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Parallel deployment of passive and composite samplers for surveillance and variant profiling of SARS-CoV-2 in sewage

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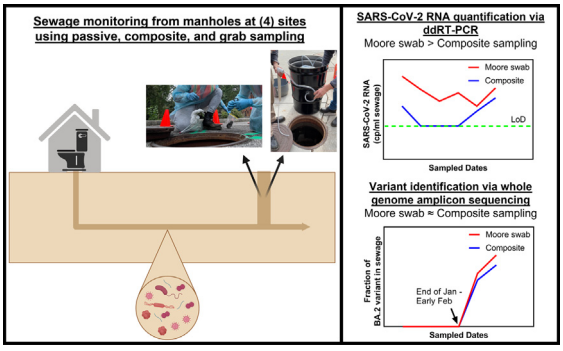
HIGHLIGHTS

- Passive sampling for SARS-CoV-2 sewage surveillance has advantages and limitations relative to composite sampling approaches.
- We conducted a wastewater sampling campaign on a college campus utilizing passive (Moore swab), composite, and grab sampling and directly compared three using ddRT-PCR and tiled amplicon sequencing.
- Moore swabs were the most sensitive of the sampling approaches and were accurate in identifying a shift in viral variants, confirmed by individual saliva testing.
- These findings reveal that Moore swabs can provide robust information for wastewater-based epidemiology (WBE) within small, campus-like communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology during the COVID-19 pandemic has proven useful for public health decision-making but is often hampered by sampling methodology constraints, particularly at the building- or neighborhood-level. Time-weighted composite samples are commonly used; however, autosamplers are expensive and can be affected by intermittent flows in sub-sewershed contexts. In this study, we compared time-weighted composite, grab, and passive sampling via Moore swabs, at four locations across a college campus to understand the utility of passive sampling. After optimizing the methods for sample handling and processing for viral RNA extraction, we quantified SARS-CoV-2 N1 and N2, as well as a fecal strength indicator, PMMoV, by ddRT-PCR and applied tiled amplicon sequencing of the SARS-CoV-2 genome. Passive samples compared favorably with composite samples in our study area: for samples collected concurrently, 42 % of the samples agreed between Moore swab and composite samples and 58 % of the samples were positive for SARS-CoV-2 using Moore swabs while composite samples were below the limit of detection. Variant profiles from Moore swabs showed a shift from variant BA.1 to BA.2, consistent with in-person saliva samples. These

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data have implications for the broader implementation of sewage surveillance without advanced sampling technologies and for the utilization of passive sampling approaches for other emerging pathogens.

1. Introduction

Sewage surveillance, also called sewage monitoring or wastewater-based epidemiology (WBE), has been utilized throughout the world during the COVID-19 pandemic. SARS-CoV-2 RNA is shed in the stool of approximately two-thirds of those that are infected with the virus, whether they are symptomatic or asymptomatic (Wölfel et al., 2020; Mesoraca et al., 2020; Gupta et al., 2020). Thus, SARS-CoV-2 RNA can be detected in sewage at different scales, including at the building, neighborhood, and wastewater treatment plant levels, as well as in both the liquid and the solid fractions of sewage (Ahmed et al., 2020; Peccia et al., 2020; Karthikeyan et al., 2021). Sewage data shows promise for public health uses as SARS-CoV-2 RNA concentrations in sewage have been shown to be correlated with clinical metrics at a variety of scales from an individual building to nationwide (Karthikeyan et al., 2021; Graham et al., 2020; Wu et al., 2021; Scott et al., 2021; Weidhaas et al., 2021; Wu et al., 2022; Nemudryi et al., 2020). While the use of sewage for disease detection has been used for decades (Metcalf et al., 1995), new tools for the current and future pandemics need to be investigated for rapid public health response.

Sampling methods for sewage surveillance have typically focused on time- or flow-weighted composite sewage samples using autosamplers. Multiple studies have shown that composite samples of sewage are preferred over grab samples as they provide more representative samples and are less variable due to diurnal fluctuations of SARS-CoV-2 RNA in sewage (Curtis et al., 2021; Gerrity et al., 2021). However, collecting composite samples is not always ideal, as autosamplers are expensive, they require a power source, secure storage, and procurement can be affected by supply chain issues, making them less ideal for low-resource settings and areas experiencing supply chain disruptions. Furthermore, low-resource settings will disproportionately feel the impacts of pandemics due to lower access to clinical testing (New WHO estimates: Up to 190,000 people could die of COVID-19 in Africa if not controlled, n.d.). Thus, evaluating low-cost passive sampling for utility in surveillance of emerging viral variants is crucial to better informed public health decision-making (Bivins et al., 2022). Passive samplers show promising scalability given their low cost and ease of deployment across diverse settings.

