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High prevalence of tomato brown rugose fruit virus in domestic wastewater as a potential viral indicator for treatment systems

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Waterborne pathogenic viruses present a critical public health challenge, particularly in potable water reuse systems where stringent safety standards must be met. Achieving the necessary Log Reduction Values (LRVs) for viruses in water treatment remains difficult due to their low prevalence and high detection limits. This study investigated tomato brown rugose fruit virus (ToBRFV) as a novel viral indicator to assess advanced treatment efficacy of advanced treatment systems in achieving target Log_{10} Reduction Values (LRVs) for pathogenic viruses. Over 13 months, wastewater samples from the Reno-Sparks Metropolitan Region (Nevada, USA) were analyzed using metagenomics and RT-qPCR to compare ToBRFV, pepper mild mottle virus (PMMoV), and MS2 bacteriophage. Results demonstrated that ToBRFV was consistently detected at high concentrations across all samples, exhibiting minimal seasonal variability. In contrast, PMMoV showed moderate fluctuations, while MS2 was detected at lower levels. The robustness and stability of ToBRFV suggest it could serve as a reliable indicator for verifying LRVs in potable reuse systems, complementing existing methods. Additionally, its plant-based origin reduces human health risks during handling. These findings support ToBRFV's potential to enhance treatment monitoring and public health safeguards. Further research should validate its applicability across diverse geographic and climatic conditions, as well as its correlation with enteric virus removal, to optimize water reuse frameworks.

Keywords ToBRFV, PMMoV, Viral indicator, High concentration, Low seasonal variability, Potable water reuse

Waterborne pathogenic viruses pose a significant public health risk in various water systems, including wastewater, drinking water, and potable reuse systems. Reuse of reclaimed water to address water scarcity and water security issues is becoming more common. Although, most water reuse systems are employed for irrigation and industrial/commercial uses, potable reuse is becoming more widespread in water deficit regions. Considering an annual risk of infection benchmark of 10^{-4}, target baseline Log_{10} Reduction Values (LRVs) of 13, 10, and 10 for viruses, *Giardia*, and *Cryptosporidium*, respectively have been suggested[1]. This implies that treatment systems must achieve a 13-log_{10} unit reduction of pathogenic viruses from untreated wastewater to the final purified water to meet safety standards for potable reuse as drinking water. A critical challenge emerges because laboratory or field data cannot conclusively demonstrate the required LRV credits due to their low pathogen prevalence in untreated wastewater and even lower concentrations in the final effluent of full-scale water reclamation facilities (WRFs). This gap highlights the urgent need for reliable viral indicators that can be consistently detected and monitored at various stages of treatment processes.

Tomato brown rugose fruit virus (ToBRFV) emerges as a promising candidate for such an indicator due to its high prevalence in untreated wastewater and detectable concentrations that mimic the behavior of pathogenic viruses[2–5]. Monitoring ToBRFV offers a novel approach to validate LRV regulations in advanced water purification systems intended for potable reuse applications. ToBRFV was first identified in some tomato plants in Jordan in 2015[6] and was subsequently reported to be found in Israel as early as 2014[7]. To date, this plant virus has been reported in over 35 countries[8]. ToBRFV, a single-stranded positive-sense RNA plant virus, has a genome of approximately 6.4 kb. The virus is encapsulated within rod-shaped virions, about 300 nm long and 18 nm in diameter. *Tobamovirus* virions are known for their extreme stability, enabling them to survive for extended

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periods in many environmental systems[8]. Natarajan et al.[3] suggested that ToBRFV, due to its high prevalence and abundance in human stool and wastewater, can serve as a reliable microbial source tracking (MST) marker. They recommend further research to explore the global applicability of ToBRFV as an MST marker and to confirm the robustness of the developed RT-PCR assays across different environmental and geographical systems. The typical concentrations of pathogens in raw wastewater used for quantitative microbial risk assessment include 4.6-$\log_{10}$ gene copies per liter (gc/L) for norovirus[9], 4.2-$\log_{10}$ cysts/L for *Giardia*, and 2.3-$\log_{10}$ oocysts/L for *Cryptosporidium*[1]. Given the average concentrations in the raw wastewater and the high detection limit of molecular biology-based methods, most pathogens in the treated water were below the detection limit[10]. Therefore, introducing ToBRFV as an indicator can directly address the challenge of using pathogen monitoring data to prove that potable reuse systems meet the LRV credits because of its high abundance in water systems.

Potential viral indicators should be consistently detectable, with fewer than 25% of source water samples falling below detection limits[11]. They must also demonstrate persistence through various treatment processes, enabling consistent tracking of their reduction. Furthermore, these indicators should be easily detectable and quantifiable, ensuring reliable monitoring throughout the treatment process[11]. These indicators should also demonstrate strong positive correlations or exhibit similar seasonal trends as other target viruses, ensuring relevance and reliability in monitoring[12]. Pepper mild mottle virus (PMMoV) has been recognized as one of the most abundant viruses in human feces for many years, a discovery initially made by Zhang et al.[13]. PMMoV is present in processed pepper products such as dried spices and sauces. When these products are consumed, PMMoV persists through the human digestive system and is excreted at high concentrations in feces, subsequently enters wastewater systems. Due to its dietary origin and stability in the wastewater environment, PMMoV serves as a significant indicator virus in wastewater studies[11,14,15]. According to a study conducted in Egypt, PMMoV was detected in approximately 94% of untreated wastewater sampled, with concentrations ranging from 4.6-$\log_{10}$ to 8.5-$\log_{10}$ gc/L[16]. Gearhart and Pagilla[17] used PMMoV and norovirus as viral indicators to assess the efficacy of soil aquifer treatment in a bench-scale study, demonstrating that the LRVs exceeded 2.72 across three different infiltration rates. Both as plant virus, ToBRFV showed high concentrations in wastewater[4] and higher persistence during different treatment[5], which make ToBRFV more promising as a viral indicator than PMMoV. While enteric viruses may have lower persistence, their low initial concentrations often prevent the empirical measurement of LRVs across treatment trains, as they fall below detection limits. The high abundance and persistence of ToBRFV ensure it remains quantifiable, making it a promising indicator for demonstrating the removal efficiency of advanced treatment processes designed to achieve stringent LRV targets. Another well-established indicator/surrogate widely applied is MS2 bacteriophage (MS2). MS2, a non-pathogenic virus that infects *Escherichia coli*, is widely used as an indicator virus in environmental and public health microbiology due to some advantageous characteristics. For example, it is highly stable under various environmental conditions, and structurally similar to many enteric viruses such as noroviruses and hepatitis A virus[18,19]. These attributes make MS2 an excellent surrogate for studying the behavior and fate of pathogens in environmental settings[20,21], but the detection can be difficult when in low concentrations, leading to false negatives and underestimating water contamination risks[22]. In addition to MS2, other bacteriophages like crAssphage have emerged as highly specific and abundant markers for human fecal pollution[23,24]. For this study, our comparison focused on the plant viruses ToBRFV and PMMoV, and the process control surrogate MS2, to evaluate ToBRFV's unique potential based on its exceptional stability and concentration.

