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RESEARCH

Detection of Dengue virus RNA in Wastewater during a Local Epidemic in Central Italy (August–October 2024)

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Abstract

Wastewater-based epidemiology (WBE) is a well-established tool for monitoring enteric and respiratory viruses. However, its potential application to vector-borne diseases, including dengue virus (DENV), remains largely underexplored. This study investigated the feasibility of using WBE to detect DENV serotype 2 RNA during the largest dengue outbreak recorded to date in Italy (216 confirmed and probable cases), that occurred in the Marche Region (central Italy) from August to October 2024. From 1 to 30 October, during the decline of the epidemic (30 reported cases), 27 wastewater samples were collected from wastewater treatment plants in the affected municipality of Fano and in the nearby urban center of Pesaro. Four viral RNA concentration methods were tested: polyethylene glycol/sodium chloride precipitation, Nanotrap® Magnetic Virus Particles, electropositive membrane filtration, and solid fraction analysis. Only solid fraction analysis detected DENV-2 RNA in 9 samples, with digital RT-PCR showing greater sensitivity than Real-time RT-PCR. Virus concentrations ranged from 6.1×10^1 to 7.9×10^2 genome copies per gram of solid material. This study highlights the feasibility of WBE as a complementary surveillance tool for vector-borne diseases. Future efforts should focus on refining detection methods and in exploring surveillance strategies for timely wastewater sampling to improve early warning and/or outbreak management.

Keywords Wastewater-based epidemiology (WBE) · Dengue virus (DENV) · Environmental surveillance · Digital RT-PCR · Vector-Borne diseases · Dengue outbreak

Introduction

Dengue virus (DENV), a member of the *Flaviviridae* family, is transmitted to humans primarily through the bites of infected *Aedes* mosquitoes, particularly *Aedes aegypti* and *Aedes albopictus* (WHO., 2024). DENV is the causative agent of dengue fever, a disease that has become a major global health concern. In recent years, the spread of *Aedes* mosquitoes to temperate regions, driven by global warming and increased urbanization, has heightened the risk of dengue outbreaks beyond tropical areas.

Both human mobility and mosquito dispersal play significant roles in the geographic spread of dengue virus. The main mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, typically have a limited active flight range (rarely traveling more than 200–500 m). However, these mosquitoes can be passively transported over much greater distances via vehicles, goods, plants, and human-related activities.

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Human movement is even more crucial for the spread of the virus itself. When an infected person travels during the viremic phase, undisclosed transport of Dengue virus to an area where competent mosquito vectors are present may occur. And, if local mosquitoes bite this subject, they can become infected and start a new transmission chain in that area. Therefore, the interplay between human movement and mosquito dispersal is a key driver behind the emergence of dengue in previously unaffected regions.

A dengue outbreak in Fano, a city in the Marche region of Italy, began in August 2024, with the first documented case reported on August 14. The epidemic reached its peak around September 13, with the latest reported case documented on October 31. A detailed description of the outbreak, including epidemiological and public health aspects, has been provided in a recent paper by Sacco et al., 2024 (Sacco et al., 2024). The paper describes 199 probable and confirmed cases, which were the total officially reported as of October 28th, prior to the final update to 216 cases. This outbreak has been the largest dengue outbreak ever documented in continental Europe and highlights the growing potential for vector-borne diseases under favorable climatic conditions.

Rapid identification of outbreaks and clusters is essential for effective infection control. Wastewater-based epidemiology (WBE) has been recognized as a powerful tool to track enteric and respiratory viruses, such as poliovirus (WHO 2003) and SARS-CoV-2 (European Commission et al., 2021). However, its application to vector-borne diseases like dengue remains underexplored. Studies have shown that DENV RNA can be shed in body fluids, including urine and feces (Hirayama et al., 2012; Wang et al., 2002), suggesting that the viral genome may be present in wastewater, offering a unique opportunity for non-invasive community-level surveillance.

To date, studies attempting to detect DENV RNA in wastewater have been limited. While some, like a 2022 study in Nepal, found no detectable RNA in any samples (Thakali et al., 2022), Wolfe et al. (2023) (Wolfe et al., 2023) identified DENV-3 RNA in 21% of samples in the United States (Miami Dade County, Florida). Similarly, a monitoring in Portugal (North, Lisboa and Vale do Tejo, and south) detected DENV RNA in 25% of samples collected during 2022–2023 (Monteiro et al., 2024). These studies indicate that while detecting DENV RNA in wastewater is feasible, significant variability exists in detection rates, and standardized methodologies are lacking.

