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Optimization of the Primary Sludge Processing Method for Wastewater Genomic Surveillance of SARS-CoV-2

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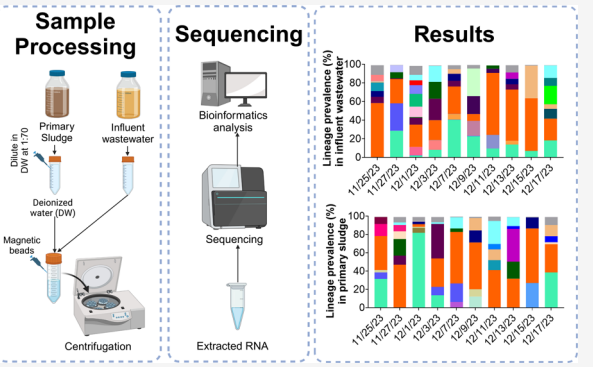
ABSTRACT: Wastewater genomic surveillance (WWGS) of SARS-CoV-2 is typically performed using influent wastewater, but the approach is challenging due to degradation as well as low target concentrations in wastewater. This could be alleviated by utilizing primary sludge; however, this matrix is prone to sequencing library failures. Our study focuses on developing a robust primary sludge-based SARS-CoV-2 genome sequencing method. The study was conducted using 30 parallel influent wastewater and primary sludge samples collected during three different time periods, under three clinically predominant SARS-CoV-2 Omicron lineages in Ottawa, Canada. Results showed that our approach consistently recovered near-complete (≥90%) SARS-CoV-2 genomes from both influent wastewater and primary sludge samples. Prevalent lineage and single nucleotide variant (SNV) profiles were identical ($p > 0.05$) between influent wastewater and primary sludge. Further analysis indicated that a similar ($p > 0.05$) number of rare SNVs were detected between influent wastewater and primary sludge. Overall, our approach enables the sequencing of the most concentrated sources of genetic material within the wastewater matrix, providing valuable insights for public health forecasting of infectious disease prevalence beyond the COVID-19 pandemic.



KEYWORDS: *wastewater and environmental surveillance, genome sequencing, single nucleotide variants, rare SNVs, lineage prevalence*

1. INTRODUCTION

The COVID-19 pandemic caused by SARS-CoV-2 has highlighted the importance of effective surveillance systems to monitor the viral spread in population levels. Clinical surveillance is the conventional method to monitor the prevalence of COVID-19; however, clinical surveillance overlooks the asymptomatic carriers as well as the individuals without access to medical facilities, leading to an incomplete picture of incidence and prevalence in the populations.[1,2] As a complement to clinical surveillance, PCR-based wastewater and environmental surveillance (WES) of SARS-CoV-2 RNA has emerged as a powerful tool for monitoring COVID-19 at various levels, offering early warning of outbreaks within the population.[3−5] With the progression of the pandemic, SARS-CoV-2 continues to evolve, leading to the emergence of multiple variants of concern (VOCs). These variants have been linked to increased transmissibility, disease severity, reinfection, and reduced vaccine effectiveness.[6−8] To monitor the evolving landscape of SARS-CoV-2 variants, clinical genomic surveillance through whole genome sequencing of patient samples is the standard practice. However, clinical genomic surveillance is also limited to symptomatic individuals and is further constrained by the high cost and resource-intensive nature of sequencing samples from individual patients.[9] In addition, as the pandemic wanes, clinical genomic surveillance becomes less capable of describing the trajectory of emerging VOCs due to widespread vaccination efforts, which has lessened the severity of infections and the need for clinical diagnosis.[10,11] Therefore, WES has become, in some regions and countries, the only remaining viable option to monitor the temporal changes of these variants and their lineages within the population. The qPCR-based WES is limited to measuring SARS-CoV-2 RNA in wastewater (except allele-specific RT-qPCR) but cannot track the prevalence of emerging variants in the population. To monitor the evolution of SARS-CoV-2 variants, WWGS has been adopted as a reliable and cost-effective method. WWGS often entails the collection of influent wastewater samples, followed by viral RNA extraction

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and sequencing to identify and characterize the distinct strains or variants of SARS-CoV-2 circulating within the population.[12−14] WWGS has demonstrated the detection of circulating, early and cryptic mutations and viral lineages in the population.[15−17] Although results of WWGS have faithfully mirrored clinical genomic surveillance in many jurisdictions, researchers routinely encounter difficulties in recovering complete genomes of SARS-CoV-2 from influent wastewater due to the fragmented nature and limited amount of the RNA target in wastewater.[18−20] This makes the detection and tracking of low prevalence or rare lineages and mutations of SARS-CoV-2 in wastewater challenging.

To resolve this issue, clarified primary sludge is theoretically a more appropriate location for sample collection compared to influent wastewater for WWGS, as SARS-CoV-2 RNA is found in higher concentrations in primary sludge as compared to influent wastewater.[21−23] However, in light of the higher concentration of SARS-CoV-2 genomic targets in primary sludge, sequencing efforts are more complex and challenging due to the anticipated presence of enriched PCR inhibitors in sludge matrices.[24] Consequently, limited efforts have been documented in the scientific literature to sequence the SARS-CoV-2 genome directly from primary sludge. Early in the pandemic, one research group attempted to sequence the whole genome of SARS-CoV-2 from primary sludge but failed to produce library for recovering near-complete (≥90%) genomes from those samples.[25] They expressed the reasons to be the presence of PCR inhibitors in primary sludge, which inhibit library preparation, while other impurities caused undesired amplification resulting in poor quality sequencing data along with incomplete genomic coverage. They also assumed that SARS-CoV-2 genome is less intact in primary sludge, which led to significant RNA degradation during direct sludge processing.[25] However, later another research group revealed the recovery of near-complete genome of SARS-CoV-2 from selected primary sludge samples (<30% of those sampled) where the cycling threshold (Ct) value was less than 31, and the genomic recovery decreased with the Ct value increased.[26] However, a critical comparison between these two studies suggests that differences in sludge processing methods may be the most influential factor affecting the successful recovery of near-complete genomes from primary sludge samples. This highlighted the need for optimized processing methods to recover near-complete genomes in primary sludge and resolve challenges associated with WWGS of SARS-CoV-2 using primary sludge samples.

