Canadian Coalition on Wastewater-Related COVID-19 Research

Design principles and protocols for a valid hypothesis-testing pilot study: Surveillance of community or institutional wastewater for SARS-CoV-2 to supplement clinical evidence about prevalence of infection

Context for the Pilot Study

There is an urgent need in Canada, prior to experiencing second or subsequent waves of COVID-19 infection, to facilitate and coordinate initiatives that allow for economic recovery while achieving focused monitoring of communities or institutions. Testing of wastewater to detect SARS-CoV-2 can provide a composite, integrated indicator of the prevalence of COVID-19 infection days in advance of testing ill patients.

Wastewater-based viral epidemiology (WBVE) has already been demonstrated to be informative for providing surveillance of poliovirus infections (Hovi et al., 2012; Lago et al., 2003). The case for WBVE to be applied to SARS-CoV-2 has been concisely argued by Daughton (2020). Several hypothesis-generating studies have recently been published (Ahmed et al., 2020; Medema et al., 2020; Nemudryi et al., 2020; Randazzo et al., 2020; Wu et al., 2020) to support the **hypothesis that wastewater surveillance can provide a cost-effective and timely indication of COVID-19 infection in a community or institution.** The Netherlands, Germany and Finland have initiated nation-wide programs of community-wide surveillance by means of monitoring for SARS-CoV-2 in wastewater.

Structuring of this protocol was conducted within the key framing of the Canadian Coalition on Wastewater-Related COVID-19 Research. As such, the ultimate intent of the work is to contribute to the overall goal of assessing the ability for WBVE to underpin the key questions faced by public health decision makers. A critical first step in testing the above hypothesis is establishing a valid protocol for sampling and analyzing wastewater to be followed by those in the pilot study, as well as those conducting outside research aiming to have those results considered with and compared to the pilot study results.

Design principles for the COVID-19 Wastewater Coalition pilot study

This preamble provides a summary of 8 design principles for the COVID-19 Wastewater Coalition pilot study, which provide a framing for the pilot study methodological protocol that follows. These 8 design principles for the COVID-19 Wastewater Coalition pilot study draw on the guiding principles for wastewater sampling programs set out by Water Research Foundation (WRF, 2020) – see Box 1.

Building on the WRF 2020 Summit and the work of other international groups and given Canada's needs in moving this important piece forward, Canadian Water Network's COVID-19 Wastewater

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For more information on the Canadian Coalition on Wastewater-Related COVID-19 Research, visit covid-19-wastewater-coalition.

Coalition developed the following design principles for the Canadian pilot study. These design principles are expanded further below. Definitions of terms used in the following text are provided at the end of this summary, before the references cited.

8 design principles for the COVID-19 Wastewater Coalition pilot study

1. **Clearly define pilot monitoring program objectives**
2. **Achieve rapid validation and adoption of a consistent Canadian sampling protocol**
3. **Confirm validity of wastewater analyses for SARS-CoV-2**
4. **Ensure potential for generalizability by fully understanding what samples represent**
5. **Maximize value of results through strategic pilot project design**
6. **Maximize potential for productive collaboration with wastewater utilities**
7. **Maximize collaboration, cooperation and knowledge exchange**
8. **Consider ultimate use and ethics of data use in public health decision-making**

Box 1. Guiding principles for wastewater sampling programs (WRF, 2020)

Summarized from the Closing Session of the Virtual International Water Research Summit on Environmental Surveillance of COVID-19 Indicators in Sewersheds (April 30, 2020)

- Baseline assumption of centralized WWTP
- Data comparability requires some consistency in practices and documentation/metadata
- Recommendations are adaptable and modifiable to best meet needs
- Intention is NOT to inhibit utility operations during a pandemic
- Balance study goals with practical considerations: resources, operator ability, freezer storage space, budget
- Some best practices for sample collection apply to all use cases, whereas others are use-case specific
- Consider worker safety in sampling and sample prep guidance
- Proof of concept to support practice and research with potential for future learning

Expansion on rationale and considerations of the design principles for development of pilot study protocols

1. **Clearly define pilot monitoring program objectives**
Identifying significant trends in the occurrence of SARS-CoV-2 signal within the receiving sewer system (sewer-sheds).

