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# A one-year study on molecular and epidemiological monitoring and analysis of enteroviruses and waterborne hepatitis throughout various stages of wastewater treatment

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ABSTRACT

Wastewater, a major contributor to waterborne disease outbreaks, is contaminated with various microorganisms, including viruses that cause hepatitis and gastroenteritis. In São José do Rio Preto, São Paulo state, Brazil, samples were collected at three stages - raw sewage (RS), post-anaerobic biological treatment (PABT) and post-chemical treatment (PCT) - over the course of one year (2022–2023) for the detection of Enterovirus (EV), Hepatitis A (HAV), and Hepatitis E (HEV) viruses, with molecular characterization when possible. The 156 collected samples were tested using in-house qPCR for EV and HAV. HEV detection utilized conventional Nested PCR. The findings were correlated with epidemiological data and physicochemical water parameters, considering seasonal and meteorological variations. EV prevalence was 98.1 % (RS), 88.5 % (PABT), and 42.3 % (PCT), while HAV prevalence was 25.0 % (RS), 23.1 % (PABT), and 13.5 % (PCT). HEV was found in two stages, with prevalences of 5.8 % (RS) and 3.8 % (PABT). EV quantification ranged from 2.20 to 4.45 $\log_{10}$ GC/mL, and HAV from 2.63 to 4.42 $\log_{10}$ GC/mL. EV was found in higher concentrations during milder temperatures (autumn and winter) across different wastewater treatment stages ($p \leq 0.05$), while no significance was observed for HAV. EV and HAV showed mean reductions of 1.74 and 2.20 $\log_{10}$, respectively, throughout the treatment process, with the greatest reduction in viral load observed after chemical treatment with chlorine. However, neither virus was completely eliminated, which may pose a potential public health concern. Phylogenetic analysis identified HAV genotype IA and HEV genotype 3, and EV genotype A was detected by Sanger sequencing. The recovery efficiency test using Echovirus 3 showed good recovery of ultracentrifugation method percentages in RS and PABT (68.0 % to 48.7 %), while PCT samples ranged from 28.1 % to 0.0 %. No statistically significant differences were found when correlating reported cases of enteric or hepatic disease with the corresponding detected viruses ($p > 0.05$). In conclusion, these findings emphasize the need for continuous monitoring of wastewater treatment to effectively assess and mitigate the risk of waterborne diseases.

## Introduction

Wastewater is a leading cause of waterborne disease outbreaks due to its contamination with a diverse array of microorganisms, including bacteria, parasites, fungi, protozoa, and viruses (Janahi et al., 2020). Consequently, it is regarded as a primary vector for viral transmission

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and a significant reservoir for these pathogens (Richter et al., 2011; Ouardani et al., 2016). Despite wastewater treatment facilities effectively reduce the concentration of most enteric viruses, some still remain and are released into the environment, potentially resulting in infection and reinfection of susceptible populations (Prado et al., 2013; Ouardani et al., 2016; Wang et al., 2020). Enteric viruses are primarily transmitted through the oral-fecal route, with viruses shed in stool and detectable in wastewater, where they generally persist in the environment for 1 to 10 months (Prado et al., 2013; Wang et al., 2020). These viruses are responsible for a range of diseases, including hepatitis and gastroenteritis (Prado et al., 2013). Viruses commonly associated with waterborne diseases include human Adenovirus species F, Aichi virus (AiV), Enterovirus (EV), Hepatitis A (HAV) and Hepatitis E (HEV) viruses, Norovirus (NoV), Sapovirus, and Rotavirus (Farkas et al., 2018; Azhdar et al., 2019; Wang et al., 2020).

EV are small, non-enveloped RNA viruses from the *Picornaviridae* family, comprising over 300 serotypes, distributed across 15 species (Tao et al., 2020; ICTV, 2023). This includes Coxsackieviruses A and B, enteroviruses, polioviruses (types 1–3), and Rhinoviruses (types A-C) (Delogu et al., 2018; Wen et al., 2019). Enteroviruses affect thousands of individuals globally each year, primarily impacting children (Pogka et al., 2017). Although these viruses predominantly replicate in the intestinal tract, they have the potential to disseminate to other organs, causing a variety of clinical manifestations that can sometimes lead to severe syndromes including non-specific acute febrile illness, skin rashes, acute respiratory distress, and severe neurological complications (Richter et al., 2011; Tao et al., 2020; Huang, 2021). Global studies have frequently detected EV in both raw and treated sewage, highlighting their resistance to wastewater treatment processes. This has been observed in countries such as Brazil (Vecchia et al., 2012); Canada (Qiu et al., 2015), France (Bisseux et al., 2018), Italy (Delogu et al., 2018 ; Pennino et al., 2018; Pellegrinelli et al., 2019) and Tunisia (Ibrahim et al., 2023).

Another member of the *Picornaviridae* family is the HAV. It is a small non-enveloped virus, and its genome consists of single-stranded RNA that encodes a polyprotein, which is cleaved into four capsid proteins (VP1, VP2, VP3, and VP4) and non-structural proteins (2A–3D) (Najarian et al., 1985; McKnight and Lemon, 2018). It is classified into seven distinct genotypes, with genotypes I, II, and III, further divided into subtypes A and B, infecting humans, while genotypes IV, V, and VI have been identified in infected non-human primates (Di Cola et al., 2021). Transmission occurs via fecal-oral route, typically via ingestion of contaminated food or water, with the liver serving as the primary site of replication (Tarek et al., 2019; Abutaleb and Kottilil, 2020). The disease spectrum varies from asymptomatic cases, especially in children, to severe illness in adults, with symptoms such as fever, fatigue, loss of appetite, nausea, and jaundice, although it generally has low mortality (Iaconelli et al., 2015). The geographical distribution of HAV is closely correlated with socioeconomic conditions and sanitary infrastructure (Ouardani et al., 2016; Pellegrinelli et al., 2019). Worldwide, HAV has been detected in wastewater from countries including Argentina (Fantilli et al., 2023), Brazil (Villar et al., 2007; Prado et al., 2011), Italy (La Rosa et al., 2014; Pellegrinelli et al., 2019), South Africa (Adefisoye et al., 2016; Rachida and Taylor, 2020), and Tunisia (Ouardani et al., 2016). It is important to highlight that both HAV and HEV are prevalent in economically less developed countries and responsible for sporadic outbreaks exacerbated by the frequent global movement of people (Farkas et al., 2018).

HEV is small with a 7.2 kb positive-sense RNA genome, belonging to the *Hepeviridae* family, which is categorized into four genera, with members of the *Paslahepevirus* genus (*Orthohepevirus A*) identified in mammals, including humans (Aslan and Balaban, 2020; Caballer-Gomez et al., 2022). It circulates as quasi-enveloped virions in blood but is non-enveloped in feces and bile (Nagashima et al., 2017). The genome has three open reading frames (ORFs) (Cancela et al., 2022). *Orthohepevirus A*, specifically *Paslahepevirus balayani*, includes eight genotypes (HEV-1 to HEV-8). HEV-1 and HEV-2 are linked to human outbreaks, while HEV-3 and HEV-4 infect humans and various animals, with pigs as major reservoirs, being zoonotic. HEV-5 and HEV-6 are found in wild boar in Japan, HEV-7 in dromedary camels with rare human cases, and HEV-8 in Bactrian camels in China (Woo et al., 2014; Takahashi et al., 2014; Smith et al., 2020; Nishizawa et al., 2021). Its transmission primarily occurs through the consumption of meat products from infected animals and through water contaminated with feces, serving as a source of infection for both animals and humans (Beyer et al., 2020). HEV replicates in the liver, usually causing a mildself-limiting disease but can become chronic in immunocompromised individuals or result in severe complications in pregnant women (Vivek et al., 2013; Iaconelli et al., 2015; Kamar et al., 2017; Cullen and Lemon, 2019). Similar to EV and HAV, HEV has also been detected in wastewater systems across several countries in Latin America [Argentina (Martínez Wassaf et al., 2014), Brazil (dos Santos et al., 2011), Uruguay (Cancela et al., 2023)] and Europe [Germany (Beyer et al., 2020), Sweden (Churqui et al., 2024), Switzerland (Masclaux et al., 2013)].

The main objective of this study was to detect, quantify, and, when possible, sequence these viruses, in raw sewage (RS) samples, post-anaerobic biological treatment (PABT) and post-chemical treatment (PCT), conducted in the city of São José do Rio Preto – SP. Although the treated wastewater of the city is discharged into the Rio Preto River and is not used as a source of drinking water or for recreational purposes, it still poses a threat to the riverside population. Given the significant threats to public health posed by these viruses, it is essential to evaluate disease exposure and transmission through sanitation systems, since environmental monitoring of these waters is crucial for managing virus spread and mitigating public health risks (Beyer et al., 2020; Lahrich et al., 2021). EV, HAV and HEV are prevalent in wastewater environments and can serve as indicators for monitoring wastewater quality to evaluate public health threats (Bisseux et al., 2018; Sigh et al., 2024).

## 2. Materials and methods

### 2.1. Characterization of the study area and sampling

Situated in the interior of São Paulo state, Brazil, the municipality of São José do Rio Preto spans an area of 431.944 km$^2$ and has a population of approximately 480,393 residents, based on the most recent data from the Brazilian Institute of Geography and Statistics (IBGE) for 2022 (IBGE, 2024). The city also has significant activities related to livestock, with companies involved in the raising and slaughtering of cattle, pigs, and poultry, all licensed by the Ministry of Agriculture, Livestock, and Food Supply (Prefeitura de São José do Rio Preto, 2022). The temperatures in the city of São José do Rio Preto - SP, typically range from 20.8 to 27.4 °C on average. Autumn (21st March to June 20th) and winter (21st June to 22nd September) are characterized by milder temperatures and drier conditions, while spring (23rd September to 20th December) and summer (21st December to 20th March) feature higher temperatures and increased rainfall, as detailed in Supplementary Table ST1. Seasonal variations were assessed to investigate the temporal dynamics of each virus in relation to fluctuations in their concentrations.