In this context, the 2024 outbreak in the Marche Region provided a unique opportunity to evaluate the feasibility of using WBE for dengue surveillance. Sampling during the outbreak demonstrated that DENV RNA could be detected in municipal wastewater, further highlighting the potential of WBE as a complementary tool for monitoring vector-borne diseases and supporting public health response efforts.

Material and Methods

Sample Collection and Processing

Between August and October 2024, an autochthonous outbreak of dengue occurred in the Marche region of central Italy. As of November 25, a total of 216 cases were reported to the National Surveillance System for Arboviral Diseases, including 143 confirmed cases and 73 probable cases, classified according to the case definition criteria of the Italian National Plan for Prevention, Surveillance and Response to Arboviruses 2020–2025 (https://www.salute.gov.it/imgs/C_17_pubblicazioni_2947_allegato.pdf). The outbreak was attributed to dengue virus serotype 2 (DENV-2). During this time, two confirmed cases of DENV-2 were also reported in the city of Pesaro, a larger urban center about 15 km from Fano.

Environmental sampling for the detection of dengue virus RNA could not begin until October 1, during the declining phase of the epidemic (Sacco et al., 2024). Between October 1 and October 30, a total of 27 wastewater samples were collected. Of these, 18 samples were obtained from two wastewater treatment plants (WWTPs) located in Fano, the city directly affected by the outbreak. The two WWTPs in Fano serve the city and its surrounding areas, including coastal and urban zones. These samples were collected twice per week. The remaining 9 samples were collected from a WWTP in Pesaro. The geographic distribution of sampling sites is illustrated in Fig. 1.

To identify the most effective method for viral concentration, four approaches were evaluated (Fig. 2 and Supplementary material):

a) Polyethylene glycol (PEG)/sodium chloride precipitation: 40 mL of wastewater were concentrated using a protocol originally adopted for SARS-CoV-2 surveillance (Rosa et al., 2022);
b) Nanotrap® Magnetic Virus Particles: a 40 mL aliquot was concentrated using Nanotrap® Magnetic Virus Particles, designed to bind viral particles (https://www.ceresnano.com/microbiome);
After the first two weeks of sampling yielded negative results, two additional methods were tested using larger volumes of wastewater:
c) Electropositive membrane filtration: 1 L of wastewater was directly filtered, without any preliminary prefiltration, through electropositive membranes (3 M™ Zeta Plus 1MDS) and directly subjected to nucleic acid extraction;

