



Significance of wastewater surveillance in detecting the prevalence of SARS-CoV-2 variants and other respiratory viruses in the community – A multi-site evaluation

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ABSTRACT

Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral genome in wastewater has proven to be useful for tracking the trends of virus prevalence within the community. The surveillance also provides precise and early detection of any new and circulating variants, which aids in response to viral outbreaks. Site-specific monitoring of SARS-CoV-2 variants provides valuable information on the prevalence of new or emerging variants in the community. We sequenced the genomic RNA of viruses present in the wastewater samples and analyzed for the prevalence of SARS-CoV-2 variants as well as other respiratory viruses for a period of one year to account for seasonal variations. The samples were collected from the Reno-Sparks metropolitan area on a weekly basis between November 2021 to November 2022. Samples were analyzed to detect the levels of SARS-CoV-2 genomic copies and variants identification. This study confirmed that wastewater monitoring of SARS-CoV-2 variants can be used for community surveillance and early detection of circulating variants and supports wastewater-based epidemiology (WBE) as a complement to clinical respiratory virus testing as a healthcare response effort. Our study showed the persistence of the SARS-CoV-2 virus throughout the year compared to a seasonal presence of other respiratory viruses, implicating SARS-CoV-2's broad genetic diversity and strength to persist and infect susceptible hosts. Through secondary analysis, we further identified antimicrobial resistance (AMR) genes in the same wastewater samples and found WBE to be a feasible tool for community AMR detection and monitoring.

Abbreviations: AMR, antimicrobial resistance; cDNA, complementary DNA; CDC, Centers for Disease Control; Ct, cycle threshold; COVID-19, coronavirus disease of 2019; DNA, deoxyribonucleic acid; DNase I, deoxyribonuclease 1; Elute, Prime, Fragment High Concentration Mix (EPH3); gc, genome copies; HCoV-OC43, Human coronavirus-OC43; ID, identification; i7/i5, Index 1/Index 2; kDa, kilodaltons; LoD, limit of detection; MD, Maryland; NCBI, The National Center for Biotechnology Information; NGS, next-generation sequencing; NT, nucleotide; NV, Nevada; PCR, polymerase chain reaction; PMMoV, Pepper mild mottle virus; PE, paired-end; QC, quality control; RNA, ribonucleic acid; RNO, Reno-Tahoe International Airport; rPM, reads per million; RSV, Respiratory Syncytial Virus; RSWRF, Reno-Stead water reclamation facility; RT-qPCR, reverse transcription and quantitative polymerase chain reaction; RVP, Respiratory viral pathogens; RVOP, Respiratory Virus Oligo Panel; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation; SE, single-end; STMWRF, Sparks Truckee Meadows water reclamation facility; TMWRF, Truckee Meadows water reclamation facility; USA, United States of America; VOC, variants of concern; VOI, variants of interest; VBM, variants being monitored; WBE, wastewater-based epidemiology; WHO, World Health Organization; WRF, water reclamation facilities.

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1. Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus, responsible for the Coronavirus Disease of the 2019 (COVID-19) pandemic, continues to be prevalent globally. In the United States of America (USA), case numbers are approaching 100 million, with a death rate that has surpassed 1.09 million persons as of 12/05/2022. [1] Infectious diseases are a significant threat to global and public health, with many drivers causing increased spread and transmission. [2] Following CDC direction, many clinical and public health laboratories, academic institutions, and private sectors have contributed significant efforts to monitor virus evolution through next-generation sequencing (NGS) technologies, which have supported tracing efforts and assisted in research on transmission dynamics and host response. [3] However, alternative and complementary technologies are also necessary for this effort as there exist barriers to the accessibility of sequencing technologies (i.e., rising costs and sizeable necessary sequencing volumes). [4,5] Furthermore, leveraging the SARS-CoV-2 monitoring to include co-occurring pathogens in the community, including other respiratory viruses that cause similar symptoms, is critical to accurately determine community health and disease prevalence.

SARS-CoV-2 viral particles are present in the feces of virus carriers and subsequently shed into sewage systems and downstream water reclamation facilities (WRFs). [6,7] Thus, wastewater-based epidemiology (WBE) within these sewage networks and WRFs can provide valuable insight into virus prevalence and variant evolution in sewershed. [8,9] Moreover, WBE shows a low risk of virus infectivity to personnel, although caution should still be used. [10,11] Wastewater samples allow for the anonymous quantification of SARS-CoV-2 viral RNA from individuals in a defined sewershed, representing the overall spatial and temporal prevalence of SARS-CoV-2 in a community at a given time point. [12–14] WBE has been shown to provide earlier detection of circulating respiratory viruses and variants of concern (VOCs), which can appear days to weeks before clinical cases are seen in healthcare settings (e.g., detection of B.1.1.519 preceding the Omicron wave). [15–17] It has also been shown that WBE of SARS-CoV-2 has a strong correlation with clinically diagnosed case numbers; [12] further, the WBE methodology is not dependent on external factors (e.g., testing and reporting rates, reporting lag, and reporting biases). [12,18,19]

In this study, we captured and enriched SARS-CoV-2 in the wastewater, reported site-specific sequencing and variant analysis, and described the implications of these data. Further, we performed a respiratory viral pathogens (RVP) panel analysis for other respiratory disease-causing viruses in these samples. Use of widespread antimicrobial agents during the pandemic could potentially trigger higher antimicrobial resistance (AMR) occurrence in the community. Thus, to contribute additional insights into community health risks due to other pathogens, this study correlated the presence of the AMR genes in wastewater with SARS-CoV-2 and other respiratory viruses. The goal was that analyzing SARS-CoV-2, other RVPs, and AMRs in the wastewater may allow for increased knowledge and clarity of impending infection waves, which may provide insight into the prevalence and evolution of respiratory viruses in the community and inform public health decision-making.

2. Materials and methods

2.1. Sample and study sites

This study was conducted in the Reno-Sparks metropolitan area in Nevada (NV), USA, between November 2021 and November 2022. Seven local sub-sewersheds and influent (pre-treatment) from three water reclamation facilities (WRF) sites were included in the study, representing wastewater collected from about 0.4 million residents. The WRFs ranged from the southern Truckee Meadows region to the northern Reno-Stead region and included the Truckee Meadows WRF

(TMWRF), the South Truckee Meadows WRF (STMWRF), and the Reno-Stead WRF (RSWRF). The Truckee Meadows WRF (TMWRF) represents sewershed catchments from both Reno and Sparks, NV, serving over 205,000 and 115,000 residents, respectively, and has an approximate 121,000 m³/day flowrate (~30 million gallons/day), accounting for about 80% of Washoe County's wastewater. The South Truckee Meadows WRF (STMWRF) serves over 52,000 residents and has an approximate 96,000 m³/day flowrate (~2.5 million gallons/day). The Reno-Stead WRF (RSWRF) serves over 18,000 residents and has an approximate 64,000 m³/day flowrate (~1.6 million gallons/day). In addition to these WRFs, we also analyzed influent from two hotel-casinos to represent Travel-Influenced Sites and combined influent from three sewer sub-catchments with inflow exclusively from residential housing areas (approximately 500 residential units each) to represent Sub-Neighborhoods (Sparks, NV). Two elementary schools, one in Reno and one in Sparks, NV, were also included to represent Elementary Schools in the region.

