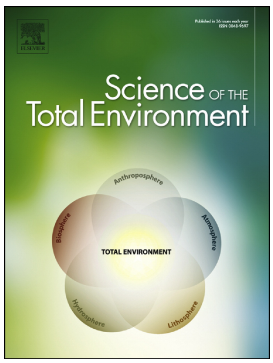

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PreK-12 School and Citywide Wastewater Monitoring of the Enteric Viruses Astrovirus, Rotavirus, and Sapovirus

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Abstract

Wastewater monitoring is an efficient and effective way to surveil for various pathogens in communities. This is especially beneficial in areas of high transmission, such as preK-12 schools, where infections may otherwise go unreported. In this work, we apply wastewater disease surveillance using school and community wastewater from across Houston, Texas to monitor three major enteric viruses: astrovirus, sapovirus genogroup GI, and group A rotavirus. We present the results of a 10-week study that included the analysis of 164 wastewater samples for astrovirus, rotavirus, and sapovirus in 10 preK-12 schools, 6 wastewater treatment plants, and 2 lift stations using newly designed RT-ddPCR assays. We show that the RT-ddPCR assays were able to detect astrovirus, rotavirus, and sapovirus in school, lift station, and wastewater treatment plant (WWTP) wastewater, and that a positive detection of a virus in a school sample was paired with a positive detection of the same virus at a downstream lift station or wastewater treatment plant over 97% of the time. Additionally, we show how wastewater detections of rotavirus in schools and WWTPs were significantly associated with citywide viral intestinal infections. School wastewater can play a role in the monitoring of enteric viruses and in the detection of outbreaks, potentially allowing public health officials to quickly implement mitigation strategies to prevent viral spread into surrounding communities.

Keywords: Wastewater-based epidemiology; Enteric viruses; Facility-level surveillance; RT-ddPCR; PreK-12 schools; Nested sampling

1. Introduction

Millions of people every year are infected with enteric viruses, a group of viruses that infect and replicate in the intestinal mucosa and can cause diarrhea, vomiting, dehydration, and can lead to hospitalization and death (Bishop and Kirkwood, 2008; Hall et al., 2013; Scallan et al., 2011; Troeger et al., 2017). These viruses are primarily transmitted through the fecal-oral route, typically through person-to-person spread or ingestion of contaminated food or water (Bishop and Kirkwood, 2008; Bouseettine et al., 2020; D'Souza, 2015; Goddard et al., 2020). The most significant enteric viruses are rotavirus (group A), norovirus (genogroups GI and GII), sapovirus (genogroups GI and GII), adenovirus (serotypes 40 and 41), and astrovirus (Franco and Greenberg, 2012; Oka et al., 2015; Page et al., 2019). These enteric viruses can cause serious infections and can lead to medically attended acute gastroenteritis across people of all ages (Burke et al., 2021).

Globally, enteric viruses pose a major threat to public health. Recent global studies have determined diarrheal diseases, such as enteric virus infections, to be a leading cause of both deaths (1.3 million deaths) and disability-adjusted life-years (72 million DALYs) (Troeger et al., 2017; Vos et al., 2020). The study by Troeger et al., 2017 specifically identified rotavirus as the leading cause of diarrheal deaths worldwide (n = 200,000 deaths) (Troeger et al., 2017). In the United States, it is estimated that 5.5 million cases (59%) of foodborne illnesses are caused yearly by viruses (Scallan et al., 2011). However, many enteric virus infections are subclinical, likely leading to unreported cases and an underestimation of infections (CDC, 2022a; Moser and

Schultz-Cherry, 2008; Parashar et al., 2013). Therefore, implementing surveillance that can monitor all types of infections is critical.

Wastewater monitoring provides an efficient way to detect enteric viruses among large populations, regardless of whether those with infections show symptoms. Enteric viruses' primary site of infection is in the intestinal mucosa, and thus these viruses are relatively abundant and detectable in feces and in wastewater (Bicer et al., 2011; Cantalupo et al., 2011; Gallimore et al., 2005; Hansman et al., 2004; Hata et al., 2013; Kauppinen et al., 2019; Meqdam and Thwiny, 2007; Phan et al., 2004; Wadell et al., 1987; Zintz et al., 2005). The ability to detect these viruses in wastewater has allowed for the monitoring of enteric viruses to track viral levels in populations over time (Bisseux et al., 2018; Cuevas-Ferrando et al., 2022; Fioretti et al., 2016; Haramoto et al., 2008; Hellmér et al., 2014). Wastewater monitoring is an effective approach for community viral surveillance because it overcomes many of the barriers faced by clinical infectious disease surveillance. Data based on clinical diagnostics likely underestimate the prevalence of enteric viruses because the data rely on health-seeking behaviors and correct diagnoses by physicians, leading to underreporting (Prevost et al., 2015; Scallan et al., 2011). In contrast, wastewater monitoring's pooled sample strategy allows for the sampling of entire communities, including individuals with subclinical cases (Hellmér et al., 2014; Xagoraraki and O'Brien, 2019). Furthermore, wastewater data have been shown to detect viral outbreaks and peaks before clinical data (Hellmér et al., 2014; Hill et al., 2023; Kazama et al., 2017; Keshaviah et al., 2023; Prevost et al., 2015; Sinclair et al., 2008; Xagoraraki and O'Brien, 2019).

While previous studies have detected and monitored astrovirus, sapovirus, and rotavirus in wastewater, targeted wastewater monitoring of these viruses in at-risk populations such as preK-12 schools is limited. Facility-level wastewater monitoring at schools could provide public health officials with crucial information on the transmission and burden of these enteric viruses in preK-12 school populations. As children are disproportionately affected by enteric viruses and are at a higher risk for serious infection, they are a critical group to monitor and mitigate transmission (Ikner and Gerba, 2017; Page et al., 2019; Wiegering et al., 2011). In addition, schools are important locations associated with fecal-oral transmission and can result in subsequent outbreaks of enteric viruses in their surrounding communities (Bishop and Kirkwood, 2008; Donaldson et al., 2020; D'Souza, 2015; Hassan-Ríos et al., 2013; Iritani et al., 2016). School wastewater monitoring of astrovirus, sapovirus, and rotavirus could also provide more targeted information about outbreaks than wastewater from wastewater treatment plants (WWTPs) that serve much larger populations. This building-level wastewater monitoring for infectious disease surveillance could enable the implementation of more targeted mitigation strategies and facilitate outbreak tracing.

Here, we report an application of wastewater monitoring of astrovirus, rotavirus, and sapovirus for preK-12 schools. We developed RT-ddPCR assays to detect these viruses and quantified the three viruses in the wastewater of 10 preK-12 schools, 2 lift stations, and 6 WWTPs in Houston, Texas over 10 weeks. We compared school wastewater detections of astrovirus, rotavirus, and sapovirus to detections of these viruses in downstream lift stations and WWTPs, as well as to syndromic surveillance of citywide viral intestinal infections.

