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Performance and suitability of wastewater based-surveillance for SARS-CoV-2 RNA in public schools

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Wastewater-based surveillance (WBS) for SARS-CoV-2 was a key strategy for epidemiological modelling and informing COVID-19 health policy during the pandemic. We assessed the capacity and performance of SARS-CoV-2 WBS in public schools. Of seventeen schools screened for participation, only four had plumbing systems that were amenable to comprehensive monitoring. From December 2020 to March 2021 composite wastewater collected twice-weekly from these four schools was compared with three municipal wastewater treatment plants (WWTPs) for SARS-CoV-2 RNA by RTqPCR and fecal biomarkers. Schools had lower rates of successful sample collection relative to WWTPs (64/79 vs. 66/66, $p < 0.001$). In a time of low COVID-19 activity, 13/64 of school samples were positive for SARS-CoV-2, versus 66/66 for WWTP ($p < 0.0001$). SARS-CoV-2 RNA in school wastewater was associated with, and often preceded, clinically confirmed COVID-19 cases among students, but showed no correlation with overall rates of student absenteeism. Levels of both SARS-CoV-2 RNA and fecal biomarkers were markedly lower in school wastewater relative to WWTPs. This work demonstrated that WBS for SARS-CoV-2 in schools can be a leading indicator of clinical disease but is technically challenging. The lower fecal biomarker levels from schools suggests children may avoid defecation at school which may further adversely impact school-based WBS for fecal-shed targets.

Keywords Wastewater, Wastewater-based surveillance, SARS-CoV-2, School, Near-to-source

Abbreviations

BCoV	Bovine coronavirus
PMMoV	Pepper mild mottle virus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
WWTPs	Wastewater-treatment plants
WBS	Wastewater-based surveillance
TS	Total solids
TSS	Total suspended solids
TDS	Total dissolved solids
TVSS	Total volatile suspended solids

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A silver lining of the COVID-19 pandemic has been the rapid evolution of healthcare innovation to meet the needs of a global pandemic. Wastewater-based surveillance (WBS) has proven especially useful in tracking and understanding the spread of disease[1]. Wastewater measured SARS-CoV-2 RNA strongly correlates with clinical case burden across a range of scales, acting as an early warning system to inform health policy[2,3]. Indeed, WBS serves as leading indicator (4–6 days) of clinically confirmed COVID-19 disease[4] and strongly correlates with hospitalizations, ICU admissions and deaths across whole communities. This approach is viable because SARS-CoV-2 RNA is shed in the stool of most symptomatic and asymptomatic infected individuals very early in the course of infection[5] allowing it to be measured using targeted RNA quantification methods applied to wastewater samples.

WBS programs for SARS-CoV-2 have been increasingly adopted in many regions, where it can complement traditional clinical surveillance methods[6–8]. Samples collected at local wastewater treatment plants (WWTPs) are analyzed to understand community disease patterns[9]. More granular monitoring programs, focused on individual neighborhoods have similarly demonstrated strong correlations with clinically diagnosed cases, with the added potential of understanding community factors associated with COVID-19 [10–13]. More granular yet, 'near-to-source' WBS focuses on specific buildings and facilities, further pushing the limits of this technology[14,15]. Initial explorations of WBS using near-to-source sampling during the COVID-19 pandemic included hospitals[16,17] and long-term care facilities[18,19] where the consequences of outbreaks are profound—both for the higher-risk residents and due to the potential disruption to the care these critical services provide to the broader community.

Another near-to-source strategy that has been explored only to a limited degree is in schools. COVID-19 transmission in pediatric and adolescent populations represents a contentious topic in epidemiological research[20–23]. Early works concluded based on clinical testing that children were responsible for less than 5% of total COVID-19 cases in general populations[24]. However, the tendency for children and adolescents to exhibit asymptomatic or pauci-symptomatic disease meant that cases were likely to be initially under reported[20,25,26]. Later studies have since demonstrated that children are a main source of viral transmission within family clusters[27]. Schools increase transmission potential between unrelated families due to the concentration of students from different households.

In this study, we initiated a 'near-to-source' pilot program to explore the viability of SARS-CoV-2 wastewater testing for detecting COVID-19 in public schools and assessed this relative to overall community disease activity as measured at municipal WWTP. To achieve this aim, we developed a framework for screening potential schools to identify suitable locations. Schools that met the required criteria enrolled in a four-month observational study whereby SARS-CoV-2 RNA was quantified in wastewater. These results were compared with clinically identified cases within the school, and with community-wide results from municipal WWTPs. Given that SARS-CoV-2 is just the tip of the iceberg for potential WBS measured analytes of public health importance (i.e., other infectious diseases; drugs or other toxins) understanding its performance in schools is essential.

Results

School selection and wastewater collection performance

Of the 17 schools that met minimal inclusion criteria, only four schools lacked any exclusion criteria for a site based near-to-source WBS pilot program (Supplementary Fig. 1). Key inclusion criteria were an amenable school administration and student population of >500. To not be excluded, a school had to have a suitable plumbing system that would enable collection of wastewater that represented the entirety of the school safely, and without disruption of student or staff duties. Detailed characteristics of the four selected schools are included in Supplementary Table 1. Between January 2021 and March of 2021, the four schools were sequentially onboarded with the goal of achieving three months of continuous sampling from each school. School #3 was an exception due to challenges in developing a comprehensive surveillance strategy which manifested in significant delays in its initiation. School #2 required two separate sampling locations (i.e., 2 A and 2B) for comprehensive sampling due to the layout of its sewer network. The timing of the study coincided with the end of the second wave of COVID-19 in Alberta, which was still characterized by the original wild type of SARS-CoV-2 variant[28]. As schools were sequentially brought online during the study period, overall numbers of samples available from each site differed. Of the total number of sampling attempts (i.e. during regular school days), 76.5% (64/79) were collected successfully. Unsuccessful collection of a composite wastewater sample was attributed to a range of issues including ragging (3/15 failures), low sanitary flow (9/15 failures), and ambient temperature excursions (3/15 failures). Sampling failures did not occur disproportionally at any specific site ($p = 0.140$, Fisher's exact test) (Supplementary Table 1). During the study period samples were collected in parallel twice per week from the three municipal WWTPs, where the rate of successful sampling was much higher (66/66) with no ragging, low sanitary flow or ambient temperature excursions ($p < 0.001$).