Moore swabs have been used since the mid-1900s for passive sampling to gather information on pathogens in communities from sewage samples (Moore et al., 1952; Sikorski and Levine, 2020). Moore swabs are simply cotton gauze that is folded to increase surface area and suspended in the sewage flow to be sampled for a certain duration, often by a string or fishing line (Moore et al., 1952). During the COVID-19 pandemic, several research groups have utilized passive sampling techniques to both detect SARS-CoV-2 RNA in sewage at variety of scales and understand COVID-19 transmission within a community. Several studies have utilized tampons (Kevill et al., 2022), cotton buds (Q-tips) (Habtewold et al., 2022), or cotton gauze (Kevill et al., 2022; Liu et al., 2022; Rafiee et al., 2021), while others have focused on passive samplers utilizing adsorptive media (Li et al., 2022a; Hayes et al., 2022), or a combination of methods (Hayes et al., 2021; Schang et al., 2021). Passive sampling approaches proved their usefulness as an early-warning tool for public health response at various scales (Kitajima et al., 2022; Corchis-Scott et al., 2021). Passive samplers that effectively capture the solid fraction of sewage may be preferred for sewage surveillance, as enveloped viruses, including SARS-CoV-2 adsorb readily to solids (Kim et al., 2021; Ye et al., 2016). Normalization with flow rate or other fecal indicator organisms and correlation with clinical metrics has had mixed success in published studies, however, highlighting the need for more data and analysis of how passive samplers can be utilized effectively for WBE during the COVID-19 pandemic. A meta-analysis found mixed sensitivity and correlations for passive relative to composite samplers from previously published studies, pointing to the need for more comparisons of sampling methodologies across diverse sites and scales (Bivins et al., 2022). Thus far, no passive sampling studies have used whole genome amplification sequencing approaches to analyze how these samplers could be used for variant detection and monitoring.

Herein, we aimed to characterize the utility of Moore swab passive samplers for WBE and understand how data obtained from Moore swab passive samplers compare with the current standard sampling approaches using autosamplers. Specifically, we aimed to answer: 1) Are Moore swab samples of building-scale sewage as sensitive as composite or grab samples from the same location for the detection of SARS-CoV-2 RNA? 2) Do data generated from Moore swab samples correlate with clinically confirmed cases of COVID-19 from the same building? and 3) Can shifts in variant prevalence in a community be captured by whole genome sequencing of passive samples?

2. Materials and methods

2.1. Experimental design

To answer our research questions, our work focused on (1) on-campus monitoring at four dormitories over six weeks from January–February 2022, to understand the sensitivity and utility of each type of sampling approach, and (2) tiled amplicon sequencing of sewage samples to analyze variants for different types of sampling methods. First, we compared previously described methods to concentrate, extract RNA, and quantify viral RNA from Moore swab samples and from composite samples, as described in the supplementary material (SM). We sampled manholes at the four sites twice per week for six weeks using grab, composite, and Moore swab sampling techniques. We quantified SARS-CoV-2 RNA targets N1 and N2 for each sampling method, as well as pepper mild mottle virus (PMMoV) RNA using ddRT-PCR. PMMoV is an abundant single-stranded RNA virus in human feces, which is known to be stable in the wastewater (Zhang et al., 2006; Rosario et al., 2009; Kitajima et al., 2014). PMMoV concentration in each sewage sample was used for normalizing the concentrations of SARS-CoV-2 RNA targets in sewage which enabled the direct comparison between samples. Finally, we utilized tiled amplicon sequencing (cat. no. 10009832, xGen SARS-CoV-2 panels, Integrated DNA Technologies, Coralville, IA) to detect variants of SARS-CoV-2 in the samples of sewage.

2.2. Biweekly dormitory monitoring

Moore swabs at each site were retrieved on Monday and Thursday between 9 and 10 AM and new Moore swabs were installed. Moore swabs were constructed in the lab the day before installation using Z-folded cotton gauze (Premium gauze bandage roll, Mighty-X) (detailed construction method in SM). After retrieval, swabs were placed into sterile Whirl-Pak bags (part no. B01195, Whirl-Pak Write On Bags 55 Oz), and subsequently stored on ice until processed in the lab within 2 h. During processing, sterile stainless steel potato ricers – a kitchen utensil designed for mashing potatoes by applying pressure through small holes – were used to squeeze absorbed liquid from each Moore swab into a sterile 1 L-glass beaker, similar to another passive sampler study (Liu et al., 2022). The Moore swabs were discarded after this single squeeze. Each swab filtrate (100 mL) was poured into two sterile 50-mL conical tubes and placed on ice. For each batch of swab processing, a negative process control was generated by pouring 100 mL of DI water through a sterile potato ricer into a sterile beaker and pouring the liquid into two sterile 50-mL conical tubes as done for swab filtrates.

Composite samples were collected at each site using a Hach autosampler (AS950) set to collect 20 mL every hour for 72 (Monday-Wednesday) or 96 h (Thursday-Sunday). Composite samples were collected in sterile Whirl-Pak bags (part. no. B01195, Whirl-Pak Write On Bags 55 Oz) placed inside the autosamplers on each sampling day. Grab samples were collected at each site in the morning between 9 and 10 AM using a sterile 50-mL conical tube tied to a fishing line that was lowered into the sewage flow and samples were poured into bags. The bags were then placed on ice during transport back to the lab until sample processing within 2 h. Composite samples were poured into two 50-mL conical tubes, for a total of 100 mL, once returned to the lab.

