was greater than 250 mg. RNA was extracted from the pellet using the RNeasy PowerMicrobe Kit (QIAGEN), following the manufacturers protocol except that 100 µL of TRIzol (Fisher Scientific) was also added to aid in cell lysis in the PM1 buffer and 2-mercaptoethanol (Fisher Scientific) step. The sample was then beaten (Bead Mill 24 Homogenizer, Fisher Scientific) for 5 min at 3.55 m/s to further lyse the cells. DNase was omitted from the procedure. Samples were extracted using a QIAcube Connect instrument (QIAGEN) with a final elution volume of 100 µL. Samples were stored in a -20°C freezer before RT-qPCR analysis.

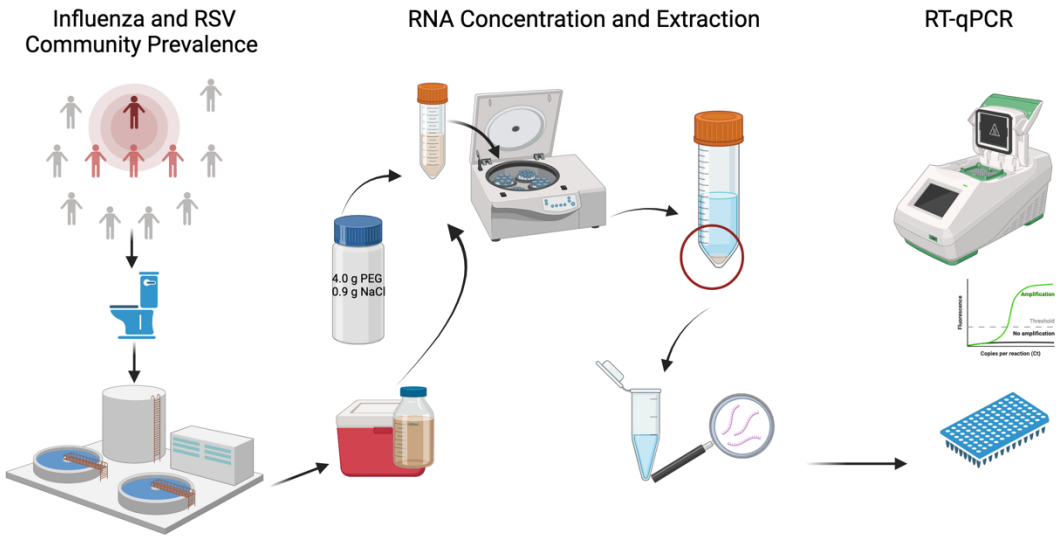


Figure 1. General workflow of the wastewater surveillance of influenza (A, B) and RSV. Figure created with BioRender.com.

2.2.3 RT-qPCR assays

The RNA extracts were quantified for influenza A (InfA) membrane protein (M-gene), influenza B (InfB) nonstructural protein (NS-gene), RSV nucleocapsid protein (N-gene), SARS-CoV-2 nucleocapsid protein (N2-gene), and PMMoV coat protein, by one-step real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). This was performed using the BioRad CFX 96 Real-time PCR Detection System (BioRad, USA). The influenza and SARS-CoV-2 assays were adapted from the (CDC, 2020a, 2020b) respectively, the RSV assay was adapted from (Hughes et al., 2022) and the PMMoV assay was adapted from (Zhang et al., 2005).

The InfA, InfB, RSV and N2 assays run on Waterloo and Kitchener samples were run in a simplex 20 µL reaction with a 5 µL RNA template and 5 µL of 4x TaqPath 1-Step RT-qPCR Master Mix (Fisher Scientific, USA). The PMMoV assay on the other hand was run in a simplex 10 µL reaction with a 2.5 µL RNA template and 2.5 µL of 4x TaqPath 1-Step RT-qPCR Master Mix. The optimized primer and probe (IDT, USA) concentrations of all the assays are listed in Table 1, with nuclease-free water making up the remaining balance for each reaction. The influenza assays (InfA and InfB) were quantified with ssRNA influenza controls (TWIST, USA), and the RSV assay was quantified with dsDNA gBlock standards (IDT, USA), and finally the SARS-CoV-2 (N2) assay and PMMoV assay were both quantified using a

linearized concatenated plasmid (ContactP) provided by (I. Yang, University of Toronto) and adapted from (Sun et al., 2024), as summarized in Table 2. The standards were quantified using dPCR (QIAcuity, Qiagen) before running the assays. A six-point standard curve was used to quantify the samples for InfA, InfB and PMMoV and an eight-point standard curve was used to quantify for RSV and N2.

The MS2 assay adapted from (Dreier et al., 2005), was also run to test for inhibition of reverse transcriptase and DNA polymerase. The master mix was spiked with ssRNA bacteriophage MS2 (*Emesvirus zinderi).* Sample extracts were plated in duplicate and assessed for a one Cq shift compared to the positive control (spiked RNase-free water) to indicate qPCR inhibition (Cao et al., 2012; Dreier et al., 2005). No qPCR inhibition (> 1 Cq shift) was observed in any of the samples.

Every plate was validated by running positive controls, non-template controls (NTCs), and non-reverse transcriptase controls (NRTs). RNA extracts, standards and controls were all plated in triplicate wells. Each plate was assessed with specific QA/QC parameters consisting of standard curves with an $R^2 \geq 0.98$, efficiency between 90% to110% and sample technical replicates that do not exceed 0.5 standard deviations away from the average. The cycling conditions for the InfA, InfB, RSV, N2 and MS2 assays were optimized to run in the following: incubation at 25°C for 2 min, reverse transcription at 50°C for 15 min, polymerase activation at 95°C for 2 min, then 45 cycles of 95°C denaturing for 3 sec and amplification at 60°C for 30 sec with an image after every cycle. The PMMoV assay had an amplification temperature of 55°C.

Table 1. Sequences of primers and probes for respiratory viruses that were synthesized by Integrated DNA Technologies (IDT) or MilliporeSigma.

Primer/Probe	Oligonucleotide Sequences (5' to 3')	Optimized Concentration (nm)
InfA forward1 primer	CAA GAC CAA TCY TGT CAC CTC TGA C	400
InfA reverse 1 primer	GCA TTY TGG ACA AAV CGT CTA CG	600
InfA probe	/FAM/TGC AGT CCT /ZEN/ CGC TCA CTG GGC ACG/3IABkFQ/	200
InfB forward primer	TCC TCA AYT CAC TCT TCG AGC G	500

InfB reverse primer	CGG TGC TCT TGA CCA AAT TGG	500
InfB probe	/YakYel/CCA ATT CGA/ZEN/ GCA GCT GAA ACT GCG GTG/3IABkFQ/	200
RSV forward primer	CTC CAG AAT AYA GGC ATG AYT CTC C	500
RSV reverse primer	GCY CTY CTA ATY ACW GCT GTA AGA C	500
RSV probe	/FAM/TA ACC AAA T/ZEN/T AGC AGC AGG AGA TAG ATC AG/3IABkFQ/	200
N2 forward primer	TTA CAA ACA TTG GCC GCA AA	500
N2 reverse primer	GCG CGA CAT TCC GAA GAA	500
N2 probe	/FAM/ACA ATT TGC CCC CAG CGC TTC AG/BHQ-1/	125
MS2 forward primer	TGC TCG CGG ATA CCC G	1000
MS2 reverse primer	AAC TTG CGT TCT CGA GCG AT	1000
MS2 probe	/HEX/ACC TCG GGT TTC CGT CTT GCT CGT BHQ-1/	500
PMMoV forward primer	GAG TGG TTT GAC CTT AAC GTT GA	400
PMMoV reverse primer	TTG TCG GTT GCA ATG CAA GT	400
PMMoV probe	/Cy5/CCT ACC GAA GCA AAT G/ BHQ-1/	250

Table 2. List of standards used in this study and their respective accession numbers with the RSV gBlocks synthesized by IDT and the influenza controls synthesized by TWIST Bioscience.

Standard	Accession Number
Twist Synthetic Influenza H1N1(2009) RNA control	NC_026431 - 026439

Twist Synthetic Influenza B RNA control	NC_002204 - 002211
RSV A gBlock	OP_890340
RSV B gBlock	OK_500264
ContactP (N-plasmid)	OR_994921

The limit of detection (LOD) and limit of quantification (LOQ) for the influenza A, influenza B, RSV and N2 assays (Table 3) were determined by evaluating the lowest concentration (copies/reaction) of the standard curves run for each assay being detected, with a detection rate of ≥ 95%, as outlined by the MIQE guidelines (Bustin et al., 2009) and by the technical guidance report on the wastewater surveillance initiative, published by the Ministry of Environment, Conservation and Parks (MECP, 2022). The LODs and LOQs for each assay were calculated based on curve fitting modeling using an R script adapted from (Klymus et al., 2020) and run on R version 4.2.2.

Table 3. The limit of detection (LOD) and limit of quantification (LOQ) for each assay used.

Assay	**Limit of Detection - LOD (Copies/mL)**	**Limit of Quantification – LOQ (Copies/mL)**
Influenza A	2.0	5.5
Influenza B	2.0	3.0
RSV	1.9	3.0
N2	1.5	3.5

2.2.4 Clinical data and correlation analysis

Clinical data was provided by The Region of Waterloo Public Health and Emergency Services. Influenza is an official Disease of Public Health Significance, therefore all confirmed positive lab results were reported to the Public Health Unit. The cases reported by episode date (date of symptom onset), broken down by city, were available for influenza A and B, but not for RSV because it is not a reportable disease.

However, hospitalization data for the entire Region of Waterloo, and not city specific, was provided for both influenza and RSV. Hospitalization data included all patients that were admitted to Cambridge Memorial Hospital, Grand River Hospital, and St. Mary's General Hospital for, or with, influenza and/or RSV. Hospitalized patients included residents of Waterloo Region, as well as non-residents from outside of Waterloo Region, who were admitted or transferred to local hospitals. The 2021 populations of Kitchener and Waterloo served in the wastewater sampling sites were approximately 256,000 and 123,000 respectively, accounting for 66% of the population of the Region of Waterloo.

The trends of the three viruses at each sewershed were investigated by plotting the weekly wastewater load (gene copies/day) against clinical metrices. The daily mass load per WWTP was calculated by multiplying the flow rate by the mean gene copies/mL (Dhiyebi, Cheng, et al., 2023). Spearman's rank correlation was used to investigate the relationships between the wastewater signal of the respiratory viruses (mass load) and the cases reported by episode date (CBED) and/or hospital admissions, using R version 4.2.2, over the span of the 2022-2023 influenza and RSV season. The data was not normally distributed based on visually assessing quantile-quantile plots, histograms and performing a Shapiro-Wilk test.