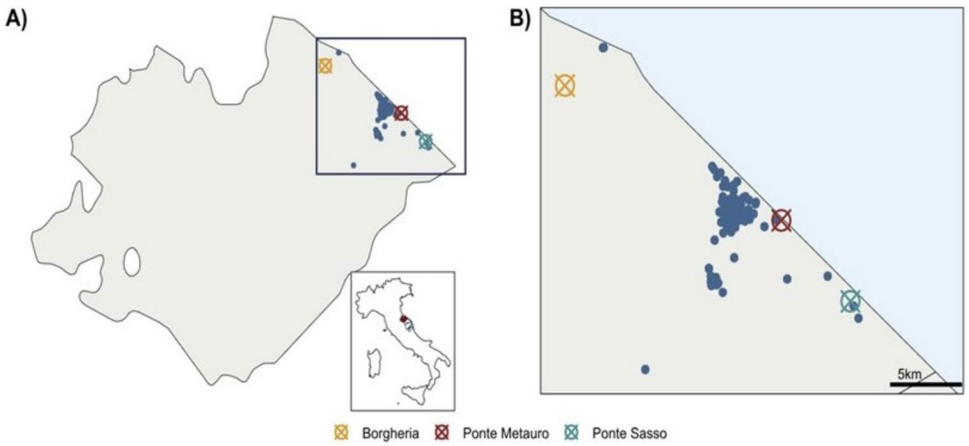

Fig. 1 Geographic distribution of the wastewater treatment facilities (Borgheria, Ponte Metauro, Ponte Sasso) and probable and confirmed autochthonous dengue cases in the Pesaro-Urbino province, Marche region, Italy. **A** The geographic distribution of dengue cases. The black square highlights the area where the cases occurred and where the wastewater treatment facilities are located. In the bottom-right corner, a map of Italy highlights the Pesaro-Urbino province in red. **B** The geographic distribution of dengue cases (blue points) by residence or exposure location and the wastewater treatment facilities in the affected area. WTPs sampled in this study: Pesaro Borgheira (capacity 116,500 p.e., load: 22,000 m3/day); Fano Ponte Metauro (capacity 60,000 p.e. load: 12,000 m3/day); Fano Ponte Sasso (15.000 p.e., load 1000–1500 m3/day in winter season; 3000–4000 m3/day in summer season)

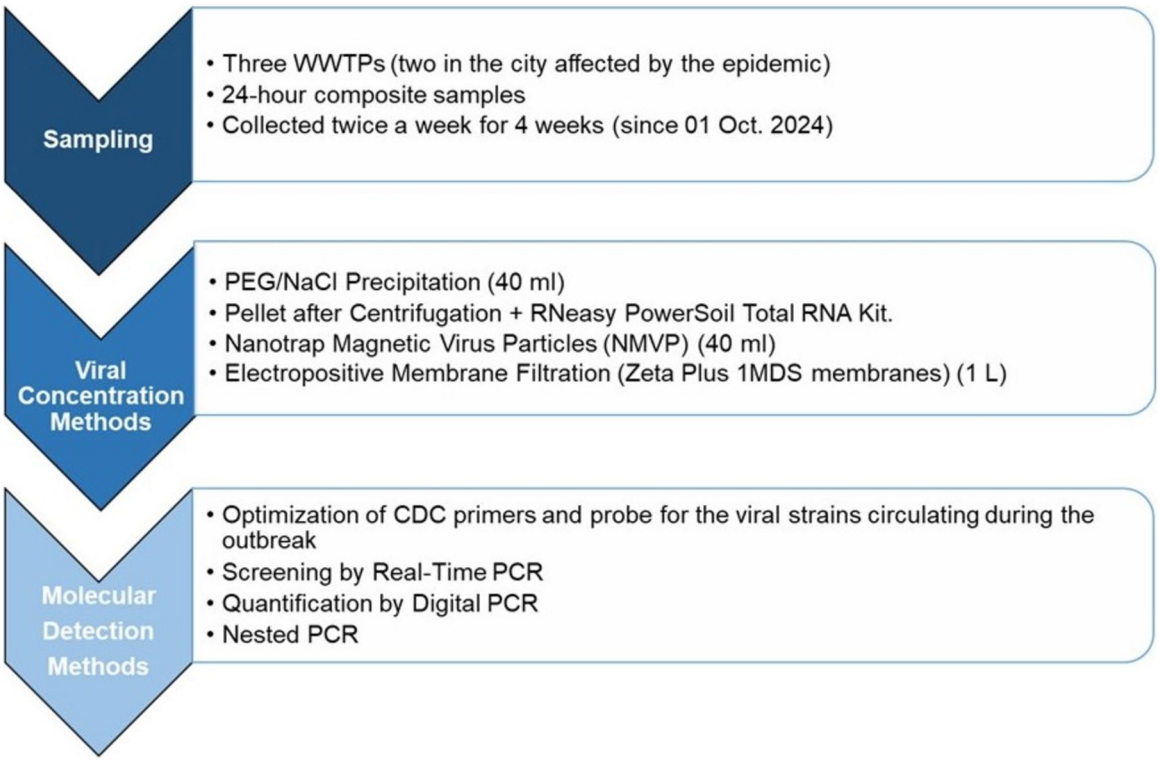

Fig. 2 Workflow for dengue virus detection in wastewater

d) Solid fraction analysis: 250 mL of wastewater was centrifuged at 4,500 × g for 30 min, and the resulting solid fraction was used for nucleic acid extraction.

