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RESEARCH

Evaluation of Trends in Influenza A and B Viruses in Wastewater and Human Surveillance Data: Insights from the 2022–2023 Season in Italy

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Abstract
Wastewater-based epidemiology (WBE) is a recognized, dynamic approach to monitoring the transmission of pathogens in communities through urban wastewater. This study aimed to detect and quantify influenza A and B viruses in Italian wastewater during the 2022–2023 season (October 2022 to April 2023). A total of 298 wastewater samples were collected from 67 wastewater treatment plants (WTPs) across the country. These samples were analyzed for influenza A and B viruses (IAV, IBV) using primers originally developed by the Centers for Disease Control and Prevention (CDC) for real-time PCR and adapted for digital PCR. The overall detection rates of IAV and IBV across the entire study period were 19.1% and 16.8%, respectively. The prevalence of IAV in wastewater showed a gradual increase from October to December 2022, peaking at 61% in December. In contrast, IBV peaked at 36% in February 2023. This temporal discrepancy in peak concentrations suggests different seasonal patterns for the two influenza types. These trends mirrored human surveillance data, which showed influenza A cases peaking at 46% in late December and declining to around 2% by April 2023, and influenza B cases starting to increase significantly in January 2023 and peaking at about 14% in March. IAV concentrations ranged from 9.80×10^2 to 1.94×10^5 g.c./L, while IBV concentrations ranged from 1.07×10^3 to 1.43×10^4 g.c./L. Overall, the environmental data were consistent with the human surveillance trends observed during the study period in the country. These results demonstrate the value of WBE in tracking epidemiological patterns and highlight its potential as a complementary tool to infectious diseases surveillance systems.

Keywords Influenza virus · Wastewater · dPCR · Surveillance

Introduction

Epidemiological surveillance is a key component of public health, providing timely and accurate data to monitor diseases. During the Coronavirus Disease 2019 (COVID-19) pandemic, wastewater-based epidemiology (WBE) demonstrated its effectiveness in tracking the spread of Severe Acute Respiratory Syndrome Coronavirus 2 virus (SARS-CoV-2), offering a complementary tool to human surveillance systems. WBE is an approach that uses urban wastewater as a source to observe the circulation of pathogens, chemicals, and antimicrobial resistance genes from human metabolic wastes that reach wastewater treatment plants (WWTPs) via the sewer system (Lorenzo & Picó, 2019). Initially applied to the study of enteric viruses, the potential of WBE has now been recognized for the study of non-enteric viruses, particularly respiratory viruses, which

Members of the 'The SARI network' are available in Acknowledgements section.

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have been a major concern, as shown in the context of the COVID-19 pandemic.

The World Health Organization (WHO) published a guidance document on environmental surveillance for SARS-CoV-2 in September 2023 (WHO, 2023). This document illustrates the uses of environmental surveillance (ES) data for SARS-CoV-2, providing cost-effective and sensitive monitoring of spatial and temporal trends in viral circulation, including variants. In addition, ES can provide early indications of changes in incidence, including peak detection, tracking case clusters, and guide human testing and vaccination priorities. It can also act as an early warning system for the emergence of new variants or strains in low-prevalence or localized contexts, such as vulnerable communities or mass gatherings, particularly in the absence of human testing. Furthermore, it helps monitor the impact of public health interventions, including adjustments to restrictions, awareness campaigns and vaccination programs.

Recently, the effectiveness of ES, not only for COVID-19 but also for other respiratory diseases, such as influenza, has been increasingly recognized. Influenza is an acute infectious disease of the respiratory tract caused by the influenza virus, an RNA virus of the family *Orthomyxoviridae* classified into types A, B, C, and D. WHO estimates that there are 3 to 5 million cases of severe illness each year, resulting in 290,000 to 650,000 deaths worldwide (WHO, 2023a). The types of influenza A virus (IAV) and influenza B virus (IBV) are typically associated with seasonal influenza, although different subtypes of IAV are also responsible for influenza pandemics (CDC, 2023). The influenza virus has a segmented ssRNA(-) genome, which gives to this virus the ability to undergo genetic reassortment. This process leads to rapid mutation of the virus and the emergence of new variants, enhancing its ability to jump between different host species, including birds and pigs (Lyons & Lauring, 2018).

Zoonotic transmission is a major concern because of the potential for new emerging influenza variants to have a significant impact on human health. Humans can become infected with influenza viruses of animal origin, including avian influenza type A H5Nx, H7N9 and H9N2 viruses and swine influenza type A H1N1 or H1N2, and H3N2 viruses. However, these animal-borne viruses are fundamentally different from human variants and are not readily transmitted between humans. Nevertheless, sporadic cases of human infection with zoonotic influenza A viruses have been documented, including subtypes such as avian influenza A viruses (H3N8, H5N1, H5N6, H7N9, H7N7, H9N2, H10N3, and H10N5) and swine influenza A viruses (H1N1, H1N2, and H3N2) (ECDC, 2023; EFSA et al., 2024; Guercio et al., 2012; WHO, 2023b). In contrast, influenza B viruses are known to circulate widely among humans only (CDC, 2023).

