Viral pathogens in water: occurrence, fate and environmental circulation

Patógenos virales en el agua: presencia, estabilidad y circulación ambiental

Programa de Doctorado en Ciencias de la Alimentación

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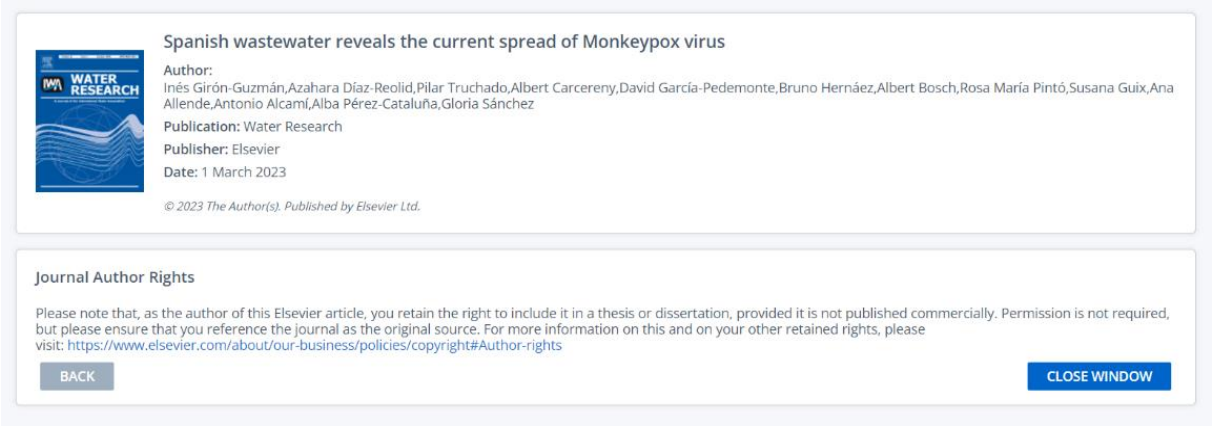
1. ***Evaluation of two different concentration methods for surveillance of human viruses in sewage and their effects on SARS-CoV-2 sequencing*** (2023) *Science of The Total Environment*, 862, 160914. Factor de Impacto de la Revista (JIF) (2022): 9,8 - Q1 (*Environmental Sciences*).

2. ***Spanish wastewater reveals the current spread of Monkeypox virus*** (2023). *Water Research*, 231, 119621. Factor de Impacto de la Revista (JIF) (2022): 12,8 - Q1 (*Environmental Sciences*).



Spanish wastewater reveals the current spread of Monkeypox virus

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3. ***Urban wastewater-based epidemiology for multi-viral pathogen surveillance in the Valencian region, Spain*** (2024). *Water Research*, 255, 121463. Factor de Impacto de la Revista (JIF) (2022): 12,8 - Q1 (*Environmental Sciences*).

Urban wastewater-based epidemiology for multi-viral pathogen surveillance in the Valencian region, Spain

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4. ***Sewage reclamation process as multifactorial public health risk concern: a longitudinal study*** (2024). Bajo revisión en la revista *npj Clean Water*.

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Dra. Irene Falcó Ferrando

A Celichurri, Carmoide, Lolita y Rob

"We could plant a house, we could build a tree"

-Kurt Cobain

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Hacer un doctorado no es un camino fácil, pero si te rodeas de las personas correctas, (casi) se te olvida.

INDEX

ABBREVIATIONS

AdV	Adenovirus
AiV	Aichivirus
AP	Aluminium-based precipitation method
ARG	Antibiotic resistance genes
ARB	Antibiotic resistance bacteria
BSA	Bovine serum albumin
CDC	Centers for Disease Control
CFU	Colony forming units
COVID-19	Coronavirus Disease
ddPCR	Droplet digital PCR
DW	Drinking water
ESBL	Extended Spectrum Beta-Lactamase
EW	Effluent water
FCV	Feline calicivirus
FDA	U.S. Food and Drug administration
GC	Genomic copies
HAV	Hepatitis A virus
HCoV-229E	Human coronavirus 229E
HEV	Hepatitis E virus
HAstV	Human astrovirus
HIE	Human intestinal enteroids
HuNoV	Human norovirus
HT-qPCR	High-throughput quantitative PCR
IAV	Influenza A virus
ISO	International Organization for Standardization
MgV	Mengovirus
MNV	Murine norovirus
mpox	Monkeypox disease
MPXV	Monkeypox virus
MP	Microplastic

NC	Negative control
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PCRU	PCR units
PEDV	Porcine Epidemic Diarrhea virus
PEG	Polyethylene glycol
PFU	Plaque forming units
PMA	Propidium monoazide
PMMoV	Pepper Mild Mottle Virus
PyV	Polyomavirus
q-PCR	Quantitative PCR
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
RV	Rotavirus
RW	Reference water
SARS-CoV-2	Severe Acute Respiratory Syndrome coronavirus 2
SMF	Skimmed Milk Flocculation
TCID50	50% Tissue Culture Infectious Dose
TNA	Total Nucleic Acid method
VACV	Vaccinia virus
VOC	Variants of Concern
WBE	Wastewater Based Epidemiology
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

ABSTRACT (ENGLISH VERSION)

Water is an essential resource for life, playing a crucial role in various human activities such as domestic use, agriculture and industry. However, its capacity to transport physical, chemical and biological contaminants, including viruses, poses significant public health risks. This thesis evaluates the presence of viral pathogens in different types of water and their stability in environmental waters. Waterborne pathogens can spread through contaminated water, impacting the water cycle and entering the food chain. Nevertheless, the contaminants in wastewater offer valuable insights through wastewater-based epidemiology (WBE), which can estimate the circulation of pathogens within populations. This approach enables rapid, cost-effective and non-invasive assessments, complementing conventional epidemiology.

In the context of WBE applied to virus surveillance, effective concentration methods are essential for virus detection and sequencing, driving the need for new approaches to enhance these analyses. Furthermore, wastewater reuse, a sustainable solution to water scarcity, demands rigorous controls to prevent the spread of pathogens and contaminants. The presence of human enteric viruses, along with respiratory and emerging viruses, at various points in the water cycle underscores the importance of studying their stability and viability in different water matrices.

In this thesis, epidemiological studies, virus stability analyses and methodological comparisons have been conducted to address the issues arising from the presence of pathogenic viruses throughout the water cycle. The results from a year-long epidemiological study for multi-pathogen surveillance (including human enteric viruses, SARS-CoV-2, influenza A virus and respiratory syncytial virus) in wastewater revealed high levels of the analysed viruses. This demonstrated the effectiveness of molecular methods, such as RT-qPCR and massive sequencing, in studying the prevalence and dynamics of viral variants. Furthermore, respiratory viruses showed a positive correlation with reported clinical data when using physicochemical parameters to normalize data and predictive models confirmed WBE as an early-warning and monitoring system.

Additionally, these techniques, routinely applied for SARS-CoV-2 analysis at the national level, proved to be powerful tools for health crisis responses, enabling the detection of emergent viruses like the monkeypox in sewage during the outbreak in Spain in 2022.

These methods were further improved in a comparative study that evaluated two concentration procedures, showing that newly developed techniques focused on viral nucleic acid capture enhanced the sensitivity of molecular methods to detect enteric, respiratory and indicator viruses.

Thus, these new methods were applied to analyse the presence of pathogenic viruses throughout the urban water cycle, in six wastewater treatment plants over the course of one year, demonstrating the presence of these viruses in reclaimed water and biosolids. This highlighted the low efficiency of the analysed wastewater treatment plants in removing pathogenic viruses and the bioaccumulation of these pathogens in biosolids.

The evaluation of viral stability in water microcosms at different temperatures showed that viruses could persist in infectious forms depending on the virus type and water conditions. Moreover, results using viability PCR indicated that it is not appropriate for monitoring virus infectivity decay in waters.

In conclusion, the presence of viruses in water and their potential to cause diseases underscore the importance of developing effective detection and treatment strategies. Enhancing water and biosolid treatment technologies is crucial to mitigate the risks associated with water reuse in agriculture and other applications.

ABSTRACT (SPANISH VERSION)

El agua es un recurso esencial para la vida y desempeña un papel crucial en diversas actividades humanas como el uso doméstico, la agricultura y la industria. Sin embargo, su capacidad para transportar contaminantes físicos, químicos y biológicos, incluidos virus, plantea importantes riesgos para la salud pública. Esta tesis evalúa la presencia de patógenos virales en diferentes tipos de agua y su estabilidad en aguas ambientales. Los patógenos transmitidos por el agua pueden propagarse a través del agua contaminada, afectando el ciclo del agua, pudiendo incorporarse a la cadena alimentaria. No obstante, los contaminantes de las aguas residuales ofrecen información valiosa a través de la epidemiología basada en aguas residuales (WBE), que puede estimar la circulación de patógenos dentro de las poblaciones. Este enfoque permite evaluaciones rápidas, rentables y no invasivas, que complementan la epidemiología convencional.

En el contexto de la WBE aplicada a la vigilancia de virus, el uso de métodos eficaces de concentración es fundamental para la detección y secuenciación de virus, lo que conlleva la necesidad de desarrollar nuevos enfoques para mejorar estos análisis. Además, la reutilización de aguas residuales, una solución sostenible para mitigar la escasez hídrica, requiere controles rigurosos para prevenir la propagación de patógenos y contaminantes. La presencia de virus entéricos humanos, así como de virus respiratorios y emergentes, en varios puntos del ciclo del agua, subraya la importancia de estudiar su estabilidad y viabilidad en diferentes matrices de agua.

En esta tesis se han llevado a cabo estudios epidemiológicos, investigaciones sobre la estabilidad de virus y comparaciones metodológicas para abordar los problemas derivados de la presencia de virus patógenos a lo largo del ciclo urbano del agua. Los resultados de un estudio epidemiológico realizado durante un año para la vigilancia de múltiples patógenos (incluyendo virus entéricos humanos, SARS-CoV-2, virus de la influenza A y virus respiratorio sincitial) en aguas residuales revelaron niveles elevados de los virus analizados. Esto demostró la eficacia de los métodos moleculares, como la RT-qPCR y la secuenciación masiva, para estudiar la prevalencia y la dinámica de las variantes virales. Además, los virus respiratorios mostraron una correlación positiva con los datos clínicos cuando se utilizaron parámetros fisicoquímicos para normalizar los datos, y los modelos predictivos confirmaron que la WBE es un sistema de alerta temprana y monitoreo.

Además, esta aproximación, aplicada de forma rutinaria en el análisis del SARS-CoV-2 a nivel nacional, demostró ser una herramienta muy útil para dar una respuesta rápida a situaciones de riesgo para la salud, permitiendo la detección del virus de la viruela del mono en aguas residuales durante el brote en España en 2022.

En el marco de esta tesis, se mejoraron metodologías mediante estudios comparativos donde se evaluaron dos métodos de concentración, mostrando que los métodos recientemente desarrollados, centrados en la captura de ácido nucleico viral, mejoran la sensibilidad de los métodos moleculares para detectar virus entéricos, respiratorios e indicadores.

Estos nuevos métodos se aplicaron para analizar la presencia de virus patógenos a lo largo del ciclo urbano del agua, en seis plantas de tratamiento de aguas residuales durante un año, demostrando la presencia de estos virus en el agua regenerada y los biosólidos. Esto destacó la baja eficiencia de las plantas de tratamiento de aguas residuales analizadas con relación a la eliminación de virus patógenos y la bioacumulación de estos en biosólidos.

La evaluación de la estabilidad viral en microcosmos acuáticos a diferentes temperaturas mostró que los virus podrían persistir en formas infecciosas dependiendo del tipo de virus y las condiciones del agua. Además, los resultados de la PCR de viabilidad indicaron que esta técnica no es apropiada para monitorizar la caída de la infectividad del virus en las aguas.

En conclusión, la presencia de virus en el agua y su potencial para causar enfermedades subraya la importancia de desarrollar estrategias efectivas de detección y tratamiento. Mejorar las tecnologías de tratamiento de agua y biosólidos es crucial para mitigar los riesgos asociados con la reutilización del agua en la agricultura y otras aplicaciones.

SUMMARY (ENGLISH VERSION)

Water is an essential element for life, both as part of our diet and for domestic use, in agriculture, aquaculture, food preparation, and industry. Ensuring a sufficient, safe and accessible water supply for the entire population is vital. However, water can also act as a vehicle for the dissemination of pathogenic microorganisms, including enteric viruses like human norovirus (HuNoV) or hepatitis A virus (HAV). These viruses can enter the food chain, contaminating facilities, packaging and the food itself. In agriculture, enteric viruses can contaminate food through irrigation with polluted water or through the use of sewage sludge as fertilizer. Both potential contamination sources originate from wastewater treatment plants (WWTPs). In aquaculture, if the water used to cultivate and harvest bivalve molluscs is contaminated, it poses a significant health risk to consumers. This is due to the filtering nature of these animals, which leads to bioaccumulation and the minimal cooking processes they undergo before consumption.

The role of water in the dissemination of enteric viruses, primarily transmitted through the faecal-oral route and the risks associated with their presence in water, have been extensively documented. However, in recent years, there has been growing evidence that respiratory viruses, which are also excreted in the faeces and other bodily fluids of infected humans and animals, can be detected and potentially spread through water. The potential transmission of these viruses via this route is a highly debated and currently under investigation topic. Additionally, the potential for water bodies to act as agents for the dissemination of zoonotic viruses due to their contact with both humans and animals has been studied, presenting a significant health risk. The interaction between humans, animals and the environment has gained increasing attention in recent years, emphasizing the importance of studying pathogens within the framework of these three fundamental pillars, an approach known as "One Health." Apart from the role of water in pathogen transmission, another crucial factor to consider is the ability of viruses to remain infectious in water. The stability of viruses depends on various factors such as the virus type, the surrounding matrix, temperature, time and pH.

The natural water cycle is a fundamental process where all water bodies, including surface waters such as rivers, lakes and seas, underground aquifers, and even ice, are interconnected and vulnerable to contamination by viruses. This contamination can stem from diverse sources, such as industrial, agricultural and urban discharges, as well as inadequate waste management practices, ultimately impacting humans, animals and the environment.

The urban water cycle stands out as a significant source of contamination, critical for water quality and safety. This cycle encompasses various stages, starting from the collection of water used by diverse population centres and industries, extending to its treatment in WWTPs for contaminant

removal. The quality of effluents and sludge produced in these treatment facilities is pivotal, directly impacting public health and the environment. The sludge generated during wastewater treatment holds potential as agricultural fertilizer, while treated effluent water finds applications in agricultural irrigation, industrial processes, or recirculation into natural water bodies like rivers and lakes. Subsequently, water undergoes purification in drinking water treatment plants (DWTPs) before distribution to the population. Upon usage, the water transitions to wastewater and re-enters the urban water cycle through WWTPs. Consequently, improper management of urban, industrial, and agricultural wastewater poses significant environmental and health risks.

Water scarcity and droughts are intensifying pressures on global water resources, a trend exacerbated by climate change and population growth. To address these challenges, policies promoting water reuse are gaining traction. These initiatives involve utilizing treated effluents from WWTPs for non-potable purposes like industrial processes and agricultural irrigation. This approach helps to reduce the demand of natural water sources such as rivers and lakes. However, while wastewater and sludge are increasingly utilized globally, a significant portion is still managed without adequate treatment or control measures, posing risks to human and environmental health. When managed effectively, adhering to established parameters like those outlined in Regulation (EU) 2020/741, the utilization of WWTP effluents and sludge offers numerous benefits. These include safer food production, enhanced resilience to water and nutrient scarcity, and the promotion of a more sustainable circular economy.

Although untreated or inadequately treated wastewater is the main source of the spread of pathogenic microorganisms in the environment, the presence of viruses in wastewater can also represent an opportunity, such as the Wastewater-Based Epidemiology (WBE). Epidemiology is defined as the study of the incidence, distribution and control of diseases in populations. There are several tools based on passive monitoring systems; however, they may present practical and economic difficulties, such as the limitation of clinical tests only to symptomatic patients, the lack of resources and medical care in certain countries, or the overload of analytical capacities during severe epidemics and pandemics. All these factors contribute to potentially inaccurate estimates of disease spread, making it necessary to develop new approaches. These novel surveillance tools must be flexible, cost-effective, scalable and capable of providing objective and comprehensive real-time data to complement the weaknesses of traditional approaches.

WBE is a powerful tool offering real-time, comprehensive and objective insights into public and environmental health status. By analysing agents excreted in feces, urin, and other fluids that ultimately enter wastewater, WBE aims to gather information about the circulation of various agents in the population, assess community health status and identify associated risk factors. Importantly, WBE helps mitigate biases stemming from socioeconomic factors or the absence of

symptoms in affected populations. The origins of WBE trace back to the 19th century when cholera cases in London were linked to contaminated water used for drinking and domestic purposes. Since then, WBE has evolved to detect a wide array of contaminants circulating in communities, ranging from chemicals like drugs, pesticides, hormones and medicines to pathogenic microorganisms such as bacteria and viruses. In recent years, the variety of pathogens that can be detected has expanded and viruses have gained increasing importance. An illustrative application of Wastewater-Based Epidemiology (WBE) is evident in the World Health Organization's polio eradication program and its role in detecting enteric viruses like HAV, hepatitis E virus (HEV), HuNoV, enterovirus, and rotavirus (RV). Notably, during the COVID-19 pandemic, WBE emerged as a vital tool for early detection of the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) and its variants, offering an effective strategy to monitor virus presence and incidence changes. The biological principle behind WBE is the excretion of viruses in faeces by infected individuals before symptom onset, facilitating early detection. As a result, WBE often identifies peaks in incidence or emerging variants of infectious agents earlier than traditional epidemiological approaches.

The detection of viruses in water typically requires a concentration step due to their low levels in water and the techniques employed for their detection. Traditional concentration methods rely on precipitation, adsorption, filtration, ultrafiltration, and ultracentrifugation. However, despite the increasing prominence of WBE in recent years, standardized and validated methods for virus detection remain lacking and the emergence of new concentration techniques necessitates further comparative studies. Historically, cell culture methods have been used for the isolation and characterization of viruses like poliovirus in water. However, the advent and adoption of molecular techniques such as polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), quantitative PCR (qPCR) and digital PCR (ddPCR) have led to an exponential increase in the number of procedures and viruses analysed in water.