2. Material and methods

2.1 Sampling Locations

We quantified astrovirus, rotavirus, and sapovirus in the wastewater of 10 Houston Independent School District (HISD) schools, 2 lift stations, and 6 WWTPs across Houston, Texas (Figure 1). The area sizes served by participating WWTPs are listed in Table A.1. The Houston Wastewater Epidemiology system includes 48 preK-12 schools in its routine wastewater monitoring program for respiratory viruses. Out of those 48 schools, the 10 HISD schools with the largest enrollments as of the 2021-2022 school year were selected for this study. The 10 selected schools included 3 elementary schools, 1 middle school, 4 high schools, and 2 combined grade-level schools. The 2 lift stations and 6 WWTPs located downstream of these 10 schools were chosen for this study to enable a nested site analysis of the relationship between school wastewater and the wastewater of their corresponding downstream site(s). Table 1 shows the schools' enrollment information and the populations served by their respective downstream sites. A raw, composite wastewater sample from each site was collected once a week between the weeks of February 27th, 2023, and May 8th, 2023, for a total of 10 weeks of sampling (excluding the week of March 13th, 2023, which was a school holiday).

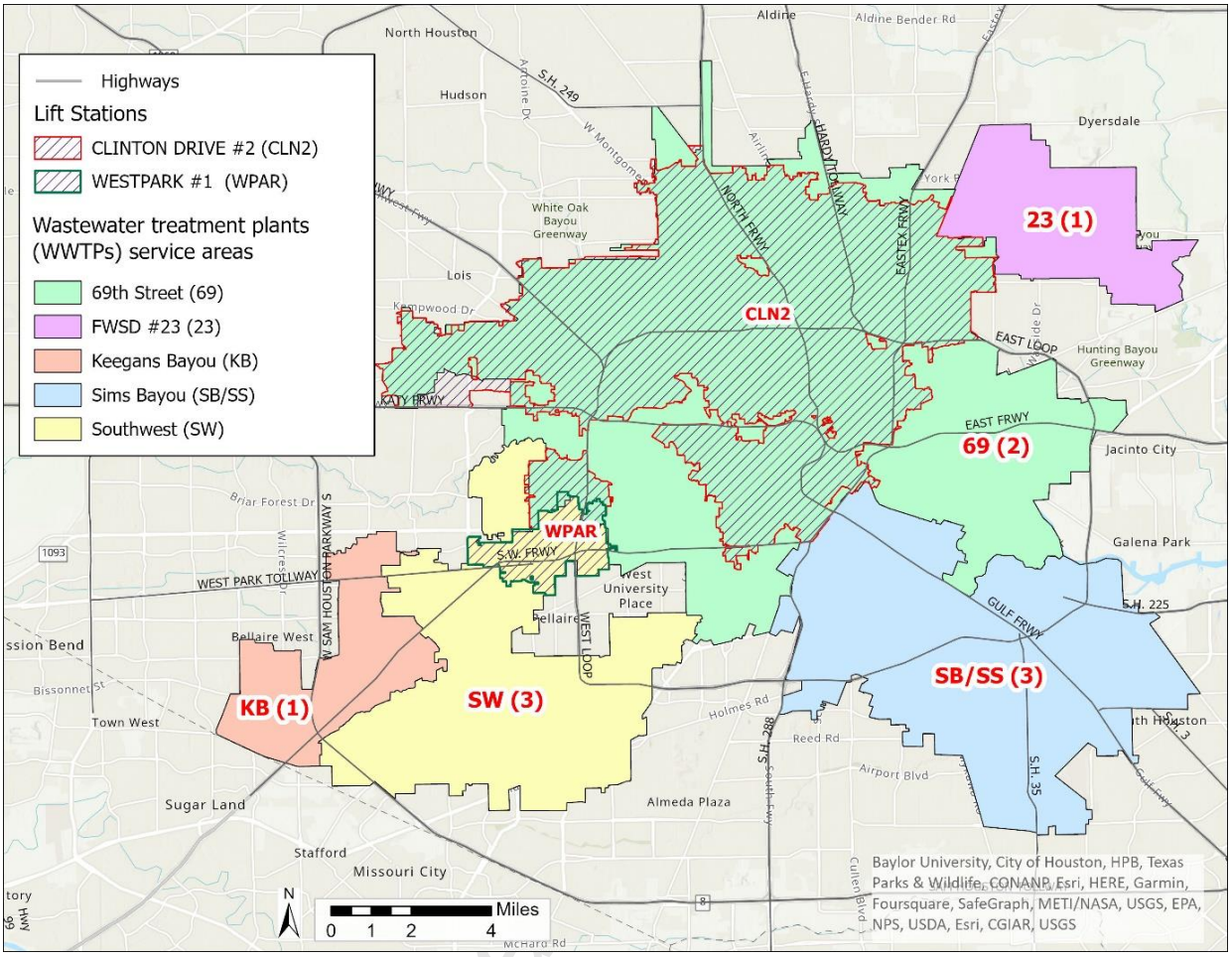

Figure 1. Sewershed boundaries for the two lift stations and six WWTPs where wastewater samples were collected and assayed for astrovirus, rotavirus, and sapovirus during this study. The colors represent different WWTP sewersheds, and the dashed areas indicate the areas served by the lift stations. The number of schools in each sewershed is indicated in the parentheticals after each WWTP sewershed label. Additional information on each school where wastewater was sampled and their corresponding downstream sites is provided in Table 1.

Table 1. School enrollment information and population size served for corresponding downstream lift stations and WWTPs. Site name codes for WWTPs and lift stations are in parentheses.

School	Enrollment as of 2022-2023 school year	Downstream lift station (if sampled)	Population served by lift station (if sampled)	Downstream WWTP(s)	Population served by WWTP(s)
1	2,769	Clinton Drive #2 (CLN2)	373,937	69th Street (69)	551,150
2	2,257	n/a	n/a	Sims Bayou North and Sims Bayou South (SB and SS)[a]	109,414
3	2,102	n/a	n/a	Sims Bayou North and Sims Bayou South (SB and SS)[a]	109,414
4	1,279	n/a	n/a	Keegans Bayou (KB)	124,000
5	1,035	n/a	n/a	Southwest (SW)	293,227
6	974	n/a	n/a	FWSD #23 (23)	40,689
7	919	n/a	n/a	Southwest (SW)	293,227

8	904	n/a	n/a	Sims Bayou North and Sims Bayou South (SB and SS)[a]	109,414
9	898	Westpark No.1 FN208 (WPAR)	43,088	Southwest (SW)	293,227
10	865	Clinton Drive #2 (CLN2)	373,937	69th Street (69)	551,150

[a]Sims Bayou North and Sims Bayou South have overlapping geographic service areas

2.2 Sample Collection

Wastewater samples were collected from each site weekly. The wastewater samples were collected using refrigerated time-weighted composite autosamplers. School wastewater samples were taken from autosamplers within or adjacent to manholes receiving wastewater from only one school. The school autosamplers collected samples once a week by taking in 250mL every 15 minutes between 6am and 12pm on the collection day. The WWTP and lift station samples were obtained once a week from 24-hour composite autosamplers that collected wastewater from 7am on Mondays to 7am on Tuesdays. Members of the Houston Health Department (HHD) obtained these wastewater samples from the composite autosamplers and delivered them on the same day on ice to the Rice laboratory where the samples were kept at 4°C until being processed within 24 hours of collection to prevent viral degradation. A total of 164 samples were collected and processed in duplicate over the 10-week period.