SARS-CoV-2 RNA detection signal in school wastewater and its relation to overall community activity

Only 13 of the 64 wastewater samples collected from schools were positive for SARS-CoV-2 RNA based on a N1 gene detection assay (20.3%; Table 1). Only six of these 13 samples were identified as positive for the N2 gene target, with all 6 having higher amounts of RNA from N1 genes as indicated by lower cycle threshold (Cq) values (37.8 vs. 40, $p = 0.0003$). Median Cq values for positive samples was 37.8 (IQR 36.6–39) for the N1 gene and 40.6 (IQR 40.1–42) for the N2 gene. Conversely, lowest Cq value observed were 34.7 and 39.1 for N1 and N2 respectively. Overall, Cq values for N1 and N2 gene RT-qPCR assays on the same wastewater samples were strongly correlated (Spearman's $r = 0.632$; $p < 0.0001$).

To understand SARS-CoV-2 burden in schools relative to the larger community from which they derived, we compared school wastewater data to that across the entire city of Calgary through samples collected at WWTPs.

Site name	Number of students with confirmed COVID-19 diagnosis	Number of WW samples positive for SARS-CoV-2 RNA	Date of positive SARS-CoV-2 RNA reading (DD-MM-YYYY)	Mean N1 detected copies/mL	Mean N2 detected copies/mL
School #1	3	3	01/02/2021	4.84	0
			10/02/2021	9.24	3.46
			01/03/2021	0.72	0
School #2	11	7	13/01/2021	1.09	1.08
			18/01/2021	0.401	0.945
			20/01/2021	0.497	0
			22/02/2021	7.14	9.29
			24/02/2021	5.38	1.07
			22/03/2021	8.94	1.91
			24/03/2021	25.9	0
School #3	3	1	24/02/2021	3.58	0
School #4	1	2	08/03/2021	0.478	0
			10/03/2021	0.601	0

Table 1. SARS-CoV-2 positive wastewater samples identified from schools [a]. [a]Calgary, Alberta, Canada wastewater samples collected from four different schools over study period. Each site was monitored for a different timespan within the study dates. Wastewater samples identified as positive for SARS-CoV-2 RNA using N1 and N2 loci RT-qPCR assays are shown (see methods for definition). School #2B not included due to lack of any positive wastewater samples.

During the study period, 66 twice-weekly samples were collected from Calgary's three WWTP (23/per). All 66 were positive for the N1 gene from SARS-CoV-2, and all but one were positive for the N2 gene. Compared to schools, municipal wastewater was much more likely to be positive for SARS-CoV-2 N1 (66/66 (100%) vs. 13/64 (20%), $p < 0.0001$). In addition to the much lower overall wastewater positivity rate, school SARS-CoV-2 levels were significantly lower than the levels in WWTPs regardless of the target gene being evaluated: N1; median Schools 0 (IQR: 0–0) genes/mL vs. median WWTP 126 (IQR: 67.8–202) genes/mL and N2; median Schools 0 (IQR: 0–0) genes/mL vs. median WWTP 66.8 (IQR: 29.2–131) genes/mL for (Fig. 1A). Similar trends were observed when SARS-CoV-2 levels were normalized using the PMMoV signal in the samples to potentially control for fecal burden: N1; median Schools 0 (IQR: 0-8.5×10^{-5}) copies/copies vs. median WWTP 1.2×10^{-2} (IQR: 6.3×10^{-3} – 2.4×10^{-2}) copies/copies, $p < 0.0001$ and N2; median Schools 0 (IQR: 0–0) copies/copies vs. median WWTP 4.8×10^{-3} (IQR: 2.1×10^{-3} – 2×10^{-2}) copies/copies, $p < 0.0001$ (Fig. 1B). Even excluding school samples that were negative for SARS-CoV-2 RNA (either the N1 or N2 target), both raw- and normalized school values were markedly lower than WWTPs (data not shown).

Clinical case information

Demographics of the included schools are presented in Table 2. Clinical case information on COVID-19-confirmed, -related and -unrelated infections as well as overall absenteeism data were collected from January 2021. The size of the student and staff population of each school varied throughout the monitoring period (Table 2) as exposures, cases (prompting exclusions) and other illnesses affected them (Supplementary Fig. 2). Between January and March, 2021 only eighteen clinically confirmed COVID-19 cases were documented amongst students and staff at the four participating schools. Weekly absenteeism rates due to confirmed COVID-19-confirmed, -related or -unrelated illnesses ranged from 0 to 0.44%, 0.04 to 27.3% and 0.19 to 16.7%, respectively (Supplementary Table 2). Absenteeism due to COVID-19 confirmed disease did not significantly change through the three-month study period ($p = 0.290$, Kruskal-Wallis test). Confirmed cases represented a very small proportion of student absences [median percent of COVID-19 confirmed 0.09 (0-0.183) vs. any other non-COVID-19 reason 9.32(7.94–12.3), $p < 0.0001$], Wilcoxon matched-pairs signed rank test) on account of rigorous exclusion policies in place in the schools to reduce secondary spread[29–31].