To each 100 mL grab, composite, or Moore swab filtrate sample, 50 μL of BCoV vaccine (1 ×) (Bovilis, Merck, code no. 156332) was added prior to sample concentration to assess the efficiency of the sample processing steps and viral RNA extraction. The BCoV vaccine was spiked into the Moore swab filtrate (instead of directly spiking onto the Moore swab) and so BCoV recovery data does not include the potential loss of viral RNA during the squeezing of Moore swabs using potato ricers. BCoV vaccine suspensions were prepared by adding 2 mL of 0.2 μm filter-sterilized 1 × PBS to each vial, inverting to mix, then refrigerating at 4 °C until use. After spiking BCoV, tubes were inverted several times and incubated on ice for 15–30 min. Then, two 50 mL samples were transferred to an autoclaved 500-mL polypropylene bottle (part. no. 361691, Beckman Coulter). Subsequently, 8 g of PEG-8000 (cat.no. P4330, Teknova) and 1.2 g of NaCl (cat.no. S640-500, Fisher scientific) were added to each combined 100 mL sample, and bottles were vigorously shaken to disperse. The final concentrations of PEG-8000 and NaCl were 80 and 12 g/L, respectively. Bottles were then placed on a shaker table at 150 RPM at 4 °C overnight. The next morning, bottles were centrifuged at 4890*g* (6,300 RPM with JLA-10.500 Fixed Angle Rotor installed in Avanti J-E centrifuge, Beckman Coulter) for 30 min to pellet samples. Supernatants were decanted from bottles, and pellets were resuspended in 2 mL of 1 × PBS (concentration factor = 50 ×). Resuspended pellets were stored at 4 °C until used for nucleic acid extractions within 5 h.

2.3. Nucleic acid extraction

Samples were blinded to extraction personnel, and nucleic acids from each sample were extracted with the Omega Bio-Tek MagBind DNA/RNA extraction kit according to the manufacturer's instructions (cat. no. M6246-03). An aliquot of resuspended pellet (0.25 mL) from each grab, composite, or Moore swab sample was added to an extraction tube consisting of 400 μL of lysis buffer (TNA lysis buffer, Omega Bio-Tek) in biological duplicates. Each batch of extractions included one negative extraction control which consisted of 1 × PBS in place of the sewage sample. Once samples were added to the tubes with the lysis buffer, samples were homogenized with 2.8-mm diameter ceramic beads, and nucleic acids were extracted within 1–5 h by a KingFisher Apex robot system (Thermofisher). The samples retrieved on Jan 10, 13, and 17th were extracted by hand due to logistical constraints. Final extract volumes were in 100 μL in DNA/RNA-free water and were stored at −80 °C for 2 months until assayed by ddRT-PCR.

2.4. ddRT-PCR quantification of viral RNA

Duplex ddRT-PCR assays for BCoV and PMMoV or SARS-CoV-2 N1 and N2 were performed as previously described using One-step RT-ddPCR Supermix for Probes (BioRad, Hercules, CA, cat.no. 1864022) (Graham et al., 2020). Primers and probes were obtained from IDT (Integrated DNA Technologies, Coralville, IA) and were suspended to 10 or 100 μM in TE buffer (cat.no. 11-05-01-05, IDT) prior to use. RT-qPCR assay information is provided in Table S1. Five μL of template was used for each reaction (in a total reaction of 20 μL) leading to approximately 0.15 mL sewage assayed per well or 0.31 mL sewage assayed per merged well sample. A positive control was run in two duplicate wells on every plate: either a 1:100 dilution of SARS-CoV-2 RNA (ATCC VR-1986D) for the N1/N2 duplex assay, or a 1:10 dilution of a sewage extract (to test for inhibition biases) and 1:1000 dilution of BCoV vaccine extract (undiluted BCoV stock concentration = 4.2 ± 0.98 × 10^5 cp/μl) for the BCoV/PMMoV duplex assay. All unknown extracts were assayed in duplicate wells and merged for data analysis. For the N1/N2 plates, all extracts were run as both undiluted and 1:10 diluted extracts to assess inhibition. The higher value of the two was considered uninhibited and thus used in data analysis. For the BCoV/PMMoV plates, extracts were run as 1:10 diluted extracts, except for three samples which were run as undiluted extracts. Quadruplicate no template control (NTC) wells were included with each plate. Thresholding procedures are given in the SM.

2.5. Tiled amplicon sequencing

A tiled amplicon sequencing approach was applied for high-throughput sequencing of the SARS-CoV-2 RNA genomes in sewage and companion clinical samples from residents of the same buildings as those sampled via sewage. RNA was freshly extracted from concentrated sewage samples stored in the lysis buffer (TNA lysis buffer, Omega Bio-Tek) at −80 °C for 3–4 months using the extraction method described above. Samples for sequencing were selected based on the Ct values from a combined N1/N2 reverse transcription-quantitative PCR (RT-qPCR) following methods described elsewhere (Gibson et al., 2021). A total of 39 sewage samples that gave Ct < 40 (Table S5) and 19 saliva samples that gave Ct < 35 were selected for sequencing. cDNA synthesis was performed immediately after RNA extraction using the SuperScript IV first strand synthesis system (cat. no. 18091200, Thermo Fisher Scientific) and stored at −20 °C for 3 weeks. This was followed by Illumina amplicon library preparation using the xGen SARS-CoV-2 Amplicon Panel (cat. no. 10009832, IDT) and subsequent normalization following the manufacturer's protocol. Libraries were sequenced on an Illumina NovaSeq 6000 at a PE150bp read length at the Georgia Institute of Technology Molecular Evolution Core.