On the other hand, massive sequencing techniques have proven to be a very useful tools for assessing temporal fluctuations and diversity of viruses in wastewater samples, as well as for detecting changes in their evolution and the emergence of new viral strains. Unlike PCR techniques, which are limited in their ability to discriminate among a wide variety of circulating lineages due to their capacity to detect only one or a few mutations of the same virus, sequencing methods offer a more comprehensive approach. While quantitative techniques such as simple and duplex PCR/RT-PCR (qPCR/RT-qPCR) can detect and quantify new virus variants, the use of sequencing techniques has proven to be crucial for monitoring variants, lineages and sub-lineages in wastewater during the COVID-19 pandemic.

For a comprehensive WBE approach, the final phase of the process includes interpreting the data by establishing connections wastewater dynamics, spatial-temporal relationships with the

population and clinical data reported from the studied area. his approach has revealed numerous correlations between WBE data and the incidence and number of patient hospitalizations across various settings. Surveillance of enteric, respiratory and emerging viruses through WBE is a valuable strategy for understanding the emergence and dynamics of these pathogens in a population by studying wastewater. However, it is important to consider that detected levels of viruses in wastewater can be influenced by factors unrelated to the sample treatment methodology. Mobility restrictions, quarantines, tourism, festivities, and dilution effects due to rainfall can all impact the estimated concentration in such studies. Data normalization enables comparisons between different samples from the same WWTP over time, or even between different populations. This is achieved by normalizing the data with physicochemical parameters or specific viruses, such as Pepper Mild Mottle Virus (PMMoV) or the crAssphage bacteriophage, which are known indicators of human faecal presence.

In this context, this doctoral thesis explored the potential of WBE for studying respiratory and enteric viruses in wastewater. Additionally, the effectiveness of this technique in responding rapidly and efficiently to the emergence of novel viruses and new health crises was evaluated. During these investigations, new methods for the analysis and detection of viruses in wastewater were assessed and validated to enhance the capabilities of detecting and characterizing viral pathogens. These analyses facilitated the study of pathogenic viruses in the urban water cycle and evaluated the capacity of WWTPs to remove viral contaminants. The results demonstrated the presence of viruses in effluent waters, leading to further studies on virus stability in different microcosms and temperature conditions.

During the pandemic caused by SARS-CoV-2, WBE experienced a worldwide surge, demonstrating its capacity as an early warning epidemiological tool, even enabling the detection of new variants introduced into the population. In the Valencian Community, this tool was widely used; however, there were no studies demonstrating the utility of WBE for the epidemiological control of other human pathogenic viruses. Therefore, within the framework of this thesis, the prevalence and temporal patterns of different enteric, respiratory and faecal indicators viruses were evaluated in wastewater samples from our region. For this purpose, wastewater samples were collected weekly at the inlet of a Valencian urban WWTP, from October 2021 to February 2023. The samples were transported to the laboratory and concentrated using the aluminium precipitation (AP) method. Genetic material extraction was automatized and the detection of different viruses was performed using RT-qPCR and qPCR for the crAssphage indicator. Thus, enteric viruses (HuNoV genogroups I and II, human astrovirus (HAstV), RV, HAV and HEV), respiratory viruses (SARS-CoV-2, influenza A virus (IAV) and respiratory syncytial virus (RSV)), and viral indicators (crAssphage and PMMoV) were detected and quantified. Additionally, the detection of somatic coliphages, as a third type of viral indicator, was performed using the plaque count technique. For

the detection and quantification of SARS-CoV-2, the nucleocapsid phosphoprotein gene (N1) region was targeted. The evolution of SARS-CoV-2 during the studied period was also evaluated by detecting its variants using duplex RT-qPCR and massive sequencing. For the detection of Variants of Concern (VoCs) via RT-qPCR, five different duplex reactions were used, revealing the prevalence of Alpha (S:69/70del), Delta (S:157/158del), Omicron BA.1 (S:214insEPE, S:69/70del) and Omicron BA.2 (S:25/27del) variants. For sequencing, a monthly sample was selected and Artic v3 (October 2021-January 2022) and Artic v4 (February 2022-February 2023) primer schemes were used for amplification of the spike region, followed by sequencing using the MinION Mk1C nanopore system (Oxford Nanopore Technologies). The obtained reads were analysed using the Freyja software. Finally, the evaluation of the prevalence and temporal patterns of human enteric and respiratory viruses in wastewater samples, as well as the identification of SARS-CoV-2 VoCs, allowed for exploring the correlation of our findings with public health records, also assessing various indicators for data normalization.

The results obtained regarding the prevalence of enteric viruses in wastewater were consistent with existing literature, showing a prevalence of approximately 100% for RV, HAstV and HuNoV GI and GII, while the prevalence of HAV and HEV was 3.77% and 22.64%, respectively. The mean concentrations ranged from 7.95 log genomic copies (gc)/L for RV to 3.05 log gc/L for HAV. Regarding respiratory viruses, SARS-CoV-2 was detected in 100% of the analysed wastewater samples, while IAV and RSV were only detected in 39.13% and 31.88% of the samples, respectively. The mean concentration values for these viruses were 5.46 log gc/L for SARS-CoV-2, 6.42 log gc/L for IAV and 3.88 log gc/L for RSV.

Despite the seasonality reported in infections caused by some enteric viruses, this study did not observe a marked seasonality for any of the enteric viruses studied, except for a slight increase in RV during the winter months. In the case of respiratory viruses, RSV showed an increase in winter, while IAV exhibited a moderate increase in winter with a much larger incidence peak during the spring months. The concentration results of SARS-CoV-2 in wastewater showed an increase corresponding with the emergence of new variants, which aligned with an increase in population incidence. The comparison of results obtained through duplex RT-qPCR for SARS-CoV-2 variants and genome sequencing indicated that duplex RT-qPCR assays achieved significantly faster results while maintaining adequate sensitivity and specificity. Although the detection of new SARS-CoV-2 variants in wastewater through sequencing methods generally requires higher concentrations of viral particles than PCR-based methods, the identification of variants was efficiently performed even in samples with low concentrations. Notably, both techniques, duplex RT-qPCR and sequencing, reliably demonstrated that the transition from the Delta variant to Omicron occurred very rapidly, in just two weeks, in Spain in December 2021.

Correlation with clinical data is another key component of WBE, as it is necessary to establish the relationship between the viral concentrations measured in wastewater and the reported clinical cases of the disease. In this doctoral thesis, statistically significant correlations were observed between confirmed clinical cases and the corresponding quantification data in wastewater for IAV and RSV, demonstrating the value of wastewater surveillance for monitoring infectious diseases in the population. The most robust correlations without normalization factors occurred between virus concentration and the number of clinical cases in the week following the sampling for SARS-CoV-2 and IAV and in the same sampling week for RSV. However, clinical data for SARS-CoV-2 ceased to be reported from April 2022 onwards, so these correlations were based on data prior to that date, which showed the best values. Once the data were normalized with different microbiological and physicochemical parameters, stronger correlations were observed when considering the number of clinical cases in the week following sampling along with the inflow rate (m^3/day) of the WWTP. For RSV, however, the strongest correlation was observed with clinical cases in the third week from sampling. Additionally, the minimum number of clinical cases of IAV and RSV required to obtain positive wastewater samples was calculated, resulting in between 110 and 205 reported clinical cases for IAV and between 40 and 120 for RSV. When reported clinical cases of IAV and RSV exceeded 205 and 210 cases, respectively, 100% of the wastewater samples tested positive for these viruses.

The implementation of WBE techniques for the epidemiological analysis of SARS-CoV-2 has exponentially increased the number of research groups, laboratories and companies capable of monitoring viruses in wastewater. This growth has been supported by governmental institutions, facilitating widespread implementation. For the first time, the mechanisms and tools necessary for controlling potential epidemics and pandemics through the study of wastewater are available. However, it remains to be demonstrated whether this surveillance system can be efficiently implemented in future health crises.

In May 2022, an opportunity arose to demonstrate the validity of this tool due to an outbreak of mpox (formerly known as monkeypox) caused by the Monkeypox virus (MPXV) in non-endemic areas, including countries like Spain. The virus is primarily transmitted through contact with the skin lesions of infected individuals, respiratory droplets and fomites. However, it has also been observed to be excreted in bodily fluids such as urine, semen and faeces. Therefore, it was decided to study this virus through the analysis of wastewater using the techniques routinely employed for the analysis of SARS-CoV-2 in the wastewater surveillance network in Spain (VATAR-COVID).

Initially, the AP concentration procedure was validated by inoculating an inactivated strain of MPXV into negative wastewater samples for the virus. The results indicated that this procedure yielded average MPXV recoveries of 31.5% ± 15.9%, demonstrating the validity of the method for

studying this pathogen. Subsequently, 312 samples from 24 WWTPs distributed throughout Spain were analysed between May 9th and August 4th, 2022. Viral particles were concentrated using the AP method, followed by automated nucleic acid extraction using paramagnetic particles. MPXV DNA was then detected and quantified using qPCR. Genetic material from MPXV was detected in 56 wastewater samples (17.9%) with values ranging from 3.35 to 4.94 log gc/L. The first detection of MPXV in wastewater was in a WWTP in Madrid during week 20 of 2022, coinciding with the first reported suspected cases in the city. Subsequently, MPXV DNA was detected in other areas such as Gran Canarias and Barcelona.

Detection in wastewater progressively increased, reaching 40% of the WWTPs analysed in the last week of June 2022 (week 26) when there were 130 diagnosed cases in Spain. The study demonstrated that MPXV DNA can be detected using qPCR in wastewater samples, reinforcing the utility of WBE as a non-invasive tool for monitoring emerging diseases. Although no anticipation of mpox clinical cases similar to that observed for SARS-CoV-2 in other studies was observed, the detection of MPXV DNA in wastewater in regions with a low number of diagnosed cases underscores the ability of WBE to reflect community circulation of the virus. Furthermore, this detection of MPXV in regions with low clinical prevalence suggests that there may be a higher number of affected individuals compared to what is indicated by reported clinical cases. Therefore, it can be concluded that the detection of MPXV DNA in wastewater and its correlation with reported clinical data during the 2022 outbreak demonstrate that WBE is a rapid, sensitive and cost-effective strategy for surveillance of emerging viral threats. In situations where stigma and discrimination may limit effective response during outbreaks, WBE represents a valuable tool for monitoring risks relevant to public health.

Taking into account the potential of WBE and the capacity to respond to new outbreaks, a study was conducted to evaluate and compare new virus concentration methods in wastewater. The aim was not only to improve viral detection and quantification but also to characterize them using massive sequencing and cell culture techniques. For this purpose, the effectiveness of two concentration methods was evaluated: the widely used AP method and a commercial direct capture system (TNA). These methods were assessed against various enteric viruses (HuNoV GI, HuNoV GII, HAstV, RV, and HEV), viral indicators (PMMoV and crAssphage) and SARS-CoV-2 using RT-qPCR/qPCR. Additionally, the impact of the methodologies on SARS-CoV-2 sequencing was examined.

For this purpose, 6 influent water samples from WWTPs were selected and inoculated with Porcine Epidemic Diarrhoea Virus (PEDV) and Mengovirus (MgV) as process controls for enveloped and non-enveloped viruses, respectively. The samples were concentrated in parallel using the AP (200 mL) and TNA (40 mL) methods. The genetic material extraction from the

concentrates of both methods was carried out using automated purification and DNA or RNA detection was performed using qPCR or RT-qPCR, respectively. To evaluate SARS-CoV-2 detection by RT-qPCR, different protocols targeting the N1 region and a region of the RNA-dependent RNA polymerase gene (IP4) were compared. In the context of this doctoral thesis, in addition to evaluating a new concentration system, the effectiveness of a duplex RT-qPCR kit for SARS-CoV-2 detection was also assessed, which involved the simultaneous detection of the N1 target along with PMMoV detection. Simultaneously, the effect of the concentration method on SARS-CoV-2 sequencing was evaluated using the primers from versions 3 and 4 of the ARTIC amplification protocol (Artic v3 and Artic v4) on the Illumina MiSeq platform. Additionally, given the limitations of molecular techniques to differentiate virus viability, this was evaluated using cell culture techniques after concentration with the evaluated methods. For this purpose, Murine Norovirus (MNV) was chosen as a model for HuNoV and the HM-175 strain of HAV was selected, using the RAW 264.7 and FRhK-4 cell lines, respectively. Cell cultures were infected with concentrates obtained with the AP and TNA procedures and the levels of infectious virus were quantified.

The results obtained through RT-qPCR indicated that the TNA method achieved a 100% detection rate for SARS-CoV-2 across all genomic regions used as targets, significantly outperforming the AP method. The AP method showed greater variability in its detection, with percentages ranging from 33.3% to 100% depending on the analyzed genome region. Regarding virus concentration levels, statistically significant differences were found for the IP4 target in samples concentrated with AP and for the N1-PMMoV duplex using the TNA method. For HuNoV GI, HuNoV GII, HEV and PMMoV, the TNA method yielded higher mean concentration values than the AP method. Additionally, the percentages of positive samples using the TNA method were also higher for HAstV (66%) and HEV (100%) compared to those observed with AP (50%). In the case of indicator viruses and RV, no significant differences ($p>0.05$) were found when comparing both methods. However, for the process control viruses, the recovery percentage of PEDV was higher using the AP method, while the recovery of MgV was higher with the TNA method. Regarding SARS-CoV-2 sequencing, the TNA method combined with the Artic v4 primer scheme, although it generated greater variability in the results, yielded a higher percentage of virus sequences, as well as greater sequencing depth and genome coverage of SARS-CoV-2.

Finally, the study also addressed how the TNA and AP methods influence virus infectivity. While the TNA method was effective for the concentration and detection of genetic material in molecular analyses, the reagents used in this technique (isopropanol and ethanol) caused almost complete lysis of the viruses. This was evidenced by partial and total reductions in infectivity in assays with HAV and MNV. In contrast, the AP method preserved viral infectivity in both viruses, with no significant reductions in the levels of infectious viruses detected after concentration. These

results underscore the importance of choosing the appropriate method according to the objective. On the one hand, the TNA method is more suitable for the molecular detection of SARS-CoV-2 and certain enteric viruses and indicators, showing greater sensitivity in RT-qPCR analyses and greater genomic coverage and performance in SARS-CoV-2 sequencing. On the other hand, the AP method is more appropriate for studies where preserving viral infectivity is important, as it better preserves the infectivity of the viruses present in the samples.

Overall, it can be concluded that concentration methods are critical for the surveillance of human enteric viruses and SARS-CoV-2. Consequently, the study results offer valuable insights into the impact of methods used in WBE studies, enabling the refinement of this tool for epidemiological use.

While the presence of pathogens in wastewater has been extensively studied, the scarcity of water and the necessity to reuse wastewater for various purposes, particularly agricultural irrigation and the biosolids generated in WWTPs, necessitates an evaluation of their microbiological quality. As a result, a study was conducted to focus on the risk assessment of reclaimed water and biosolids reuse by analysing different pathogens, as well as the effectiveness of treatments performed in WWTPs to reduce these pathogens.

Throughout the year 2022, influents, effluents and biosolids were sampled monthly from six WWTPs (n=216) in the Valencia Region (Spain) and the presence of various contaminants was analysed. These contaminants included viruses, *Escherichia coli* (both total and extended-spectrum beta-lactamases (ESBL)-producing strains), antimicrobial resistance genes (ARGs) and microplastics (MPs), although the latter were not the focus of this doctoral thesis. Influent samples (40 mL) were concentrated using the TNA method, while effluent samples (200 mL) were processed using the AP method. For biosolids, a 1:10 dilution was made in phosphate-buffered saline (PBS). The extraction of genetic material was performed using an automated system and the detection and quantification of enteric viruses (HuNoV GI, HuNoV GII, HEV, HAV, HAstV, and RV), respiratory viruses (SARS-CoV-2, IAV and RSV) and indicators (PMMoV and crAssphage) were carried out using RT-qPCR or qPCR. Levels of somatic coliphages were determined using plate counting techniques.

The average concentrations of enteric viruses in the influents ranged from 3.47 log cg/L for HAV to 8.55 log cg/L for RV. Regarding the indicator levels, mean concentration values of 5.95 log cg/L and 8.44 log cg/L were detected for PMMoV and crAssphage, respectively, and 6.54 log plaque forming units (pfu)/100 mL for somatic coliphages. Although the concentrations of all viruses decreased after treatment in the WWTPs, in the effluents analysed, the levels decreased by less than 2 log, suggesting significant persistence even after UV or chlorination treatments applied in the different WWTPs analysed. A significant reduction was observed only in the case of HEV,

which was not detected in any of the effluent samples analysed. In the case of biosolids, HuNoV GI, HuNoV GII, HAstV and RV showed the highest mean concentrations, with concentration values ranging from 5.37 (HuNoV GI) to 7.27 (RV) gc/L. The mean concentration levels of viruses in biosolids were very similar to those observed in the influents, highlighting the risk associated with their use as fertilizers in agricultural fields. Although the detection of viruses using RT-qPCR does not provide information on the infectivity of these pathogens, several publications have indicated the presence of infectious enteric viruses in reclaimed waters through tests for capsid integrity or cell culture. According to Regulation (EU) 2020/741 on minimum requirements for water reuse, the reductions for somatic coliphages are established at a minimum of 6 log. In this study, the mean reductions for this indicator were 1.83 log, a value far below the stipulated requirement in the regulation.

Regarding respiratory viruses, there has been ongoing debate in recent years about the possibility of their transmission through water and food. In influents, RSV was only detected during the winter months (except for one positive sample in one of the WWTPs in July 2022), while IAV was intermittently detected throughout the sampling year, with higher concentrations during the winter months. The average concentration values for these two viruses were 6.20 log cg/L and 4.57 log cg/L for IAV and RSV, respectively. All the effluents analysed were negative for these two viruses, while in biosolids, IAV was detected in 4.1% of the samples and RSV in 2.8%, indicating a very low risk of transmission considering the low prevalence and the fact that only traces of genetic material were detected. In contrast, SARS-CoV-2 was detected in 99% of the influent samples, with average concentrations of 5.27 log cg/L, and in 32% of the effluents, with average concentrations of 4.12 log cg/L. These results highlight the need for further studies to explore the risks posed to public health by the presence of respiratory viruses in water.

In conclusion, limitations have been observed in the current treatment processes carried out in the analysed WWTPs to eliminate the risk of virus transmission through treated water and biosolids used in agriculture. To comprehensively address the risks associated with reclaimed water reuse, it is essential to improve water and biosolid treatment technologies, as well as to adopt stricter policies to protect public health and promote sustainable practices. This work underscores the importance of continuing to research and improve wastewater treatment and surveillance systems to minimize the risks associated with their reuse in agriculture and other purposes.