As part of wastewater-based-epidemiology efforts in the Reno-Sparks Metropolitan Region (Nevada, USA), we conducted prolonged wastewater surveillance for 13 months from November 2021 to November 2022 to monitor different microorganisms and indicators in a municipal wastewater system. In this study, we found through preliminary metagenomics analyses and RT-qPCR results that the abundance of ToBRFV was consistently high with little seasonal variations. This observation prompted the focused investigation into its potential as a viral indicator. Building on previous research, this paper aims to demonstrate the high abundance of ToBRFV in untreated wastewater through long-term monitoring and to compare its correlations with other viral indicators and pathogens, ultimately evaluating ToBRFV's potential as a reliable viral indicator for advanced water treatment processes.

Materials and methods

Sample and study sites

Untreated wastewater was collected from seven sub-sewersheds in different neighborhoods and three water reclaimed facilities in Reno-Sparks metropolitan area, NV, USA. Truckee Meadows Water Reclaimed Facility (TMWRF) is the largest facility in the study area, serving over 320,000 residents with an approximate 115,000 m^3/day wastewater flow rate, covering approximately 80% residents in the study area). One liter 24-hour composite wastewater samples were collected daily from TMWRF between November 2021 and November 2022. South Truckee Meadows Water Reclaimed Facility (STMWRF) serves over 52,000 residents, with a flow rate of approximately 10,000 m^3/day. One liter grab sample was taken every Monday at around 9:00 am from STMWRF. The third sample site was the Reno-Stead Water Reclaimed Facility (RSWRF), representing over 18,000 residents with 6,000 m^3/day flow rate. One-liter composite samples were collected every Wednesday. We also collected bi-weekly grab wastewater samples from sub-sewershed sites within the region, including two travel-influenced sites (hotel-casinos), three sub-neighborhoods (residential areas), and two elementary schools. Those grab samples were collected between 9:00 and 11:00 a.m.

Viral RNA collection and quantification

The method for virus enrichment and quantification in wastewater was performed as described in our previous study[25,26]. Briefly, 250 mL wastewater was pretreated by centrifugation and sequential filtration (1.2, 0.8, and 0.45 μm) to remove large particles. Then, 60 mL of the resulting sample was concentrated by 100 kDa Amicon®

Ultra-15 Centrifugal Filter Units (Millipore Sigma, St Louis, MO, USA) to enrich total nucleic acid and virus particles in wastewater by ~ 150 fold. The concentration factors was calculated for each sample according to our previous studies[27,28]. The human coronavirus OC43 (HCoV-OC43) was used as a surrogate to evaluate virus recovery in the concentration method. A volume of 100 µL of HCoV-OC43 was spiked into 180 mL of wastewater and processed using the Amicon ultrafiltration method. The recovery rate of HCoV-OC43 from untreated wastewater was 24 ± 2%, according to our previous studies[27].The concentrated wastewater was kept and stored at -80 °C until downstream analysis unless they were analyzed the same day. We acknowledge that the initial centrifugation and filtration steps, while necessary to remove inhibitors for downstream analysis, may have resulted in the loss of some particle-associated viruses. The viral load in the solids fraction of the wastewater was not quantified in this study, and future work could explore this to provide a more complete picture of the total viral load. Total RNA from the concentrated wastewater samples was extracted by the AllPrep PowerViral DNA/RNA Kit (Qiagen, Germantown, MD, USA). To reduce data variability caused by sampling and virus enrichment, and to prevent multiple freeze-thaw cycles, weekly RNA samples (Monday to Sunday) were initially pooled together and then aliquoted into several microtubes for molecular analysis as required. ToBRFV and PMMoV were quantified using RT-qPCR, respectively.

The method of ToBRFV quantification adopted in this study was described by Panno et al.[29], with some modifications. Briefly, RT-qPCR was performed using the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The MasterMix was composed of 5 µL 4X Reliance One-Step Multiplex Supermix (BioRad, Hercules, CA, USA), 2 µL of total genomic RNA template, 0.4 µL of probes (0.2 µM), and 0.4 µL of primers (0.4 µM each), to a total volume of 20 µL for each reaction. RT-qPCR was conducted with the following program: reverse transcription for 10 min (50 °C), denaturation for 10 min (95 °C), followed by 40 cycles of denaturation for 10 s (95 °C), annealing/extension for 30 s (58 °C), and plate read, respectively. The cycle threshold (Ct) was determined using the default algorithm in the CFX Manager™ Software, Version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA, https://www.bio-rad.com/en-us/sku/1845000-cfx-manager-software). The primers (ToB5520F: GTAAGGCTTGCAAAATTTCGTTCG and ToB5598R: CTTTGGTTTTTGTCTG GTTTCGG) and an MGB probe (FAM-GTTTAGTAGTAAAAGTGAGAAT-MGB) were used in the assay. To ensure the quality of our data, positive and non-template controls were included in each RT-qPCR run. While an external process control virus was used, and results were published in our previous studies[27,28]. We calculated concentration factors for each sample to normalize for recovery and pooled weekly samples to reduce variability introduced during sampling and processing.