2.3 Sample Processing

Sample processing began with the concentration and extraction of the viral nucleic acids from the wastewater samples using an electronegative filtration and bead beating method previously described (LaTurner et al., 2021; Lou et al., 2022). The concentration in copies per liter of wastewater (copies/L) of each enteric virus was quantified using the RT-ddPCR assays designed for each virus. The individual assays (singleplex assays) were combined into multiplex assays to enable the simultaneous detection of multiple viruses. These assay combinations are discussed in more detail in Section 2.5. The viral targets were quantified in samples using a C1000 Thermal Cycler (Bio-Rad) in conjunction with the QX600 AutoDG Droplet Digital PCR System (Bio-Rad).

2.4 Design and In-Silico Validation of ddPCR Assays

To design the ddPCR assays for astrovirus, rotavirus, and sapovirus, complete genomes for each virus were downloaded from the National Center for Biotechnology Information's (NCBI's) portal for viral sequence data, NCBI Virus ("NCBI Virus," 2004). These included genomes from the NCBI Reference Sequence Database (RefSeq) release 213 and NCBI's genetic sequence database, GenBank (human astrovirus 1, taxid: 12456, 38 genomes; Human rotavirus A, taxid: 10941, 44 genomes; sapovirus GI, taxid: 515176, 22 genomes). For each virus, a multiple sequence alignment (MSA) of the viral genomes, as well as genomes from its neighboring clades, was generated with Clustal Omega v1.2.3 (Sievers et al., 2011) using default parameters. Based on the MSA, a conserved 200 base pair (bp) region within the virus's genome

but dissimilar to the neighboring clades was selected and the consensus sequence was generated for each viral target. The average nucleotide identity (ANI) for astrovirus, rotavirus, and sapovirus were 82.78%, 91.05%, and 76.81%, respectively. The consensus sequence was input to Primer3Plus (Untergasser et al., 2012), with product size 70-150bp, primer size 18-30bp (optimal 23bp), primer Tm (melting temperature) 54-56°C (optimal 55°C). Other Primer3Plus parameters were kept as default. Output primers and probes were validated with NCBI BLAST+ v2.13.0 against the November 2nd, 2022 nucleotide collection (nt/nr) database to prevent non-specific amplification (Camacho et al., 2009). The primers and probes were checked for dimerization against primers and probes from the other viruses in the multiplex assays using Olivar v0.10.0 (Wang et al., 2023). The primer and probe sequences designed for and used in this study are included in Table A.2.

2.5 Assay Development

The One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad) was used for all ddPCR testing and data collection. A gblock containing the sequences of all the target consensus sequences for astrovirus, rotavirus, and sapovirus was created to serve as a positive control for quantification. Initial testing of the assays was performed in singleplex (individually) and in 3-plex (in combination) for all three viruses using a temperature gradient on the annealing step during thermal cycling. Based on this temperature gradient, the optimal temperature for the amplification of all three viruses was chosen. The thermal cycling conditions used in this study are listed in Table A.10. The assays were then tested using wastewater sample extracts in

singleplex and in 3-plex to ensure that multiplexing the targets did not affect the quantification results.

Because each virus is present in wastewater at different concentrations, dilution factors for each virus and site type were chosen to ensure that targets fell within the appropriate dynamic range for ddPCR quantification. For example, sapovirus was very concentrated in many school wastewater samples, so it required a disproportionate amount of resources in the RT-ddPCR reaction wells. Because of this, the high concentrations of sapovirus impacted the quantification of astrovirus and rotavirus, and thus the targets were quantified in separate reactions. This disproportionate concentration of sapovirus in comparison to astrovirus and rotavirus was not seen in WWTP and lift station samples, so the WWTP and lift station samples were multiplexed in a 3-plex reaction for quantification.

Because of the differences in dilution factors across targets and sites, samples were quantified in the following manner (Table A.9): School samples were run in duplicate at a 1x dilution (raw) to detect astrovirus and rotavirus in a duplexed assay. School samples were also run in duplicate at a 100x dilution to quantify sapovirus in a singleplex reaction. WWTP and lift station samples were quantified using a 3-plex reaction and were run in duplicate at a 10x dilution to detect rotavirus and in duplicate at a 100x dilution to detect astrovirus and sapovirus. In the event that a sample was too highly concentrated with a virus to be able to threshold between positive and negative droplets, that sample was rerun at a greater dilution factor, going up to 1000x for some samples. The concentration values of diluted samples were also checked to ensure that the concentrations of diluted samples were similar to their corresponding undiluted samples after the dilution factor was taken into account. This dilution of sample extract also

helped ensure that inhibitors present in the wastewater were diluted out and thus quantification did not suffer from inhibition (Borchardt et al., 2021). Assay setup and reaction composition details are included in the Appendix.

2.6 Data Analyses

Results were classified as either positive, negative, or inconclusive for each viral target. Each sample was run in duplicate and the result for the sample as a whole was determined as follows: If both replicates had concentration measurements above the plate's limit of detection (LOD) for a viral target, the sample was deemed positive for that target. If neither measurement was greater than the LOD, the sample was labeled negative for that target. Lastly, if one replicate was above the LOD and the other replicate was below the LOD, the sample was identified as inconclusive for that target. Replicates were classified as positive or negative by thresholding based on droplet separation. Examples of droplet separation for each assay are shown for school, lift station, and WWTP samples, as well as positive and negative controls, in Figure A.1. Quality control measures and the method to calculate the final plate LOD were previously described (Lou et al., 2022; Wolken et al., 2023). The concentration factor calculations are described in Table A.11.

2.7 Citywide Viral Intestinal Infections

The citywide viral intestinal infections during the study period were aggregated by members of the Houston Health Department for use in this study. The diagnosis data was taken from the Electronic Syndromic Surveillance System for the Early Notification of Community-

Based Epidemics (ESSENCE). The data was aggregated by week with each listed date including all related cases from the previous Sunday to Saturday. This aggregated diagnosis data included the total number of citywide diagnoses of viral intestinal infections as well as the number of diagnoses for specific enteric viruses when the count was greater than 5 infections, which, out of the three viruses investigated in this study, only occurred with rotavirus. Weeks with less than 5 infections were suppressed to maintain the confidentiality of patients and the accuracy of the aggregated count data. The citywide discharge diagnosis code for viral intestinal infections included any cases of intestinal infections with viruses including, but not limited to, astrovirus, rotavirus, sapovirus, enteric adenovirus, and norovirus. Measurements of the wastewater concentrations of enteric adenovirus or norovirus were not conducted in this study. The aggregated data included diagnoses from patients of all ages from the city of Houston and Harris County hospitals, emergency rooms, and other healthcare facilities.

