Establishing Wastewater Surveillance for SARS-CoV-2 in Southern Saskatchewan

A Thesis

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SUPERVISORY AND EXAMINING COMMITTEE

Sonja Tamara Reuter, candidate for the degree of **Master of Science in Biology,** has presented a thesis titled, ***Establishing wastewater surveillance for SARS-CoV-2 in Southern Saskatchewan,*** in an oral examination held on **May 3, 2023**. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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*Attended via Zoom conferencing

Abstract

Wastewater surveillance has become a crucial part in the monitoring of the COVID-19 pandemic. Infected people shed SARS-CoV-2 in their feces, therefore, virus levels in wastewater reflect the trends in infection numbers in the population that contributes to the wastewater. Wastewater surveillance offers information about the spread of SARS-CoV-2 independent of testing strategies and individual choices and therefore better reflects population health than individual testing. In this study we compared five RNA extraction kits and reagents and different enrichment methods for wastewater surveillance of SARS-CoV-2 RNA and determined that the solids fraction of wastewater was most suitable for RNA extraction using the AllPrep PowerViral DNA/RNA Kit by Qiagen. We also examined the impact of storage on wastewater samples and determined that while short-term storage does not affect the samples significantly, longer storage changes the measured viral levels. We then used our protocol to establish a wastewater surveillance for SARS-CoV-2 viral levels in Regina and nine other locations in Southern Saskatchewan, Canada. We observed that the three waves of COVID-19 during the study time were caused by the variants Alpha, Delta and Omicron and that the SARS-CoV-2 RNA levels in wastewater were a good reflection of the reported active COVID-19 case numbers.

Acknowledgements

Sampling and lab work took place on Treaty 4 lands, which are the territories of the nêhiyawak, Anihšināpēk, Dakota, Lakota, and Nakoda and the homeland of the Métis/Michif Nation. This work was handed in by me but wouldn't have happened in this form without the help of many, many people. Firstly, I would like to thank Dr. Nicole Hansmeier and Dr. Tzu-Chiao Chao for their guidance and support throughout the time I stayed with them, both scientifically and personally. Their relentless efforts to push us to perfection may not have always been appreciated but certainly helped me grow as a researcher and I am thankful for the four years I got to spend with them and the experiences I am taking with me now.

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I would also like to acknowledge the following people for their support of my work: Alison, for her input into project planning and for transporting samples, facility management for handling the autosampler and unclogging the strainer (sorry!), the staff of the wastewater treatment facilities for collecting the samples, the National Microbiology Laboratory for providing us with sequencing results and all the other people who helped with my project in one way or the other. I would also like to acknowledge the Natural Sciences and Engineering Research Council (NSERC) and the University of Regina Faculty of Graduate Studies and Research (FGSR) for funding this project and the University of Regina for providing teaching assistantships.

Post-Thesis Acknowledgements

I would also like to acknowledge the time and effort that my committee members Dr. Jonathan Van Hamme and Dr. Christopher Yost have put into reading and improving my thesis and the interesting discussion we had during the defense.

Dedication

I dedicate this thesis to my late Grandma Hildegard, and who saw more potential in my plans than I and who always supported me on my ways, and to my other family members and friends who supported me from far away. Thank you all!

Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Meaning
ACE2	Angiotensin converting enzyme 2
Allprep kit	AllPrep PowerViral DNA/RNA Kit
BCoV	Bovine coronavirus
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus disease 2019
Ct	Cycle threshold
ddPCR	Droplet digital PCR
DMSO	Dimethyl sulfoxide
E gene	Envelope gene
E.Z.N.A kit	E.Z.N.A® Universal Pathogen Kit
FN	First-nation
GC(-MS)	Gas chromatography (-mass spectrometry)
LC(-MS)	Liquid chromatography (-mass spectrometry)
M gene	Membrane gene
Microbiome kit	RNeasy Power Microbiome Kit
N gene	Nucleocapsid gene
NML	National Microbiology Laboratory (Canada)
NSP	Non-structural protein
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PHAC	Public Health Agency of Canada
PMMoV	Pepper mild mottle virus
QiaAmp kit	QIAamp Viral RNA Mini Kit
R^2	Coefficient of determination
RNA	Ribonucleic acid
RT-(q)PCR	Reverse transcription - (quantitative) polymerase chain reaction
S gene	Spike gene
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SHA	Saskatchewan Health Authority
Soil kit	FastRNA® Pro Soil-Direct Kit
VBM	Variant being monitored
VOC	Variant of concern
VOI	Variant of interest
WBE	Wastewater-based epidemiology
WHO	World Health Organization
WWTP	Wastewater treatment plant

1. Introduction

1.1. COVID-19 Pandemic - Origin and spread of SARS-CoV-2

In the beginning of December 2019, several cases of a then unknown pneumonia-like disease were reported in Wuhan in the Chinese province Hubei (1). The pathogen causing the disease was first identified as a member of the *Coronaviridae* (1), a family of enveloped, positive strand RNA viruses, and later as a lineage within the species *severe acute respiratory syndrome-related coronavirus* (2). It was first known as 2019-nCoV (1) and later classified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2). Another known member of this species is SARS-CoV (sometimes SARS-CoV-1), a distant relative of SARS-CoV-2 (2), and causative agent of the 2002 outbreak of SARS (3). SARS-CoV-2 has a rather large genome of almost 30 kb, containing one large open reading frame (ORF1ab) encoding non-structural polyproteins, four structural proteins (Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N)) and a number of open reading frames coding for accessory genes (4,5) (Figure 1). A notable protein is the spike protein, which establishes contact to host cell angiotensin-converting enzyme 2 (ACE2) and which has accumulated many mutations over the course of the pandemic that impacted the virus' infection rate and immune evasion abilities (6).

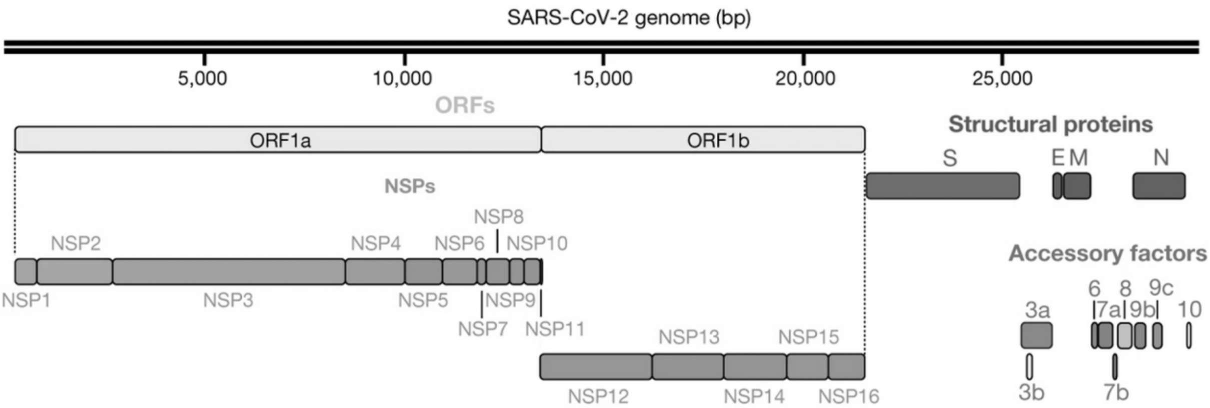
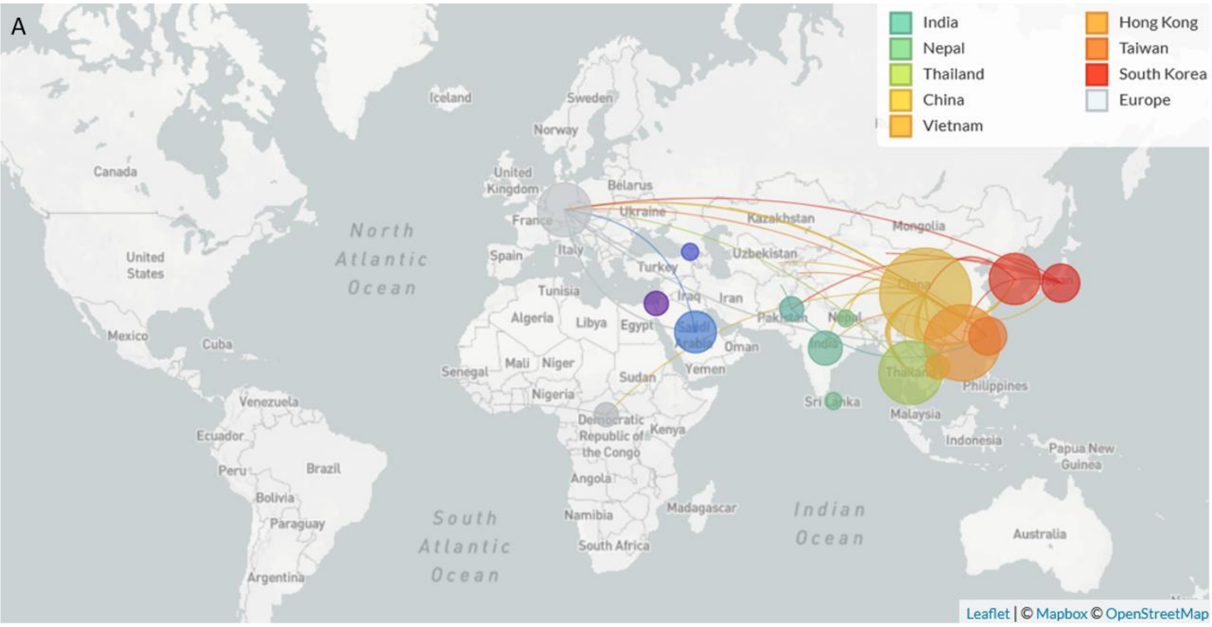


Figure 1: The genome of SARS-CoV-2 is about 30 kb long and consists of four structural proteins (spike (S), envelope (E), membrane (M) and nucleocapsid (N)) and a number of non-structural proteins (NSP). ORF – open reading frame. Figure adapted from Gordon et al. (4)

On March 11th 2020, the coronavirus disease 2019 (COVID-19) was declared a pandemic by the World Health Organization after it had spread to more than 100 countries (7). The initial spillover from bats or an intermediate species to humans likely happened in the Wuhan province in China (8) from where the disease quickly spread to several other countries (Figure 2A). As of October 2022, the virus had spread almost everywhere around the world (Figure 2B).



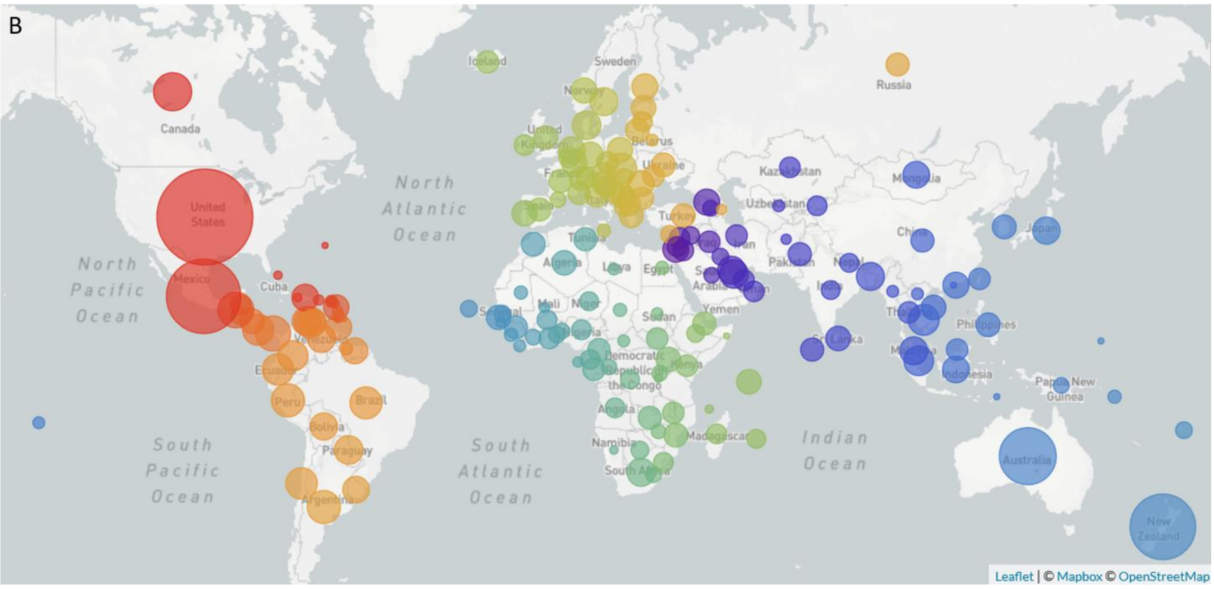


Figure 2: Within a few months SARS-CoV-2 had spread around the world. A shows the initial spread from China into other nearby countries from December 2019 to February 1st, 2020. B shows the SARS-CoV-2 presence worldwide (as of October 2022). Colour indicates the country of origin of a SARS-CoV-2 sample, the size of the circle the number of samples associated with this country. Figure generated with Nextstrain.org and data from Gisaid (9).

The strain which jumped species in 2019 has since accumulated multiple mutations (6), of which 30 are located in the spike protein alone (10). Several of those changed the structure of the receptor binding domain, which enhanced recognition of and attachment to ACE2 and also lowered the effectivity of antibody structure recognition from previous infections (10) or vaccinations (11). The accumulation of mutations led to the initial strain evolving into several variants (12). New variants are defined by distinct characteristics from its predecessor, such as a higher virulence or transmissibility (13). There are different approaches for SARS-CoV-2 nomenclature; this study follows the nomenclature used by the WHO for variants (14) and the Pango nomenclature for (sub-)lineages (15). The WHO groups newly arising variants that pose a potentially higher risk to the public than previous stages of the virus into variants of interest (VOI) and variants of concern (VOC) (14). Declared variants of concern included Alpha, Beta, Gamma, Delta and Omicron, although as of March 2023 only Omicron and its sub-lineages remain variants of concern. In Canada, the first waves of the pandemic were driven by the initial SARS-CoV-2 strain, followed by waves caused by the virus variants Alpha, Delta and Omicron (16). Figure 3 shows the course of the major waves in Canada. The Omicron wave is still ongoing as of March 2023 and therefore represents the longest wave. The first wave of the initial strain claimed the most lives in Canada, despite causing the fewest (reported) COVID-19 cases (17).

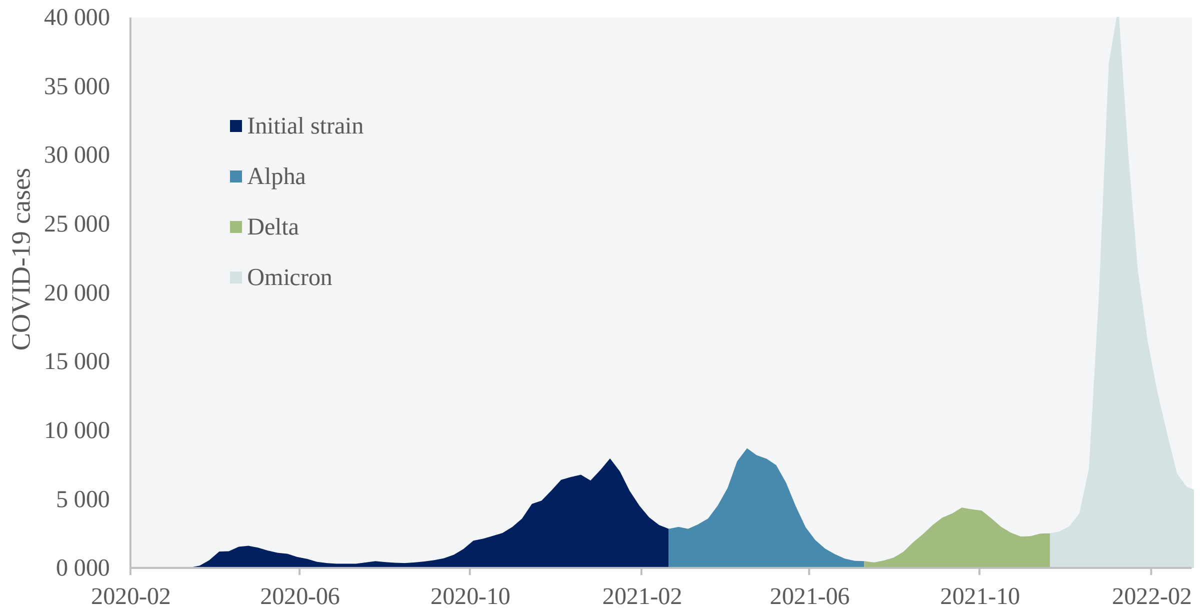


Figure 3: Canada saw four major waves of COVID-19 caused by the initial strain (dark blue) followed by the Alpha (blue), Delta (green) and Omicron (grey) variant. The graph shows the 7-day average of active COVID-19 cases in Canada. For simplicity, only the main variants triggering each wave are shown. Case data from the COVID-19 data resources (17).

As early studies indicated that SARS-CoV-2-infected people shed the virus in their stool (18,19) wastewater surveillance became of interest as a monitoring tool for the COVID-19 pandemic (20).

1.2. History and Process of Wastewater Surveillance

Wastewater surveillance, also known as wastewater-based epidemiology, was developed as a tool that could provide additional information about public health and thereby improve the responses of health authorities (21). It is based on the sampling of sewage for the detection of markers for general health that a population has shed into the wastewater. These markers can be of chemical nature, such as drugs or pharmaceuticals, or of biological origin, such as pathogens or their genetic material. For instance, infected people in a population shed markers of their infection with their urine or feces (21). Wastewater from locations such as private households or hospitals is combined and transported to wastewater treatment plants for treatment and therefore can serve as a pool of health markers of the population living in the respective catchment area (21). Wastewater surveillance can be used for long-term routine surveillance of large catchments areas (also called sentinel surveillance) or for targeted identification and management of disease outbreaks in smaller catchments areas, such as neighbourhoods (22). The information gained from wastewater surveillance can then be used by health authorities to identify problems threatening public wellbeing and to inform control measures (21).

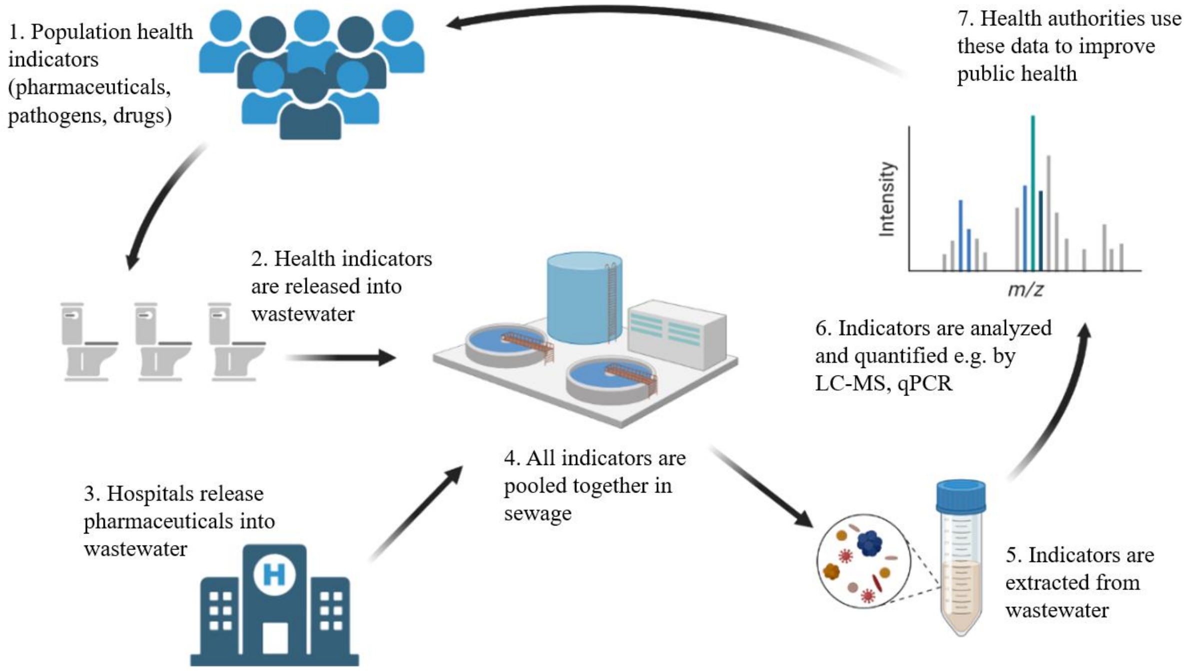


Figure 4: Wastewater surveillance provides information about public health as private households, hospitals and other living congregates release chemical and biological indicators for public health into the wastewater. The indicators can be extracted from wastewater, identified and quantified. Information can help health authorities learn about problems in the population and guide intervention if necessary. Figure created using Biorender.com

The use of wastewater surveillance has been documented as early as in the 1940s, when it was applied in New York to detect poliovirus, the agent causing poliomyelitis (polio) (23). The surveillance for polio developed into the first large-scale monitoring of a disease in wastewater and was later applied in several countries in order to guide disease eradication efforts and is still in use for this purpose (as reviewed by Ashgar et al. (24)). Polio, similar to COVID-19, generates a large proportion of asymptomatic cases, which makes it difficult to recognise spread in time to respond and also to determine whether the disease has been eradicated in a certain area. Therefore, wastewater surveillance was used to help with eradication efforts for two out of three wild polio virus serotypes (25) and confirmed that India and Egypt, two countries that previously had endemic polio, were polio-free by 2004 and 2012, respectively (24). In 2013, routine surveillance of wastewater in Israel uncovered a previously unrecognised polio spread, which enabled authorities to respond timely with enhanced testing and an immunisation campaign to counteract the spread (26).

Beyond the monitoring of polio, wastewater surveillance has become important for several other health-related problems such as tracking antimicrobial resistance and drug use (27,21,28,29). For instance, a study conducted in 2016/17 investigated antibiotic-resistant *Escherichia coli* in wastewater of ten major European cities. They observed a significant relationship between resistance genes in clinical samples and wastewater and discovered a gradient of resistance prevalence from southern to northern Europe for antibiotics, such as ampicillin, ciprofloxacin and gentamicin (30). A different study observed that antibiotic-resistant bacteria were present in wastewater in remote areas (31). It was also determined that in Nunavut the available treatment infrastructure did not remove resistance genes sufficiently and even contributed to their enrichment in the wastewater. Wastewater surveillance has also been suggested as an approach to monitor stress levels in the population (32). For chemical markers, such as pharmaceuticals, techniques involving a combination of chromatography and mass spectrometry (GC–MS, LC–MS or LC–MS–MS) have been used frequently (33). To assess the use of illicit drugs, their metabolic residues have been isolated from wastewater and identified using solid-phase extraction coupled with mass spectrometry (34).

For disease epidemiology, in addition to polio, several other viruses have been observed in wastewater (35). Viruses are, depending on the virus and environmental conditions, stable in water and solids and detectable at low quantities (35,36). In their review, Xagoraraki and O'Brien (35) described 14 virus taxa that had been successfully detected in wastewater, including adenoviruses, astroviruses, enteroviruses, hepatitis viruses, noroviruses, rotaviruses, coronaviruses and influenza viruses. Furthermore, wastewater surveillance has been suggested for several arboviruses (including the Dengue virus, Zika virus, West Nile virus, Yellow fever virus and Chikungunya virus) as they have been shown to be shed through urine (37). A study in which the Zika virus had been spiked into wastewater indicated the possibility for isolation and identification of the virus from sewage (38).

Medema et al. (20) were the first to attempt wastewater surveillance for SARS-CoV-2. They showed the general feasibility of detecting SARS-CoV-2 in sewage and were also the first to detect the virus in wastewater of a catchment area where no active COVID-19 cases had been reported, indicating the potential of wastewater surveillance as an early-warning tool for undetected outbreaks. In the following months, wastewater surveillance for SARS-CoV-2 was reported from several continents. Ahmed et al. (39) detected the virus in wastewater in Australia and started to optimise an extraction method. Furthermore, they suggested an approach to convert the results from wastewater surveillance into infection numbers but already acknowledged the problematic nature of this process due to factors such as variable shedding rate and amount.

Wu et al. (40) implemented wastewater surveillance into the COVID-19 monitoring approach in Massachusetts and further advanced the methodology by comparing extraction methods and suggesting the pepper mild mottle virus (PMMoV) as a useful normalisation marker. By now wastewater surveillance has been introduced into over 70 nations (41) (Figure 5), with 102 monitored wastewater treatment plants in Canada alone. The results of wastewater surveillance studies offer an additional piece of information about the spread of the disease to health authorities and can be made publicly available in the form of dashboards, such as by the Canadian Government or the CDC (42,43).

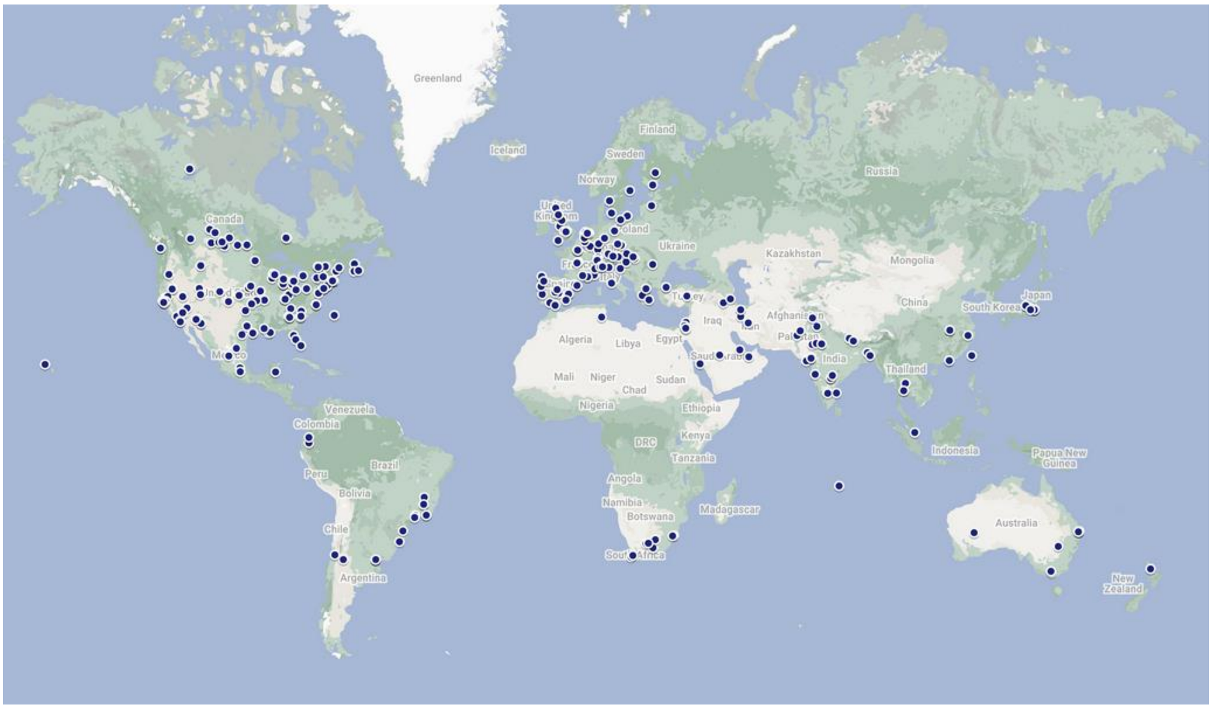


Figure 5: Wastewater surveillance has been implemented almost worldwide. This map shows a selection of wastewater surveillance sites that have been reported in publications (adapted from the COVID-19 WBE collaborative (sites as of April 44)).