SARS-CoV-2 RNA in school wastewater and its relation to clinical case burden

Comparative analysis of SARS-CoV-2 genomic signals in wastewater and clinically confirmed cases in schools started in January 2021 using complementary approaches. We looked to determine if there was a correlation between positive SARS-CoV-2 signals (N1 gene Cq < 40) measured in a specific school and the occurrence of clinically confirmed cases in the same school over the ensuing days. Using Fisher's exact test, we established a statistical association between SARS-CoV-2 signal in school wastewater and clinical cases that were confirmed in the subsequent week (Table 3). Whereas this was statistically significant when wastewater samples were collected at intervals longer than 1–3 days preceding clinical cases, there was only a trend at shorter intervals preceding clinical case occurrence.

Among the 13 positive wastewater samples, 69.2% (9/13) were followed by another positive SARS-CoV-2 N1 sample (Fig. 2) on the next closest sampling date ($P < 0.0001$, Fisher's exact test). In the other four positive samples, the sampling dates immediately following this date were all among the 15 unsuccessful sample collection events. Given the exclusion of students once COVID-19 cases had been confirmed, positive samples

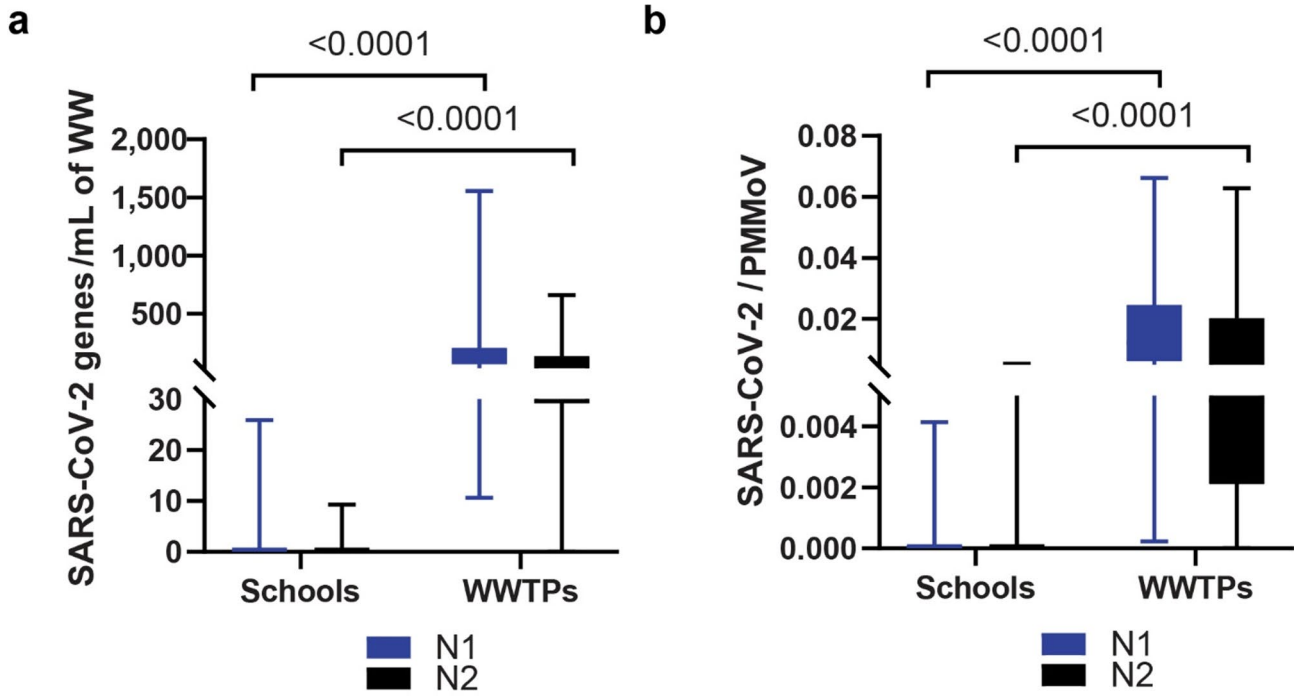

Fig. 1. SARS-CoV-2 genomic RNA in wastewater from schools is lower than city-wide wastewater. Comparison of the SARS-CoV-2 genomic RNA quantification in wastewater from schools and WWTPs between January to March 2021. RNA quantification targeted the nucleocapsid gene loci N1 (blue) and N2 (black), with results shown as direct gene abundance values (**a**) and normalized abundance relative to levels of the fecal biomarker PMMoV (**b**). Statistical differences were determined using the Mann Whitney test.

School	#1	#2	#3	#4
Category (grades)	Elementary and middle school (K-9)	High school (10–12)	High school (10–12)	High school (10–12)
Daily student population (range)	552–779	1158–1335	1285–1364	1354–1558
Daily staff population (range)	69–78	90–103	104–110	100–108
Average attendance rate (%)	87.0	92.0	86.5	92.3
Wastewater treatment plant catchment	WWTP 1	WWTP 1	WWTP 1	WWTP 2 and 3

Table 2. Demographics and characteristics of schools monitored in the City of Calgary. WWTP: wastewater treatment plant.

	Confirmed cases and a positive WBS-signal	Confirmed cases and a negative WBS-signal	Risk ratio	*P*-value
1–2 days	6/13 (46%)	15/51 (29%)	1.57 (0.76–3.24)	0.205
1–3 days	9/13 (69%)	15/51 (29%)	2.35 (1.34–4.12)	**0.011**
1–4 days	9/13 (69%)	16/51 (31%)	2.21 (1.28–3.80)	**0.015**
1–5 days	10/13 (77%)	18/51 (36%)	2.18 (1.35–3.51)	**0.008**

Table 3. SARS-CoV-2 RNA detected in school wastewater and the occurrence of confirmed COVID-19 cases in the following week.

not being followed by negative samples may represent unrecognized and/or asymptomatic secondary spread in the school identified through WBS. To assess whether overall absenteeism (measured at >5% and >10% for both students and teachers) was associated with the likelihood of detecting a positive SARS-CoV-2 N1 signal (Cq<40) in a given school, we performed Fisher's exact test. When absenteeism exceeded 5%, none of the schools demonstrated a correlation between absenteeism and SARS-CoV-2 detection (School #1, School #2A, School #2B, School #3, and School #4: $p>0.999$, $p=0.088$, $p>0.999$, $p>0.999$, and $p=0.625$, respectively). Similarly, when absenteeism exceeded 10%, no statistical association was found ($p=0.429$, $p=0.516$, $p>0.999$, $p>0.999$, and $p=0.375$, respectively).