The hospitalization data is not broken down to sewersheds but encompasses the entire Region of Waterloo. Therefore, after accounting for flow at each WWTP, the daily wastewater loads at both WWTPs were summed, and Spearman's rank correlation coefficient (r) was determined between the combined total influenza A (M-gene) and influenza B (NS-gene) loads and influenza hospitalizations. This was repeated for total RSV (N-gene) load and RSV hospitalizations, similar to that reported by Dhiyebi, Cheng, et al., (2023). The correlation coefficient r was considered to be significant at $p<0.05$. The analysis was repeated only for influenza A and influenza B using PMMoV-normalized data and not for RSV, as no clinical data was available. The normalized value was calculated by dividing the concentrations of the influenza A or influenza B by the concentration of PMMoV.

2.2.5 Partitioning of influenza A, B and RSV in wastewater

The viral partitioning of influenza A, influenza B, RSV and SARS-CoV-2 RNA in wastewater was conducted on an influent wastewater sample, collected on January 10, 2024, from the Kitchener WWTP (Figure 2). The timing of the experiment was selected to be during a surge of influenza A although the wastewater sample was also positive for influenza B, RSV and SARS-CoV-2 (N2). The sample was collected using a 24-h (4°C) autosampler composite and collected in a sterile 1 L HDPE bottle. The

sample was split into 40 mL wastewater aliquots with the bottle shaken and mixed before each pour to ensure representative replicates. The aliquots were randomly split into three treatment groups, each consisting of six biological replicates. The treatments were based on typical methods used to isolate viruses, similar to that done by Breadner et al., (2023).

Treatment Group 1 settings were chosen based on SARS-CoV-2 RNA wastewater literature methods tailored to target the liquid fraction (supernatant) (Ahmed et al., 2020; Chik et al., 2021; Torii et al., 2022). The sample was centrifuged at 4,000g for 10 min with deceleration (i.e., with brake; decel 9) and both the pellet and supernatant quantified. Treatment Group 2 settings were selected to have the same centrifugation force and time (12,000g for 1.5 h with no deceleration) as Treatment Group 3 but without the addition of overnight PEG precipitation. Treatment Group 3 was based on the routine wastewater surveillance project (Servos Lab, Ontario Wastewater Surveillance Initiative) as described above (see details in Section 2.2.2.). After centrifugation the supernatant (40 mL) in Treatment Group 1 and 2 were processed with a 10K Centricon Plus-70 device (MilliporeSigma) as described by Breadner et al., (2023). The recovered material (i.e., retentate) and centrifuged solids (i.e., pellet) were analyzed as described above and all RNA extracts quantified using RT-qPCR for influenza A, influenza B, and RSV. SARS-CoV-2 (N2) was also quantified to contrast the results obtained for the other three targets and against the literature (e.g., Breadner et al., 2023). All samples were tested for inhibition using the MS2 assay and no qPCR inhibition was detected. Comparisons among treatments for each target were analyzed with a one-way ANOVA ($p<0.05$) using copies/mL based on equivalent 40 mL starting volume. Additionally, the total (combined supernatant plus pellet by treatment) and PEG were compared with another one-way ANOVA ($p<0.05$). Pairwise comparisons were made using Tukey's post-hoc test. The assumption of normality was tested by visually assessing quantile-quantile plots, histograms and performing Shapiro-Wilk test and the assumption of homoscedasticity was assessed by performing Levene's test. All statistical analysis was conducted using R version 4.2.2.

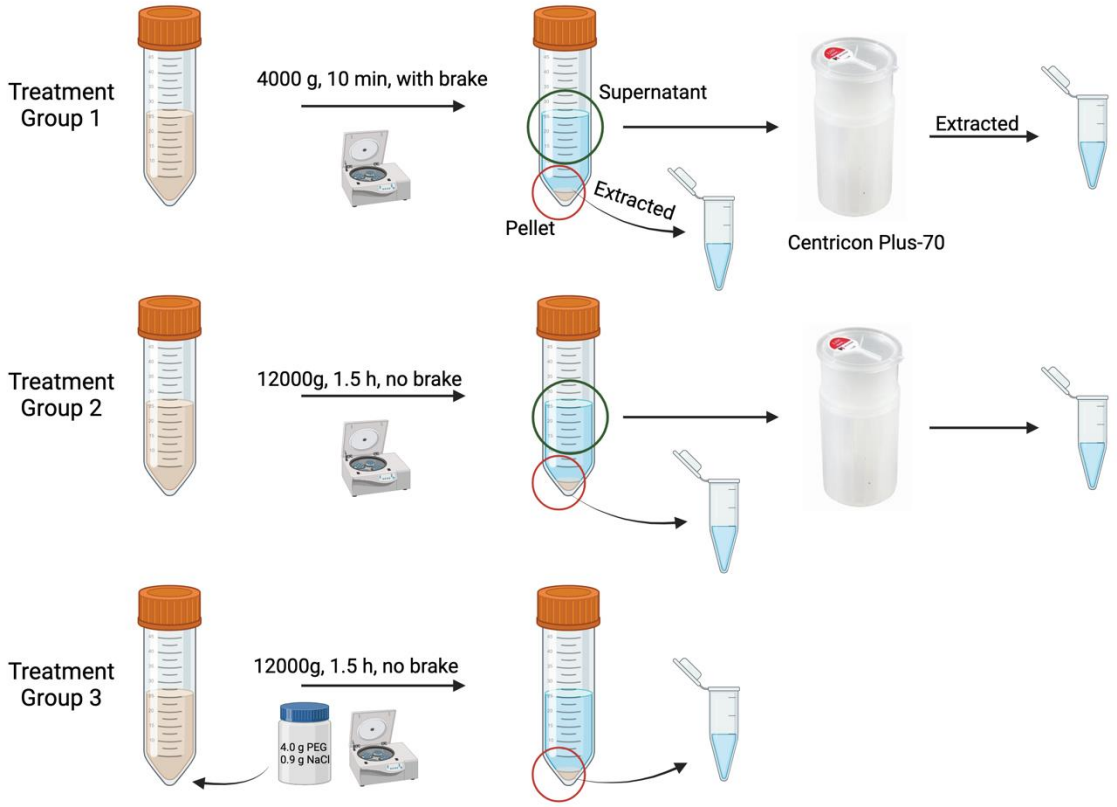


Figure 2. Summary of the viral RNA partitioning experiment setup. Figure created with BioRender.com.