1.3. Benefits of Wastewater Surveillance for the COVID-19 pandemic

Wastewater surveillance has become an important monitoring tool during the COVID-19 pandemic as samples taken from a wastewater treatment plant represent the people living in the whole catchment area (27). Therefore, only a single sample is needed to reflect the health situation in a large population (27), which is especially useful as it is more cost-effective, time-saving and less invasive than examining individual people (21,45). In addition, wastewater surveillance is less biased towards symptomatic infections as asymptomatic people contribute equally to wastewater as symptomatic carriers (22). This is especially important for diseases with a high proportion of asymptomatic cases, such as polio or SARS-CoV-2, as those could spread undetected until a cluster of symptomatic infections is recognised (27). While a single wastewater sample can be viewed as a snapshot of the current population's health status (35), continuous surveillance can show how a situation is developing, for instance to see whether the strategies to improve the situation are working. This was made use of during the COVID-19 pandemic. Furthermore, wastewater surveillance can provide information not only about the development of case numbers but also about the virus variants and (sub-)lineages present in the sampled areas. The process is simple. Sewage samples are taken at a wastewater treatment plant, a lift station, lagoon or a smaller-scale site and RNA is extracted from them. The extracted RNA is quantified by RT-PCR (reverse transcription-polymerase chain reaction) with assays specific for SARS-CoV-2. A higher signal for SARS-CoV-2 RNA in wastewater corresponds to more infected people in the wastewater catchment area (46). Sequencing or specific RT-PCR assays can add information about circulating variants and sub-lineages of the virus (47–49).

1.4. Hypothesis and Aims of the Study

In this study we aimed to establish wastewater surveillance for SARS-CoV-2 in Southern Saskatchewan to support the local health authorities. While wastewater surveillance for SARS-CoV-2 had been reported to be feasible in the beginning of the pandemic (20,40,39), at the time there had been no surveillance for our local community. We hypothesised that we could use the SARS-CoV-2 levels in wastewater determined by wastewater surveillance as an indicator for the spread of COVID-19 in the population in the sampled areas in Southern Saskatchewan. Furthermore, we hypothesized that different sample processing approaches and storage conditions would affect the surveillance results.

The following aims were pursued during the study:

1. Development of a reproducible wastewater surveillance method to enrich, extract and quantify SARS-CoV-2 RNA
2. Examining the effects of storage on virus RNA recovery

3. Monitoring of SARS-CoV-2 RNA levels in wastewater of several cities and communities across Southern Saskatchewan and the University of Regina main campus
4. Monitoring of virus variants and (sub-)lineages present in the sampled locations by sequencing

To test this, we collected wastewater samples from different locations across Southern Saskatchewan and analysed them for the quantity of SARS-CoV-2 using RT-PCR over a period of time. We compared the measured viral levels to the reported COVID-19 case numbers in Regina to evaluate the suitability of the method. Furthermore, we compared the outcomes of different RNA enrichment and extraction methods from wastewater and examined the impact of long-term storage of wastewater samples on RNA recovery. Lastly, we used sequencing data generated by the National Microbiology Laboratory (NML) in Winnipeg to track which virus variants were circulating in the sampled areas.

2. Materials and Methods

Materials, devices and software are listed in Appendix A.

2.1. Study Area and Wastewater Sample Collection

We received samples from several locations, which can be seen in Table 1. For privacy concerns, two facilities, one inside Regina and one further north, and two First-Nation communities outside the city boundaries will remain undisclosed. For most locations, 24h-composite wastewater samples were collected by autosamplers installed at the sampling sites. In the two First-Nation communities grab samples were taken once per week by a staff member after the trucks had emptied the collected waste from the community into the lagoon. All samples were transported to the lab in coolers and immediately stored at 4°C until extraction. RNA extraction took place either on the same day or up to three days after arrival of the sample.

Table 1: Sampling locations, facility and sample type from the respective location. Sampling was still ongoing at all the locations except Weyburn as of June 26, 2023. WWTP - Wastewater treatment plant

Location	**Facility**	**Sample type**	**Start of sampling**
Regina	WWTP	24h influent	08/2020
Moose Jaw	WWTP	24h influent	12/2021
Swift Current	WWTP/Lift station	24h influent/ 24h composite	12/2021
Lumsden	WWTP	24h influent	01/2022
Yorkton	WWTP	24h influent	01/2022
Weyburn	Lift station	24h composite	03/2022 - 10/2022
University of Regina campus	Manhole	24h composite	03/2022
Facility 1	Lift station	24h composite	12/2021
First-Nation community 1	Lagoon	grab	08/2022
First-Nation community 2	Lagoon	grab	08/2022

2.2. RNA Extraction Method Development

We started by comparing commercially available kits and reagents for RNA extraction from wastewater. The following kits were tested for extraction from wastewater solids: E.Z.N.A® Universal Pathogen Kit (Omega Bio-tek), FastRNA® Pro Soil-Direct Kit (MP Biomedicals), TRIzol® and TRIzol® LS (Thermo Fisher Scientific) and the QIAamp viral RNA mini kit (Qiagen). For extraction from the liquid fraction, only the QIAamp viral RNA mini kit (Qiagen) was tested. For liquid and solid extraction, 25 mM $MgCl_2$ (final concentration) was added to wastewater sample before processing (50). To enrich particles from the liquid phase, 50-200 mL wastewater was filtered through 3 kDa Amicon filters (Merck Millipore) at 4 000 *x g*. The centrifugation time depended on the sample composition and ranged mostly from 2 to 5h. Samples were then processed using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. A preceding filtration through 0.45 µm nylon syringe filters before the Amicon filtration step significantly decreased the centrifugation time needed for the Amicon filters from several hours to 45 min.

For extraction from the solid phase, 50-250 mL wastewater from 24h-composite or grab samples was pelleted at 10 000 *x g* via 35-45 min centrifugation at 4°C in a fixed-angle rotor (adapted from Ahmed et al. (50)). The wet weight of the pellets was recorded for sample comparison and samples were kept on ice until the beginning of the RNA extraction. Subsequently, RNA was isolated from the concentrated solids following the manufacturers' instructions with the following minor modifications:

QIAamp viral RNA mini kit: For RNA extractions from the solid phase using this kit, pellets were resuspended in 800 µL buffer AVL and transferred to ribolyser tubes with 0.5 mm zirconium beads (Sigma Aldrich). Samples were lyzed using a ribolyser (MP Biomedicals) with the following settings: one cycle with 6.5 m/s for 45 sec. RNA was eluted with 20-60 µL of RNase-free water.

TRIzol and TRIzol LS: Extractions with the TRIzol reagent started with a bead-beating step (3 cycles of 45 s at speed 6.5 m/s in bead tubes with 0.5 mm zirconium beads in the ribolyser). To increase the concentration of the resulting RNA, elution was done with only 20 µL RNase-free water.

The RNA extraction process for the RNeasy PowerMicrobiome Kit (Microbiome kit, Qiagen) or the AllPrep PowerViral DNA/RNA Kit (Allprep kit, Qiagen) is described in 2.4 Wastewater Surveillance.

A NanoDrop was used to measure RNA concentration and purity and unpaired Student's T-tests were conducted for direct comparisons between the extraction methods.

2.3. Comparison of Different RNA Enrichment Methods

To test the effects of different enrichment methods, we combined three wastewater samples from Facility 1 to generate a large volume sample (combined Facility 1). We spiked this sample with 375 µL of an attenuated Bovine Coronavirus vaccine (BCoV) (Zoetis) (see Wastewater

Storage Experiments) before extracting RNA using three different concentration methods: 1. enrichment of the solids fraction using centrifugation, 2. filtration of the liquid fraction and 3. PEG precipitation and subsequent enrichment of solids via centrifugation. Each method was tested in triplicates.

For the PEG precipitation, 10 mL of 0.4 g/mL PEG-8000 and 2.553 mL of 5M NaCl (final concentrations 0.076 g/mL and 0.24 M, respectively, adapted from D'Aoust et al. (51)) were added to 3 x 40 mL of wastewater in falcon tubes. The PEG precipitation samples were incubated at 4°C in a roller drum (Thermo Fisher Scientific) for 19h at 40 rpm in rotating mode to promote precipitation of viruses. After incubation, the samples were centrifuged together with three more 40 mL wastewater samples at 4°C for 10 min at 4816 *x g* in a swing-out rotor. The supernatant of the PEG samples was discarded and the pellet processed as PEG samples. The supernatant of the other samples was decanted and processed further as liquid fraction, the remaining pellet as solids fraction (Figure 6).

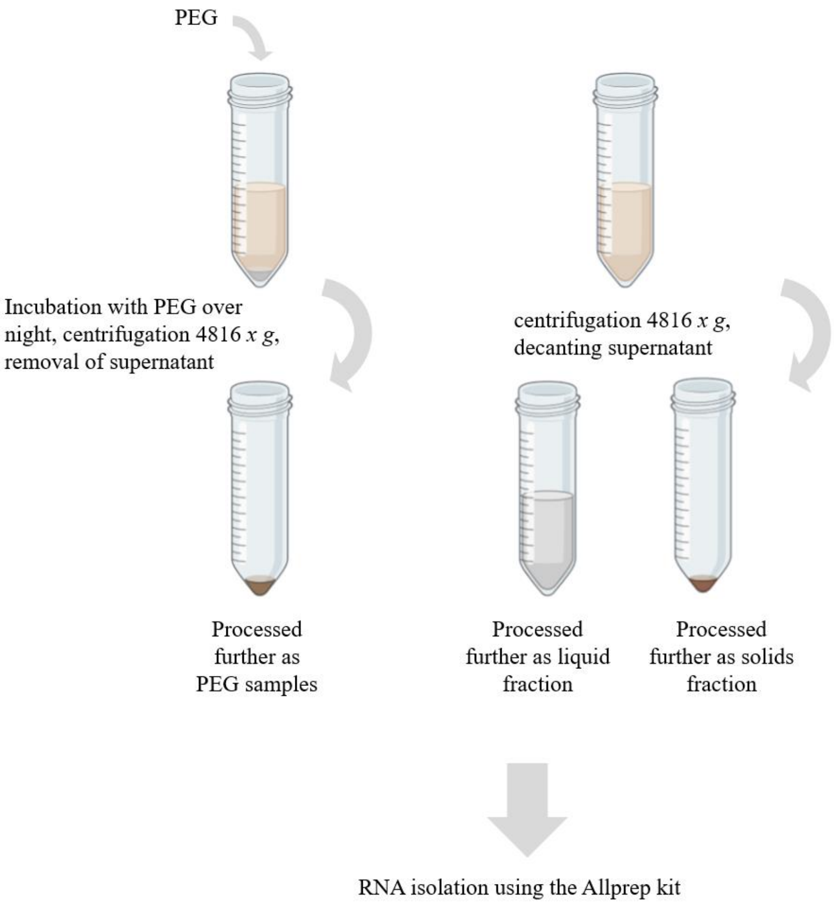


Figure 6: Process of the RNA enrichment method comparison. RNA was isolated from three replicates each of either a PEG-precipitated sample, the liquid or the solids fraction. Figure created using Biorender.com.

The pellets of the PEG samples and the solids samples were added to the PowerBead tubes of the Allprep kit and processed further as described in 2.4 with the exception that no BCoV was spiked into the pellet since the sample already contained BCoV. The liquid fractions were filtered using 10 kDa Amicon-15 filters (Merck Millipore) and the flow-throughs discarded. The particles in the filter were resuspended in the remaining 200-250 µL liquid, weighed in a fresh tube and then transferred to PowerBead tubes and processed using the Allprep kit as described in 2.4.

The final RNA samples were compared by their RNA concentration and Ct value in qPCR. Unpaired Student’s T-tests were conducted for direct comparisons between the enrichment methods.

To calculate how much PMMoV from the liquid fraction was precipitated by PEG, we assumed that all of the PMMoV that was detected in the solids fraction would also be detected in the PEG-precipitated fraction. Therefore, all additional PMMoV in the PEG-precipitated fraction had to have been precipitated from the liquid fraction. The calculation is described in the following:

Conversion of Ct values to copy numbers using a calibration curve:

Eq. 1
$$cp = 10^{(Ct\ value - y\ intercept)/slope}$$

With cp = copy number, Ct value = threshold-adjusted Ct value measured in qPCR, y intercept = y intercept of standard curve used, slope = slope of standard curve

Determination of how much PMMoV in the PEG precipitated samples originates from the liquid fraction assuming that 100% of solids-associated PMMoV is detected by PEG precipitation:

Eq. 2
$$cp_{liq(est)} = cp_{PEG} - cp_{sol}$$

With $cp_{liq(est)}$ = estimated copy number of PMMoV in PEG precipitated samples that originates from the liquid fraction, cp_{PEG} = copy number of PMMoV measured in PEG precipitated samples, cp_{sol} = copy number of PMMoV measured in solids extraction

Conversion to percentage:

Eq. 3
$$cp_{liq(meas)} = 100$$

$$\frac{cp_{liq(meas)}}{100} = 1$$

$$\%_{liq(est)} = \frac{cp_{liq(meas)}}{100} * cp_{liq(est)}$$

With $cp_{liq(meas)}$ = PMMoV copies detected in liquid fraction of wastewater, $\%_{liq(est)}$ = estimated proportion of PMMoV copies in liquid fraction that were detected by PEG precipitation

2.4. Wastewater Surveillance

For the wastewater surveillance, RNA was extracted from the solids fraction of wastewater. The volume of wastewater differed between samples depending on the density, we aimed for 250 mg of solids. For near-source samples taken directly from a building or a campus (UoR campus, Facility 1) 30-100 mL was used, for all other samples 200-300 mL was used. The volume was transferred into 50 mL falcon tubes for centrifuging at 4816 *x g* (maximum speed) in a swing-out rotor at 4°C for 10 min. The supernatant was decanted and stored at 4°C for further analysis. The pellet was transferred to a 2 mL tube and centrifuged again at 6500 *x g* to remove any residual liquid. If more liquid remained, a third centrifugation step of 2 min at 13 000 *x g* at 4°C was added. The weight of the solids pellet was noted by subtracting the weight of the empty tube from the weight of tube and pellet.

RNA was extracted from the samples using either the RNeasy PowerMicrobiome Kit (Microbiome kit, Qiagen) or the AllPrep PowerViral DNA/RNA Kit (Allprep kit, Qiagen) with the following slight modifications to the supplier's manual.

The pellets were resuspended in 650 µL pre-warmed solution PM1 and 6.5 µL β-mercaptoethanol and transferred into the PowerBead tubes after ddition of 100 µL room-temperature Phenol/Chloroform/Isoamyl alcohol (25:24:1 (v/v)) (Sigma). Afterwards, 5 µL of BCoV was spiked into the pellets as positive control (starting April 2022). The BCoV was prepared as described in Feng et al. (52). Five microliters correspond to roughly 500 000 copies (52). Homogenisation took place using a ribolyser (MP Biomedicals) at 6.5 m/s, for 45 sec for three consecutive rounds. The inhibitor removal step was not performed for samples from Weyburn, because the solution did not precipitate in those samples. A DNase digestion step was included until early 2022, then left out as the results did not differ much between extractions with and without the digest. RNA was eluted with 50 µL (until June 2022) or 100 µL (after June 2022) RNase-free water, split up into two aliquots and stored at -80°C. Until June 2021 the RNA concentration was determined using a Spectrophotometer (Thermo Fisher Scientific) and from July 2022 onwards using the Qubit RNA BR Assay Kit on a Qubit 4 fluorometer (Thermo Fisher Scientific).

2.4.1. RT-qPCR

The protocol for qPCR was also adjusted during the project.

We tested different RT-qPCR kits including the iTaq™ Universal SYBR® Green One-Step Kit (SYBR green chemistry, Bio-Rad), the UltraPlex™ 1-Step ToughMix® (Taqman chemistry, QuantaBio) and the Reliance One-Step Multiplex RT-qPCR Supermix (Taqman chemistry, Bio-Rad) using a QuantStudio™ 3 real-time PCR system (Applied Biosystems). Due to supply restrictions for probe-based assays we settled for the SYBR green technology. For the wastewater surveillance of SARS-CoV-2, we started with primers targeting the E gene and later switched to the N gene (Figure 7).

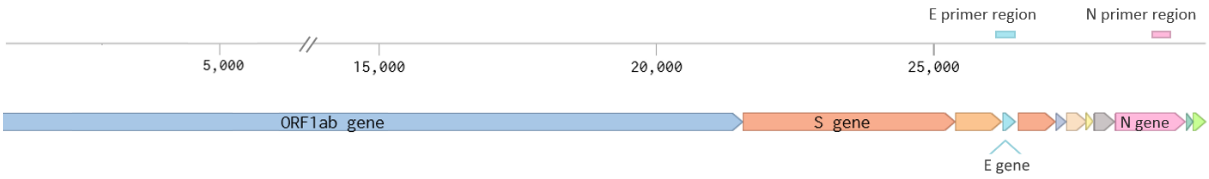


Figure 7: The genome of SARS-CoV-2 is 29903 bp long. Different assays for detection of SARS-CoV-2 RNA in wastewater have been tested. We first used primers targeting the E gene and later switched to the N gene. The sequence shows the original Wuhan strain (NC_045512) as displayed in Benchling.com.

Amplification was performed in 20 µL reactions with the following component mixture: 10 µL 2x iTaq universal SYBR green reaction mix, 0.25 µL iScript reverse transcriptase, 1 + 1 µL (forward and reverse) primers (for concentrations see Table 2), 2 µL bovine serum albumin (BSA) (4 mg/mL), 4.75 µL RNase-free water and 1 µL of 1:8-diluted RNA sample (concentration range 30-4 000 ng/µL). The following cycling conditions were used the majority of times: reverse transcription at 50°C for 15 min, initial denaturation at 95°C for 1 min followed by 45 cycles of 15 sec 95°C and 30 sec at 58°C and a melt curve using the instrument's default settings (initial denaturation at 95°C for 15 sec followed by 1 min at 60°C and then a slow heating with 1°C/sec until 95°C are reached again). Every PCR included a positive control containing an RNA standard with the respective PCR target (either SARS-CoV-2 or PMMoV) and a negative control containing water for all targets. The results were checked for amplification in the negative controls, shifted melt curves or standard deviations between the replicates >0.5. If anything unexpected happened, samples from that PCR were repeated. For each run, the thresholds for calculation of the Ct values for the three targets were manually adjusted to 0.04 for BCoV, 0.048 for N2 and 0.093 for PMMoV. BSA alleviated the effect of PCR inhibitors in a few samples and was therefore added to the PCR protocol. The addition of further PCR additives in different concentration ranges (BSA at 0.01%-0.5%, DMSO (dimethyl sulfoxide) at 0.5-10%, glycerol at 0.05-20%, combinations of BSA and DMSO) did not result in further improvement of the PCR performance.

Table 2: Sequences and concentrations of the RT-PCR primers used in this project. The use of the E gene primers (taken from Corman et al. (53)) was discontinued during the study and replaced with N2.

Primer name	**Target**	**Sequence (5'-3')**	**Concentration in PCR (nM)**	**Reference**
N2 forward	SARS-CoV-2 N gene	TTA CAA ACA TTG GCC GCA AA	500	CDC (54)
N2 reverse	SARS-CoV-2 N gene	GCG CGA CAT TCC GAA GAA	500	CDC (54)
PMMoV forward	PMMoV RdRp	GTG GCA GCA AAG GTA ATG GT	250	Hamza et al. (55)
PMMoV reverse	PMMoV RdRp	ATT TGC TTC GGT AGG CCT CT	250	Hamza et al. (55)
BCoV forward	BCoV M gene	CTG GAA GTT GGT GGA GTT	250	Decaro et al. (56)
BCoV reverse	BCoV M gene	ATT ATC GGC CTA ACA TAC ATC	250	Decaro et al. (56)
E_Sarbeco forward	SARS-CoV-2 E gene	ACA GGT ACG TTA ATA GTT AAT AGC GT	400	Corman et al. (53)
E_Sarbeco reverse	SARS-CoV-2 E gene	ATA TTG CAG CAG TAC GCA CAC A	400	Corman et al. (53)

2.5. SARS-CoV-2 Variant Surveillance

We received data about the variants present in Regina wastewater derived from whole-genome sequencing and qPCR from the Division of Enteric Diseases at the National Microbiology Laboratory (NML) in Winnipeg, Manitoba. Their full method can be found in Landgraff et al. (47).

The NML provided us with regular updates of the frequencies (in percent) of single nucleotide variation (SNV) combinations associated with certain lineages and variants. For visualisation, the frequencies for (sub-)lineages of Delta were summarised as "Delta" and the (sub-)lineages of Omicron except for BA.1,2,4 and 5 were summarised as "additional Omicron strains". Minor variants that were detected on < 4 sequencing events were summarised as "Beta, Gamma, Kappa, Eta, Lambda" for clarity. All data were checked for credibility, averaged over one week and adjusted to 100% per time point for easier comparison.

We also received qPCR data for the tracking of SARS-CoV-2 variants in wastewater from the National Microbiology Laboratory in Winnipeg. An overview over the targeted mutations and the variant they indicate can be found in Table 3. The data were adjusted as follows:

Since the original data only contain one Omicron assay for all strains and one for BA.1/4/5, the percentage of Omicron BA.1/4/5 was subtracted from the overall percentage of Omicron ("all strains") to receive a rough estimate of BA.2. Similarly, the percentages of the BA.1/4/5 assay were split between the BA.1 phase and BA.4/5 for visual distinction. The percentage for Delta was averaged from the two available Delta assays. The Alpha assay using the Sdel mutation showed signals during the Omicron wave; those were removed as they were likely wrongfully picked up and the assay was discontinued shortly after. The data were presented as weekly averages and all percentages given per time point were adjusted to 100% total.

Table 3: qPCR targets for variant surveillance (in collaboration with NML)

Mutation in SARS-CoV-2 genome	Data reported as ratio between (in %)	Indicator for SARS-CoV-2 variant
69/70 Sdel	Omicron Sdel variant and wild-type	Omicron BA.1, BA.4/5
69/70 Sdel	Sdel variant and wild-type	Alpha
D377Y	D377Y variant and wild-type	Delta
N200	S_N200_D Delta variant and S_N200_U wild-type	Delta
SN501Y	SN501Y variant and wild-type	Alpha, Beta, Gamma, Mu
SN501Y	Omicron SN501Y variant and wild-type	All Omicron strains

2.6. Wastewater Storage Experiments

To examine whether SARS-CoV-2 RNA in wastewater samples degrade during storage at 4°C we selected samples from Regina from one week to 18 months old and re-isolated RNA from them. For the re-isolations, the same volume of wastewater was used as for the initial RNA extraction. Optimisations of the protocol, that had been introduced in the mean time, were implemented in the re-isolation as long as they had been shown to not change the Cts (such as leaving out the addition of $MgCl_2$, skipping the DNase-digest and using the Allprep kit instead of the Microbiome kit). Sample dates and ages are summarised in Table 4.

The old and newly extracted samples were compared by RNA concentration, RNA integrity using the Qubit RNA Integrity and Quality Assay Kit (Qubit IQ, Thermo Fisher Scientific) and by the Ct values of N2 and PMMoV. Old and new RNA extracts were analysed on the same RT-PCR plate to prevent run-specific variations.

Subsequently, Ct values were converted into copy numbers using Eq. 1 and linear regression analyses were used to analyse the changes over time. The copy numbers were normalised against the solids content (pellet weight) and the sample volume as follows (Eq. 4):

Eq. 4

$$cp_{norm} = \frac{\frac{cp}{pellet\ weight}}{sample\ volume} * 100$$

With cp_{norm} = copy number normalised to pellet weight and sample volume, pellet weight = weight (g) of the solids pellet after centrifugation, sample volume = volume of wastewater used for RNA isolation

After seeing the results of the first degradation experiment, two further experiments were carried out to analyse the continuous changes of Ct values during long-term storage of wastewater samples. Two samples with large volumes (~1.8 L) were created by combining smaller 24h-composite or 24h-influent samples from Facility 1 and the WWTP in Regina, respectively. The samples were spiked with BCoV as extraction control and as additional target for tracking Ct shifts. Sample weights (weight of liquid with dissolved solids) and volumes of spiked-in BCoV are shown in Table 5. The amount of BCoV needed per combined sample was estimated by aiming for 8 µL of BCoV per extraction for the combined sample from Facility 1. RNA was extracted from those samples regularly, in the beginning every week, later every two weeks. The volumes used during each extraction were 40 mL for combined Facility 1 and 50 mL for combined WWTP Regina (differences due to different solids content of the samples). PCR was conducted using the standard procedure described under 2.4.1.

Table 4: Samples used to test for RNA degradation at 4°C: sampling dates, volume for RNA extraction and ages of the samples at re-isolation

Sampling date	**Extraction volume (mL)**	**Initial extraction on**	**Re-isolation on**	**Age of sample at re-isolation (days)**
2020-11-11	50	2020-11-25	2022-05-27	548
2020-11-25	50	2020-12-01	2022-05-27	542
2020-12-16	50	2020-12-22	2022-05-27	521
2021-02-14	100	2021-02-23	2022-05-27	458
2021-05-23	250	2021-05-26	2022-05-25	364
2021-06-02	250	2021-06-08	2022-05-25	351
2021-06-09	250	2021-06-15	2022-05-21	340
2021-08-25	250	2021-09-07	2022-05-25	260

Sampling date	Extraction volume (mL)	Initial extraction on	Re-isolation on	Age of sample at re-isolation (days)
2021-11-17	250	2021-11-23	2022-05-25	183
2022-01-20	250	2022-01-25	2022-05-21	116
2022-02-22	250	2022-03-01	2022-05-21	81
2022-03-17	250	2022-03-22	2022-05-20	59
2022-04-10	250	2022-04-12	2022-05-14	32
2022-04-26	250	2022-05-03	2022-05-07	4

Table 5: Samples for long-term degradation experiments were created by combining multiple 24h-composite (24h-c) or 24h-influent (24h-i) samples. Two locations (Facility 1 and WWTP Regina) were selected and the combined samples spiked with BCoV prior to storage at 4°C.

Combined Sample	combined samples made from	weight (g)	spiked-in BCoV (µL)
combined Facility 1	20220701-combF1-A (24h-c)	1875.7	375.1
	20220703-combF1-A (24h-c)		
	20220705-combF1-A (24h-c)		
combined WWTP Regina	20220809-WTP-R (24h-i)	1837.5	367.4
	20220811-WTP-R (24h-i)		

2.7. Data Analysis

The normalisation of Ct values was adapted from Isaksson et al. (57). Briefly, the Ct values measured in qPCR were converted into copy numbers using standard curves for N2 and PMMoV when available. An example for such a standard curve is shown in **Error! Reference source not found.** in Appendix B. For data obtained before standard curves were prepared, an average standard curve was generated using all standard curves for the respective target (Eq. 1). The viral load of SARS-CoV-2 was calculated by dividing the copy number of N2 by the copy number of PMMoV per PCR reaction (Eq. 5). The copy numbers were not converted to copies per milliliter wastewater as the Ct values did not correspond linearly to the volume used for elution. For normalisation using the wastewater flow rate, which was recorded for the samples from the wastewater treatment plant in Regina, the obtained ratio was divided by the deviation of the flow on the respective day (Eq. 6) from the average flow measured within the sampling period (Eq. 7). The effect of the flow rate was tested as an increasing and decreasing factor, but neither of them improved the correlation to the reported active case numbers. Therefore, normalisation against the flow was not included in the final analysis. Weekly averages were calculated as the last step after calculating the ratios.

The data for the flow rates were obtained from the wastewater treatment plant in Regina, the weather related data such as precipitation and temperature from the Government of Canada (58) and data regarding COVID-19 cases and hospitalisations from the archive of the COVID-19 situation reports (59).

