COVID-19 monitoring in rural communities: First comparison of lagoon and pumping station samples for wastewater-based epidemiology

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Abstract

Wastewater-based epidemiology is a topic of significant interest over the last year due to the application of SARS-CoV-2 surveillance to track incidence rates of COVID-19 in communities. Although SARS-CoV-2 surveillance has been applied in more than 50 countries to date, the application of this surveillance has been largely focused on relatively affluent urban and peri-urban communities. As such, there is a lack of knowledge regarding the implementation of reliable wastewater surveillance in small and rural communities for the purpose of tracking rates of incidence of COVID-19 and other pathogens or biomarkers. This study examines the correlations between SARS-CoV-2 RNA signal from wastewater samples harvested from the access port at a lagoon (waste-stabilization pond), a wastewater pumping station and the regional COVID-19 rate of incidence (measured as percent test positivity) in a small, rural community in Ontario, Canada. Real-time quantitative polymerase chain reaction (RT-qPCR) targeting the N1 and N2 genes of SARS-CoV-2 of lagoon samples demonstrate that 80% of 24-hr composite samples collected across a period of 5.5 weeks were below the limit of quantification (5 gene copies/µL). However, 100% of the 24-hr composite samples collected on the same days from the upstream pumping station were capable of not only yielding strong viral signal but once normalized for PMMoV, also predicted the increase in viral signal approximately 10-14 days prior to an increase in community’s COVID-19 reported test percent positivity. RNA concentration and integrity of samples harvested from the lagoon was both lower and more variable than from RNA harvested from the upstream pumping station that were collected on the same date, indicating a higher overall stability of SARS-CoV-2 RNA and hence a stronger viral signal that correlates to community incidence of COVID-19. In sewered small and rural communities operating wastewater lagoons, WBE samples should therefore be harvested from pumping stations or the sewershed as opposed to lagoons.

Keywords: wastewater treatment lagoon; SARS-CoV-2; wastewater-based epidemiology; COVID-19;
1. Introduction

In late 2019 mysterious cases of pneumonia were first reported in Wuhan, PRC\textsuperscript{1}. Cases of the disease, which was named coronavirus disease 2019 (COVID-19), began spreading rapidly. It became clear to public health officials across the world that this new disease, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-COV-2)\textsuperscript{2}, was rapidly becoming a pandemic-potential pathogen due to its relatively low virulence but high degree of infectiousness\textsuperscript{3}. More than a year after the first cases of COVID-19, the world is still grappling with the disease and newer and more infectious, mutant variants of the disease are rapidly spreading\textsuperscript{4-7}, causing widespread disease and death (WHO COVID-19 Dashboard \url{https://covid19.who.int/}). At the time of writing (April 22\textsuperscript{nd}, 2021), more than 1.8% of the global population (143.1 M) has been infected by SARS-CoV-2, and 2.1% of those infected have passed away (3.0 M) \url{WHO COVID-19 Dashboard}.

Now widely accepted and hailed as an essential tool to help communities monitor true COVID-19 rates of incidence in the general population\textsuperscript{8,9,18,10-17}, RT-qPCR-based wastewater-based epidemiology (WBE) efforts are underway around the world, focused primarily in larger metropolitan areas of higher income countries\textsuperscript{8}. By and large smaller communities have not had the same services afforded to them as larger communities, which is based on numerous factors that include but are not limited to: i) discrepancies in financial and material resources, ii) distance to research, academic and governmental facilities capable of carrying out the analyses and iii) capacity of the local public health unit to take in the results and act upon them. Furthermore, smaller and rural communities without larger mechanical water resource recovery facilities may not have the staff, equipment or expertise in carrying out sampling for SARS-CoV-2 viral detection in wastewater\textsuperscript{19,20}. Larger facilities are often equipped with automatic composite samplers throughout the plant, making the implementation of a WBE monitoring program in comparison relatively easy for routine purposes. Furthermore, there is a growing consensus that higher concentrations of SARS-CoV-2 viral particles are found in wastewater solids\textsuperscript{21-24} and as such several WBE efforts are now focusing on measuring signal from solid fractions of samples\textsuperscript{22}. In small and rural communities, harvesting these solids may be slightly problematic as dedicated solid separation units present in larger facilities located in urban and peri-urban communities may not exist in smaller facilities, requiring different sampling approaches.