Where might this objective lead? Among the many possibilities for useful (i.e., for guiding public health decision-making) evidence derived from reliable and effective monitoring of community or institutional wastewater for SARS-CoV-2, is the promising possibility of identifying significant trends in the occurrence of SARS-CoV-2 signal within the receiving sewer system (Daughton 2020). If that objective can be reliably achieved for a community, the logical extensions of such evidence include the possibilities of:

- Correlating the temporal trends measured in a community's wastewater with community results from collective clinical monitoring of individuals for COVID-19 infection, requiring a comparatively modest sampling and analytical burden, ultimately to determine the predictive value of observed trends in community viral load in wastewater for public health decision-makers. **Achieving this objective will require a fully validated sampling/analytical methodology and careful data analysis to ensure that clinical data covers the same catchment as the sewer system being monitored.**

- When the foregoing primary objectives have been achieved, there is potential for developing more refined and insightful evidence about levels of COVID-19 infection in a community or institution from wastewater monitoring but such potential will require understanding of published research about the magnitude and duration of viral shedding from asymptomatic, symptomatic and recovered individuals as well as persistence of SARS-CoV-2 virus markers in sewerage systems and various other site-specific factors. Research underway in other jurisdictions may assist in evaluating potential for developing such insights.

2. **Achieve rapid validation and adoption of a consistent Canadian sampling protocol**
Delivering on the program objective requires the rapid development and testing of a wastewater sampling protocol to maximize an accurate, sensitive and reproducible estimation of the evolution of the SARS-CoV-2 viral titer in the sewage system being monitored.

The protocol should ensure generalizability (design principle #4) and facilitate maximum collaboration with wastewater utilities (design principle #6). The validity of sampling will depend on many factors.

- Intuitively, the best location to collect samples at a wastewater plant will be the existing sampling location targeting raw sewage entering the plant to avoid impacts of the wastewater treatment operations on virus concentrations, but this intuition should be verified if sampling and analytical resources allow (Gundy et al. 2009).

- Sample type should be composite samples (with known frequency) if possible, either at fixed time intervals over 24 hours or according to constant flow increments, with minimum impact on existing raw sewage sampling protocols. In limited circumstances a case might be made for simple grab samples but conceptually, for this initial pilot, **composite samples are definitely preferred**. Questions about the stability of SARS-CoV-2 in wastewater over time have not been thoroughly tested. For the purpose of this initial pilot, although grab samples may be easier to obtain in some circumstances, they will require more work on the part of plant personnel to collect, unless resources for dedicated autosamplers are available, than would providing a daily aliquot from a composite sampler currently used to inform plant operations. Because flow records should be available, some interpretation of fixed time interval composite samplers may be possible, if needed, to compare with other sites using flow integrated composite samplers.

- A refrigerated composite sampler is definitely preferred and should be considered a worthwhile investment for a continuing monitoring program if not already in place.

- Spiked controls, to normalize the data, should be included – e.g., an assay for an innate fecal marker consistently present in high numbers and relevant to the enveloped SARS-CoV-2 virus, such as the enveloped, RNA plant virus, pepper mild mottled virus (PMMoV) widely used as a sewage marker (Kitajima et al., 2018) and spiking known titers of the seasonal flu coronavirus strain 229E (α-CoV) or OC43 (β-CoV) to address transport and method recovery – see QA/QC in design principle #3 below.

- Sample processing needs to address the presence of solids in samples because viral particles will be associated with solids meaning that measured viral concentrations can be expected to vary with solids content. The quantitative effects of different sample processing options for solids removal need to be evaluated and standardized as soon as possible. For example, simple pH adjustment (to pH 10.5) may provide conditions to disperse adsorbed virions from surface solids and preserve sample integrity (Conceição-Neto et al., 2015; Ye et al., 2016), but effects on SARS-CoV-2 of this pH adjustment is unknown.

- The movement of solids through a sewer system is different from the movement of liquids, resulting in longer retention times for solids. Sewer solids settle and resuspend depending on the flows going through them. Rain events may lead to sudden increases in solids loads at the inlet of the wastewater treatment plant and bring viruses that entered the sewer system much earlier. Even dry weather flow dynamics induce changes in solids transport dynamics and affect retention times in the sewer that may impact genetic marker concentration and load.