2.8 Statistical Analysis

R was used for all data analyses (R Core Team, 2023) and visuals were created using the ggplot2 package (Wickham, 2016). The concentrations of samples with zero positive droplets were imputed by setting the concentration to half of the average detection limit across all plates in this study for each virus. A Kruskal-Wallis test and subsequent pairwise comparisons using Dunn's test were employed to investigate the differences in mean concentrations of positive detections between the three facility types for each virus (Dinno, 2017). The variances in concentrations of positive detections were also compared between facility types for each virus using a modified robust Brown-Forsythe Levene-type test based on the absolute deviations from

the median (Gastwirth et al., 2023). The Bonferroni correction was applied to the p-values of both the Dunn's test and the modified robust Brown-Forsythe Levene-type test to account for the increased risk of Type 1 error that multiple pairwise tests introduce. The Bonferroni-adjusted significance level for these tests was calculated as 0.05 divided by 9 (total number of comparisons), for a significance level of 0.0056. These statistical tests were chosen instead of tests such as ANOVA or a t-test because the assumption of normality was not met. Therefore, these non-parametric methods were used.

We used linear regression in three different analyses with a significance level of 0.05. In one analysis, we used linear regression to model the relationship between the number of citywide viral intestinal infections and the school rotavirus wastewater detection rates. The proportion of the 10 schools with a positive detection of rotavirus in a given week was the independent variable and the citywide number of visits diagnosed with viral intestinal infections was the dependent variable. We then used linear regression to model the relationship between the number of citywide viral intestinal infections and the citywide viral load of rotavirus in copies/L. The citywide viral load (copies/L) was used as the independent variable with the citywide number of visits diagnosed with viral intestinal infections as the dependent variable. The citywide viral load was the concentration of rotavirus in all 10 WWTPs weighted by population. It was calculated by multiplying the concentration of rotavirus (copies/L) in a WWTP by its population served and then summing these results for each WWTP by date to obtain a weekly citywide viral load for rotavirus. Sims Bayou South (SS) was excluded from the calculation of the citywide viral load because this sample was not received on March 20th, 2023, and therefore including it in the analysis without imputation would make the citywide viral load for that week inaccurately low.

Sims Bayou North (SB) has an overlapping geographic region with SS, so the concentration of rotavirus in SB is representative of the viral load at SS. Finally, we used linear regression to model the effect of the citywide viral load of rotavirus on the number of citywide rotavirus infections. The citywide viral load of rotavirus in WWTP wastewater in a given week was the independent variable and the number of citywide rotavirus infections was the dependent variable. Since the data from each sampling site form a time series, the standardized residuals for each regression were checked for autocorrelation using the astsa R package (Stoffer and Poison, 2024) (Section A1.6).

3. Results

3.1 Newly designed RT-ddPCR assays were used to quantify astrovirus, sapovirus, and rotavirus concentrations in school, lift station, and WWTP wastewater samples

Wastewater samples were collected weekly from 10 preK-12 schools, 2 lift stations, and 6 WWTPs over a 10-week period for a total of 164 samples. The descriptive statistics and number of positive detections for each virus are listed in Table 2. Figure 2 shows the weekly concentration in log copies/L for each virus at each site over the study period. At several sites such as Clinton Drive #2 (CLN2) and Keegans Bayou (KB), sapovirus and astrovirus shared similar wastewater concentrations and trends over the course of the 10-week study period. While the concentration of rotavirus varied between sites, a general increase in rotavirus concentration over time at several WWTPs, such as Southwest (SW) and Sims Bayou North (SB), was observed. In Figure 3, the distributions of concentrations (log copies/L) of positive detections for each virus are depicted using violin plots grouped by facility type. The differences in means were

significant between schools and WWTPs for all three viruses. There were also significant differences in means between schools and lift stations for astrovirus detections, and between lift stations and WWTPs for rotavirus and sapovirus detections. The variance of the wastewater concentration for all three viruses were significantly greater for school samples compared to lift stations and WWTPs as all these pairwise comparisons had p-values less than 0.001. The variance in concentrations for lift stations and WWTPs were not significantly different from each other for any virus at the Bonferroni-adjusted 0.0056 significance level.

Table 2: Descriptive statistics for detections of astrovirus, rotavirus, and sapovirus across school, lift station, and WWTP wastewater samples.

	Astrovirus	**Rotavirus**	**Sapovirus**
Positive detections across all sites (n, %)	102 (62)	72 (44)	125 (76)
Positive detections across schools (n, %)	28 (32)	8 (9)	48 (55)
School wastewater concentration in copies/L (mean, SD)	3.9×10^7 (3.6×10^8)	4.5×10^4 (2.4×10^5)	2.4×10^7 (1.0×10^8)
Lift station wastewater concentration in copies/L (mean, SD)	3.8×10^6 (4.3×10^6)	5.2×10^4 (4.7×10^4)	3.6×10^6 (3.2×10^6)
WWTP wastewater concentration in copies/L (mean, SD)	6.4×10^6 (6.1×10^6)	1.3×10^5 (1.4×10^5)	5.7×10^6 (3.7×10^6)