Detecting viruses in effluents and biosolids implies the potential entry of these pathogens into the food chain and affecting both the population and the environment. Therefore, it is important to understand the stability of viruses in water. While there is abundant information about the stability of enteric viruses in water, studies with other viruses that may potentially be found in water are still scarce and water cannot be ruled out as a secondary transmission route. Thus, within the

framework of this doctoral thesis, the stability of enteric, respiratory and emerging viruses in effluents and drinking water was investigated.

These assays were conducted under controlled temperature conditions using reference water (RW), prepared with Milli-Q water and 7 nM NaCl, tap water (DW) from the municipal supply and effluent from a WWTP (EW). Initially, the physicochemical parameters of these samples were characterized and they were artificially inoculated with suspensions of MNV, HAV, IAV (H3N2), human coronavirus 229E (HCoV-229E), and vaccinia virus (VACV), the latter two serving as models for SARS-CoV-2 and MPXV, respectively. The samples were kept at room temperature (25°C) and refrigeration (4°C) and aliquots were collected in triplicate at different time intervals (0h, 24h, 1, 2, 4, 6, 8, 10, 12, and 14 weeks). The infectivity study was initially conducted using cell cultures, employing the corresponding cell lines for each virus included in the study. The reduction in virus levels was calculated as $\log_{10} (N_x/N_0)$, where N_0 is the concentration of infectious virus at time 0 and N_x is the concentration of infectious virus for each of the analysed times. The obtained results were modelled with linear regression over time for each temperature condition and water type and each of the decay constants, k, was compared with its 95% confidence intervals to determine if there were significant differences in the inactivation rate of each virus under the experimental conditions tested.

Finally, although cell culture is one of the most widely used techniques for assessing virus infectivity, this study also conducted viability PCR assays using the intercalating compounds propidium monoazide (PMAxx™) and platinum (IV) chloride ($PtCl_4$). The results were compared with those obtained through cell culture. For these assays, samples from DW and EW were selected at 0h, 1, 4, 8, 12, and 14 weeks at 25°C.

After cell culture analyses, it was observed that enteric viruses survived longer than non-enteric viruses, consistent with previous findings. The infectivity of MNV experienced a significant decrease after 3 weeks at 25°C, while at 4°C, it only decreased after 12 weeks. At 25°C, there was a gradual decrease in the infectivity of HAV; however, at 4°C, infectious viruses were still detected even after 14 weeks. Based on these data, decay constants were calculated, which were 0.056, 0.228 and 0.155 log/day for HAV in reference water, drinking water and effluent at 25°C, respectively. These same rates were 0.057, 0.155 and 0.083 in the same water matrices but stored at 4°C. For MNV, the decay rates were established at 0.029, 0.039 and 0.040 log/day for reference water, drinking water and effluent water at 4°C, respectively, with no significant differences observed between the k values for different water types. These rates were 0.187, 0.168 and 0.230 in the same water matrices but stored at 25°C.

In the experiments conducted with non-enteric viruses, HCoV-229E, IAV and VACV, the results showed complete inactivation between 1 and 2 weeks in DW and EW at 25°C, with more

prolonged stability observed at 4°C in reference water. For IAV, detection occurred after 84 days (12 weeks) in effluents and after 98 days (14 weeks) in reference water at 4°C. Overall, comparing the three viruses, the k values established that IAV was more stable than HCoV-229E and the latter was more stable than VACV. Additionally, all three were more stable in reference water than in drinking water or effluents, which could be attributed to the presence of the microbiota in these waters.

The assays based on molecular techniques were conducted using (RT)-qPCR to determine the persistence of the virus's genetic material and viability PCR to estimate the stability of infectious viruses through the integrity of viral capsids. These nucleic acid amplification techniques rely on the integrity of viral capsids to provide an approximation of virus infectivity. This approach primarily uses reagents that intercalate with DNA or RNA and only penetrate virions with compromised capsids. Although these methodologies have demonstrated effectiveness under extreme pressure and temperature conditions, various studies indicate that these approaches are not robust enough to reliably associate detection with infectivity. Therefore, within the scope of this doctoral thesis, we aimed to evaluate this approach for the non-enteric viruses under study, verifying its efficacy against infectivity measured through cell culture.

The results obtained, overall, showed that neither PMAxx nor $PtCl_4$ managed to decrease the signal of the genetic material belonging to non-infectious viruses. Reductions in the concentrations of IAV, HCoV-229E and VACV, measured by molecular techniques in the two types of water, were not reflected in the same way as with cell culture. While these viruses were completely inactivated after 1-2 weeks of storage at 25°C, high concentrations of genetic material were still detected using the molecular techniques tested. For example, VACV in drinking water remained infectious for only 1 week; however, its genetic material was detected even at 14 weeks, regardless of the use of viability reagents. In the case of IAV, HCoV-229 and VACV in effluents, the signal of the genetic material was completely reduced only after 14 weeks of storage, although the infectivity of the viruses was eliminated after 1 (HCoV-229E and VACV) and 2 weeks of storage (IAV).

This work has allowed us to explore the factors influencing the stability of enteric and respiratory viruses in aquatic environments. Thus, it was possible to demonstrate that the nature of the viruses, the matrix and the conditions in which they are found (type of water, temperature) affect their stability. Further studies are needed to determine if the viruses present in water can affect the health of the population and to accurately advise on the quality and safety of the water and food. Furthermore, given the low efficiency of the tested reagents, further research is required on the use of these techniques to assess capsid integrity, which would allow inferring the viability of viruses in such samples.

The studies conducted in this thesis contribute to the understanding of viruses, their presence, stability and circulation in various aquatic environments, as well as to the improvement of methodologies for detecting viruses in wastewater and reclaimed water.

SUMMARY (SPANISH VERSION)

El agua es un elemento esencial para la vida, tanto como parte de nuestra alimentación, como para uso doméstico, en agricultura, acuicultura, en la preparación de alimentos y en la industria. Por lo tanto, es crucial asegurar que su abastecimiento sea suficiente, seguro y accesible para toda la población. Además, el agua puede actuar como vehículo en la diseminación de microorganismos patógenos, entre los que se encuentran los virus entéricos, como norovirus humano (HuNoV) o el virus de la hepatitis A (HAV), que pueden entrar en la cadena alimentaria contaminando instalaciones, embalajes y el propio alimento. En agricultura, los virus entéricos pueden llegar a los alimentos a través del riego con agua contaminada o por el uso de lodos de depuradora como fertilizantes. Ambas fuentes de posible contaminación se generan en las estaciones depuradoras de aguas residuales (EDARs). En acuicultura, si el agua utilizada para cultivar y cosechar moluscos bivalvos está contaminada, representa un riesgo significativo para la salud de los consumidores debido a la naturaleza filtradora de estos animales (bioacumulación) y a los mínimos procesos de cocción que reciben antes de su consumo.

Se ha demostrado ampliamente el papel que juega el agua en la diseminación de los virus entéricos, aquellos cuya principal ruta de transmisión es la ruta fecal-oral, y el riesgo que implica su presencia en ella. Sin embargo, en los últimos años, se ha observado que los virus respiratorios, los cuales también se excretan en las heces y otros fluidos de humanos y animales infectados, pueden detectarse y propagarse a través del agua. La posibilidad de transmisión de estos virus a través de esta vía es un tema muy controvertido y está siendo estudiado en la actualidad. Además, dado el contacto que las masas de aguas tienen tanto con humanos como con animales, se ha observado la capacidad del agua para actuar como agente de diseminación de virus con capacidad zoonótica, lo que representa también un riesgo muy importante para la salud. De hecho, esta interacción entre humanos, animales y el medio ambiente ha tomado una mayor relevancia en los últimos años, evidenciando la necesidad de estudiar los patógenos desde la interacción entre estos tres pilares básicos, enfoque que se conoce como la aproximación “Una sola salud”. Además del papel que el agua tiene en la transmisión de patógenos, otro factor a considerar es la capacidad de los virus de permanecer infecciosos en el agua. Esta estabilidad de los virus depende de varios factores como el tipo de virus, la matriz, la temperatura, el tiempo y el pH.

El ciclo natural del agua es un proceso fundamental en la naturaleza en el que todos los cuerpos de agua implicados, ya sean superficiales como ríos, lagos y mares, o subterráneos, o incluso el hielo, están interconectados y son susceptibles a la contaminación por virus. Esta contaminación puede ser causada por diversas fuentes, incluidas descargas industriales, agrícolas y urbanas, así como por una mala gestión de residuos, afectando a humanos, animales y al medio ambiente.

Una de las fuentes de contaminación más importantes es el ciclo urbano del agua, por ello tiene un papel muy importante desde la perspectiva de la calidad y la seguridad hídrica. Este ciclo urbano incluye varias etapas, desde la captación de agua utilizada por parte de diferentes núcleos de población e industrias, hasta su tratamiento en EDARs para la eliminación de contaminantes. La calidad de los efluentes y lodos generados en estas plantas de tratamiento es crucial, ya que puede afectar directamente a la salud pública y al medio ambiente. Los lodos generados durante el tratamiento de estas aguas residuales pueden ser utilizados como fertilizante en agricultura mientras que el agua tratada puede ser utilizada para riego agrícola y fines industriales, o recircularse a diferentes cuerpos de agua, como ríos y lagos. Desde estos puntos se capta agua para las estaciones de tratamiento de agua potable (ETAPs), donde se lleva a cabo su potabilización y distribución a la población. Después de su uso, el agua se convierte en agua residual y regresa de nuevo a las EDARs para continuar el ciclo urbano del agua. Como resultado, una gestión inadecuada de las aguas residuales urbanas, industriales y agrícolas implica potenciales riesgos medioambientales y de salud.

Por otro lado, la escasez de agua y las sequías ejercen una presión adicional sobre los recursos hídricos globales, una situación que empeorará en los próximos años debido al cambio climático y al crecimiento de la población. En respuesta a estos desafíos, se están fomentando políticas de reutilización del agua, que implica la utilización de efluentes de EDARs para usos no potables como industria y riego agrícola. Esta estrategia ayuda a reducir la demanda de agua de fuentes naturales como, por ejemplo, ríos y lagos. Si bien las aguas residuales y lodos son utilizados globalmente, una parte significativa se emplea de manera no controlada y/o sin tratamiento adecuado, careciendo de controles suficientes para salvaguardar la salud humana y ambiental. Cuando se maneja correctamente, siguiendo parámetros como los definidos en el Reglamento (UE) 2020/741, la utilización de efluentes de EDARs y lodos puede generar numerosas ventajas, como una producción de alimentos más segura, una mayor resiliencia a la escasez de agua y nutrientes y una economía circular mejorada.

A pesar de que las aguas residuales no tratadas o tratadas inadecuadamente son la fuente principal de la propagación de microorganismos patógenos en el ambiente, la presencia de virus en aguas residuales también puede suponer una oportunidad, como es la epidemiología basada en las aguas residuales (WBE, por sus siglas en inglés Wastewater-Based Epidemiology). La epidemiología se define como el estudio de la incidencia, distribución y control de las enfermedades en las poblaciones. Existen varias herramientas que se basan en sistemas de monitorización pasivos, sin embargo, pueden presentar dificultades prácticas y económicas, como la limitación de las pruebas clínicas únicamente a pacientes sintomáticos, la falta de recursos y atención médica en determinados países, o la sobrecarga de las capacidades analíticas en epidemias graves y pandemias. Todos estos factores contribuyen a la estimación potencialmente inexacta de la propagación de las

enfermedades, lo que hace necesario el desarrollo de nuevos enfoques. Estas herramientas novedosas de vigilancia han de ser flexibles, económicas, escalables y capaces de proporcionar datos objetivos y completos en tiempo real para complementar las debilidades de los enfoques tradicionales. La WBE es una herramienta que proporciona, mediante el análisis de agentes excretados en heces, orina y otros fluidos que van a parar a las aguas residuales, evaluaciones en tiempo real, completas y objetivas del estado de salud pública y ambiental, con el fin de obtener información sobre la circulación de diferentes agentes en la población, el estado de salud de la comunidad y los factores de riesgo asociados con el agente elegido, independientemente de los sesgos causados por factores socioeconómicos o la falta de síntomas en la población afectada.

Desde el origen de la WBE en el siglo XIX, donde se vincularon los casos de cólera ocurridos en Londres con el agua contaminada utilizada para beber y fines domésticos, se han detectado diferentes contaminantes que podrían estar circulando en la comunidad: desde productos químicos, como drogas, pesticidas, hormonas y medicamentos, hasta microorganismos patógenos, como bacterias y virus. En los últimos años, la variedad de patógenos que pueden detectarse se ha ampliado y los virus han adquirido cada vez más importancia. Por ejemplo, la WBE se utiliza en el programa de erradicación de la poliomielitis de la Organización Mundial de la Salud, o para la detección de otros virus entéricos como el HAV y el virus de la hepatitis E (HEV), HuNoV, enterovirus, y rotavirus (RV). Además, durante la pandemia de la enfermedad producida por coronavirus (COVID-19, del inglés Coronavirus Disease), la detección del coronavirus de tipo 2 causante del síndrome respiratorio agudo (SARS-CoV-2, del inglés Severe Acute Respiratory Syndrome coronavirus 2) ha demostrado ser una estrategia eficaz de alerta temprana del virus y sus variantes, así como para detectar cambios en su incidencia. El principio biológico detrás de esta herramienta es el hecho de que los individuos infectados pueden excretar los virus en las heces antes de desarrollar síntomas y, por lo tanto, antes de buscar atención médica. Un pico de incidencia o nuevas variantes de un agente infeccioso pueden detectarse antes con WBE que con estrategias epidemiológicas tradicionales.

La detección de virus en aguas requiere generalmente de una etapa de concentración previa a la detección de estos patógenos. Esto se debe a los bajos niveles de virus que se encuentran en las aguas y a las técnicas que se utilizan para su detección. Estos métodos de concentración tradicionalmente se basan en métodos de precipitación, adsorción, filtración, ultrafiltración y ultracentrifugación. Sin embargo, a pesar del auge de la WBE en los últimos años, todavía no existen métodos estandarizados y validados para virus, y la aparición de nuevos métodos de concentración hacen necesario ampliar los estudios comparativos en estas técnicas. Para la detección de virus en aguas, los métodos de cultivo celular se han aplicado tradicionalmente para el aislamiento y caracterización de virus, como el virus de la polio. Sin embargo, con el desarrollo e implementación de métodos moleculares, como la reacción en cadena de la polimerasa (PCR), la

PCR con transcriptasa inversa (RT-PCR), la PCR cuantitativa (qPCR) y la PCR digital (ddPCR), el número de procedimientos y virus analizados en aguas ha crecido exponencialmente.

Por otra parte, las técnicas de secuenciación masiva han demostrado ser una herramienta muy útil para evaluar fluctuaciones temporales y diversidad de virus en muestras de aguas residuales, así como para detectar cambios en su evolución y la aparición de nuevas cepas virales. La mayoría de las técnicas específicas de PCR no pueden discriminar entre la amplia variedad de linajes circulantes porque solamente pueden detectar una o unas pocas mutaciones de un mismo virus. Mientras que las nuevas variantes de virus pueden detectarse y cuantificarse utilizando técnicas cuantitativas como PCR/RT-PCR (qPCR/RT-qPCR) simples y dúplex, el uso de técnicas de secuenciación ha demostrado ser clave para la monitorización de variantes, linajes y sublinajes en aguas residuales durante la COVID-19.

Para un enfoque integral de WBE, la fase final del proceso incluye la interpretación de los datos relacionando la dinámica de las aguas residuales, la relación espacio-temporal con la población y los datos clínicos reportados en el área estudiada. De esta forma, se han demostrado numerosas correlaciones entre los datos de WBE y la incidencia y el número de hospitalizaciones de pacientes en múltiples entornos. La vigilancia de virus entéricos, respiratorios y emergentes a través de la WBE es una estrategia muy útil para comprender mejor la aparición y la dinámica de estos patógenos en una población a través del estudio del agua. Sin embargo, un aspecto importante a tener en cuenta es que los niveles detectados de virus en aguas residuales pueden verse afectados por factores independientes del tratamiento de la muestra en el laboratorio. Restricciones de movilidad y cuarentenas, el turismo y festividades, o el efecto de dilución por la lluvia, pueden afectar a la concentración estimada en este tipo de estudios. La normalización de datos permite comparaciones entre diferentes muestras de la misma EDAR a lo largo del tiempo, o incluso entre diferentes poblaciones. Esta estrategia se lleva a cabo normalizando los datos con parámetros físico-químicos del agua o algunos virus como el virus del moteado suave del pimiento (PMMoV, de sus siglas en inglés Pepper Mild Mottle virus) o el bacteriófago *crAssphage*, conocidos por ser indicadores de presencia de heces humanas.

En este contexto, en esta tesis doctoral se exploró el potencial de la WBE para el estudio de virus respiratorios y entéricos en aguas residuales. Asimismo, se evaluó la eficacia de esta técnica para responder de forma rápida y eficiente a la aparición de virus emergentes y nuevas crisis sanitarias. Durante estas investigaciones, también se evaluaron y validaron nuevos métodos para el análisis y detección de virus en aguas residuales, con el fin de mejorar las capacidades de detección y caracterización de patógenos virales. Todos estos análisis permitieron estudiar la presencia de virus patógenos en el ciclo urbano del agua y evaluar la capacidad de las EDARs para eliminar contaminantes virales. Los resultados evidenciaron la presencia de virus en las aguas efluentes, lo

que motivó el estudio de la estabilidad de los virus en diferentes microcosmos y condiciones de temperatura.