- Ensuring sample stabilization is important, usually accomplished by ensuring that sample temperature is kept as low as possible with refrigeration or ice packs. Archiving of samples for various reasons can be very useful for cross-checking results and validating new insights. Current expectations would be that storage of samples for more than a day requires freezing at -20°C, preferably at -80°C if possible. Repeated thawing and freezing cycles must be avoided (Gundy et al., 2009).

- Care will be necessary to ensure that samples cannot be contaminated with SARS-CoV-2 by processes or individuals involved in sampling or handling of the sample.

3. Confirm validity of wastewater analyses for SARS-CoV-2

Delivering on the program objective requires demonstrating that the wastewater sample analytical protocol also provides an accurate and reproducible representation of the evolution of the SARS-CoV-2 viral titer or temporal trend of viral titer in the sewage system being monitored.

Achieving this requires determining all substantial adverse effects on the analysis, such as from sample processing and storage and inhibition of signal, caused by wastewater sample matrices. We recommend the following sampling principles:

- Inter-laboratory (round robin) testing validation among as many competent laboratories as possible is a fundamental quality assurance approach that needs to be maximized.

- Using a single reference lab to coordinate round robin testing should be included to improve the comparison of lab results, improve lab performance and support the design of studies, including a national pilot.

- Both a high analytical specificity and sensitivity must be demonstrated and verified to satisfy the program objectives. Analytical methods attempting to measure viral fragments at trace levels in a complex matrix like wastewater can lead to high levels of false negatives and false positives can be generated by contamination during field or lab handling. For these reasons appropriate controls must be applied.

- Given the complexity of the matrix and the potential for unexpected amplification from PCR (especially with simplified protocols), random tests of a number of positive results with high-throughput sequencing should be done. This will reduce the reliance on appropriate local negative field blanks that may be limited by local resources.

- Quality Assurance/Quality Control (QA/QC) requires scrutiny of false negatives and false positives by using internal and external standards to verify recovery of SARS-CoV-2 and field blanks to ensure absence of external contamination, primarily from any exposure to high concentration standards and possibly from COVID-19-infected personnel. Ideally a series of blanks and spikes would be used for each sample to assess overall sample handling, extraction efficiency (including sample preparation and filtration) and preservation. Although several different standards have been used in the literature the best control spike for an enveloped virus remains to be determined.

- Ideally a spike for matrix-control to assess overall testing/sample handling efficiency including filtration. Spiking should be done for each new testing site. The best control spike for an enveloped virus remains to be determined. Given the complexity of the matrix and the potential for unexpected amplification in PCR (especially with simplified protocols), random tests of a number of positive results should be verified with high-throughput sequencing. Use of appropriate local negative field blanks may be limited by the capacity of wastewater utility staff to take on additional duties.

- There are a number of potential causes for false negatives with RT-qPCR including poor recovery of viruses due to inadequate technique, sample degradation, matrix interference with RNA recovery and inhibition of PCR reagents. Spiking of samples with something like salmon sperm DNA can be used to detect inhibition.

- Ahmed et al. (2020), Lodder & de Roda Husman (2020), Medema et al. (2020) and Wu et al. (2020) have all demonstrated the capability to detect SARS-CoV-2 in wastewater from communities with a negligible number of laboratory-confirmed cases of COVID-19. There is evidence that recovered COVID-19 patients also continue to excrete the virus when tested negative from sputum and nasal swabs (Ali et al., 2020; Cheung et al., 2020; Xu et al., 2020), and sewage tested positive by RT-PCR has been shown to be negative in disinfected wastewater by RT-PCR and culture (Wang et al., 2020).

- Quantification of uncertainty in SARS-CoV-2 concentration estimation and variability of detection limit will be needed for quantitative microbial risk assessment (QMRA) and to identify when a real trend change can be detected (relative to the estimate of infected individuals contributing to the sewage).

- Stochastic models, using published data from China, Europe and the United States on SARS-CoV-2 human excretion variability (i.e., concentration, excretion duration along with fraction of infected individuals that have the virus in their stools) can be developed to work backwards from public health data on prevalence to provide critical limits in sewage, as well as the reverse, from monitored virus titer estimates in sewage to population prevalence including the uncertainty of that estimate.