2.2. SARS-CoV-2 specimen collection and quantification in wastewater

The methodology for virus enrichment and quantification in wastewater was performed as described in our previous study. [20] Briefly, 1 liter (L) of untreated wastewater was obtained after preliminary treatment from facilities between 9:00 a.m. to 12:00 noon and transported directly to the laboratory on ice. Samples were kept at 4 °C until further treatment. Samples were centrifuged at 3000 ×g for 15 min, and the resulting supernatants were sequentially filtered through 1.5, 0.8, and 0.45 µm sterile membrane filters to remove debris and large particles. The resulting supernatant was used to concentrate the viruses. The virus concentration was performed via ultrafiltration using 100 KDa Amicon® Ultra-15 Centrifugal Filter Cartridge Units (Millipore Sigma, St. Louis, MO, USA). Depending on the wastewater virus concentration levels, we processed 60 mL of samples (i.e., to concentrate the viruses to a detectable level). After ultrafiltration, a cartridge of ~500 µL of the concentrate was collected and stored at –80 °C for downstream analysis (unless analyzed that day).

The total RNA from the concentrated samples was extracted using an AllPrep PowerViral DNA/RNA kit, following the protocol provided (QIAGEN, Inc., Germantown, MD, USA). Reverse transcription and quantitative polymerase chain reactions (RT-qPCR) were completed using the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). N1 and N2 primers and probes were used for the RT-qPCR assay, per US-CDC recommendations. [21] The RT-qPCR was conducted by SARS-CoV-2 RT-qPCR Kits for wastewater (Promega, Madison, WI, USA) according to the kit instruction manual. Briefly, each reaction contained 10 µL GoTaq® wastewater Probe qPCR MasterMix (x2), 1 µL N1 and N2, respectively, and PMMoV Primer/Probe/IAC Mix (x20), 0.2 µL GoScript® Enzyme Mix (x50), and 5 µL of the total genomic RNA template, into a total 20 µL solution. The RT-qPCR reaction was then carried out according to the following protocol: RT at 45 °C for 15 min, RT inactivation and GoTaq® activation at 95 °C for 2 min, followed by 40 cycles of 15-s denaturation at 95 °C, 60-s annealing/extension. The plate was read after each cycle.

The RT-qPCR data were analyzed using the CFX Manager Software (BioRad, Hercules, CA, USA). The cycle threshold (Ct) values were determined by the default algorithm in the CFX Manager Software. Each run contained positive and non-template controls. Extraction RNA blanks were included monthly for field and RNA. Calibration curves (0 to 5-log range) were generated with tenfold serial dilutions of SARS-CoV-2 positive control (IDT, Coralville, IA, USA) in the range from 200,000 to 2 genome copies (gc)/µL. Correlation coefficients (R²) > 0.99 were obtained for all calibration curves, with 90% to 100% amplification efficiencies. Each qPCR assay RNA elution had a limit of detection (LoD) of >4 gc/µL, showing >50% positive signal, with Ct values of the lowest-dilution positive control.

For the endogenous biomarker target, pepper mild mottle virus

(PMMoV) was used for concentration method validation (i.e., samples positive for PMMoV but negative for SARS-CoV-2 were considered under LoD for SARS-CoV-2 virus; if both were negative, we reprocessed the samples for confirmation). For the recovery control, human coronavirus-OC43 (HCoV-OC43) was used to evaluate the recovery rate in the wastewater due to its similar envelope structure. The SARS-CoV-2 recovery efficiency was carried out as described previously by Gharoon et al. [22]

2.3. Library preparation and sequencing

Sequencing libraries for genotyping SARS-CoV-2 virus in wastewater were prepared by one of two methods. Wastewater samples for variant analysis (all WRFs and sewershed) collected from November 2021 to July 2022 were processed as previously described by our group in Hartley et al. [23] Briefly, RNA was linearly amplified into dsDNA, sheared, and ligated to Illumina-compatible sequencing adapters with the QIAGEN QIAseq FX Single Cell RNA Library Kit. These PCR amplicons were sequenced as 2×151 . These libraries were then enriched for SARS-CoV-2 sequences with an Arbor Biosciences library enrichment kit and SARS-CoV-2 specific enrichment probes. These libraries were sequenced as 2×60 . These libraries were sequenced as paired reads with the NextSeq 2000 P2 100-cycle sequencing kit. Due to decreasing coverages (described in *Limitations*) as the study period progressed, wastewater samples collected between August 2022 and November 2022 (and select samples acquired before August 2022) were processed with the QIAGEN QIAseq DIRECT SARS-CoV-2 kit according to manufacturer instructions and sequenced as paired reads with a NextSeq 2000 P1 300 cycle sequencing kit. Depending on kit availability, RVP samples were either sequenced as 2×151 or 2×60 .

For the identification of respiratory pathogens in the wastewater, RNA was extracted from samples with Ct values (range 35–37), as previously described. [12] Briefly, samples were treated with DNase I (QIAGEN, Inc., Germantown, MD, USA) for 30 min at room temperature before concentrating through RNeasy Minilute spin columns (QIAGEN, Inc., Germantown, MD, USA). Once concentrated, samples were converted into Illumina-compatible sequencing libraries using either the Respiratory Pathogen ID/AMR Enrichment Panel kit or the Respiratory Virus Oligo Panel (RVOP) according to the manufacturer's protocols (Illumina, Inc.). Briefly, RNA was first denatured using the Elute, Prime, Fragment High Concentration Mix (EPH3) for 5 min at 65 °C. Hexamer-primed RNA fragments were then reverse-transcribed to produce first-strand complementary DNA (cDNA). Second-strand synthesis was performed to complete the cDNA. AMPure XP beads were used to clean up the cDNA for fragmentation. Following the tagment step, premixed Index 1 (i7) and Index 2 (i5) adapters (Illumina, Inc.) were added to the sample and subjected to 16 PCR cycles to amplify the tagged cDNA and incorporate the adapters. Samples were again cleaned with AMPure XP beads. The cDNA was then normalized and consolidated into one-plex samples using undiluted libraries for overnight hybridization at 58 °C. Enrichment of SARS-CoV-2 and RVPs was done via the Respiratory Pathogen ID/AMR Enrichment or RVOP Enrichment Oligos (labeled with biotin), captured with streptavidin-coated beads and washed. The resulting enrichment pools were quantified and normalized using High Sensitivity D1000 ScreenTape® and TapeStation Analysis Software 3.2. Sequencing was performed using an Illumina NextSeq mid-output (2×75) or NextSeq 2000 P2100 cycle (2×50). The generated FASTQs from the sequencing reaction were subjected to the detection of RVP signatures.