In this study, we developed a robust approach by diluting primary sludge in deionized water at a 1:70 volumetric ratio and processed it using Nanotrap Magnetic Virus Particles for whole genome sequencing of SARS-CoV-2. The dilution of primary sludges likely mitigates PCR inhibitors,[27] while the high surface area and specific binding properties of Nanotrap Magnetic Virus Particles are expected to remove impurities,[28] thereby enriching and facilitating the recovery of intact SARS-CoV-2 genomes from primary sludge. In parallel, we processed influent wastewater by using the same method. We also compared the detected lineage (i.e., a genetically distinct SARS-CoV-2 genome) profiles and SNVs (i.e., single nucleotide variations across sequencing reads) counts and their genomic and temporal distributions between wastewater and primary sludge. We further evaluated the identified SNVs in wastewater and primary sludge against the Canadian and global clinical sequences during the sampling periods, as well as throughout the pandemic, to determine the presence of rare (low prevalence) SNVs in wastewater and primary sludge samples. Our approach establishes the robust recovery of near-complete SARS-CoV-2 genomes from both influent wastewater and primary sludge samples. Importantly, comparable or slightly better performance metrics were obtained in primary sludge as compared to influent wastewater samples across the study periods.

2. MATERIALS AND METHODS

2.1. Wastewater Sampling. A total of 30 pairs of 24 h-composite influent wastewater and primary sludge samples were collected in three distinct periods (first: April 02 to April 24, 2022; second: September 06 to October 01, 2023; and third: November 25 to December 17, 2023) from the City of Ottawa's Robert O. Pickard Environmental Center (ROPEC) using an autosampler (Hoskin Scientific, Burlington, Canada). Influent wastewater refers to the untreated municipal wastewater entering ROPEC for treatment, while primary sludge is the same influent wastewater following several hours of gravitational settling in the primary sedimentation basins at ROPEC. On each sampling day, 500 mL of influent wastewater (collected as ∼20 mL aliquots per hour) and 500 mL of primary sludge (collected as ∼125 mL aliquots at 6 h intervals) were obtained over a 24 h composite period. The samples were transferred to the laboratory in an ice cooler packed and stored at 4 °C for a maximum of 24 h prior to analysis.

2.2. Concentration, Nucleic Acid Extraction, and Detection of SARS-CoV-2 RNA. The primary sludge samples were processed for RNA extraction by using our optimized sludge processing methods for sequencing. Briefly, primary sludge samples were first diluted in deionized water at a 1:70 ratio by volume (0.7 mL of primary sludge with 49.3 mL of deionized water), mixed thoroughly, and incubated at room temperature for 10 min in 50 mL conical tubes. Similarly, 50 mL of wastewater was left at room temperature for 10 min to settle the larger particles. Afterward, 40 mL samples from the top were transferred to centrifuge tubes, followed by the addition of 600 μL of Nanotrap Magnetic Virus Particles (Ceres Nanosciences, Manassas, USA). The mixture was rotated at 100 rpm for 20 min at 20 °C and centrifuged for 10 min at 4 °C at 8000*g*. The centrifuge tubes were placed on a magnetic rack (DynaMag-50, Invitrogen, Massachusetts, USA), and the supernatant was discarded by pipetting without disturbing the pellet. The pellet was resuspended in 140 μL of PBS buffer (pH 7.4) and 560 μL of Viral Lysis Buffer (Qiagen, Germantown, USA). The resulting suspension was transferred to a 2.0 mL microcentrifuge tube and placed on a magnetic rack (DynaMag-2 magnet, Invitrogen, Massachusetts, USA) for 10 min to remove the magnetic particles. The lysate was carefully collected by pipet, and total nucleic acid was extracted using the QIAmp Viral RNA Mini Kit (Qiagen) and eluted in 70 μL of nuclease-free water. The SARS-CoV-2 RNA was detected by singleplex RT-qPCR targeting the N1 loci using the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher, Ottawa, Canada) on a CFX Connect qPCR thermocycler (Bio-Rad, Hercules, Canada) as previously described protocol.[29]

2.3. cDNA Synthesis and SARS-CoV-2 Tiled Amplicon Generation. First, any contaminating DNA from the extract was removed using the ezDNase Enzyme kit (Thermo Fisher), and RNA was reverse transcribed using a random hexamer and SuperScript IV First-Strand Synthesis System (Thermo Fisher). Briefly, 10 μL of DNA-free RNA was mixed with 1

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μL each of 50 ng/μL random hexamers, 10 mM dNTP mix, and nuclease-free water and incubated at 65 °C for 5 min. The mixtures were chilled on ice for at least for 2 min, and 7 μL of Mastermix composed of 4 μL of 5x SSIV Buffer, 1 μL of each 100 mM DTT, Ribonuclease Inhibitor, and SuperScript IV Reverse Transcriptase (200 U/μL) was added and serially incubated at 23 °C for 10 min, 55 °C for 10 min, and 80 °C for 10 min. Subsequently, SARS-CoV-2 cDNA was amplified through multiplexed PCR using the ARTIC primer set v4.1. Each PCR contained 12.5 μL of Q5 Hot Start Master Mix (BioLabs, New England, Massachusetts, USA), 3.6 μL of 10 μM of primer pool (IDT, Ottawa, Canada), 2.9 μL of nuclease-free water, and 6 μL of the cDNA. Samples were amplified with the following conditions: initial activation at 98 °C for 30 s, followed by 35 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 63 °C for 5 min. The amplicon size was verified in a Bioanalyzer 2100 system using the High Sensitivity DNA Kit (Agilent Technologies, Mississauga, Canada), purified with 0.8× AMPure XP beads (Beckman Coulter, California, USA), and quantified using a Qubit 4 fluorometer (Thermo Fisher) using the dsDNA High Sensitivity Kit (Invitrogen).