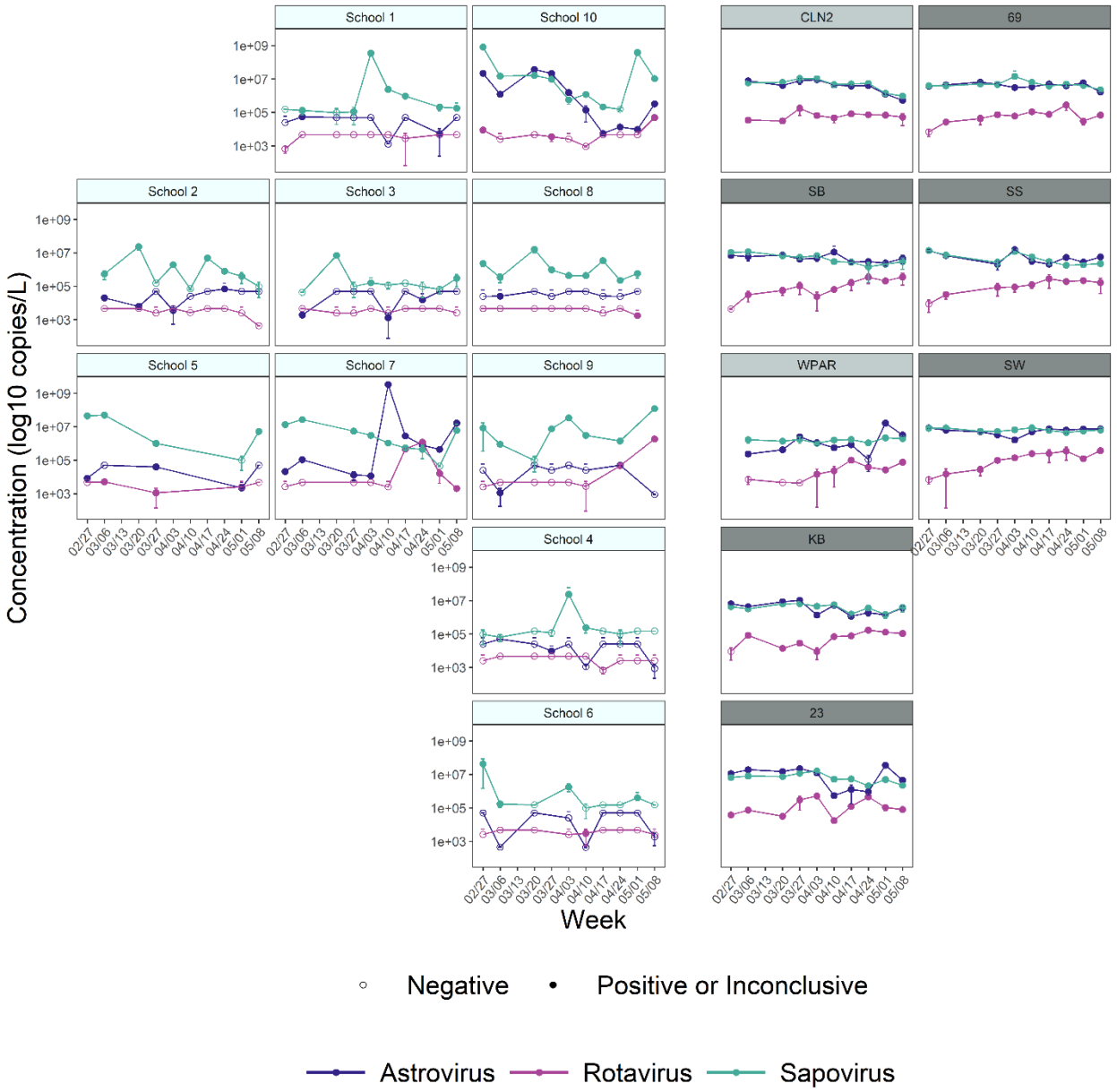

Figure 2. Time-series plot of astrovirus, rotavirus, and sapovirus wastewater concentrations in log10 copies/L from 10 schools, 2 lift stations, and 6 WWTPs (n = 164 samples) collected between the weeks of February 27th, 2023 and May 8th, 2023. Plots on left with white headers are schools, plots on right with light gray headers are lift stations and plots with dark gray headers are WWTPs. Each row represents a group of nested sites with school wastewater feeding

into the lift stations and WWTPs in their row. Error bars represent the standard deviation from the mean for each set of replicates. When there was no data shown, no samples were collected. No samples were collected the week of March 13th, 2023, due to a school holiday.

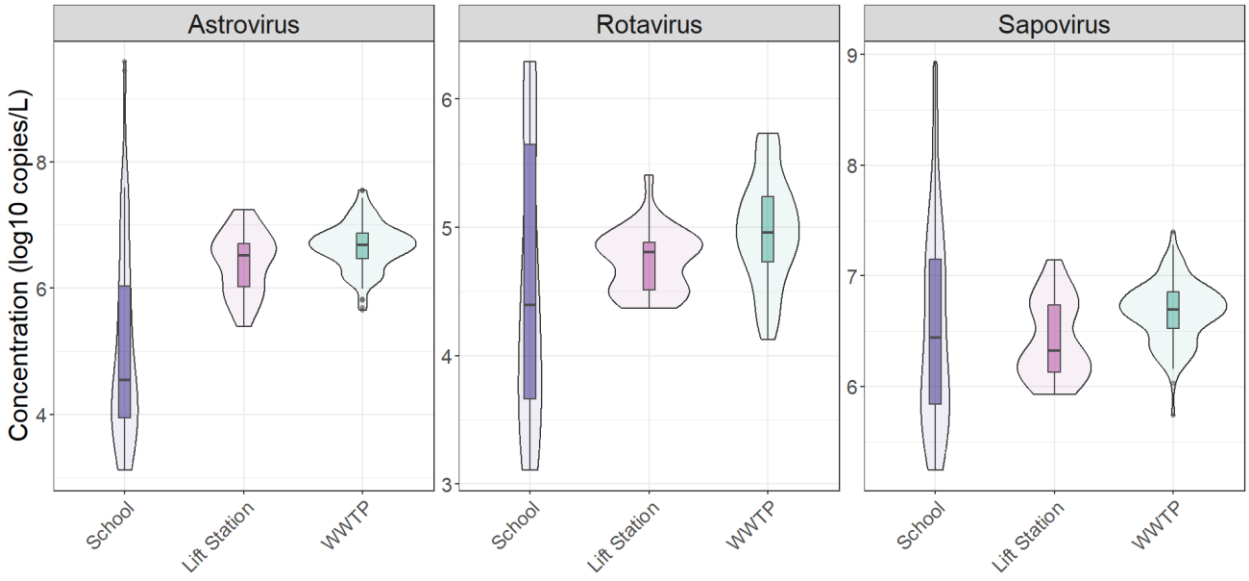

Figure 3. Violin plot of the distributions of wastewater concentrations in log10 copies/L for positive detections of astrovirus, sapovirus, and rotavirus grouped by facility type (school, lift station, or WWTP). Each violin plot is composed of a box plot and a kernel density plot.

3.2 Positive school wastewater detections were almost always accompanied by positive detections in their downstream sites in nested sampling design

Each of the 10 schools' wastewater feeds into a WWTP, and some into a lift station before reaching their downstream WWTP. The schools had fewer detections of all 3 viruses compared to lift station and WWTP detections. However, when a school was positive for a virus, at least one of its downstream sites was also positive for that virus over 97% of the time, showing that a positive school detection was almost always seen alongside a positive downstream detection for the same virus (Figure 4). There were a few exceptions when a school was positive for a virus and its downstream site was negative or inconclusive for that virus. For example, on 02/27/23, School 10 was positive for rotavirus, but its downstream WWTP (69) was negative. On 03/06/23, School 5 was positive for rotavirus and its downstream lift station (WPAR) and downstream WWTP (SW) were negative and inconclusive for rotavirus, respectively. Lastly, on 03/06/23, 04/10/23, and 04/24/23, School 7 was positive for astrovirus and its downstream lift station (WPAR) was inconclusive or negative. This indicates that school samples could provide additional information on isolated outbreaks that may be too localized to be detected at downstream sampling sites.

