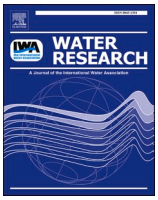
Water Research 285 (2025) 124071

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Passive sampling for genomic surveillance of SARS-CoV-2 in wastewater resource recovery facility: Insights for pandemic preparedness

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ARTICLE INFO

Keywords:
Wastewater surveillance
Genome sequencing
Single Nucleotide Variants
Lineage prevalence
COSCa-ball
Torpedo

ABSTRACT

Passive sampling provides a cost-effective alternative to conventional auto-sampling and serves as a valuable approach for wastewater surveillance in resource-limited settings. However, the feasibility of passive sampling for SARS-CoV-2 wastewater genomic surveillance (WWGS) remains underexplored in wastewater resource recovery facility (WWRF). In this study, we collected influent wastewater samples using an autosampler, COSCa-ball and Torpedo passive samplers from inlet of WWRF serving the city of Ottawa, Canada. We enriched, extracted, quantified, and sequenced samples targeting SARS-CoV-2 genome using a widely used ARTIC tiled amplicon approach. Our findings show that daily SARS-CoV-2 RNA levels were similar ($p > 0.05$) across the sampling methods. Although similar viral RNA was captured from passive samples, we found genomic recovery of SARS-CoV-2 from passive samplers was influenced by targeted sequencing read length, with shorter (300 bp) reads resulting in lower recovery than longer (600 bp) reads. Our study confirmed near-complete recovery of SARS-CoV-2 genomes ($\geq$ 90 %) from the autosampler, COSCa-ball, and Torpedo samplers using longer reads. Genome sequencing parameters such as the number of raw reads, trimmed reads, mapped reads, depth of coverage and % of genome coverage was identical ($p > 0.05$) among the sampling methods. Genomic analyses showed similar ($p > 0.05$) single nucleotide variant profiles (SNV) and lineage prevalence across sampling methods, and concordance with the available clinical surveillance. Overall, the findings suggest that passive sampling of wastewater is a viable, cost-effective alternative for population-scale genomic surveillance of SARS-CoV-2 and may allow for surveillance of other pathogens, supporting future pandemic preparedness efforts.

1. Introduction

The COVID-19 pandemic has transitioned into an endemic phase, yet it remains a formidable threat as SARS-CoV-2 continually evolves, giving rise to diverse variants that enhance transmissibility, disease severity, and evade immune defenses (Gangavarapu et al., 2023; Harvey et al., 2021). Identifying and tracking them has become critical to early and effective public health responses. However, the sensitivity of existing strategies for variant detection through clinical genomic surveillance has suffered due to widespread vaccination efforts which has lessened the severity of infections and the need for clinical diagnosis (Ling-Hu et al., 2022; Robishaw et al., 2021). Besides, clinical genomic surveillance is costly, confined to symptomatic patients, and susceptible to sampling bias due to healthcare disparities that disproportionately affect poorer and underserved communities (Lieberman-Cribbin et al., 2020; Majid et al., 2020; Reitsma et al., 2021). In contrast, WWGS has emerged as a robust tool for monitoring the emergence and spread of SARS-CoV-2 variants, effectively overcoming sampling bias and economic constraints as well as offering a non-invasive approach to track the circulation patterns of different SARS-CoV-2 variants at the population levels (Fontenele et al., 2021; Karthikeyan et al., 2021; Pérez-Cataluña et al., 2022). WWGS entails the collection of wastewater

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https://doi.org/10.1016/j.watres.2025.124071
Received 5 March 2025; Received in revised form 31 May 2025; Accepted 20 June 2025
Available online 22 June 2025

samples, followed by target enrichment, RNA extraction and sequencing to identify and characterize the distinct strains or lineages of SARS-CoV-2 circulating within the population (Ai et al., 2021; Li, Uppal, et al., 2022; Vo et al., 2022). This approach allows for the early detection of prevalent SARS-CoV-2 lineages on both regional and national scales (Amman et al., 2022; Crits-Christoph et al., 2021; Izquierdo-Lara et al., 2021), as well as demonstrated the detection of circulating and cryptic (i.e., not encountered in clinical surveillance) lineages in the population level (Jahn et al., 2022; Karthikeyan et al., 2022; Smyth et al., 2022).

WWGS has predominantly relied on composite samples, often collected using an autosampler, from WWRF or grab sampling of sewer shed access points (Nemudryi et al., 2020; Spurbeck et al., 2021; Vo et al., 2022). However, auto-sampling is expensive, and requires electricity and maintenance, while grab samples are prone to intra-day variation, making it less representative to population-level disease prevalence (Augusto et al., 2022; Harris-Lovett et al., 2021). As an alternative to traditional auto and grab sampling, passive sampling has garnered attention for its simplicity and cost-effectiveness in wastewater surveillance (Habtewold et al., 2022; Kevill et al., 2022; Vincent-Hubert et al., 2022). In passive sampling, sampling mediums (i.e., medical gauzes, cheesecloth, cellulose sponges, or electronegative membrane filters) are packed within styrene-like plastic devices and deployed in sewer networks to sorb viral particles over time (Bivins et al., 2022). The sampling mediums are then homogenized in deionized water or soluble buffer to extract total RNA for downstream analysis. This technique is simple, requires little maintenance and no mechanical components, and is particularly useful in locations with limited infrastructure and limited access to electricity, where long-term sampling is desired. Although passive sampling has demonstrated success in wastewater surveillance, it remains underutilized in WWGS of SARS-CoV-2. To date, only five studies have focused on SARS-CoV-2 genomic analysis using passive sampling. Among these, Corchis-Scott et al. (2021) and Mangwana et al. (2022) employed allele-specific RT-qPCR for the detection of lineage-specific mutations, while Cha et al. (2023), Alamin et al. (2024) and Overton et al. (2024) sequenced the SARS-CoV-2 genome for comprehensive variant identification. However, Cha et al. (2023) was unable to recover near-complete genome ($\geq$ 90 %) from any of the wastewater samples collected by Moore swabs, whereas Alamin et al. (2024) reported successful recovery of SARS-CoV-2 genome from the majority of wastewater samples collected through Tampon swabs. Despite the potential of passive sampling in WWGS, their applications were primarily limited to tracking SARS-CoV-2 lineages in congregate settings with low sewer flow rates, such as student dorms at university campuses and wastewater from airports. However, the implementation of passive sampling in larger, high-flow environments such as the inlet of WWRF for genomic analysis remains unexplored. In these settings, passive sampling may face challenges, as the high-flow, turbulent conditions could reduce the efficiency of capturing and retaining viral particles on the sampling medium. Besides, there are concerns about the integrity of RNA species in such conditions, as high-flow may degrade RNA more rapidly, potentially impacting the quality of genomic data retrieved through passive sampling. Moreover, earlier studies (Fontenele et al., 2021; Nemudryi et al., 2020) suggest that recovering SARS-CoV-2 genomes requires at least 2.8×10^5 viral copies per liter in wastewater. However, later findings indicated that genome recovery depends heavily on sample processing and concentration methods (Chen et al., 2024; Feng et al., 2023). Thus, it remains uncertain whether passive sampling in WWRF and current processing methods can be effectively utilized for genomic analysis of SARS-CoV-2. Additionally, it is unclear whether SARS-CoV-2 lineage profiles obtained from passive sampling would be comparable to those from conventional auto sampling and clinical surveillance. This underscores the need for further research to evaluate the efficacy of passive sampling at the inlet of WWRF in order to support the continued and broader application of WWGS for monitoring respiratory pathogens and emerging disease targets.

The aim of this research is to evaluate the feasibility and accuracy of passive sampling in detecting and quantifying the distribution of SARS-CoV-2 lineages from the inlet of WWRF. To validate the application of passive samplers for genomic analysis in WWRF, we sequenced and analyzed wastewater samples collected by an autosampler, a COSCa-ball, and a Torpedo passive sampler from the inlet of the Robert O. Pickard Environmental Center (ROPEC) in Ottawa, Canada. The WWRF serves a sewered population of approximately 1 million in the nation's capital, with an average flow of 4.35×10^5 m^3/day and a hydraulic residence time of the sewer network ranging from 2–35 h, averaging around 12 h.