Durante la pandemia causada por el SARS-CoV-2, la WBE sufrió un auge a nivel mundial, demostrando su capacidad como herramienta epidemiológica de alerta temprana, incluso posibilitando la detección de nuevas variantes introducidas en la población. En la Comunidad Valenciana, esta herramienta fue ampliamente utilizada, sin embargo, no se disponía de estudios que demostrasen la utilidad de la WBE para el control epidemiológico de otros virus patógenos de humanos. Por ello, en el marco de esta tesis, se evaluó la prevalencia y los patrones temporales de diferentes virus entéricos, respiratorios e indicadores virales de contaminación fecal en aguas residuales de nuestra región. Para ello, semanalmente se recogieron muestras de agua residual a la entrada de una EDAR urbana de la provincia de Valencia, desde octubre de 2021 hasta febrero de 2023. Las muestras se transportaron al laboratorio, y se concentraron mediante el método de precipitación con aluminio (AP). La extracción del material genético se llevó a cabo de manera automatizada y se realizó la detección de diferentes virus mediante RT-qPCR y mediante qPCR en caso del indicador crAssphage. De esta manera, se detectaron y cuantificaron virus entéricos (HuNoV genogrupo 1 (GI) y GII, astrovirus humano (HAstV), RV, HAV y HEV), virus respiratorios (SARS-CoV-2, virus de la influenza A (IAV) y virus respiratorio sincitial (RSV)) y virus indicadores (crAssphage y PMMoV). En paralelo, se realizó la detección de colífagos somáticos, como tercer tipo de virus indicador, mediante la técnica de recuento en placa. Para la detección y cuantificación de SARS-CoV-2 se utilizó la región del gen de la fosfoproteína de la nucleocápside (N1) como diana. También se evaluó la evolución del SARS-CoV-2 durante el periodo estudiado detectando sus variantes mediante RT-qPCR dúplex y secuenciación masiva. Para la detección mediante RT-qPCR de las variantes de preocupación (VoCs, por sus siglas en inglés Variants of Concern), se utilizaron cinco reacciones dúplex diferentes, revelando así la prevalencia de las variantes Alpha (S:69/70del), Delta (S:157/158del), Omicron BA.1 (S:214insEPE, S:69/70del) y Omicron BA.2 (S:25/27del). Para la secuenciación, se seleccionó una muestra mensual y se utilizaron los esquemas de cebadores Artic v3 (octubre 2021-enero 2022) y Artic v4 (febrero 2022-febrero 2023) para la amplificación de la región que codifica para la espícula y se realizó la secuenciación utilizando el sistema de nanoporos MinION Mk1C (Oxford Nanopore Technologies). Las lecturas obtenidas se analizaron con el software Freyja. Finalmente, la evaluación de la prevalencia y los patrones temporales de los virus humanos entéricos y respiratorios en muestras de aguas residuales, así como la identificación de VOCs del SARS-CoV-2, permitió explorar la correlación de nuestros hallazgos con los registros de salud pública, evaluando también diversos indicadores para la normalización de datos.

Los resultados obtenidos sobre la prevalencia de virus entéricos en aguas residuales están en línea con la literatura existente, mostrando una prevalencia de aproximadamente el 100 % para

RV, HAstV y HuNoV GI y GII, mientras que la prevalencia de HAV y HEV fueron de 3,77% y 22,64%, respectivamente. Las concentraciones medias se situaron entre 7,95 log copias genómicas (cg)/L para RV y 3,05 log cg/L en el caso de HAV. En cuanto a los virus respiratorios, SARS-CoV-2 se detectó en el 100% de las muestras de agua residual analizadas, mientras que IAV y RSV únicamente se detectaron en un 39,13% y un 31,88% de las muestras, respetivamente. El valor medio de las concentraciones de estos virus se situó en 5,46 log cg/L para SARS-COV-2, 6,42 log cg/L para IAV y 3,88 log cg/L para RSV.

A pesar de la estacionalidad reportada en las infecciones causadas por algunos virus entéricos, en este estudio no se observó una estacionalidad marcada para ninguno de los virus entéricos estudiados, salvo un ligero aumento durante los meses de invierno en el caso de RV. En el caso de los virus respiratorios, RSV mostró un incremento en invierno, mientras que en el caso de IAV este incremento invernal fue moderado, siendo mucho mayor el pico en incidencia observado durante los meses de primavera. Los resultados de concentración de SARS-CoV-2 en aguas residuales mostraron un aumento coincidiendo con la aparición de nuevas variantes, las cuales alineaban con un incremento en la incidencia poblacional. La comparación de los resultados obtenidos mediante RT-qPCR dúplex para las variantes de SARS-CoV-2 y la secuenciación del genoma mostró que los ensayos de RT-qPCR dúplex lograron resultados significativamente más rápidos, manteniendo la sensibilidad y especificidad adecuada. Aunque la detección de nuevas variantes de SARS-CoV-2 en aguas residuales mediante métodos de secuenciación generalmente necesita concentraciones más altas de partículas virales que los métodos basados en PCR, la identificación de variantes se realizó de manera eficiente incluso en muestras con concentraciones bajas. Cabe destacar que ambas técnicas, RT-qPCR dúplex y la secuenciación, mostraron de forma fiable cómo la transición de la variante Delta a Omicron ocurrió de manera muy rápida, en sólo dos semanas, durante diciembre de 2021 en España.

La correlación con datos clínicos es otro componente clave de la WBE, ya que se debe establecer la relación entre las concentraciones virales medidas en aguas residuales y los casos clínicos reportados de la enfermedad. En esta tesis doctoral se observaron correlaciones estadísticamente significativas entre los casos clínicos confirmados y los datos de cuantificación correspondientes en las aguas residuales para IAV y RSV, lo que demostró el valor de la vigilancia de aguas residuales para monitorizar enfermedades infecciosas en la población. Las correlaciones sin factor de normalización más robustas se produjeron entre la concentración de virus y el número de casos clínicos la semana posterior a la toma de muestra para SARS-CoV-2 e IAV, y en la misma semana de muestreo en el caso de RSV. Sin embargo, en el caso de los datos clínicos para SARS-CoV-2, éstos dejaron de reportarse a partir de abril de 2022, por lo que estas correlaciones se realizaron con los datos anteriores a esa fecha, ya que fueron los que mejores valores mostraron. Una vez normalizados los datos con diferentes parámetros microbiológicos y físico-químicos, se

observó que las correlaciones eran más fuertes cuando se tomaba el número de casos clínicos de la semana posterior al muestreo junto con el caudal de entrada (m^3/día) a la EDAR, salvo en el caso de RSV en el que los casos clínicos en la tercera semana desde el muestreo presentaron una correlación más robusta. Además, se calculó el número mínimo de casos clínicos de IAV y RSV para obtener muestras positivas de agua residual, resultando en entre 110 y 205 casos clínicos para IAV y entre 40 y 120 para RSV. Cuando los casos clínicos de IAV y RSV superaban 205 y 210 casos, respetivamente, el 100% de las muestras de agua residual resultaban positivas para estos virus.

La implementación de las técnicas de WBE para el análisis epidemiológico del SARS-CoV-2 ha aumentado exponencialmente el número de grupos de investigación, laboratorios y empresas con capacidad de monitorizar virus en aguas residuales, además de contar con el apoyo de instituciones gubernamentales para su implementación. Por primera vez, se disponen de los mecanismos y herramientas necesarios para el control de posibles epidemias y pandemias a través del estudio de las aguas residuales. Sin embargo, todavía faltaba demostrar si este sistema de vigilancia podía implementarse de una forma eficiente en futuras crisis sanitarias.

En mayo de 2022 cuando surgió la oportunidad de demostrar la validez de esta herramienta, debido a un brote de mpox (anteriormente llamada viruela del mono) causado por el virus de la viruela del mono (MPXV) en áreas no endémicas, llegando a países como España, entre otros. El virus se transmite principalmente a través del contacto con las lesiones dérmicas de personas infectadas, con gotas de secreciones respiratorias y fómites, pero también se ha observado que puede excretarse en fluidos corporales como orina, semen y heces. Por ello, se decidió estudiar este virus a través del análisis de aguas residuales mediante las técnicas empleadas para el análisis de SARS-CoV-2 utilizadas de forma rutinaria en la red de vigilancia de aguas residuales en España (VATAR-COVID).

Inicialmente, se validó el procedimiento de concentración AP, inoculando una cepa de MPXV inactivada en agua residuales negativas para el virus. Los resultados mostraron que mediante este procedimiento se obtenían recuperaciones medias del MPXV del 31,5% ± 15,9%, demostrando la validez del método para el estudio de este patógeno. Tras estos ensayos, se analizaron 312 muestras de 24 EDARs distribuidas por el territorio español entre el 9 de mayo y el 4 de agosto de 2022. Las partículas virales se concentraron utilizando el método AP, seguido de la extracción de ácidos nucleicos de forma automatizada utilizando partículas paramagnéticas. Seguidamente, se detectó y cuantificó el ADN de MPXV mediante qPCR. El material genético de este virus se detectó en 56 muestras de aguas residuales (17,9%) con valores que oscilaron entre 3,35 y 4,94 log cg/L. La primera detección de MPXV en agua residual fue en una EDAR de Madrid durante la semana 20 de 2022, coincidiendo con los primeros casos sospechosos reportados en la

ciudad. Posteriormente, se detectó el ADN de MPXV en otras áreas como Gran Canaria y Barcelona. La detección en aguas residuales aumentó progresivamente, alcanzando el 40% de las EDARS analizadas en la última semana de junio de 2022 (semana 26) cuando en España había 130 casos diagnosticados.

El estudio demostró que el ADN de MPXV puede ser detectado mediante qPCR en muestras de aguas residuales, lo que refuerza la utilidad de la WBE como una herramienta no invasiva para el monitoreo de enfermedades emergentes. A pesar de que no se observó una anticipación a la aparición de casos clínicos de mpox similar a la observada para SARS-CoV-2 en otros estudios, la detección de ADN de MPXV en aguas residuales en regiones con bajo número de casos diagnosticados subraya la capacidad de la WBE para reflejar la circulación comunitaria del virus. Además, esta detección de MPXV en regiones con baja prevalencia clínica sugiere que podría haber un mayor número de personas afectadas en comparación a lo que indican los casos clínicos reportados. Por lo tanto, se puede concluir que la detección del ADN de MPXV en aguas residuales y su correlación los datos clínicos reportados durante el brote del 2022 demuestran que la WBE es una estrategia rápida, sensible y rentable para la vigilancia de amenazas virales emergentes. En situaciones donde el estigma y la discriminación pueden limitar la capacidad de respuesta efectiva durante los brotes, la WBE representa una herramienta valiosa para la monitorización de riesgos relevantes para la salud pública.

Teniendo en cuenta el potencial de WBE y la capacidad de respuesta a nuevos brotes, se realizó un estudio para evaluar y comparar nuevos métodos de concentración de virus en aguas residuales, no sólo para mejorar la detección y cuantificación viral, sino también para caracterizarlos mediante técnicas de secuenciación masiva y cultivo celular. Para tal fin, se evaluó la eficacia de dos métodos de concentración, la precipitación con cloruro de aluminio (AP), ampliamente utilizada, y un sistema comercial de captura directa (TNA) frente a diversos virus entéricos (HuNoV GI, HuNoV GII, HAstrV, RV y HEV), indicadores virales (PMMoV y crAssphage) y SARS-CoV-2 mediante RT-qPCR/qPCR, así como el impacto de las metodologías en la secuenciación de SARS-CoV-2.

Para ello, se escogieron 6 muestras de agua de entrada de EDAR y se inocularon con el virus de la diarrea epidémica porcina (PEDV) y mengovirus (MgV), como controles de proceso para virus envueltos y no envueltos, respectivamente. Las muestras se concentraron en paralelo con los métodos AP (200 mL) y TNA (40 mL). La extracción del material genético de los concentrados de ambos métodos se llevó a cabo utilizando la purificación automatizada y se realizó la detección del ADN o ARN mediante qPCR o RT-qPCR, respectivamente. Para evaluar la detección de SARS-CoV-2 mediante RT-qPCR se compararon diferentes protocolos que tenían como diana la región N1 y una región del gen de la ARN polimerasa dependiente de ARN (IP4). En el marco de esta

tesis doctoral, además de evaluar un nuevo sistema de concentración, también se evaluó la eficacia de un kit de RT-qPCR dúplex para la detección de SARS-CoV-2, que implicaba la detección conjunta de la diana N1 junto con la detección de PMMoV. Paralelamente, se evaluó el efecto del método de concentración en la secuenciación de SARS-CoV-2 utilizando los cebadores de las versiones 3 y 4 del protocolo de amplificación ARTIC (Artic v3 y Artic v4) mediante la plataforma Illumina MiSeq. Además, dadas las limitaciones que presentan las técnicas moleculares para diferenciar la viabilidad de los virus, ésta se evaluó mediante técnicas de cultivo celular tras la concentración con los métodos evaluados. Con este objetivo, se escogió el norovirus murino (MNV) como modelo de los HuNoV, y la cepa HM-175 de HAV, utilizando las líneas celulares RAW 264.7 y FRhK-4, respectivamente. A partir de los concentrados obtenidos con los procedimientos AP y TNA, se infectaron los cultivos celulares y se cuantificaron los niveles de virus infecciosos.

Los resultados obtenidos mediante RT-qPCR indicaron que mediante el método TNA se logró detectar SARS-CoV-2 con una efectividad del 100% en todas las regiones genómicas utilizadas como dianas, superando significativamente al método AP, que mostró mayor variabilidad en su detección, con porcentajes entre el 33,3% y el 100%, dependiendo de la región del genoma analizada. En cuanto a los niveles en la concentración del virus, se encontraron diferencias estadísticamente significativas para la diana IP4 en muestras concentradas con AP y para la dúplex N1-PMMoV utilizando el método TNA. Para HuNoV GI, HuNoV GII, HEV y PMMoV, el método TNA obtuvo valores medios de concentración más altos que el método AP. Además, los porcentajes de muestras positivas en el método TNA también fueron superiores para HAstrV (66%) y HEV (100%) en comparación con los observados en AP (50%). En el caso de los virus indicadores y RV no se encontraron diferencias significativas ($p>0,05$) al comparar ambos métodos. Sin embargo, en los virus utilizados como controles de proceso, el porcentaje de recuperación de PEDV fue mayor utilizando el método AP y la recuperación de MgV fue más alta con el método TNA. En cuanto a la secuenciación de SARS-CoV-2, el método TNA combinado con el esquema de cebadores Artic v4, aunque generaba una mayor variabilidad en los resultados, el porcentaje de secuencias del virus era mayor, así como una mayor profundidad de secuenciación y cobertura del genoma de SARS-CoV-2.

Por último, el análisis también abordó cómo los métodos TNA y AP influyen en la infectividad de los virus. Aunque el método TNA fue efectivo para la concentración y detección del material genético en los análisis moleculares, los reactivos que se utilizaron en esta técnica (isopropanol y etanol) provocaron una lisis casi completa de los virus. Esto se evidenció en la reducción parcial y total de la infectividad en los ensayos con HAV y MNV, respectivamente. En contraste, el método AP, conservó la infectividad viral en ambos virus, sin reducciones significativas en los niveles de virus infecciosos detectados tras la concentración. Estos resultados subrayan la importancia de elegir el método adecuado según el objetivo planteado. Por un lado, el

método TNA es más adecuado para la detección molecular de SARS-CoV-2 y de determinados virus entéricos e indicadores, mostrando una mayor sensibilidad en los análisis mediante RT-qPCR y una mayor cobertura genómica y un mayor rendimiento en la secuenciación de SARS-CoV-2. Por otra parte, el método AP es más apropiado para estudios donde es importante conservar la infectividad viral, ya que preserva mejor la infectividad de los virus presentes en las muestras.

Con todo ello, se puede concluir que los métodos de concentración son críticos para la vigilancia de virus humanos entéricos y SARS-CoV-2. Así, los resultados del estudio proporcionan nueva información sobre los efectos de los métodos utilizados en estudios de WBE, permitiendo mejorar esta herramienta para su uso en epidemiología.

La presencia de patógenos en aguas residuales ha sido ampliamente estudiada. Sin embargo, dada la escasez hídrica y la necesidad de reutilizar las aguas residuales para diferentes fines, principalmente el riego de cultivos agrícolas, y de los biosólidos generados en las EDARs, usados en su gran mayoría como fertilizantes, hace necesario evaluar la calidad microbiológica de los mismos. Por ello, se realizó un estudio centrado en la evaluación del riesgo que comprende esta estrategia de reutilización de aguas regeneradas y biosólidos mediante el análisis de diferentes patógenos, así como la capacidad de reducción de estos patógenos tras los tratamientos realizados en las EDARs.

Durante el año 2022 se muestrearon mensualmente influentes, efluentes y biosólidos de seis EDARs (n=216) de la Comunidad Valenciana (España) y se analizó la presencia de diferentes contaminantes: virus, *Escherichia coli* (totales y productoras de 'betalactamasas de espectro extendido' (BLEE)), genes de resistencia a antimicrobianos (ARGs) y microplásticos (MPs), estos últimos no siendo objeto de estudio de esta tesis doctoral. Los influentes (40 mL) se concentraron utilizando el método TNA, mientras que los efluentes (200 mL) se procesaron mediante el método AP. Para los biosólidos se realizó una dilución 1:10 en solución salina tamponada con fosfato (PBS, del inglés Phosphate Buffered Saline). La extracción del material genético se realizó utilizando un sistema automatizado y la detección y cuantificación de virus entéricos (HuNoV GI, HuNoV GII, HEV, HAV, HAstrV y RV), respiratorios (SARS-CoV-2, IAV y RSV) e indicadores (PMMoV y crAssphage) se realizó mediante RT-qPCR o qPCR. Los niveles de colífagos somáticos se llevó a cabo mediante técnicas de recuento en placa.

Las concentraciones promedio de virus entéricos en los influentes oscilaron entre 3,47 log cg/L de HAV hasta los 8,55 log cg/L en el caso de RV. En relación a los niveles de los indicadores, se detectaron unos valores de concentración medios de 5,95 log cg/L y 8,44 log cg/L para PMMoV y crAssphage, respectivamente, y de 6,54 log de unidades formadoras de calvas (ufc)/100 mL en el caso de los colífagos somáticos. Aunque las concentraciones de todos los virus se redujeron tras el tratamiento de las EDARs, en los efluentes analizados los niveles se redujeron en menos de 2

órdenes logarítmicos, lo que sugiere una persistencia relevante, incluso después de los tratamientos de UV o cloración aplicados en las diferentes EDARs analizadas. Únicamente se produjo una reducción significativa en el caso de HEV, el cual no se detectó en ninguna de las muestras de efluentes analizadas. En el caso de los biosólidos, HuNoV GI, HuNoV GII, HAstV y RV mostraron las concentraciones medias más altas, con valores de concentración entre 5,37 (HuNoV GI) y 7,27 (RV) log (gc)/L. Los niveles medios de las concentraciones de virus fueron muy similares a los observados en los influentes, resaltando el riesgo que supondría utilizarlos como fertilizantes en campos de cultivo agrícolas.

A pesar de que la detección de virus mediante RT-qPCR no informa sobre la infectividad de estos patógenos, varias publicaciones han señalado la presencia de virus entéricos infecciosos en aguas regeneradas mediante ensayos de integridad de la cápside o cultivo celular. Según el Reglamento (UE) 2020/741 sobre los requisitos mínimos para reutilización del agua, las reducciones logarítmicas para colífagos somáticos están establecidas en un mínimo de 6 órdenes logarítmicos. En este estudio, las reducciones medias para este indicador fueron de 1,83 órdenes logarítmicos, valor muy alejado del estipulado en el reglamento.