Eq. 1

$$cp = 10^{(Ct\ value - y\ intercept)/slope}$$

With cp = copy number, Ct value = threshold-adjusted Ct value measured in qPCR, y intercept = y intercept of standard curve used, slope = slope of standard curve

Eq. 5

$$VL_{PMMoV} = \frac{cp_{\ (SARS-CoV-2)}}{cp_{(PMMoV)}}$$

With VL = viral load of SARS-CoV-2 in wastewater normalised to PMMoV, cp = copy number

Normalisation against the flow rate was tested but not deemed suitable:

Eq. 6

$$d(Q) = \frac{Q(\frac{L}{d})}{\bar{Q}\ (\frac{L}{d})}$$

With d(Q) = deviation of the flow measured at the WWTP Regina from the average flow measured on sampling dates in the period from March 2021 to September 2022

Eq. 7

$$VL_{\ flow} = \frac{VL}{d(flow)}$$

With VL_{flow} = viral load of SARS-COV-2 normalised against PMMoV and the flow

Different data normalisation methods were tested and compared using regression statistics. The R^2 values determined that the best correlation was between the weekly averages of PMMoV-normalised SARS-CoV-2 viral load and the weekly averages of the active cases, hence, these values were used for all further tests. Further normalisation approaches are described in Appendix C.

3. Results

3.1. RNA Extraction Method Development

In response to the COVID-19 pandemic, we aimed to establish a wastewater surveillance for SARS-CoV-2 in support of several communities in southern Saskatchewan. Therefore, we tested different RNA enrichment and extraction methods and the effects of long-term storage on virus detection in the wastewater samples. We looked at parameters such as the RNA yield, purity, Ct values from RT-PCR and reproducibility.

3.1.1. Comparison of Different RNA Extraction Methods from Wastewater

First we tested RNA extraction from the liquid fraction of wastewater using the QiaAmp viral RNA mini kit (QiaAmp mini kit) following the manufacturer's instructions (adjustments described in 2.2 Extraction Method Development). While RNA was successfully extracted using this kit (772 ± 101 ng/µL from 50 mL wastewater), the purity was low (Figure 8 and 9). More importantly, we did not detect SARS-CoV-2 (E gene) using RT-PCR.

Next, we tested the solid fraction of the wastewater using the QiaAmp mini kit. While RNA yields from the solid fraction were significantly lower than from the liquid fraction (142 ± 77 ng/µL, Figure 8), the SARS-CoV-2 E gene was detected in RT-PCR. However, the results were not reproducible and showed signs of inhibition in a dilution series. As other groups also indicated the presence of PCR inhibitors in RNA samples from wastewater (e.g. 60,61), we next tested the addition of TRIzol or TRIzol LS, which are suggested to remove PCR inhibitors in the phase-separation step (as reviewed by 62) and have been successfully used for RNA extraction from wastewater (e.g., 40,63). However, although the measured RNA concentrations were high (2263 ± 1388 ng/µl, Figure 8), we did not detect SARS-CoV-2 in RT-PCR. In addition, the RNA samples were opaque in colour, indicating potential contamination with humic or fulvic acids (64,65), which are known PCR inhibitors (66) and might explain the negative RT-PCRs.

Extractions with the E.Z.N.A® Universal Pathogen Kit (E.Z.N.A kit) produced fairly clean RNA samples (Figure 9) with concentrations higher than the QiaAmp mini kit (1308 ± 344 ng/µL, Figure 8). However, SARS-CoV-2 was not detected in RT-PCR.

As PCR inhibitors seemed to be prominent problem, we next tested the FastRNA® Pro Soil-Direct Kit (Soil kit), which is known to efficiently remove humic substances, one commonly known PCR-inhibiting contaminant (64,67). NanoDrop measurements, however, showed the lowest RNA purity and yield of these samples compared to all other kits (260/230 ratio: 0.1 and 23 ± 12 ng/ul, Figures 8 and 9). In addition, RT-PCRs did not result in reproducible SARS-CoV-2 detection.

Lastly, we tested the RNeasy® PowerMicrobiome® Kit (Microbiome kit), a kit optimised for wet and dry stool samples that contains a PCR inhibitor removal step. The obtained RNA yields from the solid fractions using this kit were moderate (394 ± 245 ng/uL in 50 mL) and reproducible within a 30% margin. The 260/280 ratio was 2.05 ± 0.04 indicating pure RNA and the 260/230 ratio of 2.00 suggested a low concentration of contaminants using this kit. More importantly, SARS-CoV-2 was consistently and reproducibly detected in RT-PCR.

While we started with extraction from 50 mL wastewater, we observed that the RNA yield almost doubled when increasing the wastewater volume from 50 mL to 100 mL (Figure 8), indicating sufficient RNA-binding capacity of the kit's columns. A further increase to 250 mL wastewater (up to 350 mg wet weight of solids) showed an additional 1.4-fold increase of the RNA yield. We also tested the effect of DNase treatment, which is an optional step of the Microbiome kit and is recommended to lower the background noise level in RT-PCRs.

However, in our hands we did not detect any improvement regarding background noise reduction, thus we excluded this step.

As the DNase digestion step is the only difference between the Qiagen AllPrep® PowerViral® DNA/RNA Kit (Allprep kit) and the Microbiome kit, both kits were used interchangeably for surveillance as supply chain issues during the pandemic were impacting deliveries.

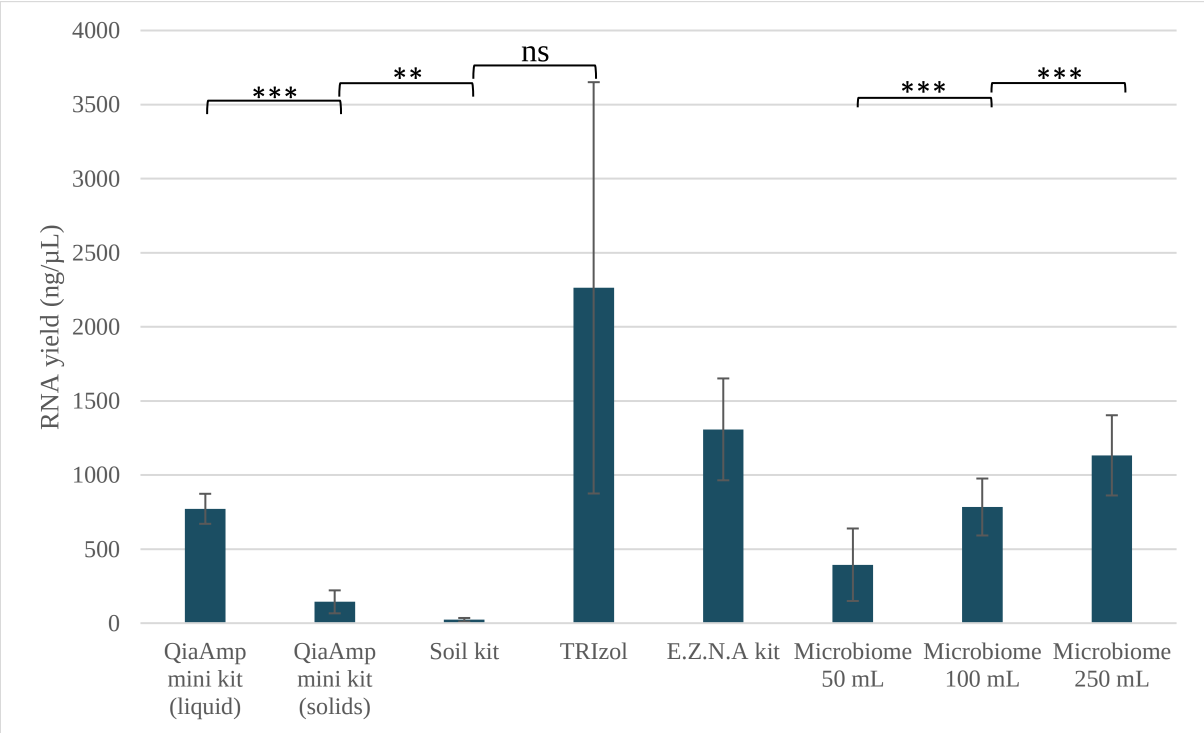


Figure 8: Comparison of RNA yields using different extraction methods. For each extraction, 50 mL wastewater were used unless further indicated. For the QiaAmp mini kit, the liquid and solid fractions were tested independently. The following kits and reagents were tested: QiaAmp mini kit solids (n=7), QiaAmp mini kit liquid (n=4), Soil kit (n=3), TRIzol (including TRIzol LS) (n=3), E.Z.N.A kit (n=2), Microbiome kit (n=29). In addition, the Microbiome kit was tested with increased wastewater volumes: 100 mL (n=19) and 250 mL (n=18). Error bars represent standard deviation between extractions per kit. t-test results: ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

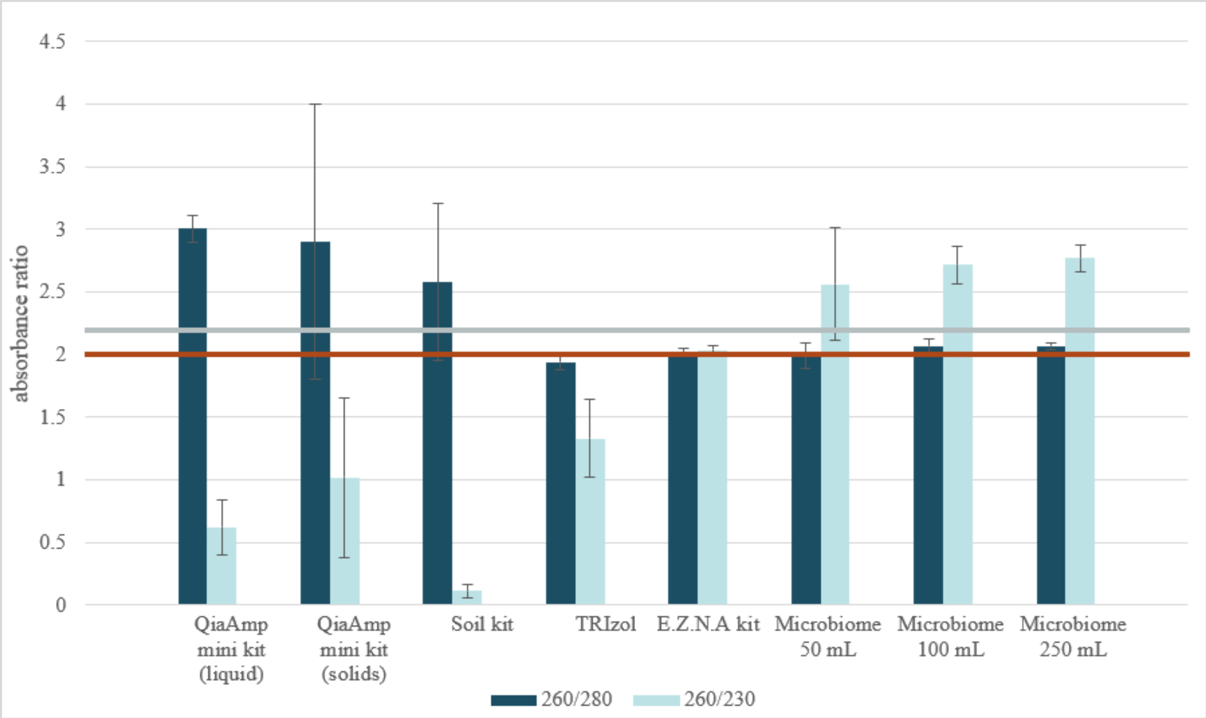


Figure 9: Absorbance ratios showed that most tested kits do not produce pure and clean RNA samples from wastewater samples. With exception to the E.Z.N.A kit, which did not produce signals in RT-PCR, only the Microbiome kit yielded pure RNA with little contamination and was upscalable to 250 mL without significant deterioration of sample quality. A 260/280 absorbance ratio of 2.0 (red line) and a 260/230 ratio between 2 and 2.2 (grey line) indicate pure, uncontaminated RNA. Error bars represent standard deviation between extractions per kit.

While we initially targeted the E gene to quantify SARS-CoV-2 in wastewater using RT-PCR, we later switched to the N2 assay for surveillance when an impaired assay sensitivity for the E gene was reported (68,69) and the CDC recommended the use of the N2 assay (54). In addition, we targeted PMMoV, a commonly used population biomarker indicative for human fecal pollution (70), that has also been suggested as a normalisation factor for SARS-CoV-2 in wastewater (71), and BCoV, which we used as a spike-in control (72).

3.1.2. Effects of Virus Partitioning and Enrichment

We then explored partitioning of the viruses SARS-CoV-2, PMMoV and BCoV into the liquid and solid fraction of wastewater and the impact of polyethylene glycol (PEG) precipitation, a commonly used method to recover viruses from environmental samples (73), on viral enrichment. We used a large wastewater sample that was spiked with BCoV (preparation described in 2.6) and extracted RNA from the solid fraction (solids), the liquid fraction (liquids) and the PEG-precipitated fraction (PEG precipitation) (for details see 2.3).

We first looked at the total RNA yield of each. The highest total RNA yield was observed in the samples extracted using PEG precipitation (612 ng/µL), followed by 454 ng/µL in the solids and 77 ng/µL in the liquid fraction (Figure 10A). As the PEG precipitated fraction is a combination of the liquid and solid fraction, the RNA yields from the liquid and solid fractions needed to be combined for a direct comparison. As shown in Figure 10B, the RNA amount of the combined liquid and solid fraction was similar to the PEG precipitated fraction (53 100 ± 3061 ng vs 61 233 ± 5254 ng, $p = 0.08$). A slight loss could be explained with the additional washing steps during RNA purification.

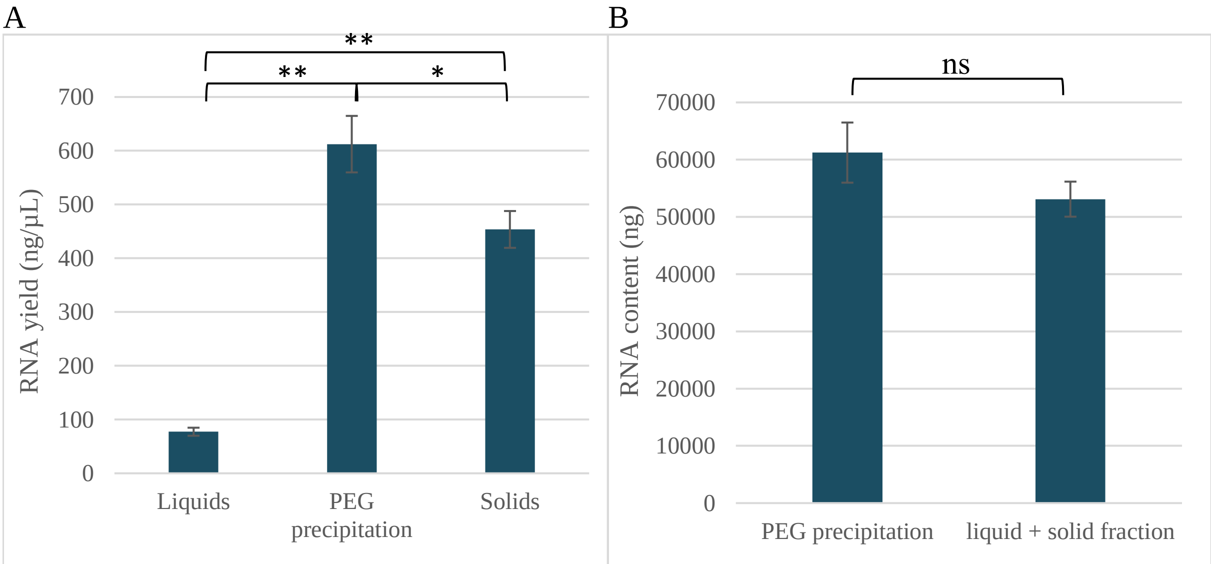
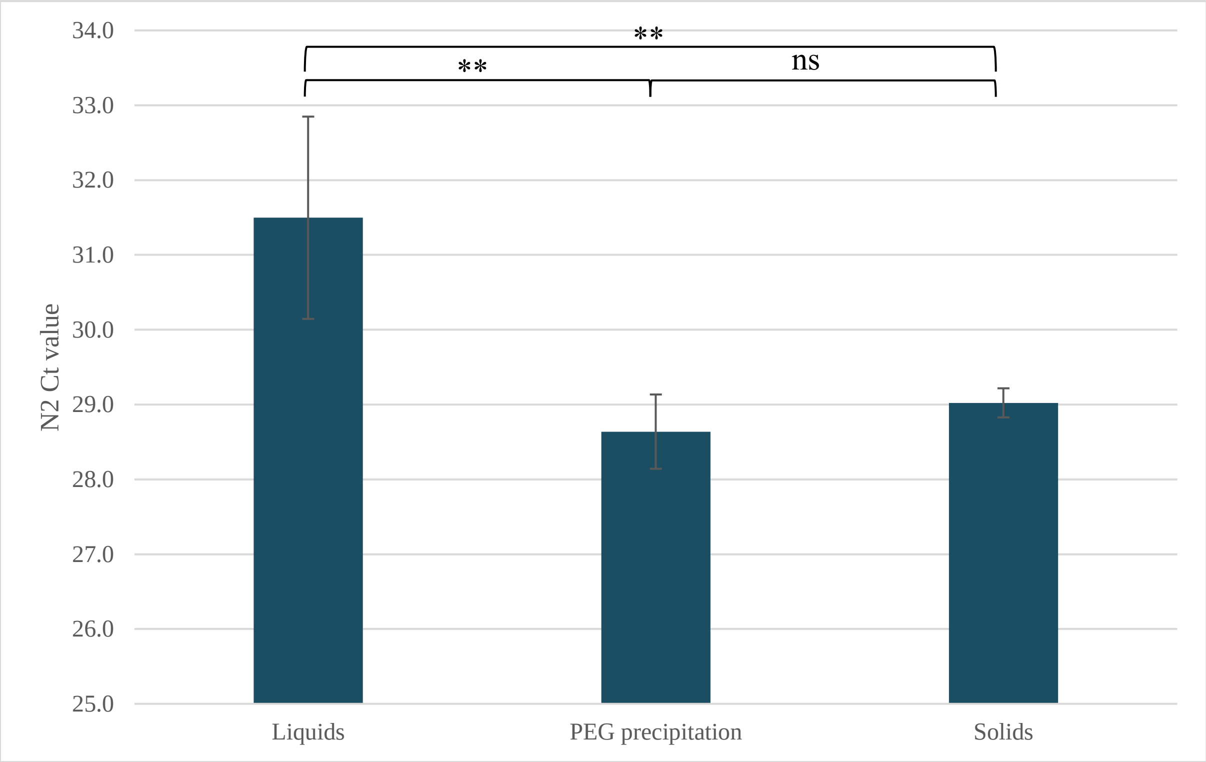


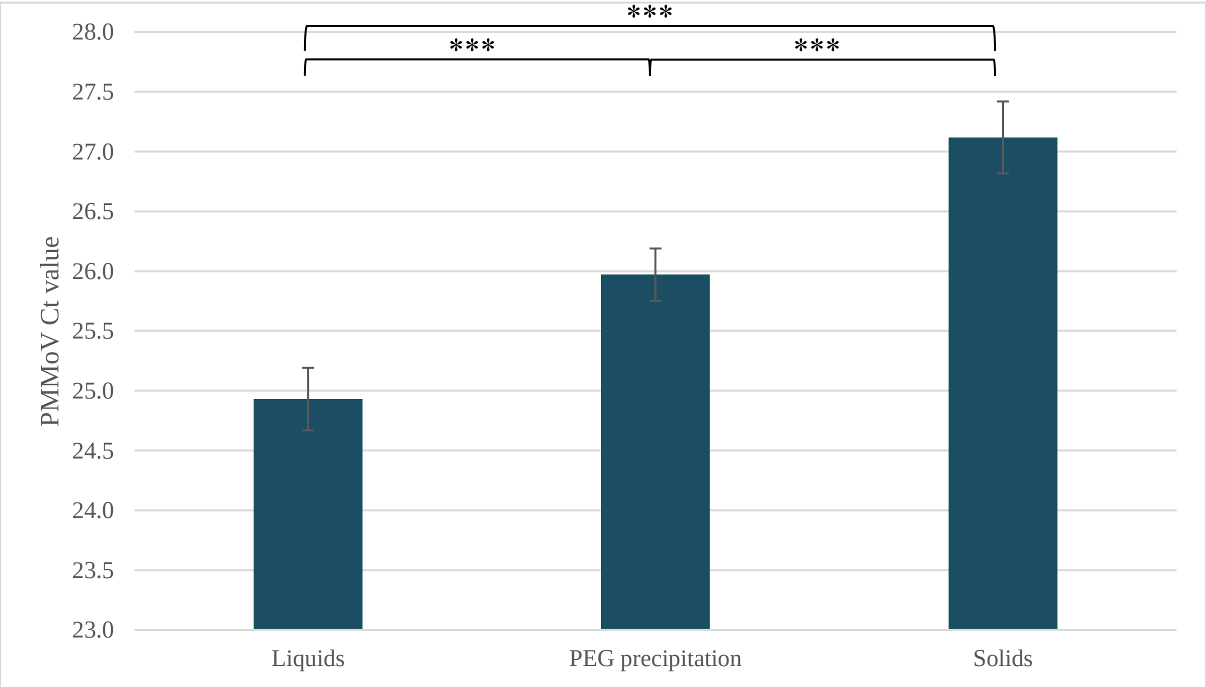
Figure 10: Comparison of RNA yield between the solid and liquid fraction and impact of PEG precipitation. RNA was extracted from the liquid and solid fraction of wastewater and after PEG precipitation using the Allprep kit. A shows the direct comparison of the RNA concentrations obtained from the three fractions, while B displays the total extracted RNA amount in the PEG fraction and the liquids and solids added. Averages of three biological and two technical replicates, error bars represent standard deviation. t-test results: ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$

Next, we quantified SARS-CoV-2, PMMoV and BCoV in the three fractions using RT-PCR. When looking at the Ct values of the individual RT-PCR targets, we see differences between the concentration methods. SARS-CoV-2 is most abundant in the solid fraction (Ct 29.0 ± 0.2) and samples extracted using PEG precipitation (Ct 28.6 ± 0.5) but partitioned less into the liquid fraction (Ct 31.5 ± 1.4) (Figure 11A). This indicates that SARS-CoV-2 accumulates on solids in wastewater.

A



B



C

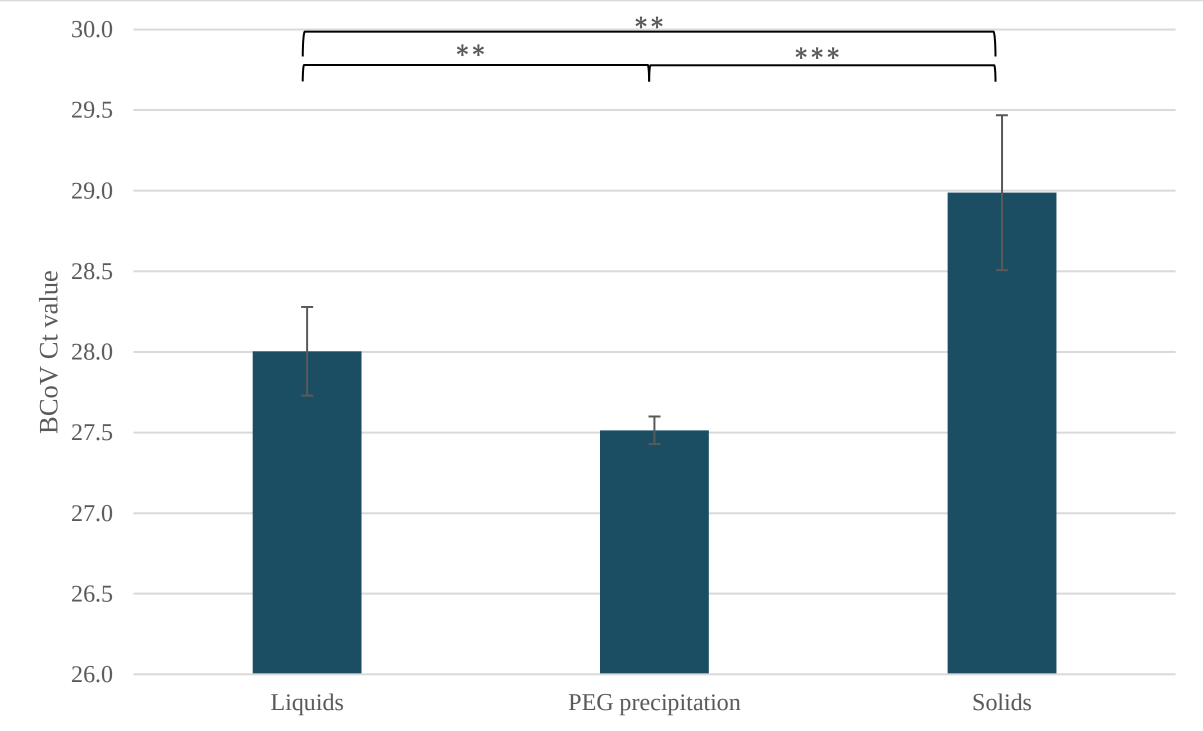


Figure 11: Comparison of the effect of PEG precipitation and liquid or solid fraction extraction on the Ct values of A: SARS-CoV-2, quantified by the N-gene; B: PMMoV, quantified by the PMMoV RdRp gene and C: BCoV, quantified by the BCoV M gene. Averages of Ct values of three biological and two technical replicates, error bars represent standard deviation. t-test results: ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

For PMMoV the results look the opposite. PMMoV is more abundant in the liquid fraction of wastewater (Ct 24.9 ± 0.3) and the lowest in the solids or PEG-precipitated fraction (Ct 27.1 ± 0.3 and 26.0 ± 0.2, respectively, Figure 11B). When comparing the yield of PMMoV from PEG precipitation with the solids and the liquid fraction we can observe that only 27% of the PMMoV in the liquid fraction was precipitated by PEG and detected using this method (calculation in 2.3).

Lastly, we analysed the partitioning of BCoV, which had been spiked into the wastewater sample before extraction as an internal processing control. Here we observed that BCoV was more abundant in the PEG-precipitated and liquid fractions (Ct 27.5 ± 0.1 and 28.0 ± 0.3, respectively) than the solid fraction (Ct 29.0 ± 0.5, see Figure 11C). This was surprising as we expected BCoV to have a similar partitioning behavior to SARS-CoV-2 due to their structural resemblance (74). However, similar results had also been observed by Juel et al. (75) and Ai et al. (76), who hypothesised that BCoV might bind less efficiently to solids than SARS-CoV-2 or that the shorter contact time of BCoV with wastewater solids results in reduced BCoV partitioning into solids.

Since SARS-CoV-2 is the main target of our surveillance, the method had to be optimised to mainly favour the enrichment of SARS-CoV-2 from wastewater samples. Under this assumption, either the PEG precipitation in combination with centrifugation or just centrifugation to collect the solids could be used. However, when a high sample throughput is desired and time becomes a limiting factor, our results point to simple centrifugation to collect the solids being the best option, which was therefore used for the surveillance program.

3.2. Effects of Wastewater Storage

Next, we analysed the impact of long-term storage of wastewater samples at 4°C on the abundance of SARS-CoV-2 and PMMoV. Although all wastewater samples were extracted at the latest two to three days after arrival, delays in sample deliveries due to holidays or bad weather could lead to extended storage times and potential target degradation.

We observed no significant differences in the Ct values of RNA samples re-isolated after up to three days of storage at 4°C. Student's t-tests of Ct values from two samples after two days of storage resulted in $p(N2) = 0.9$ and $p(PMMoV) = 0.06$ for sample 1 and $p(N2) = 0.08$ and $p(PMMoV) = 0.06$ for sample 2 (Figure 12).