Previous studies have reported elevated fecal shedding rates of IAV and IBV, with levels up to 10^5 copies/g of fecal matter (Lowry et al., 2023). Consequently, influenza viruses can be detected in a variety of water matrices. A recent systematic review described 43 articles focusing on the presence of influenza viruses in water from WWTPs and other human environments, as well as in poultry habitats and areas frequented by migratory wild birds (Kenmoe et al., 2023). The first investigation of the occurrence of influenza viruses in wastewater for epidemiological purposes was carried out in the Netherlands in 2011 (Heijnen & Medema, 2011). However, significant interest in the field only surged after the potential of WBE was recognized during the COVID-19 pandemic.

Numerous authors have investigated the occurrence and abundance of influenza viruses worldwide, including Japan (Ando et al., 2023), Canada (Mercier et al., 2022; Asadi et al., 2023; Hayes et al., 2023), Germany (Dumke et al., 2022), the Netherlands (Heijnen & Medema, 2011), the United States of America (Faherty et al., 2024; Vo et al., 2023), Greece (Zafeiriadou et al., 2023), Austria (Markt et al., 2023), Belgium (Rector et al., 2022), Spain (Toribio-Avedillo et al., 2023), Finland (Lehto et al., 2024) and Australia (Ahmed et al., 2023). In Italy, data on the presence of influenza viruses in wastewater is limited to the region of Sicily (Maida et al., 2024). Therefore, this study aims to address this knowledge gap by identifying and quantifying influenza A and B viruses in urban wastewater across the 20 regions and autonomous provinces of Italy during the 2022–2023 season (October 2022 to March 2023). For this purpose, samples were collected as part of the environmental surveillance for SARS-CoV-2, which was implemented to meet the objectives of EU Recommendation 2021/472, transposed by Legislative Decree Law No. 73 of 25/05/2021. The environmental data were compared with the human data from the RespiVirNet Integrated (epidemiological and virological) Surveillance system coordinated by the Istituto Superiore di Sanità.

The objective of this study was twofold: (i) to detect and quantify influenza A and B viruses in urban wastewater in Italy during the 2022–2023 influenza season; (ii) to compare environmental data with the RespiVirNet human virological (laboratory-confirmed) surveillance system data, to assess the consistency and complementarity of wastewater data.

Materials and Methods

Sample Collection and Processing

A total of 298 samples (24 h composite) were analyzed. The samples were collected from 67 WWTPs (Fig. 1) in 20 Italian regions and autonomous provinces during the influenza season from October 2022 to April 2023. These samples were selected from those collected by the national

Fig. 1 Distribution of WTPs in Italy. This map shows the locations of the 67 wastewater treatment plants (WTPs) across 20 Italian regions that were sampled as part of the environmental surveillance for influenza A and B viruses during the 2022–2023 influenza season

surveillance network for SARS-CoV-2 and sent monthly to the Istituto Superiore di Sanità (ISS) for the so-called '*flash surveys*' on SARS-CoV-2 variants (https://www.iss.it/en/cov19-acque-reflue). The SARI network laboratories performed the sampling process, virus concentration, nucleic acid extraction and quality assurance controls using a standardized national protocol, as previously described (La Rosa et al., 2021, 2022). After heat-inactivation at 56 °C for 30 min, 45 mL of samples were concentrated using a polyethylene glycol (PEG)-based method (Wu et al., 2020). Briefly, a 45 mL sample was subjected to PEG precipitation. Solids were removed by centrifugation at 4500×*g*. Next, 40 mL of the supernatant was transferred to new tubes and 4 g of PEG8000 and 0.9 g of NaCl were added. The mixture was stirred at cold temperature for 15 min until the PEG was completely dissolved, and then centrifuged again at 12,000×*g* for 2 h. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 2 mL of PBS and extracted. Nucleic acid extraction was then performed using commercially available, magnetic silica-based systems, and subsequently purified with the OneStep PCR Inhibitor Removal Kit (Zymo Research). Quality assurance controls (including either Mengovirus or Murine norovirus as process control virus and an inhibition control tested by real-time PCR) were included to assess viral recovery and

PCR inhibition. The purified RNAs were shipped to the ISS and stored at − 80 °C.

Multiplex Digital PCR for Influenza A and Influenza B

Samples were tested by RT digital PCR (RT-dPCR) to quantify IAV and IBV genomic copies, using the CDC protocol published in 2022 (CDC, 2022). The CDC protocol is a multiplex assay that can simultaneously detect and differentiate between influenza A, influenza B, and SARS-CoV-2 in respiratory tract specimens using a real-time reverse transcription polymerase chain reaction (rRT-PCR) laboratory test. In this study, primers and probes from the CDC protocol (Table S1) were applied in a digital PCR assay for the quantification of influenza A and B viruses in wastewater samples. This approach was selected due to the high sensitivity of dPCR, the reduced susceptibility to PCR inhibitory compounds, and the independence of quantification from a standard curve. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the method were calculated. Twist Respiratory Virus Controls (Twist Bioscience) were used, namely the Synthetic Influenza H1N1 (2009) RNA Control for Influenza A and the Synthetic Influenza B RNA Control for Influenza B.