Respecto a los virus respiratorios, en los últimos años se ha discutido si su transmisión mediante agua y alimentos es posible. En influentes, RSV únicamente se detectó en los meses de invierno (exceptuando una muestra positiva en una de las EDARs en julio de 2022), mientras que IAV se detectó de forma intermitente durante el año de muestreo, con mayores concentraciones durante los meses de invierno. Los valores promedio de las concentraciones de estos dos virus fueron 6,20 log cg/L y 4,57 log cg/L para IAV y RSV, respectivamente. Todos los efluentes analizados fueron negativos para estos dos virus, mientras que en biosólidos IAV se detectó en un 4,1% de las muestras y RSV en un 2,8%, por lo que el riesgo de transmisión, teniendo en cuenta la baja prevalencia y el hecho de que sólo se detectan trazas de material genético, sería muy bajo. Por el contrario, SARS-CoV-2 se detectó en el 99% de las muestras de influentes, con concentraciones medias de 5,27 log cg/L, y en el 32% de los efluentes, con concentraciones medias de 4,12 log cg/L. Estos resultados evidencian la necesidad de realizar más estudios con la finalidad de indagar más sobre los riesgos que supone para la salud pública la presencia de virus respiratorios en aguas.

En conclusión, se han observado limitaciones en los procesos de tratamiento actuales que se llevan a cabo en las EDARs analizadas para eliminar el riesgo de transmisión de virus a través de aguas tratadas y biosólidos de uso en agricultura. Para abordar integralmente los riesgos asociados con la reutilización del agua regenerada, es esencial mejorar las tecnologías de tratamiento de aguas y biosólidos, además de adoptar políticas más estrictas para proteger la salud pública y promover prácticas sostenibles. Este trabajo revela la importancia de seguir investigando

y mejorando los sistemas de tratamiento y vigilancia de aguas residuales para minimizar los riesgos asociados a su reutilización en la agricultura y otros usos.

La detección de virus en efluentes y biosólidos implica la potencial entrada de estos patógenos en la cadena alimentaria y la afección de la población y el medio ambiente. Por ello, es importante conocer la estabilidad de los virus en aguas. Aunque existe mucha información sobre la estabilidad de los virus entéricos en el agua, todavía son escasos los estudios con otros virus que potencialmente pueden encontrarse en el agua, y no se puede descartar el agua como vía secundaria de transmisión. Por ello, en el marco de esta tesis doctoral, se investigó la estabilidad de virus entéricos, respiratorios y emergentes en efluentes y agua potable.

Estos ensayos se realizaron en condiciones controladas de temperatura y se utilizó un agua de referencia (RW), preparada con agua Milli-Q NaCl 7 nM, agua potable (DW), procedente de la red municipal, y efluente de planta depuradora (EW). Inicialmente se caracterizaron los parámetros físico-químicos de estas muestras y se inocularon artificialmente con suspensiones de MNV, HAV, IAV (H3N2), coronavirus humano 229E (HCoV-229E) y virus vaccinia (VACV), siendo estos dos últimos virus modelos de SARS-CoV-2 y MPXV, respectivamente. Las muestras se mantuvieron a temperatura ambiente (25 ºC) y de refrigeración (4 ºC) y se recogieron alícuotas por triplicado en diferentes intervalos de tiempo (0h, 24h, 1, 2, 4, 6, 8, 10, 12 y 14 semanas). El estudio de la infectividad se llevó a cabo en primer lugar mediante cultivos celulares, utilizando las líneas celulares correspondientes a cada uno de los virus incluidos en el estudio. La reducción de los niveles de los virus se calculó como $\log_{10}$ (Nx/N_0), donde N_0 es la concentración de virus infecciosos a tiempo 0 y Nx es la concentración de virus infeccioso para cada uno de los tiempos analizados. Los resultados obtenidos se modelizaron con una regresión lineal en función del tiempo para cada condición de temperatura y tipo de agua y se compararon cada una de las constantes de decaimiento, k, con sus intervalos de confianza del 95% para determinar si había diferencias significativas en la velocidad de inactivación de cada uno de los virus en las condiciones experimentales ensayadas.

Finalmente, aunque el cultivo celular es una las técnicas más extendidas para evaluar la infectividad de los virus, en este estudio se llevaron a cabo ensayos mediante PCR de viabilidad utilizando los compuestos intercalantes propidio de monoazida (PMAxx™) y cloruro de platino (IV) ($PtCl_4$). Los resultados se compararon con los obtenidos mediante cultivo celular. Para estos ensayos se seleccionaron las muestras DW y EW a tiempo 0h, 1, 4, 8, 12 y 14 semanas a 25 ºC.

Tras los análisis mediante cultivo celular, se observó que los virus entéricos sobrevivieron durante más tiempo que los no entéricos en línea con los resultados obtenidos en trabajos previos. La infectividad del MNV experimentó una disminución significativa después de 3 semanas a 25 ºC, mientras que a 4 ºC solamente disminuyó después de 12 semanas. A 25 ºC se observó una disminución gradual de la infectividad del HAV, por el contrario, a 4 ºC se seguían detectando virus

infecciosos incluso después de 14 semanas. Con estos datos se calcularon las constantes de decaimiento, que fueron 0,056, 0,228 y 0,155 log/día para el HAV en agua de referencia, agua potable y efluente a 25 ºC, respectivamente. Estas mismas tasas fueron de 0,057, 0,155 y 0,083 en las mismas matrices de agua, pero almacenadas a 4 ºC. En el caso del MNV, las tasas de decaimiento se establecieron en 0,029, 0,039 y 0,040 log/día para agua de referencia, agua potable y agua efluente a 4 ºC, respectivamente, sin observarse diferencias significativas entre los valores k para diferentes tipos de agua. Estas tasas fueron de 0,187, 0,168 y 0,230 en las mismas matrices de agua, pero almacenadas a 25 ºC.

En los experimentos realizados con virus no entéricos, HCoV-229E, IAV y VACV, los resultados mostraron la completa inactivación de los mismos entre 1 y 2 semanas en DW y EW a 25 ºC, observándose una estabilidad más prolongada a 4 ºC y en agua de referencia. En el caso de IAV, este se detectó tras 84 días (12 semanas) en efluentes y tras 98 días (14 semanas) en agua de referencia a 4 ºC. En general, comparando los tres virus, los valores de k establecieron que IAV era más estable que el HCoV-229E, y éste a su vez más estable que el VACV. Además, los tres eran más estables en el agua de referencia que en el agua potable o en efluentes, lo cual podría atribuirse a la presencia de la microbiota de estas aguas.

Los ensayos basados en técnicas moleculares se realizaron mediante (RT)-qPCR para determinar la persistencia del material genético del virus, y mediante PCR de viabilidad para estimar la estabilidad de virus infecciosos mediante la integridad de las cápsides virales. Estas técnicas de amplificación de ácidos nucleicos se basan en la integridad de las cápsides virales para realizar una aproximación sobre la infectividad de los virus. Este enfoque utiliza principalmente reactivos que se intercalan en el ADN o el ARN y sólo penetran en los viriones con cápsides comprometidas. Aunque estas metodologías han demostrado su efectividad para tratamientos extremos de presión y temperatura, diferentes estudios indican que estas aproximaciones no son lo suficientemente sólidas como para asociar de manera confiable la detección con la infectividad. Por ello, en el marco de esta tesis doctoral, quisimos evaluar esta aproximación para los virus no entéricos en estudio, comprobando su eficacia con la infectividad medida mediante cultivo celular.

Los resultados obtenidos, en general, mostraron que ni el PMAxx ni el $PtCl_4$ lograron disminuir la señal del material genético perteneciente a virus no infecciosos. Las reducciones en las concentraciones de IAV, HCoV-229E y VACV, medido mediante técnicas moleculares en los dos tipos de aguas, no se reflejaron del mismo modo que mediante cultivo celular. Mientras que estos virus se inactivaron por completo tras 1-2 semanas de almacenamiento a 25 ºC, mediante las técnicas moleculares ensayadas se seguía detectando concentraciones muy altas del material genético. A modo de ejemplo, el VACV en agua potable se mantuvo infeccioso sólo durante 1 semana, sin embargo, su material genético se detectó incluso a las 14 semanas, independientemente

de utilizar o no reactivos de viabilidad. En el caso de IAV, HCoV-229E y VACV en efluentes, se logró reducir completamente la señal del material genético sólo tras 14 semanas de almacenamiento, aunque la infectividad de los mismos se eliminó tras 1 (HCoV-229E y VACV) y 2 semanas de almacenamiento (IAV).

Este trabajo ha permitido explorar los factores que influyen en la estabilidad de virus entéricos y respiratorios en ambientes acuáticos. De este modo, se pudo demostrar que la naturaleza de los virus, la matriz y las condiciones en las que se encuentran (tipo de agua, temperatura) afectan a su estabilidad. La continuación de estos estudios es esencial para determinar si los virus presentes en el agua pueden afectar a la salud de la población y para asesorar de forma precisa sobre la calidad y seguridad de las aguas y alimentos que consumimos. Además, dada la baja eficiencia de los reactivos ensayados, se requiere una investigación más profunda sobre el uso de estas técnicas de evaluación de la integridad de la cápside que permitan inferir la viabilidad de los virus en este tipo de muestras.

Los estudios desarrollados en esta tesis contribuyen al conocimiento sobre los virus, su presencia, estabilidad y circulación en diferentes medios acuáticos, así como a la mejora de las metodologías para detectar virus en aguas residuales y regeneradas.

INTRODUCTION

1. INTRODUCTION

1.1. Relevance of viruses in the water cycle

Water is an essential element for life, not only as part of our diet but also for numerous activities such as agriculture, aquaculture, food preparation, industry, personal hygiene, domestic use, etc. (Linderhof et al., 2021). Therefore, it is necessary for the water supply to be sufficient, safe and accessible to the entire population (World Health Organization, 2022). However, water may serve as a carrier for numerous physical, chemical and biological contaminants (Kirby et al., 2003). Among these agents are viruses, small infectious microorganisms that can only replicate using the mechanisms of a host cell. In particular, human enteric viruses are responsible for causing viral gastroenteritis, hepatitis and other illnesses primarily transmitted through the faecal-oral route. The spread of human enteric viruses is mainly associated to person-to-person contact and the consumption of contaminated food and water (Bosch et al., 2008; Oude Munnink and van der Hoek, 2016). Enteric viruses are excreted in significant quantities, up to 10^{13} viral particles per gram of stool, by both symptomatic and asymptomatic individuals. Due to their low infectious dose and high environmental stability, human enteric viruses are responsible for large outbreaks (Rodríguez-Lázaro et al., 2012). Major causative agents of waterborne viral outbreaks worldwide include rotaviruses (RVs), human norovirus genogroups I (HuNoV GI) and II (HuNoV GII), hepatitis A and E viruses (HAV and HEV), and human astrovirus (HAstV). As reported by the World Health Organization (WHO), at least 1.7 billion people used a contaminated drinking water source during 2022 (UN-Water, 2021). Certain viruses present in water and food can have a significant negative impact on the population. Notably, HuNoV and HAV cause approximately 200,000 and 30,000 deaths per year, respectively (CDC, 2015; WHO, 2019).

Waterborne illnesses are associated with the consumption of contaminated water, not only drinking and recreational water but also water used in agriculture such as crop irrigation and food processing. Contaminated water can enter the food chain and contaminate facilities, packaging and the food itself (Kirby et al., 2003; WHO and FAO, 2008). For example, if the water used to cultivate and harvest bivalve molluscs and other shellfish is contaminated, it poses a significant health risk to consumers due to the filtering nature of these animals (bioaccumulation) and the minimal treatment they receive before consumption (Bosch et al., 2008). Other fresh products such as leafy greens and berries can also become contaminated through faecal-polluted water used for irrigation or sludge generated during treatment at wastewater treatment plants (WWTPs), which are also used as fertilizer for crops (WHO and FAO, 2008). Although access to safe drinking water may not pose a significant public health issue in developed areas, safeguarding

against viral contamination of food via water remains an ongoing concern worldwide, spanning both developed and developing countries (Bosch et al., 2008).

Another crucial factor to consider regarding water- and foodborne viral infections is the stability of human enteric viruses. Viral stability depends on several factors, including temperature, time and pH, among others (Mehle et al., 2018). The infectivity and spread of the viruses are directly related to their ability to remain stable until reaching a host cell where replication can occur (Payne, 2022). By assessing viruses in water from this perspective, not only information about their infectivity is obtained, but also pave the way for developing strategies to inactivate these pathogens. Therefore, it is important to study the viruses present in different water bodies and comprehend their behaviour throughout the water cycle.

The natural water cycle is a fundamental process in nature, involving the evaporation of water from water bodies, its transportation through the atmosphere as vapor, condensation forming clouds, and eventual precipitation in the form of rain or snow (Pagano and Sorooshian, 2002). All water bodies, whether surface such as rivers, lakes and seas, or underground, or even ice, are interconnected from the atmosphere to the oceans through the hydrological cycle and are susceptible to virus contamination (Pinon and Vialette, 2019). This contamination can stem from various sources, including industrial, agricultural and urban discharges, as well as poor waste management, impacting humans, animals and the environment (Mehle et al., 2018).

Thus, from the perspective of quality and safety, the urban water cycle in industrialized countries (Figure 1) holds particular significance. It encompasses various stages, from water collection in cities and industries, to its treatment in WWTPs (Amores et al., 2013). WWTPs use a combination of physical, chemical and biological processes to remove contaminants from sewage and industrial wastewater. The goal is to produce treated effluent water that is safe for discharge or reuse and to manage the generated sludge in an environmentally sound manner. The quality of the effluents (treated waters) and sludge generated in WWTPs is crucial, as they directly affect public health and the environment. The sludge generated during the treatment of wastewater can serve as fertilizer for crop fields (Mantovi et al., 2005). Effluents maybe discharged into water bodies such as rivers, sea and lakes (Amores et al., 2013) and it is also suitable for non-potable uses, such as irrigation, industrial processes and groundwater recharge according to national regulations (Bofill-Mas et al., 2005). Moreover, water is collected from water bodies and processed in Drinking Water Treatment Plants (DWTPs) for potabilization and distribution to the population. Upon usage, it transforms into wastewater and is reintroduced into WWTPs to perpetuate the urban water cycle (Amores et al., 2013). As a result, inadequate management of urban, industrial and agricultural wastewater poses potential economic and health risks (Bofill-Mas et al., 2005; Mantovi et al., 2005).

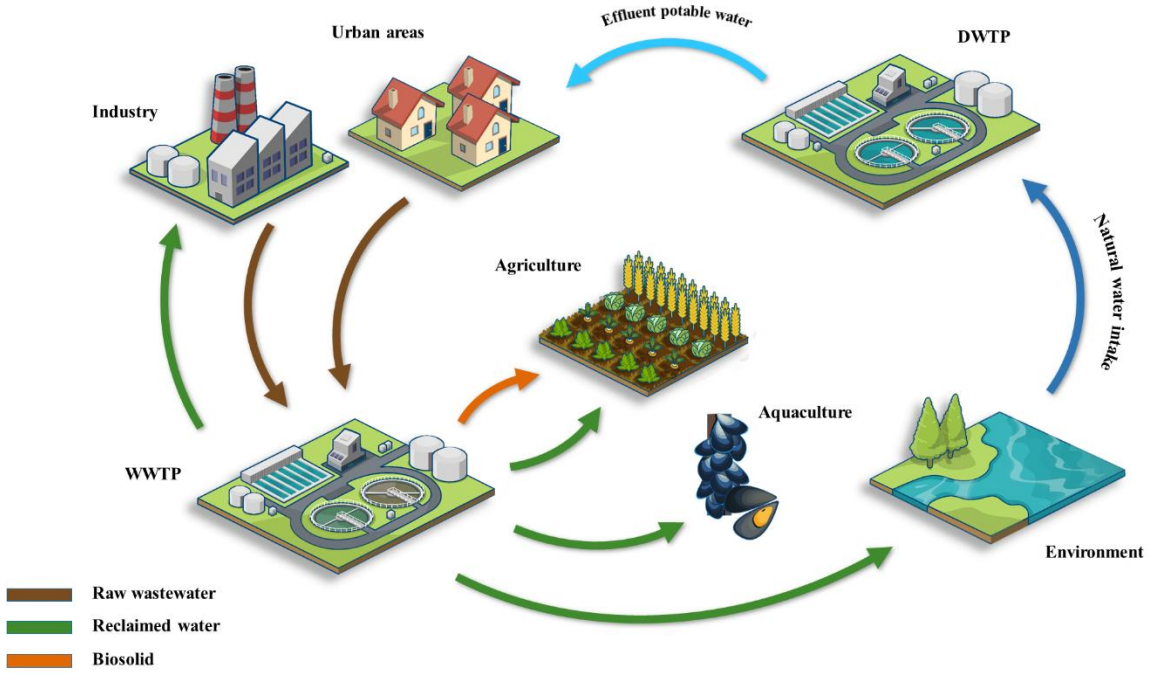

Figure 1. Schematic representation of the urban water cycle. WWTP, wastewater treatment plant; DWTP, drinking water treatment plant. Brown arrows, raw wastewater; green arrows, reclaimed water; orange arrow, sewage biosolid.

Nowadays, water scarcity and drought put additional pressure on global water resources (Shevah, 2015). In 2021, over 2 billion people resided in water-stressed countries, a situation expected to exacerbate due to climate change and population growth (UN-Water, 2021). In response to these challenges, environmental initiatives such as water reuse are being encouraged. This entails the use of treated wastewater for non-potable uses such as agricultural irrigation and industrial purposes. While the utilization of effluent wastewater and sludge is prevalent worldwide (Corpuz et al., 2020), a significant portion is informally utilized and/or inadequately treated, lacking sufficient controls to safeguard human and environmental health (Mateo-Sagasta et al., 2015).

Properly managed, the safe utilization of effluent wastewater and sludge can yield numerous advantages, including enhanced food production, heightened resilience to water and nutrient shortages and an enhanced circular economy (Regitano et al., 2022). This approach helps mitigate the demand for fresh water from natural reservoirs (Voulvoulis, 2018). However, water reuse also poses several challenges, particularly in terms of chemical and microbiological safety. Despite numerous studies detecting the presence of human enteric viruses in effluent wastewaters

(Haramoto et al., 2018), viral contamination has not been systematically considered. While Escherichia coli and other faecal indicator bacteria are commonly used to assess the microbial quality of WWTP effluents, many studies have highlighted their limitations in accurately estimating the presence of human enteric viruses (Cuevas-Ferrando et al., 2022; Farkas et al., 2020b; Truchado et al., 2021). Consequently, European Regulation (EU) 2020/741 has established a minimum requirement of ≥6 log reduction in the concentration of rotavirus or coliphages for reclaimed water to be deemed suitable for agricultural irrigation. In addition to human enteric viruses, respiratory and emerging viruses can also be excreted in the faeces and other bodily fluids of infected humans and animals, posing a potential risk of dissemination through water (Sinclair et al., 2009). While the main transmission route of some of these viruses may not be the oral-faecal route, there is a possibility of aerosol generation when contaminated waters come into contact with human (Deng et al., 2019; La Rosa et al., 2012).