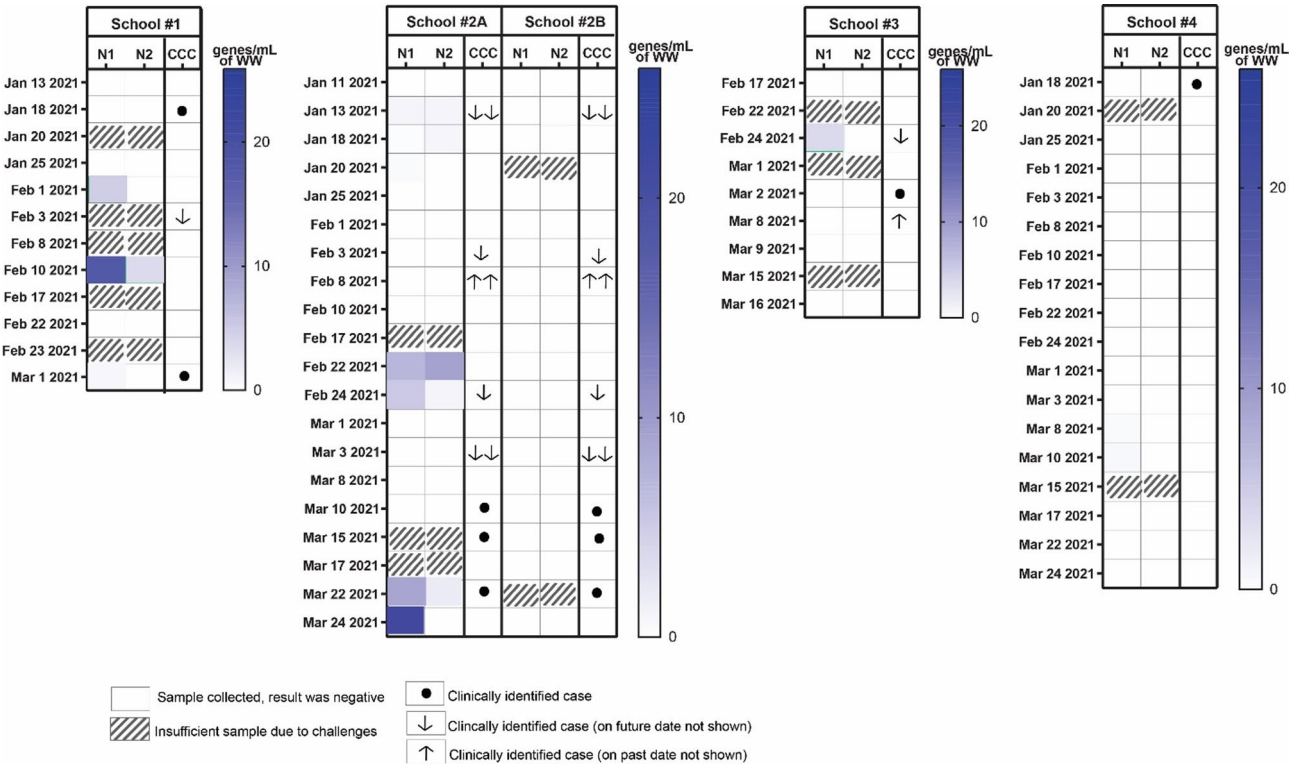

Fig. 2. Summary of RNA detection from SARS-CoV-2 in wastewater from four schools. Quantification of N1 and N2 genes plotted together with clinically confirmed cases (CCC) reported to school administrators during the same timeframe. Arrows in the CCC columns refer to clinically identified cases on dates that were different from wastewater sample collection, with the number of arrows indicating days before (upward) or after (downward) the date indicated (e.g., 2 downward arrows on January 13th indicate a clinical case on January 15th).

Finally, we sought to better understand school attendance at each site in the context of overall community viral load as determined from a city-wide N1 SARS-CoV-2 signal across each of Calgary's three wastewater treatment plants (Fig. 3A). Using Spearman correlation test we did not observe a correlation between overall attendance (within 2 days, owing to the frequency of sample collection) for all schools and the SARS-CoV-2 N1 city-wide aggregate wastewater value for the City of Calgary (Fig. 3B) (Spearman's $r = 0.136$, CI: – 0.419 – 0.616, $p = 0.629$).

Unique features of school-based WBS

To assess for differences in fecal contributions at each school, and therefore the potential to detect fecal-shed WBS targets (i.e. SARS-CoV-2) relative to the community at large we used complementary approaches. The human fecal biomarker, PMMoV, was much lower in school samples than in WWTPs (school median 2,178 [IQR: 28.1–8213] copies/mL vs. WWTP median 13,029 [IQR: 5489–19,556], $p < 0.0001$, Mann Whitney test) (Supplementary Fig. 3A-B). As school site 2B remained a significant outlier (Supplementary Table 3), we analyzed this separately as well with the same trends observed (school median 4054 [IQR: 1853–11,092] copies/mL vs. WWTP median 13,029 [IQR: 5489–19,556], $p = 0.0003$, Mann Whitney test) (Supplementary Fig. 3C). When 2B site was excluded, the median school PMMoV burden remained 3.2-fold lower than the average WWTP, suggesting a lower fecal content. Similarly, visual assessment of wastewater samples from all schools consistently showed that site 2B had little evidence of solid matter in wastewater systems (Supplementary Fig. 4). This is consistent with the difference in detection of solids at site 2B compared to the other schools (Supplementary Fig. 5 and Supplementary Table 4). In addition, chemical analysis demonstrated that total suspended solids (TSS) and total volatile suspended solids (TVSS) in wastewater from schools were also significantly lower than WWTP (Supplementary Fig. 6).

