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SARS-CoV-2 Wastewater-Based Surveillance:

A Guide for Public Health Action

Background:

The current COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a member of the *Coronaviridae* family of enveloped viruses with a single-stranded, non-segmented positive-sense ribonucleic acid (RNA) genome and the known causative agent of a range of respiratory and gastrointestinal infections.[1] Traditional infectious diseases surveillance relies on testing individuals who are ill and present for testing. The challenge for medical and public health professionals in controlling the spread of COVID-19 among others arises from infected individuals who are pre-symptomatic or asymptomatic who may not present to the healthcare system. Many of whom may be unaware of their infection status due to a mild presentation or an absence of symptoms but are nonetheless capable of transmitting the virus to others. This attribute amplifies the potential for hidden outbreaks to develop into epidemics and obfuscates public health and medical health professionals' abilities to monitor the start, spread, and evolving trends of SARS-CoV-2 infection dynamics and take effective action in a timely manner to limit or prevent spread in the community.

Traditional public health methods rely on identifying infected individuals, isolating them and tracing their contacts to find new cases. These methods, which have been effectively employed in the past to limit the transmission of infections, have been met with many challenges due to the "silent" nature of SARS-CoV-2 communicability. Identification of a biomarker or indicator that could forecast the presence of SARS-CoV-2 in a community prior to detection through clinical surveillance would provide public health officials with a viable signal to monitor and track infection trends in the community, supplying the data necessary to plan and implement judicious measures to mitigate and prevent community spread of the virus.

The clinical characteristics of COVID-19 in the early phases of the disease are varied and can include among others, gastrointestinal symptoms such as abdominal pain, nausea, vomiting, and diarrhea reported by approximately one-fifth of COVID-19 patients.[2-5] SARS-CoV-2 RNA has been detected in excreta specimens such as anal/rectal swabs and the feces of both infected and asymptomatic individuals with subsequent shedding of SARS-CoV-2 in wastewater (table

1).[3,6-13] Investigations probing the concentration of SARS-CoV-2 RNA in excreta varied by stool type, with up to 10^8 copies per gram found in feces[9,14,15] and viral RNA concentrations as high as 10^7 copies/mL in diarrhea.[3,16,17,18] Additional studies have indicated that between 41%[19] and 70%[5] of patients shed viral RNA through the gastrointestinal tract. Two studies of limited size (n=9 and n=10) have shown even higher rates of positive stool samples: 88.9% and 100%, respectively.[20,21] Prolonged shedding has been reported up to 10 weeks after first symptom onset,[5,7,10,22] despite persistent negative respiratory swab results.[10,23] These findings establish that the SARS-CoV-2 virus is shed in feces by both infected and asymptomatic individuals and confirms the detectability of a viral RNA signal in excreta signifying the prospect of monitoring wastewater as a tool for mass surveillance. It is important to highlight the fact that SARS-CoV-2 infectivity in wastewater has not yet been extensively studied. Bivins and co-authors studied the persistence of SARS-CoV-2 in wastewater[24] and determined that at room temperature, a 90% reduction in the viability of SARS-CoV-2 in wastewater was observed within one and half day. This time was substantially decreased to 15 and 2 minutes at 50 and 70 degrees Celsius, respectively.

Environmental surveillance of wastewater (WW) is not a novel concept and has been successfully employed in the past to detect viral outbreaks of measles and poliovirus, [25,26,27] the presence of a wide range of pharmaceuticals including legal and illicit substances (e.g. cannabis, opioids, cocaine, methamphetamine), psychoactive substances, pesticide, and heavy metal exposur.[28,29,30] Additionally, this approach has been used to monitor (i) endogenous biomarkers of health (e.g. oxidative stress markers), (ii) chronic conditions such as *diabetes mellitus* (through detection of metformin), (iii) the impact of lifestyle choices (tobacco, alcoholism), and (iv) the presence of antibiotic resistant bacteria.[28,31] Moreover, researchers have been studying other human coronaviruses in wastewater for nearly a decade.[32,33]

In the early months of the pandemic, a number of international research groups started testing wastewater (WW) for SARS-CoV-2 presence using a variety of protocols and methods as a means to estimate the total number of infections in the community and to monitor infection trends (see table 2). Proof of concept was demonstrated by Dutch researchers at the KWR

Water Research Institute, who were the first to report detection of SARS-CoV-2 in untreated WW in a low prevalence population, which preceded declaration of the first confirmed case of COVID-19 through clinical diagnostic testing.[34] Although this approach did not initially garner extensive use as a disease surveillance tool, it has gained traction given its capacity to establish the presence of SARS-CoV-2 before it is captured by syndromic surveillance.[35,36] Routine surveillance of wastewater provides public health practitioners with a tool for mass surveillance in instances in which clinical testing is unavailable or cost prohibitive.[10,28,37] Consequently, this strategy offers a safe (no culturable infective SARS-CoV-2 virus present in the sample), high-impact, low cost option to obtain highly representative, non-invasive biological samples from targeted populations serviced by wastewater and sewage treatment plants, as a scalable and viable compliment to clinical surveillance in controlling the ongoing COVID-19 epidemic.[28,37,38]

Implemented properly, wastewater surveillance for SARS-CoV-2 has the potential to: [39,40,41]

1. Act as an early warning signal[32,33,42-45] for SARS-CoV-2 introduction into a naïve population,
2. In the state of endemicity, demonstrate resurgence in community transmission, enabling timely deployment of public health response and conservation of scarce healthcare resources.
3. Provide reliable markers denoting progress towards SARS-CoV-2 control, independent from clinical testing regimes.
4. Provide data that are both temporally and spatially related to the emergence of clusters of infection
5. Allocate resources to high risk populations to prevent and mitigate burden of disease
6. Foster public trust and compliance with public health recommendations informed by disease tracking metrics.

Table 1: Detection of SARS-CoV-2 in human excreta specimens.

Specimen	Country	Method	Positive rate[1]	Remarks[2]	Reference
Feces or anal/rectal swab	China	qPCR	14/31 (45%)		W. Zang et al., 2020b
		qPCR	8/22 (36%)		J. Zang et al., 2020b
		qPCR	9/17 (53%)	Day 0-11; 550-1.21x10^5 gene copies/ml	Pan et al., 2020
		qPCR	8/10 (80%)	Paediatric patients, positive for a mean of 21 (range: 5-28) days	Y. Xu et al., 2020
		qPCR	5/6 (83%)	Day 3-13	Jiehao et al., 2020
		qPCR	54/66 (82%)		Jiehao et al., 2020
		qPCR	39/73 (53%)		Ling et al., 2020
		qPCR	1/1 (100%)	Asymptomatic	
		qPCR	41/74 (55%)	Positive for a mean of 27.9 (8-48) days	A. Tang et al., 2020a
		qPCR	12/19 (63%)		Y. Wu et al., 2020a
		qPCR	10/10 (100%)		Lo et al., 2020
		Cell culture	1/1 (100%)	Culturable virus isolated	Zang et al., 2020a
		qPCR	44/153 (29%)		W. Zang et al., 2020c
		Cell culture	2/4 (50%)	Culturable virus isolated	
	USA	qPCR	1/1 (100%)	Day 7	Holshue et al., 2020
	Singapore	qPCR	4/8 (50%)		Young et al., 2020
	Germany	qPCR	8/9 (89%)	Up to 10^8 copies/g-feces	Wölfel et al., 2020
	Germany	Cell culture	0/4 (0%)	No culturable virus isolated	
	France	qPCR	2/5 (40%)	6.3x10^5 - 1.3x10^8 gene copies/g-feces	Lescure et al., 2020
Urine	China	qPCR	4/58 (7%)		Ling et al., 2020
		qPCR	0/10 (0%)		
	Germany	qPCR	0/9 (0%)		Wölfe et al., 2020
	France	qPCR	0/5 (0%)		Lescure et al., 2020

[1] Based on number of patients tested
[2] Days since onset of symptoms

Table 2: Details of reported molecular detection of SARS-CoV-2 in wastewater.

Sampling Location		Water type	Virus detection methods			Detection results (Positive rate)	Reference
Country	State/City		Virus concentration method	qPCR assay	Sequence confirmation		
Australia	Brisbane, Queensland	Untreated wastewater	Electronegative membrane-direct RNA extraction; ultrafiltration	N_Sarbeco NIID_2019-nCOV	DS + qPCR products (Sanger + Miseq)	2/9 (22%)	Ahmed et al., 2020
The Netherlands	Amsterdam, The Hague, Utrecht, Apeldoorn, Schiphol, Tilburg	Untreated wastewater	Ultrafiltration	CDC N1, N2,N3 E_Sarbeco	Not done	14/24 (58%)	Medema et al., 2020
USA		Untreated wastewater	PEG precipitation	CDC N1, N2,N3	DS + qPCR products (Sanger + Miseq)	10/14 (71%)	F. Wu et al., 2020
France	Paris	Untreated wastewater	Ultracentrifugation	E_Sarbeco	Not done	23/23 (100%)	Wurtzer et al., 2020
-	-	Treated wastewater	Ultracentrifugation	E_Sarbeco	Not done	6/8 (75%)	
USA	Bozeman Montana	Untreated wastewater	Ultrafiltration	CDC N1, N2	*Re*-amplication by regular PCR followed by Sanger sequencing	7/7 (100%)	Nemudryi et al., 2020

Notable Characteristics of Wastewater-based Surveillance include:

- **Efficient use of resources**[32,33,42-45] - Pooled population sample, highly representative of large urban areas
- **Timeliness** - Results can be derived within 24-48 hours, depending on time between specimen collection and processing;
- **Early warning system** - identify rapid increases in case prevalence by up to a week in advance of traditional clinical methods;
- **Reliability and reproducibility** - Technology and expertise readily available within nearly all levels of government;

- **Expandability to many other public health issues at minimal cost**[28,30,37,38] - Opioid use, antimicrobial resistance, and emerging pathogens.

Clinical detection of SARS-CoV-2 through laboratory testing of swabs from the respiratory tracts of individuals remains the gold standard for quantifying the magnitude of SARS-CoV-2 infection in a population. Although this method yields reliable confirmed case counts, it underestimates the total number of cases in the community as it fails to account for the asymptomatic or mildly symptomatic individuals who do not present to the healthcare system. Additionally, it fails to capture and test hesitant public as well as underserved or marginalized populations who lack access to healthcare among other barriers to testing. Moreover, the lack of a perfect clinical screening test further includes the phenomenon of false positive/negative, casting some doubt in generated results. The Public Health Agency of Canada is leading a pan-Canadian collaborative to develop a comprehensive wastewater-based surveillance system to address public health issues of major significance such as opioid use, AMR, and outbreaks of communicable and emerging infections, such as COVID-19. The immediate objectives of this surveillance program are:

- Establish a national collaborative network for public health surveillance of communicable disease through wastewater
- Develop standards for appropriate data interpretation in different-sized communities (Guidance on data interpretation and implementation into public health actionable interactions)
- Serve as the national centre for evaluation and standardization of COVID-19 tests in wastewater
- Fulfill testing needs among traditional partners, Indigenous communities, Northern and remote and underserviced.
- Coordinate model development and guidance for proper interpretation of COVID-19 results in wastewater towards actionable public interactions.

This is a multi-component collaborative project involving multiple jurisdictions including multiple federal departments, provinces, municipalities and academic institutions.

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SURVEILLANCE

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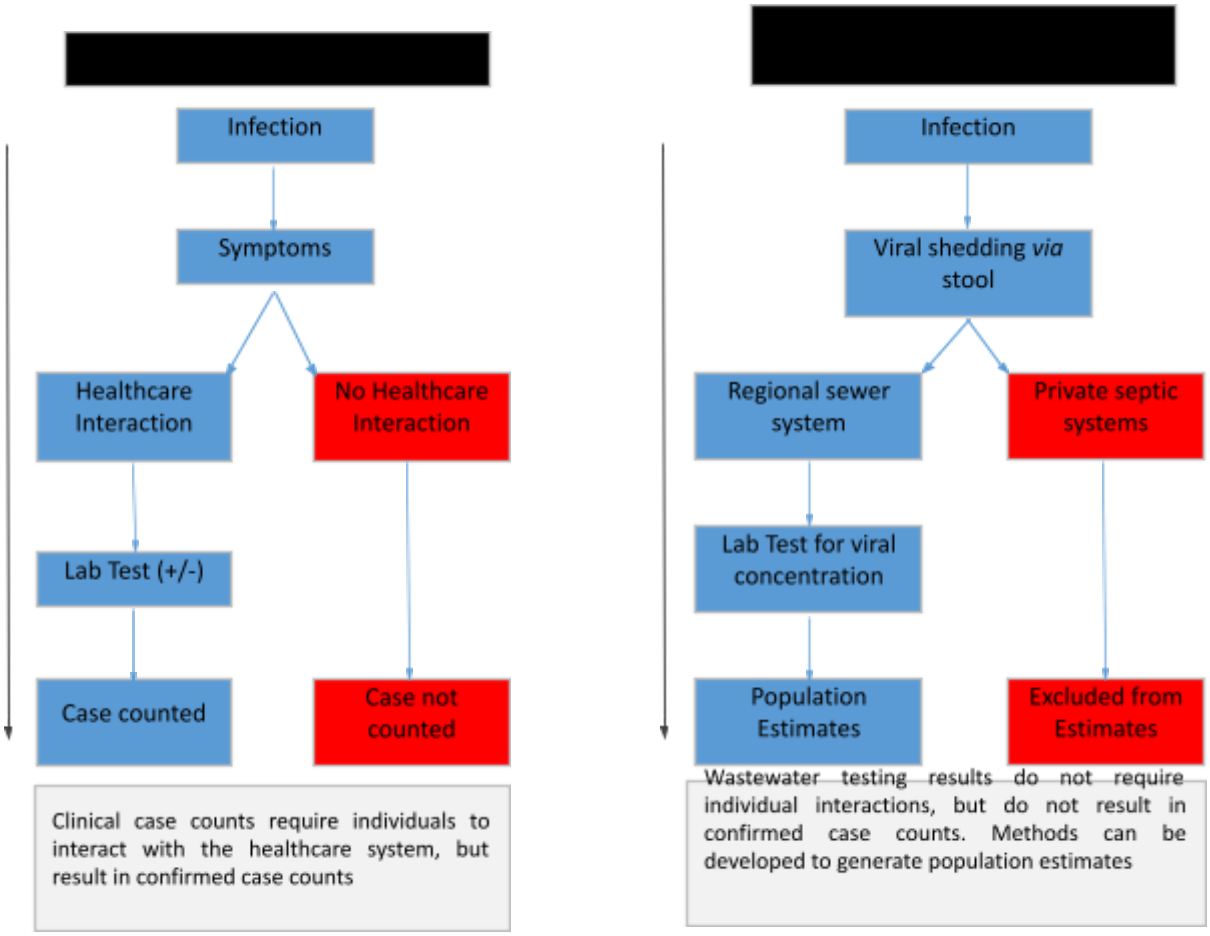
Figure 1: Comparative Illustration of clinical and wastewater-based surveillance