Moreover, water serves as an indirect transmission vehicle for certain diseases such as dengue fever, as vectors like mosquitoes inhabit water during their reproductive, larval, or pupal stages (Dom et al., 2016). Furthermore, zoonotic infections through contaminated water are possible. Sewage carries viruses originating from both humans and animals. If sewage is not properly treated, viruses such as the influenza virus can reach consumers through the water or food chain (Blagodatski et al., 2021; Christou and Kosmidou, 2013; Velkers et al., 2017; WHO and FAO, 2008)

1.2. Wastewater-based epidemiology

Epidemiology is defined as the study of the distribution and determinants of health-related conditions or occurrences, including diseases and the application of these findings in diseases control efforts (Frérot et al., 2018). Different strategies are applied for evaluating infectious disease surveillance, many of which rely on passive monitoring systems (Sims and Kasprzyk-Hordern, 2020). These methods entail gathering data from sources such as sampling and testing individuals, which offer the most accurate assessment of active transmission and disease prevalence. Nonetheless, obtaining detailed data through testing poses practical and economic difficulties for many countries (Polo et al., 2020). For instance, infectious disease testing is typically limited to a subset of patients, leaving asymptomatic individuals undiagnosed. In developing countries with limited resources, the incidence rate of a disease may not accurately reflect reality due to inadequate and/or insufficient access to healthcare services and testing resources (Olesen et al., 2021; Wang et al., 2020).

During severe epidemics, laboratory capacities can quickly be overwhelmed, resulting in the underreporting of many cases (Lefrançois et al., 2023). Moreover, the rapid pace of global urbanization and unprecedented population growth are increasing the prompt health monitoring and response, inevitably straining the current system of infectious disease surveillance and management (Markt et al., 2023). All these factors collectively contribute to the potential inaccuracies in estimating disease spread when relying only on traditional techniques. Therefore, there is a pressing need for innovative approaches to track and manage infectious diseases for early warning and prevention. These novel surveillance techniques should be flexible, cost-effective, scalable and capable of providing comprehensive and objective real-time data. Implementing such innovative methods would not only complement the weaknesses of traditional approaches but also yield comprehensive results regarding disease exposure (Mao et al., 2020; WHO, 2020).

As mentioned before, untreated or improperly treated wastewater serve as the primary source for the dissemination of pathogenic microorganisms in the environment. However, while the presence and transmission of viruses in water represent a problem, it can also be considered an opportunity (Bofill-Mas et al., 2005). Analysing sewage collected from WWTPs, septic tanks and sewers, among others, enables the cost-effective, rapid and anonymous processing of samples from a large number of individuals (Levy et al., 2023). This approach is known as Wastewater-based epidemiology (WBE). WBE is a complementary tool to conventional epidemiology that allows monitoring the presence and evolution of viruses and other pathogens in a population (Polo et al., 2020). WBE provides information on the viruses circulating at a given time and population, their evolution and can serve as an early warning system to predict peaks in disease incidence (Mao et al., 2020). It combines the detection, analysis, data processing and interpretation of excreted targets in faeces, urine and other fluids to wastewater, providing valuable information about community health (Mao et al., 2020; Polo et al., 2020; Sims and Kasprzyk-Hordern, 2020). The objective of WBE is to gather information about the circulation of a target, such as a pathogen, in the population, alongside assessing community health status and identifying associated risk factors (WHO, 2023). This information is obtained independently of biases caused by socioeconomic factors or asymptomatic cases within the affected population (Lee et al., 2022).

The origin of WBE took place in the 19th century when Jon Snow, known as the "father of modern epidemiology", linked cases of cholera occurring in London to contaminated water used for drinking and domestic purposes (Cerda and Valdivia, 2007). Several kinds of targets that could be circulating in the community have been detected in sewage samples since then: from chemicals, such as drugs, pesticides, hormones and medicines, to pathogenic microorganisms, such as bacteria and viruses (Sims and Kasprzyk-Hordern, 2020). The genetic material of some bacteria, such as Salmonella typhi (Andrews et al., 2020) and Campylobacter (Guo et al., 2022b),

parasites, like Giardia lamblia and Cryptosporidium, and fungi, such as Candida and Aspergillus, are targets for identification in wastewater (Sims and Kasprzyk-Hordern, 2020). With the establishment of methods and infrastructure for this tool, the range of pathogens that can be detected has expanded and viruses have gained increasing importance in recent years (Markt et al., 2023). For example, WBE has been used to identify outbreaks of many different human enteric viruses such as poliovirus, HAV, HEV, human norovirus, enterovirus, and RV (Benschop et al., 2017; Chacón et al., 2021; Cuevas-Ferrando et al., 2020; Guo et al., 2022a; McCall et al., 2021). However, WBE has experienced a surge in popularity due to the emergence of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and its variants during the Coronavirus disease 2019 (COVID-19) pandemic (Olesen et al., 2021; Pérez-Cataluña et al., 2021a; Randazzo et al., 2020).

Following the establishment of wastewater-based surveillance systems for COVID-19, the groundwork has been laid for WBE to be applied to antibiotic resistance genes (ARG), antibiotic resistant bacteria (ARB) (Nguyen et al., 2021; O'Keeffe, 2021; Oliveira et al., 2023), microplastics (Ding et al., 2020; Goedecke et al., 2022) and respiratory and emerging viruses excreted in the faeces and fluids of infected people, like Influenza A virus, Respiratory syncytial virus (RSV) and Arboviruses (Ahmed et al., 2023; Boehm et al., 2023; Chandra et al., 2023; Lee et al., 2023). Nowadays, surveillance systems based on WBE exist at the international, national and community levels (Maryam et al., 2023).

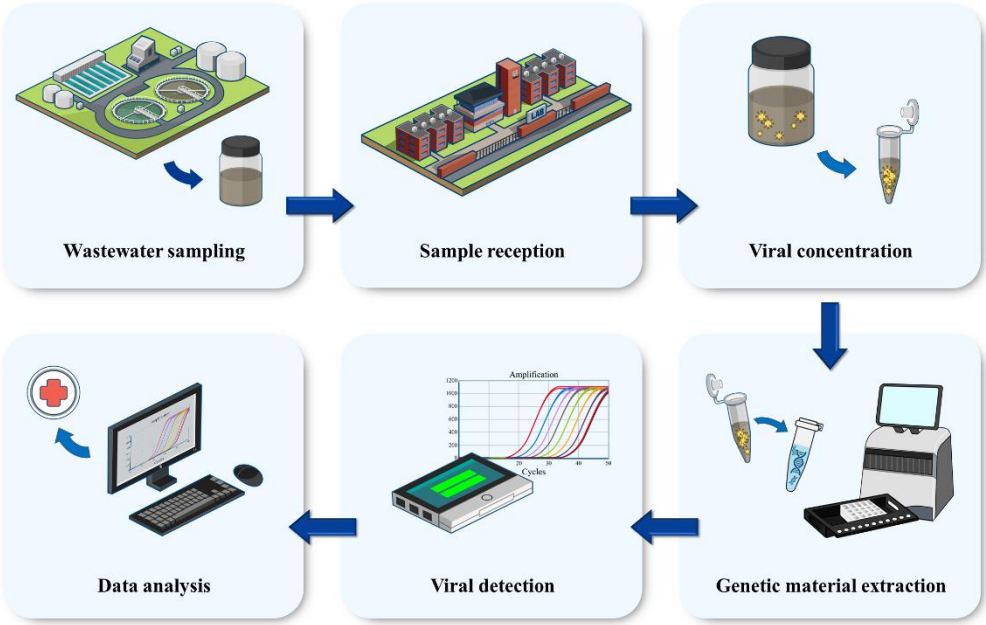

Figure 2. Workflow for the analysis of viruses using wastewater based epidemiology

To obtain accurate results, sampling is the first step and has great importance for obtaining representative results (Figure 2). Depending on the sampling point, the detected levels of virus will be representative of a larger or smaller population set. Samples can be taken from WWTPs serving one or several localities, from collectors of public institutions such as hospitals or residences, or it may even involve smaller communities, such as a single building or aircraft (Karthikeyan et al., 2022). In turn, samples can be categorized as grab if taken at a specific moment only once, or composite if, for example, several samples are collected and combined at the peak flow moment.

Detecting viral genetic material in wastewater samples is not the same as detecting it in clinical samples due to the low concentration of RNA and DNA typically present in wastewater samples. Hence, it is necessary to perform a concentration process on the samples prior to the extraction and detection of genetic material (Farkas et al., 2022). Dozens of protocols have been published, with many based on polyethylene glycol (PEG) or aluminium precipitation, as well as methods like flocculation, filtration, ultrafiltration and ultracentrifugation (Cuevas-Ferrando et al., 2020; Farkas et al., 2022; Pérez-Cataluña et al., 2021b). Additionally, due to the complexity of the matrix, detection methods such as polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) or droplet digital PCR (ddPCR) must be modified and adapted to this technique for WBE purposes (WHO, 2023).

WBE is being used to estimate not only viral trends but also the numbers of infected individuals, which requires determining accurate virus concentrations. However, these concentrations can be influenced by factors independent of laboratory sample treatment. These factors include dilution of the target in wastewater due to rain, lockdowns, weekday commuters, population migration, as well as periods such as local festivities or tourism. All of these factors significantly impact this type of studies, thereby influencing the predictions (Olesen et al., 2021; Sims and Kasprzyk-Hordern, 2020). The Center for Disease Control (CDC) and the European Commission (CDC, 2023; European Commision, 2021) recommend normalizing the data obtained by WBE before calculating trends. One approach to overcome this bias is performed using biomarkers for normalization, which can be based on physical-chemical parameters of wastewater or other viruses like pepper mild mottle virus (PMMoV) or coliphages, known to always be excreted in human faeces. Data normalization allows for comparisons between different samples from the same WWTP over time, or even across different WWTPs (Hsu et al., 2022; Markt et al., 2023).

While new viruses variants can be detected and quantified using simple and duplex quantitative PCR/RT-PCR, the use of sequencing techniques has proven to be the key for monitoring variants, lineages and sublineages in wastewater during COVID-19, as well as to

monitor temporal fluctuations and diversity of viruses (Carcereny et al., 2021; Nieuwenhuijse et al., 2020; O'Keeffe, 2021; Pérez-Cataluña et al., 2022a, 2021b). Most specific PCR techniques are unable to discriminate between the wide variety of circulating lineages because they can only detect one or limited mutations of the virus. One key application of sequencing in epidemiology is to perform phylogenetic studies and genotype isolates to examine their relationships. Additionally, genetic data analysis can elucidate the characteristics of individuals involved in an outbreak and their transmission dynamics, connecting observed patterns in the population. Sequencing enables the understanding of both current and emerging new variants' dynamic (De Salazar et al., 2022; Karthikeyan et al., 2022).

For a comprehensive approach to WBE, the final phase of data interpretation involves understanding the relationship between: wastewater dynamics, spatiotemporal relationships with populations and clinical data reported in the studied area (Mao et al., 2020). It may even be possible to identify the source of a disease and trace the carriers of pathogens by tracking pipelines and sampling sanitary lines within the building or the local neighbourhood (Armas et al., 2023) and numerous correlations have already been demonstrated between WBE data and patient hospitalizations in multiple settings (Acosta et al., 2021; Galani et al., 2022; Schenk et al., 2023).

The surveillance of enteric, respiratory and emerging viruses through WBE is a very useful strategy to better understand the occurrence and dynamics of these pathogens. The qualitative and quantitative information provided by WBE can be used to identify future trends in a disease, study local or regional outbreaks and develop action plans when peaks in incidence and saturation of healthcare resources appear. Additionally, clinical priorities can be determined during periods of high demand in the healthcare sector, case clusters can be traced, vaccination campaigns can be planned and the effect of all these measures can be evaluated (Lefrançois et al., 2023; Markt et al., 2023; WHO, 2023).

1.3. Viruses potentially present in water

Many human enteric viruses are known to be transmitted through water and are highly prevalent in influent, effluent and biosolid samples (Figure 3). Additionally, other viruses, which can be excreted in faeces or body fluids and reach sewage, were not previously considered a concern for waterborne transmission but may now be emerging waterborne pathogens due to their presence and persistence in aquatic environments. The following sections provide more details about the characteristics of the viruses studied in the framework of this thesis.

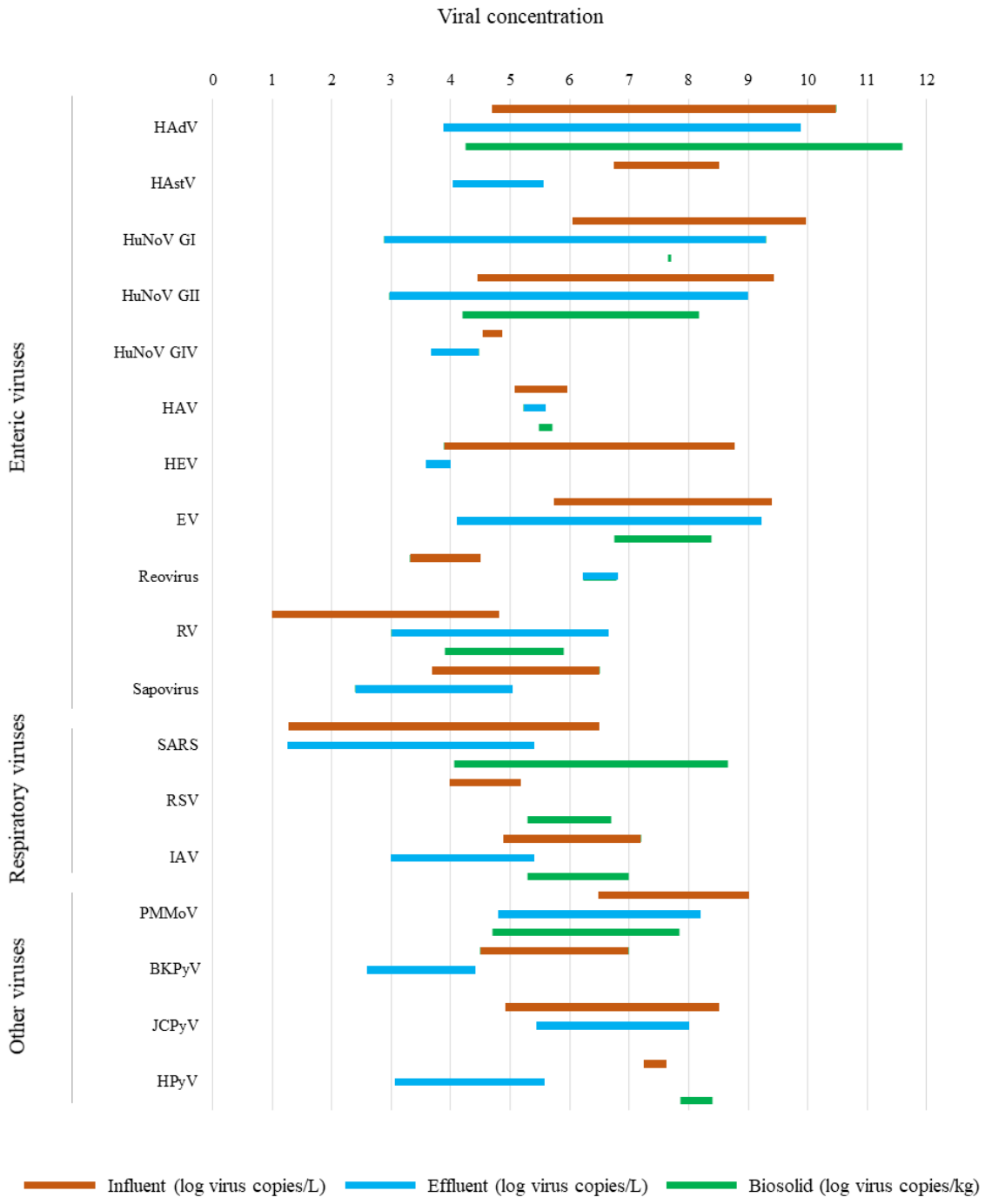

Figure 3. Levels of human adenovirus (HAdV), human astrovirus (HAstV), human norovirus (HuNoV) GI, GII, GIV, hepatitis A virus (HAV), hepatitis E virus (HEV), enterovirus (EV), reovirus, rotavirus (RV), sapovirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), respiratory syncytial virus (RSV), influenza A virus (IAV), pepper mild mottle virus (PMMoV), BK polyomavirus (BKPyV), JC polyomavirus and human polyomavirus (HPyV) found in influent (orange) and effluent (blue) wastewater and biosolids (green). Figure adapted from Corpuz et al., 2020; Haramoto et al., 2018; Toribio-Avedillo et al., 2023.

1.3.1. Human enteric viruses

Human enteric viruses are primarily transmitted through the faecal-oral route, causing viral gastroenteritis, hepatitis and other illnesses. Dozens of different human enteric viruses have been detected in various types of water and biosolids, including HuNoV GI and GII, RV, HAstV, HAV, HEV, adenovirus, enterovirus, and sapovirus, among others (Figure 3).

Norovirus

Human noroviruses belong to the Caliciviridae family, characterized by non-enveloped structure with an icosahedral capsid measuring 27-40 nm in diameter and positive-strand RNA. There are ten NoV genogroups (GI to GX) and each one is divided into a total of 49 genotypes (Khamrin et al., 2020). These viruses are primarily transmitted through the faecal-oral route, either via the consumption of contaminated food and water or through direct person-to-person contact, with a very low infection dose (Teunis et al., 2008). HuNoV causes about the 20% of the total foodborne diseases reported worldwide and its symptoms of infection include vomitus, abdominal pain, mild fever and diarrhoea (Alfieri et al., 2017; Guix et al., 2019). The most relevant HuNoV genotypes causing human infections are GII, GI and to a lesser extent, GIV. GII strain outbreaks are linked to more severe outcomes, including mortality, compared to other HuNoV strains outbreaks, particularly in elderly and immunocompromised patients (Robilotti et al., 2015). This infection may occur as a result of food contaminated during processing or serving by a food handler and it is strongly associated with the consumption of leafy greens, berries and shellfish (Hardstaff et al., 2018). HuNoV exhibit high environmental stability in various water types, with the ability to survive a broad range of temperatures, acidic pH and high salt concentrations (Seitz et al., 2011; Seo et al., 2012) Despite the recent development of an in vitro model based on stem cell-derived human intestinal enteroids (HIEs) for human norovirus replication (Costantini et al., 2018; Estes et al., 2019; Ettayebi et al., 2016), its complexity has limited its extended experimental application. Therefore, it is common to use surrogates such as feline calicivirus (FCV), murine norovirus (MNV), or Tulane virus for norovirus studies.