2. Materials and methods

2.1. Wastewater sample collection and processing

A total of 75 wastewater samples composited over a 24-hour period were collected in parallel during a period from March 14 to May 4, 2023, using an autosampler (n = 25) (Teledyne ISCO, Lincoln, NE, USA), COSCa-ball (n = 25) and Torpedo sampler (n = 25) from the inlet of WWRF, Ottawa, Canada. The COSCa-ball and Torpedo sampler housing unit were printed in polylactic acid filament using an Ultimaker 2 + 3-D printer (Ultimaker, New York, USA), following the designs described by Hayes et al. (2021) and Schang et al. (2021) respectively. The COSCa-ball and Torpedo samplers are designed with multiple entry points to allow wastewater to pass through, effectively trapping wastewater solids in the absorbing mediums secured within the cavity. In the COSCa-ball sampler, four swatches of medical gauze (Swisspers, China) were used as the absorbing medium whereas in the Torpedo sampler, two swatches of medical gauze were used, due to the smaller diameter. The gauze used was a soft, absorbent, medical-grade material composed of 100 % cotton, with a weight of approximately 90 g/m^2, individual swatch dimensions of 10 cm $\times$ 10 cm, and a thread diameter of approximately 200 μm (Swisspers). The autosampler was used to collect 24 h composite wastewater sample while COSCa-ball and Torpedo samplers were deployed for a period of 24 h. Following each sampling day, 500 mL composite wastewater sample was collected using an autosampler, and the medical gauzes from passive samplers were retrieved in 50 mL conical tubes. All samples were transported to the laboratory in ice-cooled packs. Wastewater samples collected by autosampler were allowed to settle for 30 min. All the gauzes were placed in 500 mL of deionized water and vigorously agitated with a glass rod for one minute to recover the sorbate (Fig. 1). The gauzes were subsequently squeezed using the glass rod to recover the sorbate and discarded. The mixture was then allowed to settle for 30 min to facilitate the sedimentation of larger particles. This settling step enhances the recovery of SARS-CoV-2 genetic materials from wastewater by minimizing interference from non-specific debris.

2.2. Determination of suspended solids

To evaluate the suspended solids load in the processed samples used to concentrate and extract SARS-CoV-2 RNA using the Nanotrap magnetic particles across different sampling methods, we determined Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS). Accordingly, TSS and VSS were measured from the 40 mL of supernatant obtained after a 30-minute settling, which was used as the input for RNA extraction. This analysis allowed us to assess whether variations in solids content across sampling methods influenced SARS-CoV-2 RNA extraction using Nanotrap magnetic particles. To determine the suspended solids of the input used for RNA extraction, ten paired wastewater samples were independently collected from the same WWRF using an autosampler, COSCa-ball, and Torpedo sampler between April 14 and May 6, 2024, following the procedures described in Section 2.1. The TSS and VSS were determined using standard methods (EPA, 2001). Briefly, 40 mL of the samples were filtered through glass fiber filter

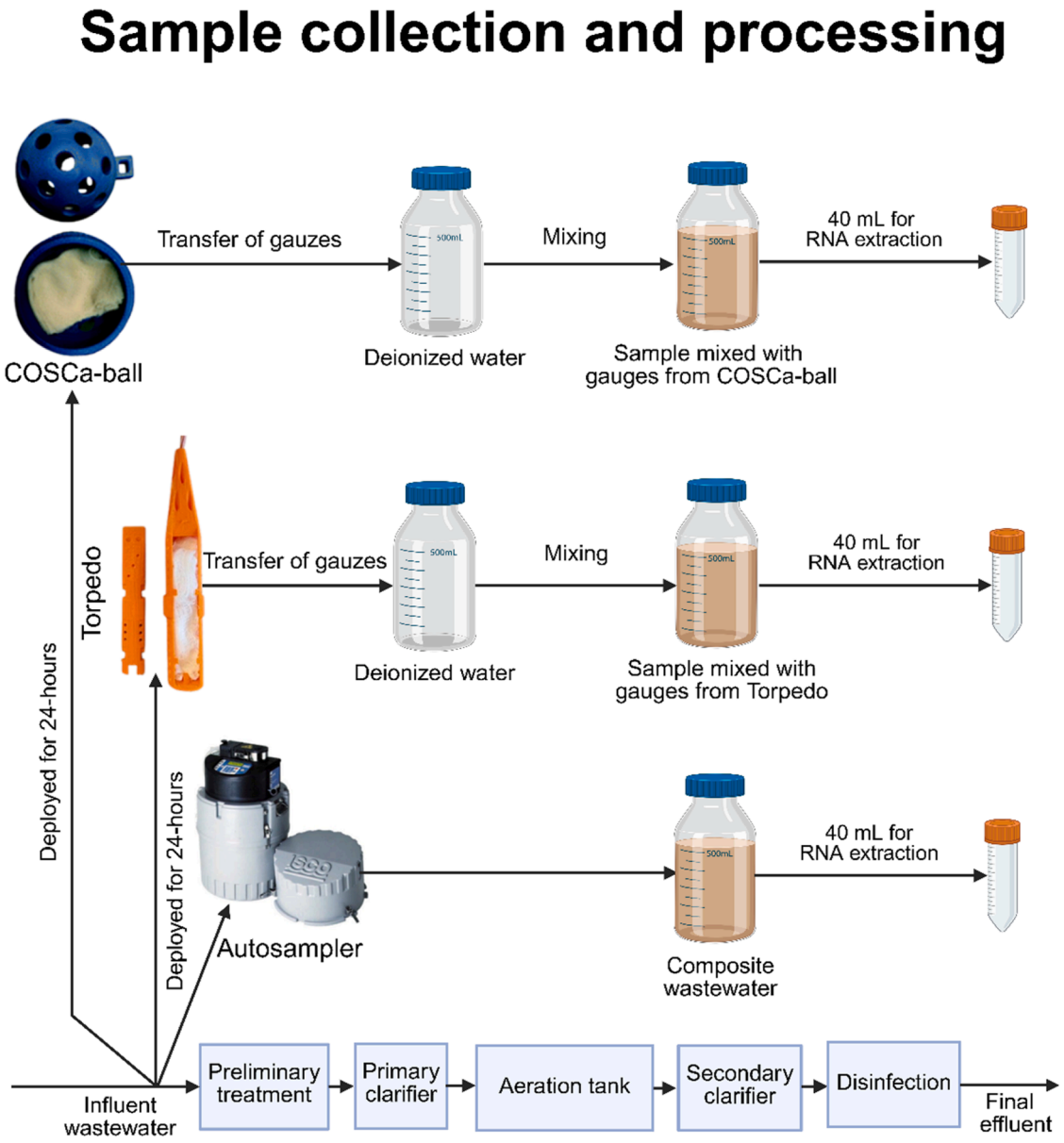

Fig. 1. Schematic displaying collection of wastewater samples using an autosampler along with COSCa-ball, and Torpedo passive samplers, followed by processing for total RNA extraction.

(Thermo-Fisher, Massachusetts, USA) using vacuum filtration (Agilent Technologies, Mississauga, Canada), the filter was then transferred to a pre-weighted aluminum weighing dish (Fisherbrand, Ottawa, Canada). The dishes were placed into a 105 °C oven (Thermo-Fisher) for 3 h, and after the incubation, they were weighed to determine the TSS content. Later, the dishes were placed into a muffle furnace (Thermo-Fisher) operating at 550 °C for 30 min, and the solids lost to ignition were determined as VSS content.