Current clinical surveillance of SARS-Cov-2 infection is highly dependent on individuals with symptoms interacting with the healthcare system. In contrast to the classical clinical communicable disease surveillance, wastewater-based surveillance results are independent of individuals seeking medical help and bypasses the medical system (figure 1). This approach relies on testing a non-invasive, pooled, biological sample from an aliquot of wastewater collected at either a centralized wastewater treatment plant servicing a community (downstream) or sentinel sites (upstream) (e.g. sewer line manholes/pump stations) in a neighbourhood and effluent access points for congregate living/working facilities. This method also provides data that are both temporally and spatially representative of infection trends at the community, neighbourhood and institutional level1.[1-3] However, there are

limitations to this approach, which include the following methodological and procedural factors:

- development of reliable, standardized viral quantification protocols,
- sensitivity and limits of detection of molecular methods used to monitor SARS-CoV-2 in wastewater,
- variability in SARS-CoV-2 shedding rates between people and within people at different stages of infection and required adjustments to account for potential differences in viral concentrations,
- unknown stability of viral RNA in wastewater,
- attribution of wastewater source and mixing that occurs in sewer lines with subsequent dilution effects, and signal degradation which limits temporal and spatial resolution based on prevalence of infection status,
- identification and determination of reliable biomarkers,
- sample normalization to control for systematic variances in wastewater flow [as well as method] recovery processes,
- determination and standardization of sampling protocols, design and assays to optimize sensitivity, frequency, periodicity and speed of testing,
- capacity to provide accurate estimates of the overall prevalence of infection at the community, neighbourhood or institution level (e.g. accounting for commuting and population transience),
- detection of SARS-Cov-2 in populations not connected to a sewage network and addressing potential risk of exposure to infectious particles for sanitation workers collecting wastewater samples.[1,2,4,5,7-14]

The methodological and procedural issues related to environmental surveillance of SARS-CoV-2 do not preclude the utility of wastewater monitoring as a semi-quantitative early detection system for trends in viral presence, absence and re-emergence[15]. Considerable progress has been made in the development of sampling protocols, targets to monitor and the sensitivity of detection methods substantiating the legitimacy of this approach to detect viral circulation in communities and at strata that are more granular (e.g. neighbourhood and congregate living settings). Wastewater surveillance can provide public health officials and

decision makers with advance warning to decide where diagnostic testing should begin, and when to introduce measures such as lockdowns to contain infection hotspots. This approach can also minimize the imposition of restrictive measures in areas where it is not required, provide supporting data and knowledge (based on post processing of the surveillance data) to inform when these measures can be scaled back to balance human and economic health, and maintain public trust.[1,2]

Wastewater-based SARS-CoV-2 Laboratory Surveillance

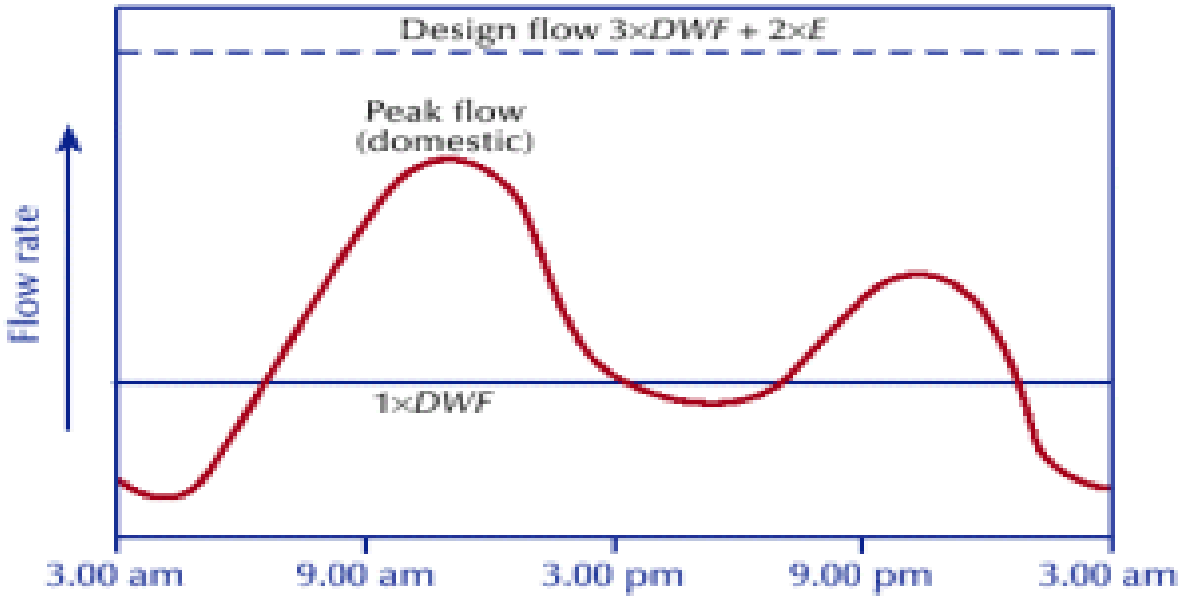
Wastewater Sampling - Principles and Best Practices

Wastewater includes any used water found in municipal sewers. It may contain human waste, household wash water, commercial, institutional, and industrial inputs, inflow from storm water and other unintended cross-connections, and infiltration from groundwater seepage through cracks and joints.

Although SARS-C0V-2 RNA signal found in wastewater is non-infective,[16] occupational safety measures are to be applied, because of the variety of potentially harmful components in wastewater, particularly human and non-human infectious pathogens. Appropriate personal protective equipment (PPE) must always be worn when collecting wastewater samples and/or working with all associated equipment and supplies. Minimum PPE are disposable gloves and safety glasses; additional PPE could include face shield, face mask, disposable coveralls, rubber boots, or steel-toe boots depending on the sampling site and potential for splashing or spraying.

Wastewater discharges from households, institutions, commercial and industrial facilities are usually variable in both flow and composition. Wastewater flow in municipal (mixed-use) sewersheds tends to follow a diurnal pattern, as illustrated in Figure 2.

Figure 2: Example of diurnal flow pattern
(Source: http://research.ncl.ac.uk/hydroinformatics/rbhd/module/c01u24.html)



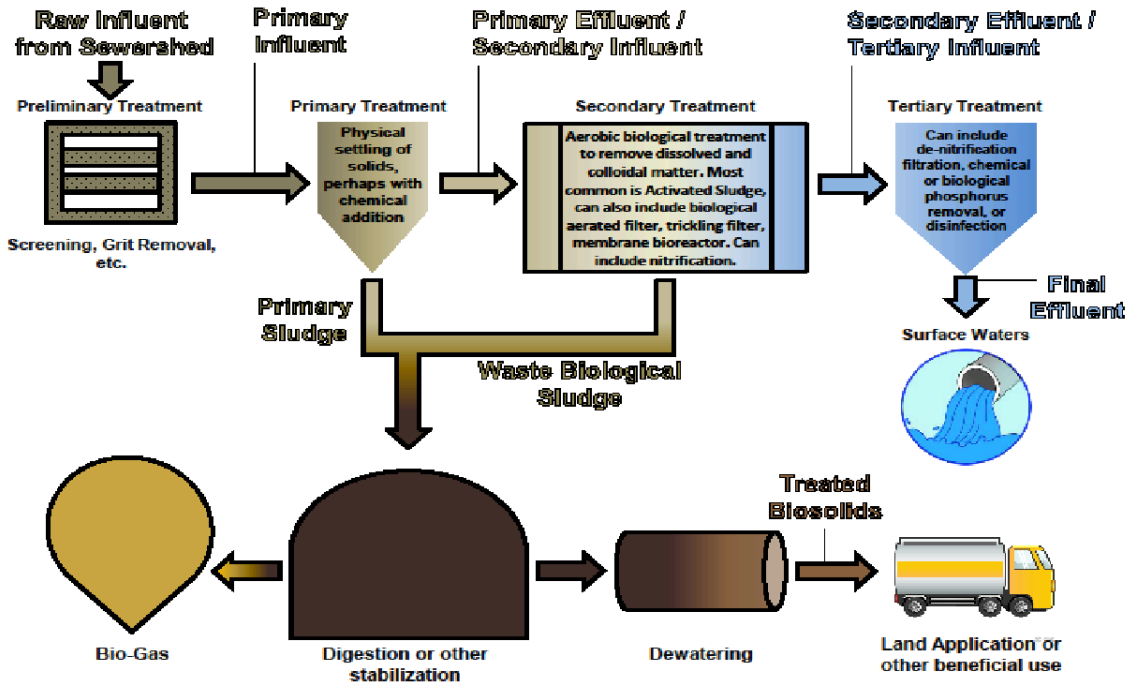
The study of either chemical or biological components in wastewater require representative, consistent sample collection in order to draw appropriate conclusions. In the early 2000s when the issue of pharmaceuticals and personal care products (PPCPs) in wastewater was emerging, there was a wide variety of sampling details and techniques included in journal publications. A comprehensive review of published sampling information[17] found that in many cases, the sampling information was insufficient to allow replication of the study and/or that inadequate design of the sampling method could lead to over-interpretation of data and misleading conclusions. Similarly, in reviewing studies of wastewater surveillance for SARS-CoV-2 RNA in wastewater, Ahmed and coworkers[18] found that details regarding auto sampler setup, as well as grab sampling time were poorly reported, which weakened the interpretation of results.

As concluded by the Ort studies,[17,19] the ideal sampling technique for wastewater is a continuous side-stream that captures the entire variation in flow and composition. However, most wastewater studies and sampling points do not lend themselves to this option. This document is intended to provide a range of practical options for collecting wastewater samples in consideration of various real-world limitations.

Wastewater Treatment Terminology

Descriptions of sampling locations must use clear and consistent terminology. There are variations in terminology between different wastewater treatment plants (WWTPs) and different regions of the world. The schematic in Figure 3 represents typical North American treatment processes and terminology.

Figure 3: Typical wastewater treatment schematic and sampling point terminology



N.B.

Sewershed and Treatment System Characteristics

Although most wastewater treatment systems include most of the unit operations and processes illustrated in Figure 3, there can be variations, e.g. absence of a primary clarifier in an extended aeration activated sludge system, or unusually long hydraulic retention times in a WWTP that was designed for higher future flows. Furthermore, every sewershed is unique, containing different proportions of residential, industrial, commercial and institutional (ICI)

inputs. Table 3 lists the general information that should be collected when assessing a sewershed or WWTP for sampling.

Other sewershed and WWTP information that may be relevant to the study include:

- Process disruptions or system maintenance (e.g. flushes in collection system, unit processes off-line in treatment plant) that could cause sample to be non-representative;
- Sewersheds that capture significant transient population (tourism, day workers), where weekday samples could be very different than weekend samples.

Sampling Techniques and Equipment

Unless the goal of the study is to examine differences in wastewater characteristics during storm events, sampling for both chemical and biological parameters should be done during dry weather and with sufficient time from the end of a storm event. Even in sewersheds with separate sanitary and storm sewers there is always some degree of inflow and infiltration, and storm events change both the flow and composition of the wastewater: storm water dilutes the parameters of interest and also flushes a higher proportion of inorganic materials (sand, grit) into the flow. After a rain event, it is important to verify with the WWTP operators that flows have returned to dry weather levels; depending on the severity of the event and the characteristics of the sewershed this can take several days.