Calibration curves were generated with 10-fold serial dilutions of ToBRFV positive control (synthetic blocks (gBlock) of double-stranded DNA, IDT, Coralville, IA, USA) from 8.7×10^8 to 8.7 gc/µL. Correlation coefficients (R^2) > 0.99 were obtained for all calibration curves, with 90–110% amplification efficiencies. The gBlock sequence is shown in the Supplementary Information (SI). The limit of detection (LoD) was determined to be 8.7 gc/µL (lowest serial dilution concentration with more than 50% amplification). PMMoV was quantified using the RT-qPCR kits for wastewater (Promega, Madison, WI, USA) following the instructions provided in the kit. To calculate the final concentration in gc/L, the total gene copies recovered from the RNA elute (instrument concentration × elution volume) were divided by the equivalent original wastewater volume. This equivalent volume was calculated by multiplying the volume of concentrated sample used for RNA extraction by the sample-specific concentration factor. The final result was then converted to gc/L. Calibration curves with correlation coefficients (R^2) > 0.99 and 90–100% amplification efficiencies were obtained. The LoD of PMMoV was determined to be ~ 2 gc/µL of RNA eluted, which showed more than 50% positive detection.

Library preparation, sequencing, and bioinformatics

Some RNA samples were converted into Illumina-compatible sequencing libraries and enriched using SARS-CoV-2-enrichment (myBaits, Arbor Biosciences, Inc.). To evaluate the effect of enrichment method, a subset of samples was enriched using the Respiratory Pathogen ID/AMR Enrichment Panel & Respiratory Virus Panel (RVP) kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions. Briefly, RNA was denatured and then reverse-transcribed. Premixed Index 1 (i7) and Index 2 (i5) adapters were added to the sample and subjected to sixteen cycles of PCR to amplify the tagged cDNA and incorporate the adapters. After normalized and overnight hybridization (50 °C), Respiratory Pathogen ID/AMR & RVP Enrichment Oligos labeled with biotin were used to enrich viral pathogens.

The resulting enrichment pools were quantified, and samples were subsequently sequenced. Libraries for the enrichment method (SC-2-enrichment using myBaits, Arbor Biosciences, Inc., Cambridge, MA, USA) were described previously Khan et al.[30]. The FASTQs from the sequencing reaction were subjected to the detection of viral signatures. We used an open-source CZ IDseq pipeline (v4.0, https://czid.org/) to analyze the presence of viral pathogens[31]. Briefly, the pipeline performs subtractive alignment of the human genome (NCBI GRC h38), followed by quality filtering with subsequent removal of cloning vectors and phage[32]. The identities of the remaining microbial reads are then queried against the NCBI nucleotide database. After background correction and filtering, retained taxonomic alignments in each sample were aggregated at the genus level, sorted by abundance, and measured in nucleotide reads per million (NT rPM).

Weather data collection and data analysis

To assess the influence of seasonal factors on viral occurrence, we collected data on temperature and precipitation from the Weather Underground website (https://www.wunderground.com/history/daily/us/nv/reno), with the data last accessed on June 1, 2023. To evaluate the seasonality of the data, four seasonal groups were categorized: Spring (March, April, and May), Summer (June, July, and August), Fall (September, October, and November), and Winter (December, January, and February). We assessed data normality using the Shapiro-Wilk test (alpha = 0.05). To determine the seasonality of PMMoV and ToBRFV, we calculated the

coefficient of variation for the monthly averages and performed an analysis of variance (ANOVA) on these averages. Differences in seasonal viability between each season were evaluated using an unpaired Student's t-test. All analyses were conducted using GraphPad Prism version 10.0.0 for Mac (GraphPad Software., Boston, MA, USA, www.graphpad.com).

Results and discussion

Concentrations of ToBRFV in wastewater and its potential as a viral indicator

To investigate the absolute gene copy concentrations of ToBRFV and PMMoV in wastewater, we utilized the RT-qPCR quantification method over a 13-month study period. The results presented in Fig. 1 and reveal that ToBRFV and PMMoV were detected in 100% of the untreated wastewater samples, confirming their persistent presence. These findings are consistent with our results from metagenomic analysis. ToBRFV showed higher concentrations, ranging from 1.86×10^8 to 7.21×10^8 gc/L with a coefficient of variation at 6%, indicating minor monthly fluctuations. Notably, the peak concentrations of ToBRFV were observed in November of both 2021 and 2022, during periods characterized by lower temperatures and minimal precipitation. In contrast, PMMoV exhibited more pronounced variability in concentrations, ranging from 2.41×10^6 to 2.04×10^7 gc/L, with a higher coefficient of variation at 175%. The peak for PMMoV was recorded in October 2021, coinciding with a month of no precipitation and moderate temperatures (average 14.4 °C, range 5.0 °C to 19.4 °C). These observations suggest that environmental factors such as temperature and precipitation may influence the variability in virus concentrations, with PMMoV showing greater sensitivity to such changes compared to ToBRFV. The high endogenous levels of ToBRFV in wastewater suggest that it could be a valuable tool for assessing water quality, fecal contamination, and the efficacy of treatment processes in wastewater and advanced treatment systems. Furthermore, utilizing ToBRFV as an indicator could enhance public health management and optimize wastewater treatment strategies.