Each reaction consisted of 40 µL of reaction mix per well, containing 35 µL mix and 5 µL RNA template, using QIAcuity Nanoplate 26k 24-well. Following optimization, primers were used at a final concentration of 0.4 µM for the InfA F1 and F2 primers, 0.6 µM for the InfA R1 primer, and 0.2 µM for the InfA R2 primer (Table S1). The remaining primers (InfB) were used at a final concentration of 0.8 µM, while all probes had a final concentration of 0.2 µM. RT-dPCR analyses were performed using the QIAcuity OneStep Advanced Probe Kit (Qiagen, Hilden, Germany) and the QIAcuity One 5-plex dPCR system (Qiagen, Hilden, Germany). Two technical replicates were performed for each sample. Nucleic acids were amplified under the following conditions: reverse transcription for 30 min at 50 °C, enzyme activation for 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 30 s at 55 °C. PCR experiments were performed following the Minimum Information for Publication of Quantitative Digital PCR Experiments (MIQE) guidelines (dMIQE Group & Huggett, 2020). The primer and probes used in this study were synthesized by Bio-Fab Research. Details of the primer/probe sequences are provided in Supplementary Table S1.

Assay Optimization

Assay optimisation was first performed to determine the optimal concentrations of primers and probes. This included the use of concentrations recommended by the CDC protocol and by the QIAcuity OneStep Advanced Probe Kit. In addition, primer and probe concentrations were alternatively varied while keeping the concentration of the other component fixed. The concentrations used for optimisation are shown in Table S2, together with the scatter plots obtained from the RT-dPCR runs (Fig. S1). Different annealing temperatures beyond those specified in the CDC protocol were also tested (data not shown), but no significant changes in the results were observed. Finally, the CDC protocol conditions (primer and probe concentrations and annealing/extension temperatures) were confirmed as optimal.

LOD and LOQ Calculation

The LOD and LOQ were calculated to validate the multiplex RT-dPCR assay using Twist Respiratory Virus Controls for influenza A and B (stock concentration 1×10^6 genome copies (g.c.)/µL for both). To simulate field conditions, serial tenfold dilutions of these controls were prepared using nucleic acids extracted from wastewater samples instead of water for dilution. These wastewater samples were collected in August 2022 outside of the influenza season, and the extracted nucleic acids were tested for influenza to confirm the absence of IAV and IBV. Dilutions D3 (10^{-3}), D4 (10^{-4}), D5 (10^{-5}), D6 (10^{-6}) were analyzed by RT-dPCR in triplicates in three independent runs under the same conditions (Doğantürk et al., 2023). The LOD95%, calculated according to Wilrich and Wilrich (2009) with the tools available at https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html, was 0.97 g.c./µL for Influenza A and 1.09 g.c./µL for Influenza B. The LOQ was calculated as the lowest standard concentration that could be quantified with a CV value of less than 35% (Klymus et al., 2020) and was 1.64 c.g./µL for Influenza A and 2.94 g.c./µL for Influenza B. The coefficient of determination (R^2) over the dilution range was calculated to be 0.97 for Influenza A and 0.98 for Influenza B.

Sample Analysis

The QIAcuity Software Suite version 2.2.0.26 was used to determine sample thresholds using positive and no-template

control (NTC) wells with the manual global threshold approach. Given the low viral concentrations inherent in environmental samples, we chose to classify samples as positive if they contained at least three positive partitions in a single replication, or if they had at least two positive partitions in both replicates. The target concentration (g.c./µL) in each sample was calculated using the instrument result and the formula:

$$\text{Influenza A or Influenza B(g.c./L)} = [\text{dPCR result (g.c.}/\mu\text{L)} \times \text{reaction volume} / \text{volume of RNA tested}] \times 100 \times 25$$

where 100 is the total volume of extracted RNA and 25 is the ratio of the volume of wastewater processed (40 mL) to the reference volume (1 L).

The quantitative data were then normalized for the flow rate of the WTP (L/day) and per 100,000 equivalent inhabitants.