As discussed by Ort and coworkers,[17,19] in the absence of continuous side-stream sampling, the cascade of preference for representative sampling of wastewater is:

1. Flow-proportional composite with high sampling frequency (<15 min)
2. Equal-volume composite with high sampling frequency (<15 min)
3. Series of grab samples, manual compositing
4. Individual grab samples

Table 3: Information Checklist for WWTP Sampling

Data elements for sewershed assessment WWTP for sampling
• Date
• Plant Name, Address, Directions
• Contacts (name, phone, email)
• Population served
• Average flowrate (m^3/d)
• % Domestic inputs
• % Industrial / commercial / institutional inputs. Any significant individual ICI inputs?
• Maximum travel time in collection system (hours)
• Screening and Grit removal (yes/no, what type)
• Influent sampler location (upstream of any chemical addition or internal recirculation)
• Primary clarification (yes, no)
• Primary effluent sampler location
• Aeration details
• Solids Retention Time (SRT, days)
• Mixed Liquor Suspended Solids (MLSS) in aeration tank
• Secondary clarification (yes, no)
• Is WWTP operated to nitrify?
• Phosphorus removal (yes/no, chemical addition points)
• Tertiary treatment (filtration, other?)
• Disinfection (yes/no, what type)
• Final Effluent sampler location
• Plant Hydraulic Retention Time (HRT, hours)
• Receiving water body
• Primary sludge (PS) details
• Waste biological sludge (WBS) details (Is WBS co-thickened in primary clarifiers?)
• PS and WBS blended before treatment?
• Raw sludge sample location
• Solids treatment type
• Dewatering (centrate or supernate recirculates where?)
• Treated biosolids sample location
• Biosolids destination

Pre-sampling preparation

1. Personal protective equipment
2. Fridge/freezer for storage.

3. Containers (tubes)
4. STP 152 Absorbent pads
5. RD plastics clear closable zip bag
6. Cooler with sufficient frozen ice packs
7. Labels (with ID, location and date/time)
8. Pre-labelled 500 ml PET containers (P500S/A bottle from Systems Plus)

Where to sample

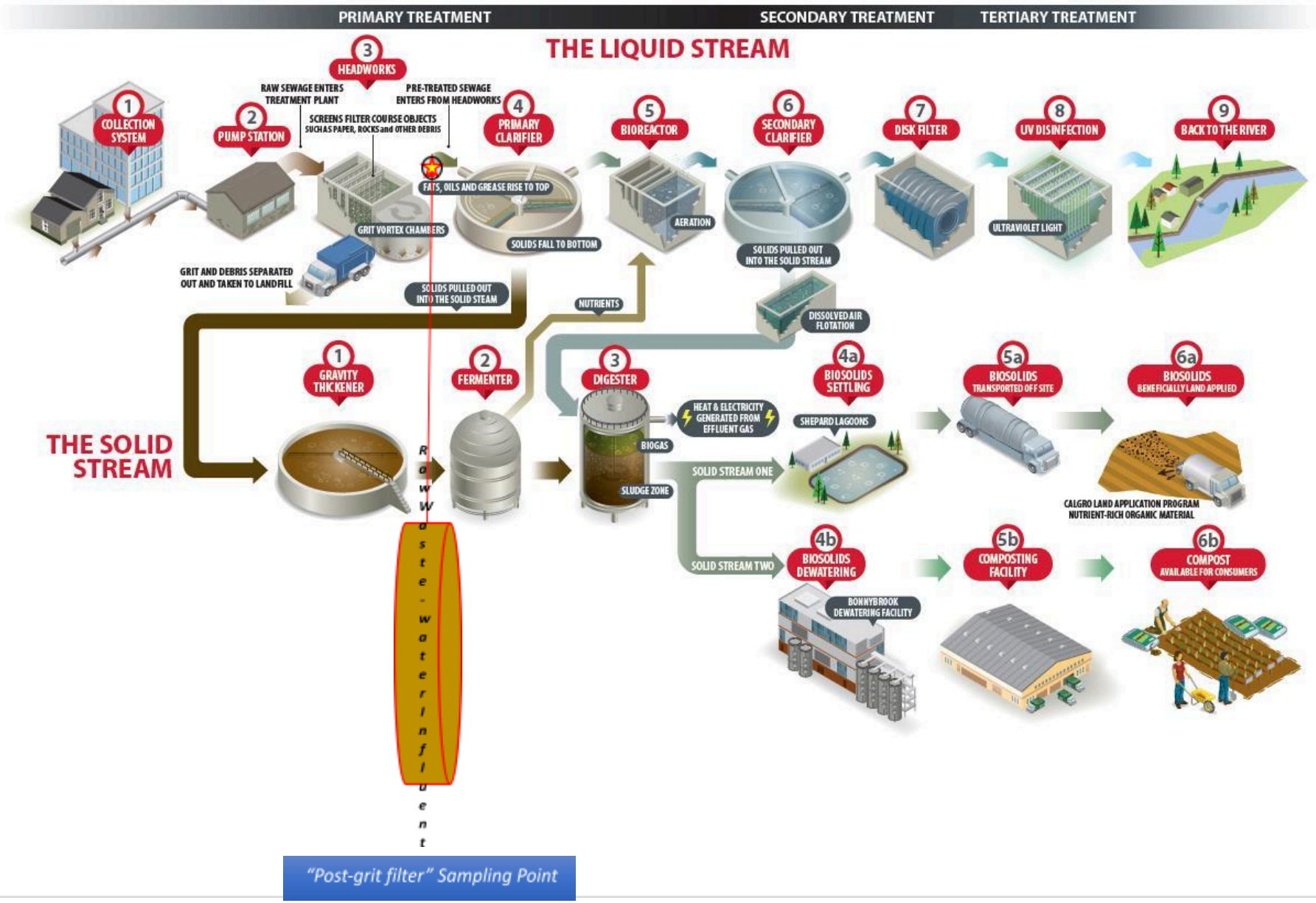
Raw untreated wastewater may be sampled from wastewater treatment plant influent (prior to primary treatment and following screening and grit removal - *Figure 4*) or upstream in the wastewater collection network (e.g., lift stations, interceptors, manholes).

Consultation with WWTP managers and operators is highly recommended to ensure that optimal representative samples are collected.

How to sample

There are two different sample collection methods used for wastewater disease surveillance: composite sampling and grab sampling. Composite sampling consists of pooling multiple grab samples collected at a set frequency over a set time period, which is generally 24 hours for wastewater surveillance. Composite samples of untreated wastewater can be collected either manually or via automated samplers with refrigeration capacity (that collect flow-weighted samples per X gallons of flow). Grab sampling on the other hand constitutes rapid collection that does not require automated equipment. Grab samples represent single moments in time and are largely influenced by daily fluctuations in the wastewater flow and its composition.

Figure 4: Schematic representation of the Wastewater Treatment Process in Calgary
(*Source: https://www.calgary.ca/uep/water/water-and-wastewater-systems/wastewater-system/wastewater-treatment-tour.html*)



Composite Sampling

Composite samples are created by collecting aliquots of wastewater and blending them over a period of time. A variety of automatic samplers are available: HACH and ISCO are two popular manufacturers. Auto-samplers are available in portable, refrigerated, and all-weather models. Flow meters can be added for flow-proportional sampling, and multi-parameter in-line sensors (e.g. conductivity, dissolved oxygen) can be added to collect additional data. Portable auto-samplers are not refrigerated but are lighter and easier to install in a wide variety of locations. All-weather auto-samplers are heavy and bulky, designed to be installed and remained in one location. Refrigerated auto-samplers can be moved between locations by a team of two physically capable people and are the preferred equipment for most wastewater sampling.

Composite sampling with automatic samplers has limitations. The sampling location (channel, tank, wet well etc.) must have continuous and sufficient flow to keep the sample line submerged at all times during the sampling period. As shown in Figure 2, flows tend to decrease drastically during the night. The sample collection tubing needs to be protected from clogging by rags and other debris, preferably by installing the auto-sampler downstream of screening (Figure 3). Although auto-samplers have battery backup devices, these do not function reliably in colder weather, and do not provide power for the refrigerator. Access to 110V power is preferred.

Grab Sampling

A grab sample is collected at one moment in time. Although replicate grab sampling is generally considered as an inferior approach to composite auto-sampling, it can be the only option in places where auto-samplers cannot be installed or when the use of auto-sampler tubing will contaminate the samples. It is also the appropriate technique when the purpose of the study is to examine differences in wastewater composition over time. Grab samples can be collected with a bucket and rope, or with an extendable “dipstick” depending on access to the wastewater.

Sampling Sludge and Biosolids

Due to the retention times of 2 to 8 hours in primary and secondary clarifiers, primary sludge and waste biological sludge can be considered composited in-situ. Similarly, the retention times of 10 to 30 days in digesters and other solids treatment processes produce biosolids that are inherently composited. Additionally, sampling access points for these streams are usually closed pipes which cannot accommodate auto-samplers. Therefore, sludge and biosolids samples are collected using grab techniques.

Sampling in the Collection System

The wastewater collection system resembles a watershed in that it includes small "feeder" pipes (tributaries), and large "main" or "trunk" pipes (rivers). Sampling in the collection system has more complications and limitations than within a WWTP. Maintenance holes can be located in streets or in parks. Depth to the wastewater flow can be quite deep. Access to the wastewater can require confined space entry with its associated hazards and required training. These characteristics can make auto-sampler installation and security very challenging or impossible, necessitating the use of grab samples instead.

What to sample

There are two main substrates for testing:

1. For wastewater-based SARS-CoV-2 surveillance, raw/untreated wastewater would be the preferred sample. Untreated wastewater includes household waste (from toilets, showers, kitchen sinks), and waste from non-household sources (rainwater, industrial use). Evidence of good correlation has been demonstrated between changes in SARS-CoV-2 RNA concentrations in samples from wastewater influent and trends in reported cases of SARS-CoV-2 infection.
2. Primary sludge that is constituted of suspended solids that settle out of wastewater during sedimentation and before chemical treatment to avoid high level of assay inhibition or poor virus recovery.

WWTP Managers and Operators

WWTP managers and operators take great pride in their work and are interested in supporting wastewater research and monitoring. They possess detailed knowledge of the collection and treatment systems and can provide real-world perspective and recommendations to help accomplish the study goals. Study personnel should always seek the advice of operators for details of auto-sampler installation to ensure it is installed in a location that (i) complies with the health and safety requirements of the WWTP, and (ii) provides the desired type of sample.

As publicly owned utilities, WWTPs operate on strict budgets. Their staff are fully occupied with their day-to-day tasks. It is essential to respect their time, i.e. do not assume that they can collect samples for your study in addition to their existing workload. If the WWTP manager agrees that their staff can collect samples, it is essential to engage directly with the operators to provide a detailed explanation of what types of samples are required and why. With this information and involvement, operators are more likely to make the additional effort to collect study samples appropriately.

How often to sample

Ideally, the sampling frequency (weekly to daily is currently being used) is dependent on the surveillance, the availability of resources, geographical location of the wastewater and sewage treatment plant, and most importantly, on the objective(s): from early warning, monitoring the trends of the SARS-CoV-2 infection in the community, to screening for SARS-CoV-2 infection at a specific target site (long-term care facility, schools, hospitals, prisons, etc.).[20-25] More frequently (daily, twice or three times a week) for early detection. Weekly sampling would be acceptable for monitoring trend in communities/institutions in which SARS-CoV-2 infections has already been detected

Sampling volume

The volume of sample to collect depends on the sample type (wastewater *versus* sludge), or requirement to repeat measurements and/or measurement of biological variability, or if any additional tests are planned. A typical 24h composite sample volume would be 360 ml.

N.B.: Recommend including the technical staff of the wastewater treatment plant in the elaboration of the sample collection protocol for guidance, and to ensure safe and secure collection of representative samples.

Sample Handling, Transportation, Storage, and Quality Control

By their nature, wastewater samples are very "active", i.e. there is a high degree of biological activity that will cause the nature of the sample to change fairly rapidly. Wastewater samples also contain suspended solids, which are an integral part of the matrix. After collection, samples should be cooled to 4°C as quickly as possible, shipped cold using natural ice or ice packs and using the most rapid available transportation. Upon arrival at the laboratory, they should be extracted as soon as possible. Standard Methods[25] recommends extraction within 7 days of collection when analyzing for trace contaminants such as semi-volatile organics. The samples must be shaken frequently and thoroughly during any sub-sampling in the field or laboratory.

Composite and replicate grab sampling include the use of consumables (tubing, bottles) and reusable containers and equipment. These containers and equipment must be tested to ensure that the sampling system is not introducing contaminants into the samples. Laboratory-grade water can be used to create Equipment Blanks by simulating a composite or grab sampling event that includes sample tubing, pump tubing, collection containers and sub-sampling containers.

WWTP Metadata and Context

As discussed above, a wastewater study must be designed in the context of the collection and treatment system realities and details. Wastewater samples should always be characterized for conventional parameters to provide the context of wastewater strength and effectiveness of the treatment process. These parameters are listed in Table 4.

Summary

Any study of wastewater constituents requires a thorough understanding of the collection and treatment system, in order to design a sample collection process that will answer the study questions. Sampling locations should be confirmed in consultation with WWTP operators, and described in details in all reports and publications. Likewise, sampling techniques (composite or grab) should be described in sufficient detail. Wastewater samples must be stored, transported, and handled appropriately to maintain their integrity.

Table 4: Conventional wastewater parameters (APHA et al 2018)

Parameter	Comments
Temperature - process	Indicator of microbial conditions for treatment
Temperature - sample	Confirmation of target sampling temperature
pH	Indicator for general chemistry and microbiology
Alkalinity	Indicator of buffering capacity and nitrification
Total Suspended Solids (TSS)	Empirical gravimetric test, indicator of wastewater strength and treatment effectiveness, can be correlated with some chemical and microbiological constituents
Chemical Oxygen Demand (COD)	Measure of material amenable to oxidation under strong chemical conditions, indicator of wastewater strength and treatment effectiveness
Biochemical Oxygen Demand (BOD)	Measure of material amenable to oxidation under specific biological conditions, indicator of wastewater strength and treatment effectiveness
Total Organic Carbon (TOC)	Measure of total organic (reduced) carbon, indicator of wastewater strength and treatment effectiveness
Total Kjeldahl Nitrogen (TKN)	Measure of total organic (reduced) nitrogen
Total Ammonia nitrogen (TAN)	Measure of nitrogen available for nitrification
Nitrate + nitrite	Measure of oxidized nitrogen, indicator of nitrification or denitrification
Measured average daily flow	Available from the WWTP, indicates the size of the system and confirms dry weather conditions or influence of storm events

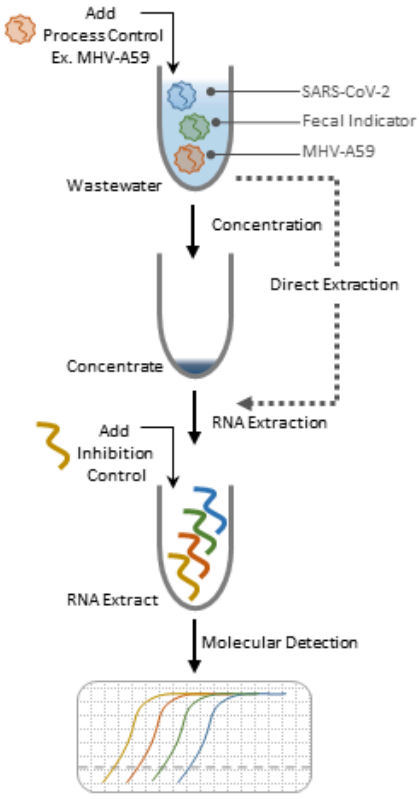
Laboratory processing of wastewater sample for SARS-CoV-2 RNA detection

Recommend that samples be processed within 24 hours of collection. Effective wastewater surveillance that aims to detect the emergence of infection relies on rapid data collection and testing. For future use, unused portions/aliquots of collected samples should be frozen at -70°C. The strength of the viral RNA signal decreases after freezing, consequently more than one freeze-thaw cycle should be avoided. Figure 3 describes the laboratory processing steps employed to detect a SARS-CoV-2 RNA signal from a wastewater sample.