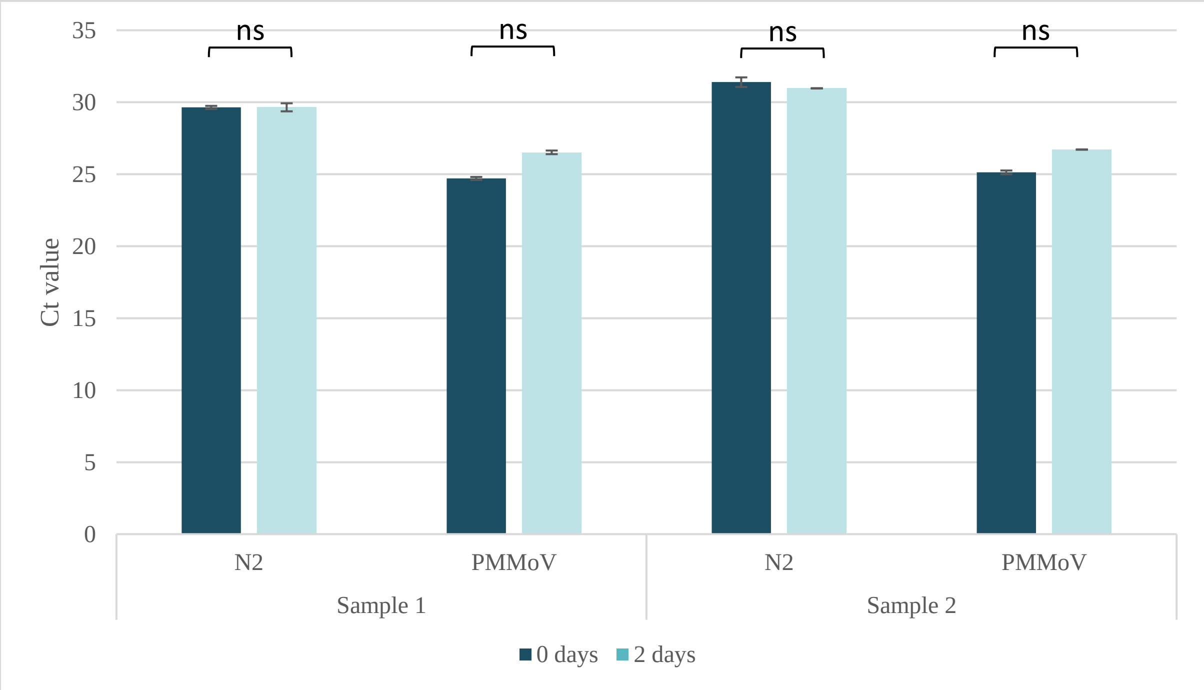


Figure 12: RNA was extracted from two wastewater samples before and after storage for two days at 4°C to examine the impact of storage on the Ct values. A comparison of the Ct values of SARS-CoV-2 and PMMoV before and after storage of the wastewater sample shows no significant differences for either target, although the differences for PMMoV appear bigger than for SARS-CoV-2 N2. Averages of Ct values of two technical replicates, error bars represent standard deviation. ns = $p > 0.05$

In order to examine the impact of longer-term storage, we selected n = 13 wastewater samples from Regina that had been stored at 4°C for up to 550 days (see Table 4). We then re-isolated RNA from them and compared the Ct values to the initial RNA extractions. We observed that for SARS-CoV-2 as well as for PMMoV the Ct values changed over time.

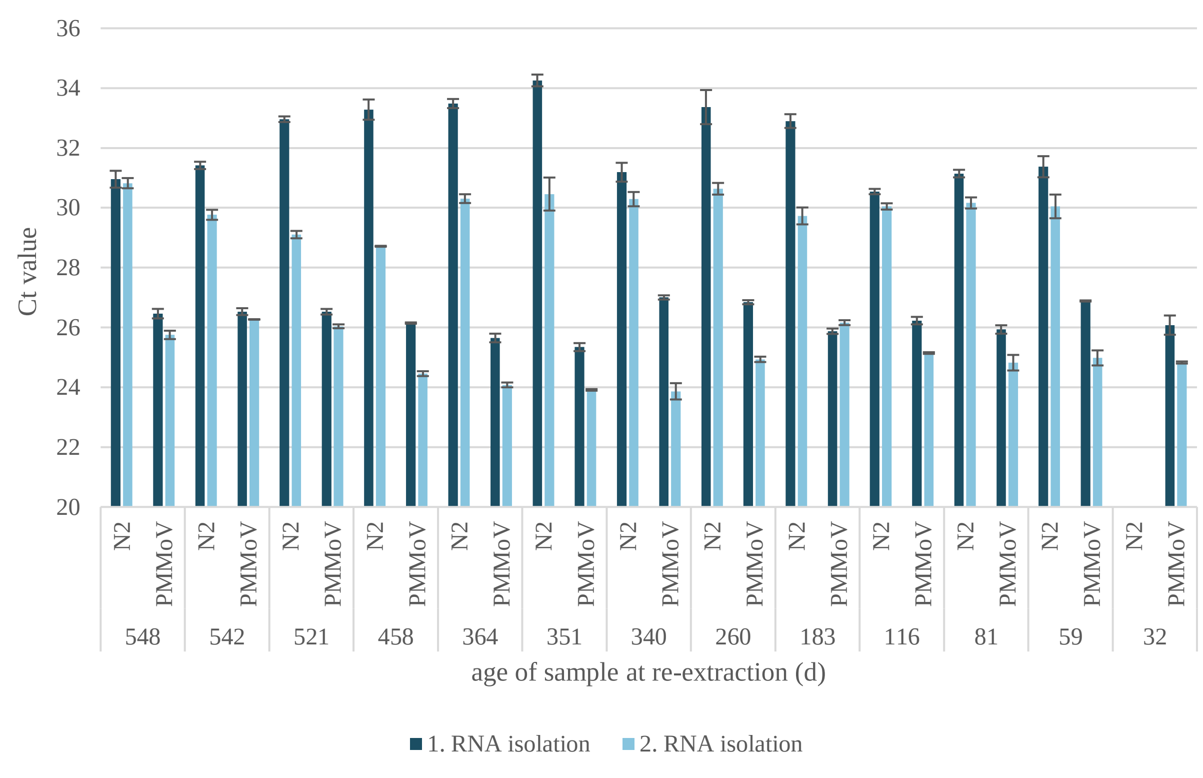


Figure 13: We isolated RNA from wastewater samples before and after storage for 32-548 days at 4°C and compared the Ct values of SARS-CoV-2 N2 and PMMoV of the first and the second isolation. With exception of one sample, in all other occasions the second RNA isolation had a lower Ct value, indicating more RNA than in the first isolation. Averages of two technical replicates, error bars represent standard deviation.

We did not observe RNA degradation for either SARS-CoV-2 or PMMoV, which would have resulted in a higher Ct value of the second isolation. Instead, we saw a shift to lower Ct values in most samples, indicating more RNA in the second extraction than in the first. In 12 out of 13 samples taken from Regina we observed a decrease in Cts ranging from 0.26 to 3.14 for PMMoV and 0.13 to 4.57 for N2 (Figure 13). While those differences appear small in Ct values, the differences become much more prominent when converting them to RNA copy numbers (see Eq. 1, Figure 14). The shifted Ct values correspond to an up to 9- or 24-fold increase in copy numbers for N2 and PMMoV during storage, respectively (Figure 14).

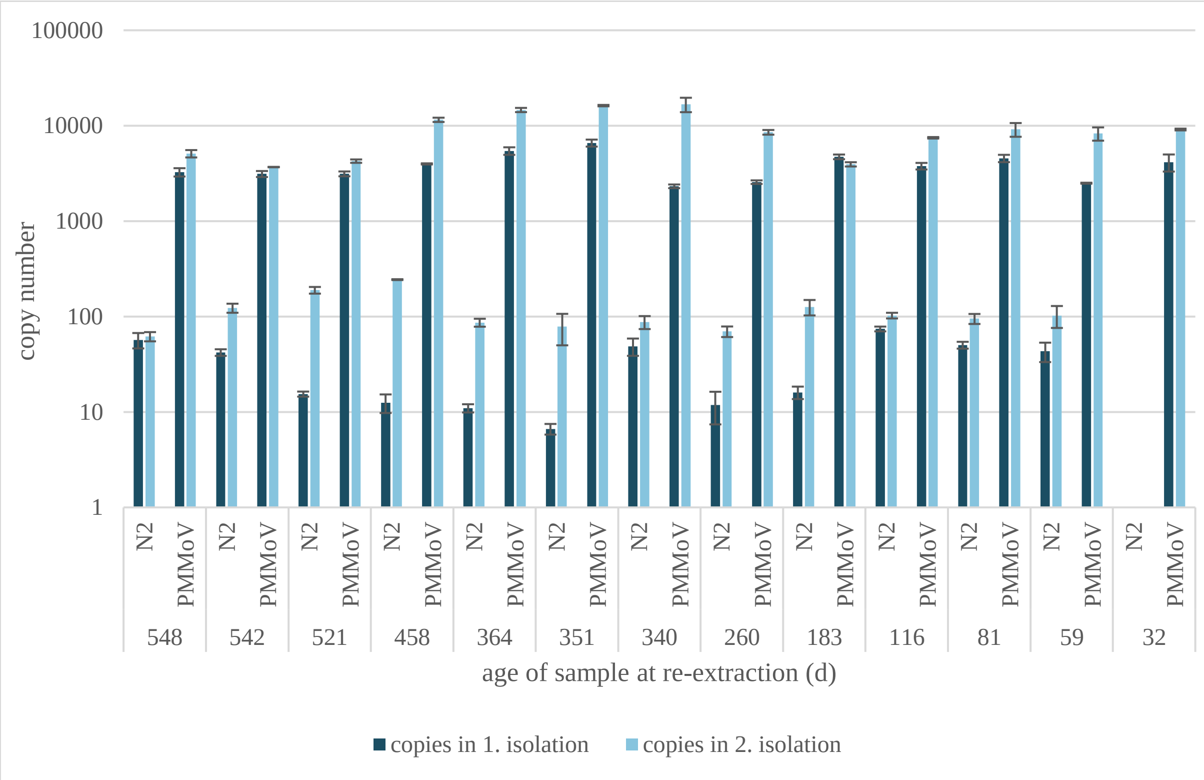


Figure 14: The Ct values from Figure 13 are converted into copy numbers using equation Eq. 1. All except one RNA isolates of the second isolation contain a higher copy number of SARS-CoV-2 and PMMoV than the first isolation. Y-axis presented in logarithmic scale to accommodate PMMoV and SARS-CoV-2. Averages of two technical replicates, error bars represent standard deviation.

There might be a correlation between sample storage time and increase of copy numbers. A linear regression between the relative increase of copy numbers and storage time shows an R^2 = 0.55 for SARS-CoV-2 N2 and R^2 = 0.33 for PMMoV (Figure 15A and B). However, when excluding the oldest samples from 2020 from the dataset, the R^2 improve to 0.77 and 0.59 for SARS-CoV-2 and PMMoV, respectively.

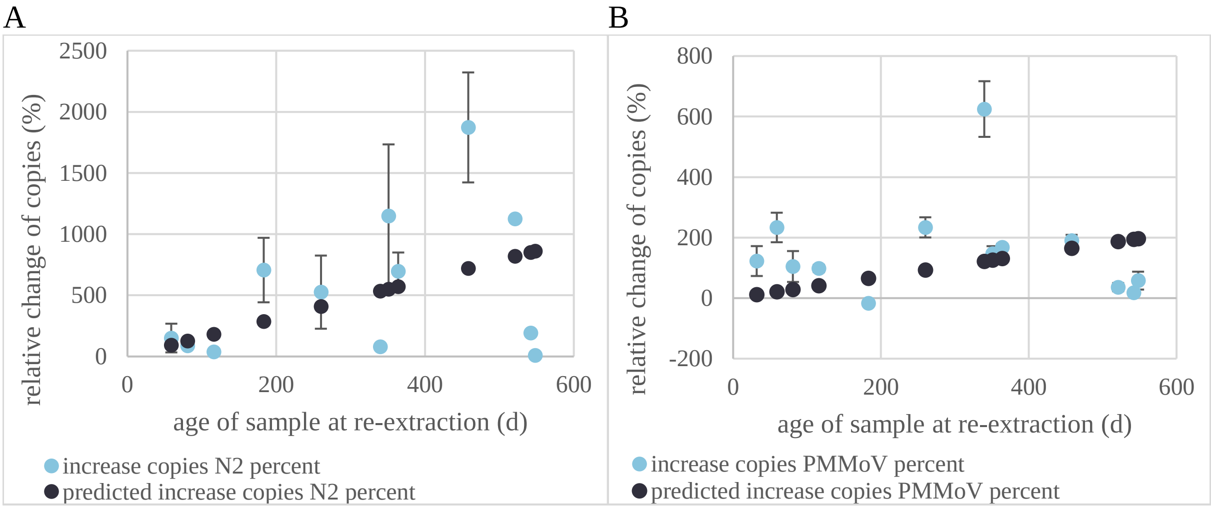


Figure 15: The copy numbers of SARS-CoV-2 (N2) (A) and PMMoV (B) increase in most samples during storage at 4°C. The increase appears to become more pronounced the longer the samples are stored, as a prediction of the relative change of copy numbers generated by linear regression shows (R^2 = 0.55 for SARS-CoV-2 N2 and R^2 = 0.33 for PMMoV). Averages of two technical replicates, error bars represent standard deviation.

In contrast to the increase of the SARS-CoV-2 copy numbers in the solids fraction over time, we observed a significant decrease of solids in the samples during storage time (**Error! Reference source not found.**), likely due to degradation of the solids.

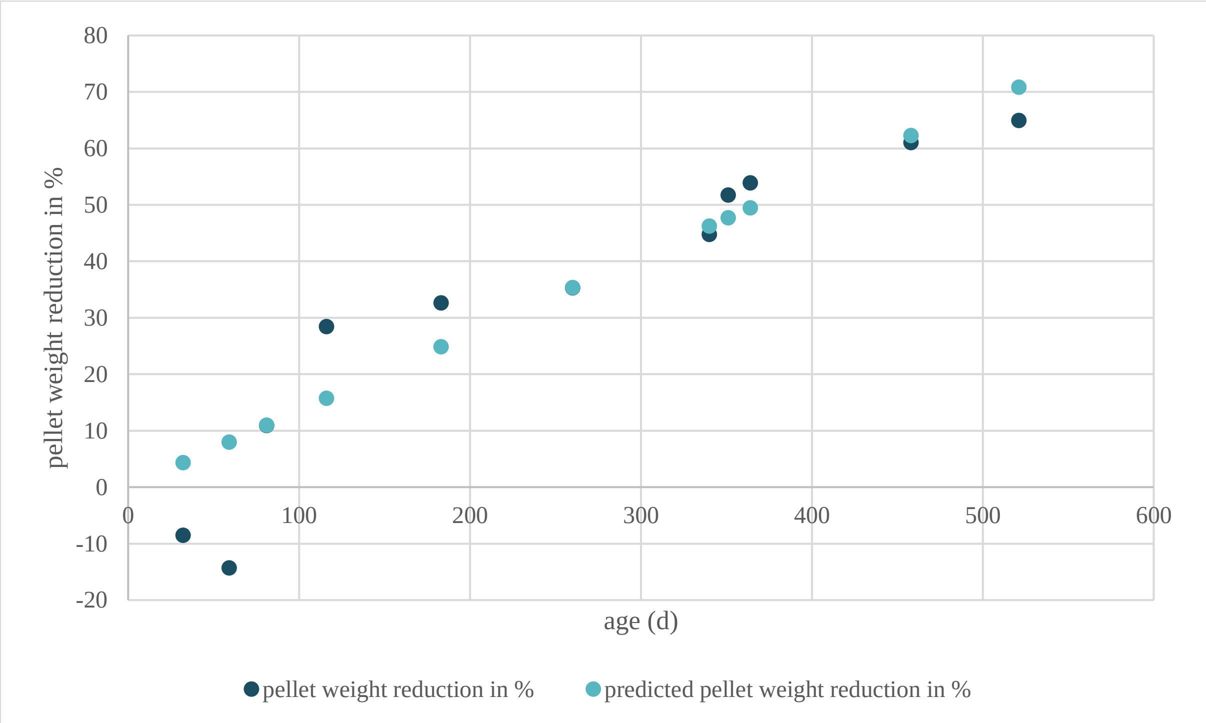


Figure 16: The solids content of wastewater samples decreases over time during storage at 4°C, as seen in the pellet weights of the wastewater samples after centrifugation. A linear regression shows $R^2 = 0.95$.

To account for this effect, we normalised the Ct values against the pellet weight and the sample volume as described in 2.6. This adjustment did, however, not change the observation that the copy numbers of SARS-CoV-2 and PMMoV increased between in first and the second RNA extraction (Figure 17). Melt curves for PCR products of the re-isolated samples did not show amplification of additional targets that could explain the lower Ct values and the results were reproducible (Ct range of 0.5).

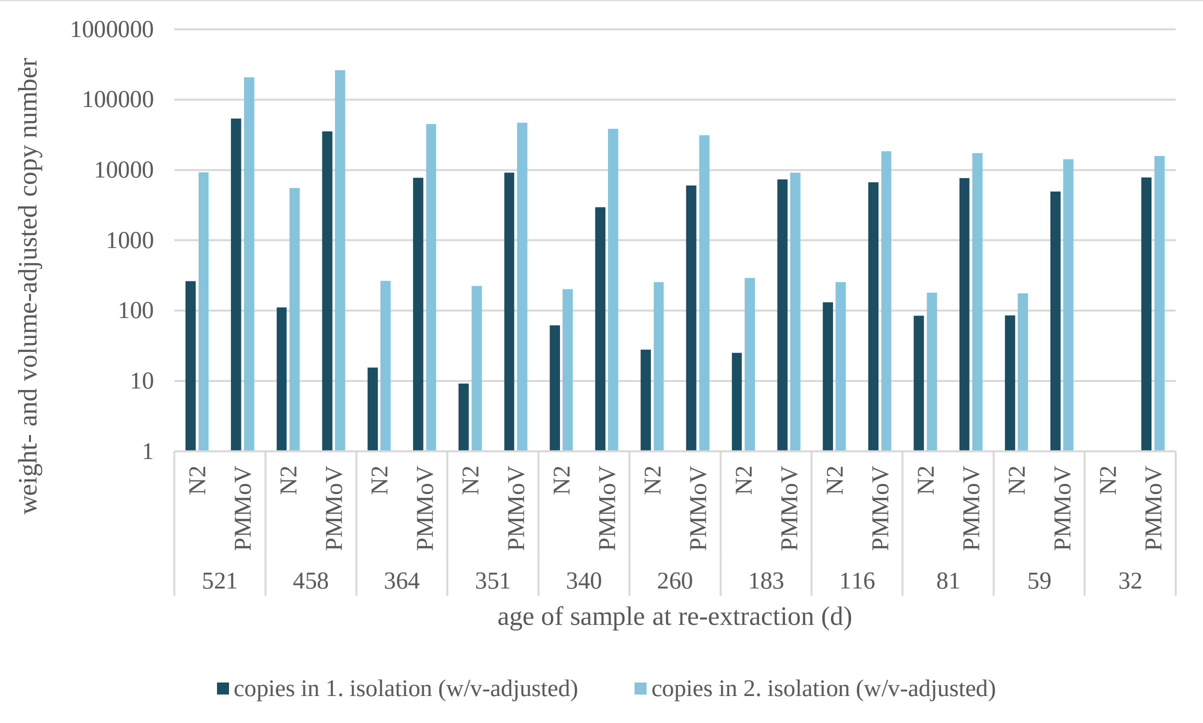


Figure 17: Normalisation of the copy numbers against the weight of the solids and the volume in each extraction does not change the trend of the 2. RNA isolations after storage at 4°C containing more RNA than the 1. isolations. No pellet weights were recorded for the first two samples (548 and 542 days of storage).

To confirm that the re-isolations did not contain more RNA than the first because the RNA of the first isolations had degraded during storage at -80°C, we ran an RNA integrity assay on both sets of samples (Figure 18). The assay distinguishes between long RNAs, pointing to intact RNA in the sample, and small RNAs, indicating degraded RNA in the sample. The samples from the first RNA isolations contained mostly long RNAs whereas the re-isolated RNA samples all contained 100% small RNAs, showing that RNA has degraded in the wastewater samples during storage at 4°C and but no degradation happened during storage of the isolated RNA at -80°C.

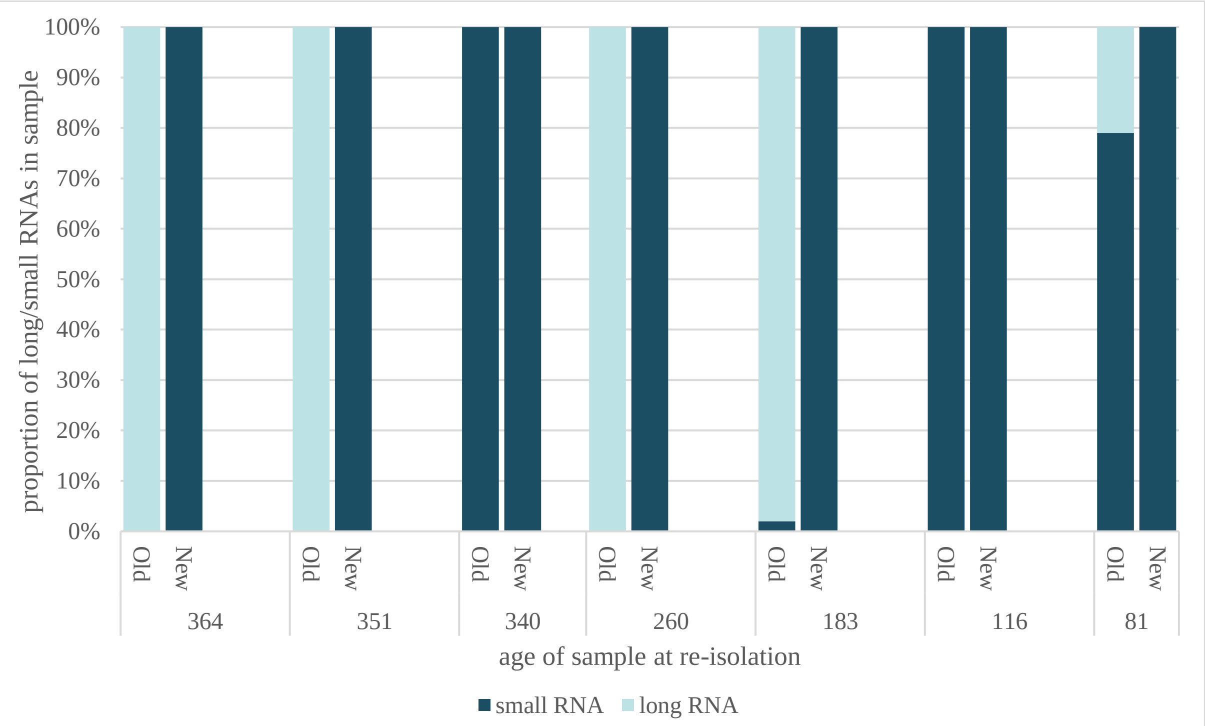


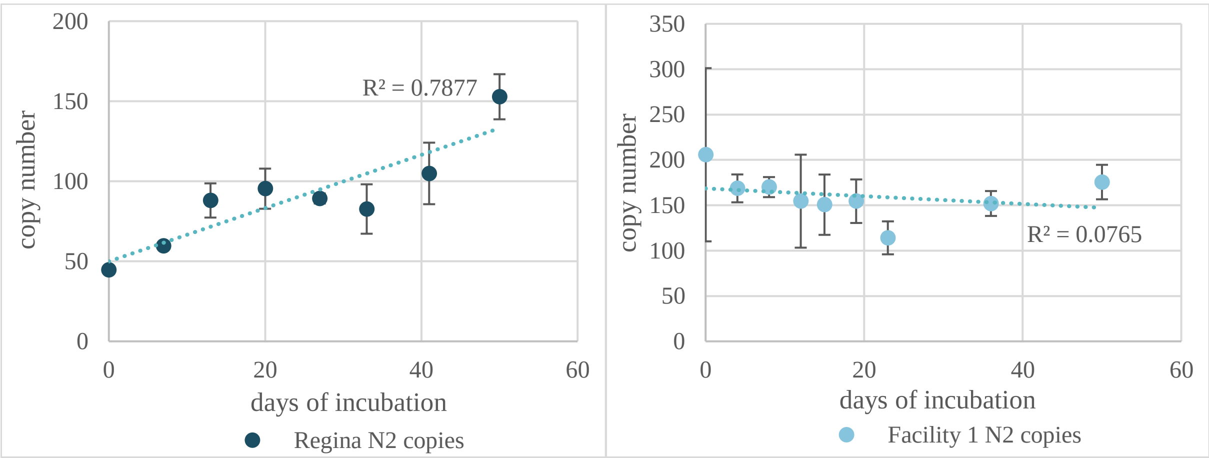
Figure 18: We re-isolated RNA from wastewater samples that had been stored at 4°C for extended periods (81-364 days) and then examined the RNA integrity of the initially isolated RNA (at 0 days incubation, termed 'old') and the RNA isolated after storage (termed 'new'). The assays showed that while the initially isolated RNA samples contain mostly long intact RNA strands, the re-isolated samples all contain small RNA fragments, indicating that RNA degradation happened in the wastewater samples during storage at 4°C but not in the RNA samples at -80°C. Failed assays due to not enough RNA in the sample are not shown.

3.2.1. Long-term Storage Effects

The unexpected results from the previous experiment made us want to investigate whether the observed effects of storage were sampling site-specific. We selected wastewater samples from two locations, the city of Regina as well as a smaller facility (Facility 1), and analysed the impact of wastewater storage at 4°C on the levels of SARS-CoV-2, PMMoV and the spike-in control BCoV. For this analysis we prepared samples with a large volume from both wastewater sampling sites that allowed us to isolate RNA from the same samples continually and thereby track changes over time. BCoV was added to the samples on the first day of incubation. The preparation of the samples is described in 2.6. The measured Ct values were converted into copy numbers using Eq. 1.

Both sampling sites were analysed over a period of 50 days (Figure 19A-E). The copy numbers for N2 and PMMoV in the Regina sample steadily increased over time by 242% and 79%, respectively, indicating an enrichment of SARS-CoV-2 and PMMoV RNA in the extract over time. For the Facility 1 sample, neither N2 nor PMMoV showed a steady trend in one direction, both fluctuated. This indicates that the effects of storage can differ between sampling sites. The copy numbers for BCoV fluctuated in both of the samples, with a possible increasing trend. In hindsight, the BCoV might have needed to be spiked into the sample earlier before starting the study to allow enough time for partitioning to take place. Since neither virus can replicate in wastewater, the apparent increase of RNA in the extract likely arises from partitioning of the viruses into the solid fraction, which is used for RNA extraction. This effect differs between sampling sites and might be caused by the site-specific wastewater matrix that can vary between sites (77,78).