Human Virological Data

The environmental data were compared with those of the human virological data from the RespiVirNet Integrated Surveillance System (https://respivirnet.iss.it/pagine/rapportoInflunet.aspx, https://www.epicentro.iss.it/influenza/respivirnet), coordinated by the Istituto Superiore di Sanità, which reported influenza virus positivity data in Italy from week 46 (14–21 November, 2022) to week 14 (3–10 April, 2023). Human samples are collected in the context of the Italian sentinel surveillance system for influenza, which integrates seasonal epidemiological monitoring of influenza-like illness (ILI) with virological surveillance of circulating influenza strains. The surveillance system is based on a sentinel network of General Practitioners (GPs) and Pediatricians reporting the number of patients with an ILI on a weekly basis. A sub-set of these GPs and Pediatricians also collect respiratory specimens from patients presenting with ILI during the surveillance season in Italy. A variable proportion of non-sentinel respiratory specimens, mainly from hospital laboratories, are also collected. Virological surveillance activities are coordinated and carried out by the National Influenza Centre (NIC) laboratory at ISS, in collaboration with a network of regional influenza laboratories, located in all the 21 Italian Regions/Autonomous Provinces. Preliminary analyses on the collected clinical specimens are performed at regional level and a representative subset of influenza virus-positive samples and/or virus isolates is sent to the NIC for further antigenic and genetic analyses.

Results

The concentration and extraction procedure had an average recovery of 28% (range: 1–100%). Regarding inhibition, 98.2% of the samples were within the acceptability criteria (ΔCq from reference reaction ≤ 2), 1.5% of the samples were not evaluated, and 0.3% showed unacceptable inhibition (ΔCq > 2).

Of the 298 samples analyzed, 19% tested positive for Influenza A by digital PCR, while 17% of samples tested positive for Influenza B. The monthly distribution of positive wastewater samples is shown in Table 1.

Detailed regional results for the seven-month period are shown in Fig. 2. In October, IAV was detected only in the Veneto region, while IBV was found in two regions (Liguria and Marche). In November, IAV was detected in three regions (Calabria, Liguria, and Veneto) and IBV in four regions (Lazio, Liguria, Marche, and Sicilia), with Liguria showing co-circulation of both viruses. In December, widespread circulation of IAV was observed in 11 regions and two autonomous provinces (Calabria, Emilia Romagna, Friuli Venezia Giulia, Lazio, Liguria, Lombardia, Piemonte, Puglia, Sicilia, Valle D'Aosta, Veneto, and APs of Trento and Bolzano), and both IAV and IBV were identified in Liguria and Veneto, indicating co-circulation in these regions. In January, while IAV persisted in 9 regions, IBV detections increased and spread to more regions (Lazio, Liguria, Emilia Romagna, and Veneto) compared to the two in December. From February to April, IBV predominated with detections in several regions, while IAV continued to be detected in a few regions.

The environmental data were compared with those of the human virological data from the RespiVirNet Integrated Surveillance System, coordinated by the Istituto Superiore di Sanità, which reported influenza virus positivity data in Italy from week 46 (14–21 November, 2022) to week 14 (3–10 April, 2023).

The percentages of IAV and IBV in both human and environmental (wastewater) settings during the study period (week 40, 2022 to week 14, 2023) are shown in Fig. 3.

Table 1 Number and percentage of Influenza virus-positive samples per month

Month	Flu A	Flu B
October 2022	1/43 (2.3%)	2/43 (4.7%)
November 2022	5/47 (10.6%)	7/47 (14.9%)
December 2022	27/44 (61.4%)	2/44 (4.5%)
January 2023	16/46 (34.8%)	5/46 (10.9%)
February 2023	4/42 (9.5%)	15/42 (35.7%)
March 2023	2/40 (5.0%)	7/40 (17.5%)
April 2023	2/36 (5.6%)	12/36 (33.4%)
Total	57/298 (19.1%)	50/298 (16.8%)