2.3. SARS-CoV-2 RNA extraction

Nanotrap® Magnetic (Ceres Nanosciences, Manassas, USA) hydrogel particles were used to enrich SARS-CoV-2 genetic material as per Kabir et al. (2025a). Briefly, 40 mL of the sample supernatant was transferred to a centrifuge tube, 600 µL of magnetic particles were added, and the tube rotated at 100 rpm for 20 min at 20 °C. Following centrifugation for 10 min at 4 °C at 8000 x *g*, the tube was placed on a magnetic rack (DynaMag-50, Invitrogen, Massachusetts, USA) to retain the particles, and the supernatant was discarded. The bound material was then lysed with 140 µL of PBS buffer (pH 7.4) and 560 µL of Viral Lysis Buffer (Qiagen, Germantown, USA), transferred to a 2.0 mL microcentrifuge tube, placed on a magnetic rack (DynaMag™−2 magnet, Invitrogen) for 10 min to complete the lysis and remove the magnetic particles. The lysate was collected without disturbing the magnetic particles, and total nucleic acid (RNA) was extracted using QIAmp Viral RNA Mini Kit (Qiagen) and eluted in 70 µL of nuclease-free water. For quality control, method blanks were included in each batch of extractions. For wastewater samples, extraction blanks consisted of 40 mL of deionized water processed identically alongside actual samples. For passive samplers, unused gauzes were processed similarly to account for potential contamination introduced during handling or processing. All the blanks consistently tested negative for SARS-CoV-2 and PMMoV RNA, confirming the absence of cross-contamination during extraction. The recovery efficiency of Nanotrap magnetic particles has been compared to various extraction methods by several studies (Banadaki et al. 2024; Karthikeyan et al. 2021), and they identified Nanotrap magnetic particles as one of the most effective methods for recovering viral RNA from wastewater. This method has also been shown to enhance sequencing

workflows by improving upon standard RNA extraction methods (Andersen et al., 2023).

2.4. Reverse transcription (RT)-qPCR and SARS-CoV-2 RNA quantification

The presence of SARS-CoV-2 N1 and N2 targets were confirmed using singleplex one-step RT-qPCR with TaqMan® 1-Step Fast Virus Master Mix (ABI) (Thermo-Fisher) on a CFX Connect qPCR thermocycler (Bio-Rad, Hercules, Canada) according to the established protocol (Kabir et al., 2025b). Briefly, 7 μL of master mix consisting of 2.5 μL of 4X TaqMan® Fast Virus 1-step mastermix (Thermo-Fisher), 0.75 μL of 500 μM primer, and 125 μM probe mixture (IDT, Kanata, Canada), and 3.75 μL of nuclease-free water was mixed with 3 μL of RNA template. The qPCR conditions are as follows: RT at 50 °C for 5 min, RT inactivation and initial denaturation at 95 °C for 20 s, 45 cycles of denaturation at 95 °C for 3 s and annealing/ extension at 60 °C for 30 s. Samples were run in triplicate with RNase-free water as non-template control and quantified using a five-point EDX SARS-CoV-2 standard curve (Exact Diagnostics, Texas, USA). The qPCR assays were performed according to the MIQE guidelines (Bustin et al., 2009), with data analysis being limited to samples that met the following quality control criteria: (a) linear standard curve ($R2 \geq 0.95$), (b) copies/well in the linear range of the standard curve, and (c) primer efficiency (90 % to 120 %). Any sample with a value ± 0.5 standard deviations from the mean of the technical triplicate was excluded from the analysis. The assay's limit of detection (ALOD) of approximately 2 copies per reaction for both the N1 and N2 targets, while the limit of quantification (ALOQ) was 3.2 copies per reaction for N1 and 8.1 copies per reaction for N2, respectively. Moreover, Pepper mild mottle virus (PMMoV) concentrations were measured at multiple dilutions (i.e., undiluted, 1:10, and 1:40) for each sample to assess the presence of PCR inhibitors. The presence of PCR inhibitors was inferred when serial dilution of the RNA extract resulted in no corresponding shift in amplification or Ct values, indicating potential inhibition of the PCR reaction. However, no evidence of PCR inhibition was observed in any of the samples throughout the study period.

2.5. Genome sequencing of SARS-CoV-2

The RNA extracts were first treated using ezDNase™ Enzyme kit (ThermoFisher), followed by reverse transcription with SuperScript IV First-Strand Synthesis System (ThermoFisher). Complementary DNA was used to amplify viral sequences through multiplexed PCR using ARTIC primer set v4.1 and Q5 Hot Start Master Mix (BioLabs, New England, USA). The thermocycling conditions are as follows: 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 expected 400 bp size of the tiled amplicons was verified using the 2100 Bioanalyzer system (Agilent Technologies), cleaned with 0.8x AMPure XP beads (Beckman Coulter, California, USA) and quantified in a Qubit 4 fluorometer (ThermoFisher) using the dsDNA High Sensitivity Kit (Invitrogen). Amplicon concentrations were normalized, and sequencing libraries prepared using Nextera XT DNA Library Preparation kit (Illumina, California, USA). Finally, the libraries were pooled, normalized and sequenced on a MiSeq platform (Illumina) with a read length of 300 bp in paired-end mode.

2.6. Bioinformatics

A customized bioinformatics pipeline (https://github.com/nf-core/viralrecon/tree/2.6.0) was used to analyze the FASTQ files for quality control and mutation detection (Ewels et al., 2020). Briefly, the quality of raw reads was assessed using FastQC (Simon Andrews, 2020), filtered using fastp (Chen et al., 2018) in order to remove adaptor sequences, ambiguous base (N), low quality reads (Phred score < 30), and small fragments (< 50 nt). Filtered reads were then aligned to the SARS-CoV-2 reference genome (accession MN908947.3) using Bowtie2 (Langmead and Salzberg, 2012) with default parameters, and Mosdepth (Pedersen and Quinlan, 2018) was used to generate coverage information along the genome. The aligned reads were sorted using SAMtools (Li et al., 2009) and consensus sequences were generated using iVar consensus algorithm (Grubaugh et al., 2019). The consensus sequences (frequency >50 %) were constructed using map reads with a coverage of $> 10 \times$ and Phred score of > 30. Finally, mutation calling was performed using iVar with minimum frequency threshold (0.01), minimum Phred score (30), and minimum read depth (10 ×).

2.7. Wastewater SARS-CoV-2 lineage estimation

To capture the dynamics of virus evolution and spread, we utilized Freyja bioinformatics tool (Karthikeyan et al., 2022) to infer relative abundance of Pango lineages in wastewater samples. 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 least absolute deviation. All samples were analyzed using Freyja, and the results were plotted against the sampling dates to observe the patterns of lineage prevalence in wastewater collected using autosampler, COSCa-ball, and Torpedo samplers.

2.8. Clinical surveillance data

Clinical genomic surveillance data was obtained from the weekly epidemiological summary of SARS-CoV-2 genomic surveillance in Ontario for the period between March 14 and May 4, 2023 (PHO, 2023). The prevalence (%) of each lineage was compiled to facilitate a comparative analysis with wastewater genomic surveillance data from the corresponding database, and lineages with a prevalence of <1 % were classified as "Other".

2.9. Statistical analysis

Linear fixed-effects model (Bates et al., 2015) was used to compare the daily variation of TSS, VSS, SARS-CoV-2 RNA, genome sequencing parameters, number of SNVs, across autosampler, COSCa-ball and Torpedo sampler. The weekly lineage prevalence was compared across the sampling methods and clinical samples using a zero-inflated model (Hall, 2000). Moreover, the relationship between SARS-CoV-2 RNA concentrations and genome sequencing parameters were evaluated using Pearson correlation analyses. The selection of the statistical test was determined based on the assessment of the normality of the data. In our statistical analysis, we considered p-values <0.05 to be significant. All statistical analyses and plotting were performed in GraphPad Prism 10.2.1 (La Jolla, California, USA), and R software packages (Version 4.3.3).

2.10. Availability of data

All the raw sequences are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the BioProject ID: PRJNA1252733.