A D



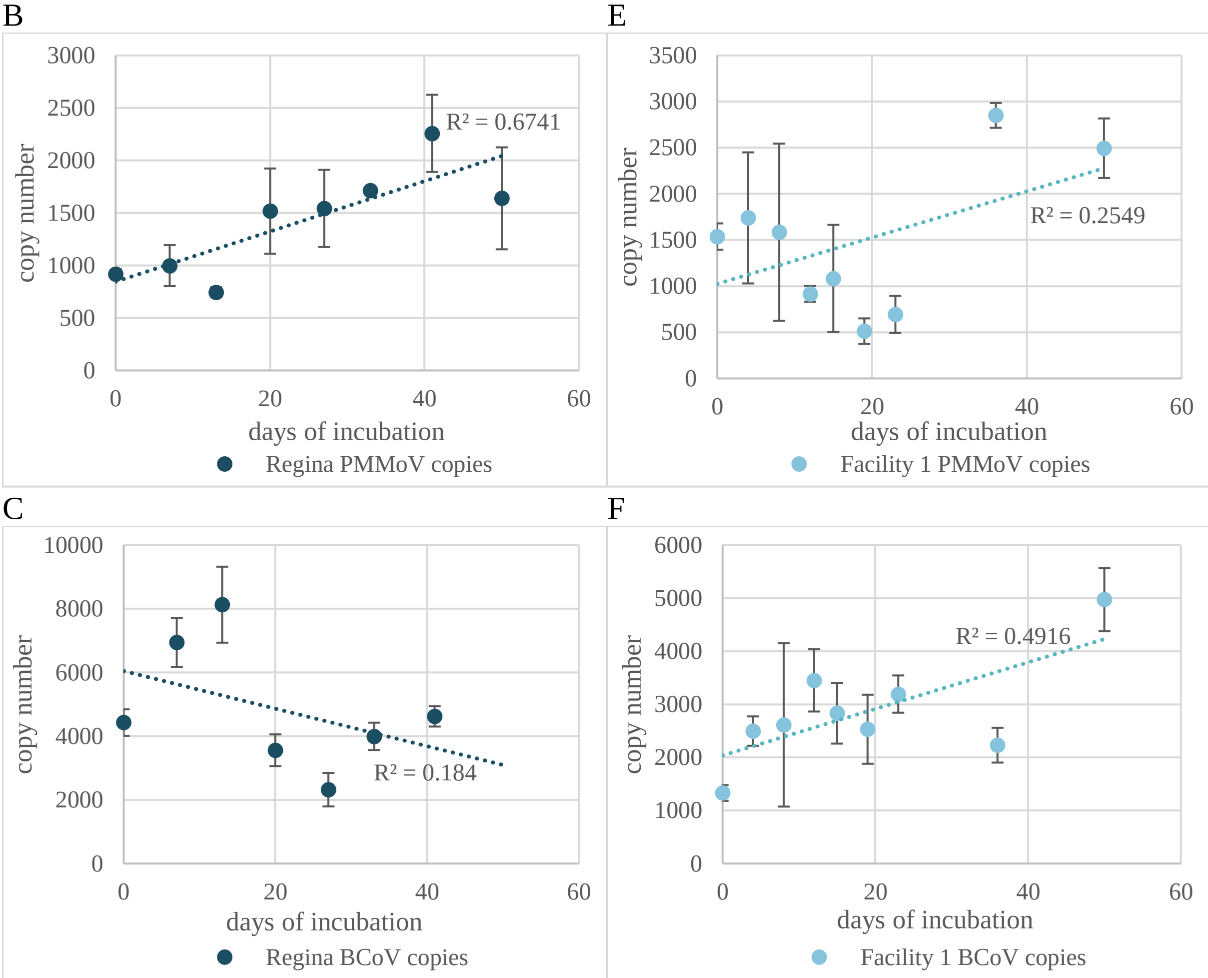


Figure 19: We continually analysed the copy numbers of SARS-CoV-2 (N2), PMMoV and the spike-in control BCoV of two large wastewater samples from Regina (A-C) and Facility 1 (D-F) for 50 days to further examine the effects of storage at 4°C. While SARS-CoV-2 (N2) and PMMoV in the Regina sample increase with incubation time, no such trends were observed in the Facility 1 sample. The spike-in control BCoV does not show a clear increase or decrease, either. Averages of two biological replicates, error bars represent standard deviation.

Similarly to the previous experiment, we observed a general decrease of the pellet size from both sampling sites over time (Figure 20A and B). In addition, we also observed a decrease of the RNA concentration (Figure 20C and D).

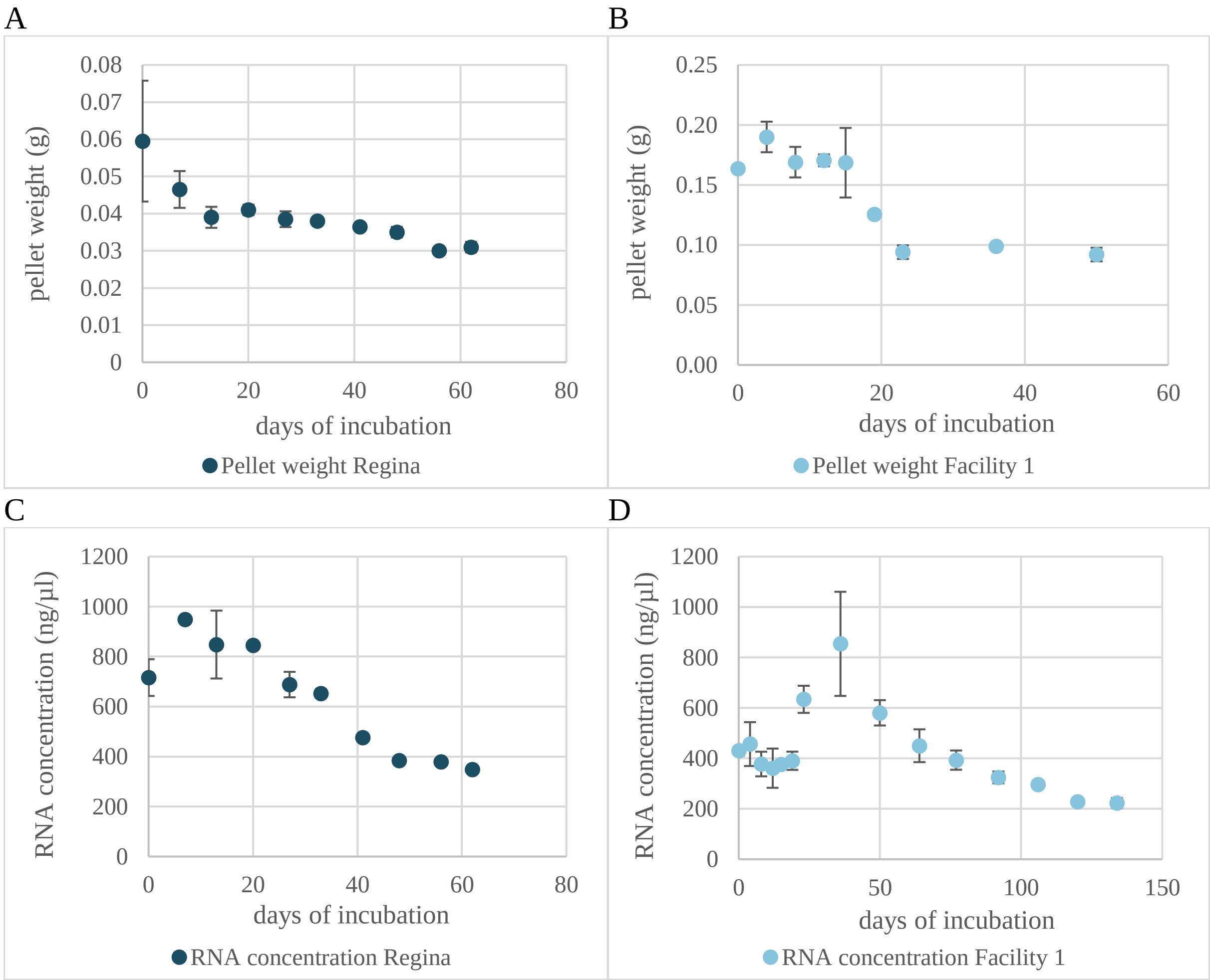


Figure 20: Two samples from Regina and Facility 1 were incubated at 4°C and continually analysed. The weight of the pellet decreased for both sampling sites (A,B) as well as the RNA concentration obtained after extraction (C,D). Averages of two biological replicates, error bars represent standard deviation.

3.3. Wastewater Surveillance

The third aim of our study was to monitor the SARS-CoV-2 levels in the wastewater of Regina and other locations in Saskatchewan and thereby track the development of the pandemic independent of clinical testing. We applied the previously established method in which we extracted RNA from enriched wastewater solids using the Allprep kit and quantified SARS-CoV-2, PMMoV and BCoV using RT-PCR. PMMoV served as normalisation factor for fecal matter and BCoV as an extraction control. The results are compared to the reported clinical data (active cases, new cases and hospitalisations). The full method can be found under 2.4 Wastewater Surveillance.

3.3.1. Observations from Wastewater Surveillance in Regina

The study on Regina wastewater was conducted from August 2020 onwards and is still ongoing as of June 26, 2023. This thesis reports about the time from March 2021 to September 2022, where a total of 304 wastewater influent samples taken from the wastewater treatment plant (WWTP) in Regina (2-3 per week) were processed.

Figure 21 shows the viral load of SARS-CoV-2 in Regina wastewater from March 2021 to September 2022 in comparison to the active COVID-19 case numbers (as reported on the Dashboard of the Government of Saskatchewan (79)). The viral load is calculated by normalizing the measured SARS-CoV-2 copy numbers against the measured PMMoV copy numbers, therefore the load has no unit.

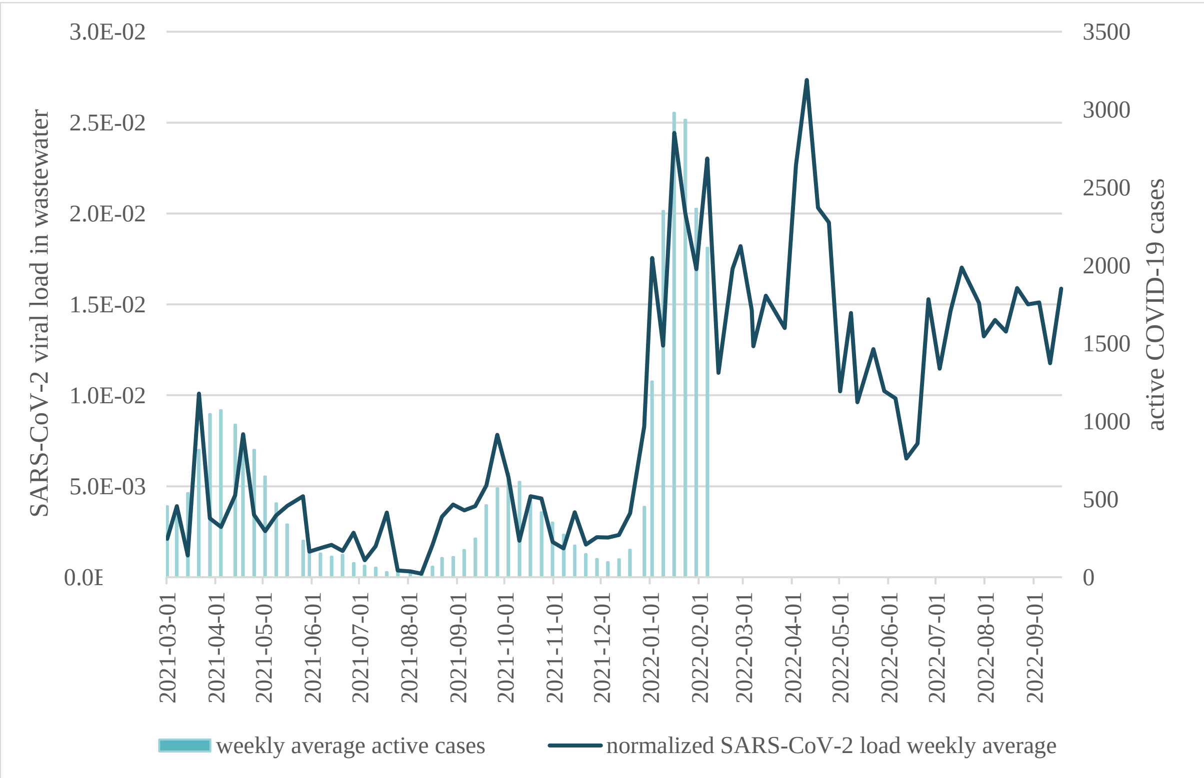
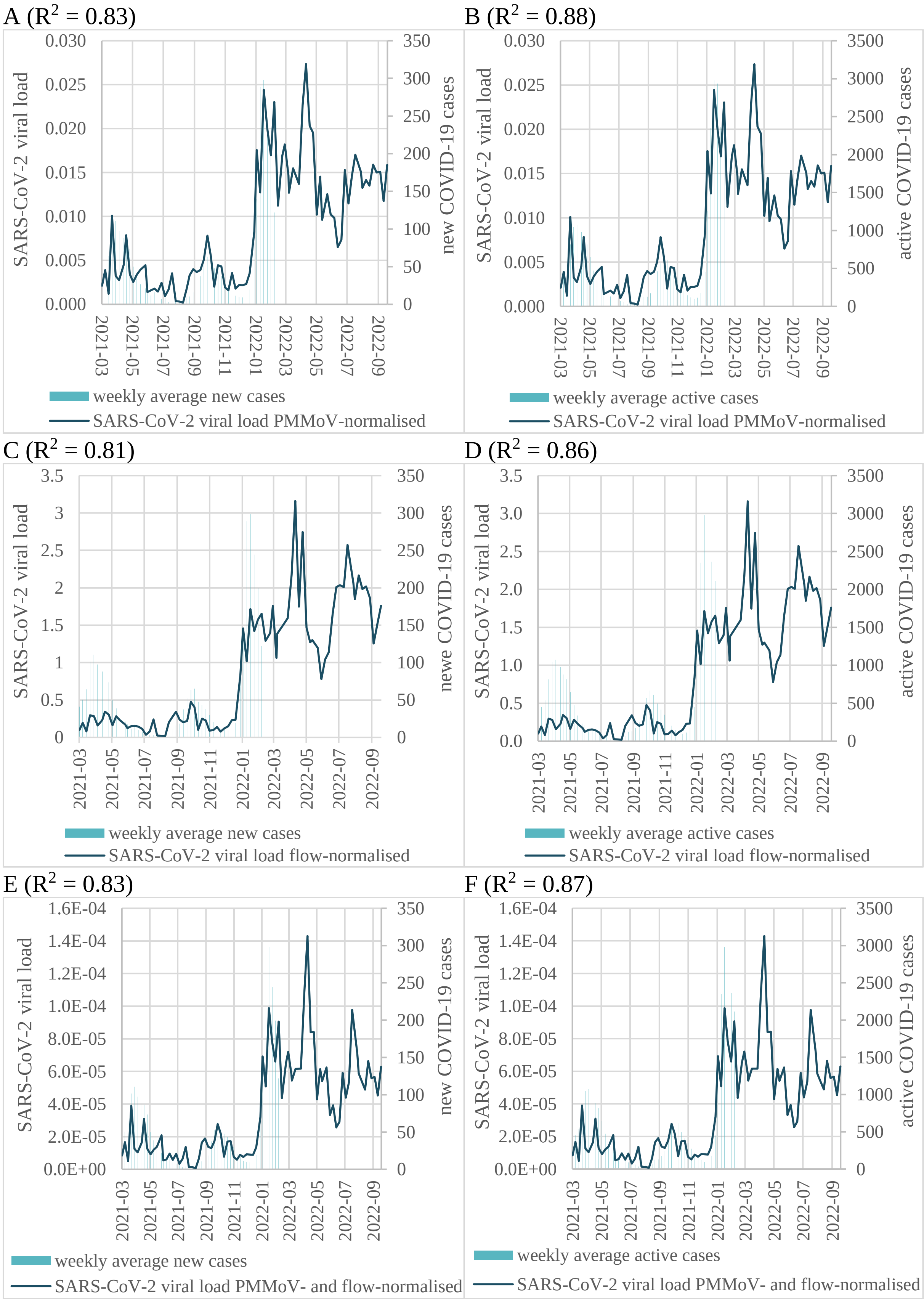


Figure 21: Time-series measurement of SARS-CoV-2 isolated from the wastewater influent collected at the Regina wastewater treatment plant from March 2021 until September 2022. The blue line represents the weekly averages of the SARS-CoV-2 viral load (PMMoV-normalised). Blue bars indicate the number of active COVID-19 cases reported by the SHA until the regular reporting of case numbers was discontinued on Feb. 6th, 2022.

The calculation for normalisation is described in 2.7. Different normalisation methods were tested including the use of PMMoV and the flow rate as described in 2.7 (Figure 22). The normalised values were compared to the reported new (A,C,E,G) and active (B,D,F,H) COVID-19 cases and linear regressions were calculated to determine which normalisation produced the best correlation to the reported data. Figure 22 A and B show the effect of normalisation against PMMoV, C and D against the wastewater flow rate with increasing effect (see 2.7). E and F show normalisation against PMMoV in combination with the flow rate and G and H show no normalisation. The best correlation was observed between PMMoV-normalised SARS-CoV-2 and the reported active cases ($R^2 = 0.88$), which was therefore used for all following graphs. Although the main trends of the pandemic are also recognizable without PMMoV-normalisation (G,H), the peaks of the Alpha and Delta wave are more pronounced with it.

The flow rate can be interpreted in two ways: a higher flow can either cause more virus particles to be picked up by the sampler and therefore correspond to a higher viral load in the sample than usual (increasing effect) or it can dilute the virus in the sample and correspond to less virus in the sample (decreasing effect) (57). Although we saw an increased flow rate after heavy precipitation (Figure 34), we found that neither normalisation against the flow rate improved the correlation of our wastewater data to the reported case numbers. Of the two flow rate approaches, normalisation against the increasing effect was slightly better (shown in C,D; comparison in Appendix C) which could be explained by the very low precipitation rates in Regina. The reported active COVID-19 cases showed better correlation to the wastewater data than the new cases (Figure 22A-H) and are therefore used for comparison from here on. The regression results of further normalisation approaches can be found in Appendix C.



A (R² = 0.83)
SARS-CoV-2 viral load
new COVID-19 cases
weekly average new cases
SARS-CoV-2 viral load PMMoV-normalised
B (R² = 0.88)
SARS-CoV-2 viral load
active COVID-19 cases
weekly average active cases
SARS-CoV-2 viral load PMMoV-normalised
C (R² = 0.81)
SARS-CoV-2 viral load
newe COVID-19 cases
weekly average new cases
SARS-CoV-2 viral load flow-normalised
D (R² = 0.86)
SARS-CoV-2 viral load
active COVID-19 cases
weekly average active cases
SARS-CoV-2 viral load flow-normalised
E (R² = 0.83)
SARS-CoV-2 viral load
new COVID-19 cases
weekly average new cases
SARS-CoV-2 viral load PMMoV- and flow-normalised
F (R² = 0.87)
SARS-CoV-2 viral load
active COVID-19 cases
weekly average active cases
SARS-CoV-2 viral load PMMoV- and flow-normalised
2021-03
2021-05
2021-07
2021-09
2021-11
2022-01
2022-03
2022-05
2022-07
2022-09

G (R^2 = 0.81) H (R^2 = 0.86)

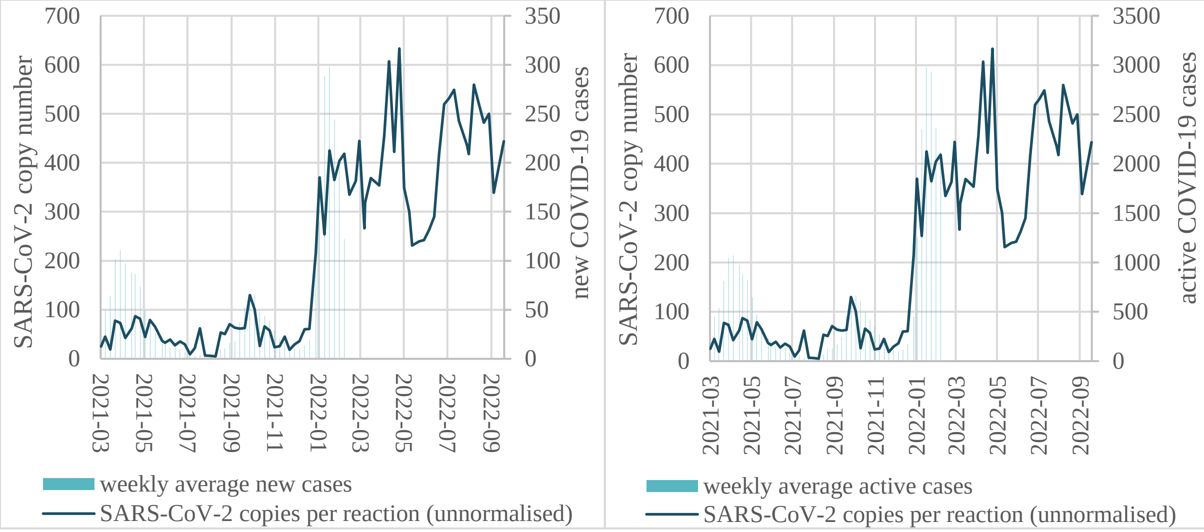


Figure 22: Different normalisation approaches for the SARS-CoV-2 levels in wastewater (lines) were tested and the best one determined by their regression statistics in comparison to the reported new and active COVID-19 cases (columns). We tested normalisation of SARS-CoV-2 against PMMoV (A,B), the wastewater flow rate (C,D), PMMoV and the flow rate together (E,F) and no normalisation other than conversion to copy numbers (G,H).

After February 2022, the active COVID-19 case numbers were not reported anymore. However, a direct comparison of the reported data until February 2022 and the SARS-CoV-2 viral loads in wastewater revealed similar trends. Both wastewater and active cases showed three distinct waves of COVID-19 in Regina (Figure 21). The first wave occurred from March to June 2021, the second from August 2021 to November 2021 and the third starting in January 2022 is still ongoing as of March 2023. The lowest viral loads in this time period were recorded in July and August 2021 and the highest during the peak of the third wave in January 2022.

In addition to the SARS-CoV-2 levels, we obtained data about the presence of SARS-CoV-2 variants in Regina wastewater starting April 2021 from the National Microbiology Laboratory (NML) in Winnipeg, who conducted tiled amplicon sequencing on SARS-CoV-2 RNA from wastewater samples. They used markers (single nucleotide variants) in the sequences as indicators for which variants were present and in what proportion. The data were adjusted and grouped to represent the variants as accurately as possible as described in 2.5.

Based on the sequencing results we can identify which virus variants were causing the COVID-19 infections in Regina. When the variant surveillance started in April 2021, the variant identified mainly was Alpha (Figure 23). Starting in late May 2021, Alpha was gradually replaced by Delta with occurrences of less abundant variants such as Beta, Gamma, Kappa, Eta and Lambda in between. In the period from July to December 2021 almost exclusively Delta sequences were present in Regina wastewater, followed by a rapid replacement of Delta with Omicron BA.1 in early January 2022. The remainder of the study period showed only the Omicron variant, with the sub-lineages BA.1, BA.2, BA.4 and BA.5 making up most of the sequences. These sequencing data confirm that the variants causing the three waves of COVID-19 in Regina were Alpha, Delta and Omicron, respectively (Figure 23).

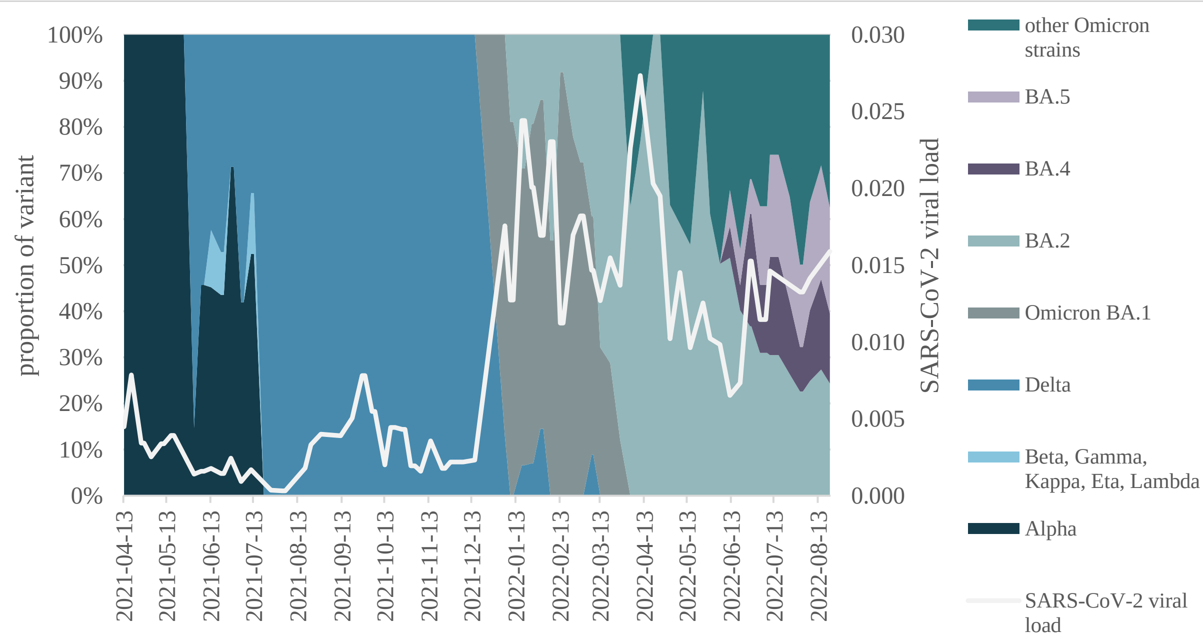


Figure 23: Sequencing provides an overview of the observed SARS-CoV-2 variants and (sub-)lineages detected in Regina wastewater compared to the measured viral load. Sequencing was conducted by the NML in Winnipeg. The variants are colour-coded, weekly averages for viral load and sequencing results.

We then compared the three waves of COVID-19 in Regina as determined by the SARS-CoV-2 viral loads in wastewater, the reported active cases or the reported new cases by measures such as the length of each wave, their maximum peak and the respective peak date (Table 6).

Table 6: Comparison of the three COVID-19-waves observed in Regina between the wastewater viral loads, active cases and new cases. Wastewater data are in arbitrary units (normalised to PMMoV), peak lengths are measured from lowest value before each peak to lowest value after each peak. The reported data were taken from the province's COVID-19 dashboard (79), data regarding the Omicron wave are as of October 2022.

		COVID-19 wave		
Data	Property	Alpha	Delta	Omicron
SARS-CoV-2 load in wastewater	wave maximum (viral load)	4.19E-05	3.57E-05	9.67E-05
	wave maximum date	2021-03-21	2021-09-29	2022-01-20
	length (days)[1]	142	152	ongoing
Reported active cases	wave maximum (cases)	1117	694	3166
	wave maximum date	2021-04-04	2021-10-10	2022-01-23
	length (days)	133	141	ongoing
Reported new cases	wave maximum (cases)	130	90	380
	wave maximum date	2021-03-28	2021-10-04	2022-01-16
	length (days)	138	128	ongoing

A direct comparison of the maxima of the reported case numbers and the wastewater viral load revealed that for the first two waves the peaks were first observed in the wastewater, followed by the new cases and lastly the active cases. Compared to the wastewater data, the peaks of the reported new and active case data were delayed by 7 and 14 days, respectively, during the Alpha wave and 8 and 14 days during the Delta wave. This delay indicates that the reported data might not have represented the state of the pandemic in Regina in real-time during the Alpha and Delta waves. During the Omicron wave, no reliable reported data are available due to a restriction of clinical testing in Saskatchewan (80).

In order to measure the length of each wave, the lowest numbers for reported active and new COVID-19 cases and SARS-CoV-2 viral loads in wastewater were determined and the time span in between them defined as length of the respective wave. According to the SARS-CoV-2 viral load in wastewater, the Alpha wave started shortly before March 01, 2021. The lowest numbers of active and new COVID-19 cases were reported on March 10. The Alpha variant

[1] The study period of Regina wastewater began after the Alpha wave had already started. Therefore, instead of the lowest value before the Alpha wave, the first day of surveillance was used to determine the length of the Alpha wave. Active or new cases are not affected as their lowest values were reported after the beginning of the study period.

was dominant in Regina wastewater from the beginning of the sequencing campaign until the end of May 2021 (Figure 23). From June to August followed a period of low case numbers when also the proportion of the less abundant variants, such as Beta, Gamma, Kappa, Eta and Lambda, became more pronounced. The lowest SARS-CoV-2 viral loads and also reported active cases were observed on July 21 with the lowest new cases following on July 26. A province-wide mask mandate, that had been in place throughout the Alpha wave, was lifted on July 11 (81). The last presence of the Alpha variant was detected on July 13. Soon after, the Delta variant became dominant and the viral load of SARS-CoV-2 as well as the reported active and new cases increased again. Even though the indoor mask-mandate was reinstated on September 17, the viral loads in wastewater increased further and reached maximum levels on September 29, the new and active cases followed on October 04 and 10, respectively. Delta remained dominant in Regina wastewater for roughly five months. After the Delta wave, the viral load in wastewater never declined to their lowest levels in July again and instead stayed at levels roughly 290% higher than the previous lowest levels. Similarly, the reported active cases only declined to levels 237% higher than the previous low on December 09. Therefore, the Omicron wave started before the Delta wave had ended. On January 05, the novel SARS-CoV-2 variant Omicron (BA.1) was detected in wastewater for the first time through sequencing and already served as consensus sequence on this day. After the first detection of Omicron, Delta sequences were only detected on five more sampling dates (until beginning of March), and most of them with frequencies below 10%, further emphasizing the rapid spread of Omicron and subsequent decline and elimination of the previous variants. The first peak of the Omicron wave reached its maximum on January 20, 2022. A second peak in April 2022 exceeded the levels measured during the first Omicron peak and was likely caused by the Omicron lineage BA.2 (Figure 23). As of March 2023, the Omicron wave is still ongoing. Compared to the previous waves, the Omicron peak was 1.3-fold higher than Alpha and 1.7-fold higher than Delta. When measuring the length of each wave, the Alpha wave was the shortest with 138 ± 4 days (133 by active cases, 138 by new cases, 142 by wastewater) followed by the Delta wave with 140 ± 12 days (141 by active cases, 128 by new cases, 152 by wastewater). The Omicron wave has not ended and is therefore the longest wave (>400 days). The differences in lengths between the Alpha and Delta wave are not significant ($p = 0.75$).