2.4. Library Preparation and Sequencing. The SARS-CoV-2 library was prepared using the Nextera XT DNA Library Preparation kit (Illumina, San Diego, USA) as per the manufacturer's instructions. Briefly, amplicons were tagmented at 55 °C for 10 min, followed by a 15-cycle PCR with the following conditions: 72 °C for 3 min, 95 °C for 30 s, and 15 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s before a single step at 72 °C for 5 min. The size distribution and quality of the resulting library were verified and quantified, as described above. Libraries were normalized and sequenced on a MiSeq Illumina platform with paired end reads of (2 × 150 bp) for the first period of sampling and (2 × 300 bp) for the second and third period of sampling.

2.5. Bioinformatics. A customized bioinformatics pipeline (nf-core/viralrecon) was used to analyze the raw sequence.[30] Briefly, FASTQ files were preprocessed using fastp[31] and aligned to the SARS-CoV-2 reference sequence (accession MN908947.3) using Bowtie2[32] to generate coverage information along the genome. Mapped genomes were subsequently processed using iVar for consensus generation and mutations (SNVs) calling with a minimum frequency threshold (0.01), minimum quality threshold (30), and minimum read depth (30×).[33] To minimize false-positive calls, SNVs detected in only one sequence were excluded from the analysis. In addition, to estimate the dynamics of virus evolution and spread, we utilized Freyja[16] bioinformatics to infer the relative abundance of lineage-defining mutations in both influent wastewater and primary sludge. We used .bam files on the Freyja workflow (v1.3.10) and packages found at https://github.com/andersen-lab/Freyja to determine lineage abundance through a regression approach that considers depth weighting and the least absolute deviation.

2.6. Determination of Species Richness and Shannon Entropy. The species richness and Shannon entropy were evaluated based on SNVs, following the protocols outlined in previous studies.[33] Specifically, species richness was defined as the total number of distinct SNVs sites identified, while Shannon entropy was calculated by using the following formula:

$$H(x) = -\Sigma p(x) \log_2 p(x)$$

where $p(x)$ is the allele frequency at the position x, and $H(x)$ quantifies the distribution of each mutation and degree of genetic diversity in the sample.

2.7. Enumeration of Rare SNVs. To enumerate rare SNVs, we analyzed uniquely detected SNVs against the Canadian[34] and global[35] clinical sequences throughout the pandemic (From Jan 01, 2020, to April 03, 2024). SNVs with a detection proportion <0.01 were considered as "low prevalence"/"rare", whereas those with a proportion >0.01 were considered as "prevalent".

2.8. Statistical Analysis. The differences in N1 Ct values, percentage of genomic coverage, depth of genome coverage, and the number of detected lineages and SNVs between influent wastewater and primary sludge were compared by using a paired *t*-test. The relationship between N1 Ct values and the percentage of genomic coverage as well as the depth of coverage was determined using the Spearman correlation coefficient. The choice of each statistical test was determined by the normal distribution of the data. The statistical analyses and graphing were performed in GraphPad's Prism 10.1 (La Jolla, California, USA).

2.9. Availability of Data. Raw sequencing data are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under BioProject ID: PRJNA1251232.

3. RESULTS

3.1. Optimization of Primary Sludge Processing for Sequencing. To develop an optimized sludge processing method for sequencing, we compared the conventional sludge processing technique[29] with our modified sludge processing approaches for SARS-CoV-2 RNA extraction. This comparative analysis aimed to overcome challenges posed by PCR inhibitors and RNA degradation typically present in primary sludge, thereby enhancing the recovery of high-quality viral RNA for reliable downstream sequencing applications. In our modified processing approaches, primary sludge was diluted with deionized water (50 mL) and Phosphate-Buffered Saline (PBS, 10× pH 7.4) buffer (50 mL) at varying volumetric ratios (1:50, 1:70, 1:100, 1:200, and 1:500), corresponding to 1.0, 0.7, 0.5, 0.25, and 0.1 mL of primary sludge mixed with 49, 49.3, 49.5, 49.75, and 49.9 mL of diluent, respectively. Subsequently, 40 mL samples from the top layer were transferred into centrifuge tubes and concentrated using Nanotrap Magnetic Virus Particles (Ceres Nanosciences), followed by total RNA extraction with the QIAmp Viral RNA Mini kit (Qiagen). After that, RNA extraction, cDNA synthesis, and amplicon generation were performed. Bioanalyzer analysis confirmed that desired amplicons (~400 bp) were successfully generated only in the clinical sample, with no amplification detected in either primary sludge samples processed using conventional methods or in the negative control (Figure S1). However, ARTIC amplification was observed at all dilution ratios in both deionized water (Figure S2) and PBS buffer (Figure S3), as well as in the positive control but not in the negative control. Subsequent analysis of the average Ct values for the N1 and N2 loci of the SARS-CoV-2 genome indicated that the lowest Ct value was achieved with deionized water at a 1:70 dilution ratio (Table S1). Thereby, we selected a 1:70 dilution of the primary sludge in deionized water for our final experiments.