3. Results and discussion

3.1. Variation in suspended solids load for SARS-CoV-2 RNA extraction

The TSS and VSS concentrations varied significantly ($p < 0.05$) among the sampling methods (Fig. 2). Post-hoc analysis indicated that the COSCa-ball samples contained significantly higher ($p < 0.05$) TSS concentrations followed by Torpedo and autosampler collected samples. Similarly, and as expected, VSS concentrations were also higher ($p <$

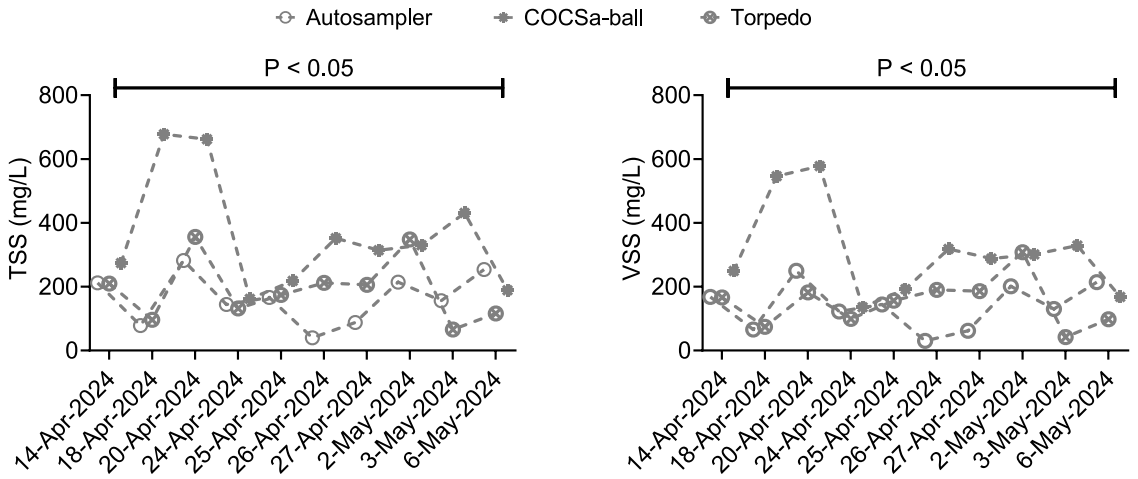

Fig. 2. TSS and VSS concentrations of the 40 mL supernatant used for RNA extraction using Nanotrap magnetic particles.

0.05) in the COSCa-ball compared to autosampler and Torpedo collected samples. In contrast, TSS and VSS concentrations were similar ($p > 0.05$) between the autosampler and Torpedo samples. These variations of TSS and VSS in the COSCa-ball samples were mainly attributed to its design and the amount of sorbent material used within the COSCa-ball casing. The COSCa-ball sampler is a hollow sphere with multiple large entry points, allowing more suspended colloids, solids, and debris to enter the samplers. Additionally, the use of four swatches of medical gauze in the COSCa-ball sampler resulted in the formation of thick layer of solids on the gauzes. When the gauzes were resuspended in deionized water, the solution became less opaque compared to that obtained from autosampler and Torpedo samples, even after 30 min of settling. Ultimately, the supernatant used for RNA extraction from the COSCa-ball sampler is herein shown to contain higher TSS and VSS concentrations compared to autosampler, and Torpedo passive sampler collected wastewaters.

3.2. SARS-CoV-2 RNA levels

The SARS-CoV-2 RNA was detected in all 75 samples (100 %) collected by autosampler, COSCa-ball, and Torpedo passive samplers. Statistical analysis indicated that daily SARS-CoV-2 RNA levels were similar ($p > 0.05$) among the autosampler, COSCa-ball and Torpedo passive sampler (Fig. 3). Our findings align with earlier research (Kabir et al., 2025b; Wilson et al., 2022) demonstrate that SARS-CoV-2 RNA levels in wastewater collected by passive samplers are often comparable to autosampler in WWRFs. However, recovery of SARS-CoV-2 RNA using different sampling methods depends on several factors, including effective deployment time of the passive sampler, selection of appropriate sampling mediums, and sample processing methods (Habtewold et al., 2022; Schang et al., 2021; LaTurner et al., 2021; Li et al., 2021). In this study, we deployed passive samplers over 24 h to align with the autosampler collection time and processed both the autosampler and passive samples in parallel using Nanotrap® Magnetic Virus Particles, a suitable method for viral RNA isolation (Antkiewicz et al., 2024; Dehghan Banadaki et al., 2024). Overall, this study showed that similar SARS-CoV-2 RNA levels are obtained between the autosampler and two different passive samplers.

3.3. Illumina platform longer read sequencing improve genomic coverage of SARS-CoV-2 from passive samplers

To obtain SARS-CoV-2 genome, wastewater samples collected using autosampler, COSCa-ball, and Torpedo samplers were simultaneously sequenced on a MiSeq platform. Initially, we sequenced one set of samples from autosampler, COSCa-ball, and Torpedo sampler using 2 × 150 bp run mode. The results showed lower genomic coverage for the COSCa-ball (48 % at 10 × depth) and Torpedo (2 % at 10 × depth) samples, despite generating approximately 2 million (M) raw sequence reads and a high proportion of SARS-CoV-2-mapped reads (Fig. 4.A and Table S1). This disparity may be attributed to differences in the shape of passive samplers and the number of gauzes used, which can affect the retention and particle size distribution of captured solids. The Torpedo sampler may preferentially retain longer SARS-CoV-2 RNA fragments, which are less compatible with short-read sequencing for generating overlapping regions necessary for full genome reconstruction. Further investigation is required to confirm the influences of shape and number

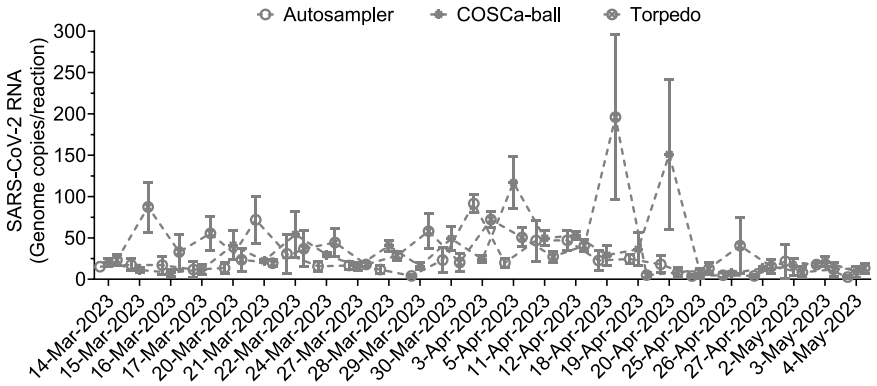

Fig. 3. SARS-CoV-2 RNA levels measured by RT-qPCR in wastewater collected using an autosampler along with a COSCa-ball, and Torpedo passive samplers. The error bar represents the standard deviation of SARS-CoV-2 RNA levels from the mean of the replicates.