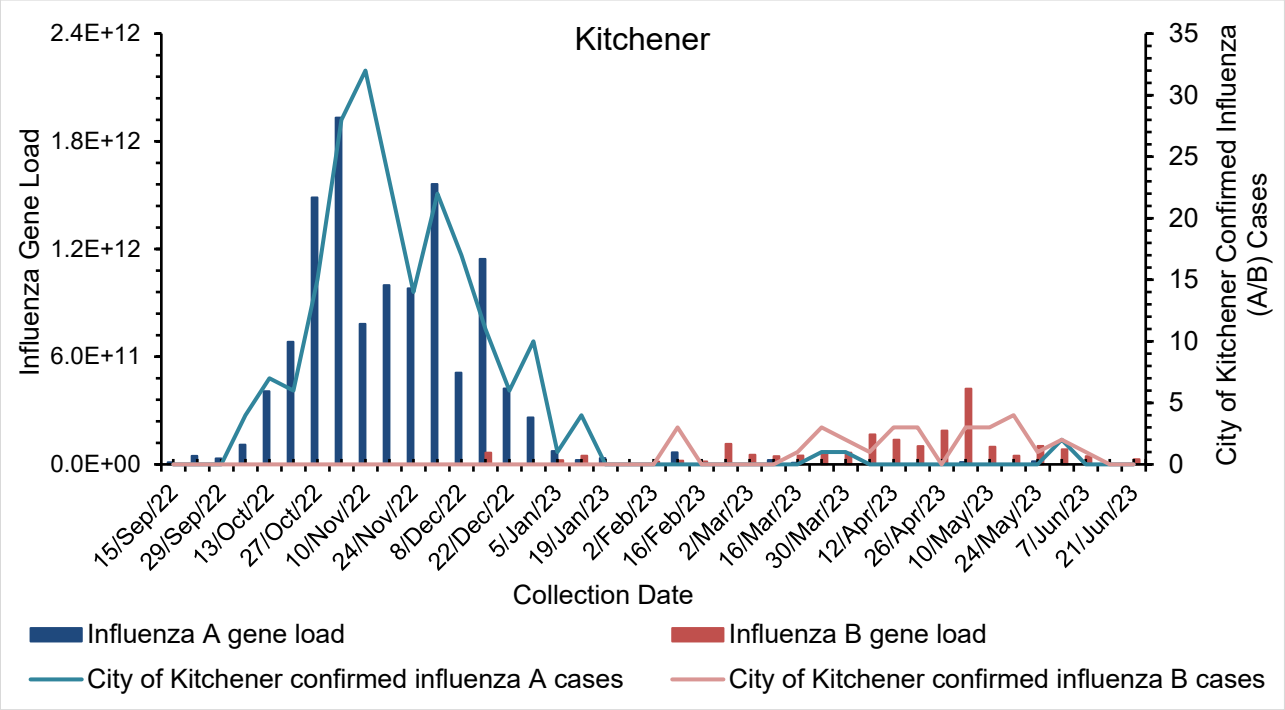
2.3 Results

2.3.1 Influenza A and B trends in wastewater relative to clinical cases

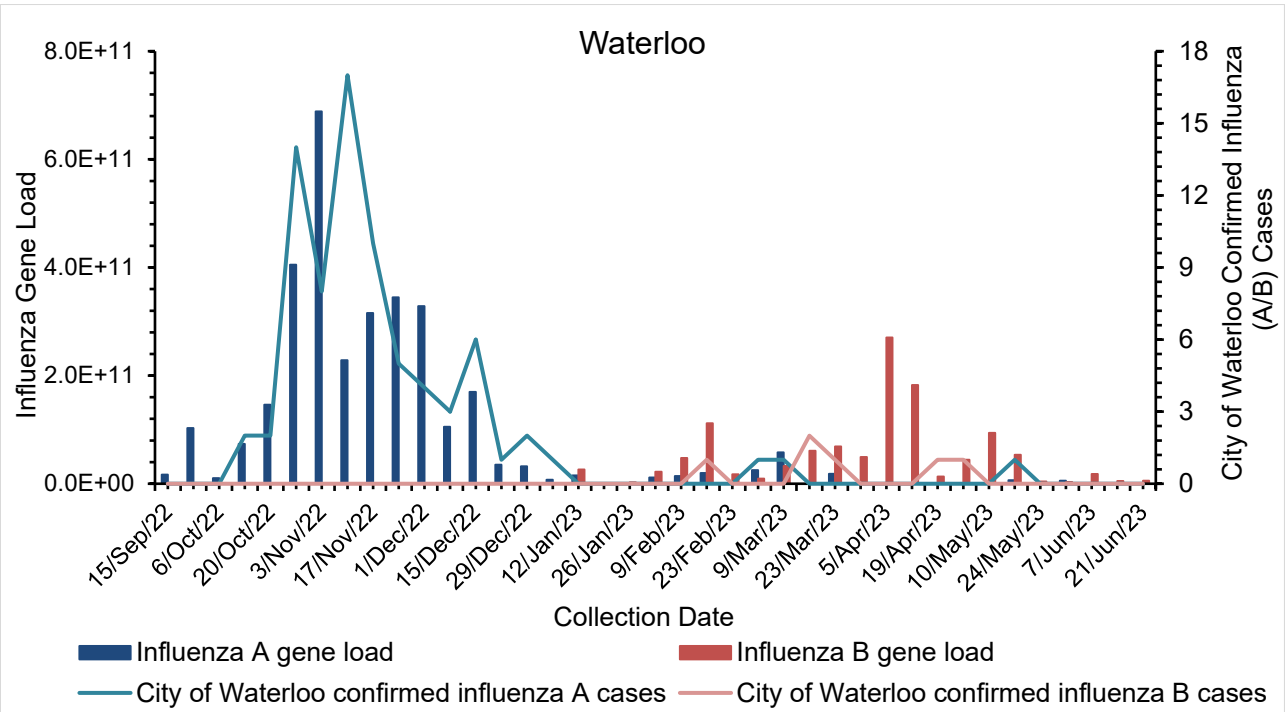
Influenza A was detected in trace amounts in wastewater on the first sampling date (September 15, 2022) at both Waterloo and Kitchener WWTPs (Figure 3). The influenza A signal in wastewater began to increase in October 2022 then peaked on November 3, 2022, and began to decline in late December 2022 into January 2023, with only trace amounts detected in February and March 2023, at both sampling sites. The influenza A wastewater wave coincided with the clinical cases seen in the cities of Kitchener and Waterloo and followed the general trends observed throughout the influenza A season (Figure 3). Influenza B was first detected in wastewater in mid-December 2022 at the Kitchener WWTP and in mid-January 2023 at the Waterloo WWTP. The influenza B signal in wastewater peaked on April 5, 2023, in Waterloo and on May 3, 2023, in Kitchener. The signal at both sites trickled down in late May into June

2023. The detection of influenza B also corresponded to the clinical cases documented in both cities (Figure 3).

The concentrations of influenza A and influenza B in wastewater over the span of the 2022-2023 season were compared to clinical cases (cases by episode date) broken down by city (Waterloo and Kitchener). The RNA concentrations in copies/mL were adjusted for flow at both wastewater sites, to represent the RNA copies in mass load (gene copies/d). The trends of both influenza A and B clinical cases, reported as cases by episode date, not only generally followed the trends of the wastewater load for both the Kitchener and Waterloo sites (Figure 3) but also statistically correlated, with sampling for this study limited to once weekly from September 15, 2022, to June 21, 2023. The relationship between the wastewater data and clinical cases for both influenza targets at both sites resulted in strong and significant correlations ($p < 0.008$; full results in Table A1). The influenza A correlations were the strongest, with a Spearman's rank correlation coefficient (r) of 0.77 at the Kitchener site and (r) of 0.83 at the Waterloo site. Influenza B also demonstrated strong and significant correlations with a Spearman's rank correlation coefficient ($r = 0.67$) at the Kitchener site and ($r = 0.43$) at the Waterloo site. Although the quantification of influenza B from the wastewater was predominantly below the limit of detection (LOD) and limit of quantification (LOQ) values there was a strong correlation with clinical cases. As previously mentioned, RSV is not a reportable disease in the Province of Ontario and as such case data was not available to perform similar analysis.


Kitchener
Influenza Gene Load
City of Kitchener Confirmed Influenza (A/B) Cases
2.4E+12
1.8E+12
1.2E+12
6.0E+11
0.0E+00
35
30
25
20
15
10
5
0
15/Sep/22
29/Sep/22
13/Oct/22
27/Oct/22
10/Nov/22
24/Nov/22
8/Dec/22
22/Dec/22
5/Jan/23
19/Jan/23
2/Feb/23
16/Feb/23
2/Mar/23
16/Mar/23
30/Mar/23
12/Apr/23
26/Apr/23
10/May/23
24/May/23
7/Jun/23
21/Jun/23
Collection Date
Influenza A gene load
Influenza B gene load
City of Kitchener confirmed influenza A cases
City of Kitchener confirmed influenza B cases

(I)


Waterloo
Influenza Gene Load
City of Waterloo Confirmed Influenza (A/B) Cases
8.0E+11
6.0E+11
4.0E+11
2.0E+11
0.0E+00
18
15
12
9
6
3
0
15/Sep/22
6/Oct/22
20/Oct/22
3/Nov/22
17/Nov/22
1/Dec/22
15/Dec/22
29/Dec/22
12/Jan/23
26/Jan/23
9/Feb/23
23/Feb/23
9/Mar/23
23/Mar/23
5/Apr/23
19/Apr/23
10/May/23
24/May/23
7/Jun/23
21/Jun/23
Collection Date
Influenza A gene load
Influenza B gene load
City of Waterloo confirmed influenza A cases
City of Waterloo confirmed influenza B cases

(II)

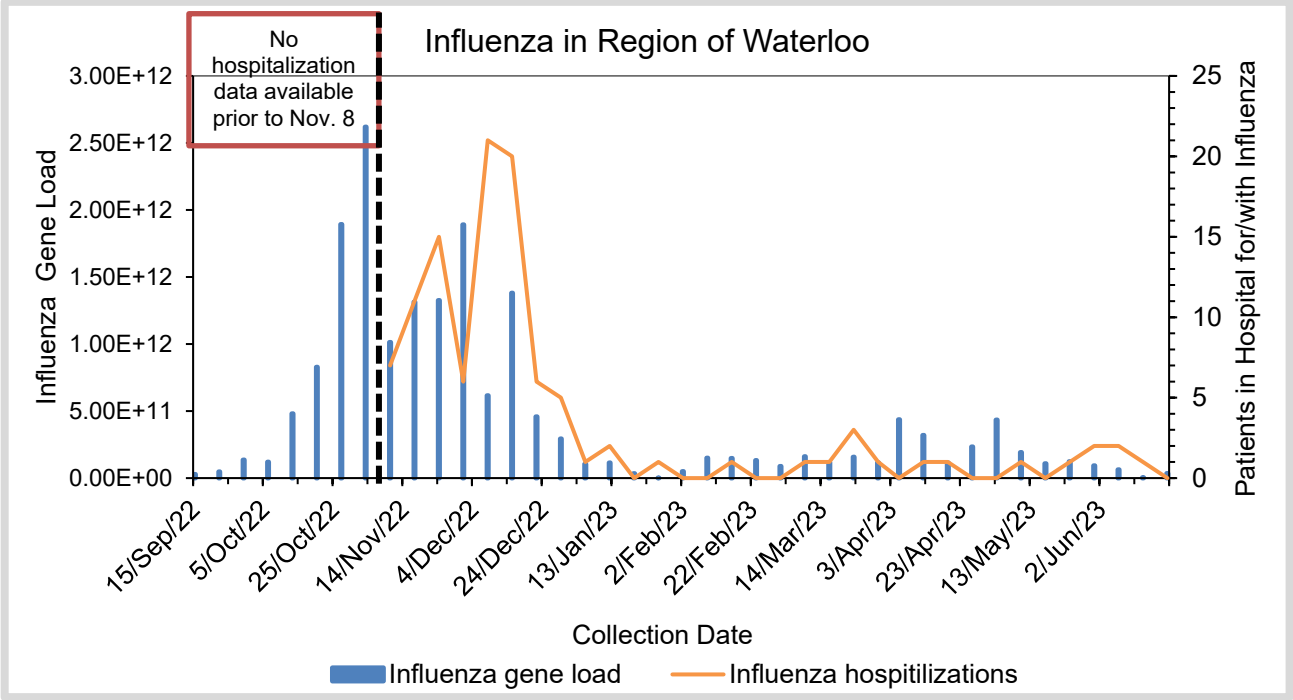
Figure 3. Weekly influenza A and B wastewater load (gene copies/d) at Kitchener (I) and Waterloo (II) plotted against confirmed influenza A and B cases by episode date.