3.2. Comparable SARS-CoV-2 Genome Coverage between Influent Wastewater and Primary Sludge.

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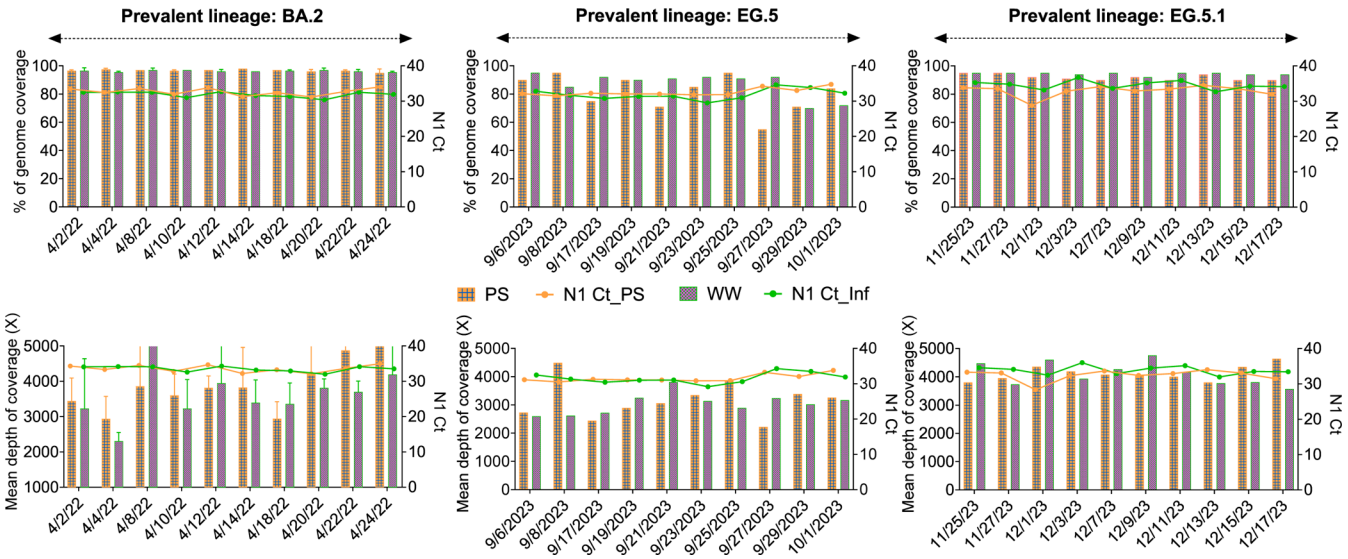

Figure 1. Longitudinal comparison of SARS-CoV-2 genome coverage in influent wastewater (Inf) and primary sludge (PS) across three different periods under the three dominant lineages of Omicron VOC. (A) The percentage of genome coverage (left axis) was plotted against the N1 Ct value (right axis) and (B) The mean depth of coverage (×) was plotted against the N1 Ct value. The percentage of genome coverage and mean depth of coverage on the leftmost graph were shown with error bars, as two technical replicates of each paired influent wastewater and primary sludge samples were sequenced during the first period of sampling.

Thirty pairs of influent wastewater and primary sludge samples were collected during three different periods under the three dominant lineages (BA.2, EG.5, and EG.5.1) of Omicron VOC from ROPEC, Ottawa, Canada. The RT-qPCR of the samples showed that the average N1 Ct values between influent wastewater (mean: 32.75 ± 1.41, range: 30.32 to 36.37) and primary sludge (mean: 32.64 ± 1.64, range: 28.28 to 36.31) were similar (paired *t*-test, $p > 0.05$) (Figure 1). Following RT-qPCR, all of the samples were processed for tiled amplicon sequencing on a MiSeq platform. In the first sampling period, we sequenced two technical replicates of each of the paired influent wastewater and primary sludge samples with paired-end reads of 150 bp, while in the second and third periods, we sequenced the samples using paired-end reads of 300 bp. Thereby, a total of 80 sequence were generated (comprising 40 from the first period and 20 each from the second and third periods), and results showed that 37 (93%) of the influent wastewater and 35 (88%) of the primary sludge samples had genome coverage higher than 90% at 30× depth (Figure 1A). The remaining three influent wastewater and five primary sludge samples with genomic coverage lower than 90% were observed during the second sampling period, which could be due to multiple cycles of freezing and thawing of those samples. These repeated freeze−thaw cycles were required by technical malfunctions in the MiSeq sequencing instrument during that time. Previous studies have also demonstrated that freeze−thaw cycles of SARS-CoV-2 RNA may impact the quality and integrity of the viral genome, potentially affecting downstream analyses such as sequencing.[36,37] However, the average genome coverage in influent wastewater and primary sludge samples was 93.5% ± 5.81% and 91.6% ± 9.02%, respectively, at 30× depth throughout the sampling periods. The calculated depth of genome coverage varied from 1420× to 7394× in influent wastewater and from 1811× to 7750× in primary sludge samples (Figure 1B). The findings indicated that our approach for processing primary sludge successfully recovers SARS-CoV-2 genomes with comparable depth to influent wastewater, overcoming the challenges encountered by other studies.[25,26]

We then explored the potential relationship between Ct values and % genome coverage as well as the depth of genome coverage (×) in influent wastewater and primary sludge samples using Spearman correlation analyses. No correlation ($p > 0.05$) was observed between the Ct value and genome coverage as well as depth of coverage in influent wastewater and primary sludge samples. Overall, our approach demonstrated the ability to recover the near-complete genomes of SARS-CoV-2 from either primary sludge or influent wastewater samples with Ct values of around 36. However, correlation analysis of the current study is aligned with the previous studies conducted using an Illumina sequencing platform via an amplification process[19,38,39] and capture-based methods.[40] The lack of correlation between Ct values and genome coverage or depth of coverage may be attributed to the fact that RT-qPCR assays target specific small regions of the genome, whereas the entire genome is susceptible to degradation in wastewaters. Another possible reason could be the presence of regions within the genome that are nonintact or not amplified to the desired depth (30×).[39]

3.3. SARS-CoV-2 Lineages Prevalance in Influent Wastewater and Primary Sludge. The SARS-CoV-2 RNA found in wastewaters is typically mixed from numerous infected individuals and provides sufficient information on circulating VOCs and lineages within a community.[41−43] To identify the prevalence of multiple VOCs and lineages in the mixed samples, we used the Freyja bioinformatics tool to efficiently recover their relative abundance in influent wastewater and primary sludge samples. Freyja shows effective recovery of variant prevalence from mixed and/or degraded samples with genome coverage as low as 50%.[16] In this study, we obtained a near-complete genome (≥90%) of SARS-CoV-2 from both influent wastewater and primary sludge (see Figure 1), and with similar genome coverage, we compared the SARS-CoV-2 lineages prevalence between influent wastewater and