Samples for the study were collected in collaboration with the São José do Rio Preto Wastewater Treatment Plant, under the management of the Municipal Water and Sewage Service (SeMAE). This facility handles 100 % of the sewage produced in the municipality, processing a total flow of around 100,826 m$^3$/day, using a conventional wastewater treatment system that involves physical, biological (both anaerobic and aerobic), and chemical processes, through disinfection using gaseous chlorine (SeMAE Rio Preto, 2025). An amount of 500 mL of twenty-four-hour composite samples were collected at three critical points within the treatment process: RS, PABT, and PCT, as illustrated in Supplementary Figure SF1, in a HACH Sigma SD900 AWRS refrigerated automatic sampler (HACH, Loveland, CO, USA), the sampling program was configured based on the input flow, collecting one sample for every

3000 $m^3$ processed by the plant, which corresponds to approximately 36 sampling events per day. Sampling occurred weekly over one year (52 weeks), from 29th March 2022 to 20th March 2023, with each season lasting approximately 13 weeks, resulting in a total of 156 samples. For internal control purposes, the samples collected in this study were named based on the detection stage, followed by the week of collection (1–52). After collection, the samples were maintained at temperatures below 4 °C (i.e., 24 h for RS samples, 8 h for PABT samples and 0 h for PCT samples) and transported in glass flasks on ice to the São Paulo State University Laboratory. Upon arrival, samples processed within two days were stored at 4 °C, while those that required longer storage were kept at −80 °C and thawed on the day of handling. A volume of 222 mL of each sample underwent initial clarification via centrifugation at 3000 × *g* and 4 °C for 20 min to remove suspended material. Following the protocol outlined by Girardi et al. (2018), with slight modifications, the samples were then concentrated using ultracentrifugation (Beckman Coulter, Indianapolis, IN, USA) at 41,000 × *g* and 4 °C for 3 h. The resulting pellets were resuspended in Tris-EDTA buffer (pH 8.0) to a final volume of 2 mL and homogenized. Aliquots of the samples were stored at −80 °C until further extraction.

### 2.2. RNA extraction and cDNA synthesis

RNA was extracted from 250 μL of each sample using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After elution in 30 μL of DEPC-treated water (Sigma-Aldrich, Saint Louis, MI, USA), the RNA was stored at −80 °C. For cDNA synthesis, the High-Capacity cDNA Synthesis Kit (Applied Biosystems, Waltham, MA, USA) was used following the manufacturer's protocol, and the resulting cDNA was stored at −20 °C.

### 2.3. Construction of the control plasmid

For HAV and HEV positive controls, two plasmids containing sequences from regions targeted by the HAV qPCR primers and HEV Nested PCR primers were acquired from Integrated DNA Technologies (IDT; Coralville, IA, USA), as detailed in Supplementary Table ST2. The reference sequences were sourced from NCBI (Accession number: MT181522.1 (HAV) and JN906976.1 (HEV)). The plasmids were purified using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific), and the concentration of the purified plasmids were determined by measuring the optical density at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

### 2.4. Virus titration by TCID50 assay for positive control

For the EV positive control, the Tissue Culture Infective Dose 50 (TCID50) assay was performed using Echovirus 3 (isolated Hu/E3/FAMERP_LR254/BRA/2017; Rocha et al., 2021). A 96-well plate with confluent Rhabdomyosarcoma (RD) cell line (ATCC—CCL-136) monolayers, each well containing $10^4$ cells, six replicates were prepared with tenfold serial dilutions of Echovirus 3, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 % fetal bovine serum (FBS) and 1 % penicillin (10,000 IU/mL)/streptomycin (10mg/mL) (Cultilab, São Paulo, Brazil). The plate was incubated at 37 °C with 5 % $CO_2$ until the appearance of cytopathic effects. To increase the virus concentration, four passages were performed. In the final passage, after 48 h, the plate was stained, cytopathic effects were evaluated, and the TCID50/mL was measured.

### 2.5. Quantitative polymerase chain reaction (qPCR)

qPCR was employed for the detection and quantification of EV and HAV. Standard curve for HAV was generated using ten-fold serial dilutions of plasmids from IDT, ranging from $1 \times 10^6$ to $1 \times 10^1$ genomic copies (GC)/μL. For EV, a standard curve was created using ten-fold serial dilutions of Echovirus 3 (Rocha et al., 2021) ranging from $1.25 \times 10^6$ to $1.25 \times 10^1$ GC/μL. QuantiNova Probe PCR Master Mix (Qiagen, Hilden, Germany) was used for the reactions. Each 10 μL reaction mixture contained 5.0 μL of 2 × Master Mix, 1:200 dilution of Dye Rox, forward and reverse primer, probe, nuclease-free water, and 1.0 μL of nucleic acid. Further details on the primers and probes concentration, and cycling parameters are provided on Supplementary Table ST2. The qPCR assays were conducted using QuantStudio 12 K Flex instrument (Applied Biosystems) with manual settings for threshold and baseline. All reactions were carried out in triplicate.

### 2.6. Assessment of qPCR inhibition

An assay was performed to assess PCR inhibition in all cDNA samples ($n = 156$) from wastewater using the *Sketa22* real-time PCR assay (Haugland et al., 2005). The cDNA samples from the wastewater control ($n = 3$) and those seeded with serial dilutions ($n = 12$) for the recovery efficiency assay were also analyzed. Each sample was supplemented with a specific quantity ($10^4$/reaction) of *Oncorhynchus keta* (*O. keta*) gBlock DNA (IDT). To evaluate potential PCR inhibition, *O. keta* DNA was added to DNase- and RNase-free water to establish a baseline mean Cq value. A shift in cycle thresholds (Ct) by more than two cycles after the addition of nucleic acids was considered indicative of inhibition (Staley et al., 2012).

### 2.7. Nested PCR assay

Nested PCR were employed for HEV detection and subsequently Sanger sequencing, while for HAV and EV, these methods were used exclusively for Sanger sequencing in positive samples detected by qPCR.

Nested PCR for HAV and HEV, were conducted using the GoTaq® Colorless Master Mix kit (Promega, Madison, WI, USA), whereas the GoTaq® Green Master Mix kit (Promega) was employed for EV Nested PCR. Each 25 μL reaction mixture contained 12.5 μL of 2 × Master Mix, forward and reverse primers, and nuclease-free water. For HAV and HEV, 2.5 μL of nucleic acid was used for both the initial PCR and the PCR product for Nested PCR. For EV, 5.0 μL of nucleic acid was used for the initial PCR and the PCR product for Nested PCR. Detailed primer sequences, concentrations and cycling conditions are provided in Supplementary Table ST2. Plasmid DNAs were used as the positive control for HAV and HEV while titled Echovirus 3 was used for EV. Ultrapure water was used as the no-template control (NTC) in place of nucleic acid. Amplification was carried out using a Veriti 96-well thermocycler (Applied Biosystems). Following amplification, PCR products were analyzed on a 2 % agarose gel containing 0.5 μg/mL ethidium bromide. Electrophoresis was performed at 90 V for 40 min, and the molecular sizes of the products were compared using a 100 bp DNA ladder (Thermo Fisher Scientific). Bands were visualized under ultraviolet light, and images were captured using the L-Pix Touch photo documentation system (Loccus, Cotia, SP, Brazil).

### 2.8. Sanger sequencing and phylogenetic analysis

The sequencing was performed based on the availability of the samples. Nested PCR amplicons from HAV and HEV positive samples were purified using magnetic beads (SpeedBead Magnetic Carboxylate Modified Particles, Cytiva, Sigma-Aldrich) according to the manufacturer's instructions. EV positive samples were initially purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. Some of these amplicons were then re-purified using magnetic beads (Sigma-Aldrich) in an attempt to improve sequencing results. Sequencing was conducted using BigDye Terminator v3.1 chemistry (Applied Biosystems). For EV, HAV, HEV, both Nested PCR primers (F and R) were used. For some EV positive samples, sequencing was repeated. The primers used are listed in Supplementary Table ST2. The Sanger sequencing was performed on the

automated Spectrum Compact CE System (Promega).

For EV, HAV and HEV, nucleotide sequences were assembled using Geneious Prime® 2023.2.1 and compared with available fragments and genomes in GenBank. The sequences were then aligned in Geneious Prime® using the MUSCLE algorithm (Edgar, 2004). For HAV and HEV, the phylogenetic tree was constructed with the IQ-TREE web service (Nguyen et al., 2015), employing the Maximum Likelihood method and the TIM2+$F$ + $I$ + G4 model selected by the program, with 1000 bootstrap replicates. The final phylogenetic tree was edited using MEGA 10.1.7. Sequences with a high number of degenerate bases or low quality were excluded from the analysis. For EV, the consensus sequence analysis revealed several gaps and degenerate bases, thereby precluding the construction of a phylogenetic tree.

### 2.9. Recovery efficiency assay

To validate the reliability of the methodology used for screening enteric viruses in wastewater systems, we proposed assessing its viability through a recovery efficiency assay, using experimental samples seeded with the Echovirus 3 reference strain (Rocha et al., 2021), which was selected based on its classification within the Enterovirus genus and its availability in the laboratory. The recovery efficiency assay was performed using inactivated Echovirus 3 treated with TRIzol (Thermo Fisher Scientific), and cultured in RD cells. One milliliter of each four serial tenfold dilutions (−1 to −4) was inoculated into 221 mL of the three different types of wastewater samples to estimate the limit. Once homogenized, the clarification and ultracentrifugation protocol, as outlined in Section 2.1, was performed. After introducing known concentrations of the virus, the contaminated wastewater samples were analyzed by qPCR as detailed in Section 2.5. Recovery efficiency was calculated using the equation provided by Ahmed et al., 2020.

### 2.10. Data analysis

Descriptive statistics, including percentages, minimum and maximum values, measures of central tendency (mean and median), and measures of dispersion (standard deviation), were calculated presented graphically using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) or SPSS V24.