4. **Ensure potential for generalizability by fully understanding what samples represent**
Delivering on the program objective requires the ability to generalize the results obtained and trends identified to accurately and reproducibly represent the system being sampled.

Careful consideration must be given to the location of sample collection, as well as the implications of the sampling methodology, for the sample results to accurately represent the information that is being sought. The initial objective is to detect trends of SARS-CoV-2 in wastewater that may be related to trends in prevalence of COVI-19 infection in the community.

- A comparative examination of wastewater temporal trends between communities in relation to their respective temporal trends in the aggregated clinical sample results must take into account the inevitable differences in respective community sewer systems (e.g., proportion of combined sewers and wet weather flow dilution, inflow and infiltration, range of travel times from source to wastewater treatment plant, variabilities in excretion loads per infected individual and dispersal of SARS-CoV-2 within the sewage matrix, impact of water temperature affecting signal decay rates during conveyance in the sewer, population demographics and variable viral excretion rates). Given all these complications, expectations for inter-community comparisons must be realistic although trends within systems may be very informative.

- Collecting very good sample metadata (flow, TSS, pH, indicators of dilution, etc.) on field and sample conditions will be important. Furthermore, these metadata should include sewer-shed descriptive variables.

5. **Maximize value of results through strategic pilot project design**
The value of information that can be generated from monitoring SARS-CoV-2 in community or institutional wastewater will be best demonstrated by selection of pilot program locations that are conceptually able to demonstrate meaningful differences in SARS-CoV-2 occurrence that are useful for informing public health decision-making.

- Identify and select large or medium-sized communities or institutions with demonstrated large differences in levels of community infection with COVID-19 based on aggregated individual clinical monitoring results to maximize the potential range of wastewater outcomes and thereby maximize potential for finding meaningful correlation between infection rates, disease and wastewater viral load (with consideration of the sewer characteristics).

- Identify viable case study locations where outbreaks with known or strongly suspected outbreak etiology have occurred to determine potential for achieving early warning at other high outbreak risk locations, and to follow secondary waves more likely to be associated with community transmission.

- Sampling a community or institution as an outbreak begins (recognizing that it will be challenging to identify early enough) or as it resolves to determine the temporal trends and expected time lags as the outbreak is controlled would be informative.

6. **Maximize potential for productive collaboration with wastewater utilities**
The full potential for wastewater monitoring to serve public health decision-making will only be realized if those conducting monitoring programs understand the needs, constraints and concerns of those operating the partnering wastewater utilities.

    - Provision of samples should, to the maximum degree possible, fit within the normal sampling programs of the wastewater utilities and seek to minimize need for additional sample handing by utility personnel.

    - Wastewater utilities understandably need to be concerned with demands on their staff and ensuring their safety. For example, provision of aliquots from routine utility composite samples is less intrusive than requesting collection of additional grab samples or supervising outside personnel to collect such samples.

    - Understanding the nature of a sewer system must rely upon gathering relevant information about the layout and function (storage, pump stations, etc.) from the wastewater utility.

7. **Maximize collaboration, cooperation and knowledge exchange**
The global impact of the COVID-19 pandemic has resulted in an unprecedented international scientific effort to apply research to address the ensuing public health challenges. Maximum utility of research in this area for informing public health decision-making will be best achieved by maximizing learning from what others are doing in Canada and globally in all relevant disciplines.

    - Research efforts to explore the utility of wastewater monitoring for SARS-CoV-2 in community wastewater have already been launched in the Netherlands, France, England, Spain, Australia, Germany, Finland, the European Union and the United States with others being contemplated. Canadian researchers can certainly benefit significantly from the experience of these pioneering efforts.

- Benefits for Canadian public health decision-makers of Canadian efforts to implement wastewater monitoring for SARS-CoV-2 will be best achieved by ensuring best practices, in all the foregoing aspects. The necessary ability to compare results among different locations within provinces and across Canada is a vital component of the pilot study approach; it will depend upon achieving comparable methods for that monitoring.

8. **Consider ultimate use and ethics of data use in public health decision-making**
The ultimate objective of informing public health decision-making demands that decision-makers are actively engaged in study planning and implementation. Full consideration must be given, from the outset, of how wastewater monitoring data is likely to be disseminated and used. This will inevitably engage ethical considerations in data collection, availability and use. These ethical issues need to be balanced with public good, and will differ in different jurisdictions.