Laboratory data management

The collation, storage and/or sharing of data generated by laboratory activities dictates the use of a reliable (safe and secured) IT infrastructure.

Figure 5: *Laboratory Processing of Wastewater sample for identification of SARS-CoV-2*



Laboratory testing Procedures

A wastewater test for SARS-CoV-2 is comprised of three major steps (Figure 5):

(i) Viral concentration,

(ii) RNA extraction, and

(iii) molecular detection.

Currently, the PHAC-NML employs a variant of an ultrafiltration-based viral concentration method previously described by the Wigginton group[26] and molecular detection of SARS-CoV-2 by RT-qPCR directed at the N1 and N2 targets as developed by the US-CDC,[3] a standard operating procedure for this method is provided in appendix 1.

There is no consensus or gold standard test for SARS-CoV-2 detection in wastewater and inter-laboratory comparisons of methods have shown that most perform comparably. In collaboration with the Canadian Water Network, PHAC-NML participated in an inter-laboratory study of SAR-CoV-2 wastewater detection methods[4]. The sample was drawn from a one of three Winnipeg WWTP and there were 85 clinical cases across the city at the time of collection. SARS-CoV-2 concentrations from most labs were within a 1-log band of each other. The Water Research Foundation[5] performed a similar inter-laboratory comparison amongst US laboratories. Grab samples from two WWTPs servicing Los Angeles County (~ 30K cases reported in the previous 14 days) were distributed to 36 laboratories for analysis. Despite methodological differences, most laboratories were within a 2-log band of each other's reported results. Importantly, the above studies show that there was no consensus methods amongst participating laboratories suggesting that most methods performed comparably.

The structure of the Canadian study revealed important considerations for laboratory methods. Eight laboratories received three sample types; samples spiked with inactivated SARS-CoV-2 at a high and low concentration (1,800 cp/mL vs. 20 cp/mL), and an unspiked sample. Firstly, only laboratories that processed the insoluble or "solids" fraction of wastewater were able to derive a signal from the unspiked sample. Because of the low number of clinical cases at the time of collection, this suggests that, to deliver early-warning

[3] https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf
[4] https://cwn-rce.ca/covid-19-wastewater-coalition/phase-1-inter-laboratory-study/
[5] https://www.medrxiv.org/content/10.1101/2020.11.02.20221622v1.full.pdf

indicators from wastewater surveillance the insoluble fraction of wastewater should be investigated. Studies of primary sludge and fractionation of wastewater influent have confirmed that the majority of the SARS-CoV-2 viral signal resides in the insoluble fraction. Secondly, the inactivated SARS-CoV-2 spiked into wastewater did not appreciably partition to the insoluble or "solids" fraction of wastewater. This suggests that the use of surrogate virus controls to monitor the overall efficiency of laboratory methods may not report on natural viral signal (See Controls, below).

Laboratory Methods

Viral concentration

SARS-CoV-2 is found at low levels in wastewater, so concentration is required for accurate analysis. This is especially true during the initial phases of an outbreak when the viral load in wastewater is low (Figure 5). Concentration is widely considered as the most influential step in the overall performance of the assay. Ahmed and co-workers have compared different concentration methods.[26] There is a variety of concentration methods, each with its own advantages and disadvantages as described in Table 3.

Some investigators employ direct extraction schemes on whole wastewater that skip a concentration step altogether (Figure 5). Typically, this involves processing about 1 mL of whole influent, which is both practical and amenable to high throughput. Direct extraction avoids sample losses associated with concentration and may improve overall yield. However, PHAC-NML cautions against using direct extraction schemes because of their unknown performance during periods of low viral load, especially when low volumes of wastewater are used as input.

Table 5. Three common concentration methods

Method	Advantages	Disadvantages
• Ultrafiltration • Viral particles are concentrated by the use of centrifugal filter device.	• Easy to use • Short turn-around time • Higher-throughput than most methods • Doesn't access solids component	• Co-concentrations of inhibitory compounds • Cost of lab ware (~$30 per device) • Supply of reagent may vary • Requires centrifuge (to 4K x g) • Filters can clog when sample turbidity is high
• Electronegative filtration • Viral particles are captured on a charged membrane by vacuum filtration	• Low Cost • Low carryover of inhibitory compounds • Low lab overhead to install test	• Low-throughput • High-hands on time • Requires extensive RNA extraction/clean-up
• PEG precipitation • A precipitating agent is added to samples and viral particles are recovered by centrifugation	• Low Cost • High supply of reagents	• Low throughput, some methods required overnight incubation • Requires centrifugation (to 10K x g) • Requires extensive RNA extraction/clean-up

As discussed above, most of the viral signal is associated with the insoluble fraction of wastewater. As such, collection and processing of wastewater solids could improve recovery dramatically. PHAC-NML has found that the viral RNA level in wastewater solids is equal to, or greater than that found in the liquids fraction. A draft Standard Operating Procedure for solids- based extraction is provided in appendix 1.

RNA extraction

There is a variety of commercial RNA extraction kits available and each should be chosen based on the type on input material. Samples with high-solids content require mechanical disruption and extensive wash steps to remove inhibitory compounds. As such, "soil" or "microbiome" extraction kits are well suited for this purpose. When the input material is clarified by centrifugation then general RNA extraction kits can be employed. If downstream detection is inhibited, then commercial clean-up kits can improve detection. RNA is unstable once extracted; therefore molecular detection should be performed the day of extraction.

Molecular Detection

Detection of the viral signal from SARS-CoV-2 is by reverse transcription quantitative polymerase chain reaction (RT-qPCR) as indicated in Figure 5. Specific primers amplify the SARS-CoV-2 genome and an intervening fluorescent probe is concomitantly consumed in this process. The viral signal is monitored by the increase in fluorescence associated with the consumption of this probe. Quantitation is achieved by measuring the number of cycles (Cycle threshold or Ct) required for the fluorescence detection of consumed probe over a baseline value "threshold", which is compared to a standard curve of known input quantities. A consistent "threshold" value should be used for all samples and the "auto-thresholding" function of the RT-qPCR instrument should be disabled. The threshold is specific to the RT-qPCR instrument and primers/probes chosen for analysis require optimization to reduce noise between replicates.

Primers/probes sequences for SARS SARS-CoV-2 and the controls, and sequences required for quantification above are provided in appendix 2. There is a variety of established primer/probe sets used to detect SARS-CoV-2 in wastewater and there is no current consensus as to which molecular targets are best detected by this test. Indeed, investigators have reported contradictory performance of the same primer/probe combinations. PHAC-NML has evaluated the *E-Sarbeco*[27] and US-CDC N1/N2 targets and found that the N1/N2 to be the most sensitive and consistent. The NML recommends using two targets to mitigate the risk of mutation.

Controls

The complex and variable nature of wastewater requires three controls run alongside the molecular detection of SARS-CoV-2 to account variations in the composition of wastewater and overall efficiency of the process.

Recovery Process control - Extraction

To account for varying efficiencies of the extraction of RNA from wastewater a spike-in control of whole-viral material is added to wastewater prior to concentration as described in Figure 3. A parallel concentration/extraction is run in PBS and the relative recovery is compared to this control and reports on the overall efficiency of concentration and extraction. The process control or surrogate is ideally a coronavirus of the same genus as SARS-CoV-2 and thus physically structured similarly to SARS-CoV-2 to best report on its recovery. Common process controls are murine hepatitis virus (MHV-A59), bovine coronavirus or one of the seasonal human coronaviruses (common cold). PHAC-NML currently adds Mouse Hepatitis Virus A59 (MHV-A59) as a process control in its assays. PHAC-NML has found that cultured MHV-A59 does not appreciably partition to the solids phase of wastewater and thus is not reflective of the natural state of the virus (as discussed above). A similar lack of solids-phase partitioning has been observed from cell-culture produced SARS-CoV-2.[28] Therefore, the utility of surrogates is likely more suited to methods that process only the liquid fraction of wastewater.

Fecal control

The fecal load of wastewater can vary across wastewater collection systems. Surface water, ground water and varying industrial and institutional inputs can dilute wastewater and introduce variance to the SARS-CoV-2 signal. A test specific for fecal load is applied to account for the varying composition of wastewater. The NML of the PHAC currently directs a RT-qPCR reaction against the Pepper Mild Mottle Virus (PMMoV), which is a naturally occurring virus that is found abundantly in edible peppers and reports on fecal load.[29] Other fecal indicators of note are the HF183 and crAssphage.[30,31]

N.B. ***Generally the use of PMMoV as fecal indicator could be variable small communities and therefore, caution is recommended when used for normalization.***

Inhibition control

Wastewater contains contaminants that are known to inhibit PCR assays. To detect the presence of inhibitors, purified RNA from a source that is not found in wastewater is added to

wastewater RNA extracts or alternatively to the wastewater concentrates. The signal intensity of this reaction is compared to the Inhibition indicator material tested alone. The Water Research Foundation inter-laboratory study suggest that a shift of less than or equal to one Ct suggests absence of PCR inhibition.[32] An alternative approach when purified RNA is not available is to dilute a wastewater RT-qPCR reaction and compare the resultant Ct value, with the expected value.[33] When inhibition is outside of one Ct of the expected range, it is suggested to dilute the wastewater prior to extraction and/or to flag the results prior to reporting.

Negative controls

Good practice with RT-qPCR based experiments is to run a RT-qPCR reaction without the addition of template. Any signal observed in this control would indicate the presence of contaminants in the RT-qPCR reagents. Mock concentration/extractions using buffer or water alone should be run periodically to identify contaminated lab ware or reagents.

Normalizing Techniques

Following quantification, the SARS-CoV-2 signal and associated controls are expressed in copies per volume of the processed wastewater (e.g. cp/mL). Adjustments should be made for wastewater losses over concentration and/or dilution of samples prior to extraction (to mitigate inhibition if observed). For instance, centrifugal filter devices have an inaccessible dead volume that is not recoverable. Estimation of the dead-volume can be made by weighing by difference the centrifugal filter device before the application of sample and after the recovery of sample. Assume that the held-up material has a density of 1 g/mL.

The quantified viral target is normalized to the quantified fecal indicator and this value alongside the un-normalized data should be considered minimal for reporting (*Equation 1*); where:

$$Vi = \frac{Viral\ target\ (\frac{copies}{mL})}{Fecal\ Indicator\ (\frac{copies}{mL})} = Fecal\ normalized\ (copies/mL) \qquad (Equation\ 1)$$

Further adjustments to the reported value can be made by incorporating the yield of the process control to overall yield. First, overall yield is calculated by using the following formula:

$$Process\ control\ yield = \frac{Process\ control\ copies\ recovered}{Process\ control\ copies\ \ in} \qquad (Equation\ 2)$$

To adjust for yield process recovery, the following formula is used.

$$Yield\ adjusted\ viral\ load = \frac{1}{Process\ control\ yield} * Fecal\ normalized\left(\frac{copies}{mL}\right) \qquad (Equation\ 3)$$

As described above, the process control may not accurately report on the overall yield of the SARS-CoV-2, especially where the solids fraction is the primary target for extraction.

Opportunities

(i) Accurate and reliable field deployable testing to detect SARS-CoV-2 in wastewater would shorten the timeline from sampling, to testing results, and public health action.

(ii)

(iii)

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Appendix 1: ***Wastewater Processing Guide Standard Operating Procedure***

National Microbiology Laboratory
December 2020

1. INTRODUCTION

1.1. This document is a guide to processing wastewater for the purpose of detection of SARS-CoV-2. This protocol describes processing of both the supernatant (Section 8.3-8.4) and solids (Section 8.5) fractions of raw influent wastewater. Raw influent is treated with detergent when processing the supernatant (Section 8.3), which could cause depletion of SARS-CoV-2 from the solids fraction, thus solids pellets from this procedure should not be used as input material for Section 8.5. This protocol uses an automated extraction platform (Roche MP96). Comparable yields were observed for the bioMérieux Nucleosens reagents on the E-mag platform. If automated extractions are not available then the Qiagen Qiamp Viral RNA mini kit showed results to similar to automated extractions but with reduced yield and consistency when using wastewater supernatant as input. The Qiagen kit was not tested for a solids based recovery. The reader is directed to dedicated soil or microbiome RNA extraction kits for the manual processing of wastewater solids derived from raw influent.

2. REFERENCED DOCUMENTS

2.1. WW Sample Receiving Log (See Table 6)
2.2. MagnNA-Pure 96 System Operator's Guide, Version 2.0
2.3. Fisher Scientific Bead Mill 24 Homogenizer User Manual

3. RESPONSIBILITIES

3.1. Section Chief or designate is responsible for ensuring all personnel using this protocol have been properly trained on this protocol and in working in a BioSafety Level 2 lab as well as all enhanced procedures needed for working with samples potentially containing SARS-CoV-2.
3.2. Laboratory Personnel trained on this procedure must understand and comply with procedures within this protocol
3.3. Laboratory personnel will use proper PPE and understand and comply with proper waste disposal procedures

4. DEFINITIONS

4.1. BSC: Biosafety Cabinet(minimum class II)
4.2. CSCHAH: Canadian Science Center for Human and Animal Health
4.3. MHV-A59: Murine hepatitis virus (MHV-A59, ATCC-764, RG2)
4.4. PPE: Personal Protective Equipment
4.5. RNA: Ribonucleic Acid
4.6. RTU: Ready to use
4.7. PCR: Polymerase Chain Reaction
4.8. PET: polyethylene terephthalate

5. POLICY

5.1. Section Chief or designate will be notified if any biosafety related issues arise.
5.2. To ensure samples are not contaminated with RNases/RNA/DNA, nitrile gloves must be worn when working with PCR mastermix/mastermix components and when handling RNA.
5.3. All surfaces, pipettes and racks in PCR Clean room and Template room will be cleaned with RNase Away followed by 70% Ethanol before and after use

5.4. Laboratory personnel will wear a designated lab coat or back closing gown in the PCR Clean room and a separate designated lab coat or back facing gown in the PCR template room, both of which are separate from the lab coat/back facing gown worn while working with the wastewater/samples prior to extraction.