The Kolmogorov-Smirnov test was used to evaluate the normality of the data. As the data did not follow a normal distribution, non-parametric tests were employed. Data normalization followed the procedure outlined in the study by Nagarkar et al. (2022). Correlation with acute diarrheal disease (ADD) and HAV cases in São José do Rio Preto, SP, as well as with hydrological data, was assessed using instantaneous correlation (lag = 0) in RS samples. The hydrological data for the samples were supplied by SeMAE, while the ADD cases were obtained from the Ministry of Health's website (https://public.tableau.com/app/profile/dda.brasil/viz/MonitoramentodasDDA/1-MonitoramentoBrasil2023). The cases of HAV and HEV were provided by the municipality of São José do Rio Preto. The cross-correlation function was used to examine the correlation of all three viruses across various lags, with each lag representing one week, in accordance with the weekly sampling schedule. To explore the association between seasonal variations, wastewater parameters, and EV and HAV concentrations, the Chi-square test was conducted. Subsequently, the Kruskal−Wallis test was employed to determine which parameters or seasons exhibited significant differences across the three wastewater treatment stages, with the Bonferroni correction applied to adjust for multiple comparisons. The Mann−Whitney test was applied to compare virus detection between rainy and non-rainy days. Results were considered statistically significant at $p \leq 0.05$, and statistical analysis was performed using SPSS V24. Samples were classified as qPCR positive if they tested positive in at least two of the three replicates, and as qPCR positive but non-quantifiable (NQ) when the quantification value was below 1 GC/reaction. Non-detectable (ND) samples were designated as negative samples or below the limit of detection (LoD). The LoD was not determined in this study, however, we relied on values reported in the original publications. For the EV assay, based on the experimental LoD values of 0.027 and 0.0105 $TCID_{50}$/mL for the EVs and EV71/CA16 channels, respectively, as reported by Zhang et al. (2014), and assuming a conservative average conversion factor of 1 $TCID_{50} = 10^4$ GC (Reetoo et al., 1999; Sachs et al., 2011), the estimated LoD values were approximately 3.15 and 1.23 GC/reaction. These values were calculated considering RNA extraction from 140 μL of sample, eluted in 60 μL, and using 5 μL of the eluate per RT-PCR reaction. For the HAV qPCR assay, the LoD was 3.00 GC/reaction (Prado et al., 2021). Furthermore, for the purpose of statistical analysis, any NQ samples were assigned a value equal to half of the smallest value observed in our study: 1.10 GC/mL for EV and 1.31 CG/mL for HAV (Helsel, 2005). $Log_{10}$ removal was calculated as the $log_{10}$ of the average concentration in RS quantifiable samples minus the $log_{10}$ of the average concentration in PCT samples. Additionally, for the calculation of treatment efficiency, non-detected (ND) samples were also assigned a value equal to half of the lowest observed concentration for each assay in PABT and PCT samples, as applied in the study by Thakali et al. (2020). Samples that were NQ in raw sewage but showed positive signals in subsequent treatment stages were excluded from this analysis, as they do not represent true log reduction pairs.

## 3. Results

### 3.1. Detection of EV, HAV, and HEV at different stages of wastewater treatment throughout the seasons of the year, along with the corresponding reported disease cases in the city

Out of the 156 samples analyzed, 119 samples (76.3 %) tested positive for EV, 32 samples (20.5 %) tested positive for HAV, and five samples (3.2 %) tested positive for HEV.

For EV, of the 119 positive samples, 51 (42.9 %) were from RS, 46 (38.6 %) from PABT, and 22 (18.5 %) from PCT. Additionally, when considering each phase independently, of the 52 samples collected throughout the year, the detection rates were 98.1 %, 88.5 %, and 42.3 % for the respective wastewater treatment stages, indicating the prevalence at each stage. RS samples exhibited high EV detection rates (100 %) in autumn, winter, and spring, with a slightly lower rate (91.7 %) in summer. PABT samples demonstrated higher detection rates (> 80 %) in autumn, summer, and spring, with a lower rate in winter (76.9 %). For the PCT samples, higher detection rates were observed in milder climates, with winter (84.6 %) and autumn (42.9 %) showing the highest rates, while lower detection rates were noted in warmer climates such as spring (23.2 %) and summer (16.7 %). A total of 6986 cases of ADD were reported in autumn, 5744 in winter, 5512 in spring, and 7258 in summer (Ministério da Saúde, 2023).

Out of the 32 positive HAV samples, 13 (40.6 %) were from RS, 12 (37.5 %) from PABT, and 7 (21.9 %) from PCT. Also, when considering each phase independently, of the 52 samples collected throughout the year, the detection rates were 25.0 %, 23.1 %, and 13.5 % for the respective sewage treatment stages, reflecting the prevalence at each stage. The highest HAV detection rate was observed in summer (33.3 %), followed by winter (30.8 %), autumn (28.6 %), and spring (23.1 %), for RS samples. For PABT samples, the detection rates followed a similar pattern, with differences in percentages seen between summer (41.7 %) and spring (7.7 %). For PCT samples, detections were limited to milder seasons, specifically winter (30.8 %) and autumn (21.4 %). Two cases of HAV were reported in both autumn and spring.

For HEV, out of the five positive samples, three samples (60.0 %) were from RS, two (40.0 %) from PABT, with none detection at PCT stage. Its prevalence, when considering the 52 samples collected throughout the year, was 5.8 % for RS and 3.8 % for PABT. Regarding seasonal variation, HEV detection in RS was consistent across winter, spring, and summer (7–9 %), with no detections observed in autumn. For PABT, detection occurred only in winter (15.4 %). No cases of HEV

were reported in the city during the study period.

Further information regarding positive samples and the number of cases can be found in Supplementary Tables ST3 and ST4.

### 3.2. Performances for EV, HAV and HEV and inhibition test for qPCR assays

For EV and HAV qPCR assays were demonstrated an efficiency of 96.4 % and 104.9 % in their standard curve, with a linearity ($R^2$) of 0.994 and 0.986, respectively. The Y-intercept were 37.102 and 40.861, and the slope were −3.412 and −3.210.The lowest sample concentrations observed in this study were 1.58 GC/reaction for the EV assay and 4.80 GC/reaction for the HAV assay, which align with the LoD values reported in the original studies and were deemed reliable for subsequent data interpretation. Although it was also attempted for HEV quantification, none of the HEV-positive samples from Nested PCR tested positive in qPCR. For the inhibition test, of the 156 wastewater samples analyzed, seven (4.5 %) exhibited inhibition. The cDNAs from these samples were then diluted 1:10 and retested, with no further inhibition observed. Among the 15 samples used in the recovery efficiency assay, none exhibited inhibition.

### 3.3. Quantification of EV and HAV in different stages of wastewater treatment

Viral quantification through qPCR was successful for EV and HAV positive samples for, but not for HEV positive samples. The data were organized by week and season, using the Southern Hemisphere as a seasonal reference, and including the quantification for each sample along with ADD cases, as depicted in Fig. 1.

Throughout the year, ADD cases exhibited a notable prevalence, with numbers ranging from 398 to 501 cases between weeks 1 and 44, excluding week 10 (May 2022), which experienced a peak of 680 cases. From weeks 45 to 47 (January and February 2023), the number of cases began to increase, ranging from 564 to 592 cases. The highest average of ADD cases in the study occurred during weeks 48 to 52 (February and March 2023), with cases ranging from 648 to 846. EVs were detected throughout the entire study period, although their concentrations varied over time (Fig. 1a). For RS samples, concentrations ranged from 2.27 to 4.45 $\log_{10}$ GC/mL. The lowest (i.e., RS-19) was observed in the winter, while the highest quantification (i.e., RS-8) occurred in the autumn season. In PABT samples, concentrations ranged from 2.29 to 4.14 $\log_{10}$ GC/mL. This time, the lowest and highest quantifications were found in