2.6. Variant profiling

Detailed procedures of quality trimming and mapping of amplicon reads to the SARS-CoV-2 reference genome (RefSeq ID NC_045512.2) are in the SM. To determine the variants present in sewage samples, the typical threshold used in the relevant studies is 50–60 % of SARS-CoV-2 genomic indices covered (or breadth, hereafter) (Karthikeyan et al., 2022; Jahn et al., 2022). These types of threshold are important since, with higher breadth of coverage, there is greater likelihood one observes a broad array of clade-defining mutations necessary for variant calling. In our dataset, 3 sewage samples had breadths >50 % and 22 samples had breadths <20 % (Fig. S5). Therefore, to retain most of the sewage samples for downstream analysis, a breadth threshold of 10 % was applied to our dataset as a minimum cutoff, which resulted in the removal of 11 low-breadth samples among the 39 total sewage samples. Additionally, retained samples were required to have a minimum average sequencing depth of 10 × (i.e., each position in the genome is covered on average by at least 10 reads). To obtain a temporal trend of the variants present in sewage, the seven mutations evenly covered by the retained 28 sewage samples were chosen for analysis (Table S3). Sequencing depths and breadths were calculated based on the iVAR-trimmed BAM files using SAMtools mpileup and mosdepth v0.3.3 (Pedersen and Quinlan, 2018), respectively.

The constellation of clade-defining mutations was obtained from cov-lineages.org (O'Toole et al., 2021). After the translation of iVAR-derived VCF files using ANNOVAR (Wang et al., 2010) with the precompiled sarscov2 database, the allele frequencies (i.e., the fraction of mapped reads carrying a single nucleotide polymorphism) of the clade-defining mutations were investigated. The variant prevalence in sewage was also independently estimated by the 'demix' tool of Freyja v1.3.6 with default options (Karthikeyan et al., 2022). The barcodes and lineage metadata for Freyja were updated on June 7th, 2022. The consensus sequence was constructed with saliva samples with coverage breadth >90 % using iVAR consensus with default options. Nextclade Web v2.3.0 was applied for the clade assignments of the consensus sequences (Hadfield et al., 2018).

2.7. Data analysis

Data from sewage samples underwent quality assurance before proceeding with data analysis: see the EMMI checklist in the SM for details on experimental design (Fig. S1) (Borchardt et al., 2021). A 60 % confidence limit of detection (LoD) for each ddRT-PCR assay was derived using serially diluted standards and values below the LoD were substituted with a value of half the LoD for data analysis. Recovery of BCoV was calculated for each sample by dividing the measured copies of BCoV for each sample by the theoretical number of BCoV copies spiked into each sample prior to the concentration step. The Shapiro-Wilk normality test was used to determine if concentrations of N1, N2, or PMMoV were normally or log-normally distributed, using $\alpha = 0.05$ significance and the correlation between Moore swab sample data, composite sample data, and/or clinically confirmed COVID-19 cases was assessed using Pearson's correlation coefficient or Spearman's rank correlation coefficient.

3. Results and discussion

3.1. Biweekly dormitory monitoring

All negative controls were evaluated to detect possible contamination and only those sewage samples that were processed with passing controls were used in the analysis (see SM for QA/QC criteria). We compared Moore swab, grab, and composite sampling methods to determine if Moore swabs could perform as well as composite sampling for sewage surveillance at four dormitories sampled biweekly over six weeks from January 6th-February 7th 2022. On the 19 sampling days that yielded data for both Moore swabs and composite samples among our sampling sites, there were 11 instances where swabs yielded positive results for SARS-CoV-2 RNA targets while paired composite samples were below the 60 % confidence limit of detection (LoD) (see SM for LoD determination). Importantly, there were no instances when swabs were negative, while parallel composite samples were positive.

N1 concentrations in swabs ranged from below the limit of detection (BLD) to 2.5×10^3 cp/ml/day (mean = 3.7×10^2 cp/ml/day, n = 29) and N2 concentrations in swabs ranged from BLD to 2.0×10^3 cp/ml/day (mean = 3.9×10^2 cp/ml/day, n = 29). Concentrations of N1 in composite samples ranged from BLD to 1.3×10^2 cp/ml/day (mean = 5.2×10^1 cp/ml/day, n = 21) and concentrations of N2 in composite samples ranged from BLD to 1.3×10^2 cp/ml/day (mean = 5.4 cp/ml/day, n = 21). Concentrations of N1 in grab samples ranged from BLD to 5.1×10^2 cp/ml/day (mean = 2.1×10^2 cp/ml/day, n = 31) and concentrations of N2 in grab samples ranged from BLD to 5.3×10^2 cp/ml/day (mean = 2.0×10^2 cp/ml/day, n = 31) (Fig. S2). Concentrations of N1 in swabs were not correlated with N1 concentrations in composite samples (Spearman's rho, p = 0.13). N1 concentrations were correlated with N2 concentrations for all the samples; thus, only N1 was used for the following data analyses (Pearson's correlation, r = 0.962, $p < 0.0001$).

We also measured a proxy for the contribution of human fecal material to the sewage flow using PMMoV, a human-associated fecal indicator, in sewage using all three sampling types to account for dilution events between sampling days due for instance to heavy rain since the manholes also receive stormwater. PMMoV concentrations in composite samples ranged from 4.8×10^3 cp/mL to 9.0×10^5 cp/mL (mean = 1.7×10^5 cp/mL, n = 21). PMMoV concentrations in swab samples ranged from 6.5×10^4 cp/mL to 4.0×10^6 cp/mL (mean = 9.1×10^5 cp/mL, n = 29). PMMoV concentrations in grab samples ranged from 6.0×10^1 cp/mL to 4.0×10^5 cp/mL (mean = 7.1×10^4 cp/mL, n = 28). PMMoV concentrations in Moore swabs and composite samples were not correlated for any of the sites (Pearson's correlation coefficient, $p > 0.05$).