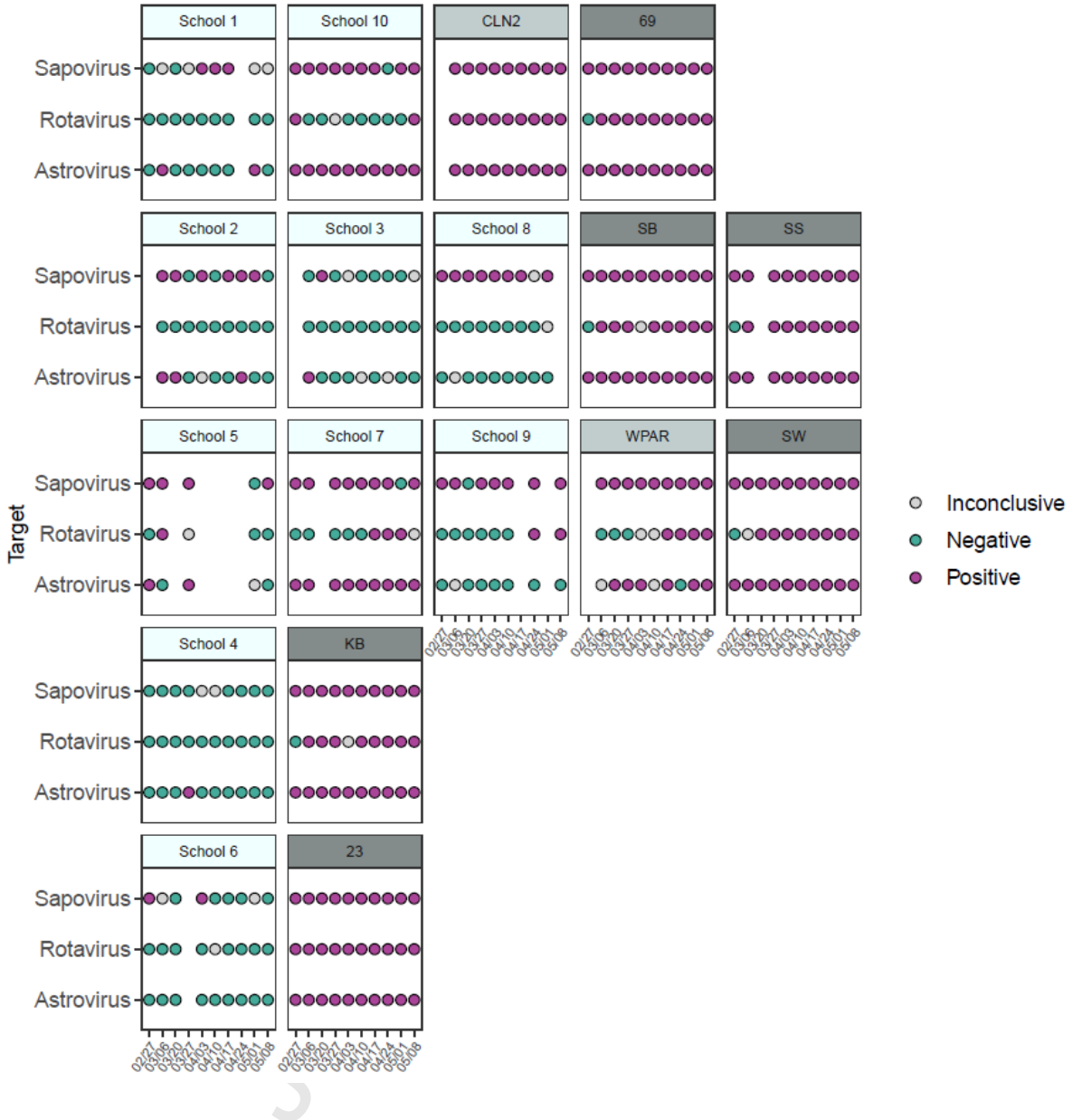

Figure 4. Strip plot of weekly astrovirus, rotavirus, and sapovirus detections for 10 schools, 6 WWTPs, and 2 lift stations (n = 164 samples) collected between the weeks of February 27th, 2023 and May 8th, 2023. Plots with white headers are schools, plots with light gray headers are lift stations, and plots with dark gray headers are WWTPs. Each row represents a group of nested sites with school wastewater feeding into the lift stations and WWTPs in their row.

3.3 School and wastewater treatment plant wastewater measurements for rotavirus were representative of citywide viral intestinal infections

Detections of astrovirus, rotavirus, and sapovirus in school wastewater were compared to the number of citywide viral intestinal infections. The number of citywide visits with a discharge diagnosis of viral intestinal infections by week over the study period is shown in Figure 5A. A significant positive relationship between citywide viral intestinal infections and the proportion of schools with positive rotavirus detections was confirmed with linear regression (Figure 5B: $\beta_1 \approx 212.51$; $p \approx 0.0351$). This means that a 0.10 unit increase in the proportion of schools with positive rotavirus detections is associated with a 21.3 increase in the number of citywide viral intestinal infections. The linear regressions for the number of citywide viral intestinal infections using the proportion of schools with positive astrovirus or sapovirus detections were not significant (data not shown).

Wastewater treatment plants serve large portions of the city of Houston and are, in turn, more representative of citywide infections. Therefore, the citywide viral loads of these viruses in WWTP wastewater were also compared to the number of citywide viral intestinal infections using linear regression (Figure 5C). This linear regression shows a significant positive relationship between the number of citywide viral intestinal infections using the citywide viral load of rotavirus in copies/L ($\beta_1 \approx 7.15 \times 10^{-11}$; $p \approx 0.041$). No clear relationships between the citywide viral loads of astrovirus or sapovirus to clinical infections were observed. In addition,

we compared citywide infections specifically diagnosed as rotavirus to the citywide viral load of rotavirus using linear regression (Figure A.2) and observed a significant positive relationship.