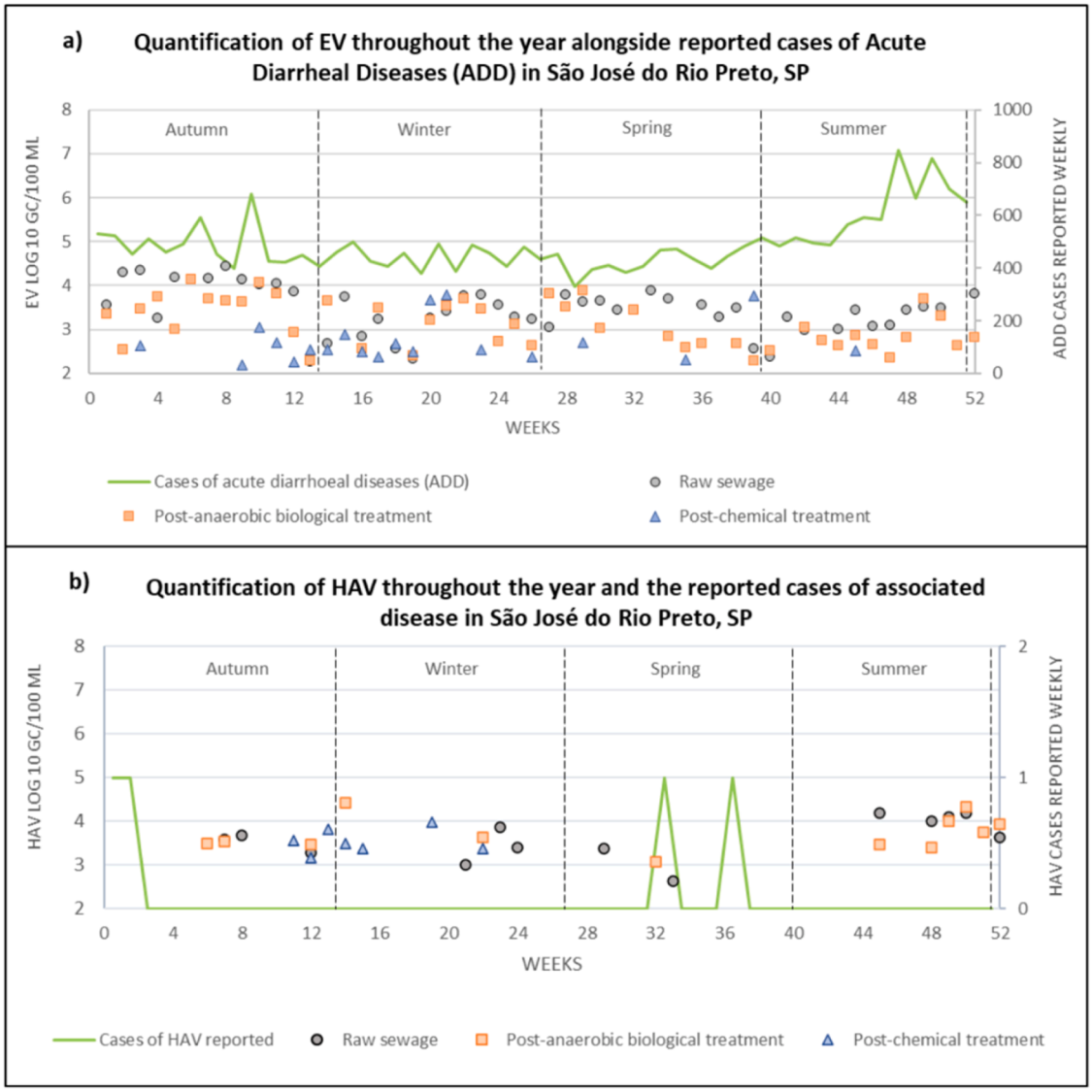


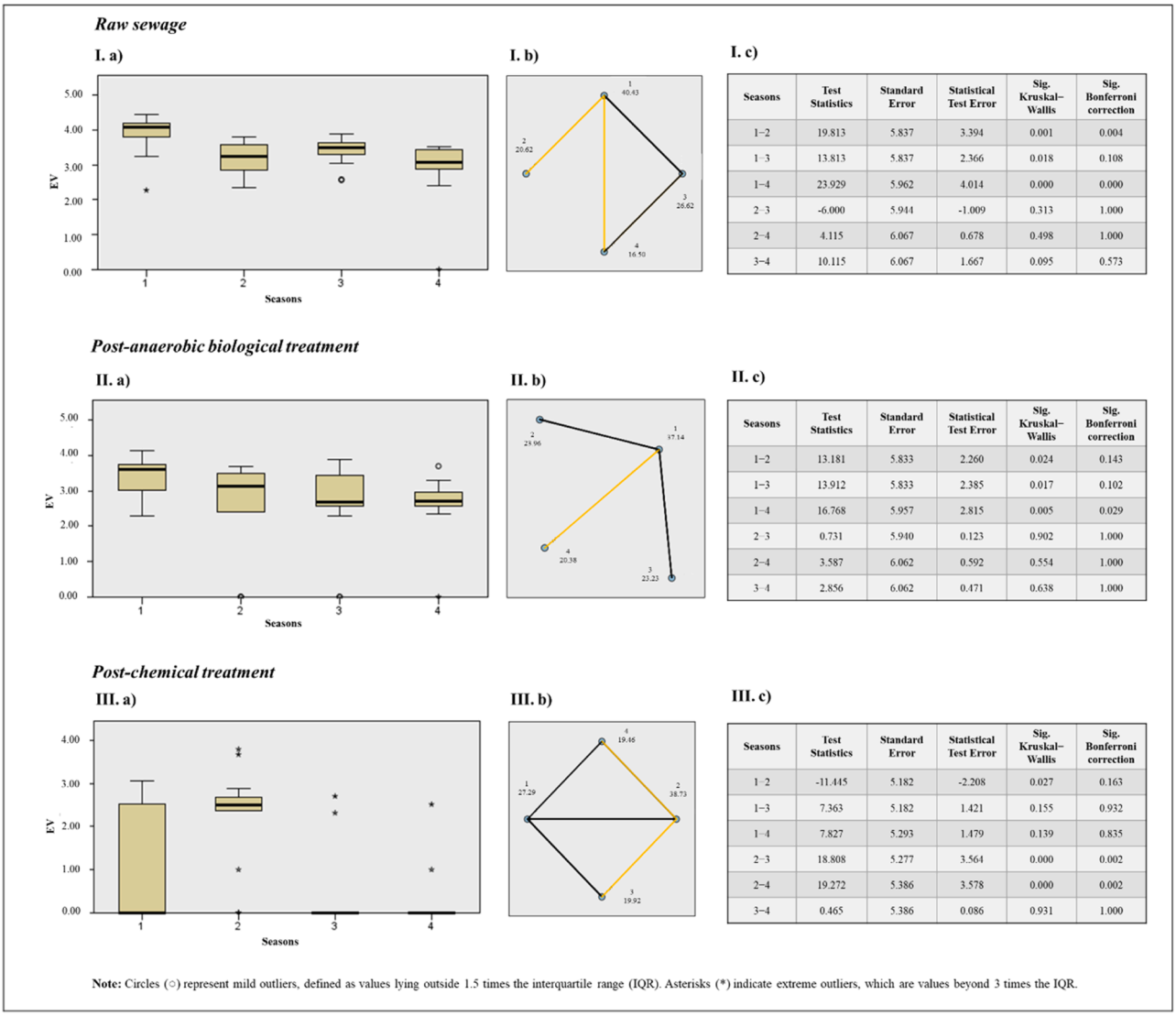

**Fig. 1.** Quantification of EV and HAV at each stage of the wastewater treatment process alongside reported cases of their respective diseases and across different seasons in São José do Rio Preto, SP. The data are presented as follows: a) Quantification of EV throughout the year alongside cases of ADD; and b) Quantification of HAV and its related disease.

the autumn, in samples PABT-13 and PABT-6, respectively. For PCT samples, quantifiable concentrations ranged from 2.20 to 3.80 $\log_{10}$ GC/mL, with the lowest quantification (i.e., PCT-9) in the autumn and the highest (i.e., PCT-21) found in the winter. However, two samples were positive but not quantifiable, observed in the winter (i.e., PCT-22) and summer (i.e., PCT-51). Overall, when ND or NQ samples were assigned a value corresponding to half of the lowest concentration detected for EV (1.10 $\log_{10}$ GC/mL), the mean concentrations were 3.43 $\log_{10}$ GC/mL in RS samples, 2.92 $\log_{10}$ GC/mL in PABT samples, and 1.69 $\log_{10}$ GC/mL in PCT samples, corresponding to an overall mean reduction of 1.74 $\log_{10}$ as a result of the treatment process.

Cases of HAV were reported in weeks 1, 2, 33, and 37 (Fig. 1.b). Samples were positive and quantifiable across all seasons, but in the spring, this was observed in only a minority ($n = 3$) of the samples. For RS samples, concentrations ranged from 2.63 to 4.19 $\log_{10}$ GC/mL. The lowest concentration (i.e., RS-33) was observed in the spring, while the highest (i.e., RS-45) occurred in the summer. In PABT samples, concentrations ranged from 3.08 to 4.42 $\log_{10}$ GC/mL, with the lowest (i.e., PABT-32) observed in the spring and the highest (i.e., PABT-14) in the winter. Finally, for PCT samples, concentrations ranged from 3.16 to 3.99 $\log_{10}$ GC/mL, with the lowest concentration (i.e., PCT-12) in the autumn and the highest (i.e., PCT-19) in the winter. No samples were identified in the spring and summer during this phase. Similarly for HAV, when ND or NQ samples were assigned a value corresponding to half of the lowest concentration detected for HAV (1.31 $\log_{10}$ GC/mL), the mean concentrations were 3.61 $\log_{10}$ GC/mL in RS samples, 1.67 $\log_{10}$ GC/mL in PABT samples, and 1.41 $\log_{10}$ GC/mL in PCT samples, resulting in an overall mean reduction of 2.20 $\log_{10}$ attributable to the treatment process.

Further details on the positive and quantified samples are provided in Supplementary Tables ST3 and ST4.

Supplementary Table ST5 presents the descriptive analyses of the association between seasons and EV and HAV concentration. For non-parametric tests, percentile values were used to evaluate the data




I. c)

| Seasons | Test Statistics | Standard Error | Statistical Test Error | Sig. Kruskal–Wallis | Sig. Bonferroni correction |
|---|---|---|---|---|---|
| 1–2 | 19.813 | 5.837 | 3.394 | 0.001 | 0.004 |
| 1–3 | 13.813 | 5.837 | 2.366 | 0.018 | 0.108 |
| 1–4 | 23.929 | 5.962 | 4.014 | 0.000 | 0.000 |
| 2–3 | -6.000 | 5.944 | -1.009 | 0.313 | 1.000 |
| 2–4 | 4.115 | 6.067 | 0.678 | 0.498 | 1.000 |
| 3–4 | 10.115 | 6.067 | 1.667 | 0.095 | 0.573 |

II. c)

| Seasons | Test Statistics | Standard Error | Statistical Test Error | Sig. Kruskal–Wallis | Sig. Bonferroni correction |
|---|---|---|---|---|---|
| 1–2 | 13.181 | 5.833 | 2.260 | 0.024 | 0.143 |
| 1–3 | 13.912 | 5.833 | 2.385 | 0.017 | 0.102 |
| 1–4 | 16.768 | 5.957 | 2.815 | 0.005 | 0.029 |
| 2–3 | 0.731 | 5.940 | 0.123 | 0.902 | 1.000 |
| 2–4 | 3.587 | 6.062 | 0.592 | 0.554 | 1.000 |
| 3–4 | 2.856 | 6.062 | 0.471 | 0.638 | 1.000 |

III. c)

| Seasons | Test Statistics | Standard Error | Statistical Test Error | Sig. Kruskal–Wallis | Sig. Bonferroni correction |
|---|---|---|---|---|---|
| 1–2 | -11.445 | 5.182 | -2.208 | 0.027 | 0.163 |
| 1–3 | 7.363 | 5.182 | 1.421 | 0.155 | 0.932 |
| 1–4 | 7.827 | 5.293 | 1.479 | 0.139 | 0.835 |
| 2–3 | 18.808 | 5.277 | 3.564 | 0.000 | 0.002 |
| 2–4 | 19.272 | 5.386 | 3.578 | 0.000 | 0.002 |
| 3–4 | 0.465 | 5.386 | 0.086 | 0.931 | 1.000 |

**Fig. 2.** Results of the Kruskal–Wallis test for EV concentration across different seasons (a), Pairwise comparisons between seasons were performed with Bonferroni correction applied; statistically significant differences are indicated by yellow lines, non-significant differences by black lines, and the absence of a line indicates that the comparison was not considered relevant. (b), and results table (c), where: Season 1: Autumn; Season 2: Winter; Season 3: Spring; Season 4: Summer; I. Results for EV quantification across different seasons in RS samples; II. Results for EV quantification across different seasons in PABT; III. Results for EV quantification across different seasons in PCT samples.

*Notes:* Asymptotic significance values (two-sided test) are displayed. The significance level is 0.05. The p-values were adjusted using the Bonferroni correction for multiple tests.

distribution or rankings.

The Chi−square test was employed to evaluate the relationship between the variables and revealed significant associations ($p \leq 0.05$) between the seasons and EV in RS ($\chi^2$= 19.01; $p = 0.000$), PABT ($\chi^2 = 9.85$; $p = 0.020$), and PCT ($\chi^2 = 17.18$; $p = 0.001$). Based on these findings, the Kruskal−Wallis test was applied to rank and analyze these variables across various stages of wastewater treatment, as shown in Fig. 2. No significant differences ($p > 0.05$) were found between the seasons for HAV in samples from RS ($\chi^2 = 2.33$; $p = 0.506$), PABT ($\chi^2 = 4.88$; $p = 0.181$), or PCT ($\chi^2 = 7.79$; $p = 0.051$).