Sherchan et al.[5] conducted an extended wastewater monitoring study from March 2017 to March 2018 in New Orleans, Louisiana, USA. The study aimed to quantify ToBRFV in wastewater and evaluate its potential as a viral indicator for monitoring water quality. During the study period, ToBRFV was detected in 77% of the influent samples, with concentrations ranging from 3.5 to 6.1 $\log_{10}$ copies/L, which were consistently lower than those observed in our study. The higher concentrations of ToBRFV observed in our study compared to[5] could be attributed to several factors. Firstly, differences in the geographical regions and climate conditions, such as the colder temperatures and lower precipitation in our study area, may have influenced virus stability and persistence in wastewater. Secondly, variations in wastewater collection methods, processing techniques, or detection sensitivities between the studies might also account for the discrepancies. Meanwhile, the presence of ToBRFV is not unique to our study area. Recent research has documented its widespread detection in wastewater across the globe, including in Southern California[4], Maryland[33], Slovenia[2], and Thailand[34]. These studies confirm that ToBRFV is a globally prevalent virus in municipal wastewater, strengthening its candidacy

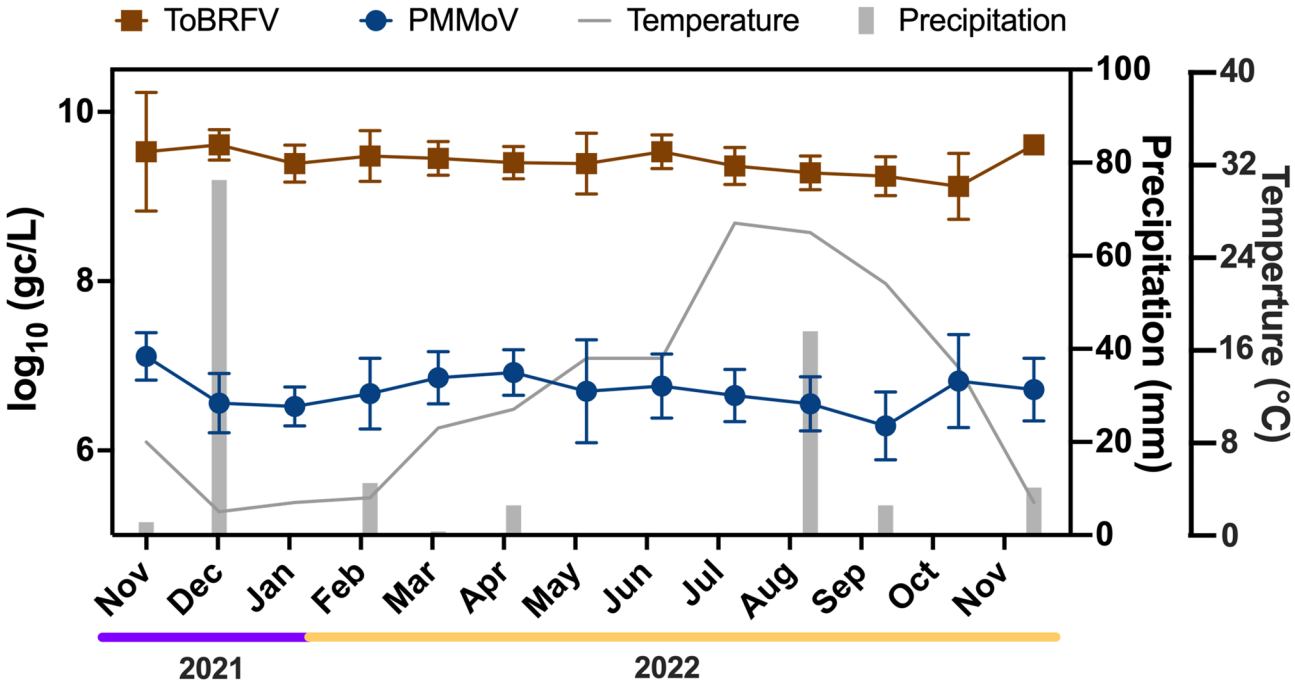

Fig. 1. Longitudinal analysis of ToBRFV and PMMoV in untreated wastewater from November 2021 to November 2022. Data points are the monthly mean of $\log_{10}$-transformed gene copy concentrations (gc/L), with error bars indicating the standard deviation. Corresponding monthly average temperature (°C, grey line) and total precipitation (mm, grey bars) are also shown.

as a universal viral indicator. Therefore, further surveillance of wastewater ToBRFV levels is essential to better understand its behavior across different geographical regions and enhance its reliability as a viral indicator for water quality monitoring.

Seasonality of ToBRFV and PMMoV

In order to investigate the seasonal pattern of the ToBRFV and PMMoV presence in wastewater, we segregated months into four seasonal categories to explore the seasonal prevalence of ToBRFV and PMMoV (Fig. 2). We conducted an ANOVA to analyze the seasonality; however, there was no significant difference in the concentrations of ToBRFV ($F = 0.4984$, $p = 0.9213$) and PMMoV ($F = 1.280$, $p = 0.3455$) across different seasons. T-tests were utilized to examine the seasonal variability between neighboring seasons to further analyze for variation. ToBRFV abundance had no significant differences between any two seasons (p = 0.2312–0.9198). Notable seasonal variation for PMMoV between spring and summer ($p = 0.0176$) suggesting significant seasonal concentration fluctuations. Our observations highlight that PMMoV demonstrated seasonal concentration variation compared to ToBRFV, which demonstrated no significant seasonality. ToBRFV concentrations remained stable, within an 8-$\log_{10}$ range, and consistently registered prominent levels throughout the year. On the other hand, PMMoV concentrations were elevated during the winter months and declined in the summer. In our study, we observed seasonal variation in PMMoV concentrations, but not in ToBRFV. These fluctuations may be influenced by environmental factors such as temperature and precipitation. While wastewater flow rates can impact viral concentrations, we did not normalize for flow because it remained relatively stable throughout the study period[35]. All flow rate data from TMWRF during the study period are presented in Table S1. Future studies should incorporate flow data to better assess viral load dynamics. Notably, the study area is served by a sanitary sewer system rather than a combined system, which reduces the influence of direct stormwater inflow.