For methods (a), (b), and (c), Mengovirus was added to each sample as a process control to assess recovery, and nucleic acids were extracted using the MagPuriX automated

extraction system (Zinexts Life Science). For method (d), nucleic acids were extracted using the RNeasy PowerSoil Total RNA Kit (Qiagen) and addition of the process control was not performed given that a representative attachment to the solid fraction cannot be secured through spiking.

All RNA samples were subsequently purified using the OneStep PCR Inhibitor Removal Kit (Zymo Research) to minimize PCR inhibitors, whose residual presence was assessed for each sample by Real-time RT-PCR, using an external inhibition control (Supplementary material).

Real-Time RT-PCR and Digital RT-PCR Analyses

Initially, we verified whether the primers and probes developed by the US CDC for detecting DENV-2 RNA (Santiago et al., 2013) were effective with the sequences of the epidemic strains obtained from clinical samples and mosquitoes (Sacco et al., 2024). While the CDC primers showed some level of functionality, we identified areas for improvement to enhance their sensitivity and specificity for the outbreak-specific viral strains. To optimize the assay, we modified the primers and probes by replacing unnecessary degenerate bases with nucleotides matching the local DENV-2 sequences and correcting a mismatch in the probe sequence. The improved primers and probes were subsequently validated for their performance. After optimization, the adjusted assay was used for nucleic acid screening by Real-time RT-PCR using the CFX96 Touch Real-Time PCR System (Biorad) and for viral quantification by digital RT-PCR, employing the QIAcuity Digital PCR System (Qiagen). Detailed information on the optimized primer and probe sequences is provided in the Supplementary material Table S1.

All dPCR experiments were conducted in accordance with the Minimum Information for Publication of Quantitative Digital PCR Experiments (MIQE) guidelines (The dMIQE Group et al. 2020). The LOD and LOQ were calculated to validate the optimized RT-dPCR assay using a local Dengue 2 viral strain (concentration 1×10^6 genomic copies (g.c.)/µL) circulating during the outbreak, kindly provided by the Department of Infectious Diseases of the Istituto Superiore di Sanità. Serial tenfold dilutions of the control virus were prepared using nucleic acids extracted from wastewater samples for dilution. These extracted nucleic acids were tested in advance for dengue virus to confirm its absence. Dilutions D4 (10^{-4}), D5 (10^{-5}), D6 (10^{-6}), and D7 (10^{-7}) were analyzed by RT-dPCR in triplicate across three independent sessions under the same conditions (Eylül Dogantürk et al., 2023). The 95% LOD, calculated according to Wilrich and Wilrich (2009) (Wilrich & Wilrich, 2009) using the tools available at https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html, was determined to be 0.799 g.c./ µL of RNA, corresponding to 3.995 g.c. / reaction. The LOQ was calculated as the lowest standard concentration that could be quantified with a CV value below 35% (Klymus et al., 2020) and was found to be 11.456 g.c./ µL, corresponding to 57.28 g.c. / reaction. The coefficient of determination (R^2) across the dilution range was 0.9745.

Sequencing attempts were made using newly designed nested PCR assays (Supplementary material Table S2). A strain of Dengue 2 (NGB strain), kindly provided by the Department of Infectious Diseases at the Istituto Superiore di Sanità, was used as a positive control for nested, Real-time RT-PCR and digital RT-PCR.

Results

In total, 27 wastewater samples were collected over a span of four weeks. The first 9 samples (collected up to October 8) were tested using two methods only: PEG precipitation and Nanotrap® Magnetic Virus Particles. The remaining 18 samples were tested using all four methods, resulting in a total of 90 RNA extractions. Results are displayed in Table 1 and Supplementary material Table S3.

The adopted concentration procedures showed different recovery rates. An average recovery of 43.4% (range: 6.4 – 83.5%), 6.1% (range: 0 – 32.2%), and 3.0% (range: 0.4 – 8.4%) was provided by PEG/NaCl precipitation, by Nanotrap® Magnetic Virus Particles, and by electropositive membrane filtration, respectively. Regarding inhibition, none of the samples extracted using the PEG/NaCl precipitation, the Nanotrap® Magnetic Virus Particles, or the electropositive membrane filtration method exhibited unacceptable inhibition ($\Delta Cq > 2$). For the solid fraction method, three samples did not meet the acceptability criteria, and showed significant inhibition. These samples underwent a second purification using the OneStep PCR Inhibitor Removal Kit (Zymo Research) to remove inhibitors before testing for dengue virus RNA. The results of concentration/extraction efficiency and inhibition verification are reported in Table S4 in the Supplementary material.