When evaluating the EV concentration across different seasons and all three stages of wastewater treatment, the Kruskal−Wallis test with a Bonferroni correction, applied to minimize false positives, identified significant differences in RS between autumn and summer, and autumn and winter, with autumn exhibiting higher viral concentrations. In PABT, significant differences were observed between autumn and summer, with autumn having higher concentrations. For PCT, significant differences were found between winter and autumn, as well as winter and summer, with winter showing higher viral concentrations.

The instantaneous correlation and cross-correlation between disease cases and the quantification of EV and HAV viruses in RS were examined to explore possible temporal links between virus detection and disease occurrence. This phase reflects the active circulation of the viruses and serves as a potential risk factor for disease. However, no significant correlation was found, as shown in Supplementary Figure SF2.

### 3.4. Wastewater parameters

Table 1 provides a description of the wastewater parameters, including average flow, total flow, sewage temperature, chemical oxygen demand, and pH at various stages, along with the quantification of EV and HAV, and their corresponding disease case data.

Disease cases, average flow, and total flow were reported as fixed values since they remained unchanged throughout the wastewater treatment process. For non-parametric tests, percentile values can help evaluate the distribution of data or rankings. The Chi-square test, which assesses the relationship between variables, showed significant results for pH ($\chi^2 = 49.20$; $p = 0.000$), COD ($\chi^2 = 105.96$; $p = 0.000$) and EV concentration ($\chi^2 = 69.41$; $p = 0.000$). Thus, the Kruskal−Wallis test was employed to rank and analyze these variables at various stages of wastewater treatment, as shown in Fig. 3.

In the comparison of the three wastewater treatment stages with the correlated variables, the Kruskal−Wallis test with a Bonferroni correction was applied to reduce the risk of false positives. Significant differences in pH and EV quantification were observed at all stages, with RS generally displaying higher values. Similarly, a statistical difference in COD was found across all stages, with the PABT stage exhibiting the highest values.

Spearman's non-parametric correlation was used to assess the relationship between wastewater parameters and EV and HAV, as shown in Table 2.

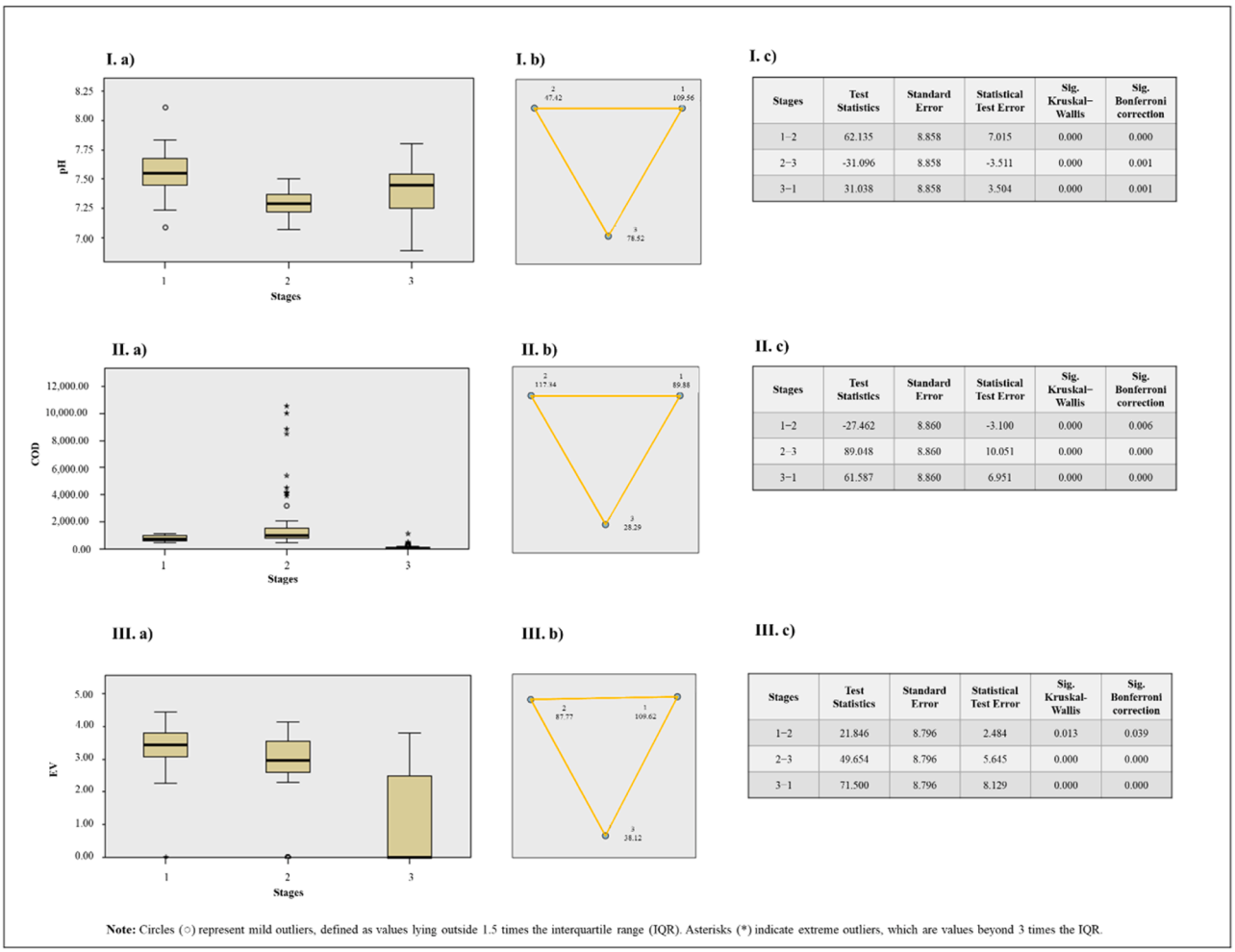
For EV, a correlation coefficient with $p \leq 0.01$ was observed for pH in RS ($R = 0.391$; $p = 0.004$) and for pH ($R = 0.440$; $p = 0.001$), temperature ($R = -0.618$; $p = 0.000$), and COD ($R = 0.452$; $p = 0.001$) in the PCT stage. For HAV, a correlation coefficient with $p \leq 0.05$ was found for HAV cases ($R = 0.290$; $p = 0.037$) in RS and pH in the PABT stage ($R = 0.333$; $p = 0.016$), while a correlation coefficient with $p \leq 0.01$ was found for pH ($R = 0.381$; $p = 0.005$) and temperature ($R = -0.572$; $p = 0.000$) in the PCT stage. Positive correlations indicate that pH and COD tend to increase with higher viral concentrations for EV, as well as pH and HAV cases with higher HAV concentrations in their respective stages of wastewater treatment. Conversely, the negative correlation observed between temperature and EV and HAV suggests that the concentrations of EV and HAV tend to decrease as the water temperature increases.

Additionally, in the Mann−Whitney test, used to observe differences between two independent variables, significant differences ($p \leq 0.05$) were found between rainy ($n = 21$) and non-rainy ($n = 31$) days for HAV at the PCT stage ($U = 252.00$; $p = 0.021$), with a lower number of occurrences observed on days without rain. No significant differences were found for HAV at the RS ($U = 325.00$; $p = 0.990$) and PABT stages ($U = 321.00$; $p = 0.909$), as well as for EV at the RS ($U = 257.00$; $p = 0.201$), PABT ($U = 227.50$; $p = 0.067$), and PCT ($U = 242.00$; $p = 0.079$).

**Table 1**
Parameters of wastewater samples at various stages, along with the quantification of EV and HAV, and their corresponding disease case data.

| | | | | | Percentiles | | |
|---|---|---|---|---|---|---|---|
| Parameters<br>Raw sewage | Minimum | Maximum | Mean | SD | 25th | 50th | 75th |
| EV ($log_{10}$ GC/mL) | 0.00 | 4.45 | 3.36 | 0.72 | 3.05 | 3.45 | 3.79 |
| ADD cases | 328.00 | 846.00 | 491.29 | 104.76 | 422.50 | 464.00 | 514.50 |
| HAV ($log_{10}$ GC/mL) | 0.00 | 4.20 | 0.90 | 1.59 | 0.00 | 0.00 | 1.97 |
| HAV cases | 0.00 | 1.00 | 0.08 | 0.27 | 0.00 | 0.00 | 0.00 |
| Average flow (L/s)[a] | 1036.00 | 1462.00 | 1182.08 | 76.13 | 1133.50 | 1165.50 | 1208.00 |
| Total flow ($m^3$/day)[a] | 88,515.00 | 124,147.00 | 102,063.52 | 6610.53 | 97,786.25 | 101,595.50 | 105,181.50 |
| Wastewater temperature ( °C) | 22.7 | 30.5 | 26.68 | 1.95 | 25.10 | 26.90 | 28.30 |
| COD (mg/L) | 456.00 | 1093.00 | 757.46 | 187.13 | 591.00 | 738.00 | 963.50 |
| pH | 7.09 | 8.11 | 7.56 | 0.18 | 7.45 | 7.55 | 7.68 |
| Post-anaerobic biological treatment | | | | | | | |
| EV ($log_{10}$ GC/mL) | 0.00 | 4.14 | 2.78 | 1.13 | 2.59 | 2.97 | 3.54 |
| HAV ($log_{10}$ GC/mL) | 0.00 | 4.42 | 0.86 | 1.59 | 0.00 | 0.00 | 0.00 |
| Wastewater temperature ( °C) | 23.10 | 30.20 | 26.99 | 1.83 | 25.50 | 27.30 | 28.30 |
| COD (mg/L) | 455.0 | 10,560.00 | 2037.13 | 2481.38 | 808.50 | 1020.00 | 1673.50 |
| pH | 7.07 | 7.50 | 7.29 | 0.10 | 7.22 | 7.29 | 7.37 |
| Post-chemical treatment | | | | | | | |
| EV ($log_{10}$ GC/mL) | 0.00 | 3.80 | 1.02 | 1.31 | 0.00 | 0.00 | 2.49 |
| HAV ($log_{10}$ GC/mL) | 0.00 | 3.99 | 0.48 | 1.22 | 0.00 | 0.00 | 0.00 |
| Wastewater temperature ( °C) | 21.10 | 30.20 | 26.47 | 2.27 | 24.40 | 26.90 | 28.30 |
| COD (mg/L) | 18.00 | 1121.00 | 128.90 | 188.19 | 35.50 | 50.50 | 115.25 |
| pH | 6.89 | 7.80 | 7.41 | 0.19 | 7.25 | 7.45 | 7.54 |

COD: Chemical oxygen demand; SD: Standard deviation; 50th percentile: median
[a] Correspond to the three stages of wastewater treatment.