Goitom et al.[36] observed that PMMoV concentrations were significantly higher during dry weather conditions, suggesting that stormwater inflow during wet weather conditions may dilute PMMoV levels in wastewater. The study also identified that factors like flow rate, temperature, and other physico-chemical parameters influenced PMMoV variability, with a weak negative correlation to flow in some instances. In our study, PMMoV concentrations peaked in summer (the dry season), aligning with Goitom et al.[36]. The seasonality in PMMoV levels compared to ToBRFV, indicates that ToBRFV might be a more robust viral indicator in wastewater.

Metagenomics analysis in wastewater from TMWRF

Samples from TMWRF were analyzed to determine the relative abundances of various viral gene copies, as shown in Fig. 3. Throughout the study period, ToBRFV consistently showed the highest abundance across all samples, ranging from 50k to 99k NT rPM as illustrated in Fig. 3A. The second most abundant virus was PMMoV, with levels between 20k and 50k NT rPM. In contrast, other plant and fruit viruses such as melon necrotic spot virus and cucumber green mottle mosaic virus were found at significantly lower levels, as low as 10k NT rPM.

Additionally, bacteriophages like MS2 and BZ13 were detected in comparable abundances to the other plant and fruit viruses, as represented in the heatmap (Fig. 3A). The inclusion of samples enriched for respiratory viruses (RVP-enriched) facilitated the identification of prevalent respiratory disease-causing viruses, allowing for a comparative analysis with results from the standard coronavirus-enriched (SC-2-enriched) samples (Fig. 3B versus Fig. 3A). Among these, *betacoronavirus*, including SARS-CoV-2, NL63, OC43, and HKU1, were detected at levels ranging from 30k to 99k NT rPM (Fig. 3B). Seasonal fluctuations were evident as *alphainfluenzavirus* (covering Influenza A subtypes H1-18 and N1-11) and *gammainfluenzavirus* (Influenza C) were primarily detected in the late spring and fall months, with abundances varying from 0 to 70k NT rPM for *alphainfluenzavirus* and 0 to 30k NT rPM for *gammainfluenzavirus* (Fig. 3B).

The results also revealed a diverse presence of human enteroviruses, encompassing 71 serotypes, in the RVP-enriched samples, ranging from 0 to 60k NT rPM. Notably, the relative abundance of enteroviruses was reduced in these samples compared to those identified using the SC-2-enrichment method (ranging from 0 to 60k versus 60 to 296k NT rPM, respectively; Fig. 3B and Supplemental Fig. 1B). In SC-2-enriched samples, *Enterovirus* was highly abundant (10 to 50k NT rPM) during the winter months (November 2021 – January 2022), followed by a significant decline during the summer months (June – August 2022).

Furthermore, noroviruses and rotaviruses, both significant causes of viral gastroenteritis, were intermittently detected in the samples. While these viruses showed a seasonal pattern, with peaks typically occurring in the cooler months, the abundance ranged from 5k to 40k NT rPM, reflecting their impact on public health due to their highly contagious nature. The sporadic detection of these viruses highlights the need for robust environmental monitoring to preempt outbreaks, particularly in settings vulnerable to rapid virus transmission such as schools and healthcare facilities.

It is important to note that NT rPM is a measure of relative abundance, and its value can be influenced by factors such as library size and the composition of the microbial community. Therefore, the sequencing results are used here to demonstrate the consistent dominance of ToBRFV relative to other viruses, while quantification for risk-relevant assessment is provided by the RT-qPCR data. The correlation analyses based on NT rPM should be interpreted as reflecting co-occurrence patterns rather than absolute concentration relationships. The longitudinal analysis illustrated in the heatmap (Fig. 3) provides a comprehensive overview of the prevalence for different pathogens and microbial indicators over 13-month period. ToBRFV exhibited sustained high abundance levels, potentially linked to environmental or vector-related factors promoting its prevalence. These findings highlight the potential of ToBRFV as a consistent fecal pollution indicator and a viral indicator of water quality for assessing the log removal reductions in wastewater treatment, given its high abundance and consistent occurrence.

Rothman and Whiteson[4] obtained raw sequencing data from the NCBI Sequence Read Archive and aligned the processed reads to reference strains of eight *tobamoviruses*. The sequencing data were collected from 275