2.3.2 RSV (A/B) and influenza (A/B) trends in wastewater overtime relative to hospitalizations

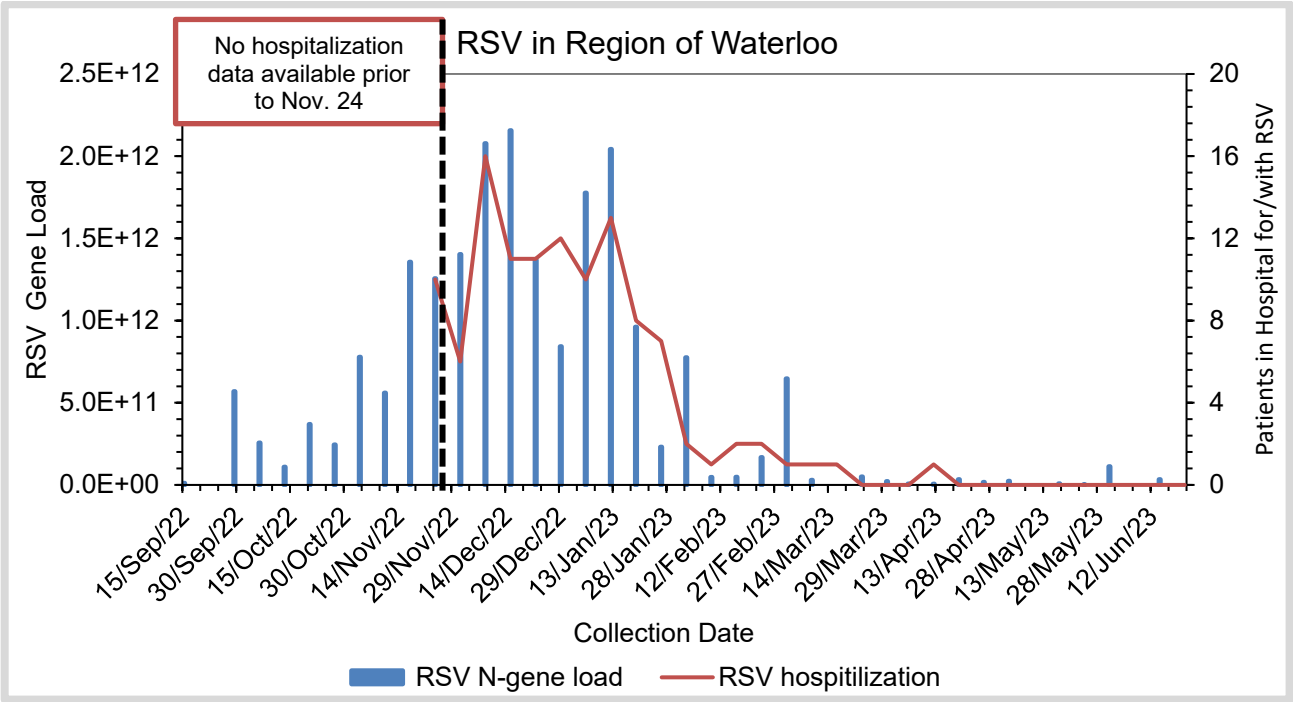
The hospitalization data provided was not broken down by city (Kitchener and Waterloo) like the reported influenza cases but encompassed the entire Region of Waterloo. The hospitalizations consisted of all patients that were admitted to Cambridge Memorial Hospital, Grand River Hospital, and St. Mary's General Hospital for or with influenza and/or RSV, so the data included individuals hospitalized from within and outside the cities of Kitchener and Waterloo. As a result of the data not broken down by city, the wastewater RNA (copies/d) were added at both sites (Kitchener and Waterloo WWTPs) to represent the Region of Waterloo, after adjusting for flow at each WWTP. This analysis was done for both influenza and RSV. Additionally, influenza hospitalization data was also not broken down by virus type (A/B), therefore the wastewater RNA copies (influenza A, B) were added together to represent total influenza load (copies/d) for the Region of Waterloo. Influenza hospitalization data was available starting November 8, 2022, and RSV hospitalization data was available starting November 24, 2022. Therefore, the correlation analysis was conducted starting on those dates until sampling ended on June 21, 2023. The lack of hospitalization admissions data prior to those dates is not a reflection that hospitalizations were not present beforehand, but a lack of monitoring and data accessibility.

The RSV signal in wastewater at both treatment plants had a similar wave to influenza A. Trace amounts of RSV in wastewater was detected in October 2022, with an increase in signal in November 2022 and the peak signals observed from mid-December 2022 to mid-January 2023 and a decline in February 2023, with only trace amounts detected in March 2023 (Figure 4). Influenza and RSV were detected in the wastewater in early October 2022 while hospitalizations were not reported till November 8, 2022, for influenza, and till November 24, 2022, for RSV. The start of the available hospitalization data (November 8, 2022, for influenza and November 24, 2022, for RSV) followed the general trends seen in the wastewater data for both viruses. Even with the incomplete hospitalization data and sampling limited to once weekly, the correlations between the wastewater data and hospitalizations for both targets revealed strong and significant relationships ($p < 0.02$; full results in Table A1), with Spearman's rank correlation coefficients of ($r = 0.85$) for RSV and ($r = 0.53$) for influenza. The wastewater data suggests

that the beginning of the RSV wave was missed as it started earlier in the 2022-2023 season, as wastewater data was showing a general increase in signal before hospitalization data was made available. However, this was not the case for influenza, as unlike RSV it is a reportable disease in the province, thus Public Health Units do not rely solely on hospitalizations to monitor, as they also have access to case data.



(I)



(II)

Figure 4. Weekly influenza (I) and RSV (II) wastewater load (copies/day) plotted against patients currently in hospital for/with influenza or RSV respectively in the Region of Waterloo. The RSV and influenza wastewater viral loads represent the copies/day combined from the Kitchener and Waterloo wastewater samples to represent the region. The influenza load and hospitalizations are not broken down by type. Influenza hospitalization data was available starting November 8th, 2022, and RSV hospitalization data was available starting November 24th, 2022.

2.3.3 PMMoV normalization

Normalization using PMMoV as a biomarker did not increase the correlation with clinical data relative to the raw influenza A or B signals in wastewater. The Spearman's rank correlation coefficients for the normalized influenza A signal at the Kitchener and Waterloo sites were ($r = 0.76$ and $r = 0.85$, respectively), in comparison with the raw influenza A signal that had correlations of ($r = 0.77$ and $r = 0.83$, respectively). The Spearman's rank correlation coefficients for the normalized influenza B signal at

the Kitchener and Waterloo sites were (r = 0.67 and r = 0.42, respectively), in comparison with the raw influenza B signal that had correlations of (r = 0.66 and r = 0.43, respectively).

2.3.4 Viral partitioning

Partitioning experiments were conducted January 10, 2024, after confirmation that the wastewater sample was positive and quantifiable for influenza A, influenza B, RSV, and SARS-CoV-2. The treatment groups differed in their concentration methods (centrifugation, ultrafiltration, PEG precipitation) and centrifugation settings (4,000g, 10 min, with brake or 12,000g, 1.5 h, no brake) (Figure 5). Both RSV and influenza B were detected at relatively low levels in the sample tested.

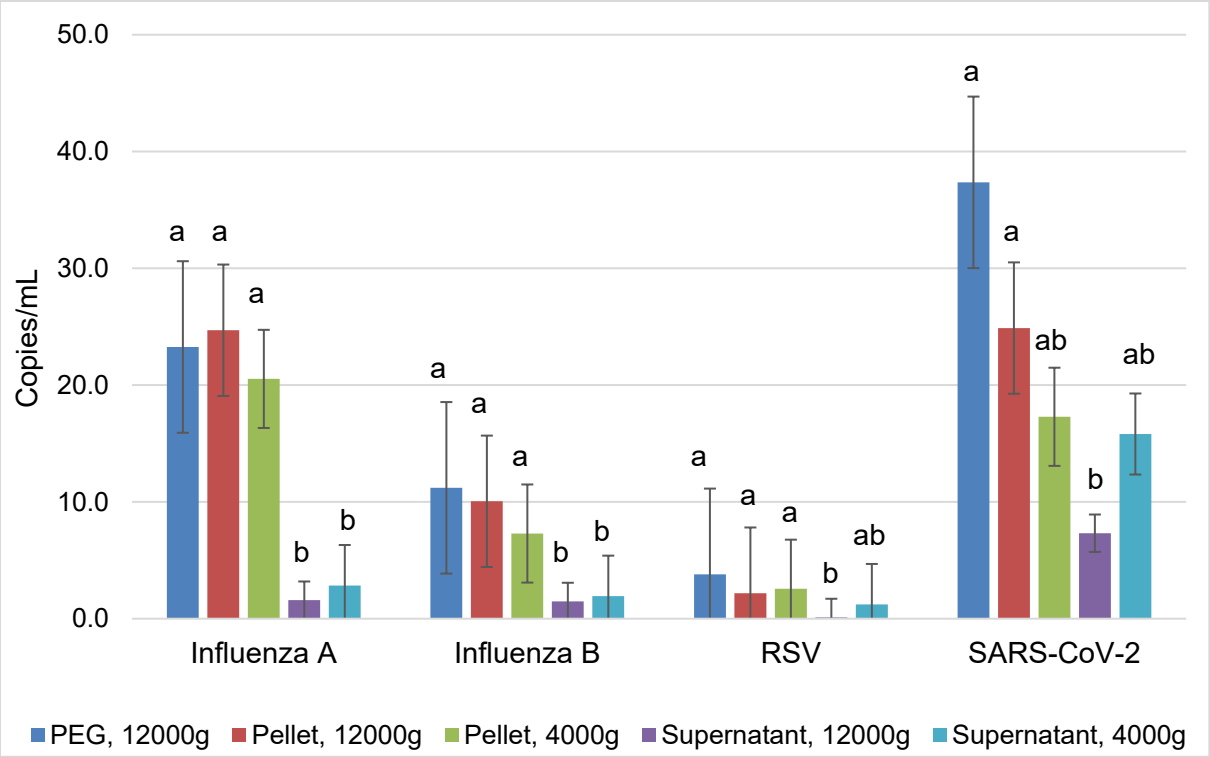


Figure 5. The average RNA concentrations (copies/mL based on equivalent 40 mL starting volume) of influenza A, influenza B, RSV and SARS-CoV-2 relative to one another under different enrichment conditions. The N2 signal is divided by 10 to better visualize the trends relative to the other targets. All Statistical Comparisons (anovas) were done only within viral targets, nothing between the targets.

The one-way ANOVA performed on the RNA copies for each treatment group resulted in significant differences between all the supernatant treatment groups and all the pellet treatment groups (including PEG) for influenza A and influenza B ($p < 0.05$). As for RSV the one-way ANOVA performed on the RNA copies for each treatment group resulted in significant differences between the supernatant treatment groups and the pellet treatment groups (including PEG) ($p < 0.05$), except between the 4,000g Supernatant -12,000g Pellet and 4,000g Supernatant-4,000g Pellet. Lastly for N2 the one-way ANOVA performed on the RNA copies for each treatment group resulted in significant differences between the supernatant treatment groups and the pellet treatment groups (including PEG) ($p < 0.05$), except between the 4,000g Supernatant -12,000g Pellet and 4,000g Supernatant-4,000g Pellet with the PEG 12,000g treatment also having significant differences with the 4,000g Pellet (Figure 5) (full results in Table A3).

In Treatment Group 1 (4,000g, 10 min, with brake) 88.0 % of the influenza A signal, 79.3 % of the influenza B signal and 68.4 % of the RSV signal were in the solids fraction, in comparison with 52.2 % of the N2 signal. Treatment Group 2 (12,000g, 1.5 h, no brake) 93.9% of the influenza A signal, 87.1 % of the influenza B signal and 96.4 % of the RSV signal were in the solids fraction, in comparison with 77.3 % of the N2 signal. Finally, Treatment Group 3 (12,000g, 1.5 h, no brake, with PEG precipitation) in comparison with the non-PEG precipitated samples did not significantly improve recovery for influenza A, influenza B and RSV, in comparison with N2 that had up to a threefold increase in signal. The increased centrifugal settings for all targets resulted in an increase in signal in the solids fraction. The influenza and RSV partitioned overwhelmingly in the solids fraction, unlike SARS-CoV-2 that had an approximately 50/50 split between the solid and liquid fractions (Figure 6).