2.4. Bioinformatics and data analysis

Single-end (SE) or paired-end (PE) FASTQ files generated from the Illumina sequencing were analyzed using a custom bioinformatics pipeline publicly available on GitHub. Details about the pipeline and setup can be obtained from its GitHub page (https://github.com/Nevada-Bioinformatics-Center/snakefile_freyja_covidwastewater). This pipeline

starts by trimming the reads using fastp (<https://github.com/OpenGene/fastp>) to remove poor-quality bases and adapter contamination. Then, the reads are classified using Kraken2 using its standard taxonomic database (<https://github.com/DerrickWood/kraken2>) to assess the quality and content of organisms sequenced in the wastewater sample. The trimmed reads are mapped to the Wuhan-Hu-1 reference (MN908947.3) using minimap2 (<https://github.com/lh3/minimap2>), and the resulting BAM files are assessed for quality control (QC) using Qualimap (<http://qualimap.conesalab.org/>). A combined QC report of all samples, including fastp trimming, Kraken2 classification, and Qualimap, is then generated by MultiQC (<https://github.com/ewels/MultiQC>). Freyja (<https://github.com/andersen-lab/Freyja>) runs on each sample to recover the relative lineage abundances from the mixed SARS-CoV-2 samples using the mapped BAM files. Freyja identifies the total coverage per sample and the abundances for each type of variant derived from the UshER global phylogenetic tree. Lastly, an aggregated report is generated, which includes viral concentration data and the relative abundances of each SARS-CoV-2 variant. This report is then used to visualize and compare the data across different sites over time and to assess the correlations between viral concentration, the presence of AMR genes, and the relative abundances of different SARS-CoV-2 variants.

2.5. Detection of RVPs and AMR genes

We used an open-source IDseq pipeline (v3.7, <https://czid.org/>) to analyze the presence of RVPs and AMR genes. [24] Briefly, the pipeline performs subtractive alignment of the human genome (NCBI GRC h38) using STAR (v2.5.3), [25] followed by quality filtering with subsequent removal of cloning vectors and phiX phage using Bowtie2 (v2.3.4). [24] The identities of the remaining microbial reads are then queried against the NCBI nucleotide (NT) database using GSNAP-L in the final steps of the IDseq pipeline. [24,26] After background correction and filtering, retained taxonomic alignments in each sample were aggregated at the genus level and sorted by abundance, measured in nucleotide reads per million (NT-rPM), independently for each sample.

3. Data sharing

The data for the study are submitted as BioSamples to NCBI Genbank under BioProject ID PRJNA772783, available at <https://www.ncbi.nlm.nih.gov/bioproject/772783>.

4. Results

In this study, we aimed to determine the presence and concentrations of SARS-CoV-2 in wastewater samples collected from the Reno-Sparks metropolitan area and understand how the concentration of SARS-CoV-2 in wastewater correlates with any emerging variants of the SARS-CoV-2 virus. We also conducted metagenomic analyses to detect other respiratory viruses and genes associated with AMR in the samples. We collected 175 wastewater samples from three wastewater treatment plants (WRFs) and seven regional sites in the Reno-Sparks metropolitan area between November 2021 and November 2022. The recovery rate of the viral genomic RNA was about 23% from the wastewater matrix. The concentration of SARS-CoV-2 gene copies ranged between the 4 gc/μL (LoD) and 8.48×10^5 gc/L (N1 gene) and 3.32×10^6 gc/L (N2 gene). We detected viral genomes with Ct values as low as 38.54 (about 1.35×10^4 gc/L).

4.1. SARS-CoV-2 concentrations in wastewater

Measuring the concentration of SARS-CoV-2 in wastewater can provide insights into the prevalence of COVID-19 in a community and help predict future outbreaks. By analyzing the concentration of SARS-CoV-2 in wastewater samples collected over a 12-month period, we were able to track changes in the virus concentration of COVID-19 in the

area. Longitudinal analysis of genomic RNA copies of the SARS-CoV-2 nucleocapsid genes, N1 and N2, are represented for TMWRF (Fig. 1 [logarithmic gc]) and all other sites (Supplementary Fig. 1 [linear gc/L]). At TMWRF, there were relatively low concentrations of N1 ($\log 3.3 \pm 0.20$) and N2 ($\log 3.67 \pm 0.27$) in the wastewater from November 2021 until early December 2021 (Delta, pre-Omicron period) (mean \pm standard deviation [SD]).

We found that the concentration of SARS-CoV-2 N1 and N2 in the wastewater varied over time. During the Omicron wave (December 2021–mid January 2022), the concentration rose sharply and then returned to early December levels. A similar pattern was observed during the Stealth Omicron wave (April–early August 2022). However, in the later months of the study (October–November 2022), we saw an increase in the N1 and N2 concentrations of SARS-CoV-2 in the wastewater. This pattern was also seen at other WRFs and sewershed. (Supplemental Fig. 1A–E).

4.2. Detection of SARS-CoV-2 variants in TMWRF sewershed

We performed SARS-CoV-2 variant analysis at TMWRF using the updated Freyja variant profiling classification analysis to classify the variants (snakemake pipeline) (Fig. 2). By depicting the variant data in a chronological manner, we visualized SARS-CoV-2 evolutionary inflection points (i.e., the presence of a variant [occurring for short – or long-term] preceding the generation of a new variant(s) that then proceed to become predominant). These inflection points were determined to be Dec 6th, 2021 (beginning of the Omicron wave) with B.1.1.529, and April 8th, 2022 (beginning of the Stealth Omicron wave), with BA.2.12.1. Thus, following these variant introductions, predominant variants during each respective wave were identified as described below. Further, these inflections can also be visualized where significant increases in N1 and N2 (log gc) proceed the Omicron and Stealth Omicron waves, respectively (Fig. 1).

During the Omicron wave (December 2021 through January 2022), the variants with great than 75% prevalence were BA.1, BA.1.1.16, BA.1.1.13, BA.1.1.12, BA.1.1.18, BA.2, and BA.2.3 (Fig. 2). Other variants, including BA.3 and its subvariants, were detected during this time period but were not predominant (e.g., BA.3 was only found in a few weekly samples at a prevalence of <25%). The introduction of the BA.2.12.1 variant led to a new Stealth Omicron wave (April 2022 through October 2022). The predominant variants (>75% prevalence) were BA during this wave. BA.2.12.1, BA.2.3.2, BA.2.12, BA.2.11, BA.5, and

BA.5.10. BA.5 subvariants were the most predominant during this time period, but their prevalence began to decrease in October 2022 (Fig. 2). BA.4 and its subvariants were detected during the Stealth Omicron wave but were not predominant. As plotted, we show respective subvariants with at least two weeks of detection (i.e., variants detected once were reclassified to their parent BA.X variant group). In our most recent TMWRF influent samples, we detected BQ (BQ.1 [24.88%] and BQ.1.1 [0.66%]), BF (mainly BF.7 and subvariants [96.3%]), and BE (BE.1.1.1 [29.68%]) variants. BQ.1 and BE.1.1.1 have established a combined prevalence of 54.31%, surpassing the declining BA.5 variants (41.52%). The prevalence of BQ.1, BE.1.1.1, and BA.5.2.1 variants are increased in recent samplings from the TMWRF sewershed, however, these variants were sparsely detected in the other studied collection sites (Fig. 2 & Supplemental Fig. 2).