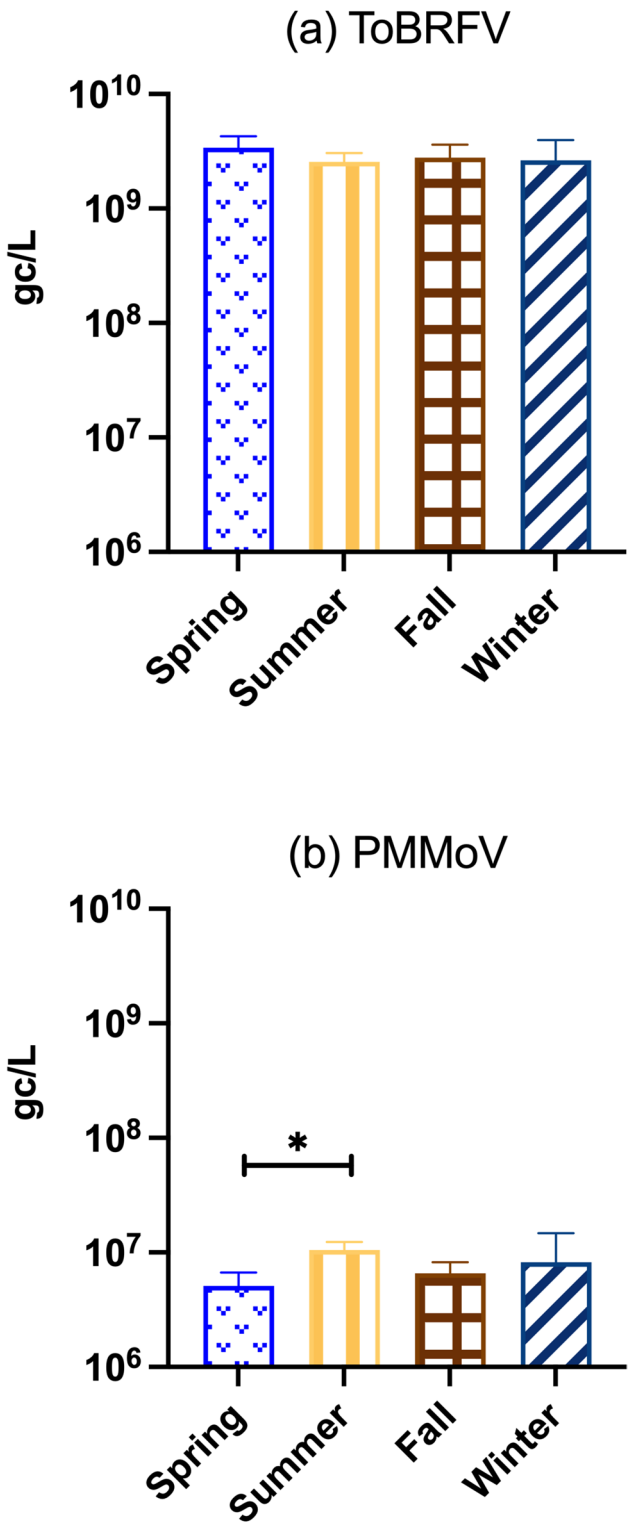

Fig. 2. Seasonal variations in the concentrations of ToBRFV and PMMoV. An unpaired Student's t-test was used to evaluate differences between seasons. A statistically significant difference was observed in PMMoV concentrations between the spring and summer seasons ($p = 0.0176$), as indicated by the asterisk. No other seasonal comparisons for either virus were statistically significant ($p > 0.05$).

samples across eight wastewater treatment plants from July 2020 to August 2021 in Southern California, USA. They found that 64% of the viral reads were from ToBRFV, making it the most abundant virus among the eight analyzed. The authors suggested that this data can be instrumental in tracking the genomic variability of these viruses, which is crucial for developing effective detection assays and managing their potential impact on public

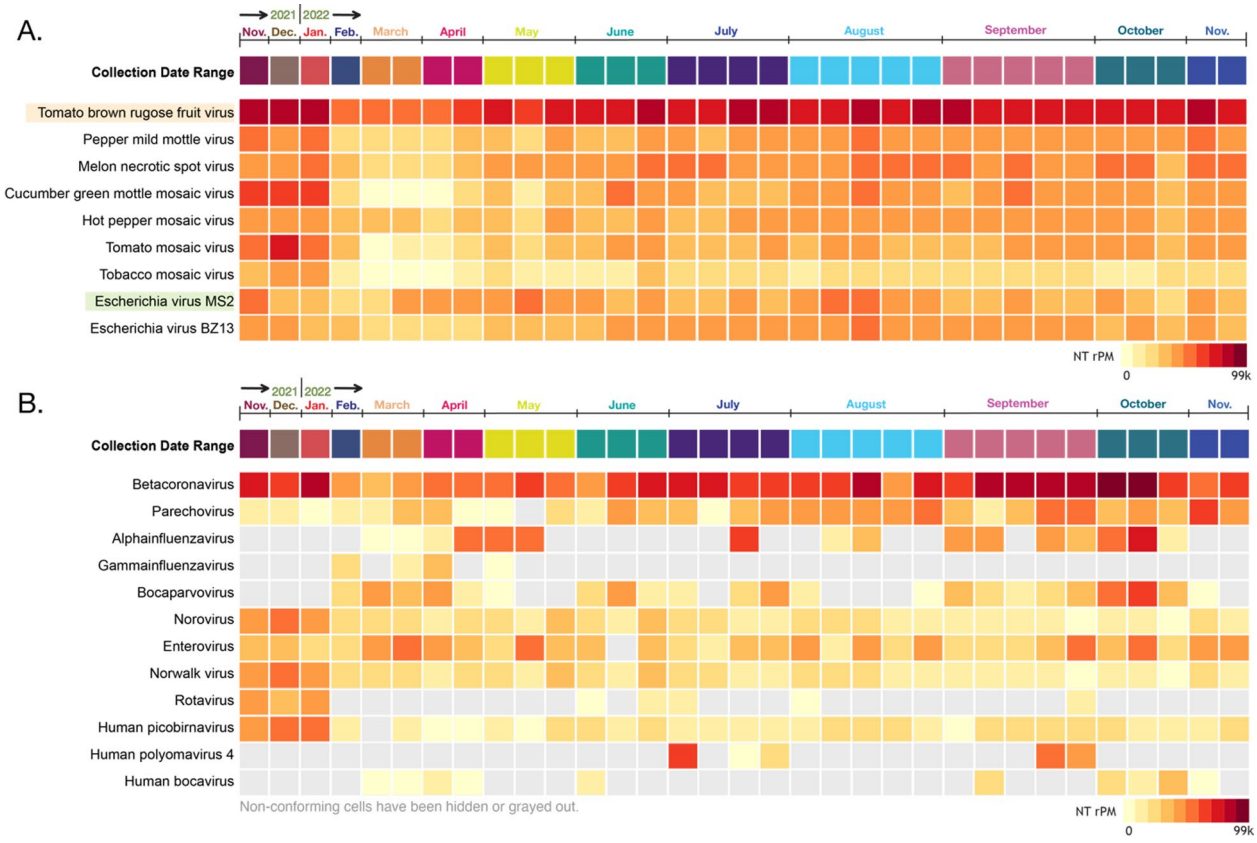

Fig. 3. Metagenomic Analysis in Wastewater from TMWRF.