The total RNA copies of the liquid and solid fractions of each treatment group were combined to represent the total wastewater sample for each of the targets (influenza A, influenza B, RSV and N2), to test if there any significant differences between the estimated overall signal for each treatment group. The one-way ANOVA performed for the total copies for each viral target did not show any significant differences ($p > 0.862$, 0.473. 0.349 and 0.672, respectively) (Fig. 6; full results in Table A2).

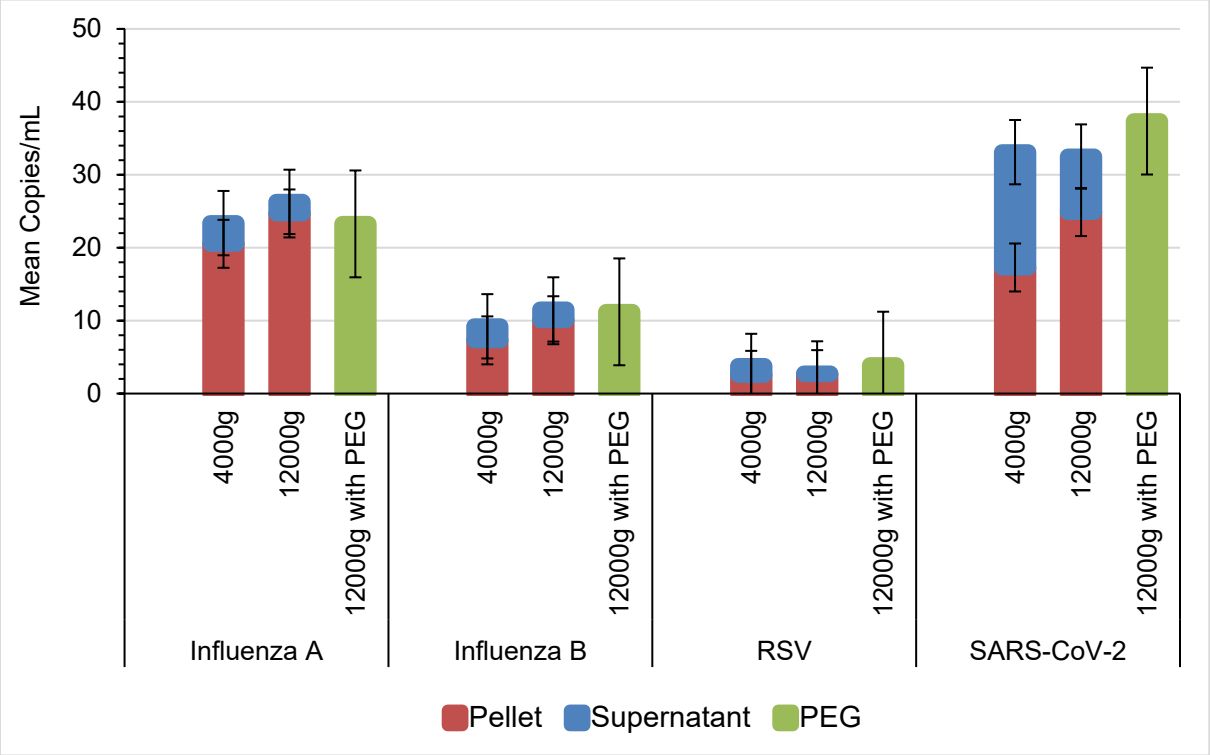


Figure 6. Effects of concentration methods and centrifugal settings on the partitioning of influenza A, influenza B, RSV and SARS-CoV-2 (N2) RNA in wastewater. The N2 signal is divided by 10 to better visualize the trends relative to the other targets.

2.4 Discussion

The 2022-2023 respiratory season showcased that influenza and RSV are optimal targets for WBS, due to their shedding characteristics and their healthcare burden. The success of COVID-19 wastewater surveillance (D'Aoust et al., 2021; Dhiyebi, Cheng, et al., 2023) has led to interest in adapting this approach for additional respiratory viruses (Mercier et al., 2022; Dumke et al., 2022; Hughes et al., 2022), to inform public health action. It has been demonstrated that respiratory viruses such as influenza A, influenza B and RSV can be shed into wastewater and detected in downstream treatment facilities (Ahmed et al., 2023; de Melo et al., 2023; Vo et al., 2023; Wolfe et al., 2022). The strong association of these viruses with organic solids in wastewaters allows for methods targeting solids to be very effective in isolating and concentrating them. The use of a PEG precipitation and centrifugation method (also used in SARS-CoV-2 surveillance) demonstrated the potential utility of wastewater surveillance for monitoring

influenza A, B and RSV in southern Ontario. The influenza A and RSV wastewater signals peaked in early November, while influenza B was lower and peaked much later in the spring (April-May) (Figures 3-4). The RNA concentrations in wastewater for all three viruses of interest exhibited strong and significant correlations ($p < 0.05$) with clinical metrices (cases reported and hospitalizations). Influenza and RSV were detected in wastewater prior to the clinical/hospitalizations data being available, specifically for RSV due to data limitations. This case study highlights some of the advantages and limitations of WBS but also demonstrated the potential of wastewater surveillance of respiratory viruses as the data was integrated into routine reporting to the local public health unit.

2.4.1 Wastewater surveillance of respiratory viruses

Wastewater surveillance of influenza A, influenza B and RSV was successful as the RNA concentrations in wastewater for all three viruses had strong and significant correlations ($p < 0.05$) with cases reported and hospitalizations. The influenza A signal in wastewater had very strong correlations with clinical metrices in this study, with both cases reported and hospitalizations (Spearman's rank correlation coefficients of ($r = 0.53$ to 0.83). This is a rapidly moving field, since the initiation of this case study wastewater surveillance of influenza A has been shown to be very successful in numerous other studies, with very strong associations between the influenza A RNA concentrations and clinical metrices (Ando et al., 2023; Bhavanam et al., 2023; de Melo et al., 2023; Dumke et al., 2022; Mercier et al., 2022; Toribio-Avedillo et al., 2023; Wolfe et al., 2022). The influenza A wastewater correlations with clinical metrices is very similar to what was seen with SARS-CoV-2 wastewater surveillance. The relatively higher concentrations of influenza A in wastewater in comparison with influenza B and RSV and its' typically more established clinical monitoring system in comparison have aided in demonstrating its' effectiveness.

Several studies that monitored influenza A and B in wastewater had substantial lower detection rates for influenza B or none at all. This could potentially be due to the low prevalence rate of the disease in communities (Ahmed et al., 2023; Boehm et al., 2023; Hirose et al., 2016; Mercier et al., 2022). Influenza B typically peaks later in the season after the initial waves of influenza A, with a lower community case count (Zhou et al., 2019). The first influenza B detection was seen in Kitchener in late-December and in Waterloo in mid-January, coinciding with the declining detection of influenza A. The lower-case counts were reflected in the wastewater signals at both sampling sites. The highest influenza B quantification was seen at the Kitchener site with a concentration of approximately 5 copies/mL, which correlated with the highest case load reported of four. Only three samples from each site (Kitchener and Waterloo) had

concentrations above the limit of detection and only two had detections above the limit of quantification. As a result, the Spearman's rank correlation coefficients of (r = 0.67) at the Kitchener site and (r = 0.43) at the Waterloo site are very strong considering the circumstances of the low detection and quantification and the low community infection incidence. There is a shortage of literature available on the quantification of influenza B in wastewater and its 'correlation with clinical metrices.

Clinical testing protocols for influenza have changed from year to year, but as clinical testing in the Region of Waterloo is now coupled with COVID-19 cases more clinical influenza cases are being detected (S. Drew, Region of Waterloo, personal communication). The wastewater signals for influenza and RSV were both showing a general increase before hospitalizations. The start of the recorded hospitalization data coincides with the peak of the viral signal observed in wastewater, this leaves no room for public health to take preventive actions, especially for RSV. The Public Health Unit of Waterloo does not rely on influenza hospital admissions to monitor the influenza waves and the circulating type and subtype, as they have access to clinical cases (cases by episode date) (M. Vythilingam, Region of Waterloo, personal communication). However, unusual surges such as the one detected in the spring of 2022 by Mercier et al., (2022) was not thoroughly documented by PHUs but was captured by wastewater surveillance. Situations that require additional public health guidance for testing and treatment would greatly benefit from WBS. The introduction of strains that are not covered by vaccines, or new influenza strains that are introduced at the animal–human interface with potentially severe public health consequences are examples of such situations (Wolfe et al., 2022). WBS was applied to the wild and vaccine-derived polioviruses in the early 2000s that were detected and tracked using WBS, which could have potentially also aided in previous influenza pandemics.

The wastewater data regarding hospitalizations only represents 66% of the population in the Region of Waterloo. Although the correlations conducted between the wastewater data for both influenza and RSV were strong and significant (Spearman's rank correlation coefficients (r = 0.53 and 0.85, respectively) it still does not encapsulate the entire communities' infection prevalence. Ideally a more comprehensive analysis with hospitalizations should be conducted that encapsulates as much of the population of the region as possible, which would require incorporating other WWTPs. However, there are numerous WWTPs therefore it is not practical to be able to represent the entire Region of Waterloo. Additionally, not all households are present in the sewer catchment serviced by WWTPs, as a portion of the population is serviced by on-site sewer systems such as septic tanks, which would also not be included in the samples collected from WWTPs. Thus, a more ideal solution would be to incorporate more WWTPs that would

encompass a wider range of a region such as the Galt WWTP (91,500), Hespeler WWTP (26,300) and Preston WWTP (24,600) which would cover a greater area in the Region of Waterloo. This inclusion would increase coverage to 79% of the Region of Waterloo and would represent the hospitalizations data collected from Cambridge Memorial Hospital that is included in the hospitalizations data but was not reflected in the wastewater data.