I. c)

| Stages | Test Statistics | Standard Error | Statistical Test Error | Sig. Kruskal–Wallis | Sig. Bonferroni correction |
|---|---|---|---|---|---|
| 1–2 | 62.135 | 8.858 | 7.015 | 0.000 | 0.000 |
| 2–3 | -31.096 | 8.858 | -3.511 | 0.000 | 0.001 |
| 3–1 | 31.038 | 8.858 | 3.504 | 0.000 | 0.001 |

II. c)

| Stages | Test Statistics | Standard Error | Statistical Test Error | Sig. Kruskal–Wallis | Sig. Bonferroni correction |
|---|---|---|---|---|---|
| 1–2 | -27.462 | 8.860 | -3.100 | 0.000 | 0.006 |
| 2–3 | 89.048 | 8.860 | 10.051 | 0.000 | 0.000 |
| 3–1 | 61.587 | 8.860 | 6.951 | 0.000 | 0.000 |

III. c)

| Stages | Test Statistics | Standard Error | Statistical Test Error | Sig. Kruskal-Wallis | Sig. Bonferroni correction |
|---|---|---|---|---|---|
| 1–2 | 21.846 | 8.796 | 2.484 | 0.013 | 0.039 |
| 2–3 | 49.654 | 8.796 | 5.645 | 0.000 | 0.000 |
| 3–1 | 71.500 | 8.796 | 8.129 | 0.000 | 0.000 |

**Fig. 3.** Results of the Kruskal–Wallis test for wastewater parameters across different wastewater treatment stages (a), pairwise comparisons between wastewater treatment stages with Bonferroni correction applied, where statistically significant pairwise differences are indicated by yellow lines (b), and results table (c), where: Stage 1: Raw Sewage; Stage 2: Post-anaerobic biological treatment. Stage 3: Post-chemical treatment; I. Results for pH; II. Results for chemical oxygen demand (COD); III. Results for EV concentration.

*Notes:* Each row tests the null hypothesis that the sample distributions for Stage 1 and Stage 2 are identical. Asymptotic significance values (two-sided test) are shown. The significance level is 0.05, with p-values adjusted using the Bonferroni correction for multiple tests.

### 3.5. Sequencing and phylogenetic analysis

Sequencing was conducted for EV, HAV, and HEV, however, phylogenetic analysis was feasible only for HAV and HEV. The likelihood trees were constructed using sequences obtained through Sanger sequencing and those available in GenBank. Bootstrap values (1000 replications) are indicated at each branch. The scales bars represent nucleotide substitutions per site. The sequences indicated in red is derived from this study.

Of the 119 samples that tested positive for EV via qPCR, 79 were tested by Nested PCR, yielding 21 positive results for genogroup A and 8 for genogroup B, with seven samples being positive for both genotypes A and B simultaneously. However, Sanger sequencing identified nine samples (GenBank acc. ns. PQ784281-PQ784289), of which six exhibited high similarity, with one sample showing 87.3 % and the others ranging between 92.9 % and 100 % to Coxsackievirus A5 (CVA5) and three to Coxsackievirus A16 (CVA16), all of which were classified within genogroup A. Additionally, eight samples yielded inconclusive results, with no similarities detected based on GenBank analysis. Further information about the analyzed samples and their respective genogroups can be found in Supplementary Table ST2.

For HAV, out of the 32 positive samples identified by qPCR, six were subsequently analyzed using Nested PCR. Of these, two samples (RS-50 and PABT-50) were positive and were further sequenced via Sanger sequencing, where they were classified as genotype IA. One of these samples (RS-50, GenBank acc. n. PQ784290) was subjected to phylogenetic analysis using a 412 bp consensus fragment, as illustrated in Fig. 4.

For HEV, of the five positive samples detected by Nested PCR and sequenced by Sanger, four (PABT-16, PABT-20, RS-28 and RS-48; 80.0 %) were identified as genotype III. As displayed in Fig. 5, two of these samples were included in the phylogenetic analysis using consensus fragments of 278 bp (RS-28) and 228 bp (PABT-20).

Additional information regarding the analyzed samples for HAV and HEV can be found in Supplementary Table ST3.

### 3.6. Recovery efficiency assay

The recovery efficiency test was conducted for each phase of wastewater treatment by seeding known concentrations of the inactivated Echovirus 3, across four dilutions, as illustrated in Fig. 6. The concentrated virus had a mean concentration of 9.20 $\log_{10}$ GC/mL ± 0.02.

The RS stage exhibited efficiencies of 68.0 %, 59.8 %, 57.2 %, and 49.7 % for each serial dilution, ranging from −1 to −4. In the PABT stage, the efficiencies were 56.7 %, 66.6 %, 51.3 %, and 48.7 % for the

**Table 2**
Spearman's non-parametric correlation between the three stages of wastewater treatment and EV and HAV.

| Raw sewage (EV) | | | | | | |
|---|---|---|---|---|---|---|
| | Average flow | Total flow | pH | Temperature | COD | ADD cases |
| Correlation Coefficient | 0.059 | 0.104 | 0.391** | −0.012 | 0.025 | −0.089 |
| Sig. (bilateral) | 0.677 | 0.465 | 0.004 | 0.931 | 0.859 | 0.532 |
| *n* | 52 | 52 | 52 | 52 | 52 | 52 |
| Post-anaerobic biological treatment (EV) | | | | | | |
| | Average flow | Total flow | pH | Temperature | COD | ADD cases |
| Correlation Coefficient | 0.220 | 0.204 | 0.075 | −0.006 | 0.080 | NP |
| Sig. (bilateral) | 0.117 | 0.147 | 0.595 | 0.964 | 0.574 | NP |
| n | 52 | 52 | 52 | 52 | 52 | 0 |
| Post-chemical treatment (EV) | | | | | | |
| | Average flow | Total flow | pH | Temperature | COD | ADD cases |
| Correlation Coefficient | −0.129 | −0.139 | 0.440** | −0.618** | 0.452** | NP |
| Sig. (bilateral) | 0.363 | 0.324 | 0.001 | 0.000 | 0.001 | NP |
| *n* | 52 | 52 | 52 | 52 | 52 | 0 |
| Raw sewage (HAV) | | | | | | |
| | Average flow | Total flow | pH | Temperature | COD | HAV cases |
| Correlation Coefficient | 0.000 | −0.136 | −0.001 | −0.065 | 0.092 | 0.290* |
| Sig. (bilateral) | 1.000 | 0.338 | 0.997 | 0.653 | 0.516 | 0.037 |
| *n* | 52 | 52 | 52 | 52 | 52 | 52 |
| Post-anaerobic biological treatment (HAV) | | | | | | |
| | Average flow | Total flow | pH | Temperature | COD | HAV cases |
| Correlation Coefficient | 0.066 | −0.024 | 0.333* | −0.027 | −0.152 | NP |
| Sig. (bilateral) | 0.640 | 0.867 | 0.016 | 0.850 | 0.283 | NP |
| *n* | 52 | 52 | 52 | 52 | 52 | 0 |
| Post-chemical treatment (HAV) | | | | | | |
| | Average flow | Total flow | pH | Temperature | COD | HAV cases |
| Correlation Coefficient | −0.054 | −0.083 | 0.381** | −0.572** | 0.214 | NP |
| Sig. (bilateral) | 0.704 | 0.556 | 0.005 | 0.000 | 0.127 | NP |
| *n* | 52 | 52 | 52 | 52 | 52 | 0 |

* . $p \leq 0.05$;.
** . $p \leq 0.01$; NP: not performed.

same dilutions. In the PCT stage, the efficiencies for dilutions −1 to −3 were 28.1 %, 24.1 %, and 19.0 %, with dilution −4 undetected at this stage.

## 4. Discussion

The rapid population growth is expected to place increasing pressure on the environment, leading to a higher demand for natural resources and increased volumes of effluents generated, which in turn contributes to the proliferation of pathogenic microorganisms responsible for outbreaks of waterborne diseases (Girardi et al., 2019; Bordoni et al., 2023). In this study, the prevalence of EV, HAV, and HEV was evaluated, along with their molecular characterization and viral concentrations at three different stages of wastewater treatment, correlating the number of reported cases in the city. The recovery efficiency for these stages was also assessed. The importance of wastewater surveillance was highlighted, as cases are often underreported and underdiagnosed, given that few diseases are subject to mandatory reporting and/or individuals seek for medical services. This makes it difficult to assess the true burden of diseases and infections attributable to various agents (Pellegrinelli et al., 2023). Additionally, the monitoring of these waters over time precedes corresponding clinical detections, sometimes by days or weeks, serving as an early warning of viral spread within communities. This helps fill epidemiological gaps and provides crucial information about the current prevalence of these infections in the human population (Singer et al., 2023; Pellegrinelli et al., 2023).

In this study, a high prevalence of EV was observed across all three treatment stages (ranging from 98.1 % to 42.3 %). On the other hand, the study by Vecchia et al. (2012) in southern Brazil found no EV in influent samples, but 37.5 % of effluent samples tested positive for the pathogen. Ibrahim et al. (2023) observed detection levels similar to ours in one of the wastewater treatment plants evaluated in Tunisia (93 % in RS and 25 % PCT). The seasonal pattern of EV infections varies according to genotype and geographical location, with outbreaks being more common in summer and autumn in temperate climates. In contrast, tropical regions maintain consistent infection levels throughout the year, facilitated by the warm climate, as observed in our findings, particularly among children (Kocwa-Haluch, 2001; Rajtar et al., 2008; Lugo and Krogstad, 2016; Vidal et al., 2019; Pellegrinelli et al., 2023). For HAV, a moderate to low detection rate was reported in this study (ranging from 25 % to 13.5 %), consistent with the findings of Prado et al. (2011), who found a prevalence of 28.5 % in Rio de Janeiro, Brazil. HAV is present worldwide, and its seroprevalence depends on hygiene conditions. In the study by Bazir et al. (2022), a correlation was observed between positive mussel samples and the rainy season, which is also in agreement with our findings, where fewer occurrences were observed on days without rain after the final effluent treatment ($p \leq 0.05$). Regarding HEV, a low detection rate was found (ranging from 5.8 % to 3.8 %), which is similar to the study by Cancela et al. (2023), who reported a prevalence of 10.9 % in wastewater in Uruguay, but not to the study by dos Santos et al. (2011), who found a prevalence of 50 % in effluent waters in Brazil. For both HAV and HEV, seasonal patterns are not well established. However, according to the review by Fares (2015), researchers believe that climatic and behavioural factors contribute to outbreaks, although evidence suggests peak occurrences in spring and summer for hepatitis A, B, C, and E.