All positive detections originated exclusively from the solid fraction of the samples (pellets obtained by centrifugation), while the other three methods yielded no positive results. The Real-time RT-PCR assay successfully detected DENV-2 RNA in 5 out of the 27 wastewater samples, although with very high quantification cycle (Cq) values, indicating low viral concentrations. Positive samples collected on October 22, 24, and 29, originated from wastewater treatment plants (WWTPs) serving the city of Fano, the epicenter of the outbreak. Two additional positive samples, collected on October 14 and 16, 2024, were from the WWTP serving Pesaro, which was not the center of the outbreak but had reported two confirmed dengue cases.

Table 1 List of samples and results of molecular tests

Sample	City	WTP	Sampling date	Real-time RT-PCR (Cq)	digital RT-PCR (genome copies/g)
5435	Fano	Ponte Sasso	01/10/2024	–	–
5436		Ponte Metauro	01/10/2024	–	–
5437	Pesaro	Borgheria	01/10/2024	–	–
5457	Fano	Ponte Sasso	07/10/2024	–	–
5458		Ponte Metauro	07/10/2024	–	–
5459	Pesaro	Borgheria	07/10/2024	–	–
5460	Fano	Ponte Sasso	08/10/2024	–	–
5461		Ponte Metauro	08/10/2024	–	–
5462	Pesaro	Borgheria	08/10/2024	–	–
5469	Fano	Ponte Sasso	14/10/2024	–	6.40E+02
5470		Ponte Metauro	14/10/2024	–	2.20E+02
5471	Pesaro	Borgheria	14/10/2024	43.38	2.37E+02
5472	Fano	Ponte Sasso	16/10/2024	–	–
5473		Ponte Metauro	16/10/2024	–	–
5474	Pesaro	Borgheria	16/10/2024	42.46	6.11E+01
5478	Fano	Ponte Sasso	22/10/2024	–	2.80E+02
5479		Ponte Metauro	22/10/2024	42.33	1.14E+02
5480	Pesaro	Borgheria	22/10/2024	–	–
5481	Fano	Ponte Sasso	24/10/2024	41.41	2.67E+02
5482		Ponte Metauro	24/10/2024	–	–
5483	Pesaro	Borgheria	24/10/2024	–	–
5484	Fano	Ponte Sasso	29/10/2024	–	–
5485		Ponte Metauro	29/10/2024	–	1.97E+02
5486	Pesaro	Borgheria	29/10/2024	–	–
5487	Fano	Ponte Sasso	30/10/2024	41.89	7.92E+02
5488		Ponte Metauro	30/10/2024	–	–
5489	Pesaro	Borgheria	30/10/2024	–	–

Using the optimized assay, digital RT-PCR demonstrated greater sensitivity compared to Real-time RT-PCR. It confirmed all five Real-time RT-PCR positives and identified four additional positive samples, bringing the total to 9. The additional positives included two samples from Fano, collected on October 14 and other two samples collected from Fano on 22 and 29 October.

Viral concentrations in the solid fraction were low, ranging from 5 to 40 copies per reaction, corresponding to a range of 6.11×10^1 to 7.92×10^2 viral copies per gram of solid**.**

Despite numerous attempts, the nested PCR did not yield amplification products, preventing the generation of sequences to compare with those obtained from clinical and mosquito samples.

In total, 9 out of 27 (33.3%) wastewater samples tested positive in October. In the same month, a total of 18 new confirmed and 12 probable dengue cases were reported in a population of approximately 60,000 (Fig. 3).