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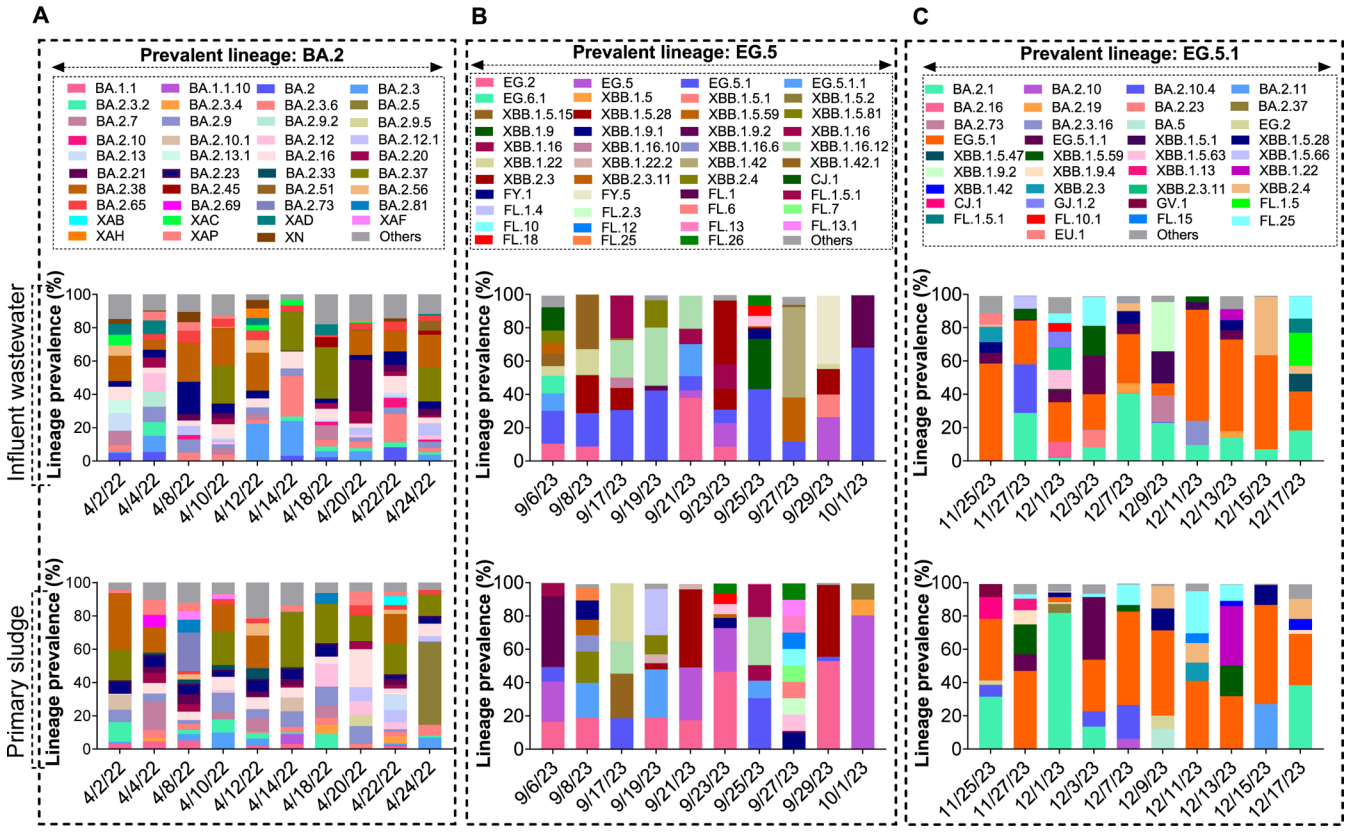

Figure 2. Prevalence (%) of different Omicron VOC lineages in influent wastewater (top) and primary sludge (bottom) was accessed using the Freyja bioinformatics tool under the three dominant lineages BA.2 (leftmost), EG.5 (middle), and EG.5.1 (rightmost). The topmost section of the figures shows the legends for each lineage associated with each sampling period.

primary sludge to determine whether these two common matrices of wastewater followed similar patterns.

In the first period of sampling, Omicron BA.2, a sublineage of Omicron VOC, was predominant in clinical genomic surveillance in the city of Ottawa, Canada,[44] and we compared the prevalence of BA.2 along with other alias of Omicron in influent wastewater and primary sludge samples. A total of 27 Omicron lineages were identified in influent wastewater and 29 in primary sludge samples, with BA.2.37 and BA.2.38 being the most prevalent lineages in both types of samples (Figure 2A). Among the detected lineages, 17 were common to both influent wastewater and primary sludge, while 10 were unique to influent wastewater, and 12 were exclusively detected in primary sludge. Later, several SARS-CoV-2 lineages of Omicron VOC became predominant in Ottawa, Canada. However, in July and August 2023, EG.5, another descendant of the Omicron variant, emerged, raising concerns about the potential for increased infections in the city.[44] In this circumstance, we started monitoring EG.5 in influent wastewater and primary sludge during the second period of sampling. In this period, 25 Omicron VOC lineages were recovered from influent wastewater and 33 in primary sludge, with 16 common to both sample types. In September 2023, EG.2, EG.5, EG.5.1, and XBB.1.5.28 were the prevalent lineages in both influent wastewater and primary sludge (Figure 2B). In late 2023, the JN.1 subvariant of Omicron, the most mutated since parental Omicron VOC, emerged in Canada with the potential for higher infection rates and more severe symptoms.[45] Due to its rapid spread, the WHO and the Public Health Agency of Canada classified JN.1 as a variant of interest.[46] Although the first clinical case of JN.1 in Canada was detected on October 9, 2023, its spread in Ottawa primarily began in late December 2023.[44] To track the early presence of the JN.1 variant in Ottawa, Canada, we began screening in paired influent wastewater and primary sludge samples during the third sampling period. JN.1 was not detected in either sample type which aligned with the clinical sequence in the city of Ottawa.[44] Instead, we identified BA.2.1 and EG.5.1 as the predominant lineages in both sample types (Figure 2C). Unlike the first and second periods, 26 Omicron lineages were found in influent wastewater and 21 in primary sludge, with only 10 lineages being shared between influent wastewater and primary sludge. Overall, throughout the sampling periods, the shared lineages between influent wastewater and primary sludge were detected with higher prevalence, while lineages unique to either influent wastewater or primary sludge were detected with lower prevalence.