3.2. Strengths and limitations of Moore swabs

Passive sampling of sewage for monitoring SARS-CoV-2 has benefits over autosampler-based sampling, as well as disadvantages that preclude its use. For sampling sewage at the sub-sewershed level, we found that passive sampling via Moore swabs can be more suitable for sewage surveillance, due to its higher sensitivity in our study area. Variable and intermittent flow conditions, especially in upstream small sized catchments, create challenging conditions for autosampler-based sampling (Medema et al., 2020; Teerlink et al., 2012; Li et al., 2022b), which are obviated through passive sampling approaches. However, the use of Moore swab passive samplers as quantitative measures (as opposed to binary measures) of SARS-CoV-2 RNA present in sewage is still being investigated (Liu et al., 2022; Rafiee et al., 2021; Wang et al., 2022a). Most notably, as also observed in our study, the volume of sewage sampled remains challenging to estimate for Moore swabs, rendering the resulting viral data as a semi-quantitative measure of concentrations in sewage. In addition, we observed that inhibition frequently affected the swab samples, especially at higher concentrations of N1 and N2, which required dilution to accurately quantify samples.

If Moore swab passive samplers are to be applied to a larger catchment, a limitation compared to the composite sampling method is the manual labor involved in the manufacturing of the Moore swabs and the sample processing (e.g., squeezing). This could be partially resolved by automating the latter, for instance introducing a paddle blender to squeeze Moore swabs (Liu et al., 2022). Another critical point to be considered is the saturation of Moore swabs by suspended solids in the sewage flow. Once the Moore swab is saturated, its capability to capture new SARS-CoV-2 RNA signal may be limited. Therefore, more research is needed to appreciate limitations of applying the Moore swab technique in larger catchment areas.

Complicating their use, the mechanisms by which Moore swabs capture SARS-CoV-2 RNA signal from sewage are not well understood. Physical filtration and adsorption to passive sampling material can contribute to SARS-CoV-2 signal, while remobilization, desorption, or decay could contribute to signal deterioration (Hayes et al., 2022; Acer et al., 2022), although the relative importance of how each mechanism might contribute to passive sampler signal and the variability of each mechanism given external factors (e.g., rainfall, ambient temperature, pH) is not well characterized. In our approach, we utilized normalization by PMMoV concentration to allow for comparisons between Moore swab, composite, and grab samples, which may circumvent several of the previously mentioned limitations of Moore swabs, assuming similar fate and transport efficiencies between PMMoV and SARS-CoV-2 viruses. Despite their relatively uncommon use, we found that Moore swab passive samplers provided at least as sensitive data as composite samples, similar to another study (Rafiee et al., 2021).

3.3. Comparison of sewage data with clinical data from the same dorms

To understand the relationship between sewage data and clinical data, we used the total number of clinically confirmed COVID-19 cases and viral concentrations in sewage at each site using COVID-19 cases in buildings connected to each manhole. Since there were no publicly available GIS maps of sewer lines, we used dye-tracing to confirm which buildings were connected to our manhole sampling sites and thus, which clinical data on campus should be used for this analysis (methods provided in the SM). For each location and sampling type (composite, Moore swab, grab), correlations between N1 concentrations in sewage and corresponding clinical positive case counts were not statistically significant (Spearman's rho, $p > 0.05$), except for Moore swabs at Dorm A (N1 concentration in swabs and clinically confirmed cases, Spearman's rho, p = 0.038). When concentrations of N1 in swab samples were normalized by PMMoV (Fig. 1) there was not a statistically significant association between sewage data and clinical data at our four sampling sites (Spearman's rho, $p > 0.05$).

However, one limitation of this analysis is that the clinically confirmed number of cases may not accurately represent the true case load at our sampling locations. While saliva tests were available on campus, testing was optional for students due to statewide policy. Additionally, there were no isolation protocols in place for students to relocate to an isolation facility after a positive clinical test, which differs from several other studies that