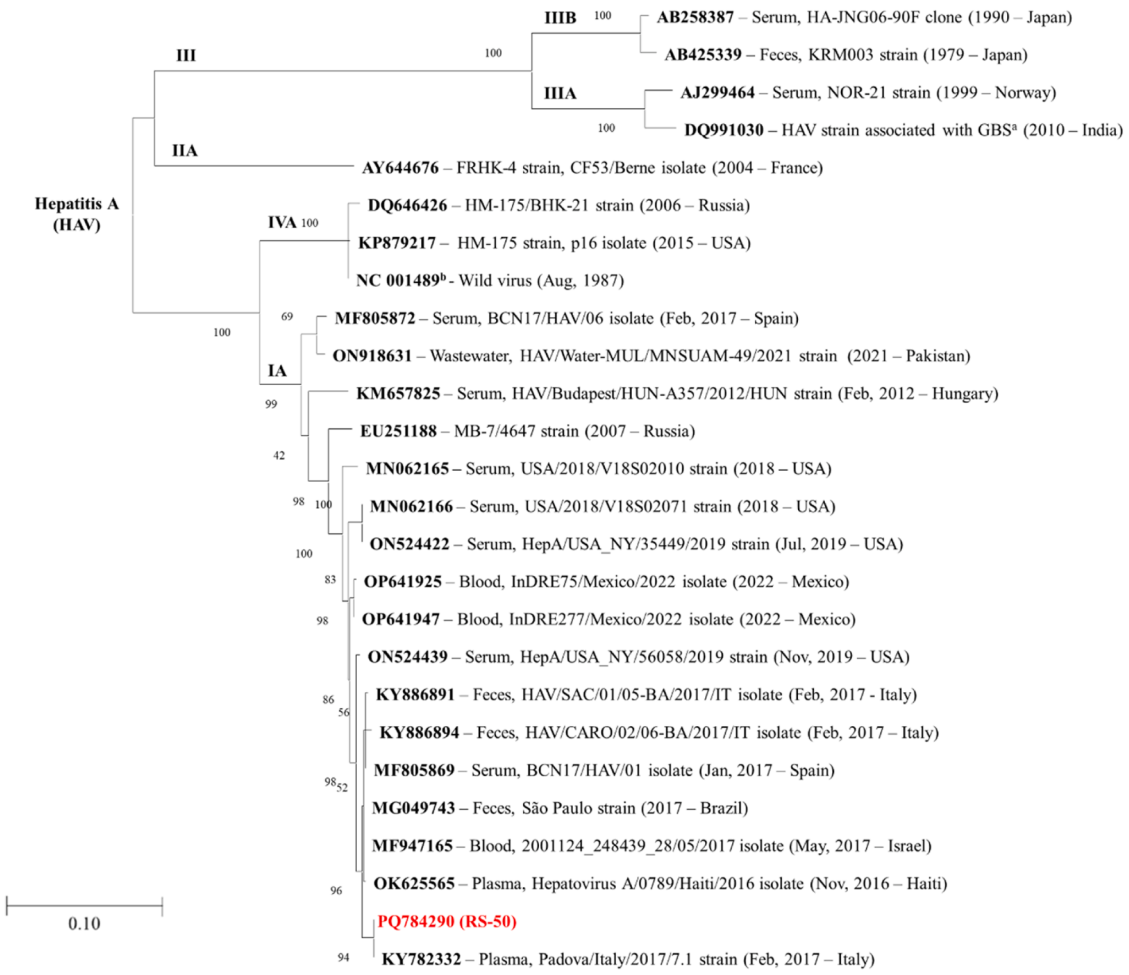



**Fig. 4.** Phylogenetic analysis of HAV was carried out based on the VP1/2A junction. [a]: Guillain-Barre syndrome (GBS); [b]: NCBI reference sequence.
*Notes:* Available references from GenBank were utilized for each HAV genotype (IA, IIA, III, and IVA). All serum, plasma, blood, or fecal samples described were from human sources. The name of the sequence written in red are from this study (i.e., RS-50; GenBank acc. n. PQ784290).

The study by Chacón et al. (2021) also correlated ADD cases with the findings of EV, but contrary to our results, which found no correlation ($p > 0.05$), it found a tendency for a higher rate of positive viral tests to be present one week before an increase in ADD cases, highlighting the importance of continuing monitoring. Compared to the study by Lizasoain et al. (2018) in wastewater samples from Uruguay, the EV concentrations found in our study were slightly higher. While in Brazil, similar concentrations were found in surface water and groundwaters (Lima et al., 2022). Our findings also indicate significant differences ($p \leq 0.05$) for higher concentrations of EV at milder temperatures, such as autumn in RS and PABT, and winter in PCT samples. For HAV, although its detection was consistent throughout the year, the highest concentrations in this study were observed during the summer in RS. Although no statistical significance ($p > 0.05$) was found regarding the virus in relation to the seasons and different wastewater treatment samples, the study by Ouardani et al. (2016) reported higher detections in the summer, suggesting that such patterns could potentially indicate the occurrence of outbreaks. A higher concentration was also observed when comparing our findings with those of Prado et al. (2021), in another city in the same state, although they detected the virus in a greater number of samples, being more consistent with the findings of Schlindwein et al. (2010) in southern Brazil. Higher concentrations were found in the PABT stage samples during the winter. No detections were observed in the PCT samples during the summer and spring. Interestingly, a significant difference was noted ($p \leq 0.01$) in this stage, suggesting that as the water temperature increased, viral concentrations tended to decrease. Additionally, supporting this result, the review by Levy et al. (2016) reports 10 analyses that described a significant negative relationship between temperature and viral diarrhea cases. However, the significant difference in pH ($p \leq 0.01$) for both EV and HAV, indicating an increase in viral concentrations at the PCT stage, can be explained by the fact that chlorine gas reacts with water to form hypochlorous acid (HOCl), which is the key component for disinfection. Higher volumes of HOCl are produced at neutral or lower pH, enhancing disinfection efficiency (Sangkham, 2021). For HEV, although our findings were unable to quantify its viral concentration, a concentration of $10^2$ GC/mL was reported in wastewater samples from Brazil (dos Santos et al., 2011).

Regarding viral reductions during the overall wastewater treatment process for EV and HAV, with mean values of 1.74 $\log_{10}$ GC/mL and 2.20 $\log_{10}$ GC/mL respectively. In studies evaluating the removal efficiencies of enteric viruses in wastewater treatment systems, Kitajima et al. (2014) observed reductions of 2.14 $\log_{10}$ GC/L for NoV GII, 1.65 $\log_{10}$ GC/L for NoV GI, 0.94 $\log_{10}$ GC/L for AiV, and 0.76 $\log_{10}$ GC/L for PMMoV in Arizona, USA. In Ontario, Canada, Simhon et al. (2019) reported a reduction of 0.3 $\log_{10}$ GC/L for EV and 0.5 $\log_{10}$ GC/L for both NoV GI and GII. All these findings are consistent with those of Ozgur & Icgen (2025), who investigated different WWTP technologies and reported that, although some reduction in viral load occurs, conventional WWTPs are generally inefficient at removing enteric viruses, as they were primarily designed to eliminate bacteria, organic matter, and chemicals. This limitation is critical, as pathogenic viruses can persist in

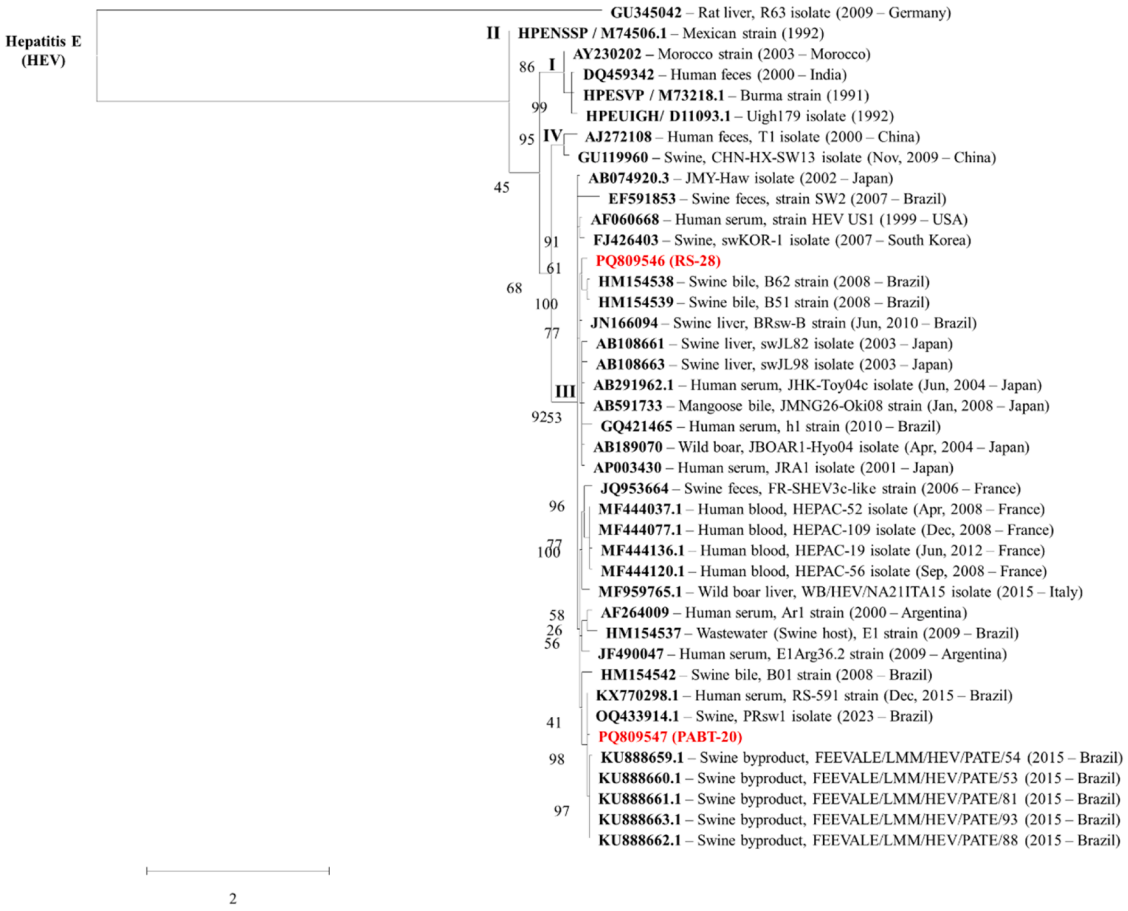



**Fig. 5.** Phylogenetic analysis of HEV was conducted based on the ORF1 region. GU345042 was used as an outgroup while other available references from GenBank were utilized for each HEV genotype (I. II. III. and IV). The names of the sequences written in red are from this study (i.e., RS-28 and PABT-20; GenBank acc. ns. PQ809546 and PQ809547, respectively).