    - Difficult decisions being made during the pandemic are at the heart of relationships between individual rights and collective societal needs. When faced with difficult societal choices, for which there is no simple black and white answer, an accepted ethical framework will provide meaningful guidance for balancing competing rights and societal responsibilities.

    - Health-related data are among the most sensitive for protecting individual privacy resulting in high levels of protection demanded for such data. Evidence about levels of community infection by COVID-19 are unlikely to result in major ethical concerns when obtained for large, diverse population areas, but as the size and/or diversity of the community population captured by a wastewater sample becomes smaller, ethical concerns about the use and dissemination of the data may correspondingly increase.

    - Investigators need to respect the reality that COVID-19 does not pose a uniform risk across society, with recognized vulnerable sectors facing clearly elevated risk. Inevitably, as the population contributing to a wastewater sample is smaller, the impact of a vulnerable sector may be expected to be reflected in the SARS-CoV-2 signal that is measured in wastewater.

    - The current crisis circumstances call for more rigor and attention to scientific detail and careful consideration of potential ethical concerns, not less.

Definitions

Many of the following definitions are as used in epidemiology and medical science (i.e., Gordis, 2000) rather than as commonly used in environmental science (i.e., Hrudey & Leiss, 2003), with terminology modified as necessary for the current application.

SARS-CoV-2: The novel, severe, acute respiratory syndrome coronavirus that is the cause of the pandemic disease known as COVID-19.

True vs. false positives: A detection of the virus when the virus is truly present in a sample is a true positive (TP) versus a detection when the virus is not present in a sample which is a false positive (FP).

True vs. false negatives: A non-detection of the virus when the virus is truly absent in a sample is a true negative (TN) versus a non-detection of the virus when the virus is truly present in a sample which is a false negative (FN).

α: The FP rate, the fraction of total positives that are false.

β: The FN rate, the fraction of total negatives that are false.

Sensitivity (Se): The conditional probability that the analysis of a sample WILL detect the virus, GIVEN that the virus IS present in the sample. Se can be quantified as: $1 - \beta = TP / (TP + FN)$. This is a subtle difference from the common understanding among environmental, analytical sciences that sensitivity is viewed as the lowest concentration of the virus that can be detected.

Specificity (Sp): The conditional probability that the analysis of a sample will NOT detect the virus, GIVEN that the virus IS NOT present in the sample. Sp can be quantified as $1 - \alpha = TN / (FP + TN)$. This is a subtle difference from the general understanding among environmental, analytical sciences that specificity, rather than being quantitatively stated as a conditional probability, is simply viewed as confidence that the method can discriminate the target virus from other non-target signals (noise).

Accuracy: The closeness of agreement between a measured quantity of the virus and the true quantity of the virus (JCGM, 2008; Taylor, 1999).

Precision: The closeness of agreement between measured quantities of the virus obtained by replicated measurements on the same sample (JCGM, 2008; Taylor, 1999).

Both accuracy and precision are important for effectively guiding decisions, but precision without accuracy, particularly in terms of accuracy of the direction of trends, can be misleading.

Etiology: The reason or explanation of the cause for an observed phenomenon, often used in the context of explaining the cause of a disease.

Method recovery: The fraction of virus detected vs. the true total content in a sample.

RT-qPCR: Reverse transcription quantitative polymerase chain reaction; a molecular biology technique for detecting and quantifying a specific genetic target from an RNA starting material.

RNA: Ribonucleic acid, single strand genetic code.

E-RNA: RNA in aqueous media.

DNA: Deoxyribonucleic acid, double helix genetic code.

C-DNA: Complimentary DNA.

E-DNA: DNA in aqueous media.

Spiking: The addition of a known concentration of surrogate for the target (virus) of interest for the estimate of method recovery (Li et al., 2019).

Variability: The inherent variation in the targeted parameter from the sample population. Further sampling will not reduce the variability, just better define it.

Uncertainty: This is quantified by a probability distribution which depends upon our state of information about the likelihood of the true parameter value, which is reduced by increased sample information.

Sewer-shed: A recently adapted term to describe the sewer network for a given community or institution.

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