Most WBE efforts attempt to provide some prediction of trends of epidemiological metrics of COVID-19 in the general population by predicting increases and decreases\textsuperscript{11,13,25-27} in the rates of clinical cases of COVID-19. These studies will often focus on collecting samples within the raw influent or primary sludge due to the relatively high concentration of solids in these wastewater streams\textsuperscript{27}. However several municipalities operating smaller types of treatment systems such as waste stabilization ponds, also known as wastewater treatment lagoons, do not have direct
access to raw influent or primary sludge. Smaller communities may however have direct access to the waste stabilization ponds and pumping/lift stations. Due to the nature of lagoons, solids separation occurs due to the slowing of flow velocities, leading to particle settling, particularly in the same area of the lagoon that oxidizes carbonaceous deleterious substances or in lagoon areas or isolated lagoon units designed specifically for solids sedimentation. As such, harvesting of wastewater solids in lagoon treatment systems with the goal of performing WBE is very difficult due to potentially substantial retention times in lagoons, the degradation of RNA targets and the difficulty of selectively collecting “fresh” solids in a lagoon which represent current incidence of COVID-19 in the community. Furthermore, as lagoon systems are located outdoors and are uncovered/unheated, in locales where air temperatures can dip below freezing (0°C / 32°F) these systems may become highly difficult to sample due to the presence of ice-cover. As a result, smaller communities may not have evident sampling locations to collect wastewater samples containing SARS-CoV-2 viral particles which can accurately represent incidence of the disease in the community. Another factor in the ease of implementation of WBE program in small and rural communities is whether the community is sewered or not. In communities that are not sewered and where centralized sampling points are not available, sewage brought to lagoons can be sampled from sewage and sludge trucks while they are being emptied at the facility.

In preparation for the application of a WBE/SARS-CoV-2 surveillance initiative in a small sewered community in Eastern Ontario (Canada), wastewater samples were collected from the lagoon access/sampling point, situated between the first and second cells, and from the last pumping station on the sewer network. The specific objectives of this study were to: i) juxtapose SARS-CoV-2 signal at both sampling locations and compare them for strength of the RNA viral signal and RNA integrity and ii) compare existing community epidemiological data with longitudinal data of SARS-CoV-2 viral signal to ascertain the ability of WBE efforts to track and predict trends in rates of incidence of COVID-19 in small and rural communities.
2. Experimental methods

Experimental site description

The sites investigated in this study are located in a small community of less than 5,000 inhabitants in Eastern Ontario (Canada). Two sites were sampled regularly during this study: i) the last pumping station in the sewer network before the wastewater treatment lagoon, and ii) the lagoon access/sampling point, situated between cells 1 and 2. The lagoon sampling location and the pumping station are located approximately 1.3 km from each other. The sampling points are shown below in Figure 1.

![Figure 1: Location of the sampling locations, with the pumping station and lagoon location highlighted.](image)

Sample collection

24-hr composite samples of wastewater were collected from October 16th, 2020 and March 14th, 2021 using two Isco 6700 series automatic samplers (Teledyne Isco, Lincoln, NE, USA). The composite samples were comprised of 50 mL aliquots collected hourly by the Isco autosamplers. One sampler was installed at the pumping/lift station just upstream of the lagoon treatment system, and the second sampler was installed at an access manhole allowing sampling of the lagoon, located between the first cell and second cells of the lagoon. Five samples were collected every 3 to and 7 days from the pumping station and the waste stabilization pond for comparison between December 3rd, 2020 and January 11th, 2020. The 24-hr composite samples were collected within 24 hours of the completion of the automatic collection and were transported on ice to the laboratory. Once in the laboratory, samples were rapidly concentrated and the resulting pellets were frozen and processed within 14 days. After January 11th, site access became difficult due
to low outdoor temperatures, freezing of the lagoon, and snowed in conditions, making access to the automatic sampler highly difficult, which lead to the cessation of sampling at the lagoon.