The success of wastewater surveillance of RSV was also seen in other studies, with strong associations between the RSV RNA concentrations and different clinical metrices (hospitalizations and clinical cases) (Hughes et al., 2022; Mercier et al., 2023). The early RSV detection in wastewater and the strong correlation with hospitalizations was reported by Mercier et al., (2023) in two cities in Ontario, Canada (Ottawa and Hamilton). The wastewater data had a lead time of 12 days in comparison with the pediatric RSV hospitalizations data. The RSV wastewater data preceded the clinically determined provincial start date in the city of Ottawa by 36 days and the city of Hamilton by 12 days. However this was an unusual RSV season after the COVID-19 restrictions were lifted in the province with RSV circulating earlier than in typical seasons prior to the pandemic. Ahmed et al., (2023) in Queensland, Australia also had a significant lead time in RSV detection (17 days) in wastewater in comparison with RSV clinical cases. They also had strong Spearman's rank correlations of ($r = 0.39$ to 0.95) between RSV (A and B combined) individual WWTP catchments and clinical RSV cases. RSV is a notifiable disease in Queensland, Australia therefore they had access to clinical RSV cases to conduct their study, unlike in Ontario where RSV is not a reportable disease in the province, thus only hospitalizations are available. RSV is a reportable disease to public health officials in some locations in the United States, but not all (Hughes et al., 2022). Hughes et al., (2022) in the state of California also successfully detected RSV in the wastewater ahead of clinical cases and showcased strong correlations of concentration of RSV in settled solids of wastewater (Kendalls tau = 0.65–0.77, $p < 10–7$) with clinical positivity rates for RSV at sentinel laboratories in the state. These studies support the notion that monitoring RSV in wastewater can provide a comprehensive picture of disease transmission at the community level, particularly in provinces/states that classify RSV as a non-reportable disease (and therefore clinical data is not available).

RSV primarily causes mild to moderate respiratory illness, with the severity and spread of the disease between RSV A and B being quite similar (Ciarlitto et al., 2019). The subtyping of RSV in wastewater samples may be beneficial but not necessary to inform public health action. The application of various assays needs to be assessed carefully in terms of sensitivity and specificity to ensure comparability of the

results. Although, an RSV assay can be multiplexed this needs to be assessed thoroughly with the overall surveillance objective in mind. All assays in this study were run as simplex assays to ensure sensitivity and avoid potential cross reactivity (although this could be achieved in a well-designed multiplex assay). RSV is not a reportable disease in Ontario, despite its potential for hospitalizations, particularly in infants, and its impact on emergency department utilization. In the absence of clinical data, WBS can provide valuable information to public health as an independent indicator of community spread and a leading indicator of hospitalizations.

The seasonal pattern of RSV shifts from year to year across Ontario, as was observed in the earlier years of the COVID-19 pandemic. The COVID public health measures put in the first two years of the pandemic may have altered the prevalence of various respiratory diseases including RSV, in comparison with the 2022-2023 respiratory season when mandates had been lifted (Bourdeau et al., 2023; Zulli et al., 2024; Zven et al., 2023). WBS has the potential to be particularly useful in monitoring the different transmission patterns observed each respiratory season as well as capturing unusual seasonal trends like the one observed during the 2022-2023 respiratory season. Moreover, as RSV in this study was detected in wastewater overwhelmingly before any increases in associated hospitalizations, it can be used in the future to inform evidence-based decision-making for public health interventions. Hence, if an increase in signal is observed, public health officials can implement measures such as public health messaging, timely vaccination, or other preventive strategies to mitigate the impact of RSV on the population (M. Vythilingam, Region of Waterloo, personal communication). In conclusion, early detection of RSV through wastewater surveillance is possible and can help ensure that the healthcare system is better prepared for future waves, as it is not a reportable disease in the province of Ontario and thus lacks an established reporting system.

Hospitalization data for RSV in Waterloo Region was provided from the Ministry of Health's Capacity Planning and Analytics Division (CPAD). The 2022-2023 respiratory year, the RSV hospitalizations data was more accessible to The Region of Waterloo Public Health and Emergency Services (S. Drew, Region of Waterloo, personal communication). PHUs have access to different levels of clinical data when it comes to RSV, as even though the RSV hospitalizations data in the Region of Waterloo in the 2022-2023 respiratory year was more accessible in comparison with previous years it was still not complete with the start of hospitalization data availability on November 8, 2022 for influenza and November 24, 2022 for RSV (S. Drew, Region of Waterloo, personal communication). WBS would allow the various PHUs across the country to have access to a baseline monitoring system that can inform them of their

communities' infection incidence. The Ontario Wastewater Surveillance Initiative has recently shifted (i.e., 2024) to utilize the tools developed for the SARS-CoV-2 program to detect and monitor influenza and RSV within communities.

As viral signals in wastewater are highly variable, there have been efforts to use other biomarkers (e.g., chemicals, bacteria, or viruses) to normalize the data to better detect trends (Dhiyebi, Abu Farah, et al., 2023; Tandukar et al., 2020). PMMoV is often recommended for use as a normalizing biomarker, due to its stability in wastewater and its association with human feces. Although PMMoV has high and relatively stable concentrations in wastewater, its' diet dependency and seasonal variability poses challenges for its use for normalization of viral targets (Dhiyebi, Abu Farah, et al., 2023; Greenwald et al., 2021). Normalization using PMMoV as a biomarker in this study did not increase the correlation with clinical testing data relative to the raw influenza signal in wastewater, for either influenza A or B at either sampling site. Other studies have concluded that the raw viral signal of SARS-CoV-2 often has stronger correlations with clinical testing data than the normalized signal, especially when flow rates are relatively stable, possibly due to the additional variability introduced by extra viral target (Dhiyebi, Abu Farah, et al., 2023; Feng et al., 2021). Hughes et al., (2022) reported that the correlation of RSV in wastewater to clinical data was not improved or hindered by PMMoV normalization, as was seen in this study here with the influenza A and influenza B signals. The effectiveness of fecal normalization may be dependent on the characteristics of the sewershed and wastewater (Dhiyebi, Abu Farah, et al., 2023) The use of fecal biomarkers for normalization in sewersheds, such as Kitchener and Waterloo WWTPs, with relative stable wastewater flows (e.g., minimal infiltration, etc.) may offer no advantage and may in fact add additional variability (Dhiyebi, Abu Farah, et al., 2023). The normalization of influenza A data in the current study did not improve the correlations with the cases by episode date reported. However, fecal biomarkers and normalization of viral targets may be more applicable in systems that do not have stable wastewater flow rates, fecal inputs, or sampling practices that create more variability (grab vs. composites). The fecal biomarker chosen needs to behave similarly to the target to account for the loss or dilution of signals in sewers, sample collection/storage, and laboratory processing (Greenwald et al., 2021). Although CrAssphage has been used in many studies (Dhiyebi, Abu Farah, et al., 2023; Tandukar et al., 2020; Wilder et al., 2021), it is a DNA virus while influenza and SARS-CoV-2 are RNA viruses. PMMoV is rod-shaped, non-enveloped single-stranded RNA virus (Symonds et al., 2018) that has been shown to differ in its partitioning behaviour relative to the enveloped coronaviruses (Breadner et al., 2023). Although normalization has many potential advantages, including being a good process control, it

may introduce additional variability when used for signal normalization (Dhiyebi, Abu Farah, et al., 2023; Greenwald et al., 2021).

2.4.2 Wastewater partitioning of respiratory viruses.

Understanding the apparent partitioning of viruses in wastewater can support the development of appropriate and sensitive methods and aid in the interpretation of wastewater viral data. Optimizing the viral concentration step allows for consistent and sensitive detection of the viruses of interest. The apparent partitioning of influenza A, influenza B and RSV in wastewater was affected by the concentration methods used (e.g., centrifugation speed, filtration). The apparent partitioning of SARS-CoV-2 (N2) included in this study for comparison (sample from Kitchener, January 2024) was consistent with that reported previously by Breadner et al., (2023). The SARS-CoV-2 (N2) signal in Treatment Group 1 (4,000g, 10 min, with brake) paralleled their findings, with the signal approximately spilt 50/50 between the solid and liquid fractions. Centrifugation at a higher speed and longer time increased the amount of SARS-CoV-2 detected in the pellet (with a corresponding decline in the amount detected in the supernatant) such that 77% was found in the pellet (based on total mass). When the two fractions are added, the totals are not significantly different, suggesting that the observed shift are not an artifact. In contrast, Breadner et al. (2023) showed a more even split between the pellet and supernatant fractions, even with an increase in centrifugal force (12,000g). The pre-treatment of the sample with overnight PEG/NaCl precipitation increased the amount detected in the pellet but not to the 2-3-fold previously reported by Breadner et al. (2023). These subtle differences between studies could potentially be related to the variability among samples as wastewater is a complex matrix that can change between sites as well temporally.

Several studies have previously suggested that many viruses, including SARS-CoV-2 are strongly associated with particles, with reported K_d (concentration liquid/concentration solids) ranging from 350-4100 mL/g (Graham et al., 2021; Roldan-Hernandez & Boehm, 2023). Roldan-Hernandez & Boehm, (2023) centrifuged influent samples at 24,500g for 20 min, then extracted 200 µL of the supernatant directly. They reported mean K_d from six treatment plants ranging from 3000-12,000 (mL/g dry weight). Considering that there were approximately 150 mg/mL solids (wet weight) in the Kitchener influent the K_ds are similar. B. Li et al., (2021) reported 82.5-92.5% of the mass of SARS-CoV-2 being in the solid phase after centrifugation (13,000g, 30 min), but they assessed the supernatant concentration using PEG precipitation which is likely less efficient than the ultrafiltration (Centricon-70) used in this study. Kim &

Boehm, (2023) compared the solid phase (pellet after settling and centrifugation at 24,000g 30 min) to the supernatant (after centrifugation at 4,100g 10 min) measured with an 0.45 µm electronegative membrane. Although they reported that the SARS-CoV-2 was predominantly in the solids the use of a relatively large pore filter may not have efficiently captured the virus in the supernatant. Many studies have reported strong recovery of SARS-CoV-2 in sludge (high solids content) which is consistent with a strong affiliation of this virus with solids (Graham et al., 2021). Differences in the wastewater across sites and time, sample handling, and concentration protocol will alter the apparent partitioning of SARS-CoV-2 and other viruses. Despite the large number of studies conducted on the detection of SARS-CoV-2 RNA in wastewater, the methods for concentration and extraction of viral RNA and subsequent quantification are yet to be standardized (Chik et al., 2021). However, a major proportion of the SARS-CoV-2 viral signal remains in the supernatant based on total mass (as the mass of solids is proportionally much lower than the mass in the solution) which may be why such a diversity of methods appear to work well to quantify SARS-CoV-2 in wastewater influents. As the concentration of solids increases, such as in sludge relative to influent, the proportion in the solids phase will increase and therefore the mass of the SARS-CoV-2 detected. Comparisons are often done based on concentration without consideration of the solids' concentration in the original sample (i.e., mass), making it appear that most of the virus is in the solids when in fact a major proportion can be in the liquid phase. Typically, the Kitchener effluent has <150 mg/mL of solids (wet weight). In the current study, assuming 20% solids in the pellet, the K_d for SARS-Cov-2 would be 1960 mL/g which is similar to that reported in other studies.