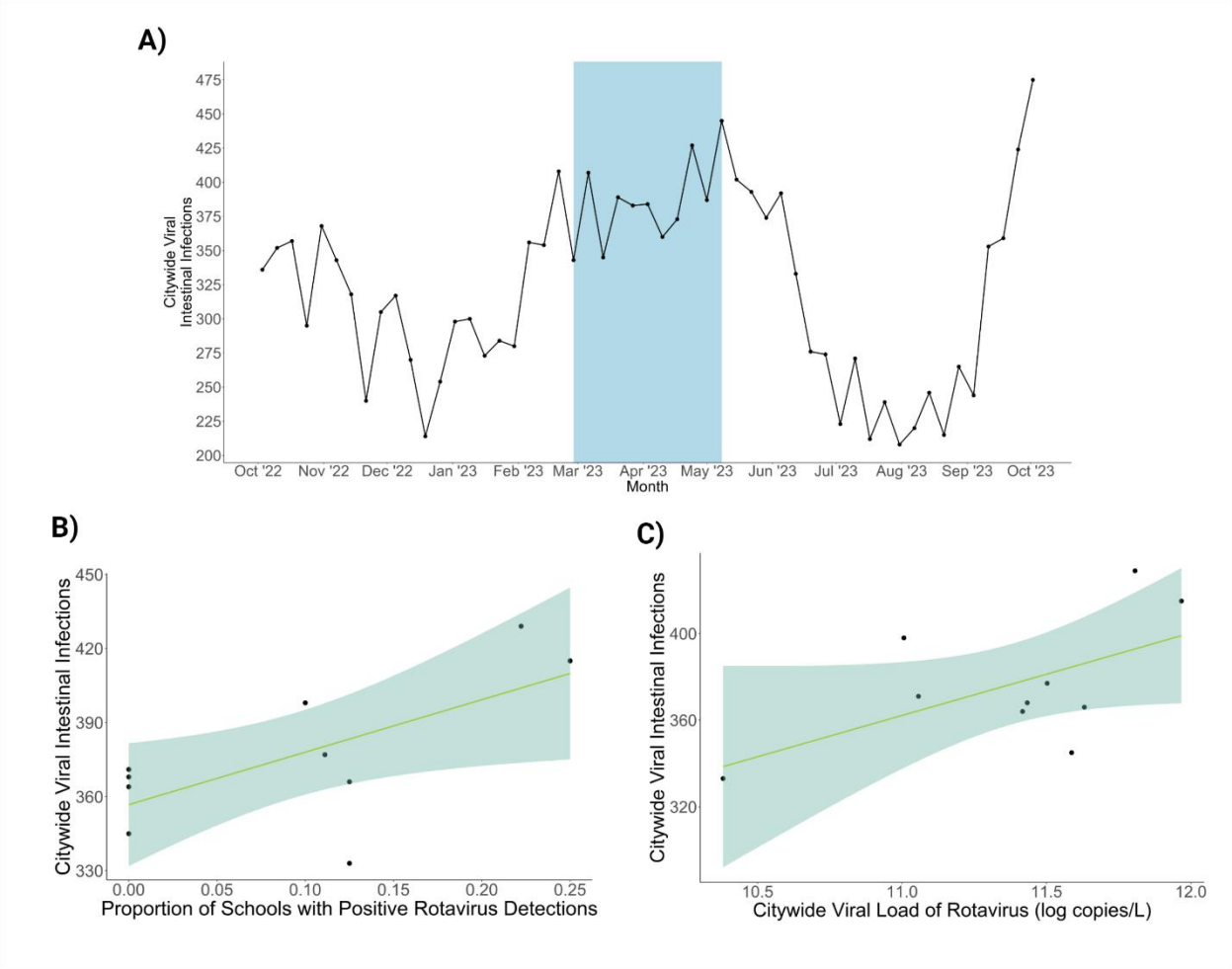

Figure 5. A) Time series of the number of citywide viral intestinal infections by week from October 3rd, 2022 to October 2nd, 2023. The blue-shaded region depicts the period in which wastewater was tested for astrovirus, rotavirus, and sapovirus. B) Linear regression of the number of citywide viral intestinal infections estimated using the proportion of schools with

positive wastewater detections of rotavirus ($\beta_1 \approx 212.51$; $p \approx 0.0351$). C) Linear regression of the number of citywide viral intestinal infections estimated using the citywide viral load of rotavirus in log10 copies/L. The green shaded regions of both linear regressions represent the 95% confidence intervals of the estimated regression lines. The slope coefficient and p-value for the regression between viral intestinal infections and wastewater viral load in copies/L are $\beta_1 \approx 7.15 \times 10^{-11}$ and $p \approx 0.041$.

4. Discussion

Enteric viruses are an important group of pathogens to survey as many infected individuals do not seek medical attention, leading to an underreporting of enteric virus infections by clinical surveillance data (Miranda and Schaffner, 2019; Prevost et al., 2015; Scallan et al., 2011). As enteric viruses are often spread person-to-person or through contaminated food or water, schools represent a surveillance location with the long-term potential of decreasing the likelihood of transmission among school-goers and into the surrounding communities (Bouseettine et al., 2020; Donaldson et al., 2020; D'Souza, 2015; Hassan-Ríos et al., 2013; Iritani et al., 2016). In this study, we show that school, lift station, and WWTP wastewater monitoring can be used to detect enteric viruses that largely go unreported in communities (CDC, 2022a; Moser and Schultz-Cherry, 2008; Parashar et al., 2013). This information can complement existing disease surveillance systems based on clinical testing. The wastewater monitoring protocols described in this study could be easily adjusted to detect other pathogens, including other major enteric viruses such as adenovirus group 40/41 and norovirus.

We used a nested sampling design that included 10 preK-12 schools, 2 lift stations, and 6 WWTPs, and spanned a period of 10 weeks. The study period took place during the spring, which coincided with the end of the peak season for astrovirus and sapovirus infections and during the peak of rotavirus infections (American Academy of Pediatrics, 2021; CDC, 2021; Dey et al., 2012; Moser and Schultz-Cherry, 2008; Thwiny et al., 2022; Zhuo et al., 2021). While some sites shared similar trends in virus concentrations over time, there was significant variability between sites, facility types, and viruses. For instance, school wastewater concentrations were generally more variable than the concentrations of their downstream lift station and WWTP sites that serve larger populations. This increased variability in facility-level samples compared to lift station and WWTP samples has also been seen in previous studies with viruses such as pepper mild mottle virus (PMMoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Corchis-Scott et al., 2021; Sharkey et al., 2021). These differences may occur because facility-level samples have less people contributing to them and are thereby more influenced by fluxes in the number of people infected. This increased variability in school wastewater concentrations could mean that school wastewater provides more sensitive information on changes in concentration levels than lift stations or WWTPs, potentially making it easier to determine when outbreaks are occurring in these schools.

Further, positive school wastewater detections were almost always accompanied by positive detections in their downstream sites. Lift stations and WWTPs were almost always positive for all three viruses over the 10-week study period, suggesting that these viruses were prevalent in the community. In rare instances, school wastewater samples were positive for viruses that were not detected in the schools' downstream sites. This could be due to isolated

outbreaks in schools that did not affect the larger community enough to be detected in the wastewater of downstream sites. The collection of unrepresentative samples could have also resulted in these differences as autosamplers collect imperfect composite wastewater samples and may have resulted in a false negative. These rare instances may also indicate that schools can provide additional detection information that enables the monitoring of outbreaks that would otherwise go undetected. This building-level surveillance approach allows for the detection of local outbreaks within the school community. Monitoring school wastewater samples could enable the rapid detection of outbreaks and, in turn, the timely implementation of mitigation strategies to protect vulnerable populations. Several studies discuss mitigation strategies that have been implemented based on wastewater detections of pathogens. These strategies include directing resources to communities for clinical testing and vaccinations, messaging with community members to increase awareness and encourage behavior changes, communication with school leadership, and the creation of public interactive dashboards that summarize recent wastewater data aggregated by community (Hopkins et al., 2023; McClary-Gutierrez et al., 2021; O'Keeffe, 2021). Enteric virus detections could be used to inform school staff when to increase messaging around specific infectious diseases, initiate more intensive cleaning procedures, promote more frequent hand-washing, and in the case of vaccine-preventable diseases, provide information about immunization (Bhatta et al., 2020; Jin et al., 2022).

While no previous studies have reported on astrovirus, sapovirus, or rotavirus in school wastewater, some studies from across the world have monitored these viruses in WWTP samples (Bisseux et al., 2018; Cuevas-Ferrando et al., 2022; Fioretti et al., 2016; Haramoto et al., 2008). Overall, our results were consistent with prior studies in terms of virus concentration and

detection frequency. Most of the results from previous studies showed similarly high positivity rates and concentrations in WWTP samples when compared to the results of the present study. While there were some differences among the positivity rates and concentrations of sapovirus (Fioretti et al., 2016; Haramoto et al., 2008), differences are expected as wastewater concentrations are a function of the number of infected individuals in a community. Differences in methods, including concentration, extraction, assay design, and quantification methods could also lead to differences in observed wastewater concentrations across studies.