**Sample concentration, extraction, and PCR quantification**

The composite samples were concentrated by allowing the samples to settle at 4°C for an hour, followed by the decantation of the supernatant to isolate the settled solids fraction. 40 mL of remaining solid fraction was then transferred to a 40 mL centrifuge tube and samples were then centrifuged for 45 mins at 10,000 x g at 4°C to isolate the centrifuged pellet. Sample pellets which could not be immediately processed were frozen at -30°C for a period of up to 14 days before being extracted. RNA was extracted and purified from the resulting pellet using the RNeasy PowerMicrobiome kit (Qiagen, Germantown, MD, USA) using a QIAcube Connect automated extraction platform, with the protocol modifications specified in an earlier study\(^1\). The SARS-CoV-2 signal in the samples was assayed using a singleplex one-step RT-qPCR targeting the N1 and N2 gene regions of SARS-CoV-2 genome. The signal of an internal biomarker, pepper mild mottle virus (PMMoV) was also measured in each of the samples (samples were diluted 1/10 for measurements of the biomarker). In each PCR reaction, the reaction mix consisted of 1.5 µl of RNA template, 500 nM of each of forward and reverse primer (IDT, Kanata, Canada) in 4x TaqMan\textsuperscript{®} Fast Virus 1-step Mastermix (Thermo-Fisher, USA) with 125 nM probe (IDT, Kanata, Canada) in final volume of 10 µl. The samples were run in triplicates with non-template control and five-point gradient of SARS-CoV-2 RNA standards (Exact Diagnostics, USA). Reverse transcription (RT) was performed at 50°C for 5 minutes followed by RT inactivation and initial denaturation at 95°C for 20 seconds. This was followed by 45 cycles of denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 30 seconds with a CFX Connect qPCR thermocycler (Bio-Rad, USA). The limit of detection of the method was previously assessed\(^2\) and determined to be approximately 2 copies/reaction.

**Assessment of RNA Integrity**

Samples were assessed for RNA integrity using an Agilent 2100 Bioanalyzer. RNA (2 µL) of each sample was loaded on an RNA 6000 Pico Chip (#5067-1513). Data analysis was performed using Agilent’s proprietary 2100 Expert software (version B.02.10.SI764).

**Collection of epidemiological data**

Epidemiological data was obtained from the Eastern Ontario region of Canada via the ICES COVID-19 dashboard (https://www.ices.on.ca/DAS/AHRQ/COVID-19-Dashboard) and the Eastern Ontario Health Unit (EOHU) dashboard (https://eohu.ca/en/covid/covid-19-status-update-for-eohu-region).
Correlation of epidemiological data with wastewater RNA signal

Prior to performing a correlation analysis between wastewater RNA signal and available epidemiological data, the viral RNA signal was normalized for an internal normalization biomarker, pepper mild mottle virus (PMMoV). SARS-CoV-2 N1 and N2 gene region viral signals were normalized by dividing the N1 and N2 gene copies per reaction by the PMMoV gene copies per reaction, as a means of normalizing the N1 and N2 signal by the quantity of fecal matter in the sample\textsuperscript{13,21,24,32}. 
3. Results & discussion

Comparison of sampling sites: pumping station vs. lagoon

During the side-by-side comparison of both sampling locations, all samples (5/5) collected from the pumping station showed strong signal for N1 and N2 gene regions of SARS-CoV-2 (Figure 2), as well as for the PMMoV internal biomarker. Ct values for the N1 and N2 gene regions in the samples collected from the pumping station varied from 33.0 ± 0.3 to 38.2 ± 0.3 and 34.8 ± 0.3 to 37.8 ± 0.2 Ct for the N1 and N2 gene regions. Meanwhile, Ct values of composite samples collected at the lagoon sampling manhole between cells #1 and #2 of the lagoon were unable to be detected or quantified in four of the five samples for either SARS-CoV-2 gene regions. N1 and N2 viral signal was tentatively detected in only one of the lagoon samples (Jan. 11th, 2021) with Ct values of 40.1 ± 0.5 and 40.0 ± 0.5, for N1 and N2 respectively. During the same short side-by-side test period, the internal biomarker (PMMoV) was only observed in three of the five lagoon samples. The Ct values of PMMoV in the lagoon samples were of 34.5 ± 0.2, 39.4 ± 0.4 and 29.3 ± 0.1, for Dec. 3rd, Dec. 6th, and Jan. 11th, respectively. The lack of PMMoV signal in some of the lagoon samples could potentially signify that no fecal material was collected from the samples, or that significant RNA degradation of the samples occurred in the lagoon. When comparing Ct values from the RT-qPCR analyses for the N1 and N2 SARS-CoV-2 gene regions and the PMMoV fecal biomarker, pairwise comparisons clearly outline a stronger presence of RNA from the SARS-CoV-2 gene region in the samples collected from the pumping station (Figure 2).
Wastewater samples collected from the pumping station yielded stronger viral signals than samples collected directly from the lagoon. As samples from the pumping station are located upstream of the lagoon, it is likely that the wastewater is relatively undegraded, leading to better RT-qPCR signal. Samples collected in lagoons may have experienced more degradation due to mixing/holding than those collected in the final pumping station directly upstream of the treatment facility, and may also be subject to UV degradation once in the lagoon itself\(^{33}\). Since SARS-CoV-2 viral particles appear to partition preferentially to wastewater solids in typical wastewater conditions, a significant portion of the viral signal may be lost when sewage enters the inlet structure of lagoons as flow velocities immediately decrease, causing rapid settlement of a large portion of solids. Furthermore, as this facility doses alum post pumping-station (but pre-lagoon), chemical additions may contribute to a more rapid settling of solids, and may have other, unforeseen, or currently unknown effects of viral particle partitioning or RNA degradation effects\(^{33}\). Finally, the relatively long retention times (and largely unknown time spent by samples) within the lagoon cell prior to the lagoon access/sampling manhole may explain the low perceived signal. Analysis of the sample RNA using an Agilent 2100 Bioanalyzer was performed and electropherograms for samples collected from the lagoon and pumping station are shown below in Figure 3. Total RNA concentrations are distinctly lower in lagoon samples as compared to pumping station samples. This is likely due to the presence of less biological material and greater degradation in the material collected in the samples collected in the lagoon.