Finally, sources more proximal in the sewershed have greater potential for molecular inhibition from chemical and other molecular factors. To assess if differences might explain our data (i.e., single versus dual sites for sampling locations), we compared recovery of spiked-BCoV into wastewater from the schools and WWTPs samples. There was a small but statistically significant difference in the spiked external positive control BCoV median copy numbers between schools and WWTPs (694,914 [IQR: 55,703–1,675,777] copies/mL vs. 1,217,451 [IQR: 774,211–1,934,018], $p = 0.005$, Mann Whitney test) (Supplementary Fig. 7A). However, school 2B was a significant outlier and had significantly lower detection (~ 10,000-fold) of BCoV (Supplementary Table 3). While this suggests the potential for substantial signal interference due to molecular inhibition (Supplementary

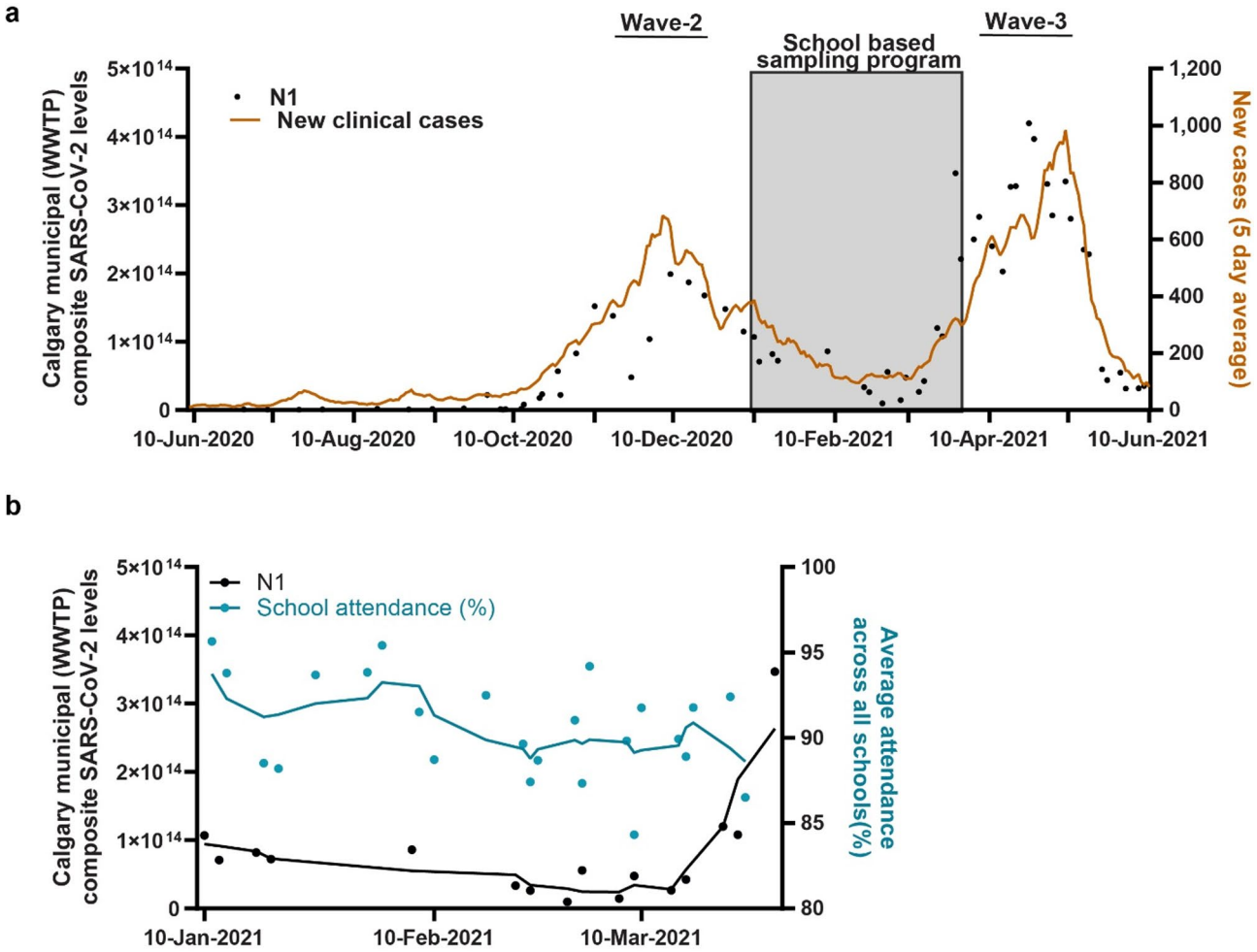

Fig. 3. Total mass flux of SARS-CoV-2 genomic material across the city of Calgary relative to attendance patterns in the four individual schools monitored by WBS. (**a**) Daily city-wide dynamics of the mass flux of SARS-CoV-2 levels (N1 gene target) from June 2020 to June 2021 (black dots). Clinically confirmed COVID-19 new cases (5-day average) are represented by the orange line. The sampling period for the four schools is highlighted in grey, occurring during a period of low COVID-19 activity between waves 2 and 3. (**b**) Lines represent the line of best fit plotted with second order smoothing for the average attendance rate (staff and students) across all four schools (blue dots and line) plotted together with community burden of SARS-CoV-2 as measured in Calgary's wastewater (same data as in panel A; black dots and line) from January to March 2021.