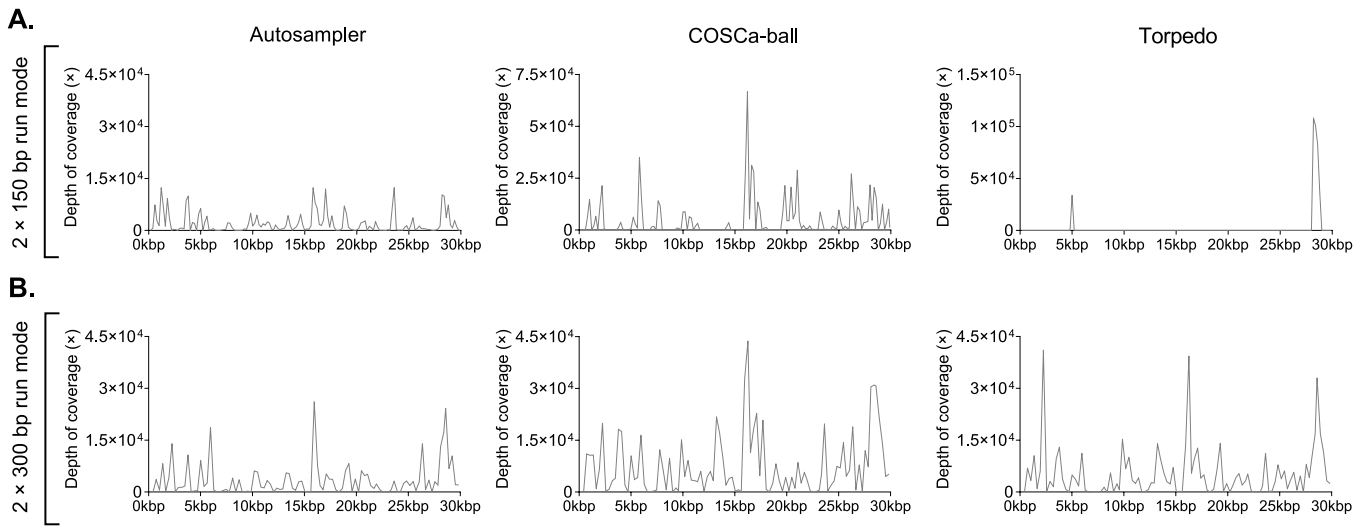

Fig. 4. Genomic coverage of 150 bp paired-end run mode (A) and 300 bp paired-end run mode (B) across autosampler, COSCa-ball, and Torpedo samples. The 150 bp paired-end run mode yielded lower genomic coverage, with COSCa-ball achieving 48 % at 10 × depth and Torpedo only 2 % at 10 × depth, while the near-complete genome (≥ 90 %) was recovered from autosampler, COSCa-ball, and Torpedo samplers using the 300 bp paired-end mode.

of sorbent materials used in passive samplers on sequencing performance using short-read platforms. Later, we subsequently re-sequenced the same set of samples on a MiSeq platform in 300 bp paired-end run mode and successfully recovered near-complete consensus genomes (≥ 90 %) with nearly the same number of raw reads as the 2 × 150 bp run from the autosampler, COSCa-ball, and Torpedo samples (Fig. 4.B and Table S1). The difference in genomic coverage between the 150 bp and 300 bp paired-end run mode suggests that sequencing length may have a significant role in SARS-CoV-2 genome recovery from passive samplers. This is possibly due to the fact that longer sequencing read lengths (i.e., 2 × 300 bp) can enhance genome assembly by providing greater overlap between reads, improving the resolution of complex or variable regions, and increasing overall coverage across the genome.

3.4. *Overview of SARS-CoV-2 genome sequencing parameters across sampling methods*

We sequenced all 75 samples of this study on a MiSeq platform using a 2 × 300 bp run mode, and results showed that the average number of raw reads obtained from autosampler, COSCa-ball, and Torpedo samplers were 2.61 $M \pm 0.43$ M, 2.56 $M \pm 0.38$ M, and 2.41 $M \pm 0.38$ M, respectively (Fig. 5.A). On average, 38.5 % of the reads were filtered out for each sample, yielding high-quality trimmed reads: 1.62 $M \pm 0.38$ M for auto, 1.58 $M \pm 0.31$ M for COSCa-ball, and 1.46 $M \pm 0.26$ M for Torpedo sampler. An average of 1.53 M (94.5 %) trimmed reads from the autosampler, 1.51 M (95.6 %) from the COSCa-ball, and 1.34 M (91.9 %) from the Torpedo sampler were mapped with the SARS-CoV-2 reference genome sequence (accession MN908947.3). The number of mapped reads varied from 0.55 M to 2.12 M (mapping rate: 46.49 % to 99.82 %) for the autosampler, 0.85 M to 2.07 M (mapping rate: 80 % to 99.81 %) for the COSCa-ball, and 0.82 M to 1.86 M (mapping rate: 54.29 % to 99.87 %) for the Torpedo sampler (Table S2). After mapping to the reference genome, the average genome coverage of SARS-CoV-2 at 10 × depth was 93.68 % ± 6.52 % (range: 69 % to 99 %) for the autosampler, 95.28 % ± 3.06 % (range: 88 % to 99 %) for the COSCa-ball, and 96.12 % ± 1.98 % (range: 90 % to 98 %) for the Torpedo sampler (Fig. 5.B). Among the 25 samples collected using each type of sampler, near-complete genome (≥ 90 % coverage) at 10 × depth was recovered from 84 % of the autosampler samples, 92 % of the COSCa-ball samples, and 100 % of the Torpedo samples. The calculated median depth of coverage varied between 1168 × to 6392 × for the autosampler samples, 1391 × to 6396 × for the COSCa-ball and 1810 × to 5682 × for the Torpedo sampler (Fig. 5.C). However, no significant differences ($p > 0.05$) were observed in the total number of raw reads, trimmed reads, mapped reads, percentage of genome coverage, and depth of coverage among autosampler, COSCa-ball, and Torpedo samplers. Overall, the

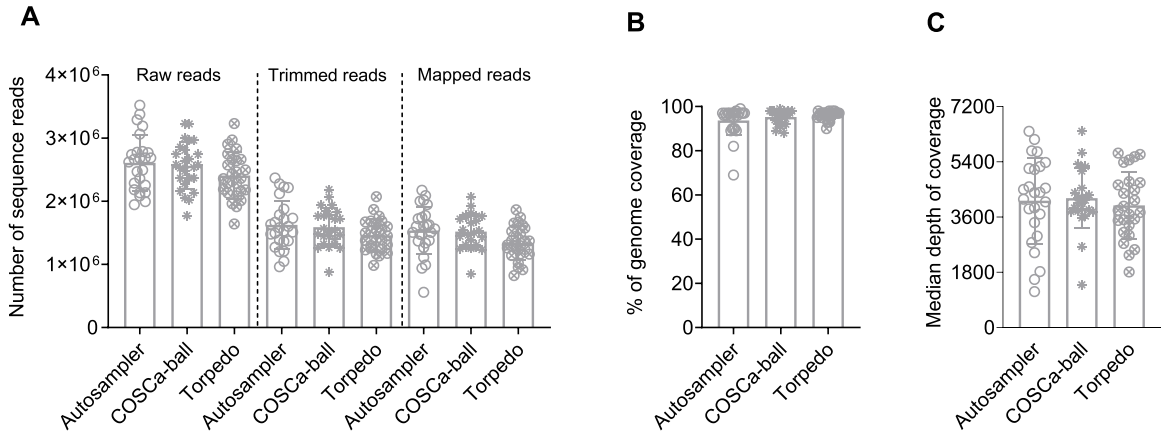

Fig. 5. SARS-CoV-2 genome sequencing parameters in wastewater across three different sampling methods. (A) Total number of reads, trimmed reads, and SARS-CoV-2 mapped reads, (B) % of genome coverage at minimum 10 × read depth, (C) median depth (×) of coverage.

results of this study suggested that SARS-CoV-2 genome sequencing parameters were consistent across different sampling methods, indicating that genomic data were not limited by the sampling technique.

We further investigated the potential relationship between SARS-CoV-2 RNA concentrations and sequencing parameters using Pearson correlation analyses, and no significant correlation ($p > 0.05$) was observed across the different sampling methods (Table S3). This demonstrated that genome sequencing parameters, particularly genome coverage, were not dependent on SARS-CoV-2 N1 or N2 RNA concentrations, as near-complete SARS-CoV-2 genomes were successfully recovered from the autosampler at SARS-CoV-2 RNA concentrations as low as 1.29×10^3 copies/L (corresponding to a Ct value of 38). Similarly, near-complete genomes were recovered from COSCa-ball and Torpedo samplers at the lowest SARS-CoV-2 RNA concentrations of 4.17×10^3 and 2.91×10^3 copies/L (corresponding to Ct values ~36), respectively. Our findings contrast with earlier studies (Fontenele et al., 2021; Nemudryi et al., 2020) showing that recovering SARS-CoV-2 genomes from wastewater required a minimum SARS-CoV-2 RNA concentrations of 2.8×10^5 copies/L. This suggests that low SARS-CoV-2 RNA concentrations (specifically N1 or N2 genomic regions) do not adversely affect sequencing parameters; rather, the sample processing method may play a more dominant role. Our hypothesis is in accordance with a recent finding by Feng et al. (2023), which evaluated different sample processing methods and suggested that sample processing is one of the predominant factors influencing success/failure of amplicon sequencing. Moreover, advanced sequencing technologies can enable the recovery of high-quality data, even from samples with very low SARS-CoV-2 RNA concentrations. The assumptions aligned with earlier research, where robust sequencing data were derived from low concentration samples using Illumina platforms via amplification process (Fontenele et al., 2021; Izquierdo-Lara et al., 2021; Pérez-Cataluña et al., 2021), and capture enrichment method (Crits-Christoph et al., 2021).