Discussion

The detection of dengue virus RNA in wastewater samples, though limited to low concentrations, provides valuable epidemiological insights. While these figures provide important context, the detection of the virus in wastewater is likely to reflect a broader and more complex dynamic. In addition to the reported cases, it is plausible that individuals with asymptomatic or mild infections who did not seek medical care also contributed to virus shedding. Furthermore, due to the prolonged viral shedding documented by Humaidi et al. (2021) (Humaidi et al., 2021), the sewage signals may include contributions from individuals infected in earlier weeks, possibly as far back as September. Finally, some cases reported in late October may not have started shedding the virus yet, as viral excretion in urine typically peaks several days after symptom onset (Supplementary material Figure S1 and S2). It is

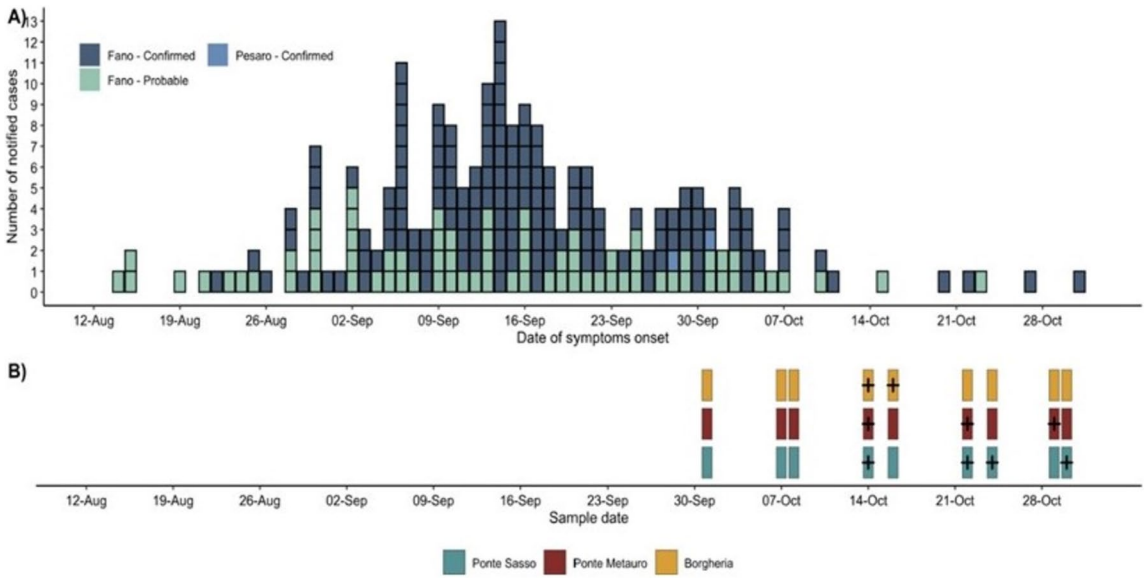

Fig. 3 Notified cases by date of symptom onset and wastewater sampling dates. **A** Confirmed dengue cases are shown in blue for Fano and azure for Pesaro, while probable cases are shown in light green for Fano. Dengue cases by date of symptom onset are presented. **B** Wastewater samples are shown by sampling date and wastewater treatment plant of collection (Ponte Sasso, Ponte Metauro, and Borgheria). Positive wastewater samples are marked with a plus sign (+)

also important to consider that the viral signals detected could include contributions from non-residents who have traveled to Fano for work, education, or other activities. Population mobility, both within and outside the city, plays an important role in shaping the dynamics of wastewater monitoring. Interestingly, we also identified two positive samples at the wastewater treatment plant in Pesaro, a city located 15 km away from Fano. While Pesaro was not the epicenter of the epidemic, two cases had been reported there. These detections could correspond to those cases, as well as to asymptomatic individuals, or to infected subjects resident in Fano and commuting to Pesaro for work or study.

All these factors highlight that the viral signals detected in wastewater are not necessarily solely attributable to the dengue cases reported in October, underscoring the complexity of interpreting wastewater-based epidemiology in dynamic populations.

Our analysis detected positive samples – though all at low concentrations – in soil-derived samples collected starting October 14. Between October 14 and 30, when these positives were identified, only six confirmed and probable cases had been recorded. This detection confirms the sensitivity of the approach and underscores the potential of environmental surveillance as a tool for monitoring dengue circulation in the population, while acknowledging the challenges and limitations discussed earlier. Notably, the World Health Organization (WHO) estimates that systematic environmental sampling for poliovirus can detect one individual excreting the virus among 10,000 inhabitants, illustrating the capability of this approach to capture even low-level circulation (WHO, 2003).