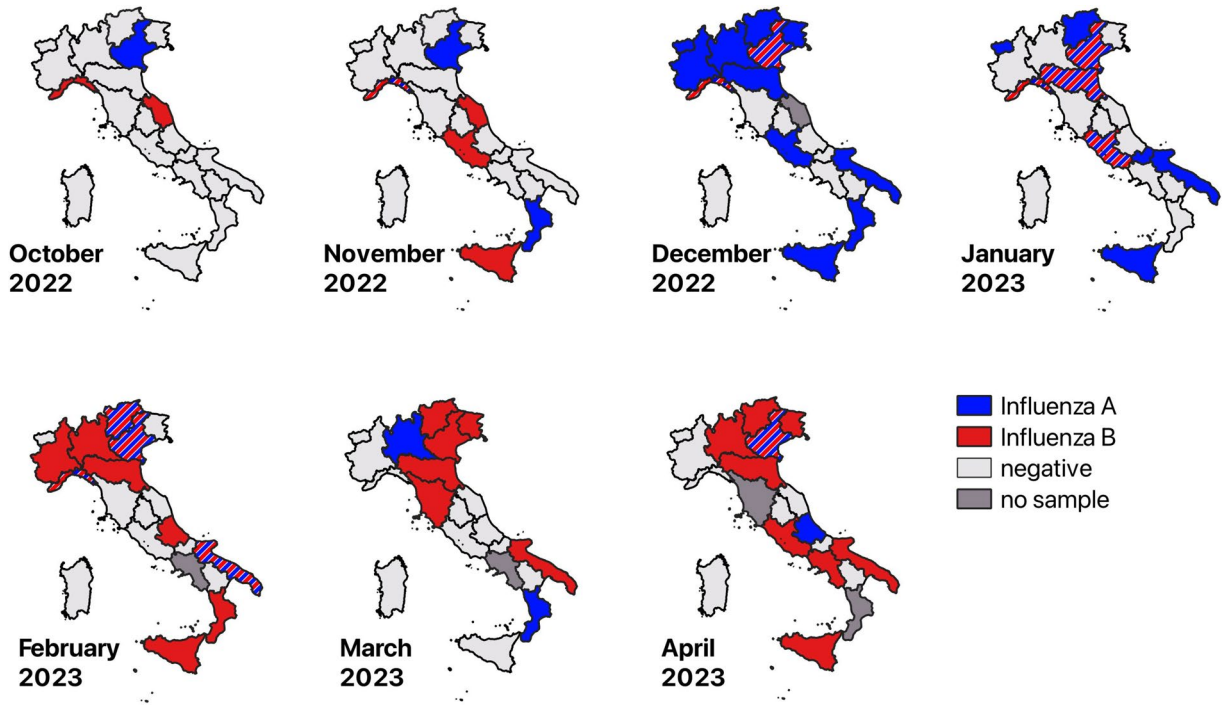

Fig. 2 Results for environmental data over the seven-month period. This figure illustrates the detection of Influenza A and B viruses in wastewater samples across different regions of Italy from October 2022 to April 2023. Each color-coded region represents the presence of either Influenza A (in blue) or Influenza B (in red) viruses, based on the percentage of positive samples collected monthly (Color figure online)

Human surveillance data were collected weekly, whereas the environmental data were collected monthly.

For influenza A, the percentage of positive clinical cases increased significantly, peaking at around 46% in week 48 of 2022. After this peak, the percentage gradually decreased to around 2% in week 13 of 2023. Similarly, in wastewater samples influenza A showed an increasing trend with a peak percentage of about 61% in December 2022 (week 48), followed by a decrease to about 6% by April 2023 (week 14). Despite being collected monthly, the environmental data captured the same seasonal increase and subsequent decrease observed in the human samples.

Influenza B showed a raise in the percentage of positive clinical cases starting from week 3 in 2023, peaking at up to 14% in week 9 in 2023. The percentages then gradually declined to around 7% by week 14 of 2023. This indicates a slightly delayed but prolonged period of influenza B activity compared to influenza A. In wastewater samples, influenza B also showed an increasing trend, with a peak of 36% in February 2023 (week 6). By week 10 of 2023, the percentage of positive cases in wastewater decreased to around 18%, before increasing further to about 33% in April 2023 (week 14). Notably, there was a peak in week 45 of 2022 with a percentage of 15%, which could indicate a small cluster of influenza B cases. However, direct comparison with human data for this period is not possible as human data collection started in week 46.

IAV concentrations ranged from 9.80×10^2 to 1.94×10^5 g.c./L, while IBV concentrations ranged from 1.07×10^3 to 1.43×10^4 g.c./L. For IAV an outlier was identified in October 2022, with considerably high concentrations (1.94×10^5 g.c./L). The average concentration of IAV, excluding the outlier, was 8.33×10^3 g.c./L while the average concentration of IBV was 3.56×10^3 g.c./L. The virus concentrations obtained during the study period, normalized to population equivalent and WTP flow rate, are detailed in Supplementary Table S3.

The scatter plot in Fig. 4 illustrates the concentrations of influenza A and B viruses in wastewater samples collected from October 2022 to April 2023, expressed in copies per 100,000 equivalent inhabitants per day.

Each data point represents a single measurement, with blue dots representing IAV concentrations and red dots representing IBV concentrations. The graph shows clear temporal trends for both viruses, with influenza A concentrations peaking in late November/early December (week 48–2022) and influenza B concentrations peaking in early February 2023 (week 06–2023). Excluding the exceptionally high value of IAV of 5.77×10^{12} copies per 100,000 equivalent inhabitants per day in October (week 40), which could possibly be attributed to a cluster of cases in the area served by