3.5. Evaluation of SARS-CoV-2 SNVs and INDELs across sampling methods

Genomic analysis showed on an average 105 ± 10, 102 ± 9, and 99 ± 7 individual mutations (SNVs and Indels) detected in the autosampler, COSCa-ball, and Torpedo samplers respectively in comparison with a SARS-CoV-2 reference sequence (Fig. 6.A). Temporal trends exhibited similar ($p > 0.05$) SNVs profile across the sampling methods, with the exception of the autosampler on March 29, 2023, which was possibly due to lower genomic coverage (69 %) on that date (Table S2). This finding aligns with previous research that demonstrated correlations between SARS-CoV-2 mutations and genomic coverage. A study conducted by Crits-Christoph et al. (2021) found that higher sequencing genome coverage improved the resolution of mutations detection in wastewater samples. Similarly, Jahn et al. (2022) showed that genomic coverage influences the ability to detect and quantify mutations of SARS-CoV-2 in wastewater, where higher coverage facilitates more reliable identification of lineages and their defining mutations.

The majority of detected SNVs and Indels in SARS-CoV-2 genome across the autosampler, COSCa-ball, and Torpedo samplers were found in the ORF1ab (40 %) and S (40 %) regions, with the remaining identified in the N region (~7 %), followed by ORF3a (3.5 %), M (3 %), ORF8 (2.25 %), E (2 %), ORF6 (2 %), and ORF10 (0.1 %) of the genome respectively (Fig. 6.B). Of the detected SNVs, ~70 % were missense (resulting in amino acid changes in protein sequences) across all sampling methods, while 21 % were synonymous (not altering the amino acid sequence) in autosampler and Torpedo samples, and 16 % in COSCa-ball samples. Similarly, 5 % of Indels were identified in autosampler and Torpedo samples, with 7 % detected in COSCa-ball samples (Fig. 6.C). It was worth noting that deletions were more common than insertions across all genes across the sampling methods. We categorized four types of SNVs: Nonsense (premature stop codon), nonstop (point

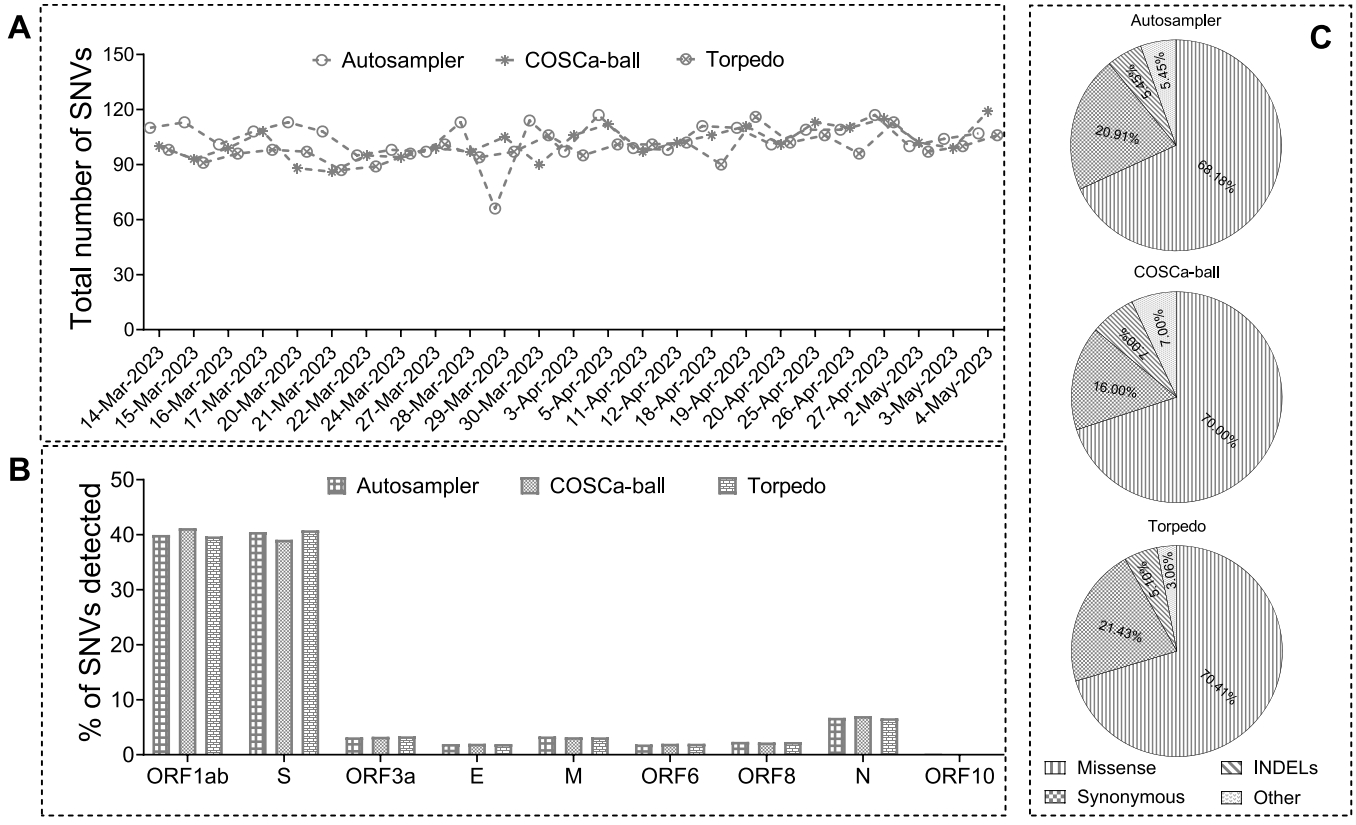

Fig. 6. SARS-CoV-2 SNVs and Indels in wastewater samples collected using autosampler, COSCa-ball, and torpedo sampler. (A) Longitudinal assessment of the total number of detected SNVs in wastewater per sample, (B) their location and (C) type of the detected SNVs and Indels in the genome.

mutation in a stop codon leading to continued RNA translation), upstream (towards 5′ UTR), and downstream (towards 3′ UTR) as "others". Fig. 6.C shows the distribution of these other SNVs across the autosampler, COSCa-ball, and Torpedo samples. Notably, nonsense SNVs (mostly detected in ORF8) were found only in RNA extracted from autosampler and COSCa-ball samplers, none in Torpedo samples. Furthermore, two upstream and one downstream SNVs were detected across the samples. The findings of this study align with clinical sequence analyses regarding the locations and types of SNVs and Indels detected. An early study (Gálvez et al., 2022) conducted in Cambodia during the initial phase of the pandemic revealed that the majority of SNVs and Indels were located in the ORF1ab and S genes, predominantly as missense or synonymous mutations. Subsequently, evaluations of clinical sequences available in the GISAID database corroborated these observations, indicating similar patterns in SNVs and Indels distribution and types throughout the world during five major VOCs (Saldivar-Espinoza et al., 2023). Overall, the number, locations, and types of SNVs and Indels detected in SARS-CoV-2 genome were identical ($p > 0.05$) across the autosampler, COSCa-ball, and Torpedo samplers.