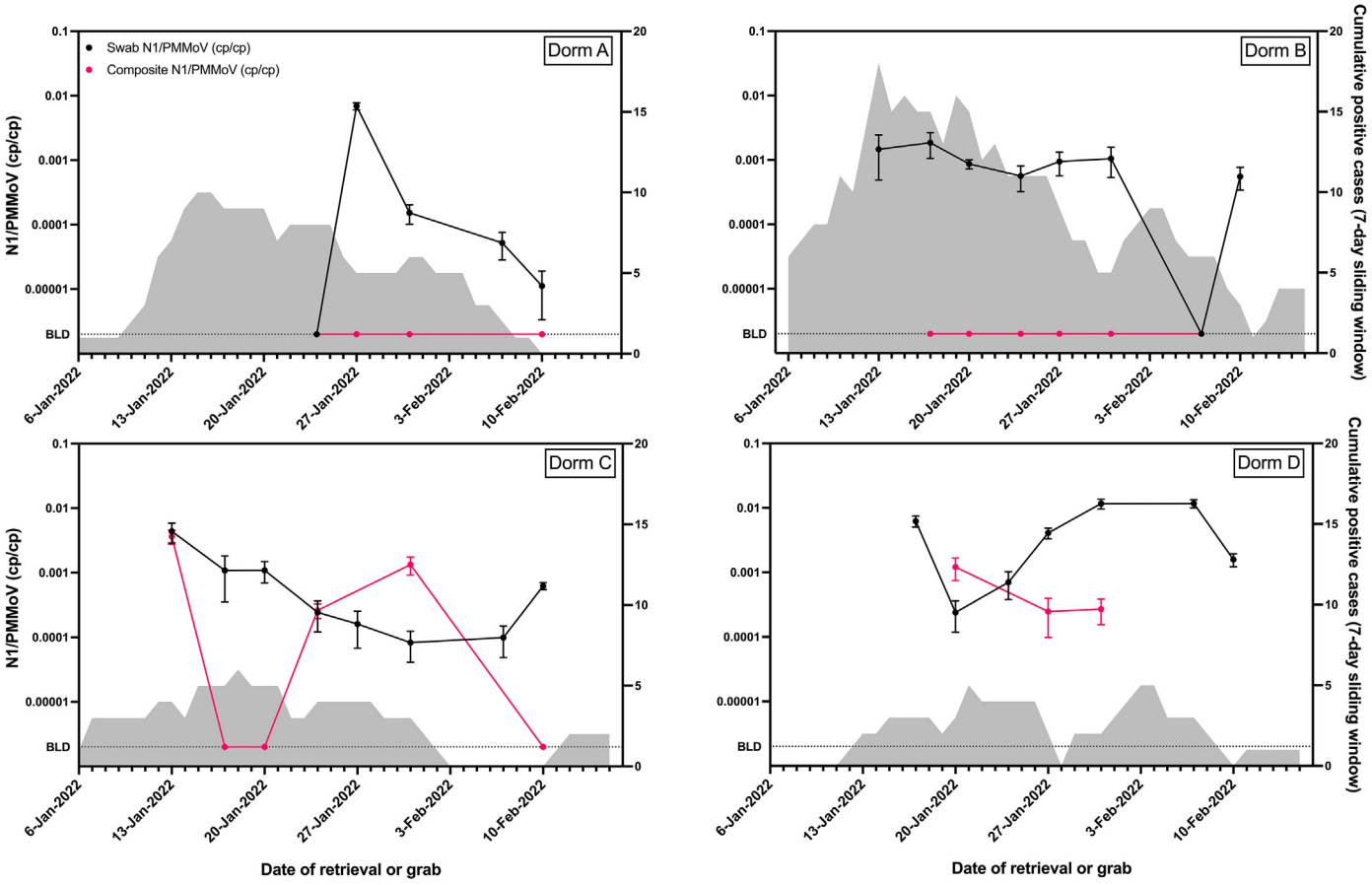

Fig. 1. Passive and composite sample N1 concentrations normalized by PMMoV concentrations at the four sample locations over the study period. The secondary y-axes enumerates the cumulative number of positive cases confirmed by voluntary saliva testing during a seven-day sliding window preceding the sample collection (or start of sample collection) date (in gray). Data points plotted on the "BLD" dotted line represent data that were below the empirically defined 60 % confidence limit of detection.

analyzed sewage data with clinical data from mandatory testing (Karthikeyan et al., 2021; Corchis-Scott et al., 2021; Wang et al., 2022a; Reeves et al., 2021).

3.4. Allele frequency profiling of clade-defining mutations

One of the key objectives of sewage-based genomic surveillance is profiling the circulating SARS-CoV-2 variants in the community. In the United States during the period of study (Jan-Feb 2022), 21 K (Omicron BA.1) was the most dominant variant clade followed by 21 L (Omicron BA.2) and 21 J (Delta) (Hadfield et al., 2018), which was in line with the clade assignments of our sequenced clinical saliva samples. We used the previously determined constellations of mutations, based on the global efforts to track variants (Shu and McCauley, 2017), to define those variant clades (O'Toole et al., 2021; Rambaut et al., 2020). Considering the low coverage breadth of amplicon sequences from sewage samples, our efforts were first focused on determining the likelihood of the existence of the dominant clades in these samples. The allele frequencies of 21 J, 21 K, and 21 L clade-defining mutations in 28 sewage samples with breadth >10 % and minimum average sequencing depth >10× were determined (Fig. S3). Two sewage samples from Dorm A did not satisfy the breadth of coverage threshold, and thus were excluded from further analysis. To derive the temporal change of likelihood of existence for each variant clade in our sewage samples, seven clade-defining mutations with loci covered by >70 % of the sewage samples (i.e., > 20 samples) were defined (Table S3). The allele frequencies of those seven mutations are assumed to represent the likelihood of the existence of a corresponding variant clade (Fig. S6). The two mutations - nuc:C25584T and N:RG203KR - shared between 21 K and 21 L clades were detected in most sewage samples with a high average frequency of 0.89 ± 0.24, which was a strong signal of the Omicron variant being present in sewage sampled from Dorm B, C, and D (Fig. S4). The 21 K-specific mutation nuc:T13195C was found in high frequency during January but was no longer detected in the first week of February. In contrast, the trend in the frequency of the 21 L-specific ORF1ab:L3201F mutation was the opposite. Even though the date of the first observance of the 21 L variant (BA.2) differed by as much as a week in different sampling locations (Dorm D: Jan 24th, Dorm C: Jan 27th, and Dorm B: Jan 31th), the likelihood of the variant being detected in the three dorms turned out to be low during the month of January contrasting with clear detection from Dorm B and D in the February 7th sewage samples. On the other hand, sewage samples that yielded positive mean mutation frequencies of 21 J-defining mutations were sporadically distributed throughout the monitoring period.

3.5. Variant prevalence estimation

In order to cross-validate the likelihood of the existence of 21 J, 21 K, and 21 L clades, the variant prevalence in sewage was also estimated by linking the presence of lineage-defining mutations with sequencing depth at the corresponding loci using Freyja (Karthikeyan et al., 2022). The estimated prevalence was further compared to the associated clade of sequenced clinical saliva samples for the assessment of the efficacy of the sewage genomic surveillance. Twenty-eight sewage and 18 clinical saliva samples with coverage breadth higher than 10 % were included in the variant analysis. Since the sequenced saliva samples were either from Dorm C or D, the comparison between sewage and clinical saliva was done using samples from those two sites (Fig. 2).