Fig. 7B), it may also be related to the consistently low fecal content and reduced levels of suspended solids observed at site 2B (Supplementary Fig. 5 and Supplementary Table 4), which would reduce RNA recovery efficiency and impair target detection (Supplementary Fig. 7B). When we removed site 2B as a significant outlier, school median BCoV did not differ from the WWTPs (Supplementary Fig. 7C).

Discussion

To understand the potential for near-source WBS in schools, we analyzed the spatial and temporal differences among SARS-CoV-2 among four large public schools in the city of Calgary. Despite the sporadic patterns of wastewater flow in individual buildings such as schools, RNA from SARS-CoV-2 genomes can be identified. Other studies have confirmed that the incidence of SARS-CoV-2 in wastewater from schools corresponds with the community disease burden[15,32–34]. The present study takes this a step further by demonstrating the correlation between clinical disease occurring in student and staff populations and corresponding wastewater SARS-CoV-2 signals from the schools during a period of low COVID-19 community transmission (i.e., after the second wave but before the third wave). Augmenting this analysis with overall community SARS-CoV-2 burden through parallel testing of municipal WWTPs, this study provides a more comprehensive, holistic perspective of disease activity against which to understand the potential of WBS in schools. Indeed, this complements other school-based WBS studies in the UK, USA, Czech Republic, Canada, and Thailand[15,32–39]. Some of these other studies found that the levels of SARS-CoV-2 signal in school wastewater correlated with incident COVID-19 cases[33,34] and in some instances provided a leading signal[15,36,39]. In contrast, other school WBS studies reported only

moderate correlations between SARS-CoV-2 signal in wastewater and clinical cases of COVID-19, specifically during periods of low prevalence[37]. Sampling four schools during a period of low community activity in the present study demonstrated that WBS, performed twice a week, provided an up to five-day lead relative to clinical case diagnoses. This is similar to Kappus-Kron et al. (2024) who identified a one day lead time[39]. Similar to other studies that have evaluated school-based WBS, this study monitored SARS-CoV-2 RNA signals in wastewater from schools encompassing a broad student age range (K–12) and population size[15,32–39]. The sampling frequency (i.e., twice per week) also aligns with other near-source wastewater surveillance studies, which ranged from one to four times weekly, especially during periods of high community transmission[15]. The size of the school populations in this study (i.e., 552–1558 students) were also comparable to those studies[32,33,37,38]. In contrast, the positivity rate observed in our study (20.3%) was lower than the average reported across comparable studies (mean = 42.2%, SD = 23.1%)[15,32–39]. Some factors may account for this difference. First, the timing of sample collection may have played a role where this study was conducted during a period of relatively low community transmission (i.e., post second wave of COVID-19), while other studies were conducted during high community COVID-19 prevalence[33] or during winter seasons. Second, differences in sampling methodology may have contributed; this study used composite sampling which provides a time-averaged signal that may differ from 'grab sampling' used in some other studies which represent a single point in time[35,37].

WBS in other near-to-source settings such as colleges, university campuses and residence halls generally demonstrate a stronger correlation with clinical diseases than results reported for schools here and in the studies mentioned above[40–46]. One likely explanation for this discordance relates to behavioral factors such as toileting[10,36,47]. As SARS-CoV-2 is almost exclusively shed in the stool[48]successful monitoring is dependent on toileting behavior of the population using the facilities in the buildings being sampled. This appears to represent a critical barrier for WBS programs in schools. An average of 63% of students between the ages of 6 and 16 years refuse to defecate while they are at school[49]. This avoidance behavior is most pronounced in high school students. Relative to community-wide WWTP samples, significantly lower levels of fecal biomarkers in wastewater including PMMoV, TSS and TVSS observed in this study overall—and especially in high schools—supports this. This represents an important limitation in the potential of school WBS programs for fecal-shed targets such as SARS-CoV-2 and may similarly impact attempts at monitoring pathogens such as Influenza, Respiratory Syncytial Virus (RSV) and viral causes of gastroenteritis. Other targets of interest such as measles and other viruses that are shed in high amounts in urine, and which have all been previously identified in sewage[50–53] may be less affected and more amenable to WBS.

School-based WBS applications come with significant technical challenges. Less than a quarter of the schools screened for potential participation in this study had infrastructure that enabled a comprehensive program that was capable of sampling the entire school's wastewater from ≤ 2 sampling sites while excluding wastewater from the surrounding neighborhoods. While multiple complementary sampling devices operating in parallel could in many instances overcome this shortcoming, such an approach would substantially increase the work and costs associated with WBS[36]. Once installed in schools, wastewater autosamplers did not successfully collect samples in all instances (unlike the consistency observed at WWTPs that did not encounter any failed sampling events) and were more likely to experience complications requiring specific maintenance (i.e. ragging and blocking). These challenges have been observed in other near-to-source sampling programs and highlight the need for on-site experts capable of autosampler maintenance and sample management.