3.6. Comparable SARS-CoV-2 lineages prevalence across sampling methods

As wastewater contains a complex mixture of SARS-CoV-2 genetic material contributed from many different infected hosts and different viral lineages were in circulation during the sampling period, we evaluated the effectiveness of passive samplers in capturing the Pango lineages distribution compared to autosampler. To estimate the prevalence of lineages in RNA extracted from autosampler, COSCa-ball, and Torpedo samplers, we employed the Freyja bioinformatics tool (Karthikeyan et al., 2022). The analysis showed the detection of two parental Omicron lineages B.1.1.529* and CH.1.1 (alias B.1.1.529.2.75.3.4.1.1.1.1) along with five recombinant lineages from BJ.1 and BM.1.1.1 namely XBB, XBB.1.5, XBB.1.9, XBB.1.16, and XBB.2.3 in wastewater samples collected using various sampling methods throughout the study period (Figure S1).

Among the detected lineages, XBB.1.5 was identified as the dominant lineage across all sampling methods, closely aligning with available clinical surveillance throughout the sampling period (Fig. 7). Notably, while clinical sequences did not report XBB during the sampling period, the wastewater samples identified its presence at approximately 30 % prevalence throughout the study period in nearly all wastewater samples across the three sampling methods. XBB.2.3 was detected in wastewater but not clinical sequences, although these detections were sporadic regardless of sampling method, and were at low prevalence among sampling methods. The prevalence of the Omicron (B.1.1.529*) lineage in clinical surveillance was approximately 20 % at the beginning of the sampling period and gradually declined. In contrast, the prevalence of Omicron in autosampler collected samples were consistently detected at a higher prevalence throughout March 2023, while a lower prevalence was observed for the COSCa-ball and Torpedo samples, although prevalences reached ~75 % in early-to-mid April samples. The prevalence of XBB.1.9 in wastewater collected using autosampler, COSCa-ball, and Torpedo samplers closely aligned with that observed in clinical sequences. XBB.1.16 was first detected in mid-March in wastewater at lower prevalence in samples collected using COSCa-ball and Torpedo samples, while it first detected in both wastewater samples collected by autosampler and clinical samples in early April 2023. Thereafter, its prevalence gradually increased in both wastewater and clinical sequences. The prevalence of "other" lineages in wastewater is shown to gradually increase with time across the sampling methods as well as in clinical sequences. The early detection of XBB.1.16 using COSCa-ball and Torpedo samplers highlights the potential of passive sampling for early identification of emerging SARS-CoV-2 lineage. Earlier studies (Alamin et al., 2024; Overton et al., 2024) have demonstrated the utility of passive sampling as an early warning tool for the detection of SARS-CoV-2 lineages prevalence across various scales. However, longitudinal analysis of lineage estimates revealed some variation in the detection of low-prevalence lineages across the different sampling methods. This variability is likely attributable to differences in the nature and composition of the wastewater solids captured by each sampler. Specifically, certain sampling approaches may have preferentially collected solids shed by individuals infected with particular lineages, while others may have missed them due to spatial and temporal heterogeneity in wastewater composition.

To evaluate the differences in lineages prevalence across autosampler, COSCa-ball, Torpedo and clinical surveillance data, a zero-inflated model was employed. The Zero-inflated model is suitable for datasets

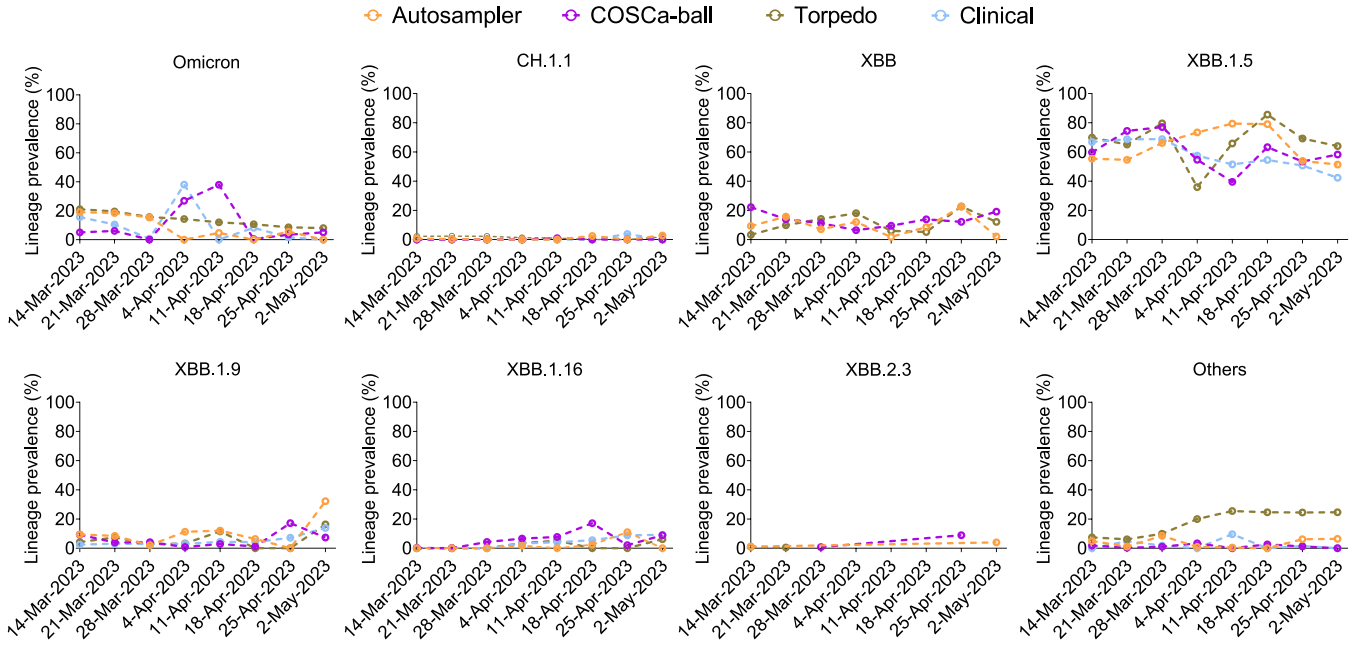

Fig. 7. Weekly SARS-CoV-2 lineage prevalence across autosampler, COSCa-ball, Torpedo, and clinical surveillance data. The lineage prevalence in wastewater across the sampling methods was presented weekly, as the Ontario clinical surveillance data (PHO, 2023) were available on a weekly basis.

with an excess of zeros, as they can accurately fit the mean and variance while distinguishing between true zeros and counts modeled by a negative binomial distribution (Hall, 2000). The analysis showed that lineage prevalence across the sampling methods was comparable ($p > 0.05$) and concordant with the clinical surveillance data. However, although not statistically significant, the prevalence of each lineage in wastewater captured by the autosampler, COSCa-ball, and Torpedo samplers was consistently higher than that observed in clinical sequences throughout the sampling period. This may be attributed to the fact that WWGS reflects lineages prevalence across an entire community, capturing SARS-CoV-2 RNA from both symptomatic and asymptomatic cases, whereas clinical surveillance generally reports only symptomatic individuals (Gupta et al., 2023; Li, et al., 2022). As the lineages prevalence observed in passive sampling was comparable to those obtained through autosampler, this study highlights the potential of passive sampling as a cost-effective alternative to high cost autosampler in large-scale WWRF settings for genomic surveillance of future pandemics beyond COVID-19.