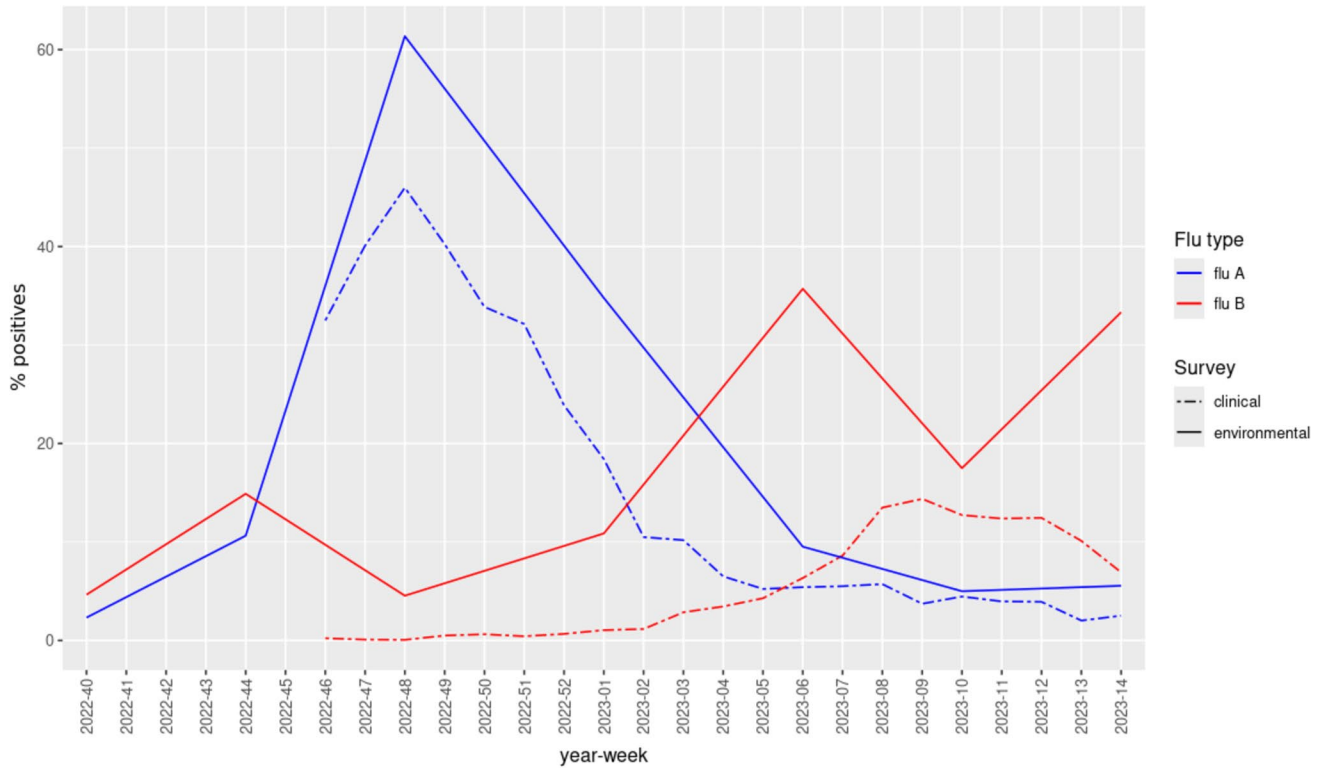

Fig. 3 Percentage of IAV and IBV positive samples over time: human and environmental data. This figure compares the percentage of positive samples for Influenza A and B viruses detected in wastewater and human samples over time from October 2022 to April 2023. The environmental data (solid lines) were collected monthly, while the human data (dashed lines) were collected weekly (data from the RespiVirNet Integrated Surveillance System)

the tested WTP, influenza A concentrations ranged from a minimum of 3.13×10^9 to a maximum of 1.58×10^{12} g.c. per 100,000 equivalent inhabitants per day. Unfortunately, human data to explain such a high value in October were not available, as the human surveillance started in November. The concentrations of influenza B ranged from a minimum of 1.38×10^9 to a maximum of 2.27×10^{11} copies per 100,000 equivalent inhabitants per day.

Discussion

The aim of this study was to detect and quantify influenza A and influenza B viruses in wastewater during the 2022–2023 season, and to describe the onset of the epidemic and its trends over several months (October 2022–April 2023). Environmental results and human surveillance data, available through RespiVirNet, the Integrated Surveillance System (epidemiological and virological) for cases of influenza-like syndromes and respiratory viruses (formerly InfluNet), were evaluated.

The detection rates of Influenza A and Influenza B viruses in the wastewater samples indicate a significant prevalence of both viruses in the country, with, overall, 19.1% of samples testing positive for IAV and 16.8% for IBV. In Europe, similar prevalences of IAV in wastewater were detected in Germany (23.4%) (Dumke et al., 2022), Belgium (11.2%) (Rector et al., 2022) and Spain (10.7%) (Toribio-Avedillo et al., 2023). Higher prevalences were found in Greece (97.6%) (Zafeiriadou et al., 2023), whereas lower prevalences (0%) were reported in the Netherlands (Heijnen & Medema, 2011). These variations may be due to differences in regional virus circulation, local public health interventions, sampling periods and detection methods. For IBV, except for a 46.9% prevalence in Germany (Dumke et al., 2022) and 63% in Greece (Zafeiriadou et al., 2023), other studies consistently reported prevalences lower than in our study: Austria 0% (Markt et al., 2023), Belgium 0% (Rector et al., 2022) and Spain 0.8% (Toribio-Avedillo et al., 2023). These discrepancies may reflect variations in IBV circulation in different regions, differences in vaccination coverage, and the timing of sample collection relative to peak influenza activity. On the other hand, the higher detection rate in Germany and in Greece could indicate a localized outbreak in the tested area, or analytical differences increasing detection rates. In addition, the number of samples analyzed in these studies varies considerably, ranging from 10 to 273 for IAV