4.3. Detection of RVPs in TMWRF sewershed

In addition to analyzing the SARS-CoV-2 variants, we also performed metagenomic analysis to identify other co-occurring human respiratory disease-causing pathogens which allowed us to assess the prevalence of multiple respiratory viruses in the community. (Fig. 3). The results of the metagenomic analysis showed that the prevalence of SARS-CoV-2 was consistently high throughout the study period, while the prevalence of other viruses had a seasonal pattern. For example, the prevalence of human Mastadenovirus increased during the early winter months, while Paraechovirus and Parvovirus (NIH-CQV) had a higher prevalence during the summer months. Low levels of enteric viruses, such as Mastadenovirus (F) and Enterovirus sp. (B and C), were detected throughout the year as expected. Other human respiratory disease-causing viruses that were detected in the metagenomic analysis included Paraechovirus (A), Mastadenovirus sp. (A, B, C, D, E, and G), Influenza (A and C), Rhinovirus sp. (A, B, and C), human Polyomavirus (1), HMO Astrovirus (A), and human Bocavirus.

4.4. Detection of AMR genes in TMWRF sewershed

To investigate the presence of AMR genes in the TMWRF region, we conducted a secondary analysis to identify genes that confer resistance to various antibiotic classes (Table 1). We detected AMR genes that confer resistance to macrolide, beta-lactamase, tetracycline, sulfonamide, glycopeptide, fluoroquinolone, trimethoprim, aminoglycoside, phenicol, and colistin antibiotics. These AMR genes had coverage ranging from 2.34% to 94.28%, and AMR gene reads (per million) ranging from 0.01 to 1911.99 RPM. The most predominant AMR genes in our samples belonged to the macrolide and tetracycline antibiotic classes, followed by beta-lactamase and aminoglycoside. The sulfonamide antibiotic class had two AMR genes detected at a prevalence of >90%. The glycopeptide, fluoroquinolone, trimethoprim, phenicol, and colistin antibiotic classes had fewer AMR genes detected, with 1 or 2 dominant AMR genes in the respective antibiotic class (40–77% prevalence).

4.5. Detection of SARS-CoV-2 variants in RSWRF, STMWRF, and sub-sewersheds

We analyzed the prevalence of SARS-CoV-2 variants at all the tested sites to understand the distribution and evolution of the virus in the community. The results showed that all the sites had a similar prevalence of variants, with slight variations in the order of appearance (Fig. 4). BA.5 was the predominant variant at TMWRF from April 8th, 2022 (inflection point for the Stealth Omicron wave). However, our sample collection at the non-TMWRF sewershed began on May 3rd, 2022. RSWRF showed an increasing prevalence of BA.5 (range 43–99%) over the collection period, with a single sample with a high prevalence of BA.2.12.1 (33.64%) on July 13th, 2022. The first overall detection of BF and respective subvariants occurred on August 3rd, 2022, at RSWRF

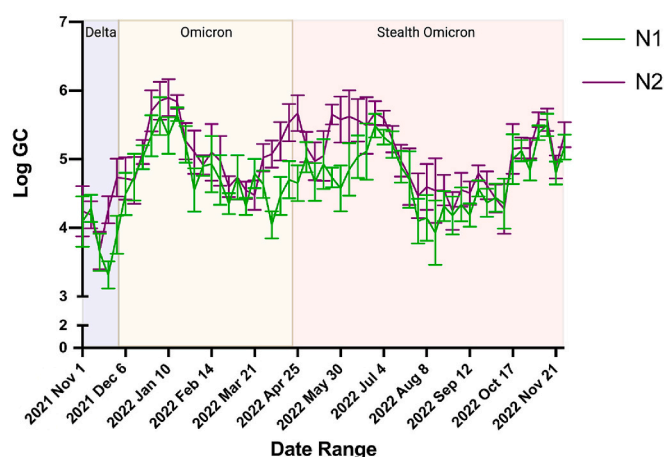


Fig. 1. Longitudinal analysis of genomic RNA of SARS-CoV-2 nucleocapsid genes, N1 and N2. Influent was collected from November 2021–November 2022. RT-qPCR results for N1 (green) and N2 (purple) are plotted over time. Weekly mean logarithmic N1 and N2 GC is plotted with error bars representing standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