This study adds to the growing body of evidence supporting the effectiveness of WBS for near-source monitoring such as school settings. WBS can be efficiently used to detect disease hotspots or rising transmission at a very granular scale[46]. WBS in school settings could help to provide near real-time disease burden estimates on school populations that is unbiased, objective, and comprehensive of every individual[1]. Previous studies have shown that WBS can detect SARS-CoV-2 RNA before or concurrently with clinically confirmed cases in children, making it a valuable early warning system[54] and can be used to assess school-targeted public health interventions[55,56]. To improve WBS implementation in schools, future efforts should include the prioritization of infrastructure assessments during school renovations or partnering with school boards to streamline access to sewer systems. Additionally, improvements are needed in how WBS-derived epidemiological data are communicated to school boards and public health agencies to support timely and targeted actions[1,56,57]. By demonstrating both the feasibility and limitations of near-source WBS in school settings, our study supports its broader integration as a complementary tool to clinical surveillance not only for SARS-CoV-2 but also potentially for other pathogens of public health relevance[34,57]especially in school age-populations where clinical testing is often inconsistent, and symptoms may be mild or lacking entirely, likely leading to an underreporting of infections[58]. Near-to-source WBS can support public health decision-making by enabling timely actions like focused testing, isolation, and changes to school/campus protocols to help prevent outbreaks[32,59]. Also, WBS can have impactful effects on public health, particularly in densely populated locations or remote communities[55]. However, in this WBS study, several limitations reduced its feasibility for meaningful public health impact (see details below).

This study has several limitations that may reduce its impact: First, this study was conducted after Alberta's second wave of COVID-19 at the end of the 2020–2021 winter season[60] during a time of very low overall community COVID-19 activity[10]. Strict public health measures (including a province-wide mask mandate, encouragement to work from home, prohibition of social gatherings[61] were in place, enabling schools to resume in-person learning in January after the winter holiday break. Had this study been conducted during a period of higher COVID-19 incidence, the performance of school-based testing may have been different. Second, while composite samples were collected differently for schools vs. WWTPs (8-h vs. 24-h collection), this was intentional to try to focus on hours of school attendance, yet this still gave rise to SARS-CoV-2 levels that were much lower than corresponding WWTP testing—suggesting differences in toileting patterns. Third, there were limitations related to the spatial resolution of sampling within school buildings, particularly at the School #2B location,

where samples collected from this site consistently showed a reduced fecal content and lower levels of suspended solids compared to other locations. This likely compromised the efficiency of RNA recovery using the 4 S process and impaired the detection of both internal controls and SARS-CoV-2 targets[62,63]. Differences in water usage patterns among the schools and the presence of solids could affect the concentration and detectability of viral RNA in the wastewater samples. Physicochemical conditions (i.e., temperature, pH, electric conductivity, and turbidity) of wastewater samples could likewise influence the detection of SARS-CoV-2 RNA[64]. Fourth, given the limited study duration, we were unable to assess SARS-CoV-2 wastewater temporal trends among all schools. Finally, due to infrastructure constraints within schools, this study was conducted with a modest number of participating sites (~25% of those that were assessed) which could limit the applicability of findings to other school settings—settings that may also have different demographics, community behaviors or infrastructure. Appropriate sewer access points for routine monitoring for SARS-CoV-2 in wastewater is a key aspect for WBS programs[65].

Conclusion

WBS performed in schools successfully identified incident cases of COVID-19 before they were diagnosed clinically. However, significant limitations exist. The plumbing network in most schools did not allow for comprehensive surveillance that was non-disruptive and sample collection is technically challenging relative to conventional WWTP-based programs. Perhaps most importantly, student reluctance to defecate at school will limit the potential for this technology to be used effectively for SARS-CoV-2 and other fecal shed WBS targets is reduced.

Methods

Public school and community wastewater sampling

In partnership with Alberta Health Services (AHS) Medical Officers of Health, and a Calgary-based school board, we first identified candidate schools based on criteria that included a population of ≥ 500 students and a school administration amenable to WBS. This was followed by a process of identifying appropriate wastewater sampling sites that included a series of screening steps used to exclude sites (Supplementary Fig. 1). First, each building's sewer network mechanical drawings were assessed to determine if plumbing access ports allowed for wastewater collection from all toilets, sinks and other source locations in the building from ≤ 2 sampling sites, while simultaneously avoiding external sources from the surrounding neighborhood. The second step involved a physical evaluation of potential plumbing access ports to check for safety hazards or a hindrance to student or faculty activities during installation or ongoing sample collections and maintenance. The final screening was an extensive in-person physical review of internal plumbing of each facility to check relevant infrastructure for any obstructions or unavoidable structural hindrances and to identify where wastewater could be safely collected without disrupting teaching or administrative functions. If within-building collection was not possible, nearby municipal access points were reviewed to see if they could be effectively substituted, without impeding road traffic. C.E.C autosamplers were deployed at the sewer access port(s) of participating schools, as detailed in Supplementary Table 1. Autosamplers were programmed to operate continuously. Collection from school #3 was impeded by a physical obstruction identified after passing all steps, however, an outdoor municipal sewer access port exclusively and comprehensively serving the school building was available in a green space. A wastewater sampling routine was developed in which wastewater was collected only during times in which students and faculty were present at schools. Details of individual autosampler programming are available in Supplementary Table 1.

All methods were carried out following University of Calgary institutional guidelines and regulations, and protocols were approved by the Conjoint Regional Health Ethics Board (REB 20-1544). The study was granted a waiver of consent exemption as are all WBS studies there was no contact with any participants, collecting individual consent would not be feasible and only aggregate case data was collected and no information on individuals was collected.

To compare the burden of SARS-CoV-2 in school populations relative to the community, raw wastewater from each of Calgary's three wastewater treatment plants (WWTPs) was collected up to three times per week by City of Calgary Water Services staff as previously described[10]. Briefly, ISCO 5800 and ISCO 6712 portable autosamplers were programmed to collect and store 24-h composite samples. WWTP-1 samples were flow-weighted and samples from WWTP-2 and WWTP-3 were time-weighted to create a single city-wide metric (see below for details)[10].

School and WWTP samples were transported to the University of Calgary's Advancing Canadian Water Assets (ACWA) laboratory on ice for sample concentration and nucleic acid extraction. Samples that were not successfully collected were categorized based on complication type: ragging (abundance of fibrous material blocked sampling inlet tubing), low sanitary flow, or temperature excursions (defined as sample freezing or autosampler malfunction related to ambient temperature) as detailed in Supplementary Table 1.