3.4. Comparative Analysis of SARS-CoV-2 SNVs in Influent Wastewater and Primary Sludge. SARS-CoV-2 SNVs were detected in influent wastewater and primary sludge samples using an iVAR tool with a minimum quality threshold (30) and minimum read depth (30×).[33] To ensure consistency and minimize interrun variability of SNV calling, paired influent wastewater and primary sludge samples were sequenced within the same Illumina MiSeq run. Furthermore, sequencing was performed in distinct MiSeq runs corresponding to different predominant periods of SARS-CoV-2 Omicron lineages, each characterized by unique mutational signatures. As a result, most SNVs were inherently specific to their respective sequencing runs; however, approximately 40% of

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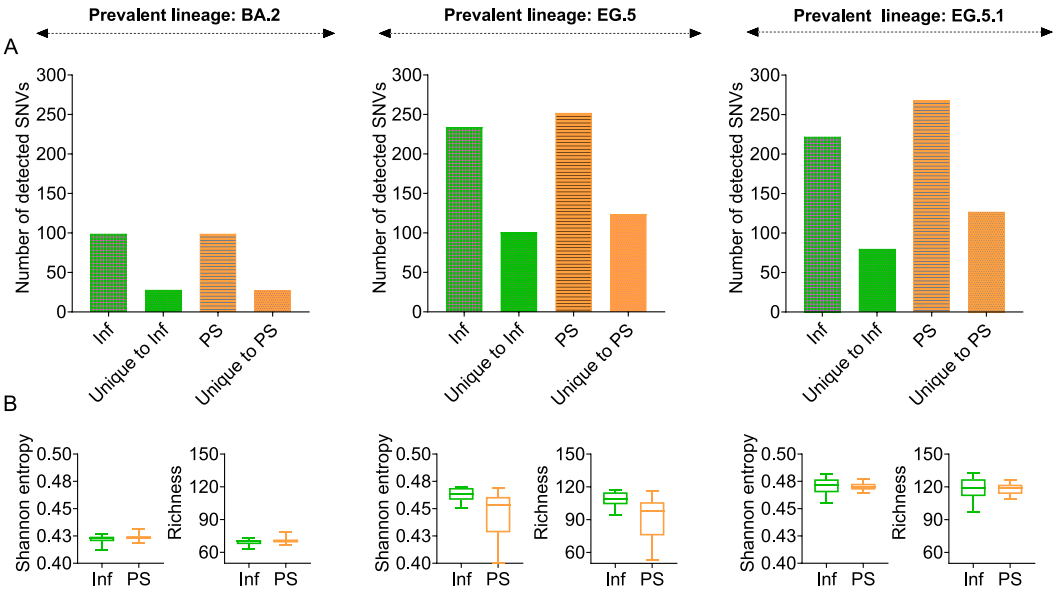

Figure 3. SARS-CoV-2 genetic diversity in influent wastewater (Inf) and primary sludge (PS) samples. (A) Total and unique (detected exclusively in either sample type) SNVs counts in influent wastewater and primary sludge samples across the three sampling periods. (B) Boxplots of Shannon entropy (leftmost of each lineage type) and richness (rightmost of each lineage type) during the periods dominated by lineages BA.2, EG.5, and EG.5.1. The edges of the box represent the first (bottom) and third (top) quartiles, the solid line indicates the median, and the whiskers show the maximum and minimum values.