We compared school and WWTP wastewater detections of enteric viruses to citywide clinical data. Our results show that school and WWTP wastewater detections of rotavirus were associated with citywide viral intestinal infections. This indicates that schools can reflect community-level trends and thus represent an important sentinel site for wastewater monitoring for infectious disease surveillance, as was previously shown for respiratory viruses as well (Castro-Gutierrez et al., 2022; Wolken et al., 2023). Significant relationships between the wastewater detections of sapovirus and astrovirus and the number of citywide viral intestinal infections were not found. This is potentially due to the stable levels of these viruses in wastewater compared to the more variable levels of rotavirus. Other studies have also compared wastewater detections of enteric viruses to clinical data. Boehm et al., 2023 monitored norovirus GII levels in wastewater solids at 145 WWTPs across the United States and found a significant positive association between wastewater measurements and clinical positivity rates for norovirus GII (Boehm et al., 2023). Additionally, McCall et al., 2021 investigated the concentration of another enteric virus, hepatitis A virus, in WWTP samples and saw a significant positive correlation between the number of reported hepatitis A cases and the concentration of hepatitis A

in the wastewater 7 days before (McCall et al., 2021). Several studies have also discovered correlations between the circulating strains of rotavirus in the wastewater and the strains of rotavirus found in clinical samples (Carducci et al., 2006; Kamel et al., 2010; Sdiri-Loulizi et al., 2010). These studies as well as the one presented here show that wastewater measurements of enteric viruses can reflect clinical infections and thereby provide insight on infections that are often underreported.

While school and nested site wastewater monitoring provide many benefits to public health, there are limitations. Wastewater is a complex matrix that contains a mixture of biological and chemical constituents that can lead to measurement and sample variability (Pulicharla et al., 2021). Because of these uncertainties, it can be difficult to use wastewater concentrations of a pathogen to determine how many infected individuals are in an area or population. These infected individuals across schools could have potentially been infected by the same contaminated sources, which could affect the validity of the linear regression comparing the proportion of schools with positive rotavirus detections to the number of citywide viral intestinal infections. Additionally, even if a sample is negative, or below the limit of detection, it does not clear a population of infections. Infections corresponding to low viral loads in the wastewater could also be missed due to the sample collection process, such as choice of sampling day(s), sample collection methods (grab vs. autosampler), etc. (CDC, 2022b). In this study, sample extracts were often diluted to enable thresholding between positive and negative droplets. However, diluting samples could have potentially led to results that fell below the LOD as samples with low viral concentrations may have been diluted to the point that the final concentration is under the limit of detection. Viral divergence could also cause a virus to be

reported as undetected when present (Schussman et al., 2022; Silva et al., 2022; Thakali et al., 2024). Additionally, there is intrinsic temporal variability among time series data. While autocorrelation was explored in this study, time series analysis methods are more impactful for data sets that span larger periods of time. Other sources of variability associated with the present analysis include variability and uncertainty of toilet use behavior, especially when sampling from schools, and fecal shedding of the different pathogens. Children may be less likely to use the bathroom at school than at home, so wastewater samples may not be representative of all students (Zemer et al., 2023). Additionally, sick individuals may use the bathroom more than healthy individuals (Rao et al., 2014). Further research is needed to investigate how these factors differ between groups of people and how they could affect the comparability of results from different studies.

5. Conclusions

The results of this study show how astrovirus, rotavirus, and sapovirus can be monitored in preK-12 school wastewater and how a positive school detection of one of these viruses was almost always paired with a positive downstream detection of the same virus. This study also demonstrates how these wastewater measurements for rotavirus were reflective of citywide clinical data. Further research, including more longitudinal wastewater monitoring data and paired clinical testing at individual facilities is needed to understand the relative contributions of the viruses investigated in this study and other major enteric viruses, such as noroviruses, to enteric infections in preK-12 schools. The pooled sample approach of wastewater monitoring allows for the cost-effective and sustainable surveillance of groups of individuals without the

need for individuals to be symptomatic or to exhibit health-seeking behaviors. School wastewater monitoring for enteric viruses can provide schools and public health officials with timely and specific information on the presence of enteric viruses in their communities. This information could be especially helpful in high-transmission areas such as preK-12 schools. The implementation of wastewater monitoring in schools coupled with public health mitigation strategies could ultimately lead to reduced transmission and burden of disease among children and surrounding communities.

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Table 1. School enrollment information and population size served for corresponding downstream lift stations and WWTPs. Site name codes for WWTPs and lift stations are in parentheses.

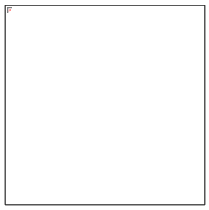
School	Enrollment as of 2022-2023 school year	Downstream lift station (if sampled)	Population served by lift station (if sampled)	Downstream WWTP(s)	Population served by WWTP(s)
1	2,769	Clinton Drive #2 (CLN2)	373,937	69th Street (69)	551,150
2	2,257	n/a	n/a	Sims Bayou North and Sims Bayou South (SB and SS)[a]	109,414
3	2,102	n/a	n/a	Sims Bayou North and Sims Bayou South (SB and SS)[a]	109,414
4	1,279	n/a	n/a	Keegans Bayou (KB)	124,000
5	1,035	n/a	n/a	Southwest (SW)	293,227
6	974	n/a	n/a	FWSD #23 (23)	40,689
7	919	n/a	n/a	Southwest (SW)	293,227

8	904	n/a	n/a	Sims Bayou North and Sims Bayou South (SB and SS)[a]	109,414
9	898	Westpark No.1 FN208 (WPAR)	43,088	Southwest (SW)	293,227
10	865	Clinton Drive #2 (CLN2)	373,937	69th Street (69)	551,150

[a]Sims Bayou North and Sims Bayou South have overlapping geographic service areas

Table 2: Descriptive statistics for detections of astrovirus, rotavirus, and sapovirus across school, lift station, and WWTP wastewater samples.

	Astrovirus	**Rotavirus**	**Sapovirus**
Positive detections across all sites (n, %)	102 (62)	72 (44)	125 (76)
Positive detections across schools (n, %)	28 (32)	8 (9)	48 (55)
School wastewater concentration in copies/L (mean, SD)	3.9×10^7 (3.6×10^8)	4.5×10^4 (2.4×10^5)	2.4×10^7 (1.0×10^8)
Lift station wastewater concentration in copies/L (mean, SD)	3.8×10^6 (4.3×10^6)	5.2×10^4 (4.7×10^4)	3.6×10^6 (3.2×10^6)
WWTP wastewater concentration in copies/L (mean, SD)	6.4×10^6 (6.1×10^6)	1.3×10^5 (1.4×10^5)	5.7×10^6 (3.7×10^6)



Highlights

- Astrovirus, rotavirus, and sapovirus were quantified in a nested sampling design
- Positive school detections were paired with positive detections downstream
- Rotavirus school wastewater detections were reflective of citywide infections
- Citywide rotavirus wastewater viral load was reflective of citywide infections