Sample concentration and nucleic acid extraction

Wastewater samples were processed in real-time following a previously described methodology[16]. Briefly, each sample was thoroughly agitated to ensure maximum homogeneity, then a 40 ml aliquot was spiked with 200 µl of a bovine coronavirus (BCoV) exogenous control (final concentration of 2500 TCID50/ml) and then subjected to the sample processing and nucleic acid purification steps of the modified 4 S (Sewage, Salt, Silica and SARS-CoV-2) silica column purification method[16,62]. An extraction blank (i.e., UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen)) was included in each batch of processed samples. Extracted nucleic acids were transported on dry ice to the University of Calgary's Health Sciences Center for subsequent molecular analysis.

Using separate laboratory locations minimized the potential for contamination. Residual wastewater samples were stored at – 20 °C for chemical analysis.

Molecular analysis

Gene targets assessed by RT-qPCR included SARS-CoV-2 nucleocapsid gene loci N1 and N2, the exogenous control BCoV, and the fecal biomarker PMMoV (for population normalization purposes), which were all quantified using a QuantStudio-5 Real-Time PCR System (Applied Biosystems) using protocols described previously[16]. RT-qPCR raw data is in located in supplementary material 1. Each RT-qPCR reaction was performed in triplicate as per MIQE guidelines[66]. Samples were classified as positive for SARS-CoV-2 if either the N1 or N2 gene targets yielded a threshold quantification cycle (Cq) less than 40 [16,67,68]. City-wide measurement of SARS-CoV-2 burden (copies/day) in wastewater from Calgary's three WWTP was calculated as the sum of the mass flux from each of the three WWTPs, where it is the product of the SARS-CoV-2 concentration (C, copies/ml) and the daily volumetric flow[10]. PCR inhibition was assessed using a spike and dilution method as previously described[10] using a representative 40 µl purified nucleic acid sample derived from the school wastewater (School #2B) extracted without the addition of the internal control (i.e., BCoV) and a control sample of Ultrapure™ DNase/RNase-free distilled water (ThermoFisher). Wastewater samples with a ≥ 2-Cq delay relative to controls were considered to have experienced RT-qPCR inhibition [69].

Chemical analysis

Calculating total solids (TS), total suspended solids (TSS), total volatile suspended solids (TVSS) and total dissolved Solids (TDS) in wastewater samples used standard methods[70]. Briefly, to determine TS, homogenized 50 ml aliquots were placed in pre-weighed crucibles and dried at 104 °C for 12 h to evaporate liquid content, then weighed after cooling to yield TS. To determine TSS, homogenized 20 ml aliquots were filtered through 1.5 µm pore size Grade 934-AH® RTU glass microfiber filters (Whatman). Residue retained on the filter was dried at 104 °C for 12 h and weighed to calculate TSS. To determine TVSS, the residue from the TSS step was dried again at 550 °C for two hours to drive off volatile solids in the sample, which was then cooled and re-weighed to give TVSS. To determine TDS the filtrate from the TSS step was evaporated and dried at 104 °C for 12 h, and the residue was weighed. Chemical raw data is in located in supplementary material 2. Chemical analysis of WWTP samples was performed by the staff of City of Calgary following the same standard methods.

Clinical case data

Clinically confirmed cases of COVID-19 of students attending each school were identified in real-time by Alberta Health Services (AHS) using established protocols and were reported to each school by AHS Medical Officers of Health. Daily aggregate case counts including new/incident cases of COVID-19 in students were shared with the study team. Individuals with confirmed COVID-19 were excluded from attending school for 10 days after their symptoms began. At the time of the study, if a case was identified in any individual class, all other class members were excluded from attending school for the next 14 days. Daily aggregate attendance counts for student/staff were recorded and daily student/staff enrollment were shared with the study team.

Statistical analysis

Spearman correlation tests were used to test associations between cycle threshold (Cq) values for N1 and N2 detection by RT-qPCR assays within the same wastewater samples. Differences in SARS-CoV-2 RNA N1 & N2, BCoV and PMMoV between schools and communities (WWTPs) were determined using the Mann Whitney test. Dunn's Multiple Comparison Test was used to determine which specific school sites differed from others for WBS targets. Differences in student absenteeism rates due to confirmed COVID-19 cases versus other reasons were determined using the Wilcoxon matched-pairs signed rank test. To compare student absenteeism percentages due to confirmed COVID-19 cases during the study period for all schools Kruskal-Wallis testing was performed. Cases confirmed within 1 to 5 days before/after wastewater sample collection were compared using Fischer tests to assess if positive school wastewater associated with confirmed cases in schools. Correlation between the SARS-CoV-2 wastewater-N1 (Calgary) with the overall attendance percentage was assessed using the Spearman correlation test. Additionally, we conducted a series of comparisons between absenteeism with the likelihood of getting a positive SARS-CoV-2 wastewater result at each school using the Fischer test. Statistical analyses were performed using GraphPad Prism version 10.3.1 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

N.A. and A.B.B. prepared the original draft of the paper. N.A. and A.B.B wrote the main manuscript text. N.A., A.B.B, N.S., P.P., L.M, Jo.H., M.A.B, B.J.W., Ja.M, M.P. and J.C. performed investigation and methodology. N. A. performed visualization and prepared figures. N.A., A.B.B., R.C., K.F., C.R.J.H.; and M.D.P led the study conceptualization. N.A., A.B.B, P.W., Jo.M., G.A., M.C.R, J. Hu, J.L.C., C.R.J.H., K.F; and M.D.P performed the formal analyses. All authors reviewed and provided critical feedback to the manuscript. R.C. led the project administration. M.D.P acquired funding and supervised the project.

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Declarations

Competing interests
The authors declare no competing interests.

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