6. SAFETY INSTRUCTIONS

6.1. Appropriate PPE will be worn at all times. When working with wastewater lab coat, back facing water resistant gown, double nitrile gloves, respirator and goggles will be worn. After RNA is extracted only gloves and a lab coat are required.

6.2. Technicians will only open wastewater sample bottles inside the BSC.

6.3. BSC will be lined with an absorbent underpad to aid in reduction of aerosolization of the pathogen if a spill occurs

6.4. After waste water samples have been concentrated one must use RTU Actril Cold Sterilant Liquid to decontaminate the outside of the samples and in procedure that may cause the concentration of fungal spores. Otherwise one may use 70% Ethanol to decontaminate samples and materials.

6.5. Spray bottles with RTU Actril Cold Sterilant Liquid and 70% Ethanol will be present in the BSC for surface decontamination of BSC and items (centrifugation buckets, racks, wastewater samples, conical tubes etc)

6.6. A pipet tray with 5% Microchem will be prepared and placed in BSC in which all pre PCR activities are carried out. This will be to decontaminate all pipet tips and serological pipettes

6.7. Centrifugation will be carried out in an aerosol tightbiosafe centrifuge rotor.

6.8. RTU Actril Cold Sterilant Liquid treated items will be treated for a minimum of 2 minutes before removal from the BSC

6.9. Due to use of RTU Actril Cold Sterilant Liquid, all waste to be autoclaved will be held for a minimum of 24 hours prior to autoclaving to ensure breakdown of RTU Actril cold sterilant liquid

6.10. Liquid waste (with the exception of anything containing guanidinium) will be treated to a final concentration of 0.525% sodium hypoclorite (bleach), incubated overnight, adjusted to 5<pH<11 and disposed of into the sanitary sewer

6.11. Respiratory protection is to be worn when working with wastewater, supernatant and solids prior to lysis

7. EQUIPMENT AND MATERIALS

Absorbent underpad
Biohazard bag (small and large)
RTU Actril™ Cold Sterilant Liquid (Cat No:78400-258)
Infrared Thermometer
Aluminum foil
15 mL, 50 mL conical tubes (Falcon Tube or equivalent)
10 mL, 25 mL Serological pipettes
Pipet boy/man
5 mL tube
Waste bottle
Bleach (10.8% Sodium Hypoclorite)
70% Ethanol
5% Microchem
Rack to hold 50 mL falcon tubes
RNAse Away
2.0 mL screwcap centrifuge tube

0.5 mm of zirconia/silica beads
Buffer RLT (Qiagen cat#: 79216)
2-mercaptoethanol (cat#: Millipore-Sigma 63689-25ML-F)
Pipettes (20 µL, 200 µL, 1000 µL) and tips- including 200 µL extended length tips
E4 Electronic Pipet
Pipet tray
Metal autoclave bins
Kim wipes or Wypall
Amicon Ultra-15 10 KDa centrifugal filter (Millipore, UFC9010) or similar filter
Tween-80
Sterile Nuclease Free Water
MHV-A59 (ATCC-764, RG2)
1.5 mL LoBind tubes
Carrier RNA (Sigma-Aldrich cat#: R5636-1 mL)
Magnapure 96 Processing cartridge (Roche cat#: 06241603001)
MagNA Pure 96 External Lysis Buffer (Roche cat# 06374913001)
Magna Pure 96 DNA & Viral NA Large Vol Kit (Roche cat#: 06374891001)
Transfer box
Biohazard safe centrifuge
Vortex
Microcentrifuge
Biosafety Cabinet
Bead Mill (Fisherbrand™ Bead Mill 24 Homogenizer or similar)
PPE: nitrile gloves, lab coat, back closing reusable water resistant gown, Respirator (3M™ Full Facepiece Reusable Respirator 6800, 3M™ 7500 Series Half Facepiece Respirator or equivalent), safety glasses or goggles
Cold Chain Complete indicator (SpotSee or equivalent)
exacto knife/utility knife or similar
MicroAmp™ fast Optical 96 well Reation plate (0.1 mL)
MicroAmp™ Optical Adhesive Film
RT qPCR Machine (QuantStudio or equivalent)
4°C Fridge
-80 °C Freezer

8. INSTRUCTIONS

8.1. Sample receiving

8.1.1. JC Wilt shipping and receiving will notify the Wastewater group via email once samples arrive

8.1.1.1. Shipments must be sent to CSCHAH for x-ray scanning, which will then be forwarded to JC-Wilt

8.1.1.2. Shipments received daily at 8:30am and 1:30pm.

8.1.2. When samples arrive, obtain specimen receiving cart from J1103 and retrieve samples from shipping and receiving (J1262)

8.1.3. Return to J1103 and ensure that proper PPE is donned prior to opening coolers/boxes.

8.1.4. Place ‘Do Not Enter’ signs on all doors

8.1.5. Don laboratory coat, gown, respirator, safety glasses, and double gloves

8.1.5.1. Note: reusable surgical gowns must be laundered daily

8.1.6. Open the cooler/box and place samples in a BSC lined with an absorbent underpad. Each cooler should contain:

8.1.6.1. Sample list

8.1.6.2. 500 mL PET bottle (containing wastewater) wrapped in absorbent material in a large resealable bag

8.1.6.3. Cold Chain Complete temperature indicator

8.1.6.3.1. Freeze indicator

8.1.6.3.2. Warm indicator

8.1.6.4. Cooling packs

8.1.7. Record the following on the WW Sample Receiving Log: date received, sample names/site code, temperature of sample, sample collected date, status of the Cold Chain Complete temperature indicator and the Autosampler last cleaned date and time if available.

8.1.7.1. Note in the comments section any irregularities i.e. frozen samples, not enough absorbent material, leaks etc.

8.1.8. Write on each Cold Chain Complete indicator the current date, sample location, and indicate if the freeze or warm indicator is good (white/green) or has gone out of temperature range (red). Indicate with a '√' (in range) or an 'X' (out of range).

8.1.9. When handling the Sample Receiving Log and/or Cold Chain Complete indicators, ensure you are wearing clean gloves

8.1.10. Discard absorbent material and resealable plastic bag in biohazard bag in the BSC

8.1.11. Take the temperature of the sample using an infrared thermometer

8.1.12. Write the temperature and Sample code on the lid. If the sample is part of the Pilot study, also write the week on the lid.

8.1.13. Spray wastewater bottles with RTU Actril Cold Sterilant Liquid (henceforth referred to as RTU Actril) and allow for two-minute contact time. Dry bottles, wrap in aluminum foil and store in a secondary container in the 4°C fridge.

8.1.13.1. Alternately, you may omit the aluminum foil if you have a fridge that has all the outside light blocked out.

8.2. Storage of Samples

8.2.1. Prepare BSC with absorbent underpad, biohazard bag for waste and waste bottle for liquid waste

8.2.2. Prepare six 50 mL conical tubes per sample

8.2.3. Create labels for one 50 mL conical tube which will contain wastewater and one 5 mL tube which will contain only the pellet

8.2.4. Don appropriate PPE and place 'Do Not Enter' signs on all doors

8.2.4.1. PPE: laboratory coat, gown, respirator, safety glasses, and double gloves

8.2.5. Swirl sample bottle to create a homogeneous mixture.

8.2.6. Transfer 50 mL of wastewater into each of the six 50 mL conical tubes using a serological pipette and a pipet boy/man.

8.2.7. Label one of the 50 mL tubes and freeze as is.

8.2.8. Clarify the other five 50 mL aliquots in a centrifuge for 20 min at 4,000-4,200 x g, 4˚C.

8.2.8.1. Use a Biohazard safe centrifuge

8.2.9. Ensure centrifuge buckets are cleaned with appropriate disinfectant after each use and at the end of each day

8.2.10. Pour off supernatant into a waste bottle, keeping pellet and a small amount of supernatant (no more than 500 µL)

8.2.11. Combine pellets into 5 mL tube, label and freeze

8.2.12. The 50 mL tube and 5 mL tube will be frozen at -80°C in J1103

8.3. Clarification of wastewater prior to concentration:

8.3.1. Prepare BSC with absorbent underpad and biohazard bag

8.3.2. Prepare one 50 mL conical tube for each sample being processed, by adding 15 µL of Tween-80 (0.1% v/v).

8.3.2.1. To prepare Tween-80 (0.1% v/v)

8.3.2.1.1. Heat Tween-80 in 60°C water/bead bath

8.3.2.1.2. Add 1 mL Tween-80 to 15 mL conical tube

8.3.2.1.3. Add 9 mL sterile water to tube

8.3.2.1.4. Vortex until thoroughly mixed

8.3.2.1.4.1. You may need to alternate between 60°C water/bead bath and vortexing to create homogenous mixture

8.3.3. Don appropriate PPE and place 'Do Not Enter' signs on all doors

8.3.3.1. PPE: laboratory coat, backward facing gown, respirator, safety glasses, and double glove

8.3.4. Thoroughly mix wastewater samples by inversion or swirling to ensure that the organic matter is equally distributed.

8.3.5. Transfer 15 mL of wastewater to a prepared conical tube

8.3.6. Thaw an aliquot of MHV-A59 Batch 2'-1/100 dilution

8.3.7. Add 10 µL of MHV-A59 'Batch 2'-1/100 dilution to the wastewater sample

8.3.7.1. To prepare MHV-A59 1/100 dilution:

8.3.7.1.1. Dispense 99 mL sterile Nuclease Free water into an autoclaved 100 mL Glass container

8.3.7.1.2. Add 1 mL of MHV-A59 cell culture stock to the water

8.3.7.1.3. Mix thoroughly

8.3.7.1.4. Dispense into 1.5 mL Lo-Bind tubes to be used as one time use aliquots (volume of aliquot will depend on amount needed for each day)

8.3.7.1.5. Freeze all aliquots

8.3.7.2. Vortex and centrifuge MHV-A59 before use

8.3.7.3. NOTE: MHV-A59 cannot contact the high concentration of Tween-80 as it could disrupt its structure and performance. Tween-80(0.1% v/v) and MHV-A59 must not contact prior to the addition of wastewater.

8.3.8. Vortex each conical tube at maximum speed for full 20 seconds after Tween-80 addition, wastewater and MHV-A59 (1/100 dilution) have been added.

8.3.9. Spray tubes with 70% Ethanol or RTU Actril. Allow for 2-minute contact time if using RTU Actril. Dry and transport tubes to centrifuge.

8.3.10. Clarify samples by centrifuging at 4,000-4,200 x g for 20 minutes at 4˚C.

8.3.10.1. While samples are spinning, gown, respirator and goggles can be doffed.

8.4. Concentration of Supernatant

8.4.1. If only processing the solids proceed directly to 8.5

8.4.2. Label one Amicon Ultra-15 10 KDa centrifugal filter (or similar) per sample being processed.

8.4.2.1. If PPE was removed, don proper PPE before next step

8.4.3. After spin is complete, remove swinging buckets from centrifuge and place into BSC. Gently remove tubes from centrifuge bucket and place into a rack.

8.4.4. Spray centrifuge buckets with 70% ethanol, remove from BSC and place back into the centrifuge to cool.

8.4.5. Decant supernatant into an Amicon Ultra-15 centrifugal filter ensuring that the pellet is not disturbed.

8.4.5.1. If the pellet is dislodged, re-centrifuge sample.

8.4.5.2. A 10 mL or 25 mL serological pipet with aid of pipetman/boy may also be used to remove supernatant and transfer to Amicon Ultra-15 centrifugal filter

8.4.6. Decontaminate tubes with 70% Ethanol or RTU Actril. If using RTU Actril allow for 2 min contact time.

8.4.7. Centrifuge at 4,000-4,200 x g for 35 minutes at 4°C

8.4.7.1. Approximately 200 µL of wastewater concentrate will be obtained per sample

8.4.8. Once spin is complete, remove tubes from centrifuge and place into BSC. Gently remove tubes from bucket and place into a rack.

8.5. Preparation of Solids

8.5.1. If only testing supernatant proceed to 8.6

8.5.2. Preparation of Bead Beating Tubes

8.5.2.1. On a clean bench or in the BSC, transfer approximately 200 µL of 0.5mm zirconia/silica beads to a 2.0 mL screwcap centrifuge tube

8.5.2.2. Add 700 µL of Buffer RLT containing 1% 2-mercaptoethanol (buffer stable for 90 days following addition)

8.5.3. Solids extraction

8.5.3.1. Clarify 30 mL of sample as described in Section 8.3, but do not add Tween-80.

8.5.3.2. Bring centrifuge buckets into the BSC and spray the outside with 70% Ethanol.

8.5.3.3. Use a 10 mL serological pipet to remove liquid supernatant leaving approximately 500 µL of liquid plus solid pellet

8.5.3.4. Using a 1000 µL pipet with "sawed-off" tip, vigorously pipet mix and transfer to a 2.0 mL bead beating tube mixture

8.5.3.4.1. To create 'sawed-off' tip take 1000 µL tips and cut of the end (approx. 2 cm) with an exacto knife/utility knife. This allows easier transfer of pellet.