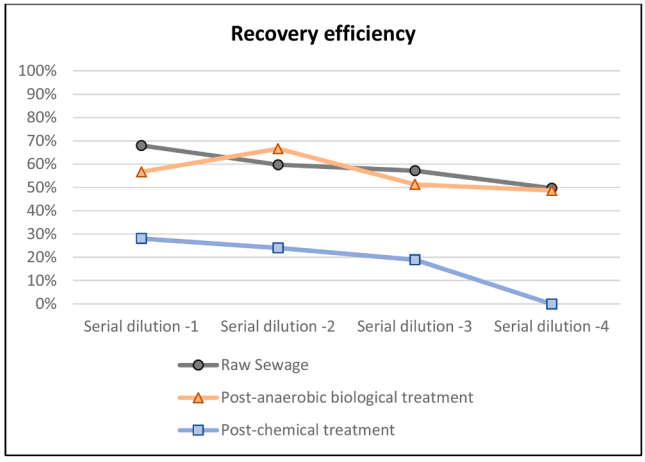



**Fig. 6.** Results of the recovery efficiency test ( %) across the three different wastewater matrices from each treatment stage, using four serial dilutions of the inactivated Echovirus 3.

treated effluent, posing a public health concern, as demonstrated in our previous study where infectious AiV was detected post-treatment in the same sewage plant (do Nascimento et al., 2025).

Currently, both live attenuated and inactivated HAV vaccines are available worldwide, with Brazil using the inactivated whole-virus vaccine (Ministério da Saúde, 2013; Zhang, 2020). For HEV, there is only one available vaccine, Hecolin, which is an inactivated vaccine and had its first reported use during an outbreak in 2022 (WHO, 2023). It is highly unlikely that vaccine-derived viral signatures would be detectable in excreted body fluids or wastewater due to the nature of vaccine mechanisms. Vaccine-derived viral shedding typically occurs with vaccines containing replicative viruses, such as live attenuated or live replicative viral vaccines, which can replicate in the body. However, shedding is not common with vaccines that do not use replicating viruses. In contrast, for the poliovirus vaccine, which uses a live attenuated virus, shedding can occur through feces from 7 to 60 days post-vaccination (Armas et al., 2023). In this way, it is important to emphasize that, although for EV some of the cases may or may not be of vaccine origin, also due to the difficulty in sequencing these samples, the cases reported here for HAV and HEV have a high likelihood of not being of vaccine origin.

Hand, Foot, and Mouth Disease (HFMD) is one of the most prominent outbreaks caused by EVs, a common and contagious viral infection primarily affecting infants and young children. It is mainly associated with infections by EV-71, CV-A16, or CV-A6. While most cases are mild and last less than a week, some can lead to severe or even fatal complications (Lugo and Krogstad, 2016; Xie et al., 2024; Jartti et al., 2024). Findings from clinical samples analyzed by Sousa et al. (2018) in different regions of Brazil also identified CV-A16 and CV-A6 as the main causative agents of HFMD, while Lizasoain et al. (2018) in Uruguay also found CV-A16 in 8 out of 11 samples from genogroup A in wastewater samples. Our findings, thus, further confirm the circulation of these strains in Brazil and South America, while in Africa, all the wastewater samples ($n = 25$) analyzed were from genogroup B (Bero et al., 2022). For HAV, this study showed genotype IA circulating, consistent with the

two reports from different cities in southeastern Brazil (Prado et al., 2011, 2021) and Latin America (Ré et al., 2024) in wastewater samples, while genotype IB was observed in Iran (Nasiri et al., 2022). For HEV, our findings correlate with those of Treagus et al. (2023) in wastewater samples from England, even showing genetic similarities between swine and human samples. Finally, the study by Lo Castro et al. (2023) in Argentina also corroborated our findings for HAV and HEV, with all HAV isolates belonging to genotype IA and HEV isolates belonging to genotype 3, the most prevalent genotypes in South America.

The recovery efficiency test was conducted with Echovirus 3 at three different sewage treatment points, using various dilutions and employing the ultracentrifugation method, as in the study by Gularte et al. (2021), which also observed a tendency for recovery to decrease as concentrations decreased in dilutions −1 to −4, using HAdV, a DNA virus, in samples without prior concentration methods before ultracentrifugation, although they observed a lower recovery rate compared to our study (ranging from 12.3 % to 1.76 %). Similarly, we observed a higher recovery efficiency compared to the study conducted by Ahmed et al. (2020), which used a murine hepatitis virus (MHV), an enveloped RNA virus, and employed ultracentrifugation, reporting a recovery efficiency of only 33.5 %. However, enveloped viruses are much more easily inactivated compared to non-enveloped viruses (Sangkham, 2021). On the other hand, the study by Prata et al. (2012) analyzed samples after secondary (biological) treatment, using ultracentrifugation, but this time as a secondary concentration step. They observed a recovery efficiency ranging from 66–72 % in this type of wastewater, without analyzing different dilutions but instead focusing on different viruses. This is particularly interesting, considering that in our findings, the recovery efficiency ranged from 48–66 %, without prior concentration or different dilutions. Last but not least, the low recovery efficiency (28–0 %) highlighted how the effluent matrix PCT with chlorine can influence virus detection, depending also on its concentration, as shown in the findings of this study. This result is considered satisfactory, suggesting that the wastewater treatment processes in the city of São José do Rio Preto, SP, appear to be effective. Chlorine inactivates viruses by reacting with viral biomolecules, disrupting replication processes, but the viral susceptibility also can vary significantly, even within the same genus, due to differences in viral structure and composition (Qiao et al., 2022). Simonet and Gantzer (2006) demonstrated that Poliovirus 1 inactivation by chlorine dioxide ($ClO_2$) is dose-dependent, with varying resistance across genome regions and fragment sizes. They found that while infectivity was completely lost after 3 min at 5 mg $L^{-1}$ $ClO_2$, exposure to 0.5 mg $L^{-1}$ for 120 min did not compromise the integrity of the targeted RNA fragment. However, it is important to note that the infectivity test was not conducted in this study. Therefore, the detection of viral sequences may only indicate the presence of nucleic acids and does not confirm whether the virus remains viable or capable of causing infection. In addition, according to the study by Choi & Jiang (2005), chlorination is also able to inactivate virions without degrading the virus structure or genome integrity.

Lastly, an interesting observation in this study was that one sample (i.e., 51T) was negative for EV in raw sewage but positive in both subsequent treatment stages. Similarly, nine samples that were negative for HAV in raw sewage (i.e., 6T, 10T, 11T, 13T, 14T, 18T, 22T, 32T, and 51T) were positive in PABT and/or PCT. Because inhibition and recovery controls were performed across this study and showed satisfactory results, and because sampling was performed as 24-h composites, we consider it unlikely that PCR inhibition or sampling bias alone explain these findings. Instead, these discrepancies may reflect concentrations in the influent that were below the assay LoD or nuclease-mediated degradation of target RNA in the complex influent matrix. The study by Cheshomi et al. (2025) also reported similar observations when comparing different viral concentration methods, supporting the interpretation that detection in effluents despite non-detects in influent can result from methodological and matrix-related factors.

In summary, the type of virus studied, as well as the different samples and their matrices, concentration methods, extraction processes, and detection techniques, or even the differences between each methodology used, can influence the detection and/or quantification of pathogens. Therefore, despite the existence of various studies, the importance of conducting recovery efficiency tests for each flowchart used in different laboratories is emphasized. We also reaffirm the importance of monitoring wastewater samples in different regions, as cases of underreporting are commonly observed. Ultimately, these efforts contribute to a more accurate understanding of pathogen dynamics and support public health surveillance strategies.

## 5. Conclusion

- For EV and HAV, mean reductions of 1.74 $\log_{10}$ and 2.20 $\log_{10}$, respectively, were observed from the beginning to the end of the treatment process. Although present in lower concentrations and smaller proportions, the detection of these viruses in samples collected from the PCT of the wastewater treatment process highlights the importance of implementing environmental monitoring focused on waterborne viruses to assess water quality and environmental contamination levels for surveillance purposes. Furthermore, despite the resistance of viruses to treatment and their potential threat to public health, the results demonstrate the positive impact of final chemical treatment with chlorine in reducing viral loads.
- For HEV, although few detections were observed, the findings suggest that the virus continues to circulate within the studied population. This underscores the importance of proper food preparation, particularly with regard to meat, as a preventive measure, in addition to water treatment.
- The findings of this study show statistical significance ($p \leq 0.05$) for higher EV concentrations in milder seasons, such as autumn and winter, compared to other seasons. For HAV, no statistical significance was found ($p > 0.05$).
- The findings of pathogens and their respective associated diseases in populations should be further explored, in conjunction with wastewater parameters. This approach would enhance our understanding of their behavior and help determine whether there are any patterns across different populations.
- The recovery efficiency test demonstrated good recovery percentages using the ultracentrifugation methodology across four different dilutions for both RS and PABT samples (ranging from 68.0 % to 48.7 %). However, for the PCT samples, the chlorine-treated effluent showed a significant decrease, with recovery efficiency dropping by approximately threefold or more, ranging from 28.1 % to 0.0 %. This underscores the importance of conducting tests under various flowcharts in laboratories, taking into account different matrix types and the pathogens being studied.

## CRediT authorship contribution statement

**Mariah C.A. do Nascimento:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura S.R. Souza:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Camila R. Rosa:** Methodology, Investigation, Data curation, Conceptualization. **Meriane Demoliner:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pamela J.P. da Conceição:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniela M. Quevedo:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dayla B. Geraldini:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adriana Luchs:** Writing –

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## Declaration of competing interest

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.temicr.2025.100037.

## Data availability

Data will be made available on request.

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