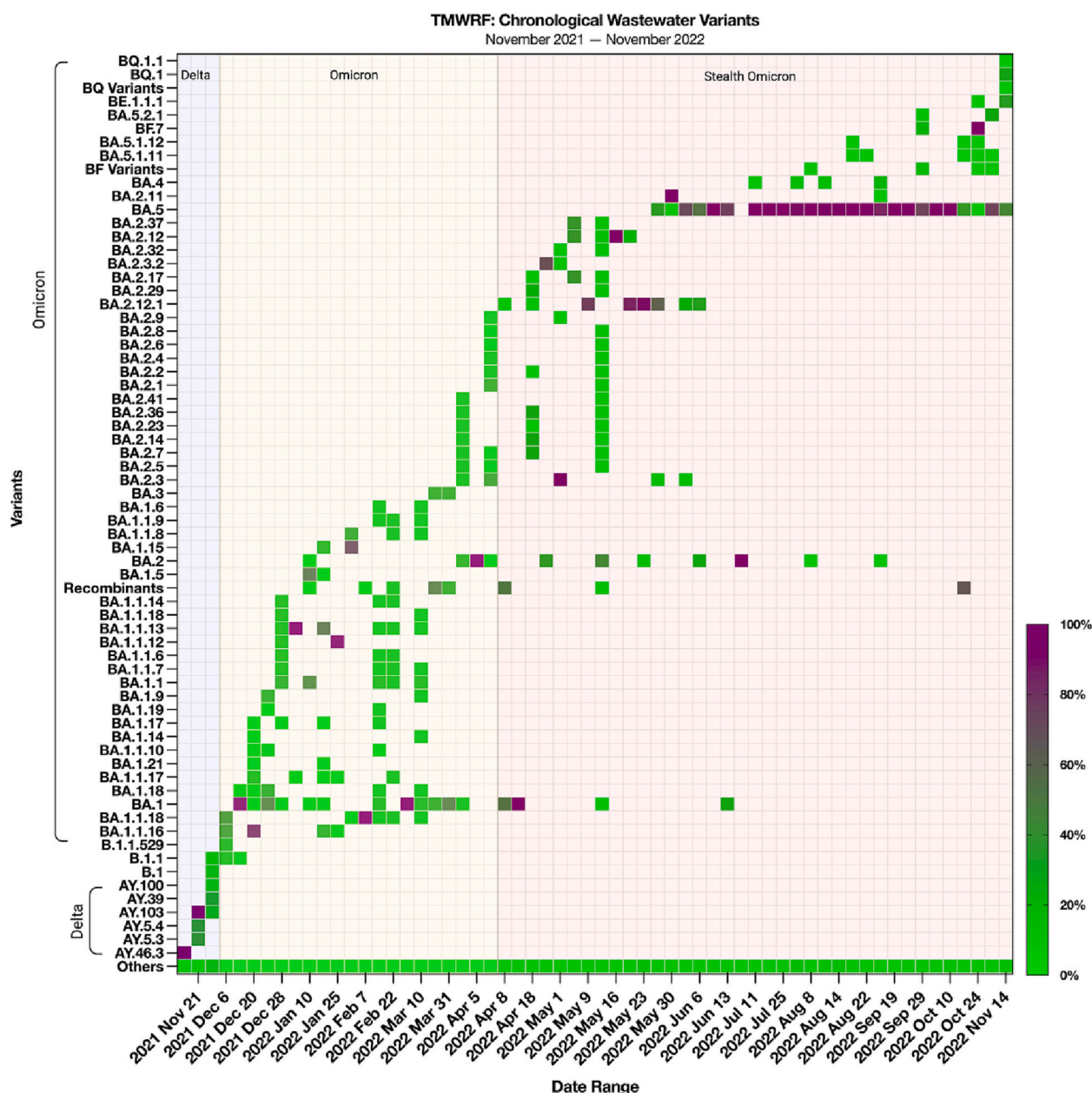


Fig. 2. Chronological detection of SARS-CoV-2 variants in TMWRF sewershed. Influent was collected from November 2021–November 2022. The relative proportion of variants was determined by Freyja, a SARS-CoV-2 variants analysis pipeline. B.1.1.529 (Omicron) was first detected on Dec. 6th, 2021, before the identification of a clinical case of Omicron in Washoe County. BA.2.12.1 (Omicron) was first detected on Apr. 8th, 2022.

*TMWRF geographic location is represented in Fig. 4.

(4.32%), with subsequent detections at STMWRF on October 3rd, 10th, 24th, and November 7th (0.35%, 0.44%, 0.44%, 65.81%, respectively), Travel-Influenced sites on August 9th and 10th, 2022 (10.76%, 55.45%) and Elementary Schools on October 19th, 2022 (74%). There were no detections of BF variants at Sub-Neighborhoods. BG and respective subvariants were detected in the effluent collected from the Elementary Schools on May 3rd, 2022 (97.4%), followed by Sub-Neighborhoods on Jun 14th, 2022 (0.16%), and at STMWRF on Jun 20th, 2022 (0.65%). There were no detections of the BG variants at TMWRF. There was only one low trace detection of BE and respective subvariants in STMWRF on August 29th, 2022; however, there has since been no detection of BE at any non-TMWRF site (as of October 24th, 2022). BQ and respective subvariants were detected as early as October 31st, 2022 in STMWRF, confirming the introduction of this new variant.

5. Discussion

WBE for SARS-CoV-2 analysis is a recent global endeavor to mitigate low testing frequencies and reporting information to gather more comprehensive data on the current status of COVID-19 in a community. [20,27–30] Moreover, WBE has been effective in detecting key variants responsible for the evolution of SARS-CoV-2 infectivity and pathogenicity. [13,15,20,31] In this study, we analyzed multiple WRFs and local collection sites in the Reno-Sparks metropolitan area using RT-qPCR to quantify the presence of SARS-CoV-2 and its variants. To contribute additional insight to the literature, the present study sought to independently assess different regional WRFs and local collection sites for the quantification of SARS-CoV-2 and respective variants via frequent sample collection and by using an up-to-date variant classification bioinformatics analysis. Furthermore, our study is strengthened through

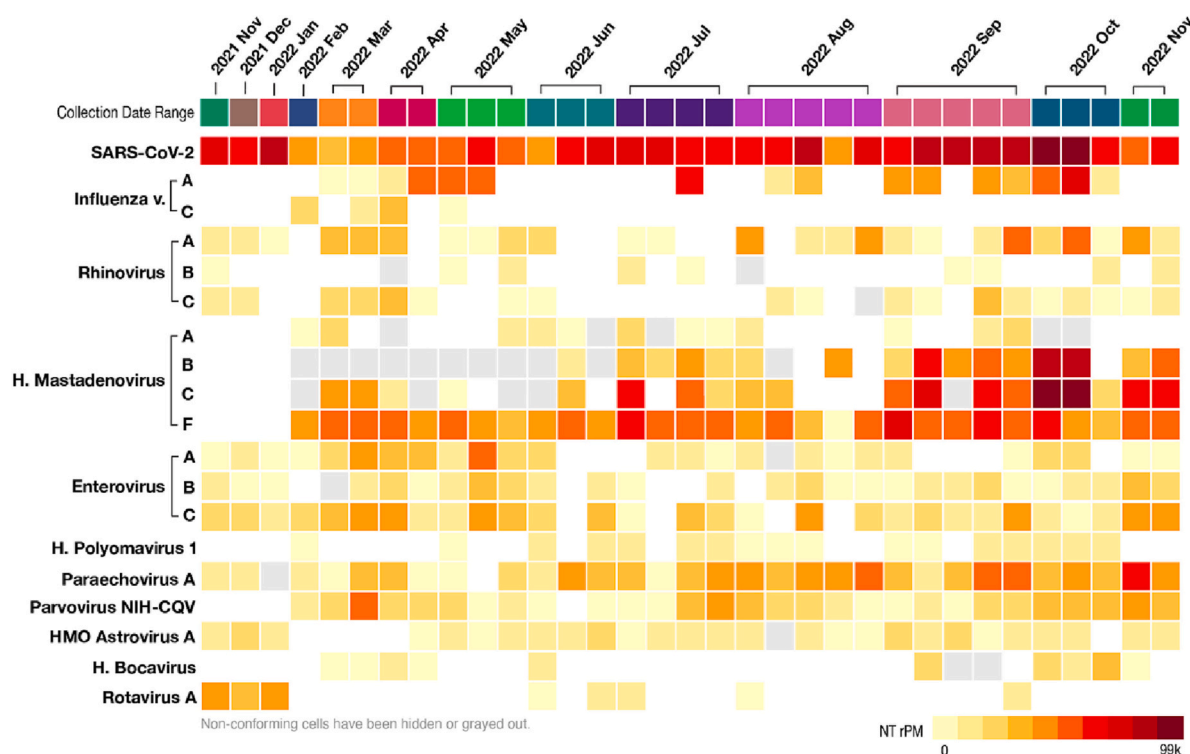


Fig. 3. RVP metagenomic analysis of TMWRF sewershed. Influent was collected from November 2021–November 2022. The relative proportion of SARS-CoV-2 and other RVPs were conducted through sequencing of genomic RNA using an RVP kit (Illumina). The sequences were analyzed through the Pathogen Detection tool of [CZiD.org](https://czi.org). A heatmap was generated based on the number of total reads per million (NT rPM) of each pathogen detected relative to their overall abundances.

secondary analysis and identification of other respiratory disease-causing viruses and AMR genes not included in other respective studies. Our findings provide valuable information on the COVID-19 status in the Reno-Sparks area and demonstrate the usefulness of WBE in detecting circulating viruses. Our data showed detections of new variants. We noted a recent rise in viral genome concentrations that was not reflected in the number of clinically diagnosed cases, possibly indicating a discrepancy between testing and reporting at the community level.