The BA.1 sublineage was estimated to be the most widespread variant in sewage during the month of January in both Dorm C and D based on clade-defining mutation profiling and variant prevalence estimation analysis (Fig. S6 and Fig. 2). This was consistent with the clade assignments of consensus sequences from clinical saliva samples, i.e., 15 out of 18 total sequenced saliva were BA.1 and the remaining three were BA.2. Further, the identification of the introduction of a novel variant into the monitored community, which in our case was Omicron BA.2 sublineage, was achieved from both clade-defining mutation profiling and the variant prevalence estimation. As shown in the Fig. 2, Freyja estimated the dates of BA.2 variant emergence in sewage from Dorm C and D sites as January 24th and January 27th, respectively, which coincided with the result from allele frequencies of BA.2-defining mutations (composite sample from Dorm C on 27 January 2022 samples and swab sample from Dorm D from 24 January 2022). However, none of the sequenced saliva samples from Dorm C residents were assigned to the BA.2 sublineage despite both clade-defining mutation profiling and Freyja estimating the presence of the BA.2 clade in the sewage sampled from Dorm C in the end of January. In addition, XF, XD, and XS lineages, which were grouped as 'Delta + Omicron' in Fig. 2, were estimated to be present in sewage from both Dorm C and D. These recombinant lineages were suspected to be the result of co-infections with both the Delta and Omicron variants similar to infections reported in United States in January 2022 (Bolze et al., 2022; Lacek et al., 2022). We were unable to further test this hypothesis due to the fragmented nature of the amplicon sequence data.

Bioinformatic tools that facilitate variant deconvolution analysis using whole genome amplicon data from sewage often require that genome coverage breadth to be higher than 50–60 % (Karthikeyan et al., 2022; Jahn et al., 2022) to facilitate the detection of constellations of discriminatory mutations, particularly among closely related variant clades (e.g., Omicron BA.1 and BA.2). Breadths of coverage below 50–60 % may result in unreliable estimations for the prevalence of different variants within a sewage sample since more than half of the SARS-CoV-2 genome will contain no mapped reads. In our case, the average breadth of genome coverage of the sewage samples was 22.6 %, and 11 out of 39 samples were below 10 %. Although this is low relative to clinical saliva samples, this is not peculiar for tiled amplicon sequencing data from sewage (Lou et al., 2022; Sapoval et al., 2022; Wang et al., 2022b), and is presumably due to potentially decayed SARS-CoV-2 RNA in sewage and/or non-specific amplification as observed in this study (e.g., amplification of DNAs belonging to other members of the sewer microbiome by Xgen SARS-CoV-2 panel). Considering that the commercial SARS-CoV-2 amplicon panels were constituted of multiple sets of primers, which were initially developed for reconstructing the consensus genome from clinical samples to determine the corresponding variant lineages and discriminate from co-occurring human DNA, those are not necessarily the best options for amplifying SARS-CoV-2 genomic RNA in the sewage matrix. Increasingly specific primers capable of warding against possible non-specific amplification of sewage-associated microbial genomes are urgently needed to improve the quality of sequencing data produced from sewage sampling and to thereby enable better identification of both prominent and emerging variants therein. Additionally, optimization of sequencing library prep such as applying DNase treatment prior to cDNA synthesis could improve the quality of amplicon sequences (Mondal et al., 2021).

For the variant analyses presented herein, we took a combined analysis approach by evaluating (1) the probability of existence of a certain variant from clade-defining mutation profiling (Fig. S6) and (2) prevalence estimation of those variants (Fig. 2). Cross validation of estimated presence and prevalence by each workflow corroborated the introduction of the Omicron BA.2 variant in Dorm C at the end of January, which a voluntary clinical saliva testing program failed to capture. Furthermore, several types of Omicron/Delta recombinant lineages such as XF, XD, and XS, which differ based on the position of a breakpoint on the genome (e.g., XD has two breakpoints, one at the beginning of spike gene and the other at the beginning of ORF 3a gene) and the putative parental lineage (Lacek et al., 2022; Simon-Loriere et al., 2022) were estimated by Freyja to be present in both