8.5.3.5. Add 10µL of MHV-A59 to the resuspended solids

8.5.3.6. Spray all bead beating tubes with 70% Ethanol and transfer to Bead Mill sample collar

8.5.3.6.1. Ensure tubes are labeled on top and side

8.5.3.6.2. At least 4 positions of the bead mill tube carriagerotor must be occupied (1-7-14-21 positions) to maintain balance

8.5.3.6.3. Hand tighten tube carriage locking ring , and set to "lock" position when fully tightened

8.5.3.7. Close Bead Mill lid and run Cycle Program: 4 cycles of 30 sec @ 6m/s with 20 sec. pause

8.5.3.8. When complete, remove samples from the Bead Mill and centrifuge in Microcentrifuge at full speed for 3 minutes

8.5.3.9. Transfer sample tubes to the BSC

8.6. MP 96 Extraction:

8.6.1. If using MP96 proceed with this protocol. If using an alternate protocol follow the manufacturers protocol and 8.8 for PCR instructions

8.6.2. Prepare a 96-well MagNA Pure 96 (MP96) Processing Cartridge by adding 2 µL of carrier RNA to each well that will contain a sample.

8.6.2.1. It is important to note that the instrument works in columns (A1-H1) not rows (A1-A12).

8.6.2.2. An E4 Electronic Pipet may be used to dispense Carrier RNA into Processing Cartridge

8.6.3. If using supernatant add 700 µL of external lysis buffer to each well that will contain a sample

8.6.4. To add concentrated supernatant to Processing Cartridge (from 8.4.8) use an extended length 200 µL pipette tip, remove wastewater concentrate directly from the Amicon filter to the processing cartridge. Discard the used filter into an autoclave bag.

8.6.5. If using solids (from 8.5.3.7) proceed to gently aspirate 1000 µL (or as much as you can) of lysate supernatant to a prepared MP96 processing cartridge containing 2µL (~20mg) carrier RNA

8.6.5.1. When aspirating be careful not to remove beads or pellet. Only transfer lysate

8.6.5.2. For the wells with the solid sample you do not need to add lysis buffer as that has already been done in the solids preparation portion

8.6.6. Once all of the samples are added, cover the processing cartridge with an aluminum foil plate seal. Spray with RTU Actril (if you have concentrated wastewater in the BSC) and wait two-minute contact time. Before removal from the BSC, wipe down residual RTU Actril. If you are only processing solids you can use 70% Ethanol

8.6.7. Briefly centrifuge processing cartridge using the deep-well plate adaptor to ensure reagents are at the bottom of the plate.

8.6.8. Place processing cartridge in a secondary container and transport to the extraction room J1109.

8.7. MP96 Interface

8.7.1. Open the MP96 program by double clicking the program icon on the desktop

8.7.1.1. User: Admin

8.7.1.2. Pass: MNAPure96

8.7.2. Click on the WORKPLACE tab and check the following fields:

8.7.2.1. Method: Purification

8.7.2.2. Kit name: DNA/Viral NA LV 2.0

8.7.2.3. Protocol: Viral NA Plasma ext lys LV 4.0

8.7.2.4. Sample: 1000

8.7.2.5. Elution: 100

8.7.3. Fill each sample name field with “x” or any other chosen character, entry, or sample identifier

8.7.4. Click the SAVE icon (yellow save button)

8.7.4.1. Name format: DATE (yyyy-mm-dd) PROJECT NAME/INITIALS

8.7.5. Click the NEXT>> button

8.7.5.1. The next display will determine the amount of consumables required for your run

8.7.6. Open the bottom door flap by flipping it down and ensure system fluids are adequate

8.7.6.1. Check to ensure there is adequate system fluid in the white container (left)

8.7.6.2. Ensure the yellow waste fluid receptacle (right) is either empty, or below 1/3 full. If not, empty into the white MP96 waste pail under the bench

8.7.7. Obtain Reagent Tray 1 & 2, and 2 bottles of magnetic beads per 96 sample kit

8.7.7.1. One partial kit + a new reagent kit can be used if there is not enough left in an old kit

8.7.8. Load bead bottles into the bottle tray with the barcode facing OUT

8.7.8.1. Ensure the numbers under the barcode are visible

8.7.9. Push the bottle tray into the left most tray slot until the bottle tray window is highlighted

8.7.9.1. Be VERY CAREFUL to not damage the plastic tabs on the bottle tray

8.7.10. Place a clean processing cartridge into instrument trays 1 AND 2 as shown on the diagram on the computer

8.7.11. Load Reagent Tray 1 & 2 into instrument tray 2 with the barcode facing OUT (LEFT)
8.7.12. Load an appropriate number of tip racks into tray 3
8.7.12.1. Use up incomplete tip racks first and supplement with full tip racks
8.7.13. Load Source Tray (samples) & Output plate into instrument tray 4
8.7.13.1. Output plate goes into the metal segment of instrument tray 4
8.7.14. Check to ensure all barcodes are facing LEFT for consumables in instrument trays 1 -4 and bottle tray
8.7.15. Place a black waste tip funnel rack in to the waste tray top most position
8.7.15.1. clean funnel racks are located on the shelves or in the white box on the middle shelf of the left cupboard
8.7.16. Place clean exhausted tip racks into the empty spots in the waste tray to be used as tip holders on the waste rack for used (dirty) tips
8.7.16.1. It does not matter which way the barcodes of the clean exhausted tip racks are facing
8.7.17. Insert waste rack into the instrument in the last slot on the right
8.7.18. Once all trays are filled and in the instrument, close the flap and allow the instrument to scan
8.7.18.1. the instrument will run a self detection and consumables check which will take a few minutes
8.7.19. Once the green light becomes solid again, you can perform the daily maintenance if this is the first run of the day
8.7.19.1. This can be done by either clicking the MAINTENANCE button OR clicking the INTRUMENT menu, then choose the MAINTENANCE & SERVICE tab
8.7.19.1.1. check DAILY MP96 MAINTENANCE, then press start
8.7.19.1.2. Allow the instrument time to prime before use
8.7.20. Once the instrument is done priming go to the WORKPLACE tab
8.7.21. Click START
8.7.21.1. Run time will be displayed on the screen
8.7.22. When the extraction is done click “OK”
8.7.22.1. Go to the “WORKPLACE” tab
8.7.23. Open the bottom door flap of the instrument, remove the elution plate and seal it with the silver aluminum plate cover
8.7.24. Mark the date and experiment on the side and place in 4°C fridge in the Template room
8.7.25. Remove the bottle rack and trays 1 & 2.
8.7.25.1. Record the date and # of runs left in each reagent tray if applicable
8.7.25.2. seal the reagent trays with the thin **GOLD** aluminum sealing foil
8.7.26. Remove magnetic bead bottles from the bottle rack
8.7.27. Store magnetic bead bottles and Reagent Trays together in the 4°C cooler
8.7.28. Throw used MP96 Processing cartridges in the biohazard bin
8.7.29. Remove Tray 3
8.7.30. Retain empty clean tip racks and store on the shelf
8.7.31. Spray bottle rack and trays 1,2 and 3 with 70% Ethanol and leave on a clean surface to dry (you may put bench liner down on an empty bench and place them there)
8.7.32. Remove trays 4 and 5
8.7.32.1. Toss wasted tips, keep empty tip racks to be used as waste holders
8.7.33. Spray waste funnel rack and tip racks with 3% Microchem and place in sink for minimum 5 min contact time OR place in 3% Microchem bath for a minimum of 30 minutes, then rinse with water and spray with 70% EtOH

8.7.34. Spray Tray 4 and 5 with 3% Microchem and place in sink for minimum 5 min contact time, then rinse with water and spray with 70% EtOH place on clean surface to dry

8.7.35. Close the bottom flap of the instrument

8.7.36. The instrument should be cleaned once per day. To clean follow the below steps. If not cleaning skip to 8.8

8.7.36.1. Turn off the MP96 instrument

8.7.36.2. Open top and bottom door flaps of the instrument

8.7.36.3. Insert the white tray slot cover into slot #4 on the instrument to protect the magnetic tray and heat block (optional)

8.7.36.4. Wipe all inside surfaces (tray slots, instrument bolts, tip park) with a kimwipe soaked in T36 Disinfex or other appropriate cleaning agent, then wipe clean with a kimwipe soaked in 70% EtOH

8.7.36.5. Check needles and clean with 70% EtOH if needed

8.7.36.6. Close both flaps and turn instrument back on

8.7.36.7. Go to instrument tab on the interface

8.7.36.8. Select UV DECON

8.7.36.9. Set for 30 minutes and click START

8.8. RT qPCR

8.8.1. Prepare PCR plate map and print off

8.8.2. Don lab coat or back closing gown designated for PCR Clean room and nitrile gloves

8.8.3. Enter PCR Clean room with PCR plate template

8.8.3.1. Place PCR template somewhere that it will be visible while working in the BSC

8.8.4. Spray gloves with RNAse away

8.8.5. Retrieve master mixes that are needed from the freezer and place on heat block to thaw

8.8.6. Clean BSC and pipette with RNAse Away, followed by 70% Ethanol

8.8.7. Place new MicroAmp™ fast Optical 96 well Reation plate (0.1 mL) in BSC (hence forth called PCR Plate)

8.8.8. Once master mixes have thawed place in BSC.

8.8.9. Dispense 15 µL of appropriate master mix into wells using a multi-dispenser pipette

8.8.9.1. Currently there are separate master mixes for N1 target, N2 target, PMMoV and MHV (Appendix 1A - 1F) for preparation of mastermix

8.8.10. Place PCR plate map and PCR plate in pass through window

8.8.11. Place unused master mix back in freezer box

8.8.12. Clean BSC and pipette with RNAse Away, followed by 70% Ethanol

8.8.13. After exiting room, remove lab coat or back closing gown and proceed to PCR template room

8.8.14. Don lab coat or gown designated for PCR Template room and nitrile gloves

8.8.15. Retrieve PCR plate map and PCR Plate from pass through window

8.8.15.1. Place PCR plate in BSC and PCR plate map in visible spot

8.8.16. Clean pass through window with RNAse Away, followed by 70% Ethanol

8.8.17. Retrieve RNA elution plate (see 8.7.23 and 8.7.24) from fridge and place in BSC

8.8.18. Dispense 5 µL of RNA into appropriate wells

8.8.19. Cover PCR plate with MicroAmp™ Optical Adhesive Film

8.8.19.1. Ensure cover is sealed well to avoid well to well contamination

8.8.20. Reseal RNA elution plate with silver aluminum foil cover and return to fridge

8.8.21. Clean BSC and pipette with RNAse Away, followed by 70% Ethanol

8.8.22. Remove lab coat or gown and dispose of gloves

8.8.23. Don new nitrile gloves and take PCR plate to RT qPCR machine

8.8.24. Spin PCR plate to ensure mastermix and template is at the bottom of the wells
8.8.25. Place in RT qPCR machine
8.8.26. Select appropriate cycling program (See Appendix 1A - 1F) and assign targets to wells in program
8.8.27. Run program

Table 6: Wastewater Sample Receiving Log

Date Received	Location / Code	Spot See		Temp	Sample Collection		Auto-sampler Last Clean		Comments
		Freeze	Warm		Date	Time	Date	Time	

Appendix 1A - 1F: Polymerase Chain Reaction (PCR)

A - SARS-CoV-2 N1 gene

N1 Primers and Probe	Sequence (5'→3')	Concentration
2019-nCoV_N1-Forward	GAC CCC AAA ATC AGC GAA AT	0.5µM
2019-nCoV_N1-Reverse	TCT GGT TAC TGC CAG TTG AAT CTG	0.5µM
2019-nCoV_N1-Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	0.125µM

CDC Protocol: *https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf*

B - SARS-CoV-2 N2 gene

N2 Primers and Probe	Sequence (5'→3')	Concentration
2019-nCoV_N2-Forward	TTA CAA ACA TTG GCC GCA AA	0.5 µM
2019-nCoV_N2-Reverse	GCG CGA CAT TCC GAA GAA	0.5 µM
2019-nCoV_N2-Probe	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	0.125 µM

CDC Protocol: *https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf*

C - SARS-CoV-2 E -gene

Detection of the SARS-CoV2 viral E gene target

N2 Primers and Probe	Sequence (5'→3')	Concentration
E-Sarbeco_F	ACA GG TAC GTT AAT AGT TAA TAG CGT	0.4µM
E-Sarbeco_R	ATA TTG CAG CAG TAC GCA CAC A	0.4µM
E-Sarbeco_P1	FAM-ACA CTA GCC ATC CTT ACT GCG CTT CG-BBQ	0.2 µM

Source: Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR (Corman et al., 2020) Eurosurveillance, 25, 2000045 (2020). https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045

D - SARS-CoV-2 RdRp -gene

Note: Primers and probes were optimized by the BC CDC

N2 Primers and Probe	Sequence (5'→3')	Concentration
BCCDC_RdRp_F	TGC CGA TAA GTA TGT CCG CA	0.4µM
BCCDC_RdRp_R	CAG CAT CGT CAG AGA GTA TCA TCA TT	0.4µM
BCCDC_RdRp_P	FAM-TTG ACA CAG ACT TTG TGA ATG-MGB/NFQ	0.2 µM

E - Pepper Mild Mottle Virus (PMMoV)

- *Detection of a viral coat protein of the pepper mild mottle virus gene target*

PMMoV Primers & Probe	Sequence (5'→3')	Concentration
PMMV-FP1-rev:	GAG TGG TTT GAC CTT AAC GTT TGA	0.4 µM
PMMV-RP1:	TTG TCG GTT GCA ATG CAA GT	0.4 µM
PMMV-Probe1:	FAM-CCT ACC GAA GCA AAT G-BHQ1	0.2 µM

Zhang (2005) Protocol: *"RNA Viral Community in Human Feces: Prevalence of Plant Pathogenic Viruses", PLoS Biology 2006; 4(1): e3*
PPMV-FP1-rev: (revised: Haramoto, 2013: DOI: 10.1128/AEM.02354-13)

F - Murine Hepatitis Virus (MHV)