Our RT-qPCR data on the N1 and N2 genes in wastewater show early signs of all new variants (Fig. 1), and continual monitoring for variants allowed for early and specific detection of new VOIs and VOCs. WBE is particularly useful in times of low testing frequencies and can provide comprehensive, up-to-date data on the COVID-19 status of a community, including individuals who are not being tested. This is particularly valuable in rural areas where access to testing may be limited. Previous research has shown that SARS-CoV-2 cases tend to spike in the summer and winter months, which may lead to the emergence of new variants, which, based on the current pattern, could be driving the BE, BF, or BQ variants. [32,33] We also note a recent rise in viral genome concentrations; however, we do not see the same trend in clinically diagnosed cases, alluding to a discourse between testing and reporting at a community level. Thus, WBE of SARS-CoV-2 is once again proving to be an effective tool during times of low testing frequencies and reporting, and further, can be used as a more effective and inclusive (i.e., acquisition of virus prevalence amongst testing and non-testing persons) diagnostic in rural settings to provide public health and city officials of up to date community data, especially as the process becomes increasingly more streamlined and broadly accepted. [34,35]

5.1. Variants and genomic surveillance

There are nationally recognized entities that exist to provide updated information on variants of interest (VOIs), variants of concern (VOCs),

and variants being monitored (VBMs) (e.g., Centers for Disease Control [CDC], World Health Organization [WHO]). [36,37] Current CDC guidelines report pre-Omicron variants as VBMs (e.g., Alpha, Delta, Wuhan strains). One variant is currently reported as a VOC (B.1.1.529); however, no VOIs are currently established. [37] During the beginning of this study (November 2021), almost all the signatures detected were of the Delta (AY) variant, which got replaced following the introduction of the B.1.1.529 Omicron variant (Fig. 1). Since their disappearance in the wastewater, we have not re-detected their signatures (Fig. 2). This was a similar WBE result seen by Galani et al. in Greece (e.g., the Delta variant being replaced by B.1.1.519, signifying the start of the Omicron wave). [38] The B.1.1.529 (Omicron) variant was first detected in our study on December 6th, 2021, which preceded the Omicron wave by one week, and consisted of predominantly BA.1, BA.1.1.18, BA.1.1.12, BA.1.1.13, and BA.2 variants.

Preceding the Stealth Omicron wave of BA.2 and respective sub-variants, we saw the highest prevalence of BA.2.12.1, which we then hypothesized to be the predominant variant in all our study sites to precede BA.5 in high concentrations (Fig. 2). This followed early data reporting that US sequences submitted to GISAID accounted for 26% of BA.2.12.1 and that BA.4 and BA.5 comprised >90% of the genomes sequenced in South Africa. [39] Furthermore, BA.2.12.1 has been observed to overcome immunity from earlier Omicron infections, suggesting that it may cause re-infections and increase infectivity. [40,41] In our TMWRF samples, which represent the most extensive collection area, BA.2.12.1 was detected seven weeks prior to the first sample containing BA.5. Although sample collection dates at non-TMWRF sites are limited, we did see BA.2.12.1 as the predominant variant at two other sites (Sub-Neighborhood and Travel-Influenced Sites) preceding BA.5; however, BA.2.12.1 was predominant alongside BA.5 in two other sites (STMWRF, RSWRF) (Fig. 4). In contrast to B.1.1.529, which had a brief prevalence preceding the BA.2 Omicron wave, BA.2.12.1 had a longer time course before BA.5.

The detection of BF and respective subvariants in our sub-sewershed

Table 1
Antimicrobial resistance gene analysis from wastewater influent.

Antibiotic class	Gene	Sample counts	Avg. coverage of AMR gene (%) [*]	Avg. RPM of AMR gene
Macrolide	MphE	31	89.43	530.71
	MsrE	31	88.66	1193.98
	MefA/Mel	31	66.34	105.50
	MsrD	31	53.02	0.94
	ErmF	30	83.78	382.78
	ErmG	30	79.10	92.04
	Mef(B)	29	38.23	0.52
	ErmB	28	70.91	163.25
	LnuC	28	58.07	71.07
	OXA-7	31	73.61	94.27
Beta-lactamase	OXA-2	31	62.42	204.32
	TEM-1D	31	67.07	104.44
	CfxA	30	70.58	1.11
	OXA-211	27	49.24	99.91
	OXA-5	22	27.93	1.86
	CARB-5/ BlaRTG-2	19	33.71	12.59
	CEPH	15	23.85	1.77
	TetC	31	36.30	0.52
	TetQ	30	94.28	1483.71
	TetX	30	86.70	974.86
Tetracycline	TetW	30	81.13	1911.99
	TetO	30	76.17	566.78
	TetM	30	38.20	65.54
	TetB-P	30	40.45	0.83
	TetA-P	30	38.90	0.60
	Tet-32	29	38.42	126.82
	Tet-39	29	49.00	0.61
	Tet-44	29	53.15	1.16
	Tet-40	26	27.79	1.62
	Sull	30	64.05	173.47
Sulfonamide	SulII	28	52.85	77.65
Glycopeptide	VanA-G	16	20.97	0.08
	VanRD	1	13.59	0.14
	VanTC	2	3.33	0.05
	VanSA	1	2.25	0.10
	VanYD	1	8.15	0.05
	VanRF	1	6.03	0.02
Fluroquinolone	QnrVC5	13	48.67	21.10
	OqxB	24	6.14	0.23
	QnrVC1	8	20.09	5.52
	QnrD	8	38.02	22.66
Trimethoprim	DfrA5	13	46.30	21.41
	DfrA16	8	32.75	3.65
	DfrA3	6	23.79	0.81
	DfrA1/Dfr1	6	39.30	6.84
Aminoglycoside	Aph3-III	30	49.13	116.08
	ANT(6)-Ib	27	54.03	67.68
	ANT(6)-Ia	24	42.64	43.85
	Aac3-I	20	33.91	17.87
	APH(3'')-Ia/ AphD2/AphE	15	20.76	0.14
Phenicol	FloR	3	5.79	0.06
	Cmr	2	9.86	0.03
	CmlB1	2	2.77	0.04
	CatA2	1	17.13	0.03
	CatB	1	4.09	0.11
Colistin	MCR-4.1/ MCR-4	2	3.88	0.03
	MCR-1.1/ MCR-1	1	2.34	0.01

^{*} AMR genes >50% Avg. Coverage Bolded.

sites on August 3rd, 2022 is noteworthy (Fig. 4 and Supplemental Fig. 2). Despite the early detection and rapid drop of these BF and respective subvariants, these variants have the potential to become the predominant strain in the region, as evidenced by the increasing prevalence observed in our largest site (TMWRF): August 8th, 2022, with BF.8 (0.8%), followed by BF.7 (15%) on September 29th, and BF.7 (96.35%) on October 24th, 2022. The last TMWRF sample with the BF variant was on October 31st, 2022, with BF.4 (0.44%). Other sub-sewershed sites also showed detections of BF variants, with RSWRF

detecting BF.3.1 (4.03%) and BF.1 (0.21%) on August 3rd, 2022, and Travel-Influenced sites detecting BF (10.76%) and BF.2, BF.4, and BF.18 (26.14% each, respectively) on August 9th, 2022. Elementary schools also detected BF.12, BF.7, and BF.28 (24.5% each, respectively) on October 19th, 2022, where these BF subvariants were more predominant than BA.5. These data collectively indicate that BF may also become the predominant variant in the region, especially when considering rising abundance at TMWRF.