4. WWGS using passive sampling for pandemic preparedness

WWGS has emerged as a valuable tool for monitoring circulating SARS-CoV-2 lineages within population and is based on the recovery of viral RNA fragments found in wastewaters (Khan et al., 2023; Wurtz et al., 2021). Due to its non-invasive nature in tracking the spread and evolution of circulating lineages, many countries have started using WWGS as a complementary surveillance method for population-level screening (Tiwari et al., 2023; Wardi et al., 2024). Despite the promising potential of WWGS for monitoring SARS-CoV-2 lineages, its implementation faces significant challenges arising from the mixed origin (i.e., contributions from multiple individuals), degraded state, and low concentration of genetic material present in these sample types. Additionally, matrix and context-specific factors, such as the presence of PCR inhibitors, can critically affect its success. These complexities highlight the need for improved sample processing and more downstream haplotype phasing methods. Besides, It is also important to recognize the fast-paced progress of this research area throughout the pandemic with significant progress being made in both sample processing and data analysis. However, meaningful challenges remain, particularly for implementing WWGS in countries with limited funding resources. Besides, as the COVID-19 pandemic transitions toward endemicity, funding for WWGS has been gradually declining worldwide, limiting the ability of many regions to maintain robust surveillance efforts. To sustain wastewater surveillance through genome sequencing in a cost-effective manner, it is crucial to address key methodological issues, especially in the fundamental step of wastewater sampling.

WWGS primarily relies on composite wastewater though conventional auto sampling, which is costly, due to high capital, and ongoing operations and maintenance costs, and is limited to locations amenable to these devices (e.g., accessible to a maintenance crew, secure, temperature-controlled, and with adequate flow). As a cost-effective alternative, researchers have piloted passive sampling in small congregate settings, demonstrating its potential as a low-cost method for variant tracking (Corchis-Scott et al., 2021; Mangwana et al., 2022, 2023). However, current funding for WWGS remains directed towards tracking SARS-CoV-2 lineages at the national and city level, often using WWRFs to provide community snapshots for millions of individuals with a single sample. While passive sampling could further reduce costs in these settings, its implementation in high-flow, influent inlets of large-scale WWRFs present significant challenges. High flow rates, fluctuating wastewater composition, and diverse catchment areas can contribute to the dilution of SARS-CoV-2 viral particles and introduce variability. Additionally, SARS-CoV-2 RNA is often fragmented and degraded in wastewater (Anand et al., 2021; Zhakparov et al., 2023), raising further questions about whether passive sampling in such environments can capture sufficient genomic material for sequencing and variant detection. Lastly, methodological standardization remains a challenge, as variations in sampling, processing, and sequencing protocols can lead to inconsistent results, further complicating large-scale deployment of passive sampling.

Our study demonstrated that daily SARS-CoV-2 RNA levels measured by N1, and N2 RT-qPCR were similar among autosampler, COSCa-ball, and Torpedo samples. However, our findings suggested that genomic recovery of SARS-CoV-2 from passive samplers may depend on sequencing read length, as longer read lengths enhanced genome recovery. When sequenced with longer reads (i.e., 2×300 bp) on an Illumina platform, the genome recovery and sequencing parameters for SARS-CoV-2 were comparable across sampling methods. Genomic analyses further revealed that mutation-level (i.e., SNVs and Indels) profiles and lineages prevalence were consistent across sampling approaches and concordant with available clinical genomic surveillance, underscoring the capability of passive samplers to effectively capture viral RNA in high-flow wastewater treatment plants. This indicated that passive sampling is able to sufficiently replicate and preserve SARS-CoV-2 RNA signatures. Overall, similar mutations and lineages profiles emerged across sampling methods highlighting the potential of passive samplers for whole genome sequencing of emerging pathogens in high-flow wastewater contexts.

The 21st century has witnessed the vulnerability of global public health to an array of epidemics and pandemics, including SARS, MERS, 2009 H1N1 influenza, Ebola in West Africa, yellow fever in Angola, Cholera, Zika, Tuberculosis, Mpox, and the continuing HIV/AIDS and COVID-19 pandemics (GPMB, 2024). Additionally, climate change has accelerated the spread of vector-borne diseases such as malaria, chikungunya, Japanese encephalitis, dengue, kala-azar, and lymphatic filariasis, as warmer temperatures and shifting ecosystems create favorable conditions for novel or non-endemic pathogens to emerge or re-emerge as public health threats (WHO, 2024). Meanwhile, antimicrobial resistance (AMR) is increasingly recognized as a silent pandemic due to the global proliferation of antibiotic-resistant genes (ARGs), which threaten to undermine current medical treatments and complicate the control of bacterial infections (Prestinaci et al., 2015). These health emergencies underscore the rising risk of future pandemics, which is evident by recent developments like the spillover of avian influenza H5N1 to cattle and humans which increases the risk of a zoonotic events (Venkatesan, 2023) and the emergence of a novel strain of a more pathogenic Mpox virus in East Africa with potential for global dissemination (Olufadewa et al., 2024). Besides, rising human interconnectedness and mobility facilitate the rapid transmission of endemic pathogens and the emergence of novel variants worldwide. In light of this evolving threat landscape, a proactive and adaptable approach to preparedness is essential. WWGS can therefore serve as a viable option for monitoring the spread and evolution of future pandemics at the population level, if the causative agents (i.e., RNA or DNA) of the diseases are excreted in significant quantities through feces and urine or other means and are able to adsorb be persistent in wastewaters. Implementing WWGS through passive sampling can enhance pandemic preparedness in a cost-effective manner, and findings of this study provide crucial evidence of its applicability of passive sampling in population levels. This reinforces the role of passive sampling may not only as a cost-efficient preparedness strategy but also as a means to advance the One Health approach, which integrates human, animal, and environmental health monitoring to address global health risks comprehensively.

5. Conclusion

This study demonstrated the viability of passive sampling for SARS-CoV-2 genomic surveillance at a high-flow WWRF. Our findings show that daily SARS-CoV-2 RNA levels were similar between conventional autosampler and passive sampling techniques. Although similar viral RNA was captured from passive samples, the recovery of genomes was

influenced by sequencing read length, with longer reads enabling near-complete genome recovery. Genomic analyses revealed that SARS-CoV-2 mutation and lineage profiles were consistent across sampling methods and aligned closely with clinical surveillance data. The simplicity, affordability, and versatility of passive samplers make them a practical alternative to conventional composite sampling for SARS-CoV-2 WWGS and may also be applicable for other targets of public health concern. Overall, these findings support the use of passive sampling as a feasible and effective tool for wastewater genomic surveillance, positioning it as a valuable method in future pandemic preparedness.

Funding

This study was funded by the CIHR Applied Public Health Chair in Environment, Climate Change, and One Health, awarded to Dr. Robert Delatolla. The authors are also grateful to Ontario's Ministry of Environment, Conservation, and Parks SARS-CoV-2 surveillance initiative (awarded to the University of Ottawa) and to the Children's Hospital of Eastern Ontario (CHEO). The funding sources had no involvement in the study design, data collection, data analysis, data interpretation, nor the writing or decision to submit the paper for publication.

CRediT authorship contribution statement

Md Pervez Kabir: Writing – original draft, Validation, Methodology, Formal analysis, Writing – review & editing, Visualization, Software, Investigation, Conceptualization. **Julio Plaza-Diaz:** Software, Investigation, Writing – review & editing, Methodology. **Élisabeth Mercier:** Investigation, Writing – review & editing. **Shen Wan:** Investigation, Writing – review & editing. **Nada Hegazy:** Writing – review & editing. **Chandler Wong:** Writing – review & editing. **Felix Addo:** Writing – review & editing. **Elizabeth Renouf:** Writing – review & editing, Software. **Opeyemi U. Lawal:** Validation, Writing – review & editing, Software. **Lawrence Goodridge:** Software, Writing – review & editing. **Tyson E. Graber:** Writing – review & editing, Supervision, Formal analysis, Validation, Methodology. **Robert Delatolla:** Validation, Resources, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that no known competing financial interests or personal relationships influenced the work reported in this manuscript.

Acknowledgments

The authors wish to acknowledge the support and assistance provided by the City of Ottawa's wastewater treatment plant, and Public Health Ontario, as well as all their employees involved in this study. Their time, facilities, resources, and insights were instrumental in the success of this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.124071.

Data availability

All the raw sequences are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the BioProject ID: PRJNA1252733.

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