We then compared the presence of the different SARS-CoV-2 variants in Regina wastewater to the reported inpatient hospitalisation numbers for the city of Regina (as reported by 79) (Figure 24). The most hospitalisations per day during the study period were observed during the Alpha wave with >70 per day, followed by the Delta and Omicron wave with >60 per day. As of the latest report of the Community Respiratory Illness Surveillance Program (CRISP) in March 2023, Omicron, including the sub-lineages XBB.1.5 and BQ.1, is still the major driver of the pandemic. Hospitalisations with COVID-19 have been increasing during the last reporting period (82).

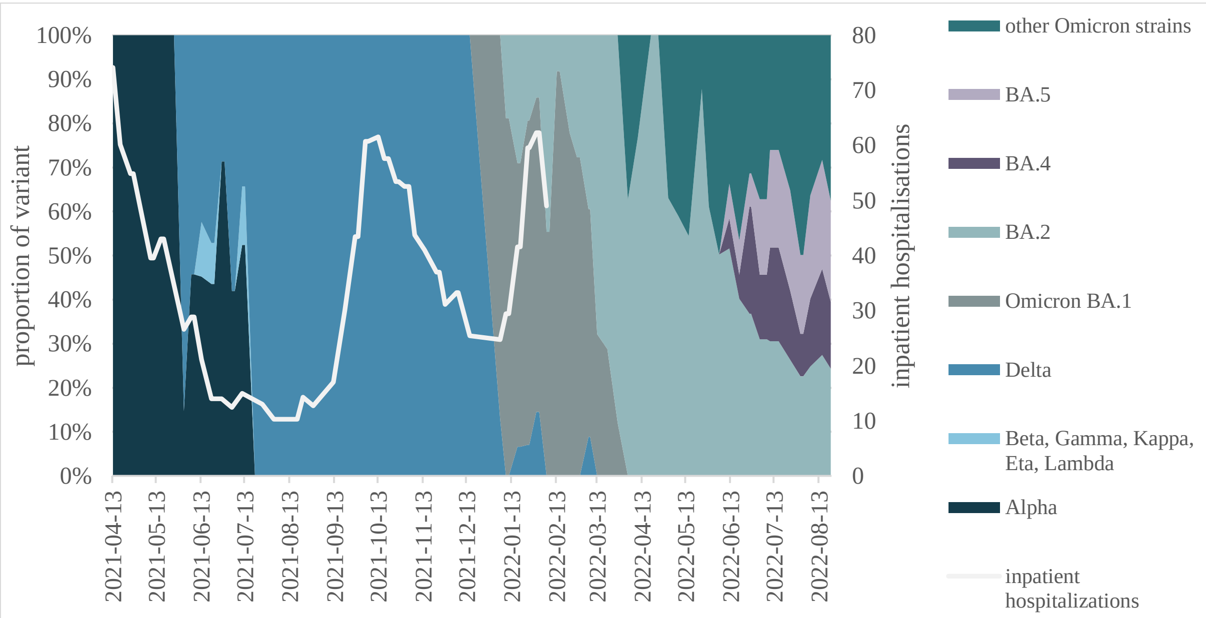


Figure 24: A comparison of the different SARS-CoV-2 variants in Regina wastewater and the inpatient hospitalisations indicates which variant caused the most hospitalisations during the study period. Of the three observed waves, the most hospitalisations at the same time (as reported by the Government of Saskatchewan (79)) happened during the Alpha wave. Sequencing was conducted by the NML in Winnipeg. The variants are colour-coded, weekly averages for the inpatient hospitalisations in Regina and the sequencing results.

Lastly, we compared detection of SARS-CoV-2 variants in wastewater measured by sequencing with detection by qPCR assays. Both data sets were provided by the National Microbiology Laboratory in Winnipeg. Their qPCR assays are described in 2.5.

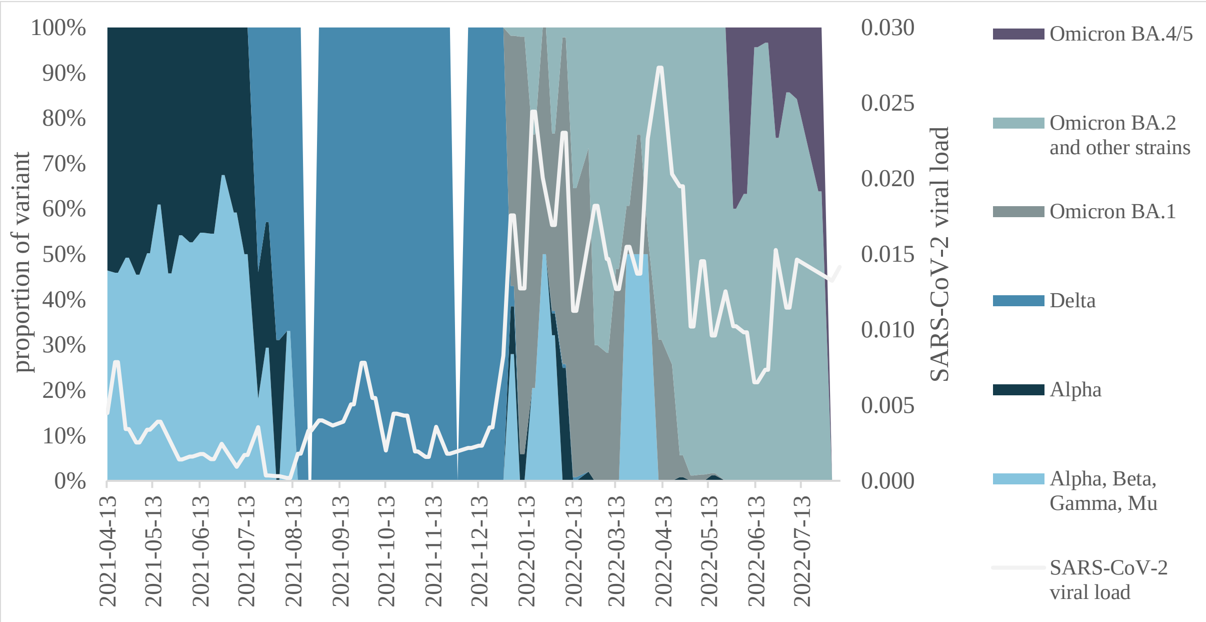


Figure 25: The virus variants present in Regina wastewater were also tracked by qPCR assays conducted by the NML. The results are similar to the sequencing data in Figure 24 but show slight differences in timing and resolution on the lineage level. Some assays target mutations indicative of more than one variant or lineage. Gaps show missing data but are presumed to be Delta.

Both approaches for variant detection showed similar results (Figure 23 and Figure 25). Both first detected the Alpha variant; however, sequencing was able to distinguish between Alpha and the concurrently present variants Beta, Gamma, Eta, Kappa and Lambda (summarised in Figure 23, separate in the data) whereas qPCR required a separate assay for Alpha and one combined assay for Alpha, Beta, Gamma and Mu. This explains why the proportion for (only) Alpha looks much smaller in the qPCR graph than would have been expected during the Alpha wave. The Alpha wave was followed by several months of exclusively the Delta variant in wastewater, as shown by sequencing and qPCR. Sequencing detected Delta one month before qPCR. However, it is not known whether the qPCR assays for Delta were negative before the first report or if the new assay was just started one month later. The latter is likely as the assay had to be developed and in addition the first report of Delta by the qPCR assay already showed a frequency of 100% (raw data not shown). In early 2022, both approaches showed the Omicron variant BA.1 rapidly replacing Delta, and then the Omicron variants BA.2, BA.4 and 5 following after BA.1 (Figure 23 and Figure 25). While both approaches show the same trends, sequencing data provide a higher resolution as they include proportions of sequences down to the sub-lineage level. For clarity and an easier comparison between the two methods, some variants and (sub-)lineages detected using sequencing were combined in Figure 23 (see 2.5). A complete list of all 36 variants and (sub-)lineages that were identified in Regina wastewater by sequencing can be found in listed in. Sequencing data show that more different sub-lineages were detected during the Omicron wave than during the Delta wave. However, several Delta AY lineages coexisted for a long time during the Delta wave whereas when Omicron emerged there was mainly one lineage, BA.1, for over two months, indicating a higher sequence diversity during the Delta wave than during the early Omicron wave. In contrast to the sequencing results, the number of variants identified by qPCR was limited to six (Table 3), which only distinguished between Alpha; Alpha, Beta, Gamma and Mu; two assays for Delta; Omicron BA.1; BA.4/5 and one assay for all Omicron strains, thereby providing no information about the sequence diversity during the Delta wave. Another problem with the qPCR data were unexpected signals in the Alpha and Alpha, Beta, Gamma, Mu assays during the Omicron wave (Figure 25), which likely arose from overlapping mutations between the Alpha and Omicron variants.

3.3.2. Observations from Wastewater Surveillance in other Cities in Saskatchewan

In addition to Regina, SARS-CoV-2 was also monitored in wastewater of several sites in southern Saskatchewan, including Swift Current, Weyburn, Moose Jaw, Yorkton and Lumsden as well as two facilities within Regina and two First-Nation communities in the area. The locations were selected to cover different health regions as well as reflect communities with different population sizes ranging from small communities (Lumsden with fewer than 2 000 inhabitants), mid-sized towns (e.g., Yorkton with 16 000 inhabitants) to large cities with >200 000 inhabitants. Figure 26 shows the location of the communities in southern Saskatchewan and their distance from Regina. The University of Regina campus and Facility 1 are located within the city of Regina and the two First-Nation communities are within a 70 km radius of the city. These locations were studied from their respective start of sampling until October 2022 (for details see Table 1). A total of 1112 samples were collected from the different sampling sites, of those 304 for Regina (2-3 per week), 131 for Swift Current (2-4 samples per week), 110 for Weyburn (3-5 per week), 95 for Moose Jaw (1-2 per week), 148 for Yorkton (2-3 per week), 52 for Lumsden (1-2 per week), 134 for Facility 1 (2-3 per week), 108 for the University of Regina campus (1-3 per week), 17 for the First-Nation community 1 and 13 for the First-Nation community 2.

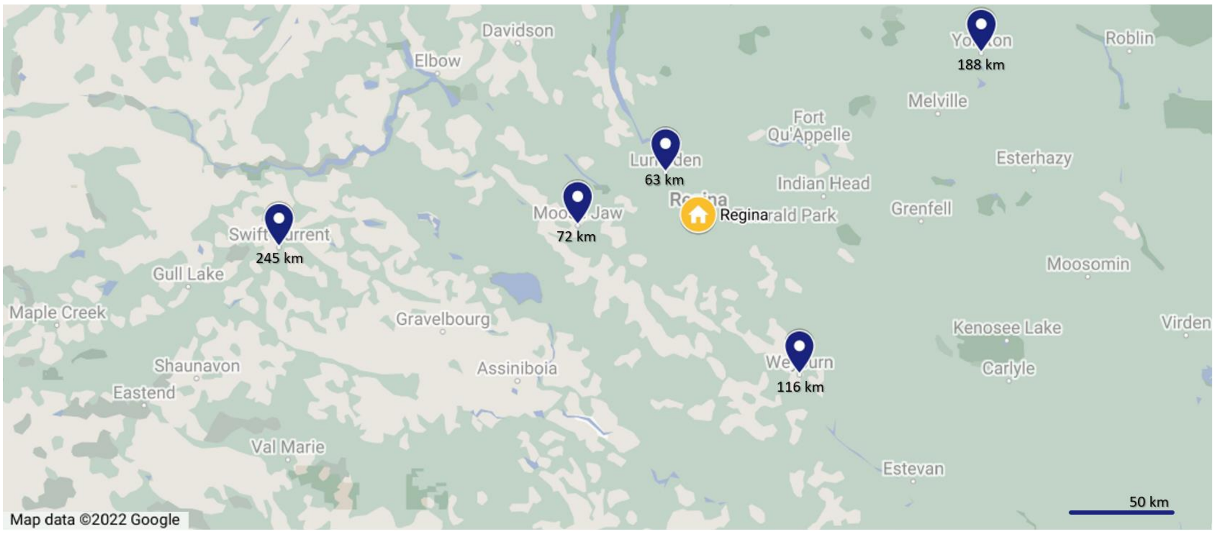


Figure 26: Overview of SARS-CoV-2 wastewater surveillance locations in southern Saskatchewan. Included are cities such as Swift Current (16 604 people), Moose Jaw (33 890), Lumsden (1 824), Regina (228 000), Yorkton (16 343) and Weyburn (10 870). Two smaller locations, the university of Regina campus and Facility 1, are located within Regina and two First-Nation communities are located within a 70 km radius around the city (not specified due to privacy concerns).

While we were generally able to use the same established protocol for RNA extraction from wastewater for all monitored locations, we had to make a few adjustments to accommodate different wastewater qualities. Firstly, we adjusted the volume used for RNA extraction to the solid matter content of the wastewater from each location accordingly to receive pellet wet weights of 200-250 mg per sample. Figure 27 shows the volumes of wastewater used for centrifugation and the weight of the pellet. On average, samples from Moose Jaw contained the highest amount of solid particles whereas samples from Swift Current hardly contained any.

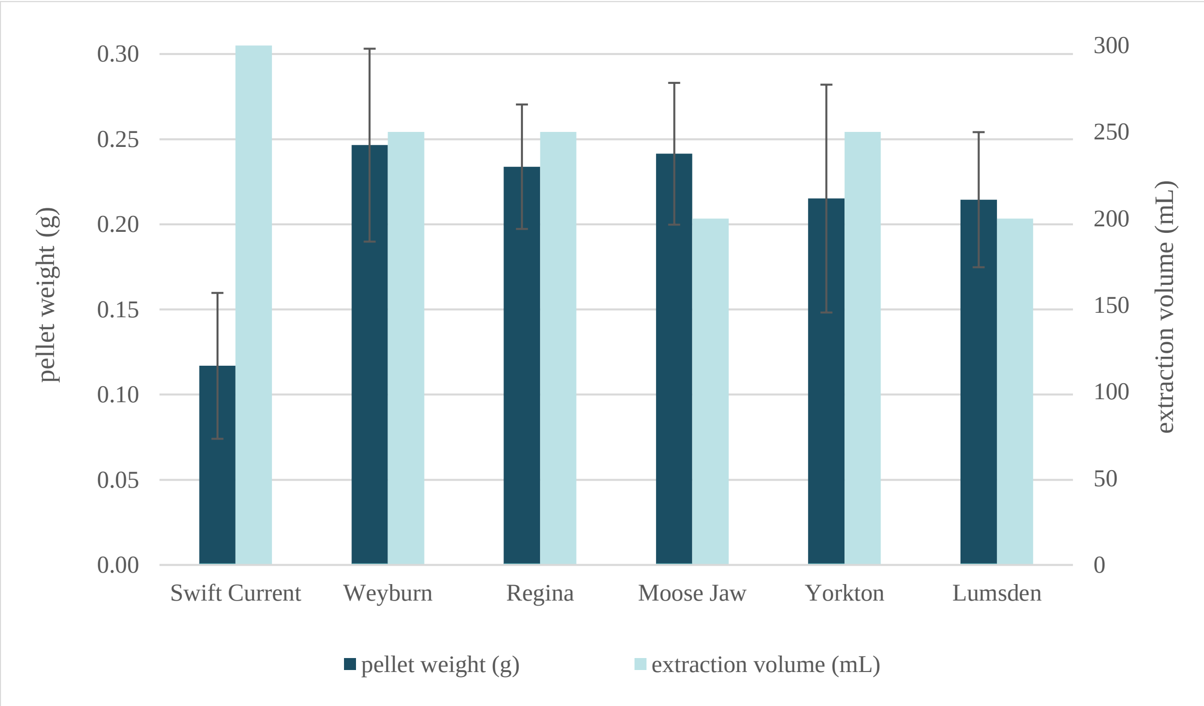


Figure 27: Different volumes of wastewater were used for the different sampling locations due to differences in solids contents. The pellet weights of samples from each location were averaged and compared to the corresponding sampled volume. Averages of pellet weights (n=15-40), error bars represent standard deviation. The wastewater of Swift Current did not contain enough solids to reach our aspired pellet size of 200-250 mg.

In addition to pellet sizes, also the RNA yield varied between the different sampling sites. While wastewater samples from Swift Current contained only half the solids of the other locations ($p < 0.001$), the RNA yield was similar to other locations such as Yorkton and Lumsden. In contrast, wastewater samples from Weyburn contained similar amounts of solids to most other sampled locations but only half as much RNA ($p < 0.001$) could be extracted from them (Figure 28).

Furthermore, wastewater samples from Weyburn were also different from the others in that the inhibitor removal solution (IRS) used during RNA extraction did not precipitate in them entirely and therefore could not be removed before RNA elution. This resulted in failed RT-PCR runs, i.e., no signals were obtained or the Ct values were beyond the quantification limit. As SARS-CoV-2 and PMMoV detection in RT-PCR were reproducible in Weyburn samples also without the IRS step, it was omitted in the processing of those wastewater samples. The reason for RT-PCR inhibition when using IRS remains unknown, along with whether there is a correlation between the low RNA yield and the omission of the IRS step.

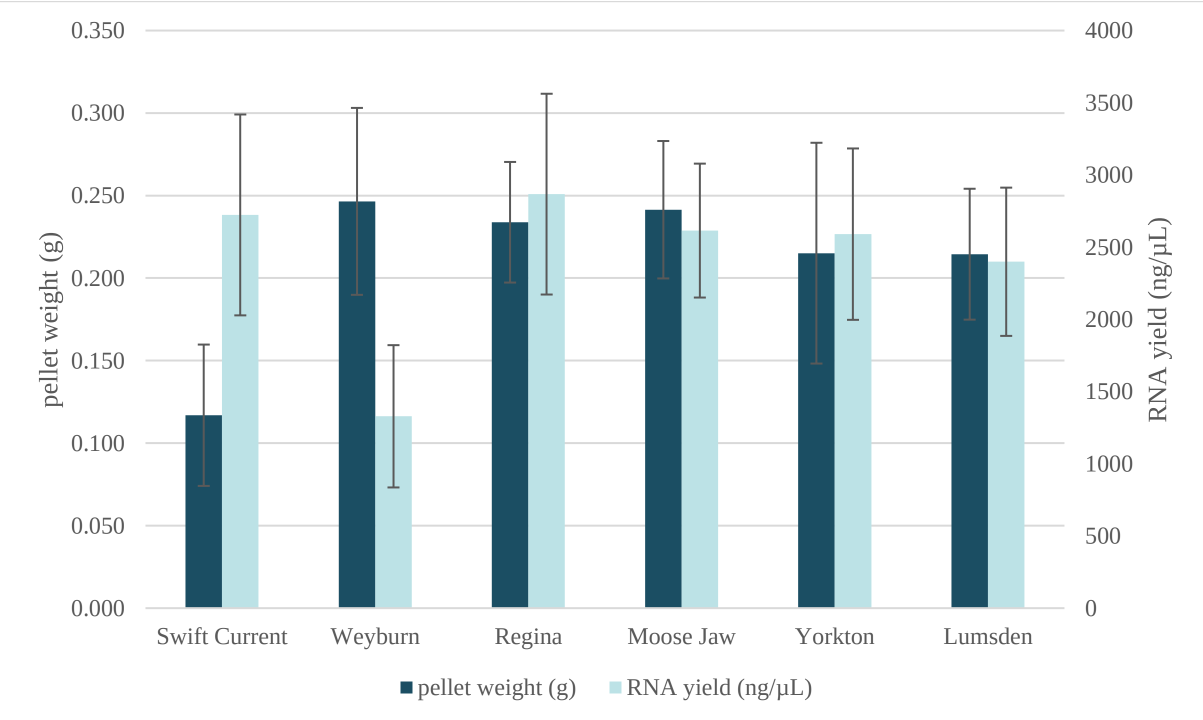


Figure 28: A comparison between the average pellet weight of each location and the respective RNA yield shows that the RNA yield is not solely dependent on the amount of solids in the sample. Averages of pellet weight and RNA yield (n=15-40), error bars represent standard deviation.

For all other sampling sites, RNA extraction and data normalisation were conducted as described in 2.4 and 2.7. Due to a lack of reported active case data during the Omicron wave, the SARS-CoV-2 levels in wastewater of the different locations are compared to the levels measured in Regina, which is the biggest of the sampled cities with > 200 000 inhabitants. To allow for cross-comparison between cities with very different absolute viral loads, the SARS-CoV-2 levels in wastewater are displayed as percentage of the respective highest measured value for each location. The data are not adjusted to flow rates as those were not available for all the locations. We also received frequent updates about the variants present in the locations' wastewater from the NML in Winnipeg.

Our wastewater surveillance of the city of Moose Jaw started in December 2021, just before the beginning of the Omicron wave. Moose Jaw is a mid-sized city with 33 890 inhabitants located 72 km west of Regina (Figure 26). We observed that after a short period of low SARS-CoV-2 prevalence in Moose Jaw wastewater in early December 2021, the levels increased sharply in the last week of December (Figure 29A). This increase correlates timewise with the sharp increase also seen in the Regina wastewater levels. Moose Jaw and Regina are located roughly an hour driving away from each other and likely see a lot of commuting between them, particularly over the December holidays. The highest SARS-CoV-2 viral loads in wastewater during the study period were measured in the second week of January 2022, concurrently with the first peak of the Omicron wave in Regina. While we observed a second peak of the Omicron wave in mid to late April 2022 in Regina, this was not the case in Moose Jaw. Instead, viral loads in Moose Jaw fluctuated until September 2022 at around 40% of the peak with a slightly increasing tendency from June onwards (Figure 29A).

For Yorkton, wastewater surveillance started shortly after Moose Jaw in late January 2022. Yorkton has 16 343 inhabitants, which is about half the size of Moose Jaw, and is located 190 km north-east of Regina (Figure 26). Here the SARS-CoV-2 levels were already high, showing that the Omicron wave began prior to the surveillance and possibly at similar times as in Regina and Moose Jaw (Figure 29B). The first sharp increase of SARS-CoV-2 in Yorkton that our data showed happened roughly two weeks after the first peak seen in Regina and Moose Jaw but coincided with a second peak observed in Regina. Similar to Moose Jaw, Yorkton also showed its highest viral loads in January 2022. Sequencing data confirmed that this first peak was caused by the emergence and rapid takeover of the Omicron lineage BA.1. A second peak was observed in May 2022; however, the levels did not reach the previous peak. The lowest levels in Yorkton wastewater were measured in the beginning of August 2022, about two months after the lowest levels measured in Regina and two and a half months after the lowest levels measured in Moose Jaw. At the very end of the study period the SARS-CoV-2 levels in Yorkton increased sharply again to a level higher than the peak in May but lower than the first peak in February (Figure 29B). The rapid increase of viral levels in Yorkton coincided with the first detection of a mutation that indicates the presence of BE.1.1.1, a novel sub-lineage of Omicron and the parent strain of BQ.1 and BQ.1.1, which in turn are suspected to possess immune evasive traits (83,84). BE.1.1.1 was also detected in Moose Jaw on August 31 and September 07 but did not lead to a sharp increase of viral levels in wastewater there.

Swift Current is a city similar in size to Yorkton with 16 604 inhabitants and is situated 245 km to the west of Regina (Figure 26). Here we started SARS-CoV-2 wastewater surveillance in December 2021, just before the first peak of the Omicron wave became apparent (Figure 29C). We saw that the initial increase of viral load during the beginning of the Omicron wave happened at the same time as in Regina. We also saw the values decrease after the initial peak in January only to increase again to a second peak in April. The second peak coincided with the peak seen in Regina wastewater and the lowest values were also measured during the same time as in Regina. The increase after the low levels in June was, however, more pronounced in Swift Current than in Regina, Moose Jaw or Yorkton. While the levels in Regina increased by about 25%, in Swift Current the increase spans more than 200% (Figure 29C is cropped for better resolution of the initial sampling phase). In both cities the Omicron lineage BA.5 became dominant around the same time (middle of July 2022), which is therefore likely the driver of the increase of SARS-CoV-2 levels in both locations afterwards. In the following months, the dominant lineages alternate mostly between BA.5 and its sub-lineages in both cities (lineages including BA.4, BA.5, BA.5.1, BA.5.2, BA.5.2.1, BA.2.12.1, BF and BE.1.1.1).

Our surveillance of Weyburn, which is 116 km south of Regina and about one-third smaller than Swift Current and Yorkton, started at the end of February 2022. For the first two weeks of surveillance the viral loads in the wastewater of Weyburn decreased from 40% to 5% of the maximum, suggesting that the initial peak of the Omicron wave had already passed (Figure 29A-D). The subsequent rapid increase to over 40% after the lowest values in the middle of March could have been caused by a change of the dominant SARS-CoV-2 lineage. While BA.1 was dominant in the middle of March, it was replaced by BA.2.3 by the end of March. BA.2.3 is a sub-lineage of BA.2, which has been shown to be more transmissible than BA.1 (85). From June onwards, the SARS-CoV-2 levels increased further to levels 70% and up. Interestingly and in contrast to the other surveyed cities, the initial increase of SARS-CoV-2 levels in Weyburn from June onwards seemed not to be driven by the emergence of the BA.4 or BA.5 lineage, which were commonly observed in other Canadian cities during this time. In Weyburn wastewater, the BA.4 and BA.5 lineages were first detected in July 2022, almost one month after the SARS-CoV-2 viral loads started to rise. Instead, the spike in June and early July seemed to have been caused by BA.2.12.1, which was the dominant variant at that time. Lineage BA.2.12.2 has been shown to evade antibodies built after vaccination or infection with BA.1 in a similar manner as BA.4 and 5 (86). When BA.4 and 5 were first detected in wastewater in early July 2022, they immediately became the dominant lineages. This is also in accordance with Cao et al. (86), who showed that BA.4 and 5 exhibit even greater immune evasion than BA.2.12.1.