The influenza A partitioning findings from this study are similar to the findings of Mercier et al., (2022) that suggest that influenza A strongly partitions to the solids fraction. Several studies have suggested that enveloped viruses (such as SARS-CoV-2, influenza and RSV) tend to have high affinity for the solids phase (Roldan-Hernandez & Boehm, 2023; Ye et al., 2016). The analysis conducted by Mercier et al., (2022) detected 84.6% of the influenza A viral signal in the settleable solids of municipal wastewater and 88.1% of the influenza A viral signal in the settleable solids of primary sludge. After centrifugation (10,000g, 45 min) of either sludge or influent, Mercier et al., (2022) reported minimal recovery of influenza A in the retentate after the sample was passed through a 15 mL 30K Amicon filter. In the current study, Treatment Group 1 (4,000g, 10 min, with brake) had 88.0 % of the influenza A signal in the solids while Treatment Group 2 (12,000g, 1.5 h, no brake) had 93.9 % of the influenza A signal detected in the solids. The addition of overnight PEG/NaOH precipitation had no effect on the concentration of influenza A or B. This was similar to that observed by Mercier et al., (2022) as they did

not see an increase in recovery of influenza A with the addition of PEG/NaCl to sludge. These observations are consistent with the low levels of influenza A and B detected in the supernatant. Using ultrafiltration (Centricon-70) of the supernatant after centrifugation at 12,000g, 1.5 h only 5.6% of the influenza B mass was detected while at 4,000 g, 10 min, 11.4% of the influenza B mass was detected. Unlike SARS-CoV-2, where there is considerable virus remaining in the supernatant (22.7%), influenza is primarily in the pellet (centrifuged solids) and therefore not influenced by additional centrifugation or PEG/NaCl precipitation.

Although the RSV concentrations were low, it was found predominantly in the solids fraction of wastewater in this study, which paralleled the findings in Roldan-Hernandez & Boehm, (2023). In the current study, 68.4 % of the RSV signal was in the solids fraction in Treatment Group 1 and once the centrifugal force was increased from 4,000g for 30 min to 12,000g for 1.5 h, 96.4 % of the RSV signal was found in the solids fraction. Higher concentrations of viral RSV RNA were also observed in solids fraction under various conditions by Roldan-Hernandez & Boehm, (2023) although on a mass equivalent basis, partition and distribution coefficients were similar across temperature conditions. The current findings are therefore corroborated by other similar studies that showed that RSV has a strong association with the solids fraction in wastewater, thus quantification methods that enrich solids may improve the sensitivity of wastewater methods.

The low levels and temporal variability of the endogenous viral signal in the wastewater makes partitioning studies very challenging. The endogenous virus can be highly variable temporally and often trace concentrations limit detection and therefore determination of reliable ratios. Therefore, many studies have examined viral surrogates spiked into wastewaters, with only a few enveloped viruses such as MHV and nonenveloped bacteriophages such as MS2 (Titcombe Lee et al., 2016; Ye et al., 2016). The presence of a lipid envelope outside the protein capsid is thought to impact the solid–liquid partitioning of viruses. Ye et al., (2016) measured the adsorption of MHV and MS2 in wastewater samples and found that enveloped virus MHV strongly associated with solids in comparison with the non-enveloped virus MS2. However, the assumption that the virus of interest will behave similarly to their respective model virus (surrogate) may not be valid. There has been no study to date that has thoroughly investigated the apparent partitioning of influenza B, but the assumption is that it should partition similarly to influenza A, as was seen in this current study. Boehm et al., (2023) measured the concentrations of various respiratory viruses in wastewater solids, including influenza A, influenza B, RSV A, RSV B, and seasonal coronaviruses. However, the study was limited to only examining settled solids that were collected from

the sludge line and then dewatered. The results from Boehm et al. (2023) support that influenza A, influenza B and RSV A are strongly associated with the solids fraction, although no direct experimental comparisons were done. Several studies have focused on sludge as it has often been able to effectively detect various viruses, likely because of their high solids' concentration and the strong affinity of the viral particles for solids. Samples collected from six wastewater treatment plants by Roldan-Hernandez & Boehm, (2023) found that the viral RNA concentrations were higher in the solid fraction (copies/g dry weight) than the liquid fraction (copies/mL) of wastewater by approximately 3–4 orders of magnitude. There are a lot of issues that impact partitioning: viral structure, wastewater matrix, separation, and concentration methods (Basha, 2020). Although additional studies are needed, solids-based approaches are well suited for analysis of enveloped viruses such as SARS-CoV-2, influenza and RSV. However, many other factors, such as inhibition, may also be important considerations in method selection.

2.5 Conclusion

Various methods targeting either the solids or liquid fraction of wastewater can quantify SARS-CoV-2 since it is present in both fractions. However, influenza and RSV partition almost exclusively in the solids fraction of wastewater so targeting the solids will improve sensitivity and possibly precision and accuracy of methods (assuming there is no inhibition or other factor). Further research is still required to better understand the partitioning of viruses of interest in wastewater. A solids-based concentration method that was already in use for SARS-CoV-2 surveillance was applied to the respiratory viruses: influenza A, B and RSV. The wastewater signal of influenza A, B and RSV derived using this method correlated well with clinical measures, including hospitalizations in the Waterloo Region. Limitations in clinical data may therefore be partially addressed through application of wastewater surveillance. WBS holds promise as an early indicator of RSV activity in communities (as a currently non reportable disease in Ontario) and can potentially serve as an early warning for potential increases in hospitalizations. Although influenza clinical testing is well developed, wastewater surveillance may further inform public health agencies on the spread and trends of this disease. The combination of multiple respiratory diseases (e.g. SARS-CoV-2, influenza, and RSV) within wastewater surveillance may be helpful in creating early warning of the co-occurrence of these diseases and inform public health planning and action. The tri-pandemic experience in Ontario in 2022- 2023 is an example of where wastewater surveillance may support public health in following an unusual pattern that overwhelmed some hospitals (Zulli et al., 2024). The monitoring of these respiratory viruses in wastewater is currently being applied throughout

Canada and the world and were recently added for routine monitoring in the Ontario Wastewater Surveillance Initiative. Continued wastewater surveillance can possibly detect new pathogens or variants that could lead to additional waves or even another pandemic. Interpreting influenza and RSV wastewater signals, like SARS-CoV-2, is most valuable when considered alongside other community surveillance indicators. Despite considerable experience during the COVD-19 pandemic further development of wastewater surveillance is essential to ensure effective future monitoring and public health protection.

Chapter 3
Conclusions and Recommendations

3.1 Conclusion

This study showcased the applicability and success of WBS for influenza and RSV, and its capability to inform and support Public Health Units. In early 2022 the potential to apply WBS to other respiratory viruses beyond COVID-19 arose, and since then it has been widely applied to target an array of viruses. WBS was effective in monitoring influenza and RSV, it defined their trends in wastewater that strongly correlated with clinical data and hospitalizations in the Waterloo Region. This proof of concept demonstrated the effectiveness of WBS with reporting conducted in almost real time, even with limited weekly sampling, low viral copies in wastewater, and only partial clinical data availability. This study highlighted the feasibility and importance of monitoring multiple respiratory viruses using wastewater. The partitioning experiments conducted on influenza A, influenza B and RSV indicate that these viruses almost exclusively partition in the solids fraction of wastewater, especially under higher centrifugation settings. Methods focused on the supernatant may therefore have lower sensitivity and higher variability for these viruses compared to SARS-CoV-2 that is more evenly distributed. The fate of influenza and RSV may also differ considerably from the that of PMMoV that is often used to normalize viral signals, possibly limiting the effectiveness of this approach. Moving forward, further investigation and validation of methods and their ability to track trends in community cases and hospitalizations are required. However, this cases study demonstrated the utility of applying WBS to additional viral targets. WBS is a powerful tool, with potential to support and inform public health action in the future with other existing or emerging pathogens or substances of concern.

3.2 Limitations

Wastewater surveillance of influenza and RSV was successfully applied in the Waterloo Region and demonstrated that WBS is a very effective approach for tracking community trends in infection and hospitalizations. Nevertheless, several scientific challenges remain.

One of the main challenges in the current study was accessing clinical data, particularly when it came to RSV. The RSV clinical data available was limited to hospitalizations for the entire region. Acquiring more specific clinical data by city or ideally by sewershed would have been extremely helpful in further validating the methods. It is likely that the data exists but is not available in this format. Changing how

the data is collected and reported would allow for better validation of the WBS approach. Even with this limitation, wastewater surveillance results correlated very well with hospitalizations and showed many advantages for surveillance of this disease.

Inhibition during RT-qPCR is another issue that occurs during the sampling process that hinders the reliability of signals reported. The samples used in this study were tested for inhibition using the MS2 assay previously described and none of the samples were inhibited. In most sites tested in the WSI program inhibition is rare, but at a few sites inhibition is regularly detected (M. Servos personal communication). For example, the Galt Wastewater Treatment Plant site in the Region of Waterloo has the addition of rotary drum thickener filtrate return (RDT filtrate) that causes RT-PCR inhibition. RDT is used in primary treatment to separate solids and liquids in the influent to then treat the wastewater, therefore this substance cannot be removed from the process (Boake, 2006). This is why despite representing a large proportion of the Waterloo Region population (e.g., 91,000 people), this site was avoided when conducting this case study. Although dilution is often used to reduce inhibition, the low copy numbers for the viral target in wastewater (influenza, RSV) means that the signal would be reduced below LOQs. Clean-up of the sample is an alternate option but would result in an additional loss of signal as well. Addressing inhibition is therefore a continuing challenge that needs to be considered in the future monitoring programs.

The fecal biomarker is ideally expected to behave similarly to the target of interest to account for the loss or dilution of signals in sewers, sample collection/storage, and laboratory processing. PMMoV has often been recommended for use as a normalizing biomarker, due to its stability in wastewater and its association with human feces. However, environment factors such as rainfall and snow melt may not affect PMMoV in the same manner as it does to influenza or RSV, which results in shifts in the normalized values that are not consistent with the raw signals. PMMoV is found predominately in the liquid phase (supernatant) while influenza and RSV are predominantly in the solids (pellet) phase suggesting that their fate may differ significantly. Although PMMoV has high and relatively stable concentrations in wastewater, its' diet dependency and seasonal variability poses challenges for its use for normalization of viral targets (Dhiyebi, Abu Farah, et al., 2023; Greenwald et al., 2021). The diet dependency of PMMoV will ultimately impact the normalized signal reported in a community. PMMoV is an RNA virus with a dietary origin from peppers and their products, therefore, even though it is one of the most abundant human fecal viruses, some communities do not consume peppers and hot sauce, which was found to be the case in northern Ontario. At least for the two sites tested in this case study the use of

PMMoV to normalize the signal did not improve correlations with clinical cases or hospitalizations. However, PMMoV can serve as a good process control and indicator of changes occurring in the influent/sewershed.