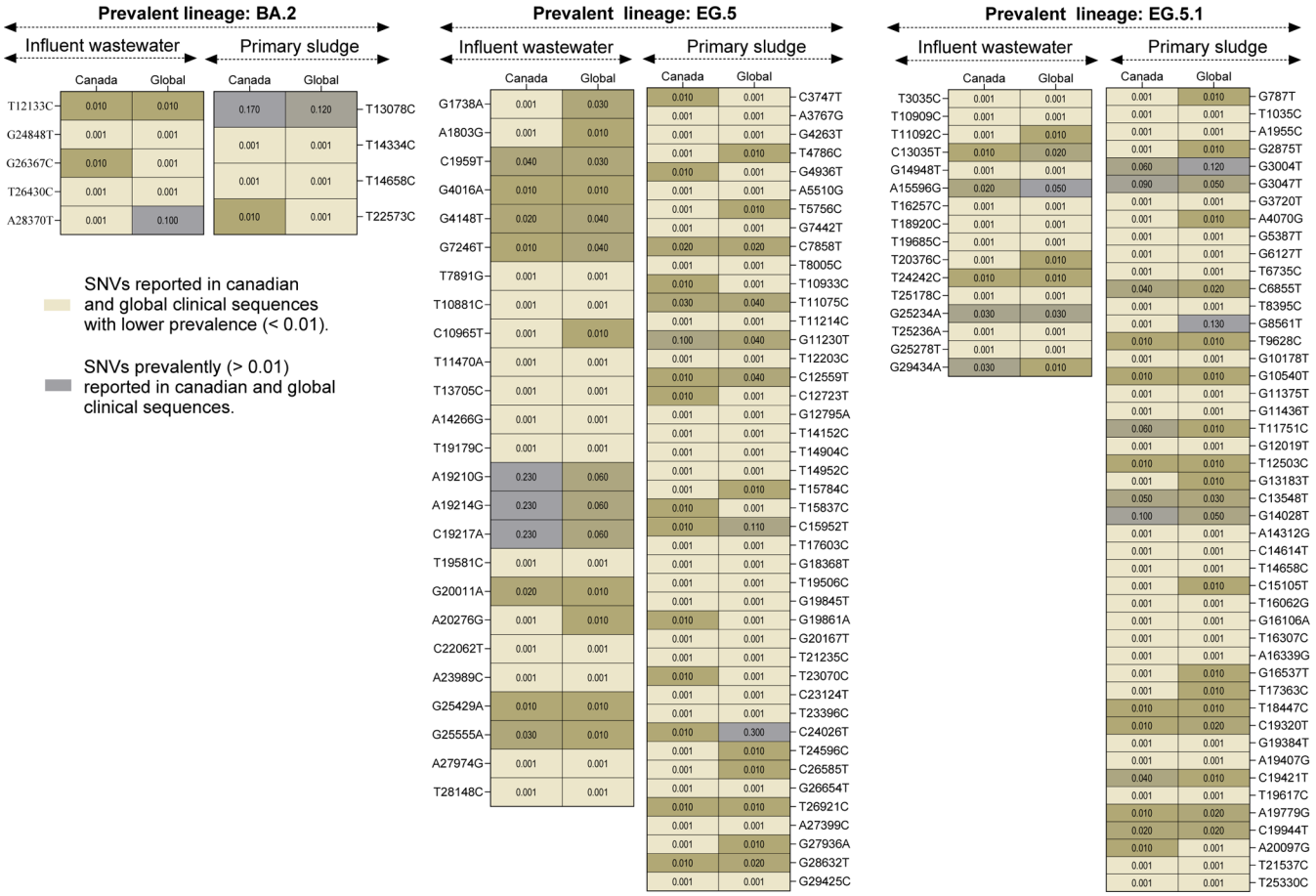

Figure 4. Evaluation of nondetected SNVs in influent wastewater and primary sludge during the sampling periods against the Canadian and global clinical sequences to determine their prevalence. The figure denotes rare SNVs using olive green-colored cells, while commonly reported SNVs in Canadian and global clinical sequences are represented by ash-colored cells.

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SNVs were consistently detected across all of the runs, reflecting a conserved set of mutations shared across multiple SARS-CoV-2 lineages. Overall, results showed that over the course of the monitoring, a total of 555 and 619 SNVs were detected in influent wastewater and primary sludge samples, respectively (Figure 3A). During the first period of sampling, ∼100 SNVs were reported in both influent wastewater and primary sludge samples. In subsequent periods, influent wastewater and primary sludge samples showed higher counts of SNVs in both the second (234 and 252) and third periods (222 and 268), although this trend was not statistically significant ($p > 0.05$). To further define the SARS-CoV-2 genetic diversity between influent wastewater and primary sludge, we employed two commonly used metrics: Shannon entropy, which quantifies the uncertainty in randomly sampling an allele, and richness, which measures the number of SNVs.[33] We observed a statistically comparable ($p > 0.05$) genetic diversity of SARS-CoV-2 between influent wastewater and primary sludge samples throughout the monitoring periods (Figure 3B). Later, we assessed each SNV to determine whether they were common between influent wastewater and primary sludge or exclusively present in either influent wastewater or primary sludge. The results showed that ∼39% (214) SNVs were found exclusively in influent wastewater, whereas ∼45% (278) unique SNVs were identified only in primary sludge. Similar to total SNV counts, comparable ($p > 0.05$) counts of unique SNVs were detected between the two sample types throughout the sampling periods. To access the reproducibility of the sequencing data, we monitored variation in mutation profiles between technical replicates and found consistent SNVs calling between replicates across the paired samples (Figure S4).

3.5. Similar Number of SARS-CoV-2 Rare SNVs Detection between Influent Wastewater and Primary Sludge. We evaluated the detected SNVs in influent wastewater and primary sludge against the Canadian[34] and global[35] clinical sequences during the sampling periods. Results indicated that common SNVs between influent wastewater and primary sludge were detected in both global and Canadian clinical sequences during the sampling periods. However, a total of 46 unique SNVs (5, 25, and 16 in each of the three periods, respectively) in influent wastewater and 93 unique SNVs (4, 43, and 46 in each of the three periods, respectively) in primary sludge were not detected in Canadian clinical sequences during the sampling periods (Figure 4). We then analyzed these unique SNVs, undetected in Canadian clinical sequences during the sampling periods, against the global and Canadian clinical sequences throughout the pandemic (From Jan 01, 2020, to April 03, 2024). The analysis showed that 63% (29 out of 46) of the SNVs unique to influent wastewater and 66% (61 out of 933) in primary sludge were rarely (prevalence <0.01) found in Canadian clinical sequences either before or after the sampling periods. The remaining unique SNVs in both influent wastewater (37%) and primary sludge (34%) were detected in Canadian clinical sequences with higher prevalence (>0.01). All of the unique SNVs (i.e., rare and prevalent) found in influent wastewater and primary sludge samples were detected in global clinical sequences.

4. DISCUSSION

In this study, we demonstrated that our approach of processing primary sludge for sequencing allows the recovery of near-complete SARS-CoV-2 genomes, comparable to influent wastewater. Primary sludge is usually processed using direct sludge processing methods (i.e., centrifugation), resulting in higher viral RNA concentrations compared to influent wastewater.[21−23] Despite the higher concentrations of SARS-CoV-2 RNA extracted from primary sludge, the RNA may contain significant amounts of nontarget genetic material leading to background noise that complicates the identification and assembly of SARS-CoV-2 genomes. To resolve the difficulties in primary sludge sequencing, we diluted primary sludge in deionized water at a 1:70 volumetric ratio, which might remove the impurities as well as potential inhibitors from the primary sludge. We then concentrated on pairing diluted primary sludge and influent wastewater samples using Nanotrap Magnetic Virus Particles and extracted viral RNA using the Qiagen Viral RNA Mini Kit. More exclusively, the beads and dilution may negatively select for PCR inhibitors (polymeric substances, possibly low polarity) or the beads may positively select for a narrow range of size/charge, thus excluding nontargeted particles. The high surface area and specific binding properties of Nanotrap Magnetic Virus Particles likely facilitated the recovery of SARS-CoV-2 genomes from both influent wastewater and primary sludge. In the downstream analysis, we processed all paired samples in parallel, including cDNA synthesis, amplicon generation, and library preparation, and sequenced them in the same MiSeq run. Finally, the percentage of SARS-CoV-2 genome coverage and the depth of coverage were calculated using Mosdepth with default parameters and found to recover a similar SARS-CoV-2 genome from influent wastewater and primary sludge samples. Considering the overall similarity of the sequencing methodologies and bioinformatics pipelines employed for influent wastewater and primary sludge, our approach demonstrates effective mitigation of background noise and impurities from the extracted RNA of primary sludge, leading to the successful recovery of a comparable SARS-CoV-2 genome from influent wastewater and primary sludge.