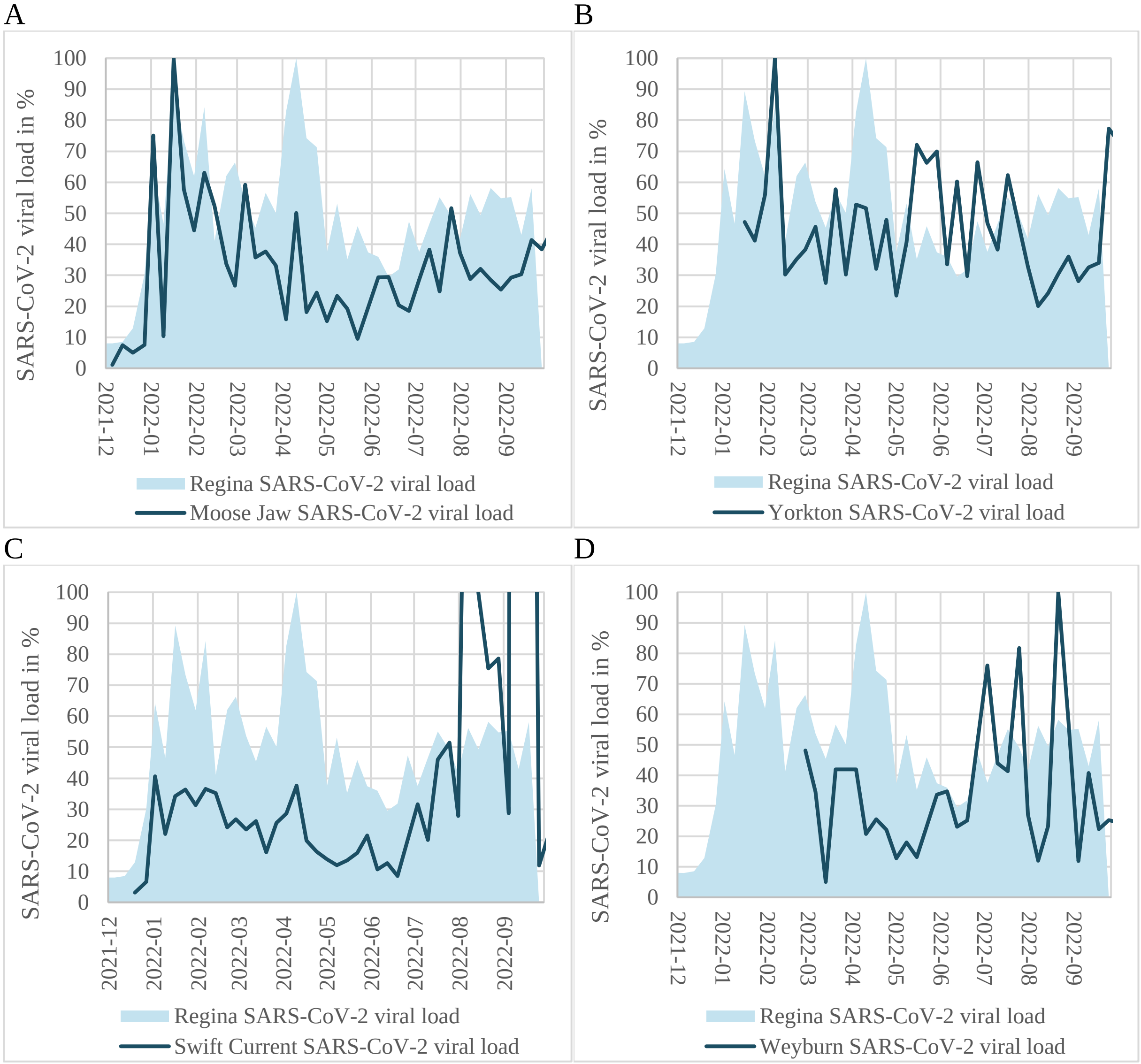


Figure 29: Overview of the SARS-CoV-2 wastewater surveillance from A) Moose Jaw, B) Yorkton, C) Swift Current and D) Weyburn. The graphs show the weekly averages of SARS-CoV-2 viral loads measured in the different locations (lines) in comparison to the levels detected in Regina at the same time (blue shaded area). The data are weekly averages of the viral loads in wastewater and converted into percentage of the maximum SARS-CoV-2 viral load (A,C,D) or a relatively high load (B) measured during the covered time period at the respective location for easier comparison between the locations. The highest peaks in Swift Current would be at 230% in August and 1329% in September but were cropped in order to get better resolution of the trends in the beginning of the surveillance.

The surveillance of wastewater for the presence of SARS-CoV-2 in Lumsden started in the second week of January 2022, when the viral levels measured in Regina had already peaked once during the Omicron wave. Lumsden is a small rural community with just 1824 inhabitants, 63 km north of Regina (Figure 26). Here the course of viral levels differed from the other cities in regards that instead of showing the highest peaks in early 2022 and then a decline of SARS-

CoV-2 levels until June, in Lumsden we observed four peaks between January and June with the first three of them have similar virus levels (Figure 30A). The lowest viral levels were observed in Lumsden in early July. Two weeks later, they increased sharply and peaked at the end of July with viral levels more than three times as high as measured during the previous peaks. This peak was likely caused by the rapid spread of the Omicron lineage BA.5, which was dominant in Lumsden wastewater during this time. The outbreak, which is supported by RT-PCR as well as sequencing data provided by the NML, ended after roughly two weeks, which was the shortest duration of any outbreak seen in our surveyed locations. After a short decline, viral levels steadily increased from August until the end of the study period (Figure 30A). Interestingly, sequencing data revealed that the Omicron sub-lineage BA.5.6 was the dominant variant during the highest peak in Lumsden. BA.5.6 had also been detected in Regina at low levels (below 30%) during this time but, as opposed to Lumsden, never became dominant there or in any other surveyed location. This indicates that in small communities like Lumsden the dynamics of primary lineages can differ from those in larger communities, even if they are in close proximity.

The wastewater samples from the two First-Nation communities (short FN community 1 and 2, Figure 26) were collected from lagoons. We started their surveillance in August 2022 and observed large fluctuations of the otherwise stable PMMoV levels throughout the two months. This led to immense fluctuations of the PMMoV-normalised SARS-CoV-2 viral loads that were deemed unlikely (Figure 30B). Further data will be required to determine whether this problem arises from sampling at lagoons.

Lastly, we extended wastewater surveillance for SARS-CoV-2 to two facilities within the city of Regina. Facility 1, which hosts around 1 000 people, was monitored from December 2021 onwards and the University of Regina (UoR) campus, of which wastewater from two dormitories and one classroom building were sampled, from March 2022 onwards. For both facilities, autosamplers were deployed either in a manhole (UoR) or in a lift station (Facility 1). On the UoR campus, we observed low levels of SARS-CoV-2 in wastewater for the first couple of months until June 2022 (Figure 30C). On June 01, 2022, the university removed the masking mandate after lifting the vaccination mandate at the end of April. Soon after, SARS-CoV-2 levels in wastewater began to increase. On July 22, wastewater sequencing detected the Omicron variant BA.5 on campus, which was about one month after the variant had been detected in the city of Regina's wastewater. In contrast to the other sampled locations, on the UoR campus BA.5 did not become dominant as soon as it was detected for the first time. On August 05, two weeks after the first detection of BA.5 in campus wastewater, sequencing indicated a high abundance of the Omicron sub-lineage BA.2.3.5. Wastewater samples from the city only showed BA.5 and its sub-lineages during this time (and trace presence of BA.2.12.1) but not BA.2.3.5, which might indicate that the Omicron lineage BA.2.3.5 entered and spread solely on campus.

The highest viral loads on campus were measured in September 2022, coinciding with the start of the winter semester in the last week of August. During the winter semester, the vast majority of classes were offered only in-person and dorm life had returned on campus without vaccination or masking mandates. These factors have most likely contributed to the peak of SARS-CoV-2 levels in wastewater in the beginning of the fall semester. In addition, mutations indicating the presence of BA.5 sub-lineages that are suspected to be more infectious (84) were also detected during the time of the peak (i.e., BQ.1, BQ.1.1). Unfortunately, we experienced a particularly low flow rate at the manhole during this time, which prevented us from collecting 24h-composite samples and may have skewed the observed viral loads.

Sampling at Facility 1 happened at a lift station with no flow-rate related problems. We started the surveillance in December 2021 and observed relatively low viral loads with some short intermittent spikes until June 2022 (Figure 30D). At the beginning of June 2022, SARS-CoV-2 levels in the wastewater started to increase rapidly. Sequencing data from the middle of July suggest that the lineage causing this increase could have been BA.4. In Regina wastewater, mainly BA.5 was dominant during this time. Since the introduction of BA.5 in Regina, the virus levels in Facility 1 fluctuated but seemed to be increasing at a similar rate as in Regina until the end of the study period (Figure 30D).

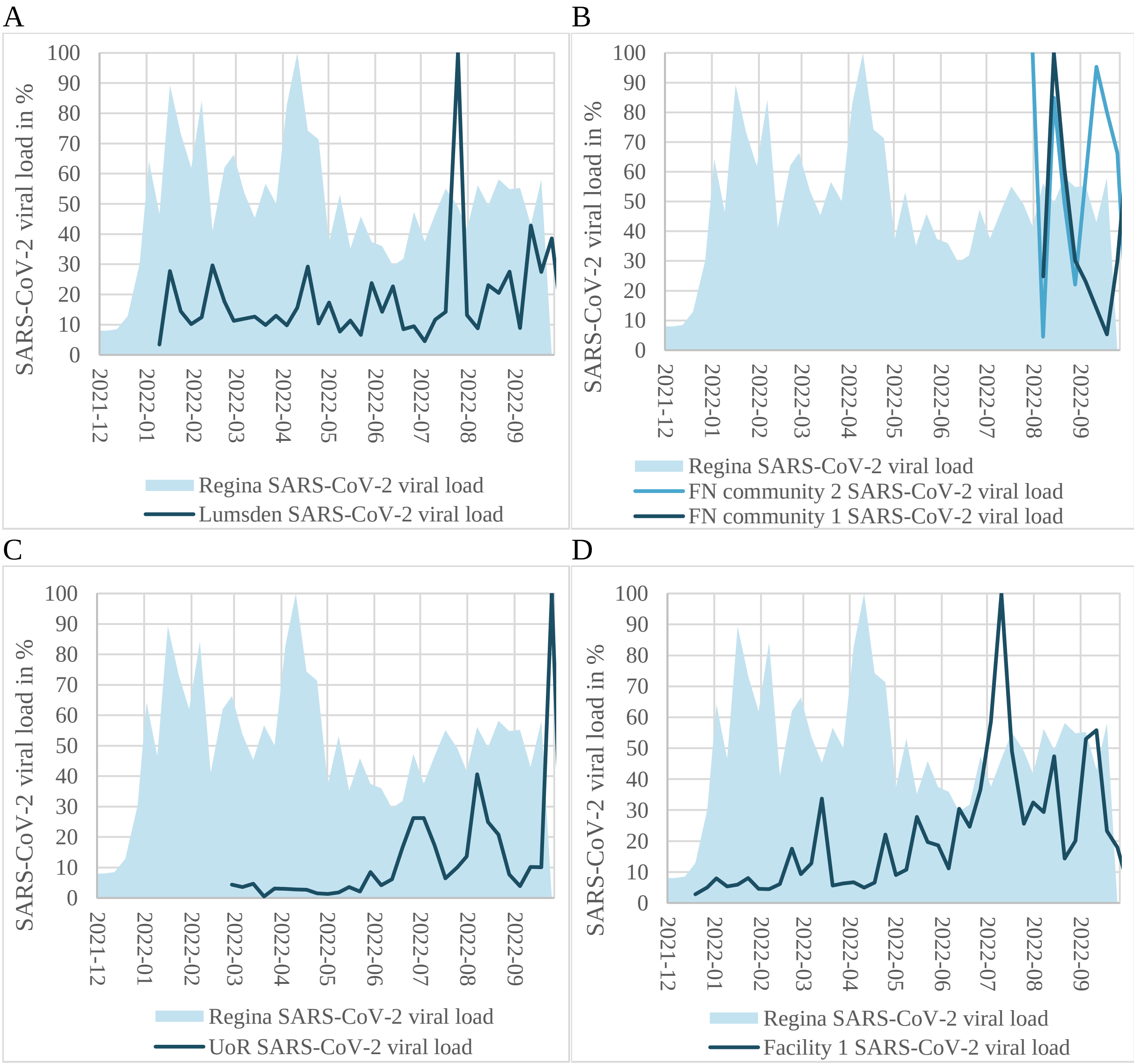


Figure 30: Overview of the SARS-CoV-2 wastewater surveillance from A) Lumsden, B) the First-Nation communities 1 and 2, C) the University of Regina campus and D) Facility 1. The graphs show the SARS-CoV-2 viral loads measured in the different locations (lines) in comparison to the levels detected in Regina (blue shaded area). The data are weekly averages of the viral loads in wastewater and converted into percentage of the maximum SARS-CoV-2 viral load measured at the respective location.

4. Discussion

Although wastewater surveillance has been performed long before the COVID-19 pandemic, extraction and surveillance protocols still need to be optimised for every source and target due to the complex matrix of wastewater (e.g., 87,88). We tested different experimental approaches, including using the liquid or solid fraction of wastewater, and different RNA enrichment and extraction methods. We further investigated the effect of sample storage over time and lastly determined the optimal data normalisation approach to use for our SARS-CoV-2 surveillance of several sites in southern Saskatchewan.

4.1. Effects of RNA Extraction Methods on Wastewater Surveillance

After testing several RNA extraction kits and reagents we decided to use the RNeasy PowerMicrobiome Kit (Qiagen) as it gave us the most reproducible results. Notably, the microbiome kit did not produce the highest RNA yields of the compared kits, indicating that SARS-CoV-2 detection is not solely dependent on overall RNA concentration. Similar observations have been made by Tomasino et al. (89), who observed that an RNA sample with concentrations as low as 11.7 ng/µL can still show Ct values below 29 for SARS-CoV-2.

Furthermore, when comparing the total RNA yields between the solid and the liquid fraction of wastewater received from the same sample and the same extraction method, we found that roughly 8 times more RNA was isolated from the solids fraction than the liquid. For SARS-CoV-2 specifically, we observed five times more N2 copies in the solids. Based on these results we determined that the solid fraction was the most suitable for wastewater surveillance of SARS-CoV-2. Several other studies came to similar conclusions (e.g, 90–92). Li et al. (92) used a similar surveillance method to ours and reported 3 500-10 000 times more SARS-CoV-2 in the solids than the liquid fraction (dependent on location and qPCR target gene). Graham et al. (72) also reported an abundance of SARS-CoV-2 in solids 100-1000 times higher than in the liquid fraction, however using a different extraction and quantification method. Both studies reported a higher level of SARS-CoV-2 in solids than we observed but also high deviations between the two, indicating large variances of SARS-CoV-2 ratios in solids to liquids (72,92). Other enveloped viruses have also been shown to partition to a higher proportion into the solid than the liquid fraction (93), likely because of the hydrophobic lipid bilayer that surrounds them (93,94). In contrast, we observed the non-enveloped PMMoV to be 5 times more abundant in the liquid fraction than the solids. A similar partitioning behaviour of PMMoV has been observed by Hasing et al. (95) and Kitamura et al. (91).

Early in the pandemic, other research groups focussed on the liquid fraction of wastewater (e.g. 20) and some observed that the liquid fraction possessed less variation of RNA yield and contamination than the solids from the same location (89). While Li et al. (92) reported that fluctuations in liquid and solids extractions were almost the same, we generally did not observe statistically significant variation between biological replicates from the solid fraction. For samples taken from wastewater treatment plants or lift stations we also did not encounter unexpectedly large fluctuations between Ct values of samples taken within a short time from each other. We did, however, observe that samples taken from near-source locations with small catchment areas, such as the University of Regina campus, occasionally showed large fluctuations between samples. The fluctuations might have been enhanced by the autosampler getting clogged within the 24h sampling period, thus reducing the 24h-composite sample to less than 24h. It has been shown previously that a single flushing of the toilet can be crucial when looking at low concentration compounds, such as drugs, in wastewater (96) but the concept might also be the case for SARS-CoV-2. The fewer cases are expected, the higher the sampling frequency has to be in order to not miss positive signals, especially when looking at single-building level (97).

Initial attempts to apply wastewater surveillance in two First-Nation communities with lagoons showed high fluctuations of the PMMoV levels, which we observed to be relatively stable in the other sampled locations. Further optimizations were not part of the study. Similar observations regarding fluctuating PMMoV levels were made by D'Aoust et al. (98), who suggested there could be increased RNA degradation in lagoons. In addition to the observations made by us and D'Aoust et al. (98), there are more instances reported in which PMMoV did not improve the correlation of SARS-CoV-2 RNA in wastewater to the reported case numbers (52,99). Since PMMoV is not an endogenous marker for human waste but introduced through the consumption of infected pepper plant products, its presence in wastewater is influenced by the eating habits of the population and might therefore differ between sampling sites (100). In our study, PMMoV did improve the correlation of the SARS-CoV-2 N2 gene in wastewater to the reported case numbers for surveillance data from Regina.

4.2. Effects of Wastewater Storage on Wastewater Surveillance

Sample storage is a critical factor for wastewater surveillance as changes happening during storage could lead to under- or overestimation of virus prevalence in wastewater. We shipped and stored our wastewater samples at 4°C and generally processed them within 2-3 days after arrival, as it had been shown early on that storage at room temperature causes significant degradation of SARS-CoV-2 in wastewater (101). We did not see a significant decrease of SARS-CoV-2 signal within 2-3 days, which is in accordance with several studies showing that short-term storage at 4°C causes little to no degradation (102,103). The same was observed for storage at 4°C for slightly longer (14 days) by Islam et al. (104). Although storage at 4°C for longer times has been observed to cause a reduction of SARS-CoV-2 (84 days, Hokajärvi et al. (105) and 122 days, Simpson et al. (103)), it was determined that the loss of SARS-CoV-2 during storage at 4°C is still lower than at -20°C, likely due to effects of freeze-thawing. Therefore, storage at 4°C was described as the preferable storage condition (103,104). While this appears to be the consensus for SARS-CoV-2, Islam et al. (104) and Hokajärvi et al. (105) both observed that it does not apply to all viral targets. The norovirus GII, which had been used as a non-enveloped reference in one of the studies, decreased by 1 $\log_{10}$ unit between 29 and 84 days at 4°C (105). PMMoV, which is frequently used as a normalisation factor, has also been seen to act differently than SARS-CoV-2 during storage by Islam et al. (104), who observed that PMMoV degraded faster than SARS-CoV-2 and that this degradation was more dependant on time than temperature. More research is needed to determine whether storage of the sample has different effects on SARS-CoV-2 and on PMMoV, and whether this would cause data normalisation using PMMoV to become biased with extended storage time (104). When we tested storage for longer times, we observed differences between the sampled locations. For samples coming from the wastewater treatment plant in Regina, we did not observe degradation; instead, in 12 out of 13 samples we observed an increase of the SARS-CoV-2 and PMMoV signals after long-term storage. The same was observed for a continuously run experiment with a sample from Regina. Samples from Facility 1, however, did not show such an increase and instead either fluctuated or decreased for the duration of the experiment (up to 50 days). Differences of storage effects on different locations, including increasing signals, have also been observed by other studies. Simpson et al. (103) noted that in two out of four samples from four different locations PMMoV did not degrade in the same way as SARS-CoV-2. They also reported that in (different) two out of their 16 tested samples the SARS-CoV-2 signal increased within 2 days of storage at -20°C by 107 ± 44%. Islam et al. (104) also observed an increase in SARS-CoV-2 and PMMoV levels from 7 to 14 days in one location and decreases or no changes in all the others. A significant increase of SARS-CoV-2 RNA over time has also been observed by Khan et al. (106). They used the liquid fraction of wastewater and explained the increase of signal with virus particles in the solid fraction lysing and releasing their RNA into the liquid fraction (106). This does not explain the increase of SARS-CoV-2 and PMMoV we observed in Regina as we used the solid fraction for our experiments. An increase of the SARS-CoV-2 or PMMoV signal after storage has to be related to the extraction or quantification process since neither virus can replicate in wastewater. It is possible that longer storage time gives suspended

SARS-CoV-2 in the samples more time to settle and attach to solid particles. SARS-CoV-2 has been shown to partition more into the solid phase than the liquid (90). In the sewer lines SARS-CoV-2 and the solids are under constant motion and this might detach virus particles from the solid particles or prevent the attachment in the first place. An unrelated study by Juel et al. (75) has shown that a short sonication step of the wastewater sample before RNA extraction improves recovery of SARS-CoV-2 and BCoV when extracting RNA from the liquid fraction. They suggest that sonication detaches the viral particles from the solids and prevents their loss during the removal of the solids from the sample. Vice versa this could mean that not moving the sample for some time could improve attachment of virus particles to the solid particles. Is has been suggested that longer contact time with the solids could result in higher recovery of SARS-CoV-2 (in the solid fraction). However, tests with BCoV, another enveloped virus suggested to behave similarly to SARS-CoV-2 (107), did not show improved recovery when incubated with the sample for 48h compared to just 30min (108). It is still possible that a longer storage time is needed to see differences as neither we nor other groups observed significant changes in short-term storage (up to 3 days) at 4°C. PMMoV has been observed to partition into the liquid fraction of wastewater (91,95), however, we also found it to increase in the solids over time. Kim et al. (90) reported that on a per-mass basis PMMoV is enriched in the solids fraction, similar to SARS-CoV-2, and D'Aoust et al. (109) suggested that it might attach itself to solid particles based on electrostatic and hydrophobic forces, as described for other viruses in wastewater (110). It is therefore possible that extended storage time might have similar effects on PMMoV and SARS-CoV-2.

4.3. Impact of Wastewater Surveillance on COVID-19 in Canada

Wastewater surveillance has been extensively used during the COVID-19 pandemic worldwide. In Canada alone, there are at least 65 sites across all provinces and territories using wastewater surveillance (as of October 27, 2022, 111) and surveillance data of 15 locations are published twice a week by the Government of Canada (111). We used wastewater surveillance to observe the progression of the COVID-19 pandemic at ten locations in southern Saskatchewan. The SARS-CoV-2 levels in wastewater of locations with smaller populations (Lumsden, the First-Nation communities, UoR campus and Facility 1) mostly show a pattern of random short peaks scattered throughout the surveillance period. In the bigger cities (Regina, Moose Jaw, Yorkton, Swift Current) we observed similar trends throughout the Omicron wave. The SARS-CoV-2 levels in wastewater sharply increased in early 2022, followed by a decrease until the early summer 2022 and then again a slight but steady increase until October (end of the study period). When we compare the pattern seen in the bigger cities in Saskatchewan to the viral levels measured in other cities in Canada, we see similarities to some degree. Figure 31 shows four additional locations in Canada (Edmonton, Saskatoon, Winnipeg and Toronto) that applied wastewater surveillance during the COVID-19 pandemic and whose data were reported publicly (42).

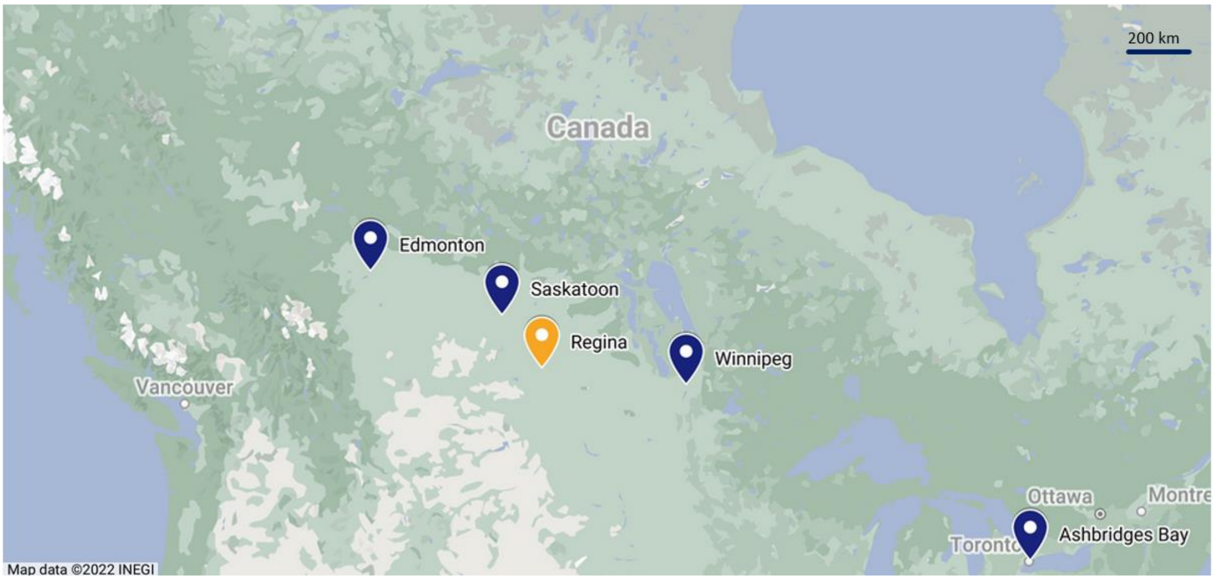


Figure 31: Several cities across Canada implemented wastewater surveillance into their COVID-19 monitoring, including Edmonton, Saskatoon, Winnipeg and Toronto.

In all locations we can see an increase of virus levels during the time of the Alpha wave (Figure 23, Figure 32A-D). In Regina, Edmonton and Toronto it started in the beginning of March, in Saskatoon and Winnipeg slightly later. However, the extent of the wave differs between the locations. While the virus concentrations in Regina, Edmonton and Toronto (Ashbrigdes Bay) came to roughly 100 copies/mL, they were more than four times higher than in Winnipeg. Reported active case data confirm that there were more infected people at the peak of the Alpha wave in Winnipeg than in Regina (over 3000 compared to roughly 1000, respectively (112)).

The Delta wave, which followed after the Alpha wave and peaked around in September 2021 in all cities, is clearly visible in the virus levels in wastewater in Regina, Edmonton, Saskatoon and Toronto (Figure 23, Figure 32A,B,D). We observed the peak in Regina wastewater in the last week of September 2021. It seems not to have been as severe in Winnipeg as there was hardly any increase in wastewater virus levels. Manitoba had an indoor mask mandate in place until August 07 and then re-instated it on August 28 (81), which resulted in just 20 days in which the virus could spread easily indoors and which might have contributed to the comparatively smaller outbreak. Saskatchewan on the other hand did not have an indoor mask mandate in place for almost two months from July 11 to September 17, 2021, which made indoor transmission more likely during the first phase of the Delta wave in Regina (81). This not only shows the benefit of masking, which has been shown before to reduce the spread of the virus (113), but also confirms that the significantly smaller Delta wave in Manitoba compared to Saskatchewan was actually due to fewer cases and not undertesting in Manitoba. Vaccination likely has not had a major impact on the missing Delta wave in Manitoba since (as of July 01, 2021) Saskatchewan had more vaccines delivered per person than Manitoba (114). During the Delta wave we observed many different AY-lineages in Regina simultaneously. A similar diversity of AY-lineages, 15 in total with four found in all three cities, has also been reported for Saskatoon, Prince Albert and North Battleford (48). The four sub-lineages that were found in all three of those cities (AY.12, AY.25, AY.27 and AY.93) were also found in Regina. Interestingly, only one further lineage, AY.6, which Oloye et al. detected only in Saskatoon, was also found in Regina as consensus sequence. The lineage AY.4, which was reported in Saskatoon and Prince Albert by Oloye et al. (2022), was found as a sub-consensus sequence in Regina. No lineage that was unique to North Battleford was detected in Regina either as consensus or sub-consensus sequence. This indicates that, except for the four most common lineages that are observed in all surveyed cities, Regina lineages have the most overlap with Saskatoon, followed by Prince Albert, but no overlap with North Battleford. Considering the location of the four cities, this could be a reflection of the travel between them with Saskatoon being the closest to Regina (and also the biggest of the four cities) and North Battleford being the smallest and farthest away from Regina. This is supported by Oloye et al., who observed that North Battleford saw new variants (Delta in August 2021 and Omicron BA.1 in January 2022) later than Saskatoon and Prince Albert (48) and Regina according to our data set.