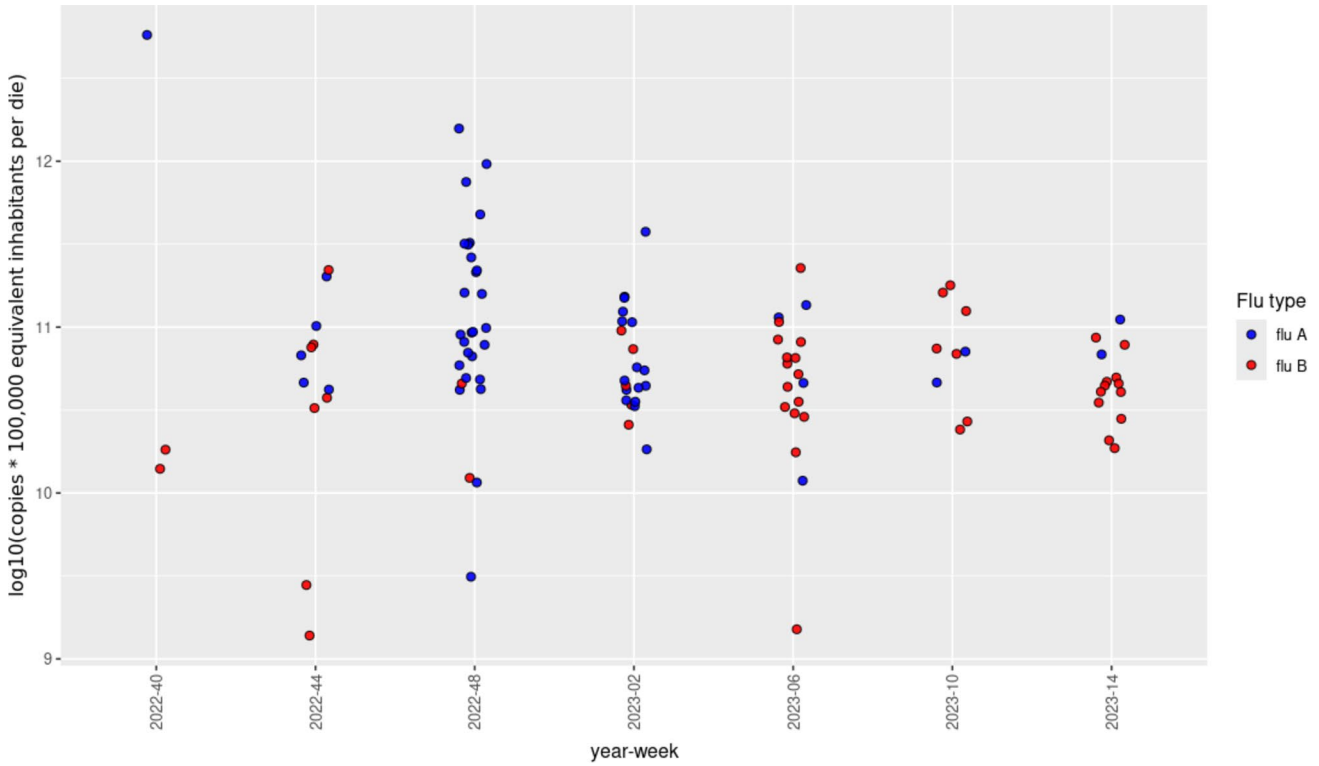

Fig. 4 Concentrations of Influenza A and B viruses in wastewater samples. The scatter plot illustrates the concentrations of Influenza A (blue dots) and Influenza B (red dots) viruses in wastewater samples collected from October 2022 to April 2023 (week 40–2022 to week 14–2023). The data are expressed in genomic copies per 100,000 population equivalent per day (Color figure online)

and from 89 to 538 for IBV. This wide range in sample size may also contribute to the variability in detection rates, as studies with fewer samples may not capture the full extent of virus prevalence compared to studies with larger sampling sizes.

The monthly distribution and concentration of positive samples revealed key insights into the prevalence of influenza A and B viruses during the study period. In October 2022, both viruses showed low prevalence (2.3% for IAV and 4.6% for IBV), consistent with early season patterns. By November, the number of positive samples increased, marking the start of heightened influenza activity. December saw a significant rise in influenza A positive samples, indicating its predominance. In January 2023, IAV remained active while influenza B began to spread more widely. From February to April, influenza B predominated, although influenza A was still detected in some regions, particularly in February and April. Influenza A concentrations peaked in December, with the highest values aligning with clinical data showing a 46% peak in cases. From January onward, influenza A detections declined, reaching 2% in human data and 5% in wastewater by April. Influenza B was detected in wastewater from October, with a peak in February at 36%. Human cases of influenza B began increasing in January, peaking at 14% in March. By April, human sample positivity was 8%, while wastewater samples remained higher at 30%. This indicates a slight lag in the clinical detection of influenza B compared to wastewater data.