- Detection of a membrane (M) protein of the murine hepatitis virus gene target

MHV Primers & Probe	Sequence (5'→3')	Concentration
MHV-FP1:	GGA ACT TCT CGT TGG GCA TTA TAC T	0.3 µM
MHV-RP1	ACC ACA AGA TTA TCA TTT TCA CAA CAT A	0.3 µM
MHV-Probe1	FAM-ACA TGC TAC GGC TCG TGT AAC CGA ACT GT-BHQ1	0.4 µM

Ahmed (2020) Protocol: *"Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater", Science of the Total Environment 2020; 739(2020): 139960*

Reaction Mix

Reaction Mix prepared as per product insert specifications, following above listed primer and probe final concentrations.
15µL reaction mix + 5µL template = 20µL final volume

Cycling parameters:

25°C for 2 minutes (UNG incubation)
50°C for 15 minutes (RT incubation)
95°C for 2 minutes (enzyme activation)
95°C for 5 seconds, 60°C for 30 seconds with optics on (40 cycles)
60°C for 30 seconds with optics on (end read)

Reagents:

Life Technologies/Thermofisher Scientific/Applied Biosystems
TaqPath™ 1-Step RT-qPCR Master Mix, CG
A15299 5 × 1 mL
A15300 1 × 10 mL

Life Technologies/Thermofisher Scientific/Invitrogen
UltraPure™ BSA (50 mg/mL)
AM2616 1 x 50 mg
AM2618 5 x 50 mg

DNA Quantification standards

- **N1**

AAATTCCTCCCTTTCCCTTTGACCCCAAAATCAGCGAAATTTTACCCCGCATTACGTTTGGTGGACCTTTCAGATTCAACTGGCA
GTAACCAGATTTCCCAGGTTTCCTTTTGTTGTTATGGCCA

- **N2**

AAATTCCTCCCTTGCCCTTTACAAACATTGGCCGCAAATTTACAATTTGCCCCCAGCGCTTCAGTTTTTCTTCGGAATGTCGCGCT
TTCCTTTTGTTGTTATGGCCATTTCCATTTAACCCTTTA

- **E gene**

CATTCGTTTCGGAAGAAACAGGTACGTTAATAGTTAATAGCGTACTTCTTTTTCTTGCTTTCGTGGTATTCTTGCTAGTCACACTA
GCCATCCTTACTGCGCTTCGATTGTGTGCGTACTGCTGCAATATTGTTAACGTGAGTTTAGTAACCCAAAGACCACATTGGCACC
CGCAATCCTAATAACAATGCTGCCACCGTGCTACAACTTCCTCAAGGAACAACA

- **RdRP**

ATGATTCAATGAGTTATGAGGATCAAGATGCACTTTTCGCATATACAAAACGTAATGTCATCCCTACTATAACTCAAATGAATCTTA
AGTATGCCATTAGTGCAAAGAATAGAGCTCGCACCGTAGCTGGTGTCTCTATCTGTAGTACTATGACCAATAGACAGTTTCATCAA
AAATTATTGAAATCAATAGCCGCCACTAGAGGAGCTACTGTAGTAATTGGAACAAGCAAATTCTATGGTGGTTGGCACAACATG
TTAAAAACTGTTTATAGTGATGTAGAAAACCCTCACCTTATGGGTTGGGATTATCCTAAATGTGATAGAGCCATGCCTAACATGCT
TAGAATTATGGCCTCACTTGTTCTTGCTCGCAAACATACAACGTGTTGTAGCTTGTCACACCGTTTCTATAGATTAGCTAATGAGT
GTGCTCAAGTATTGAGTGAAATGGTCATGTGTGGCGGTTCACTATATGTTAAACCAGGTGGAACCTCATCAGGAGATGCCACAA
CTGCTTATGCTAATAGTGTTTTTAACATTTGTCAAGCTGTCACGGCCAATGTTAATGCACTTTTATCTACTGATGGTAACAAAATTG
CCGATAAGTATGTCCGCAATTTACAACACAGACTTTATGAGTGTCTCTATAGAAATAGAGATGTTGACACAGACTTTGTGAATGA
GTTTTACGCATATTTGCGTAAACATTTCTCAATGATGATACTCTCTGACGATGCTGTTGTGTGTTTCAATAGCACTTATGCATCTCA
AGGTCTAGTGGCTAGCATAAAGAACTTTAAGTCAGTTCTTTATTATCAAAACAATGTTTTTATGTCTGAAGCAAAATGTTGGACT
GAGACTGACCTTACTAAAGGACCTCATGAATTTTGCTCTCAACATACAATGCTAGTTAAACAGGGTGATGATTATGTGTACCTTCC
TTACCCAGATCCATCAAGAATCCTAGGGGCCGGCTGTTTTGTAGATGATATCGT

- **PMMV**

GGTTTCAAATGAGAGTGGTTTGACCTTAACGTTTGAGAGGCCTACCGAAGCAAATGTCGCACTTGCATTGCAACCGACAATTAC
ATCAAAGG

- **MHV**

TTCCTTAAGGAATGGAACTTCTCGTTGGGCATTATACTACTCTTTATTACTATCATACTACAGTTCGGTTACACGAGCCGTAGCATG
TTTATTTATGTTGTGAAAATGATAATCTTGTGGTTAATGTGGCCAC

Outline of Wastewater Surveillance Data Interpretation

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Appropriate public health interpretation of wastewater surveillance data depends on the primary goal and scope of the surveillance system. At the regional or municipal level, wastewater surveillance data can be used to (i) monitor the presence of infected individuals contributing to a wastewater treatment plant from which positive samples are collected; (ii) track infection trends within the community contributing to the sewer collection system and measured at the wastewater treatment plant (also known as a "*sewershed*").

Although SARS-CoV-2 has been successfully detected in low prevalence settings such as dormitories, there are a number of challenges related to monitoring for viral presence at more granular sites, (e.g. institutions, specific buildings) due to limitations related to the sensitivity of current molecular methods when the case count is low, necessitating collection of larger volumes of WW and additional steps to concentrate and recover SARS-CoV-2 RNA.[1-8] Accounting for commuting behaviour or transience of individuals visiting a building or institution using the restroom but residing in another locale is another issue that needs to be addressed, as well as the presence of a resident who previously had COVID-19 but continues, to shed SARS-CoV-2 leading to a positive signal in the waste stream. These situations can be resolved through the establishment of a baseline reading, followed by a sampling protocol and frequency that is either flow proportional or time dependent based on the occupancy cycle of the building that facilitates trend analysis and reduces reliance on a result based on a single collection point in time.[9-11]

N.B.: Wastewater treatment plants in tourist areas where the population is predominantly transient may provide highly variable RNA viral signals that need to be taken into accounted.

Data normalization

Validating comparisons of SARS-CoV-2 RNA wastewater concentrations over time, necessitates normalizing these concentrations with daily wastewater flow. This necessary step accounts for changes in wastewater contributions, and the population size within the target catchment

area of the wastewater treatment plant. Normalization results in the use of “units of viral gene copies per person contributing to the sewershed per day.”

As the number of individuals contributing to the sewershed changes over the surveillance period (due to tourism, weekday commuters, temporary workers, etc.), normalization of the human fecal load becomes important as it allows for meaningful interpretations and concordant and standardized longitudinal comparisons between SARS-CoV-2 levels in wastewater. Fecal normalization entails the use of organisms/compounds specific to human feces that are quantifiable in wastewater and can be used as an estimate of fecal content. It has been suggested that normalization can be achieved by using the ratio of non-normalized wastewater concentrations over the human marker concentrations

Meaning of individual test result

Wastewater surveillance can detect the aggregate viral load of pre-symptomatic/asymptomatic and symptomatic shedding days before the rise in cases are identified through clinical surveillance.[12,13-15] Environmental surveillance has limitations related to its threshold of detection and inability to provide information at the individual level, which precludes its use as a sole source tool to inform the public health response; however, its sentinel function compliments traditional surveillance. Detection of SARS-CoV-2 in wastewater depends mainly on the sensitivity level of the test used, the sampling design, and the amount of SARS-CoV-2 excreted by infected individuals in the target community. A positive wastewater SARS-CoV-2 RNA signal means there is at least one individual in the target community shedding SARS-CoV-2. This method cannot make distinctions regarding an individual’s infection status, i.e. discern if an individual is infectious or symptomatic. Furthermore, a low viral RNA concentration reading level from a community wastewater sample might indicate either a small number of infected individuals in the catchment area serviced by a particular wastewater treatment plant shedding virus into the sampled wastewater, or a low amount/viral load being shed per infected individuals in the target community. In reference to the limits of detection associated with the molecular methods used to detect SARS-CoV-2 RNA

and interpretation of findings, an assay result indicating that a viral RNA signal was not detected, might signify one of two results. Either an absence of SARS-CoV-2 in the sampled community or the viral concentration in the sampled wastewater was below the assay's threshold of detection. The minimum number of infected individuals shedding SARS-CoV-2 into the wastewater treatment system needed to detect a viral RNA signal in wastewater is yet to be established.

Trend analysis

Trend classification of wastewater-based surveillance data is the statistical analysis of changes observed in the normalized concentration of SARS-CoV-2 in wastewater. The concentration of virus detected in wastewater has been shown to correlate well with the burden of infected individuals contributing to the wastewater and sewage treatment plant[16-19]. The data necessary to estimate the number of infections represented by specific viral concentrations isolated from wastewater is currently available. Normalized wastewater surveillance data can be used to estimate trends in SARS-CoV-2 infections (both reported and unreported) in the target community if tracked over time. Trends can be monitored for direction of change and duration.

While the comparability of individual results from different wastewater and sewage treatment plants may not be possible due to differences that include population size and wastewater volume. Trend analysis of wastewater test results are comparable and if executed in a timely manner, can forecast and track trends in reported SARS-CoV-2 infection. Trends can be calculated using linear regression analysis with the slope representing the trend, and the estimate (e) set as the independent variable (if estimate of change is to be assessed daily). Using weighted least squares regression produces an estimation of wastewater data that is more precise as it accounts for variability in the sampling, processing, and quantification steps. A log transformation of SARS-CoV-2 normalized concentrations is recommended prior to trends analysis and/or other statistical analyses. Trend classification is semi-quantitative in nature, because it uses a broad categorization based on the duration ("short- and long-term,

sustained" defined by the sample frequency) and the direction ("increase, decrease, or plateau").

Estimates of SARS-CoV-2 infection in a community

Estimates of SARS-CoV-2 infection in the community depends largely on clinical data, although this is often an underestimation as only those with significant symptoms seek medical help and are tested. Thus, correlating the results from wastewater detection of SARS-CoV-2 is challenging due to the unknowns such as the variability in the amount and duration/persistence of viral shedding by individuals over the clinical course and the caseload. The concordance between detection of viral RNA in wastewater and community infection prevalence is challenging and point to the critical need for clinical data to facilitate the analysis.

Assuming the application of reliable and representative sampling and testing methodologies, as described above, the (a) interpretation of results from a SARS-CoV-2 infection wastewater surveillance system and (b) the subsequent public health actions are largely defined by the objectives set by the participating jurisdictions - most of which generally relate to the following two scenarios:

1. The identification of SARS-CoV-2 infection introduction to a specific population (e.g. isolated or remote communities who have no sustained community transmission, targeted sampling of vulnerable populations), and;
2. Securing reliable time-series data on the normalized concentration of virus detected in wastewater (e.g. urban areas with sustained community transmission) as a means to infer relative variation of the prevalence of SARS-CoV-2 infection in a population (e.g. establishing baseline trends to detect escalation and de-escalation).

While a wastewater surveillance system for SARS-CoV-2 infection carries numerous advantages, there are some important limitations to consider. For example:

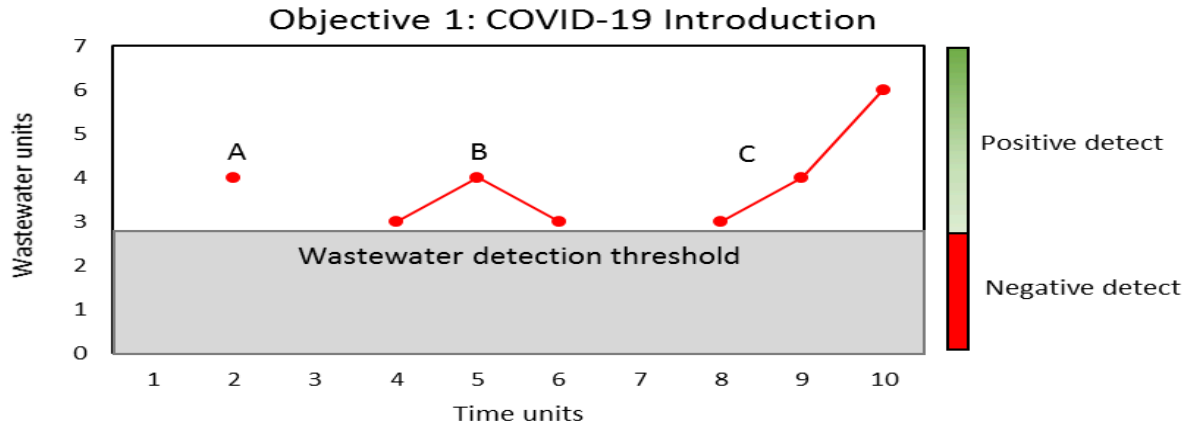
- The wastewater viral signal, even when normalized, is often highly variable, owing to numerous factors (e.g. precipitation events that impact wastewater through the treatment facility)
- Jurisdictions interested in the use of wastewater surveillance as an early warning system must commit to a sufficient sample collection frequency and have access to rapid laboratory results
- The lower limit of detection (i.e. the wastewater detection threshold) is not well understood and therefore, wastewater surveillance cannot always be used as evidence of absence of SARS-CoV-2 infection
- Wastewater surveillance may not be able to determine the overall prevalence of SARS-CoV-2 infection at the population level (however, some applications do exist to estimate ranges)
- Wastewater surveillance may not be able to determine zero transmission of COVID-19 in a population (however, some applications do exist within smaller targeted populations)

The following scenarios describe examples of COVID-19 wastewater signal interpretations and how these signals can be used to inform public health action.