With comparable genome coverage of SARS-CoV-2 between influent wastewater and primary sludge, we utilized Freyja[16] to analyze the prevalence of SARS-CoV-2 lineages and iVAR[33] to determine the presence of SNVs in the genome. Genomic analysis indicated that a similar ($p > 0.05$) number of SARS-CoV-2 lineages and SNVs were identified between influent wastewater and primary sludge. The prevalent lineages and SNVs were common between influent wastewater and primary sludge; however, some SNVs were exclusively found in either influent wastewater or primary sludge samples. The detection of these SNVs demonstrates the effectiveness of WWGS using influent wastewater and now primary sludge, consistent with findings from earlier research.[13,16,17] Our study also showed a similar number of rare SNVs detection between influent wastewater and primary sludge. The detection of rare SNVs in both influent wastewater and primary sludge samples may be attributed to the evolving nature of the pandemic. As the pandemic wanes and vaccination rates increase, the severity of the disease diminishes, often presenting as mild flu-like symptoms, leading to fewer individuals seeking clinical diagnosis.[17,47] Thereby, the detection of these SNVs in wastewater and primary sludge confirmed the presence of a lower number of infected individuals in the community who do not undergo clinical diagnosis. In addition, as Ottawa serves as the capital city of Canada and hosts numerous historical landmarks, attracting visitors year-round, these individuals may

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contribute to the introduction of circulating, low-prevalence SNVs into wastewater networks.

Viral particles in influent wastewater are generally present at low concentrations due to dilution, but they accumulate in primary clarified sludge within the settling tanks of wastewater treatment plants through gravitational sedimentation. Therefore, SARS-CoV-2 genomic surveillance should prioritize primary sludge over influent wastewater due to the inherent advantages of sludge as a sample matrix. However, more impurities and enzyme inhibitors may also concentrate in primary sludge, making its sequencing more difficult and challenging. Our approach for processing primary sludge in SARS-CoV-2 genome sequencing overcomes these barriers, allowing for the detection of rare SNVs in primary sludge. Furthermore, sludge provides a more aggregated and representative snapshot of population level viral loads, reducing variability introduced by flow rate changes and rainfall dilution in influent wastewater. These advantages make primary sludge an optimal matrix for robust SARS-CoV-2 genomic surveillance, improving the sensitivity, consistency, and alignment with clinical epidemiological data to better inform public health decision-making. Overall, our approach demonstrates the potential applicability of primary sludge sequencing for recovering whole genomes to detect different variants of emerging pathogens. These insights provide critical information for robust public health strategies during the COVID-19 pandemic and future public health emergencies.

5. CONCLUSION

In this study, we developed a robust approach for processing primary sludge for whole genome sequencing of SARS-CoV-2. Using our approach, we successfully recovered near-complete genomes from approximately 90% of both influent wastewater and primary sludge samples. This demonstrates that our approach can provide valuable genomic data for WWGS, despite the challenges posed by RT and PCR inhibitors, impurities, lower target concentrations, and viral RNA degradation in wastewater. In addition, we identified various lineages and SNVs in SARS-CoV-2 sequences from each influent wastewater and primary sludge sample. Some of these, found at higher prevalence, were common to both influent wastewater and primary sludge, while others, with lower prevalence, were unique to either influent wastewater or primary sludge. The lower prevalent SNVs provided insights into rare mutations in both influent wastewater and primary sludge samples. As a collective, the approaches we have outlined in this study can be utilized within public health settings to inform infectious disease mitigation measures for numerous pathogens and their associated variants, particularly in scenarios in which obtaining clinically derived sequences is challenging.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.5c00142.

Proof of ARTIC amplification at conventionally extracted RNA from primary sludge, extracted RNA from diluted primary sludge at deionized water, extracted RNA from diluted primary sludge at PBS buffer, variation of technical replicates between influent wastewater and primary sludge, and detailed comparison of Ct value between deionized water and PBS buffer (PDF)

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Author Contributions

Md Pervez Kabir: developed research question, experimental design, sample collection, laboratory experiments, bioinformatics analysis, data analysis, prepared and revised the manuscript. **Julio Plaza-Diaz**: assisted in performing laboratory experiments, data analysis, bioinformatics analysis, and revised the manuscript. **Élisabeth Mercier**: supported laboratory experiments and revised the manuscript. **Patrick M. D'Aoust**: supported laboratory experiments and revised the manuscript. **Lawrence Goodridge**: supported bioinformatics analysis and revised the manuscript. **Opeyemi U. Lawal**: supported bioinformatics analysis and revised the manuscript. **Shen Wan**: revised the manuscript. **Nada Hegazy**: revised the manuscript. **Tram Nguyen**: revised the manuscript. **Chandler**

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Wong: revised the manuscript. **Felix Addo**: revised the manuscript. **Elizabeth Renouf**: reviewed statistical analysis and revised the manuscript. **Tyson E. Graber**: supervised the study, reviewed experimental design and data analysis, review, and revised the manuscript. **Robert Delatolla**: supervised the study, reviewed experimental design and data analysis, funding acquisition, review and revised the manuscript.

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Notes

The authors declare no competing financial interest.

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