health. Our sequencing results align with this research, though our study was conducted in Northern Nevada, USA. Similar results were observed in Maryland, USA, through a wastewater surveillance study conducted from December 20, 2020, to November 16, 2021. ToBRFV was detected alongside other RNA viruses, such as PMMoV and cucumber green mottle mosaic virus, indicating that ToBRFV was among the most frequently detected RNA viruses in the wastewater samples. This study points out the presence of ToBRFV in the environment and its potential role as a marker in wastewater surveillance[33].

To investigate the abundance of ToBRFV in other sewersheds, we analyzed wastewater samples from two additional facilities, RSWRF and STMWRF, and sub-sewersheds from different neighborhoods, travel-influenced locations and schools between April and September 2022. This analysis highlighted a consistent high prevalence of ToBRFV across all sites and date ranges, with concentrations ranging from 67k to 167k NT rPM (Figure S1). Although other viruses, including plant, fruit, enteric, and respiratory types, as well as coliphages, were also detected, their abundances were significantly lower compared to ToBRFV (range 0–134k NT rPM, Figure S1).

Correlations among ToBRFV and other human pathogenic viruses at the species level

We utilized non-parametric Spearman's r correlation analysis to explain the varied occurrence of a spectrum of viruses, including both plant-specific and human-specific pathogens and bacteriophages (coliphages) like MS2 and *Escherichia* virus BZ13. Table 1 presents correlations between various viruses at the species levels, focusing on their relationships with ToBRFV, PMMoV, MS2, and norovirus. These correlations help understand how these viruses behave and interact within wastewater environments.

Notably, ToBRFV shows very strong correlations with other *Tobamoviruses*, such as PMMoV ($r = 0.89$) and hot pepper mosaic virus ($r = 0.83$), suggesting similar environmental or transmission pathways. Additionally, ToBRFV exhibits a moderate correlation with Rotavirus A ($r = 0.45$) and a very weak correlation with norovirus ($r = 0.18$), indicating limited shared factors influencing their presence in wastewater. Conversely, ToBRFV shows very weak to moderate negative correlations with *Enteroviruses*, such as *Enterovirus* C ($r = 0.07$) and *Enterovirus* A ($r = -0.44$), suggesting differing behaviors or transmission routes compared to ToBRFV.

Additionally, PMMoV shows a weak correlation with norovirus ($r = 0.26$), suggesting some overlap in their environmental presence. Meanwhile, MS2 exhibits a moderate correlation with Enteroviruses (e.g., *Enterovirus* B, $r = 0.43$) and a weak correlation with norovirus ($r = 0.20$). This confirms MS2's utility as a surrogate for studying enteric viruses, commonly used in assessing viral contamination and the efficacy of wastewater treatment processes. Norovirus, as a common studied enteric virus, shows moderate correlations with PMMoV ($r = 0.54$) and Rotavirus A ($r = 0.45$), suggesting that while it shares some commonalities with these viruses, it may be influenced by different environmental factors or conditions. These findings highlight the need for using multiple viral indicators to capture a comprehensive view of viral presence and behavior in environmental

Species level	Genus level	ToBRFV	PMMoV	MS2	Norovirus
Tomato brown rugose fruit virus	*Tobamovirus*	1.00	0.89	−0.03	0.18
Melon necrotic spot virus	*Gammacarmovirus*	0.52	0.52	0.08	−0.05
Pepper mild mottle virus	*Tobamovirus*	0.89	1.00	0.17	0.26
Hot pepper mosaic virus	*Tobamovirus*	0.83	0.93	0.15	0.14
Escherichia virus BZ13	*Levivirus*	0.78	0.80	0.28	0.10
Cucumber green mottle mosaic virus	*Tobamovirus*	0.72	0.78	0.01	0.13
Tomato mosaic virus	*Tobamovirus*	0.72	0.73	−0.06	0.13
Escherichia virus MS2	*Levivirus*	−0.03	0.17	1.00	0.20
Tobacco mild green mosaic virus	*Tobamovirus*	0.64	0.76	0.19	−0.08
Tomato mottle mosaic virus	*Tobamovirus*	0.67	0.74	0.05	0.16
Enterovirus C	*Enterovirus*	0.07	0.03	0.25	0.14
Norwalk virus	*Norovirus*	0.18	0.26	0.20	1.00
Tobacco mosaic virus	*Tobamovirus*	0.83	0.80	−0.04	0.25
Pepper mild mosaic virus	*Comovirus*	0.37	0.31	0.09	0.54
Rotavirus A	*Rotavirus*	0.45	0.35	−0.16	0.45
Enterovirus A	*Enterovirus*	−0.44	-0.41	0.31	0.20
Enterovirus B	*Enterovirus*	0.01	0.08	0.43	0.01
Enterovirus sp.	*Enterovirus*	−0.01	0.12	0.09	−0.22

Table 1. Correlation analysis of viruses with ToBRFV, PMMoV, MS2, and Norovirus in untreated Wastewater.

samples. Despite the weak correlations between ToBRFV and certain enteric viruses such as Norovirus ($r = 0.18$) and Enterovirus A (r = -0.44), ToBRFV remains a promising viral indicator due to its remarkable environmental persistence, high concentrations in wastewater, and widespread detection across geographic locations.