Interpreting the data has also posed a challenge to PHUs in instances where the wastewater signals for the different respiratory viruses do not align with other surveillance indicators. For example, when the wastewater signal increases, but there is no corresponding increase in hospitalizations or outbreaks. The possible variability between the different surveillance systems is challenging to interpret and thus hard to effectively communicate to the public, without alarming them unnecessarily. Improvements to clinical testing and reporting as well as more intensive wastewater testing (i.e. at least 3 days per week) would greatly enhance assessment and reliability of WBS.

Lastly, even if wastewater surveillance provides an early signal, PHUs may not always be able to take specific or targeted actions in response to short-term changes (M. Vythilingam, Region of Waterloo, personal communication). So, challenges in what are the appropriate, feasible and realistic measures to be put in place needs to be assessed, as well as how and when to share this information effectively without causing alarm to the public.

3.3 Recommendations

There are several recommendations and considerations that can be implemented based on the case study conducted:

1. Wastewater surveillance of respiratory viruses should be conducted throughout the year, not just during their expected season, given the variability and shifts in their occurrence from year to year. Extensive sampling and processing may not be required in times outside the typical respiratory season, but this will be dependent on the objectives of the surveillance program.
2. Multiple municipalities and wastewater sampling sites (WWTPs) should be incorporated when analyzing the trends and behaviors of each virus. The incorporation of multiple sites will allow for better interpretation of the changes of the viral signals in wastewater and linkages to clinical/hospitalization data.
3. Specific protocols and QA/QC criteria should be defined to ensure consistency and reliability of the methods used in the WBS program, similar to that done in the SARS-CoV-2 surveillance programs (e.g. WSI). This should be applied to not only influenza and RSV but to any other potential targets

that may be incorporated as well. The methods should be validated and compared between laboratories (e.g. interlab studies) to ensure reliability of the data reported to PHUs and ultimately the public.

4. It will be important in the future to establish the best gene targets for each virus so that direct comparisons can be made across studies and ensure interpretation of the data. The subtyping of influenza A in wastewater would distinguish whether the circulating influenza A is avian influenza (e.g. H5N1) or the commonly circulating human influenza A (H3N2 and H1N1). Should RSV A and B be done separately or as a single assay is an area of debate.
5. Surveillance programs should include sequencing of selected samples, when possible, to determine patterns in the viral genome. However, sequencing typically is not as sensitive as RT-qPCR, so the two approaches are complementary.
6. More extensive research should be prioritized to better understand the partitioning behavior of viruses in wastewater. Replicating the series of partitioning experiments at different virus surges and at different WWTPs will allow for a more comprehensive understanding of their partitioning. Viruses peak at different times in the respiratory season and to capture the surge of each target a repeat of the experiments at those times at different WWTPs will be needed to confirm the outcomes in this study, as was done in Breadner et al., (2023) for SARS-CoV-2.
7. Factors that could potentially hinder and degrade the viral signal in wastewater should be investigated. These factors will affect the quantification and ultimately the detectability of the viruses of interest in wastewater, resulting in inaccurate reporting. We assume the viral targets are stable in the sewershed, but we currently do not know if they are stable or degrade during transit. In a similar way, we currently do not have direct evidence that these respiratory viruses are stable during sample storage. Although this has been studied for SARS-CoV-2 it remains a gap for other respiratory viruses.
8. Utilization of WBS should be done with the goal to support PHUs and the public. Although it may be possible to measure many viral targets in wastewater, there needs to be an assessment of the best way to use limited resources to both develop WBS but also serve the communities.
9. Clinical data availability and reporting need to be enhanced. Clinical data should be broken down by city or if possible sewershed (e.g., postal code), to allow direct comparisons to clinical cases or

hospitalizations. If this is not possible then a sufficient part of the population needs to be included in the wastewater surveillance to ensure the results are representative.

10. WBS is the most effective when considered alongside other surveillance systems such as clinical cases and hospitalizations. Incorporating multiple surveillance systems will provide a more comprehensive snapshot of the community's infection prevalence.
11. Wastewater sampling should be conducted a minimum of three times weekly. The CDC recommends a minimum sampling frequency of three days a week and the Ontario Wastewater Surveillance Initiative has been sampling five days a week for key sites, to capture the incidence of the disease in a community. If samples are only collected once weekly there would be limited data points available for trend analysis.
12. Wastewater surveillance programs should incorporate key respiratory viruses (SARS-CoV-2, influenza, RSV) that may co-occur and represent a threat to public health and the health care system. As of the spring of 2023, influenza wastewater surveillance has been incorporated in the Ontario program and as of the spring of 2024 RSV has been incorporated.
13. Further research and validation of WBS is needed to continue its acceptance and application as a public health tool. Wastewater surveillance has shown to be very effective and likely to remain as a prominent approach in the future. However, further research will only allow it to become an even more reliable and powerful tool that can serve generations to come.

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Appendices

Appendix A
Statistical Analysis

Table A 1. Summary of Spearman's Correlational Analysis results between the viral RNA copies and clinical metrices.

Relationship	Site	S	p -value	Rho(r)
Influenza A cases and influenza A copies in wastewater	Kitchener	2609.6	3.246e-09	0.7726819
Influenza A cases and normalized influenza A copies in wastewater	Kitchener	2725.5	6.86e-09	0.7625854
Influenza A cases and influenza A copies in wastewater	Waterloo	1660.1	5.292e-11	0.8319697
Influenza A cases and normalized influenza A copies in wastewater	Waterloo	1448.5	5.144e-12	0.8533858
Influenza B cases and influenza B copies in wastewater	Kitchener	3847.8	2.128e-06	0.6648247
Influenza B cases and normalized influenza B copies in wastewater	Kitchener	3819.9	1.893e-06	0.6672575
Influenza B cases and influenza B copies in wastewater	Waterloo	5650.7	0.006557	0.4280673
Influenza B cases and normalized influenza B copies in wastewater	Waterloo	5737.1	0.00788	0.4193238
RSV hospitalizations and RSV copies in wastewater	Region of Waterloo	483.65	1.673e-08	0.8523652
Influenza hospitalizations and influenza copies in wastewater	Region of Waterloo	2825.3	0.001594	0.5278518

Table A 2. Summary of one-way ANOVA results for total RNA copies between each treatment for each gene target (influenza A, influenza B, RSV, N2).

Test	Target	Df	Sum sq	Mean sq	F value	p value
Total RNA copies between centrifuge conditions	Influenza A	2	18.6	9.32	0.149	0.862
Tukey's post hoc	4,000g – 12,000g					0.890
	PEG – 12,000g					0.879
	PEG – 4,000g					0.999
Total RNA copies between centrifuge conditions	Influenza B	2	20.43	10.22	0.787	0.473
Tukey's post hoc	4,000g – 12,000g					0.603
	PEG – 12,000g					0.979
	PEG – 4,000g					0.487
Total RNA copies between centrifuge conditions	RSV	2	4.758	2.379	1.132	0.349
Tukey's post hoc	4,000g – 12,000g					0.542
	PEG – 12,000g					0.342
	PEG – 4,000g					0.927
Total RNA copies between centrifuge conditions	N2	2	5614	2807	0.416	0.672
Tukey's post hoc	4,000g – 12,000g					0.994
	PEG – 12,000g					0.691
	PEG – 4,000g					0.751

Table A 3. Summary of one-way ANOVA results for RNA copies between each treatment for each gene target (InfA, InfB, RSV, N2).

Test	Target	Df	Sum sq	Mean sq	F value	p value
RNA copies between each treatment condition	Influenza A	2	2915	728.7	16.67	1.16e-06
Tukey's post hoc	12,000g Supernatant – 12,000g Pellet					0.0000273
	4,000g Pellet – 12,000g Pellet					0.8088255
	4,000g Supernatant – 12,000g Pellet					0.0001616
	PEG – 12,000g Pellet					0.9954564
	4,000g Pellet – 12,000g Supernatant					0.0004033
	4,000g Supernatant – 12,000g Supernatant					0.9915546
	PEG – 12,000g Supernatant					0.0000685
	4,000g Supernatant – 4,000g Pellet					0.0021425
	PEG – 4,000g Pellet					0.9507332
	PEG – 4,000g Supernatant					0.0003937
RNA copies between each treatment condition	Influenza B	4	450.9	112.72	15	2.84e-06

Tukey's post hoc	12,000g Supernatant – 12,000g Pellet					0.0001305
	4,000g Pellet – 12,000g Pellet					0.4279151
	4,000g Supernatant – 12,000g Pellet					0.0008543
	PEG – 12,000g Pellet					0.9478961
	4,000g Pellet – 12,000g Supernatant					0.0095451
	4,000g Supernatant – 12,000g Supernatant					0.9861560
	PEG – 12,000g Supernatant					0.0000219
	4,000g Supernatant – 4,000g Pellet					0.0445992
	PEG – 4,000g Pellet					0.1309957
	PEG – 4,000g Supernatant					0.0001523
RNA copies between each treatment condition	RSV	4	48.77	12.191	8.914	0.000147
Tukey's post hoc	12,000g Supernatant – 12,000g Pellet					0.0068532
	4,000g Pellet – 12,000g Pellet					0.9998913
	4,000g Supernatant – 12,000g Pellet					0.4537974

	PEG – 12,000g Pellet					0.3828963
	4,000g Pellet – 12,000g Supernatant					0.0097014
	4,000g Supernatant – 12,000g Supernatant					0.3257120
	PEG – 12,000g Supernatant					0.0000757
	4,000g Supernatant – 4,000g Pellet					0.5361629
	PEG – 4,000g Pellet					0.3096286
	PEG – 4,000g Supernatant					0.0166010
RNA copies between each treatment condition	N2	4	200675	50169	12.63	0.000107
Tukey's post hoc	12,000g Supernatant – 12,000g Pellet					0.0111107
	4,000g Pellet – 12,000g Pellet					0.4602230
	4,000g Supernatant – 12,000g Pellet					0.2968552
	PEG – 12,000g Pellet					0.0847543
	4,000g Pellet – 12,000g Supernatant					0.2431043
	4,000g Supernatant – 12,000g Supernatant					0.3887735

PEG – 12,000g Supernatant	0.0000631
4,000g Supernatant – 4,000g Pellet	0.9971230
PEG – 4,000g Pellet	0.0032801
PEG – 4,000g Supernatant	0.0017414