![Electropherograms](image)

**Figure 3:** Electropherograms for samples from the lagoon and the pumping chamber, showing drastic differences in the RNA profiles. Total RNA concentrations were calculated using Agilent's 2100 Expert software (version B.02.10.SI764).

When comparing PMMoV-normalized viral signal to available epidemiological data from the Eastern Ontario Health Unit (EOHU) (Figure 4), preliminary analysis indicates that in this instance SARS-CoV-2 viral signal in wastewater preceded percent positivity of clinical cases in the community by 10-14 days. Additionally, when compared to the average new daily cases/100K pop., major increases in viral signal in wastewater appear to precede increases...
in new cases of COVID-19 in the community by approximately 10-14 days, which showcases the usefulness and ability of WBE to track and even predict COVID-19 rates of incidence, even at a relatively low sample collection frequency. A limitation on the interpretation of this data is that epidemiological data was not available for the community itself, but rather is for the whole geographical region covered by the EOHU. As a point of reference, population-wise, the studied small community (~4,000 people) represents approximately 2% of the whole population administered by the EOHU (~200,000 people). This limitation in the available epidemiological data may slightly change localized patterns in data however if the assumption that this town and its citizens do not behave in dramatically different ways than the rest of the residents under the purview of the EOHU, trends and conclusions on the predictive abilities of WBE efforts are not expected to change significantly. As outlined with these results, samples harvested from the pumping station of the community provide strong pathogen signal than those collected at the lagoon in the context of a WBE program, making the pumping station an objectively better sampling location.

![Graph](image)

Figure 4: Overlay of the 3 data-point moving average for PMMoV normalized SARS-CoV-2 viral signal, along with the overall weekly COVID-19 test percent positivity in Eastern Ontario, and the average new daily cases per 100,000 pop.
4. Conclusions and recommendations

As many locations throughout the world have now recently experienced new widespread resurgences in COVID-19 cases due to the appearance and rapid spread of more infectious and deadlier variants of concern, it has become even more important for communities to rapidly implement effective COVID-19 wastewater monitoring programs which can rapidly detect and predict upcoming resurgences in COVID-19 cases. Such programs may afford communities the time to enact public health measures to limit the spread of COVID-19, potentially preventing their local health units from being overrun, possibly saving lives. The conclusions of this study are as follow:

- In municipalities with wastewater lagoons, surveillance of pathogens such as SARS-CoV-2 in the general population is possible. However, where possible, sampling of wastewater should be done upstream of the lagoon, in a pumping station or wet well.
- In this study, samples collected from the lagoon facility demonstrate significant weaker viral signal in the lagoon samples, likely due to preferential partitioning of SARS-CoV-2 viral particles to solids in normal wastewater conditions, and degradation of viral particles.
- PMMoV-normalized SARS-CoV-2 viral signal can predict important upcoming epidemiological events, such as increases in new cases of COVID-19 in the community, and indirectly increase in test positivity, even in low-frequency WBE applications.
Declaration of competing interests

The authors declare that no known competing financial interests or personal relationships could appear to influence the work reported in this manuscript.

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