Schmitz et al.[12] investigated correlations between various virus surrogates, including PMMoV and Norovirus (Genotype II, GII), and found significant relationships between them, suggesting their utility in monitoring waterborne pathogens. In contrast, our study focused on Norwalk virus, a Norovirus of Genotype I (GI), which showed weak correlations with PMMoV and ToBRFV. These findings emphasize the genotype-specific behavior of Noroviruses in relation to other viral indicators. Further research is essential to explore how ToBRFV correlates with other pathogens across different environments and treatment stages, as understanding these interactions could enhance our ability to monitor and manage water quality effectively.

A key finding of this study is the significantly higher concentration and lower variability of ToBRFV compared to PMMoV. Several factors may explain this observation. ToBRFV is an emergent plant pathogen that has spread rapidly since 2014, infecting tomato crops globally. Tomatoes and tomato-based products are a major dietary staple, and the recent, widespread nature of the infection may lead to higher viral loads in food products compared to PMMoV in pepper products. While both are highly stable *Tobamoviruses*, potential differences in their persistence through the human digestive tract or in the wastewater matrix could exist. Ultimately, the difference likely reflects a combination of current agricultural epidemiology and regional dietary habits, highlighting ToBRFV's current dominance in the wastewater virome of our study region.

A potential consideration in this study is the initial sample pretreatment, which involved centrifugation and filtration to remove particles larger than 0.45 µm. It is well-established that viruses can associate with solids in wastewater, and this step could theoretically lead to an underestimation of the total viral load if a significant fraction is particle-bound. However, this is unlikely to have biased our findings regarding the relative abundance of ToBRFV and PMMoV. First, both are non-enveloped, highly stable *tobamovirus*es with similar physicochemical properties, making a drastic difference in their partitioning behavior improbable. Second, recent research has shown that RNA viruses, including PMMoV, norovirus, and enterovirus, were detected in significantly higher concentrations among the smallest particles (< 0.45 µm) and as free viruses in the filtered water[37], the fraction analyzed in our study. Finally, the observed ~ 100-fold higher concentration of ToBRFV compared to PMMoV is of a magnitude that cannot be reasonably explained by partitioning losses alone, which for non-enveloped viruses are typically minor. Therefore, we conclude that the observed difference is a robust biological signal, likely reflecting the high prevalence of this emergent pathogen in consumed food products, a finding consistent with other recent studies proposing ToBRFV as a highly abundant fecal indicator.

Prospective challenges and opportunities

Our study has successfully assessed the presence of ToBRFV, PMMoV, and bacteriophages within various wastewater systems in Northern NV, USA. However, it is crucial to recognize that the distribution and persistence of these viral indicators may vary significantly across different geographical regions. This variance underlines the necessity of extending the research to encompass a more diverse array of locations, which would enhance our understanding of ToBRFV's applicability as a viral indicator across various environmental settings. Furthermore, it is important to consider the primary source of ToBRFV in wastewater. As a plant virus, its presence is tied to the human consumption of infected tomato products, after which it is excreted in feces[4]. While contributions from other sources like agricultural runoff or food processing facilities are possible, the ubiquitous and high

concentrations observed in domestic wastewater globally point to human dietary intake as the principal source. This dietary origin is what makes ToBRFV a strong candidate as a human fecal indicator, similar to PMMoV.

There remains a substantial gap in knowledge regarding the abundance and distribution of ToBRFV both throughout the US and globally. Addressing this gap is essential for determining its viability as a universal indicator. Future research should therefore not only aim to establish standardized methods for the detection and quantification of ToBRFV in wastewater but also explore the correlation between ToBRFV levels and other pathogens, particularly in terms of resistance to treatment and overall treatment efficiency. This includes examining its persistence and behavior under varying environmental conditions to fully understand its dynamics. By doing so, correlations between ToBRFV and other waterborne pathogens at the treatment level can be established, which will help in meeting LRV regulations more effectively. Such studies are imperative for validating ToBRFV's effectiveness and reliability as a robust viral indicator in diverse wastewater systems and geographic settings.

Conclusions

This study effectively demonstrates ToBRFV's suitability as a potential novel viral indicator for assessing wastewater systems, because of its consistently high concentrations in wastewater. Our investigation revealed ToBRFV's stability and high detection rates across a 13-month monitoring period within the Reno-Sparks Metropolitan Region, Nevada, USA. This characteristic is important for its role in verifying whether wastewater treatment processes can achieve the required LRV credits, making it an invaluable tool for ensuring desired water quality.

The study also identified PMMoV and MS2 bacteriophage as reliable indicators, yet ToBRFV stands out due to its consistent abundance and potential for broader application. Through metagenomics analysis and RT-qPCR, we confirmed ToBRFV's predominant presence compared to other viral indicators, showcasing its effectiveness in real-world wastewater systems.

Future work should focus on refining the application of ToBRFV as a viral indicator across various geographical and operational environments, developing standardized methods for its detection, and quantifying its presence to better understand its dynamics in wastewater systems. By advancing ToBRFV's role as a primary indicator, we can enhance the verification of wastewater treatment effectiveness and contribute significantly to public health protection and environmental conservation. Furthermore, future research should aim to directly compare the performance of ToBRFV with other widely accepted human fecal indicators that are not of plant origin, such as the crAssphage bacteriophage. Such studies would help to comprehensively position ToBRFV within the broader suite of tools available for water quality monitoring and microbial source tracking.

Data availability

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.

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Author contributions

LL, Data Curation, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing—Review & Editing; MK, Validation, Formal Analysis, Investigation, Writing—Original Draft; LH, Data Curation, Writing—Review & Editing; SCV, Project Administration, Funding Acquisition, Resources, Conceptualization, Validation, Formal Analysis, Investigation, Data Curation, Writing - Original Draft, Writing—Review & Editing; KP, Project Administration, Funding Acquisition, Resources, Conceptualization, Validation, Writing—Review & Editing.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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