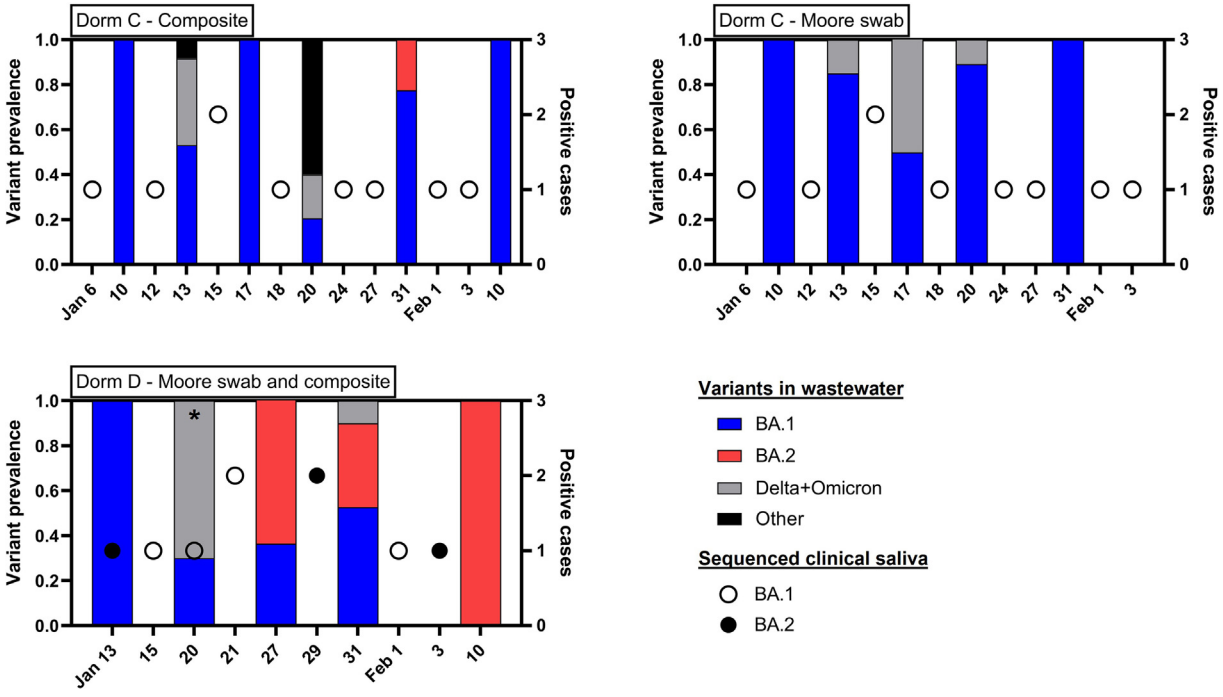

Fig. 2. Estimated variant prevalence in sewage sampled from Dorm C and D during January to early February 2022 and the clade assignments of the corresponding clinical saliva samples. The underlying data is the variant lineage estimation by Freyja summarized by WHO designations (e.g., Omicron BA.1 or BA.2 clade) of sewage samples (columns) and the clade assignments of consensus sequences from clinical saliva samples (circles). The estimate of variant prevalence of Dorm D from January 20th was from a composite sample (marked with an asterisk) and the rest were from Moore swabs. The predicted presence of Delta/Omicron recombinant lineages such as XF, XD, and XS are grouped into a 'Delta + Omicron' group. The 'Other' class incorporates both the B.1.1.221 and B.1.1.225 lineages. The deconvolution estimation in grab samples did not capture any new variant clades.

sewage sampled from Dorm C and D in January 2022 (Fig. 2). However, considering that the clade-defining mutation profiling was unable to discriminate between the recombinant lineages from individual variant clades (e.g., XF versus BA.1 versus Delta) and due to the relatively low breadth of coverage obtained from our sewage samples, confirmation of the presence of the recombinant lineages as opposed to the presence of genomic RNA fragments derived independently from Delta and Omicron variant was not feasible. Nonetheless, considering that none of the SARS-CoV-2 genome sequences submitted to the GISAID database during our period of study (early January to February 2022) were assigned to those recombinants, the presence of those recombinant lineages in our sewage is considered unlikely and mixed infections probably underlie the results observed.

4. Conclusions

This study highlights the utility of sewage surveillance, especially low-cost passive samplers like Moore swabs, in areas where clinical testing is not mandatory and catchment areas are small. Saliva testing for SARS-CoV-2 on this college campus was not mandatory over the study period, leading to potentially confounding the relationships between clinical and sewage data. However, both Moore swab passive samplers and time-weighted composite samplers applied for on-campus sewage monitoring were able to ascertain the introduction of a newer variant (BA.2) and the maintenance of an older variant (Delta) within the sampling area between the end of January and early February 2022, when clinical surveillance was not robust enough to detect it. The two-track approach applied in this study (clade-defining mutation profiling and variant prevalence deconvolution) may be suitable for identifying the transition of prevalent circulating variant lineages from SARS-CoV-2 whole genome amplicon datasets with relatively low (non-ideal) genome breadth of coverage. Future developments of SARS-CoV-2 amplicon primer sets to prevent non-specific amplification with sewer microbiome organisms and determination of viral RNA capture mechanisms of passive samplers would assist in finer variant identification during sewage surveillance.

CRediT authorship contribution statement

Gyuhyon Cha: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Katherine E. Graham:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Kevin J. Zhu:** Methodology, Investigation, Writing – review & editing. **Gouthami Rao:** Methodology, Investigation, Writing – review & editing. **Blake G. Lindner:** Methodology, Investigation, Writing – review & editing. **Kumru Kocaman:** Investigation. **Seongwook Woo:** Investigation. **Isabelle D'amico:** Investigation. **Lilia R. Bingham:** Investigation. **Jamie M. Fischer:** Investigation. **Camryn I. Flores:** Investigation. **John W. Spencer:** Investigation. **Pranav Yathiraj:** Investigation. **Hayong Chung:** Investigation. **Shweta Biliya:** Investigation. **Naima Djeddar:** Investigation. **Liza J. Burton:** Investigation. **Samantha J. Mascuch:** Investigation. **Joe Brown:** Conceptualization, Funding acquisition, Methodology, Validation, Writing – review & editing. **Anton Bryksin:** Resources, Validation, Writing – review & editing. **Ameet Pinto:** Validation, Writing – review & editing. **Janet K. Hatt:** Resources, Validation, Writing – review & editing. **Konstantinos T. Konstantinidis:** Conceptualization, Project administration, Funding acquisition, Resources, Methodology, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.161101.

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