Scenario 1 – The identification of COVID-19 introduction to a specific population

Jurisdictions using wastewater surveillance to detect the introduction of COVID-19 to a specific population can efficiently monitor the entire sewershed, but face a unique challenge: a positive detect of SARS-CoV-2 is evidence of introduction, but trends over time may be obscured by the detection threshold.

Figure 1



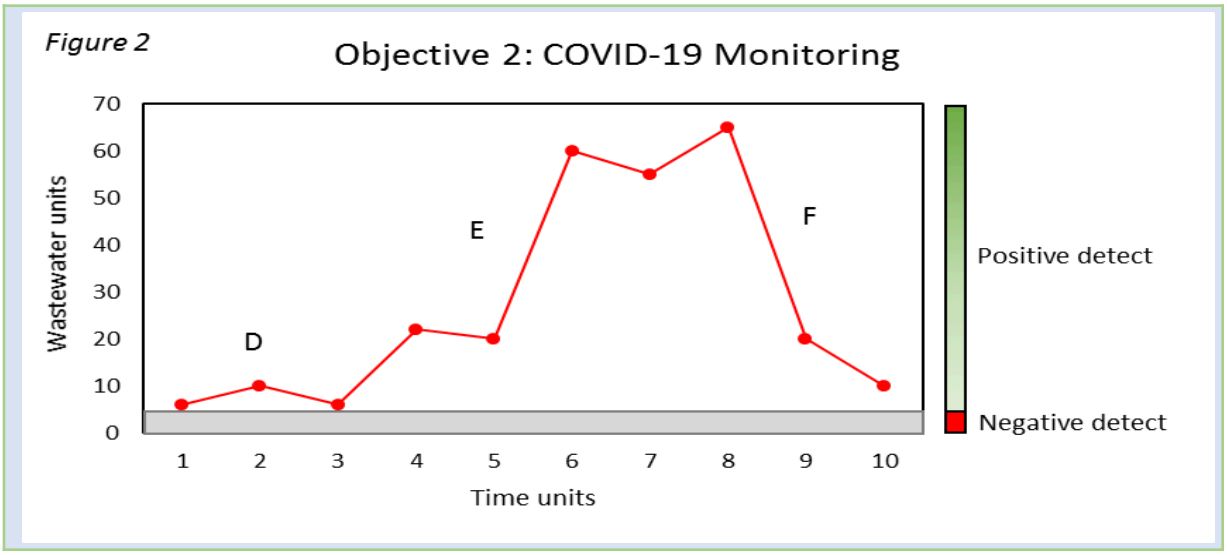
Marker	Interpretation(s)	Example public health action(s)
A	A single wastewater detect is the weakest evidence required to suggest the introduction of COVID-19 to a targeted population; however, this result may be explained by local epidemiologists (e.g. known positive cases returning from travel and self-isolating).	Increase wastewater testing methodology for the rapid identification of escalation.
B	Three wastewater detects may represent stronger evidence that COVID-19 has been introduced to a targeted population; however, these results may be explained by local epidemiologists (e.g. known self-isolating travel-related positive cases causing secondary infections in household contacts).	Ensure the full compliance of self-isolation for all known positive cases and close contacts, while investigating the presence of asymptomatic infections.
C	Three or more escalating wastewater detects may represent the strongest evidence that COVID-19 has been introduced to the targeted population, especially when unexplained by local epidemiologists (e.g. no escalation in known or expected case counts).	Establish clear public health messaging (e.g. immediately limit non-essential activities, encouraging individuals to present for testing if symptomatic). Escalate contact tracing of all identified positive cases.

An example of wastewater data relating to scenario 1 is provided in Appendix A.

Scenario 2 – The monitoring of SARS-CoV-2 infection over time

Jurisdictions using wastewater surveillance to monitor variations in the viral signal over time are well positioned to rapidly identify trends in COVID-19 transmission at the population level. These trends can reliably identify resurgence, as well as measure the success of public health interventions (e.g. the closure of high-risk settings).

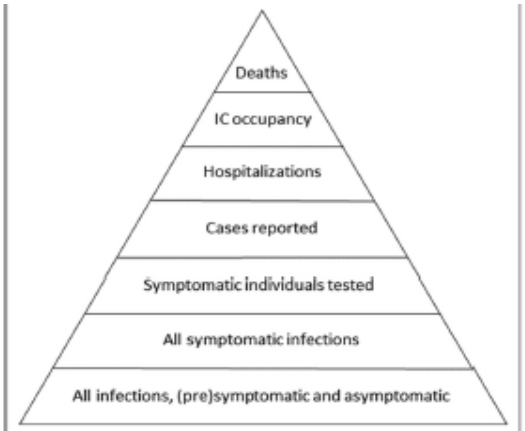
An example of wastewater data relating to scenario 2 is provided in Appendix B.



Marker	Interpretation(s)	Example public health action(s)
D	A series of wastewater detects of approximately equal value at the low-end of the spectrum suggests that the transmission of COVID-19 remains low and controlled.	• Maintain preventative messaging regarding routine public health interventions (e.g. masking, distance and avoiding non-essential activities). • Maintain a high degree of contact tracing for all identified cases.
E	A series of escalating wastewater detects is strong evidence of a rapid increase in the transmission of COVID-19; however, these results may be explained by local epidemiologists (e.g. known outbreaks or jurisdiction-specific factors).	• Establish clear public health messaging (e.g. immediately limit non-essential activities, encouraging individuals to present for testing if symptomatic). • Implement mass public health action, such as limiting exposure to high-risk settings and minimizing the number of non-household contacts.
F	A series of de-escalating wastewater detects is strong evidence that the transmission of COVID-19 is declining, suggesting public health interventions were successful.	• De-escalate public health intervention. • Promote clear public health messaging emphasizing the success of the intervention(s) while maintaining increased vigilance.

Integrating SARS-CoV-2 wastewater and laboratory surveillance

Figure 6: Implementation of Environmental Surveillance for SARS-CoV-2 virus



To date, Canadian jurisdictions primarily utilize traditional laboratory surveillance to detect and monitor SARS-CoV-2 infection trends over time - a methodology that generates confirmed case

counts by jurisdiction. While the limitations associated with laboratory surveillance are well documented, it is widely recognized that not all infections are captured (figure 3).

Conversely, wastewater surveillance methodologies for the detection and monitoring of SARS-CoV-2 infection are insufficient to identify infected individuals or establish population-level prevalence estimates; however, results are not dependent on the manifestation of SARS-CoV-2 infection symptoms or individuals presenting for testing.

As a result, many jurisdictions have demonstrated the predictive utility of wastewater surveillance two-days to two-weeks prior to laboratory surveillance.

Given these characteristics, the proper implementation and integration of these two complimentary surveillance systems may generate the most robust, reliable and timely data available to public health authorities.

The following matrix illustrates how the integration of wastewater surveillance results and laboratory surveillance results can directly inform the escalation and de-escalation of public health interventions.

Table 7: Integration matrix of Clinical & Wastewater SARS-CoV-2 Surveillance results

		LABORATORY SURVEILLANCE SIGNAL		
		Trending down	Baseline	Trending up
WASTEWATER SURVEILLANCE SIGNAL	Trending down	● Strong evidence of decline in prevalence, the transmission of COVID-19 is controlled	● Some evidence of a decline in prevalence	● Conflicting evidence (requires local interpretation)
	Baseline	● Some evidence of decline in prevalence	● Contextual result (requires local interpretation)	● Some evidence of an increase in prevalence
	Trending up	● Conflicting evidence (requires local interpretation)	● Some evidence of an increase in prevalence.	● Strong evidence of a true increase in prevalence, the transmission of COVID-19 is not controlled

Legend:

Green	-	*Decrease stringency of public health intervention*
Yellow	-	*Plan for the increased stringency of public health intervention*
Red	-	*Increase the stringency of public health intervention*

The integration of wastewater surveillance results and laboratory surveillance results may require additional local expertise or tools to interpret results that appear to be unclear or contradictory. A high degree of coordination between jurisdictional experts (e.g. epidemiologists, wastewater operators and laboratory personnel) can assist with the identification of additional elements required for local interpretation (e.g. methodological problems).

Using wastewater surveillance to inform the COVID-19 public health response

SARS-CoV-2 wastewater surveillance can provide an early indicator of the presence and/or trends of SARS-CoV-2 infection in a community. As such, it can be used to trigger clinical testing to determine the magnitude of SARS-CoV-2 infection within a community. Other uses of SARS-Cov-2 wastewater surveillance include monitoring the trend in infection levels to guide public health communication about preventative measures, community mitigation strategies, and to measure the impact of public health interventions.[20,21]

The interpretation of SARS-CoV-2 wastewater surveillance data into appropriate public health response (Table 3) requires a minimum set of data on the:

(i) wastewater treatment plant (treatment process, service area and corresponding population size),

(ii) sampling procedure including (type of sample (grab or composite), time, date of collection, location, as well as the wastewater flow rate during sample collection),

(iii) testing procedure to ensure comparability of results from different sewersheds,

(iv) the flow rate,

(v) etc.

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An example of wastewater data relating to scenario 1 is provided in Appendix A.

N.B. Obtain consent and publish actual wastewater data with high-level interpretation that augment Scenario 1 and 2

An example of wastewater data relating to scenario 2 is provided in Appendix B.

N.B. Obtain consent and publish actual wastewater data with high-level interpretation that augment Scenario 1 and 2

Mathematical Modelling of SARS-CoV-2 in Wastewater

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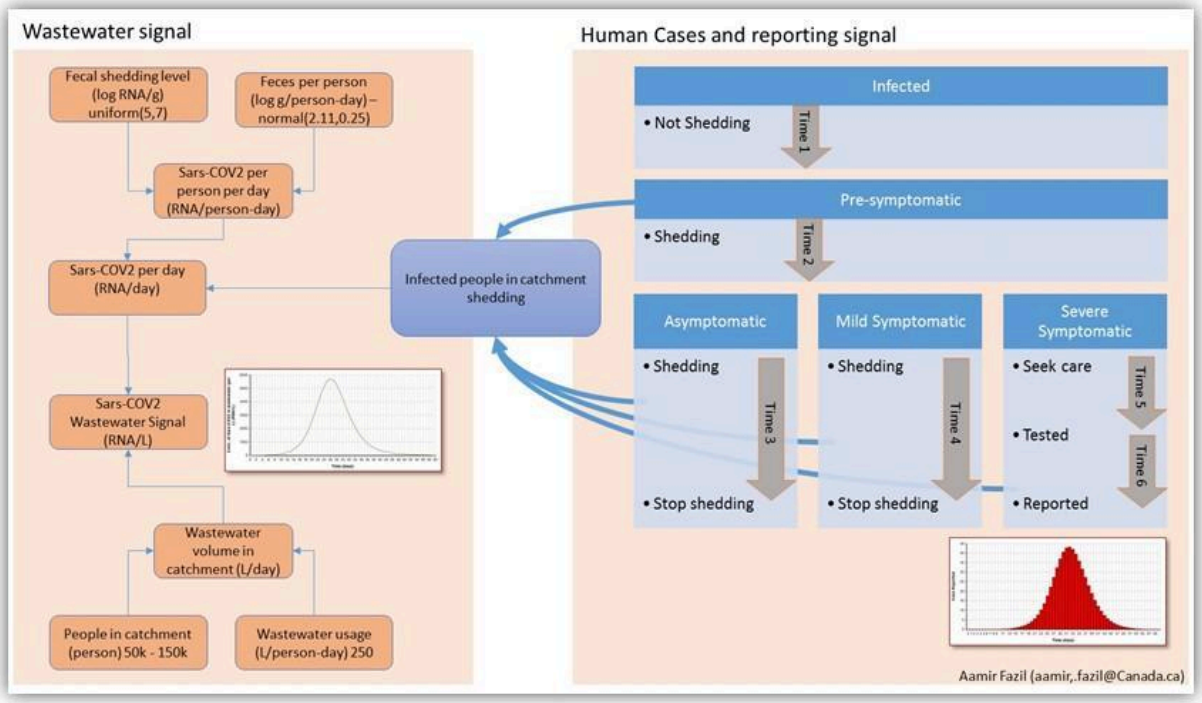
Mathematical Modelling of the dynamic of SARS-CoV-2 infection

Wastewater laboratory testing can reliably predict the presence/absence of SARS-CoV-2 and its concentration in wastewater but is limited in its ability to determine the total number or percentage of infected individuals in a community. More data on SARS-CoV-2 concentrations in the feces of infected individuals are needed in order to model the relationship between SARS-CoV-2 RNA concentrations in wastewater and the number of infected individuals in the targeted sewershed. Mathematical modelling of infectious diseases has been used to study disease spread, forecast short- and/or long-term patterns, the magnitude and the anticipated impact to facilitate advanced public health planning and the development of control measures. The Wastewater Modelling Consortium convened for the purpose of this surveillance project is comprised of individuals with strong mathematical modelling expertise and extensive background in public health and/or health sciences. The consortium has explored several questions exploring the possibility of the following options:

(i) use of wastewater data to develop dynamic model(s) to predict the pattern of COVID epidemic over time in specific or mega jurisdictions, or

(ii) the development and application of mechanistic/theoretical models to explain how human infection data can be translated to correspond with waste water signals as illustrated in the example in Figure 5.

Figure 7: *Mechanistic model on how human infection translate to wastewater signals*



Other areas of interest to the Modelling Consortium for this surveillance project include the following:

(a). What are the overall correlations between wastewater signal and human illness outcomes

(b). Can wastewater serve as an early warning signal

(c). Can wastewater serve as a measure of overall infection in the community

(d). Can wastewater serve as a signal for the effect of interventions

(e). What are the factors that impact all these

(f). What are the preferred normalization techniques to use

(g). Identify a reference standard to measure the performance of the wastewater signal against (i.e. the human case data may not be the appropriate gold standard to use under all conditions, in these circumstances the wastewater data may be telling us something that may not correlate with the case data).