Our TMWRF data suggests that BQ.1 and BE.1.1.1 are beginning to establish strong predominance in the region, with the exception of STMWRF, which continued to show a higher prevalence of BA.5.2 and BA.5.2.1 subvariants in recent samples (October 24th – November 7th, 2022). The higher prevalence of BQ.1 is in accordance with the CDC COVID-19 variant foresight and prevalence tracker, which uses a NOWCAST model to estimate and predict proportions of circulating variants based on recently circulating variant proportions, is predicting that BQ.1, BQ.1.1, and BF.7 variants to become the predominant future variants in the region including NV (Region 9). [42] Our data indicate that these emerging variants with rising prevalence are consistent with data on major variants circulating in the US, but that smaller sub-regions may be slower to pick up this detection (e.g., BQ variant); thus, large, central wastewater collection is key to the early detection of emerging predominant variants in a defined region. The variant dynamics seen in our study are generally consistent with the trends observed nationally, reinforcing the significance of wastewater surveillance in capturing the regional prevalence of SARS-CoV-2 variants. [3,42]

5.2. RVP and AMR analysis

This WBE analysis consistently detected high levels of SARS-CoV-2 throughout the year compared to other respiratory viruses (e.g., Influenza), which varied seasonally (Fig. 3). This may suggest that SARS-CoV-2 was constantly present in the community but caused fewer clinical cases in the summer months. Further, regional health data from 2021 to 2022 in Washoe County (Reno, NV) showed that Influenza A (93.8%) was more prominent during the same study period in hospitalized patients; however, Influenza C (6.2%) was not identified, whereas Influenza B was prevalent, although in low amounts. [43] Of these hospitalized patients, only 35.6% were vaccinated with the seasonal flu vaccine. Interestingly, other regions in the US did report circulating Influenza B, suggesting that Influenza B and C's viral patterns are consistent with seasonal, and perhaps regional, patterns and may be influenced by factors such as vaccination. Moreover, in our study, the detection of SARS-CoV-2 sub-variants and the seasonal patterns of other respiratory viruses closely align with the CDC's reports on the persistence of SARS-CoV-2 and the seasonal fluctuations of respiratory viruses such as adenoviruses and influenza. [44] This further supports the utility of wastewater surveillance as a reliable tool for monitoring the prevalence of respiratory pathogens in communities.

Cold temperatures are known risk factors for respiratory infections, particularly in the winter, when they can cause irritation and inflammation of the airways, making it easier for respiratory viruses to enter and infect the body (e.g., irritation and inflammation of the airways, making it easier for respiratory viruses to enter and infect the body). [45,46] Furthermore, studies have found that respiratory infections are higher during the winter months as people are more likely to be exposed to cold temperatures (e.g., the rate of Influenza infections in the US was significantly higher at colder temperatures and climates compared to warmer temperatures and climates). [47,48] Another study associated lower temperatures with higher rates of SARS-CoV-2 infection. [32] WBE, paired with secondary analyses for respiratory disease-causing viruses, provides a comprehensive assessment of the total microbial burden in a community and can inform healthcare responses. [49,50]

The introduction of antibiotics has played a crucial role in the treatment of diseases globally, but the simultaneous delivery of these antibiotics has allowed microbes to develop antibiotic resistance,