In this study, no positive samples were detected in the three sampling dates of October 1, 8, and 9, despite the higher number of reported cases during those weeks compared to the second half of the month. However, for those sampling dates, testing of the solid fraction – the only analytical approach that yielded positive results among the four tested in this study – was not performed and sample's residuals were not available for retrospective analysis. Therefore, this absence of detection is most likely due to methodological limitations.

All positive detections originated exclusively from the solid fraction of the samples. This is likely due to the affinity of DENV, an enveloped virus, for particulate matter. Like other enveloped viruses, DENV exhibits a tendency to adsorb onto particulate matter in wastewater, possibly due to interactions between the viral envelope and organic or inorganic solids. This partitioning phenomenon has been observed for other viral targets and is supported by findings from Wolfe et al. (2023), who reported consistent detection of DENV-3 RNA from the solid fraction only. Moreover, the solid fraction method allowed us to process the equivalent of 250 mL of wastewater in a concentrated form, which, after centrifugation, results in a smaller, more enriched matrix for nucleic acid extraction, improving the chance of detection, especially in settings with low prevalence or limited shedding. Despite the advantages of targeting the solid fraction,

some methodological limitations should be acknowledged. The separation process may lead to loss of viral particles, as the liquid fraction might still harbor some of them, and variability in particulate matter content among samples could affect detection sensitivity. Additionally, the solid fraction typically contains higher amounts of organic material, which may carry PCR inhibitors; although extraction kits include purification steps, complete removal is not always achieved, potentially reducing amplification efficiency. Future work should aim to optimize protocols that consider both fractions to improve overall detection reliability.

This study is affected by a significant limitation, the delayed start of sampling, which occurred during the declining phase of the outbreak. This prevented the ability to track the epidemic in Real-time RT-PCR and to evaluate whether wastewater viral concentrations correlated with case numbers during the peak of the outbreak. The lack of data from the earlier phases of the epidemic limited our ability to describe its progression or assess temporal trends in viral concentrations relative to case notifications. In addition, due to the low levels of DENV found in the samples, it was not possible to sequence the virus in the wastewater, despite numerous attempts.

These findings underline the importance, in a preparedness perspective, of establishing pre-emptive WBE systems that can be rapidly deployed. Wastewater surveillance approaches should be explored when transmission risk is highest for early warning and/or at the onset of an outbreak to support monitoring. Such systems would enable the timely collection of wastewater samples, that could provide critical insights into transmission dynamics and support early detection of circulation. In conclusion, this study demonstrates the technical feasibility of detecting DENV RNA in wastewater using optimized methods and highlights the value of including the solid fraction in dengue virus processing protocols. Despite the challenges posed by initiating sampling when the epidemic peak had already passed, DENV RNA was successfully detected in wastewater samples. This underscores the potential of WBE as a complementary surveillance system for vector-borne diseases serving both as an early warning system in the absence of reported cases and, following case detection, to evaluate epidemic trends. Future research should focus on understanding the factors influencing virus detection in wastewater, including shedding dynamics, environmental persistence, and matrix-specific sensitivity. With these advances, WBE could become a powerful tool for monitoring dengue and other vector-borne pathogens, informing health policy applications, and supporting the implementation of targeted interventions and strategies to reduce the spread of these diseases.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12560-025-09658-y.

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Author Contributions This work was carried out in collaboration between all authors. Mancini P. and Veneri C. contributed to investigation, methodology, validation, visualization, writing – original draft, and writing – Review Editing. These two authors contributed equally to this work; Bonanno Ferraro G., Franco A., Iaconelli M., Brandtner D. were involved in investigation, methodology, and writing—review and editing; Lucentini L. conducted investigation, supervision, and writing – review and editing; Venturi G, Mancuso E., Marsili G., Del Manso M., Sacco C., Bella A., Riccardo F., and Pezzotti P. performed data curation, formal Analysis, and writing—review and editing; Bolognini L.and Magi S. contributed to investigation, resources, and writing—review and editing; Filippetti F. and Liverani A. participated in investigation and writing – review and editing; Suffredini E.: methodology, supervision, validation, and writing – review and editing; La Rosa G. contributed to conceptualization, funding acquisition, methodology, supervision, writing – original draft, and writing – review and editing. All authors reviewed the manuscript.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interests The authors declare no competing interests.

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