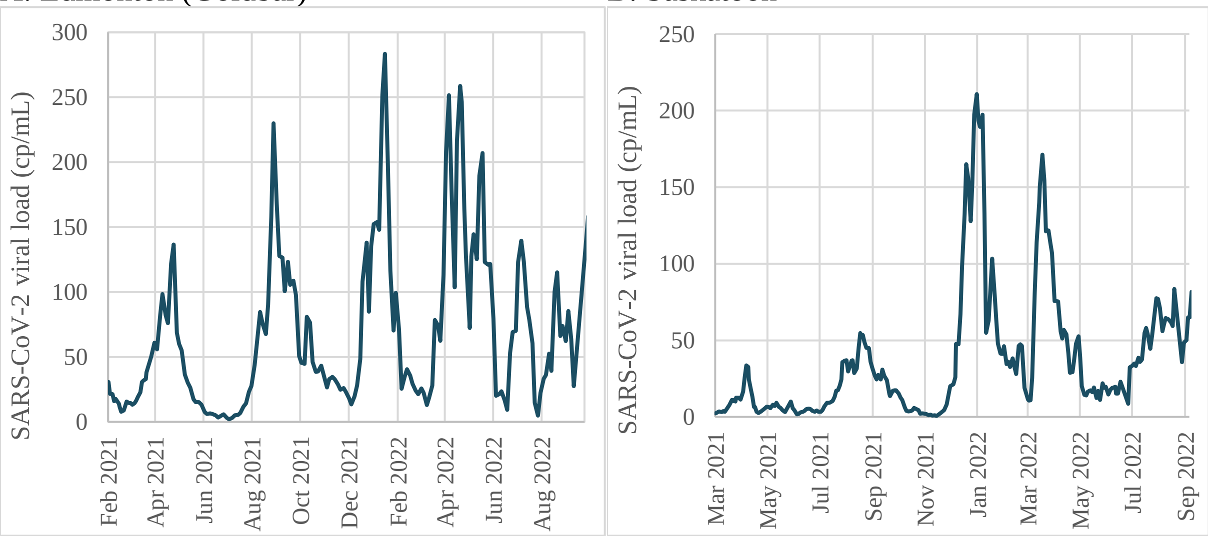
The Delta wave in Toronto started slightly earlier than in the other locations and did not reach the extent seen in the much smaller cities Saskatoon and Regina (Figure 32B,D, Figure 23). Since Toronto is a major travel hub and the biggest city population-wise, it is likely that a new variant would reach there before reaching the smaller, less populated areas. This had been observed during the first SARS pandemic (115). Ontario had an indoor mask mandate in effect throughout the entire Delta wave, which likely limited virus transmission. However, since the Toronto area is much more populated than smaller cities such as Regina, direct comparisons of the absolute wastewater data have to be drawn with care. Toronto has more than one wastewater treatment plant, so the data taken from one of them do not necessarily reflect the spread of the virus in the entire city. In addition, the different sewer system configurations and characteristics of the wastewater can skew comparisons of surveillance data from different locations. Nevertheless, since bigger cities experience new variants and their spread earlier than the smaller and more remote locations, we can look at the surveillance data of bigger cities in order to get an idea of what is to be expected in the smaller cities (48,116).

The highest SARS-CoV-2 levels in all cities surveyed by us and also in the reported data from other locations have been measured during the Omicron wave (Figure 23, Figure 32A-D). Omicron had first been detected in southern Ontario wastewater in the last week of November 2021 and two weeks later had already mostly replaced the Delta variant in that area (117). A different study found that the replacement of Delta with Omicron happened almost simultaneously at six different locations in Ontario (118). In Regina wastewater, we did not detect Omicron until the first week of January, further supporting the observation that bigger cities saw changes earlier than the smaller ones. The infection numbers of the Omicron variant surpassed Delta and all the previous variants and the initial strain by far (119), which can also be seen when comparing the virus levels measured in wastewater during the first Omicron wave with the levels seen before then. During the peak of the Omicron wave in Regina, we measured viral levels 2.7-fold higher than the peak of the Delta wave. Similarly, in Saskatoon the Omicron values were reported as 2-fold higher than Delta (48). The reason for this is likely the increased transmissibility and immune evasion of Omicron (10), which caused rapid spread across the world. In addition, the ongoing holiday season in late December brought many people together and thus likely contributed to the spread of the new variant.

When comparing the graphs in Figure 32 it appears that the first Omicron wave peaked more or less at the same time in all selected locations across Canada in January 2022. We observed the highest peak of the first Omicron wave (BA.1) in Regina on January 20, 2022 and in the same week the levels also peaked in Moose Jaw (Figure 23, Figure 29A). In Toronto, the highest levels were measured in the first and second week of January 2022, depending on the area in the city (42). In contrast to that, the second Omicron wave, which according to our sequencing results and also the data published on CoVariants.org (120) was caused by BA.2, only appeared as a clear peak in Regina, Edmonton and Saskatoon in May 2022. In Regina we found the 7-day average levels during the BA.2 peak to even exceed the initial Omicron peak by 12%. In

Winnipeg and Toronto, the viral levels increased slightly but did not reach the levels seen during the initial Omicron wave. It is not known why BA.2 failed to cause major outbreaks in Toronto and hardly caused the virus levels to rise at all in Winnipeg. We can speculate that the magnitude of the first Omicron wave, which was much higher in Winnipeg and Toronto compared to the other locations, conferred immunity to a larger proportion of the population. A recent BA.1 infection has been shown to generate antibodies which also neutralise BA.2 and BA.3 (121) and might therefore have limited the spread of BA.2 in locations that saw large BA.1 outbreaks. As opposed to this, the previous Delta wave did not prevent large-scale outbreaks of Omicron BA.1 (122) in any surveyed location. Lundberg et al. (122) conclude that a previous wave of infections does not protect from the next wave if the virus has significantly altered its antigens and that instead the same conditions that benefitted the initial wave, such as restricting policies or the lack thereof, could also become drivers of the next wave.

A: Edmonton (Goldbar) B: Saskatoon



C: Winnipeg (North End)

D: Toronto (Ashbridges Bay)

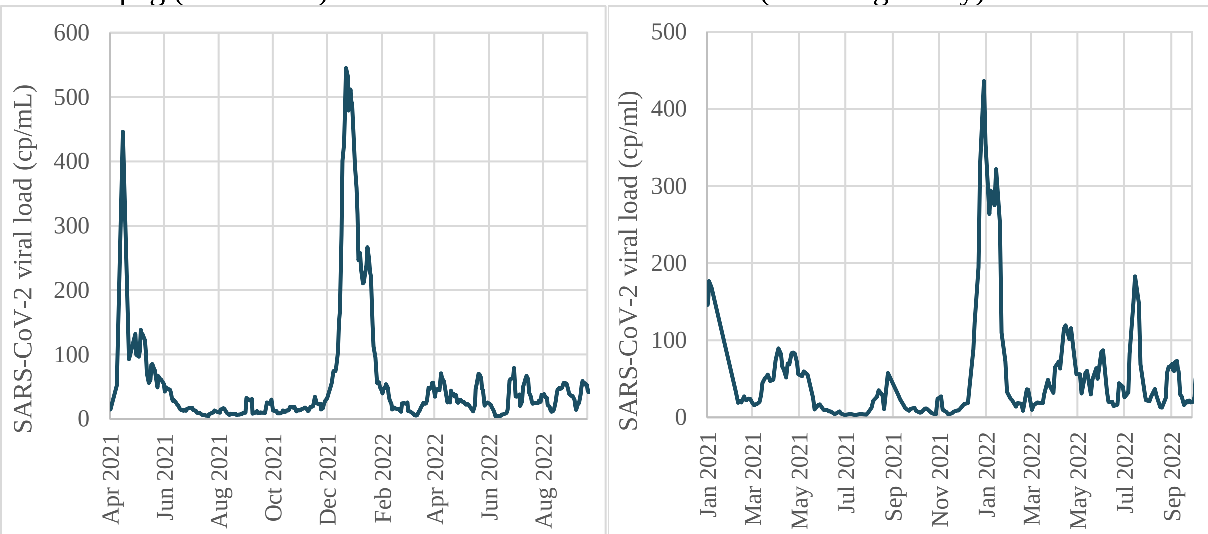


Figure 32: Wastewater surveillance has been conducted in several cities across Canada. The surveillance results for Edmonton (A), Saskatoon (B), Winnipeg (C) and Toronto (D) are provided on the COVID-19 wastewater surveillance dashboard (42). The surveillance for each location was conducted by the University of Saskatchewan (Saskatoon), the city of Winnipeg and Province of Manitoba (Winnipeg) and the Canadian Wastewater Survey (Edmonton and Toronto), respectively. The viral levels are shown as 7-day rolling averages of SARS-CoV-2 copies per milliliter wastewater. The locations in brackets shows the sampling location for cities with several treatment plants.

4.4. Challenges of Wastewater Surveillance and Future Perspectives

Even though wastewater surveillance is now commonly in use, it still faces challenges. Despite the countless studies conducted on method comparisons, no standardised protocol for sampling, RNA extraction, quantification and normalisation is available yet. For instance, we had to adjust the RNA extraction protocol for samples from Weyburn, even though the city is size- and location-wise very similar to the other sampled locations. The reason likely lies within the sample itself: different conditions and characteristics of wastewater from different locations require adjusted protocols in order to yield good-quality data (123). The sample composition also differs between locations, some samples contain PCR inhibiting substances that effect the downstream analysis of the sample if they are co-isolated with the RNA (124). From a global point of view, wastewater surveillance still faces challenges in low- and middle-income countries. While the methods have been tailored to work for centralised sewer systems and treatment plants that are mostly found in high-income countries, additional research will be needed to make the surveillance suitable also for the infrastructure available in less developed regions worldwide (125). The same issues have been characterised for less populated and remote regions in Canada, as they often do not have treatment plants but use septic tanks and lagoons, which have shown to be difficult to use for wastewater surveillance (126,98). Spiked-in process controls can be used to account for variations in the extraction process, however, the controls will not necessarily be affected by variations in the same way as SARS-CoV-2. For example, we observed that our process control BCoV is affected differently by storage than SARS-CoV-2. Similarly, Graham et al. (72) spiked BCoV into their extractions and noticed that the recovery of BCoV was not significantly correlated to the concentration of SARS-CoV-2. Lastly, there are also challenges in interpreting wastewater surveillance data. Several studies have attempted to infer the absolute number of infected people from wastewater data (e.g., 127). This is problematic as many of the factors that determine how the number of infected individuals is represented in wastewater are highly variable, including but not limited to the fecal shedding amount and duration and degradation during travel through sewer lines (128). It is known that some infected people will shed virus particles in their stool; however, it is unknown how many people do so. The mean prevalence has been reported as 43% (95% CI 34%–52%) in April 2021 (129). In addition, the number of viral particles in stool and the shedding duration vary (130,129,131). Cevik et al. determined that the mean shedding duration was 17.2 days; however, much longer fecal shedding has also been reported (>90 days, 132,7 months, 133). These uncertainties pose the current limitations to the interpretation of the results from wastewater surveillance (126).

While the COVID-19 pandemic is slowly subsiding and transitioning from a pandemic to an endemic, the public responses to the situation is also changing (134). Once testing and sequencing of clinical samples have ceded, wastewater surveillance including the tracking of newly emerging variants through sequencing will become the major tool for tracking COVID-

19 (134). It has been shown that wastewater data represent the infection numbers better than the clinical data during times when the test positivity rates are high, indicating undertesting of COVID-19 infections (135). We observed this in Saskatchewan during the Delta wave, as free PCR testing was restricted to symptomatic people in October 2021 and in December 2021 to vulnerable people (80). Under these circumstances it is likely that, depending on frequency and timing of the wastewater testing, peaks become apparent earlier in the wastewater data than in the reported case numbers, simply because everybody contributes to the wastewater and thus no cases can be missed due to not enough testing. As an alternative to regular testing of large sewer sheds at wastewater treatment plants, Wu et al. (134) suggested that samples could be collected from smaller catchment areas, such as neighbourhoods, schools or even single buildings. This could determine more accurately where outbreaks are happening and enable authorities to take action tailored to a specific locality (134). This has already been applied in care homes, prisons and several university dormitories (136–138), including the University of Regina. In our case, the surveillance data from our university campus are made publicly available to the campus community, so people have access to up-to-date information about the current situation and can make decisions about protective measures such as wearing a mask based on those. In addition to monitoring the tendencies of infection numbers, wastewater can also show us the prevalence of virus variants and thereby indicate which development of case numbers is to be expected in the future.

While wastewater surveillance has gained a lot of popularity during the COVID-19 pandemic, its use will not be limited to tracking SARS-CoV-2 in the future. The developed techniques and experiences made during the COVID-19 pandemic will help expand wastewater surveillance to further health concerns. Since wastewater surveillance is independent of local restrictions and testing standards, it is especially useful for the tracking of diseases with high rates asymptomatic cases (37), such as e.g., polio. Recently, wastewater samples that had initially been collected for routine monitoring of SARS-CoV-2 in New York showed presence of poliovirus type 2 (139). Additionally, wastewater surveillance is currently being introduced for monitoring of influenza (140) and has been suggested for the surveillance of arboviral diseases (37). Surveillance of the spread of sexually transmitted diseases such as Mpox (formerly Monkeypox) (141) and even antibiotic resistance genes have also been reported and will likely be expanded (142). For these reasons, wastewater surveillance will continue to be used as a monitoring tool to improve public health in the future.

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6. Appendix

Appendix A

The following tables show the consumables, devices and software that were used over the course of this project.

Table 7: Chemicals and kits used for RNA extraction and RT-PCR

Consumable	**Supplier**	**Catalogue number**
AllPrep® PowerViral® DNA/RNA Kit	Qiagen	28000-50
BeadBug™ prefilled tubes, 2.0 mL capacity	Sigma Aldrich	Z763772
Bovine serum albumin (BSA)	Thermo Fisher Scientific	B14
Calf-guard® Bovine Rota-Coronavirus vaccine (modified live virus)	Zoetis US	GCA-00448
Chloroform	Thermo Fisher Scientific	C298-4
E.Z.N.A® Universal Pathogen Kit	Omega Bio-tek	D4035-01
Ethanol	Greenfield Global	P006EAAN
FastRNA® Pro Soil-Direct Kit	MP Biomedicals	1.16E+08
Isopropanol	Fluka	34965-1L
iTaq™ Universal SYBR® Green One-Step Kit	Bio-Rad Laboratories	1725150
$MgCl_2$ (hexahydrate)	Thermo Fisher Scientific	BP214-500
Phenol/Chloroform/Isoamyl alcohol (25:24:1, v/v)	Sigma	77617-100ml
Polyethylene glycol (PEG)-8000	VWR	0159-500G
QiaAmp viral RNA mini kit	Qiagen	52904
RNase-free water	Ricca Chemical Company	R9145000-1G
RNeasy® PowerMicrobiome® Kit	Qiagen	26000-50
TRIzol	Sigma	T9424-200ML
TRIzol LS	Ambion	10296028
Various oligonucleotides	Thermo Fisher Scientific, Sigma, Invitrogen, Bio-Rad	
β-Mercaptoethanol	VWR	0482-100ML

Table 8: Devices used for RNA extraction and RT-PCR

Name	Device	Supplier
Airstream Horizontal Laminar Flow Clean Bench, Gen 3	Laminar flow bench	ESCO
Amicon® Ultra-15 Centrifugal Filter Units (3 and 10 kDa)	Amicon filters	Merck
Centrifuge 5418 R	Centrifuge	Thermo Fisher Scientific
Class II A2 Biosafety Cabinet (1387)	Biosafety bench	Thermo Fisher Scientific
Disruptor Genie	Vortex	Scientific industries
Eppendorf Reference® 2	Pipettes Eppendorf	Eppendorf
Eppendorf Research® plus	Pipettes Eppendorf	Eppendorf
FastPrep-24	Ribolyser	MP Biomedicals
Isotemp general purpose waterbath	Waterbath	Thermo Fisher Scientific
Mini tube rotator US plug	Roller drum	Thermo Fisher scientific
MultiTherm shaker	Thermoshaker	Mandel
NanoDrop 1000	Spectrophotometer (NanoDrop)	Thermo Fisher Scientific
Practum® Precision Balance, 10 mg	Balance	Sartorius
QuantStudio™ 3 Real-Time PCR (96-Well 0.1ml Block)	qPCR machine	Applied Biosystems by Thermo Fisher Scientific
Qubit 4	Fluorometer (Qubit)	Thermo Fisher Scientific
Sorvall Legend XTR	Centrifuge	Thermo Fisher Scientific
VWR® High Performance Pipettor Kit	Pipettes VWR	VWR

Table 9: Software used for data viewing, analyses and figure generation

Name	Software	Publisher
Benchling.com (accessed 2022)	DNA sequence viewer	Benchling
Biorender.com (accessed 2022)	Figure generation	BioRender
Quantstudio™ design and analysis software version 1.5.1	RT-qPCR analysis	Thermo Fisher scientific

Appendix B

In order to convert the Ct values into copy numbers and for quality control purposes we prepared standard curves using RNA standards for SARS-CoV-2, PMMoV and BCoV. An example for such a standard curve from 40 to 40.000 SARS-CoV-2 RNA standard copies is shown in **Error! Reference source not found.**. The efficiency in this case was 96.9%, calculated using the slope of the standard curve.

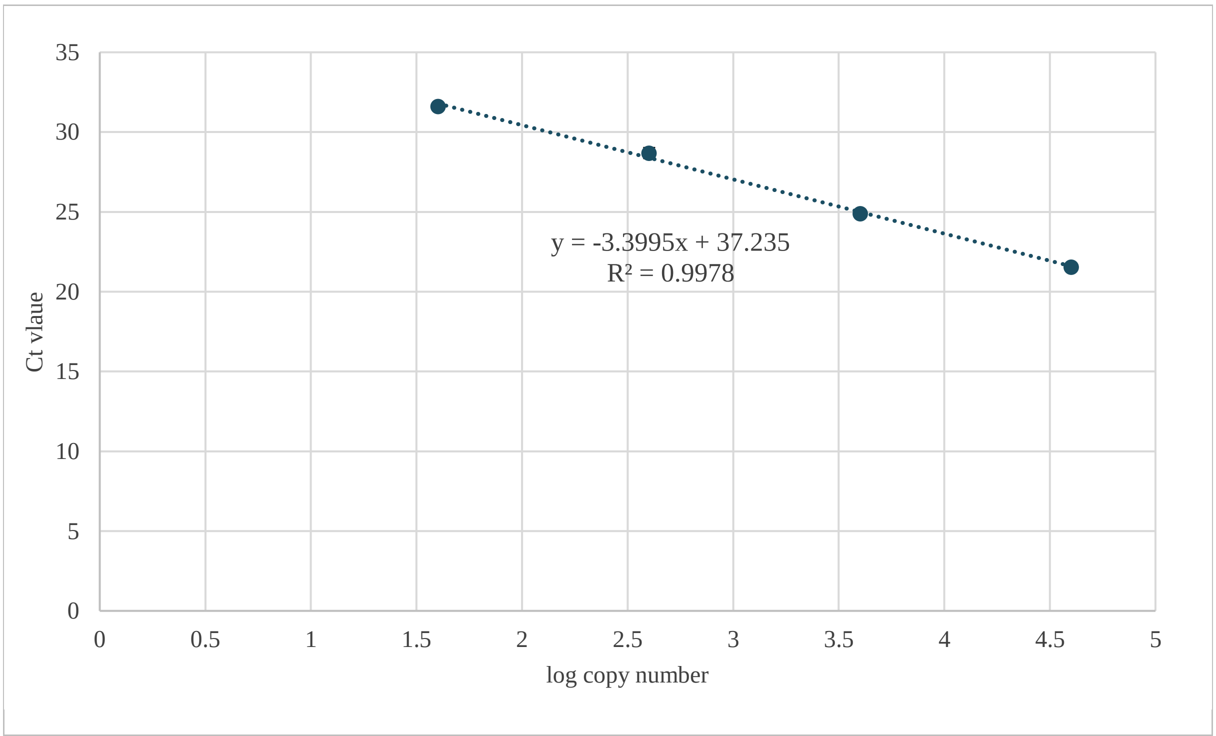

Figure 33: For quality testing of our RT-PCR runs we conducted efficiency tests with RNA standards. This example shows a standard curve with an RNA standard of the SARS-CoV-2 N gene. The efficiency was 96.9% with an R^2 of 0.9978.

Appendix C

We tested different normalisation approaches for the SARS-CoV-2 levels in wastewater, compared those to the reported clinical data (active cases, new cases and hospitalisations) and then ran linear regressions to rate the degree of correlation. We further examined the impact of shifting the reported active and new cases numbers by 7 and 14 days, respectively, to account for delays in data reporting. Table 10 shows the statistics received by linear regression. The best correlation was found between weekly averages of the SARS-CoV-2 levels normalised against the PMMoV copy numbers and the reported active cases.

Table 10: Outcomes of linear regressions to determine the best normalisation approach based on correlation to the reported data. Correlations are between the SARS-CoV-2 levels in wastewater (either normalised or as copies/reaction) and a data set of the reported data (active cases, new cases or hospitalisations), the tested data sets were either the full data set or weekly averages. For normalisation we used the PMMoV copy numbers in wastewater, the wastewater flow rate measured on the day of sampling (tested with increasing and decreasing effect, see 2.7 Data Analysis), both together or none.

Correlation between	Data set	Normalisation	Multiple R	R^2	Adjusted R^2	Data points
viral load and active cases	Weekly averages	PMMoV	0.936	0.877	0.856	50
viral load and active cases	Weekly averages	PMMoV, flow (increase)	0.935	0.874	0.853	50
viral load and active cases	Weekly averages	PMMoV, flow (decrease)	0.932	0.868	0.848	50
N2 copies and active cases	Weekly averages	none	0.926	0.858	0.837	50
viral load and active cases	Weekly averages	flow (decrease)	0.925	0.856	0.835	50
viral load and active cases	Weekly averages	flow (increase)	0.925	0.856	0.835	50
viral load and new cases	weekly averages, shifted by 7 days	PMMoV, flow (decrease)	0.919	0.844	0.823	47
viral load and new cases	Weekly averages	PMMoV	0.912	0.831	0.811	50
viral load and new cases	Weekly averages	PMMoV, flow (increase)	0.911	0.829	0.809	50
viral load and new cases	Weekly averages	PMMoV, flow (decrease)	0.907	0.822	0.802	50
N2 copies and new cases	Weekly averages	none	0.902	0.813	0.793	50
viral load and new cases	Weekly averages	flow (increase)	0.901	0.812	0.792	50
viral load and new cases	Weekly averages	flow (decrease)	0.900	0.810	0.790	50
viral load and active cases	Full data set	PMMoV	0.832	0.692	0.687	184
viral load and active cases	Full data set	PMMoV, flow (increase)	0.832	0.692	0.687	184

Correlation between	Data set	Normalisation	Multiple R	R^2	Adjusted R^2	Data points
viral load and active cases	weekly averages, shifted by 14 days	PMMoV	0.831	0.690	0.668	47
viral load and active cases	Full data set	flow (decrease)	0.825	0.681	0.675	184
viral load and active cases	Full data set	PMMoV, flow (decrease)	0.816	0.666	0.661	184
viral load and new cases	Full data set	PMMoV	0.797	0.635	0.630	184
viral load and new cases	Full data set	flow (decrease)	0.791	0.626	0.620	184
viral load and new cases	Full data set	PMMoV, flow (decrease)	0.779	0.607	0.602	184
viral load and hospitalisations	Weekly averages	PMMoV, flow (decrease)	0.705	0.497	0.476	50
viral load and hospitalisations	Weekly averages	PMMoV	0.694	0.481	0.461	50

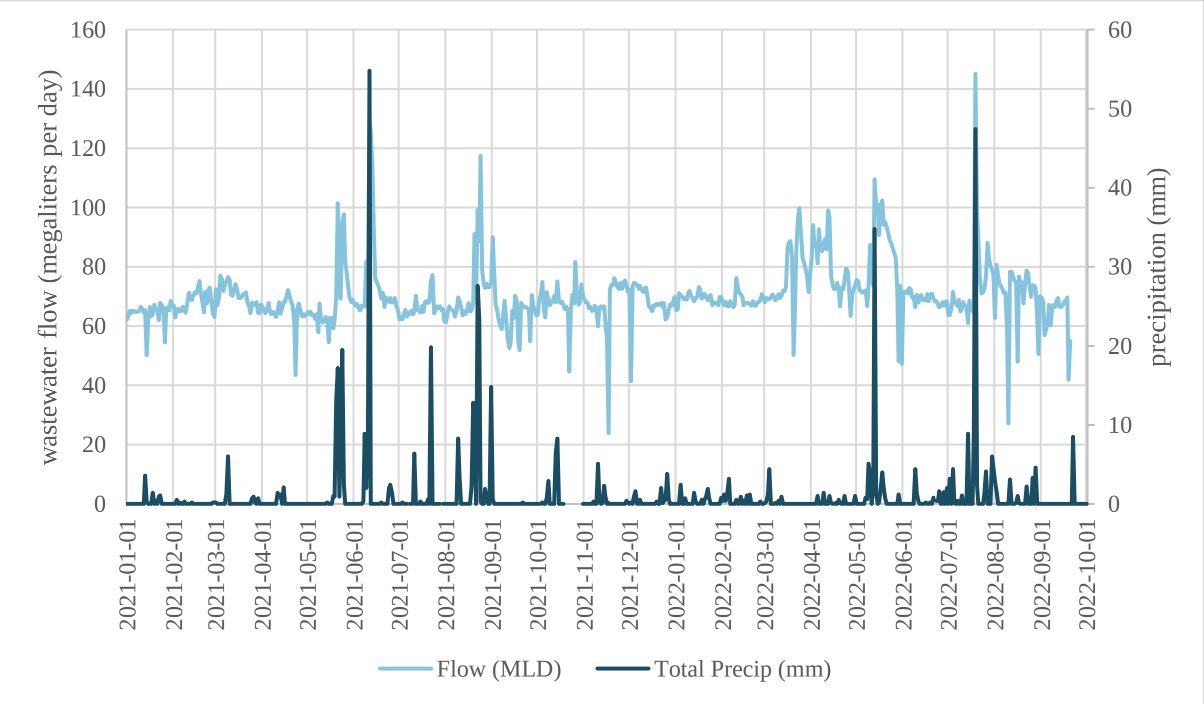

Figure 34: Heavy precipitation correlates timewise with an uptick in the flow rate in Regina wastewater. However, adding the flow rate into normalisation does not improve the correlation of viral loads in wastewater to active cases.

Appendix D

Table 11: All 36 SARS-CoV-2 variants and sub-lineages detected in Regina wastewater by sequencing between April 2021 and August 2022 and their current CDC classification (143). VBM: variant being monitored, VOC: variant of concern, blank: not classified. CDC classifications are updated regularly, here as of October 15, 2022.

Detected variants	CDC classification
A.2.5	
A.23.1	
B.1.1.7 (Alpha)	VBM
B.1.351 (Beta)	VBM
B.1.525 (Eta)	VBM
B.1.526 (Iota)	VBM
B.1.616	
B.1.617.1 (Kappa)	VBM
B.1.617.2 (Delta)	VBM
B.1.617.3	VBM
B.1.621 (Mu)	VBM
BA.1 (Omicron)	VOC
C.37 (Lambda)	
P.1 (Gamma)	VBM
P.3 (Theta)	

Detected Delta lineages	CDC classification
A.2.5	VBM
A.23.1	VBM
AY.11	VBM
AY.12	VBM
AY.23.1	VBM
AY.25	VBM
AY.25	VBM
AY.27	VBM
AY.27	VBM
AY.28	VBM
AY.3	VBM
AY.3.1	VBM
AY.33	VBM
AY.4	VBM
AY.4.2	VBM
AY.44	VBM
AY.56	VBM
AY.6	VBM

AY.86	VBM
AY.93	VBM

Detected Omicron lineages	**CDC classification**
BA.1.1	VOC
BA.1.16	VOC
BA.2	VOC
BA.2.12.1	VOC
BA.2.13	VOC
BA.2.3	VOC
BA.2.3.2	VOC
BA.2.48	VOC
BA.2.9	VOC
BA.4	VOC
BA.4.1	VOC
BA.5	VOC
BA.5.1	VOC
BA.5.2	VOC
BA.5.2.1	VOC
BA.5.6	VOC
BE.1	VOC
BE.1.1	VOC
BF.10	VOC