In terms of viral concentration in wastewater, Influenza A showed significant variation throughout the study period, ranging from 9.80×10^2 to 1.94×10^5 g.c./L. A key observation is the identification of an outlier in October 2022, with remarkably high concentrations of Influenza A. It's worth noting that human surveillance data only starts at week 46, which means that there is a gap in the comparison of environmental and human data for October, making it difficult to verify this particular epidemic. After the peak in December, Influenza A concentrations began to decline in January and continued to fall, reaching much lower levels by the end of the study period in April. Influenza B concentrations in wastewater also varied over the study period, and were in general lower than those found in IAV. Viral loads ranged from 1.07×10^3 to 1.43×10^4 g.c./L of wastewater. High viral load was observed in November, with concentrations around 1.18×10^4 g.c./L. Concentrations fell slightly in December but started to rise again in January. The highest Influenza B concentrations were recorded in February, reaching up to 1.43×10^4 g.c./L. After February, Influenza B

concentrations gradually decreased but remained relatively higher than Influenza A until the end of the study period in April. Influenza A and B concentrations fell within the concentration ranges observed worldwide. Indeed, according to a recent systematic review, reported concentrations ranged from 4.9×10^{1} to 2.5×10^{10} g.c./L for Influenza A and from 7.2×10^{2} to 2.1×10^{7} g.c./L for Influenza B (Kenmoe et al., 2023).

This study demonstrates that wastewater-based surveillance is a highly effective tool for monitoring influenza viruses within the community, offering significant value for public health and complementing national sentinel surveillance programs.

A key aspect of this research is the optimization of the CDC assay in multiplex RT-dPCR for detecting influenza A and B viruses in wastewater samples. This provides a robust methodological tool for the surveillance of Influenza viruses in wastewater. This advancement is particularly important in light of the revision to the Urban Wastewater Treatment Directive 91/271/EEC, which includes Article 17 mandating Member States to monitor public health parameters in urban wastewater, such as pathogens like SARS-CoV-2, poliovirus, and influenza viruses. This highlights the increasing recognition of the importance of using wastewater-based epidemiology for influenza surveillance, as part of broader public health monitoring initiatives.

A limitation of the study is the geographic coverage, as one region did not participate, and some regions had missing samples for certain months. This lack of consistent sampling across all regions and months might have affected the comprehensiveness of the data. Additionally, the measurements were taken only on a monthly basis, which might have missed more granular trends in the prevalence of influenza A and B viruses. Despite these limitations, the study still achieved good correlations with human surveillance data. In this study, our focus was on assessing the feasibility of detecting and quantifying influenza viruses without delving into sample sequencing. However, it's also important to note the significance of understanding the diversity of circulating subtypes, especially if a novel subtype emerges that has not previously circulated in humans, potentially leading to large outbreaks outside the typical flu season. Seasonal influenza viruses evolve continuously, exposing individuals to multiple infections throughout their lives. As a result, the components of seasonal influenza vaccines are regularly reviewed (currently twice each year, in February and in September, respectively for Northern and for Southern Hemisphere vaccine recommendations) and regularly updated to ensure vaccine effectiveness (WHO, 2018). In the future, WBE may provide additional information on circulating influenza virus strains useful for determining vaccine composition. It could also provide support to national influenza prevention and control programs with insights into the timing, impact and severity of seasonal outbreaks.

In conclusion, this study successfully demonstrated the technical feasibility of detecting influenza A and B viruses in wastewater and showed that trends in wastewater samples closely mirrored the patterns observed in human surveillance data, confirming the seasonal behavior of both viruses. However, a limitation of this study is its relying on archival samples, collected monthly as part of the national surveillance network for SARS-CoV-2. This collection frequency does not allow for detailed temporal analyses, such as assessing lag times time between the environmental data and clinical data and calculating precise correlations between wastewater and clinical data. To address this limitation, we are currently conducting a pilot project funded by the Ministry of Health to collect samples on a weekly basis, which will allow the type of comprehensive analyses needed to improve wastewater surveillance as a strong complement to clinical surveillance systems.

Overall, the results underscore the potential of WBE as a complementary tool to traditional surveillance methods, providing valuable insights for public health monitoring and response. Future research should continue to refine detection methods and explore the integration of WBE with other surveillance systems to enhance public health strategies.

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Study period: October 2022–April 2023

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Author Contributions This work was carried out in collaboration between all authors. GLR, ES: writing—original draft, methodology, formal analysis, conceptualization, project administration, funding acquisition. PM: writing—original draft, methodology, review & editing. GBF, CV, MI: methodology, formal analysis, data curation, review & editing. DB, AM: visualization, validation, software, review & editing. SP, MF, GDM, PS, LL, the SARI network: methodology, investigation, review & editing.

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Data Availability All data have been included in the main text and in Supplementary Material.

Declarations

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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