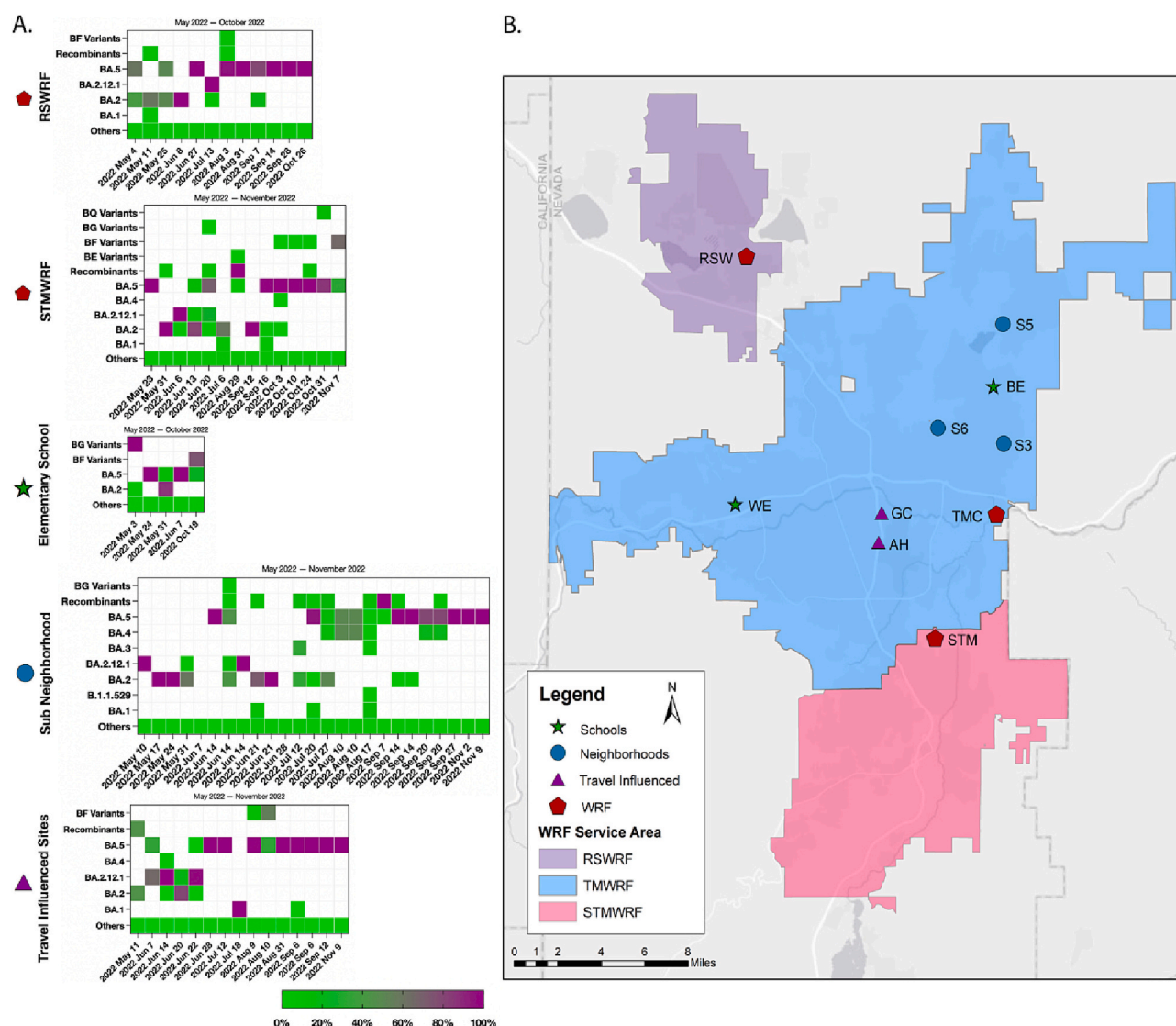


Fig. 4. Detection of SARS-CoV-2 variants in STMWRF, RSWRF, and other sub-sewershed. Influent was collected from May 2022 – October 2022 at two WRFs and 3 sewershed (A). A map of the Reno-Sparks metropolitan area depicting WRFs and sewer shed locations and servicing areas is shown (B). Collective occurrences of the main variants of SARS-CoV-2 Delta and Omicron (e.g., AY.X, BA.1.X, BA.2.X, BA.3.X, BA.4.X, BA.5.X, BE.X, BF.X, BG.X, and BQ.X) were calculated. The relative proportion of variants was determined using Freyja, SARS-CoV-2 variants analysis pipeline. BA.2.12.1 was excluded from summation to depict the significance of detection.

*TMWRF variant data is shown in Fig. 2.

reducing their therapeutic effect in humans and leading to difficulties in the management of many infectious diseases. [51] In addition, the rise in AMR (and associated AMR genes) is responsible for over 700,000 worldwide deaths yearly. [52] Brumfield et al. performed secondary metagenomic, metatranscriptomic, and targeted SARS-CoV-2 analyses on wastewater samples as cases were rising, and found the presence of other pathogens and important spike mutations, as well as potential coinfection insights and AMR genes. [7] Similarly, our study identified significant macrolide and beta-lactamase AMR genes.

A recent study investigated the levels of beta-lactam antibiotics via the *mecA* gene by comparing wastewater samples from hospitals and other healthcare facilities to other sources, and a higher prevalence of the *MecA* gene in hospitals and healthcare facilities. [53] In our study, the most abundant beta-lactamase AMR genes were *MphE* and *MsrE* (89.43% and 88.66%, respectively), suggesting that the increased use of antibiotics may be related to secondary bacterial infections associated with SARS-CoV-2 and may have implications for changes in the human gut microbiome. [54,55] While we found high levels of AMRs for many

classes of antibiotics we also identified classes of antibiotics with lower levels of detected resistance, which may have a potential therapeutic effect against antibiotic-resistant pathogens.

5.3. Study limitations

WBE provides valuable insight into regional virus prevalence and can provide data into circulating viral variants and antimicrobial resistance genes. However, it does not provide specific information on persons infected, and tracing efforts are difficult as wastewater samples are pooled at WRF sites (unless a specific sub-sewershed is used). WBE is a relatively new research approach to the public health and medical field, and a few ethical concerns must be considered. For example, there is the potential for discrimination or stigmatization of individuals or communities based on the results of the research, which may lead to social and economic consequences leading to undermined trust in research and public health efforts (e.g., if there is a high abundance of a particular RVP in a community, they may be targeted and blamed). Thus,

anonymizing or aggregating data is important, as well as developing and following strict protocols for handling and sharing sensitive information. Furthermore, although the more significant sample regional area site (TMWRF) provides more generalizable data, there were slight differences between the smaller sub-sewershed, which indicates a decrease in the degree of generalizability provided by more extensive sample-area data. We also noted a gradual worsening in coverages in our variant analysis as the study period progressed, which required us to switch our amplification method (Qiagen). This could be partly due to the original enrichment oligos not working well due to evolution in the viral sequence, quality issues with the wastewater samples themselves, or degradation of reagents. Lastly, our sample collection, processing, and sequencing methodology were continuously analyzed and updated throughout the study period, which may contribute to some sample variation between early and recent data.

6. Conclusion

6.1. Utility of wastewater-acquired variant data and site-specific monitoring strategies

Overall, our findings highlight the values of WBE for monitoring the presence and dynamics of SARS-CoV-2 in an urban setting, employing multiple fields and expertise in the process (e.g., virology, bacteriology, epidemiology, public health research, and surveillance). We emphasize that WBE can be an essential tool for monitoring the transmission of pathogens between humans, animals, and the environment, providing valuable insights into the community prevalence of SARS-CoV-2 variants as well as other respiratory viruses and AMRs.

By collecting samples from multiple sites, we were able to identify regional differences in virus prevalence and detected the emergence of new variants which preceded the inflections of the two dominant Omicron waves and showed that near-proximity regional differences in virus prevalence exist (i.e., different variants are more prevalent in certain areas). The CDC-NOWCAST model's prediction of the BQ.1, BQ.1.1, and BF.7 variants becoming the predominant future variants in the region aligns with our findings, further supporting the effectiveness of WBE in detecting and tracking the spread of SARS-CoV-2 and its variants. Moreover, this method does not rely on the requirement for persons to self-report or depend on testing infrastructure, as our samples are community-pooled wastewater, which provides better insight into community virus prevalence and supports WBE as a sufficient and acceptable alternative to clinical-based testing surveillance.

Our analysis also identified the presence of other RVPs and AMR genes, providing insight into the dynamics of SARS-CoV-2 co-occurring viruses, which can provide valuable information for healthcare teams. We observed a wave-like pattern of occurrence for respiratory disease-causing viruses aside from SARS-CoV-2 and Mastadenovirus F, as well as the identification of dominant AMR genes, such as MphE and MsrE. Additionally, our analysis revealed the seasonality of certain viruses (e.g., Influenza), with SARS-CoV-2 and Mastadenovirus (variants B, C, and F specifically) levels remaining consistently high throughout the year, while other viruses showed higher prevalence in specific seasons.

CRedit authorship contribution statement

Majid Khan: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Lin Li:** Data curation, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Laura Haak:** Data curation, Writing – review & editing. **Shannon Harger Payen:** Data curation, Validation, Investigation, Writing – original draft. **Madeline Carine:** Data curation, Validation, Investigation, Writing – review & editing. **Kabita Adhikari:** Data curation, Validation, Investigation. **Timsy Uppal:** Writing – review & editing, Validation, Supervision. **Paul D. Hartley:** Data curation, Validation, Formal analysis. **Hans**

Vasquez-Gross: Data curation, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Juli Peterreit:** Data curation, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Subhash C. Verma:** Project administration, Funding acquisition, Resources, Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Krishna Pagilla:** Project administration, Funding acquisition, Resources, Conceptualization, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.onehlt.2023.100536>.

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