Rotavirus

Rotaviruses are members of the Sedoreoviridae family (Matthijnssens et al., 2022). These non-enveloped viruses exhibit an icosahedral capsid with a diameter ranging between 70 and 100 nm and a segmented, double-stranded RNA genome (Sadiq et al., 2018). This segmented nature genome introduces the potential for genomic re-assortment between human and animal RV (Greening and Cannon, 2016). Classified into ten groups (A-J) (Matthijnssens et al., 2022), group A rotaviruses are commonly associated with infections in humans, cattle and other domestic animal species, while groups B and C are infrequently reported. RV are typically excreted in human stools and transmitted via the faecal-oral route, causing diseases in both humans and animals (Dhama et al., 2009). While primary transmission occurs person-to-person, contamination of fomites and aerosols is also possible (Bass et al., 2015; Ganime et al., 2016). Group B rotaviruses, in particular, can infect adults and have sporadically contributed to food and waterborne outbreaks (Chitambar et al., 2011). Although RV can affect all age groups, it is generally considered a mild infection in adults but can cause acute gastroenteritis in young children, posing a significant threat in areas with limited access to medical services (Bernstein, 2009). In many parts of the world, RV remain a major cause of severe acute gastroenteritis, contributing to over 200,000 deaths in children under 5 years in developing countries (Tate et al., 2016). Rotaviruses exhibit limited tolerance to extreme conditions compared to other enteric viruses, but they can survive for several hours on hands and weeks in river water at 4 and 20 °C (Greening and Cannon, 2016). However, cooking temperatures are usually sufficient to inactivate RV. These viruses are found in water and sewage and many of them can be cultured in cell lines derived primarily from monkey kidneys such as MA-104 cell line (Peña-Gil et al., 2023).

Hepatitis A virus

HAV is classified in the Picornaviridae family, Hepatovirus genus. This non-enveloped virus with icosahedral capsid measures 27–32 nm and has a genome consisting of a single-stranded RNA of positive polarity. HAV strains are grouped into three genotypes (I, II and III) and six subgenotypes (IA, IB, IIA, IIB, IIA and IIIB) (Van Damme et al., 2023). HAV causes hepatitis A, an infectious necro-inflammatory liver disease, is transmitted primarily through the faecal-oral route, with person-to-person contact being the primary one. Outbreaks of foodborne disease are associated with contamination, either pre-harvest or during food handling. Shellfish, fruits and vegetables are common sources of contamination, with sewage often identified as the irrigation water pollution source. Infected food handlers and processors, shedding the virus before

symptoms appear, contribute to HAV contamination (Previsani et al., 2003; Sánchez, 2015). HAV infection occurs worldwide, especially in children from developing countries who are asymptomatic in most cases (Franco et al., 2012). Symptoms of hepatitis A include nausea, fever, dark urine, light stools and diarrhoea. Viremia and jaundice appear one to two weeks after first non-specific symptoms. Chronic liver disease is not associated with HAV but this infection may produce fatal disease (Matheny and Kingery, 2012). The onset of symptoms occurs weeks after infection, making it unlikely to have the suspected food available for analysis. HAV exhibits remarkable environmental stability, retaining its infectivity for several months under refrigeration and freezing conditions (reviewed by Greening and Cannon, 2016). Additionally, HAV remains stable in various environments, including marine sediment and seawater and exhibits resistance to desiccation and low pH (McKnight and Lemon, 2018). When applying cell culture techniques to HAV cell-culture adapted strains, cultures in primate cell lines such as fetal rhesus monkey kidney cells (FRhK-4) are characterized by slow growth and a low yield compared to other picornaviruses (Cromeans et al., 1989).

Hepatitis E virus

HEV is a non-enveloped virus characterized by its icosahedral capsid, a diameter ranging from 27 to 30 nm and positive single-strand RNA. Classified within the Hepeviridae family, HEV belongs to the Hepevirus genus and its genotypes exhibit distinct geographic distributions. HEV is divided into four main genotypes, namely HEV1, HEV2, HEV3 and HEV4 (Usuda et al., 2024). Geographic prevalence plays a significant role in the manifestation of the disease, with HEV1 and HEV2 primarily causing infections in regions where water quality and sanitation are inadequate, causing waterborne outbreaks and secondary person-to-person spread (Teshale et al., 2010). The clinical manifestation of HEV infection results in Acute Jaundice Syndrome (AJS), exhibiting symptoms similar to those caused by HAV. Viremia, nausea, fever, abdominal pain, arthralgia, dark urine and general malaise may precede the classic onset of jaundice after an incubation period of 2–8 weeks (Gerbi et al., 2015). HEV transmission occurs primarily through the faecal-oral route. As a zoonotic pathogen, animal meat is susceptible to contamination through liver infection or contact with infected faeces during animal dressing or meat processing. Epidemiological investigations strongly suggest that human infections often result from the consumption of pork products and game meat (reviewed by Greening and Cannon, 2016). Moreover, HEV has been detected worldwide in diverse environmental sources such as raw sewage, river water, shellfish and seawater (Beyer et al., 2020; Crossan et al., 2012; Takuissu et al., 2022). The presence of faecal contamination in run-off water from pig farms or from areas where untreated pig manure has been spread may lead to the contamination of surface and

irrigation waters (Christou and Kosmidou, 2013). Water contaminated with human or swine waste can introduce HEV into the food chain, particularly if used for shellfish production or irrigation, with the ensuing contamination of fruits, vegetables and shellfish with HEV (Takuissu et al., 2022; Treagus et al., 2021). Raw or undercooked shellfish consumption poses a significant risk for HEV infection and sporadic cases in Europe and Southeast Asia have been linked to this practice (reviewed by Greening and Cannon, 2016). Experimental evidence also indicates the potential for HEV contamination through irrigation water, as seen with strawberries irrigated with contaminated water (Brassard et al., 2012). Recently, HEV has begun to be considered an emerging pathogen, making its study even more important (Ricci et al., 2017). Since the cell culture of HEV is difficult to standardize and different cell culture models for efficient replication are still under study (Chew et al., 2022), techniques like RT-qPCR are utilized for monitoring the virus (Davis et al., 2021; Jothikumar et al., 2006; Shukla et al., 2011). In fact, there is an international project to stablish an ISO method for detecting HEV in meat products (ISO/TC 34/SC 9/WG 31).

Human Astrovirus

HAstV are non-enveloped viruses with a diameter of 28–30 nm, exhibiting an icosahedral structure. They belong to the family Astroviridae, which comprises two genera: Mamastrovirus, infecting mammals; and Avastrovirus, affecting birds. These viruses are characterized by a positive-sense single-stranded RNA genome (Bogdanoff et al., 2017). There are three different groups within HAstV: two emerging groups, HAstV-MLB and HAstV-VA/HMO and the classic HAstV group, which includes eight serotypes (1-8), being the serotype HAstV-1 the most prevalent worldwide (Vu et al., 2017). The potential for cross-species transmission of HAstV between animals and humans represents a significant concern. While the pathogenic strains affecting animals and humans appear distinct, recent studies utilizing both traditional sequencing and next-generation sequencing (NGS) techniques suggest the possibility of interspecies transmission (Bosch et al., 2014). The primary manifestation of HAstV infection in both humans and animals is self-limiting gastroenteritis, contributing to approximately 20% of all sporadic non-bacterial gastroenteritis cases in humans, primarily affecting children under 2 years old (Karlsson and Schultz-Cherry, 2013). Co-infections with other enteric pathogens are common and the virus is also implicated in gastroenteritis cases among the elderly, often in conjunction with viruses like rotavirus or calicivirus (De Benedictis et al., 2011). Symptoms of HAstV infection include diarrhoea, fever, nausea and general malaise, occasionally accompanied by vomiting after an incubation period of 3–4 days. While severe cases and fatalities are rare, they can occur (Cortez et al., 2017). High-risk environments include nurseries, childcare centres and hospitals,

with outbreaks commonly reported in institutional settings, particularly paediatric wards. Potential transmission through contaminated shellfish and water has also been reported (Suffredini et al., 2020; Wohlgemuth et al., 2019). HAstV exhibit remarkable resistance to environmental conditions and various chemicals, including chloroform, alcohols and non-ionic and anionic agents. As reviewed by Greening and Cannon (2016), they can also remain viable at low pH values and high temperatures, withstanding 50°C for 1 hour. While applying cell culture techniques to wild-type HAstrV is challenging, certain HAstV-MLB demonstrate efficient replication in Caco-2 and HuH-7 cell lines (Vu et al., 2019).

1.3.2. Emerging Viruses

Emerging viruses are those that have appeared or been recently identified, often due to the development of new techniques for pathogen detection and identification. Additionally, these viruses are characterized by an expanded geographical range and/or a significant increase in incidence or prevalence in recent decades (Bofill-Mas et al., 2005; Morse, 1996). The emergence of viruses can be caused by different circumstances. RNA viruses have a great capacity for adaptation to the environment due to the high error rate of their polymerases (Elena and Sanjuán, 2005). The appearance of a new virus may result from changes in the environment, climate, virus control systems, or directly, the evolution of an unknown variant. Crossing the species barrier, or from a small population sample (human or animal) to a larger one, can lead to the emergence of new emerging viruses (Burrell et al., 2017).

The diversity of emerging viruses is related to the variability of their biological cycles, transmission routes and pathogenicity (Parrish et al., 2008). A large portion of these viruses are zoonotic, linked to ecological cycles that depend on one or more animal hosts. The risk posed by these viruses lies in their ability to jump between species, affecting populations that lack immunity to the new pathogen (Burrell et al., 2017).

The appearance or resurgence of a virus arises from the interaction of three factors: the virus, the susceptible population and the environment where they are found. In addition to this, there are other elements that can influence the evolution of emerging viruses, such as international travel and trade, social inequalities and lack of prevention and hygiene measures, among others (Ka-Wai Hui, 2006). Likewise, changes associated with the development of agriculture and different uses of water are also relevant concerning emerging viruses (Sutherst, 2004).

Severe Acute Respiratory Syndrome Coronavirus 2

SARS-CoV-2 is an emerging virus belonging to the Coronaviridae family, which can cause diseases in animals and humans. The structure of SARS-CoV-2 is characteristic of coronaviruses, with a lipid envelope and a positive-sense single RNA strand genome. It has spike proteins on its surface, which allow it to adhere to human cells, especially those in the respiratory tract (N. Zhu et al., 2020). SARS-CoV-2 is the etiologic pathogen of COVID-19 pandemic. COVID-19 can present different degrees of severity, from asymptomatic infection to severe cases that can lead to respiratory complications and death, especially in older individuals or those with pre-existing medical conditions (Feng et al., 2020). Symptoms can vary including fever, cough, difficulty breathing, fatigue, headache, loss of taste or smell, muscle aches and gastro-intestinal problems (reviewed by Zhu et al., 2020). Some infected individuals may be asymptomatic, making virus detection and control challenging. Since the beginning of the COVID-19, multidisciplinary experts have worked to learn more about SARS-CoV-2 and its spread. Although the primary route of SARS-CoV-2 transmission is through respiratory droplets and aerosols during human-to-human interactions (Lamers and Haagmans, 2022), the role played by fomites in this transmission was also been investigated (Arienzo et al., 2023). SARS-CoV-2 can remain stable in the environment for variable periods of time, depending on factors such as temperature and humidity (Riddell et al., 2020). This has led to discussions about the role of environmental contamination and the potential risk of food as a carrier of the virus. Research on cold-chain transportation within the frozen food industry has shown the potential for reinfection, as the SARS-CoV-2 was successfully isolated from the surfaces of seafood packaging (Liu et al., 2020). The detection of infectious SARS-CoV-2 on surfaces, along with the gastrointestinal symptoms caused by the virus and its presence in the faeces of infected individuals, led to concerns about the possibility of oral-faecal transmission at the beginning of the pandemic (Cuicchi et al., 2021; Xiao et al., 2020). Although this route of transmission has been ruled out, the detection of SARS-CoV-2 in faeces and other fluids from sick patients, both symptomatic and asymptomatic, has aided the implementation of the WBE for COVID-19 surveillance (Lodder and de Roda Husman, 2020). During the COVID-19 pandemic, the primary detection methods for SARS-CoV-2 in clinical samples have been RT-qPCR and rapid antigen tests. In addition to molecular techniques and immunoassays, cell culture using Vero cells has also been employed to study SARS-CoV-2 behaviour (Steiner et al., 2024). Due to the risks associated with working with infectious SARS-CoV-2, which must be cultivated in a BSL-3 laboratory, different surrogates have been used to assess its stability and environmental resistance. Some of these models include porcine epidemic diarrhoea virus (PEDV), murine coronavirus (MHV) and human coronavirus 229E (HCoV-229E)

tested on Vero, DBT, MRC-5 and HuH-7 cell lines (Bhavanam et al., 2022; Contrant et al., 2023; Neuman et al., 2004).

Monkeypox Virus (MPXV)

The Monkeypox virus (MPXV) is an enveloped, double-stranded DNA orthopoxvirus, considered one of the largest and most complex known viruses. It belongs to the Poxviridae family. Two distinct MPXV clades are differentiated, each endemic to a specific region of Africa: a highly virulent clade in the Congo Basin (Clade I) and a less virulent clade found in West Africa (Clade II) (Americo et al., 2023; Pastula and Tyler, 2022). During the illness, mpox (formerly known as monkeypox) manifests, during 2-4 weeks, symptoms such as headache, myalgia, fever and skin lesions, typically appearing 4-17 days after exposure. However, it is worth noting that some patients may remain asymptomatic. While mortality caused by Clade II MPXV is low, Clade I is associated with a mortality of up to 10% (Altindis et al., 2022; Americo et al., 2023; Pastula and Tyler, 2022). The recent increase in cases of MPXV infection, including the global outbreak in 2022, has raised concerns about this emerging zoonosis. Factors that may have influenced this increase in incidence include the expansion of natural animal reservoirs and increased human contact with them. Additionally, the decrease in collective immunity to poxviruses, following the eradication of smallpox virus in 1980, could also be a contributing factor (Sklenovská, 2020). African rodents and primates can serve as natural reservoirs of this virus, with zoonotic transmission occurring through direct contact with these animals. Additionally, transmission via fomites and human-to-human contact, including direct contact, sexual contact, or respiratory droplets, is possible. Consumption of meat or other products from infected animals could also pose a transmission risk (Altindis et al., 2022; Nakhaie et al., 2023). MPXV stability depends on factors such as the matrix in which the virus is found, the surface and environmental conditions. For example, MXV has been found to exhibit prolonged infectivity in various body fluids and retain infectivity for nearly a week in untreated wastewater (Yinda et al., 2023). Detection of MPXV presence in water can be determined by qPCR, sequencing and cell culture techniques. Various cell lines, including those derived from monkeys, guinea pigs, rabbits, bovines, mice, monkeys and certain human tissues such as lung fibroblasts, support MPXV growth and exhibit different cytopathic effects when infected with the virus (Nakhaie et al., 2023). The classification of MPXV as a biosafety level 3 virus has led to the widespread use of surrogates for its study. Among these surrogates, vaccinia virus (VACV) is commonly used and can be cultured in the HeLa cell line (Atoui et al., 2023).

1.3.3. Other viruses

Influenza virus

Influenza virus is an enveloped virus that belongs to the Influenzavirus genus, within the Orthomyxoviridae family and is characterized by a segmented genome of single-stranded negative-sense RNA, with an approximate size of 80-120 nm in diameter (Van Reeth et al., 2012). There are four types of influenza viruses: A, B, C and D (Krammer et al., 2018), although most cases are caused by Influenza A (IAV) and B viruses (Van Reeth et al., 2012). Influenza A virus (IAV) infect not only humans but also pigs, horses and poultry. Additionally, IAV is found in wild migratory birds, with more than 100 species, including ducks, swans, gulls and various wild aquatic birds, being recognized as natural hosts (Krammer et al., 2018). The most outstanding characteristic of influenza viruses is their rapid evolution, especially in the case of IAV, being responsible for regular epidemics and pandemics (Krammer et al., 2018). IAV zoonotic infections from animal reservoirs like pigs and birds have been reported worldwide (AbuBakar et al., 2023; Van Reeth et al., 2012). Some influenza viruses, particularly those classified as highly pathogenic, such as H5 and H7, have the capability to infect and spread among birds as well as wild and farmed mammals (Blagodatski et al., 2021). This inter-species transmission, coupled with the capacity for IAV to be transmitted from mammal to mammal, underscores the virus's potential to spark pandemics, as evidenced by current concerns regarding IAV H5N1 (Shi et al., 2023). Typical symptoms include fatigue, fever, muscle pain and headaches, with serious illness occurring less frequently due to secondary bacterial infections or exacerbation of cardiovascular and respiratory diseases (Krammer et al., 2018). Immunocompromised individuals, older adults, infants and pregnant women are also vulnerable groups for this virus. Nonetheless, prophylactic vaccination is possible to manage the extensive clinical IAV burden both in animal and human (Van Reeth et al., 2012). The transmission of the virus primarily occurs through the respiratory pathway, involving respiratory droplets and aerosols. However, the virus's RNA can also be detected in the stools of symptomatic patients, with detection rates ranging from 7.2% to 47% (Markt et al., 2023). Depending on environmental conditions, such as humidity and temperature, IAV can survive for several hours, even in waters at low temperatures, being vulnerable to heat and low pH. Nevertheless, IAV lipid envelope makes it highly susceptible to damaging environmental impacts like detergents and commonly used antiviral disinfectants (Blut, 2009). In addition to RT-qPCR, IAV can be detected and quantified using cell culture methods, particularly through the use of the MDCK and Vero cell lines (Youil et al., 2004). This method not only serves as a complementary technique to RT-qPCR but also provides valuable insights into the IAV infectivity and replication dynamics.

Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is an enveloped virus belonging to the genus Orthopneumovirus, family Pneumoviridae, order Mononegavirales (Sanz-Muñoz et al., 2024). RSV is called after its ability to cause cell fusion, leading to the formation of large multinucleated syncytia (Jha et al., 2016). This virus is characterized as a negative-sense, single-stranded, non-segmented RNA genome and has been classified into two distinct groups (A and B) based on its antigenic variability. These groups are distinguished by the reactions of two major surface proteins to monoclonal antibodies (Sanz-Muñoz et al., 2024). RSV A and RSV B have the same pathogenicity (Ciarlitto et al., 2019) and often co-circulate, although generally one predominates due to the periodic emergence of new RSV genotypes and its tendency to replacing the circulating strain of the virus (Hause et al., 2017). RSV transmission is typically produced by respiratory pathway and direct contact, being highly contagious with an incubation period of 4 to 7 days (Kaler et al., 2023). Moreover, RSV can remain infectious on skin, clothing and other objects for prolonged periods, promoting its dissemination (Hall, 2001). RSV is one of the most pathogenic infections in childhood, associated with significant morbidity and mortality, particularly among infants six months old or younger (Munro et al., 2023). This virus is the leading cause of bronchiolitis and pneumonia in infants, as well as the major cause of hospitalization during infancy. The possibility of reinfection with RSV has been demonstrated in 30-75% of children and long-term sequelae can appear after severe infections (Borchers et al., 2013). Moreover, RSV affects various at-risk adults, such as elderly individuals and immunocompromised persons, being an important cause of death due to lower respiratory tract infections in resource-limited settings (reviewed by Jha et al., 2016). Various techniques can be used to detect RSV, including cell culture, direct immunofluorescence assay and RT-qPCR (Hu et al., 2003). Recently, due to the rise of WBE for respiratory viruses, RT-qPCR is being used as a detection technique for RSV surveillance in wastewater. Although knowing the levels and subtypes of these viruses circulating in the population is essential for global health, it should be noted that during COVID-19, resources were stretched thin and there may have been an underestimation of cases. Additionally, the use of non-pharmaceutical interventions to tackle the pandemic indirectly affected RSV cases. For example, the use of masks and quarantines contributed to a 10% decrease in RSV cases in 2019 and 2020, compared to cases reported in 2020 and 2021(Ando et al., 2023).

1.3.3. Viral indicators

Tracking indicator microorganisms in water is a widely used strategy. Traditionally, bacteria such as E. coli have been used as indicators of faecal contamination in water. However, research indicates that viruses are more resistant and stable in the environment compared to bacteria (Gerba et al., 2013). Therefore, current water monitoring programs include viruses as faecal contamination indicators, as the Regulation (EU) 2020/741 that included rotavirus or coliphages as an indicator of reclaimed water in addition to considering E. coli levels. The presence of viral indicators is not only useful for assessing the quality of water and determining faecal contamination but also to check the effectiveness of different treatments applied to wastewater and biosolids generated in WWTPs, seawater, freshwater and potable water (Kitajima et al., 2014). Additionally, the detection and quantification of viral indicators are tools used to normalize data for WBE (Dhiyebi et al., 2023).

Viral indicators must not only exhibit persistence in the environment and resistance to wastewater treatments similar to targeted viruses in the environment, but also possess additional characteristics that enable their use as reliable indicators. As reviewed by Farkas et al., 2020, viral indicators may be source-specific, meaning that their association with human excretion has to be clear to relate the data obtained to the population, without possible errors when choosing viruses that could also be excreted by animals. Additionally, indicator must be globally distributed and be stable over time. The global distribution of viral indicators is important because if these viruses can be detected and quantified worldwide, data comparison between different communities is possible. Finally, the virus must be present in wastewater at high concentrations and be easy to detect and quantify (Scott et al., 2002). From a technical standpoint, it would be advantageous for viral indicators to be in high concentrations and to be easy to detect and quantify with simple and affordable concentration and detection methods.

Several viruses, such as adenoviruses (AdVs), polyomaviruses (PyVs), aichiviruses (AiVs), PMMoV, bacteriophages, Bacteroides phages and crAssphage meet the requirements to be good indicators of human faecal contamination (Farkas et al., 2020b). AdVs, AiV, crAssphage and PMMoV are detected more frequently and at higher concentrations in wastewater and contaminated water bodies. PyVs and bacteriophages are also present in high concentrations in wastewater, but they degrade rapidly. CrAssphage and PMMoV, found in the human gut, are not infectious to humans, facilitating their detection in the laboratory without risk of infection. From a molecular assay perspective, DNA viruses (AdV, PyV and crAssphage) may be easier and more cost-effective to monitor than RNA viruses (i.e., AiV and PMMoV). Other viruses like AdV and bacteriophages are amenable to being studied through culturing, thereby also providing information about their infectivity.

Bacteriophages

Bacteriophages are viruses that infect and replicate within bacteria cells. The diversity of these microorganisms exhibits an immense range in size, morphology and genomic organization and all of them are ubiquitous and abundant in the environment (Kasman and Porter, 2022). Within the group of bacteriophages, coliphages are viruses that infect coliforms such as E. coli. These viruses have symmetric protein capsids with icosahedral and helical shapes, among others, which encapsulate the viral genetic material, which can be single or double-stranded DNA or RNA (Sanz-Gaitero et al., 2021). Coliphages are increasingly being used as water quality indicators due to the association with the presence of viral pathogens suggested by their presence and faecal contamination, indicating a potential risk to public health (Lin and Ganesh, 2013). Coliphages are divided into two groups: somatic coliphages and F-specific RNA coliphages (Singh et al., 2022). Somatic coliphages are commonly found in water environments exposed to human excreta, including natural waters contaminated with wastewater (Toribio-Avedillo et al., 2021). In the context of wastewater, coliphages detection can be used as an indicator of the effectiveness of wastewater treatment processes. The presence of coliphages in treated wastewater may indicate a failure in treatment processes and the possible presence of faecal contaminants and pathogens, underscoring the importance of continuously monitoring and improving wastewater treatment systems to protect public health and the environment (Jofre et al., 2021). Within the group of bacteriophages, crAssphage belongs to the Bacteroides family, with a single-stranded circular DNA genome that owes its name to the Cross-Assembly software that was used for its discovery (Park et al., 2020). This group is very heterogeneous and ubiquitous and its detection is associated with human faeces because it is typically found in the human gut virome. Like somatic coliphages, it is widely used as an indicator of water quality and has also been used in several studies to normalize virus data (Dhiyebi et al., 2023; Holm et al., 2022). Bacteriophages can be detected and quantified by affordable, rapid and simple methods based on molecular and plaque assay by double- or single-layer agar methods (Jofre et al., 2021). Somatic coliphages culture methods are rapid and useful strategies for reporting viability. Thus, it is possible to know the concentration of actual infectious viruses in the sample. Detected levels can vary from 10 plaque-forming units (PFU) to 108 PFU per gram of human faeces or litre in the case of wastewater samples. In the case of crAssphage, levels of this virus have been detected in wastewater samples of up to 1012 genomic copies (GC) per litre (Dhiyebi et al., 2023; Farkas et al., 2020b).

Pepper Mild Mottle Virus (PMMoV)

PMMoV, a member of the Tobamovirus genus and Virgaviridae family, is a rod-shaped plant virus characterized by its single-stranded RNA genome with and a length of approximately 312 nm (Kitajima et al., 2018; Lin and Ganesh, 2013). PMMoV infects many pepper varieties and the symptoms of its presence range from small white mottles to systemic infections. However, infected peppers often display no symptoms or only mild foliar damage, allowing the virus to propagate unnoticed (Kitajima et al., 2018). PMMoV has been identified in aquatic environments worldwide, including rivers, aquifers, irrigation systems and coastal waters, often at higher levels than human pathogenic viruses (Farkas et al., 2020b). Potential sources of PMMoV include faecal reservoirs, contaminated plants and food sources. The consumption of PMMoV-infected peppers and processed products is a significant factor contributing to its presence in human excreta, which in turn leads to pollution of water bodies. Intact PMMoV particles can persist through food processing and may end up in human faeces after ingestion (Kitajima et al., 2018). Furthermore, PMMoV exhibits remarkable stability in various environmental conditions and its seed-borne nature facilitates its global dissemination, making PMMoV a potential conservative indicator of faecal pollution (Kumari et al., 2023). The CDC and the European Commission have recommended PMMoV as a biomarker to standardize data related to WBE (Hsu et al., 2022). PMMoV concentration range from 106 to 1010 per litter in wastewater (Farkas et al., 2020b). These concentrations highlight the potential utility of PMMoV as a robust marker for tracking viral loads in wastewater, aiding in the assessment of community transmission and the effectiveness of public health interventions (Symonds et al., 2018).

1.4. Methods for analysing viruses in water

One major limitation in understanding virus transmission thought water cycle is the lack of standardized and validated methods (Pino et al., 2021). While standardized or validated methods exist for the detection and quantification of some human enteric viruses in sewage (WHO, 2003), bottled water (e.g., ISO 15216-1:2017 and ISO 15216-2:2019 standards) and drinking water (US EPA, 2014), limited methodologies are available for detecting other viruses that may be present in water samples.

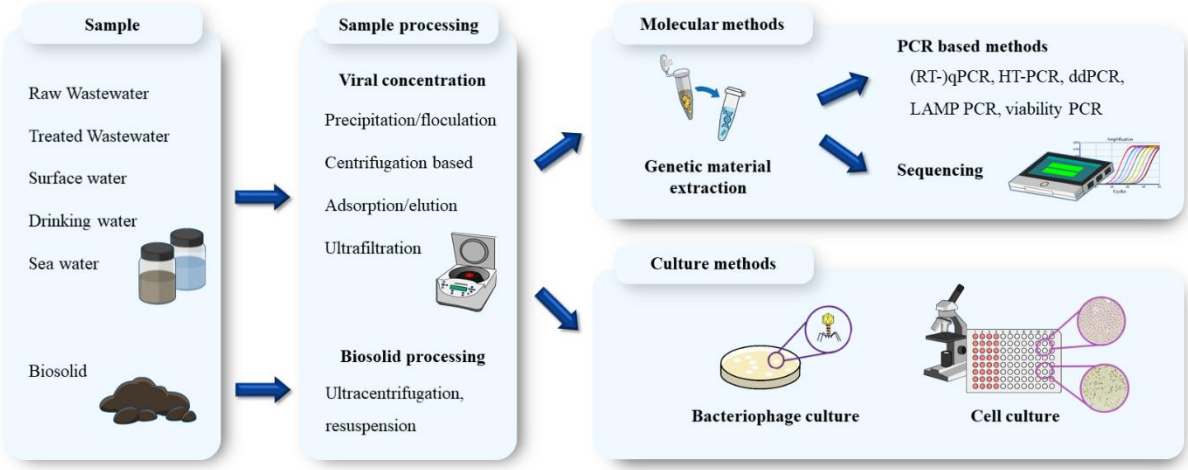

Figure 4. Schematic representation of the samples that can be collected throughout the water cycle, along with the most common processing methods used for sampling processing and analysis.

The methods used to concentrate viruses from water (Figure 4) vary depending on the type of water being analysed. In sewage, where virus concentration is typically high, the volume to be analysed generally ranges from 40 to 200 ml, although the presence of inhibitors and interfering substances is anticipated to be high (Cashdollar and Wymer, 2013; Ikner et al., 2012). Since the beginning of the COVID-19 pandemic, a significant number of laboratories worldwide have been involved in wastewater monitoring programs, with dozens of protocols for SARS-CoV-2 detection published (Mohapatra et al., 2023). Many of these protocols have been adapted from methods previously used for detecting human enteric viruses in sewage (Michael-Kordatou et al., 2020).

In natural water resources such as reclaimed, surface, drinking and sea waters, procedures for detecting human enteric viruses are more limited. This is primarily because virus concentration in these waters is expected to be low, requiring the use of large volumes of water for analysis. Additionally, challenges such as the co-concentration of PCR inhibitors (e.g., salt), the presence of suspended solids and logistical and cost constraints in delivering water samples to laboratories further complicate the detection process (Cuevas-Ferrando et al., 2021).

1.4.2. Viral concentration methods in sewage and non-sewage samples

Numerous approaches are available for detecting viruses in wastewater samples, including ultrafiltration, filtration with electronegative membranes, ultracentrifugation, precipitation with polyethylene glycol (PEG), aluminum polychloride flocculation, skimmed milk flocculation, among others (Bofill-Mas and Rusiñol, 2020; Farkas et al., 2022). Despite numerous comparisons of concentration methods in wastewater, standardization remains lacking (Barril et al., 2021; Farkas et al., 2022).

Due to low viral concentration in non-sewage samples, larger volumes and two-steps concentration are required. For instance, Borgmästars et al., (2017) performed a primary concentration using Dead-End Ultrafiltration (DEUF) method based on single-use filters. Subsequently, a secondary concentration was performed through PEG precipitation to further reduce the sample volume, thereby achieving even higher genetic material concentration). This method has been validated for viruses such as bacteriophages MS2 and PhiX174 (Kahler et al., 2015; Mull and Hill, 2012) and coronaviruses (Monteiro et al., 2022). Other methods for virus concentration are based on glass wool or electropositive cartridges, which also require a second-step concentration (Blanco et al., 2019; Deboosere et al., 2011; Lowther et al., 2019; US EPA, 2014).

Each concentration method has its own advantages and limitations (Table X). The choice of the most suitable method depends on factors such as the type and quantity of viruses present in the sample, operational conditions, specific equipment and cost (Bofill-Mas and Rusiñol, 2020; Cashdollar and Wymer, 2013; Ikner et al., 2012).

Concentration principle	Concentration method	Water type and volumes	Advantages	Drawbacks
Precipitacion/Floculacion (Chemical precipitation)	PEG/Dex	500 mL	Efficient as secondary concentration Useful for large volume samples	Intra-assay variability
	SFM	50 - 500 mL	Field deployable	Time-consuming
		10 - 20L	One step method	
		10 L	Useful for large volume samples	Skimmed milk can add inhibitors to qPCR
	Ammonium sulfate	20 - 200 mL	Efficient for dirty samples or as secondary concentration	High cytotoxicity
		20 - 200 mL		Inhibitory to RT-PCR enzymes
	Aluminium polychloride flocculation	35 - 100 mL	Low cost Useful for turbid samples	Cannot be used for large volume samples Require preconditioning/centrifugation
	Organic flocculation	500 mL - 1L	Efficient for dirty samples or as secondary concentration	Beef extract is inhibitory to RT-PCR enzymes
Centrifugation based (Physical sedimentation)	All		Good recoveries Expensive equipment needed	Cannot be used for large volume sample Useful for turbid samples
	Ultracentrifugation	15 - 42 mL	No preconditioning One step method	Only useful for small volume samples
		36 mL	Efficient as secondary concentration	Costly
Ultrafiltration (Particle size separation)	TFUF	1 L	Useful for large volumes	It can require filter conditioning
		10 - 50L	Can be automatised	Time-consuming
	DEUF	10 - 6000 L	One step method depending on sample type Good recoveries for clean samples	Costly
		10 - 100 L	Simultaneous concentration of several pathogens	Cannot be used for turbid samples
Adsorption–elution methods (Ionic charge)	Negative filters	1 - 5 L	Low cost	No useful for turbid samples
		50mL - 20L	Direct extraction can be done directly from membrane	Requires preconditioning
		100 mL - 20L	Good recoveries	No useful for large volume samples
	Positive Filters	1 - 20L	Useful for large volumes Field deployable	No useful for turbid samples
		5 - 20L	Good recoveries	Costly
	Glas powder	20 L	Low cost	
		20 L		Fragile apparatus
		20 L	Good recoveries	
	Glass wool	5 - 20 L	Field deployable	Time consuming Recovery variability
Freeze-drying	Lyophilisation	50 mL	Efficient for dirty samples or as secondary concentration May remove RT-PCR enzymes inhibitors	Costly Time-consuming
Immunoaffinity	Magnetic beads	50 mL	Good recoveries from small volumes	Requires specific assay for each virus Costly Limited data available

Table 1. Advantages and drawbacks of concentration methodologies for viruses in wastewater (orange), sea (green) and regenerated and surface water (blue). PEG/Dex, polyethyleneglycol dextran precipitation or two phase method; SMF, skimmed milk flocculation; TFUF, tangential flow ultrafiltration; DEUF, Dead-end ultrafiltration. Figure adapted from Bosch et al., 2008; Bofill-Mas & Rusiñol, 2020; Farkas et al., 2022; Gajardo et al., 1991; Villena et al., 2003; Oh et al., 2022.(Bofill-Mas and Rusiñol, 2020; Bosch et al., 2008; Farkas et al., 2022; Gajardo et al., 1991; Oh et al., 2022; Villena et al., 2003)

1.4.3. Virus detection and quantification

Traditionally, cell culture-based methods have been used in clinical virology for viral detection and quantification. However, these methods have considerable limitations when applied to environmental samples due to co-contamination by multiple virus species, the absence of permissive cell lines for certain viruses the low number of viral particles and the cytotoxic effects of wastewater in cell culture (Gerba et al., 2018; Lodder et al., 2010). Even though, cell-culture methods have been used for EV, poliovirus, AdV and RV isolation and quantification (Bosch et al., 2008; Mahalanabis et al., 2010; Mocé-Llivina et al., 2004; Thorley and Roberts, 2016). Abad et al. (1997) (Abad et al., 1997) also employed culturing methods and describe a mixed technique that integrates cell culture and RT-qPCR (ICC-RT-qPCR) to detect astrovirus present in water. This has led to a reduction of the time required for conventional cell culture techniques (Farkas et al., 2020a). In recent years, novel approaches utilizing cell culture systems such as human intestinal enteroids (HIE) have emerged as promising alternatives for the detection and study of enteric viruses in environmental samples, including water (Carmona-Vicente et al., 2024; Desdouits et al., 2022; Kennedy et al., 2023; Shaffer et al., 2022). HIE are three-dimensional structures derived from intestinal stem cells that closely mimic the architecture and function of the human intestine and provide a physiologically relevant model for studying the interaction between the intestinal epithelium and enteric viruses such as HuNoV, AdV, HAstV and RV (Carmona-Vicente et al., 2024; Saxena et al., 2015).

As commented above, due to the challenges in propagating viruses in cell cultures, which are considered the gold standard for assessing virus infectivity, the detection of viruses has traditionally relied on molecular methods that do not distinguish between infectious and non-infectious viruses (Li et al., 2009). Established methods for virus detection in water samples, such as those outlined by the International Organization for Standardization (ISO) and the Food and Drug Administration (FDA) procedures, utilize RT-qPCR and are highly specific and sensitive (FDA, 2021; ISO 15216-1:2017.).

In the last decades there has been a surge in the development and application of molecular techniques for detecting viruses in water. In addition to (RT)-qPCR techniques, various methodologies have been developed and adapted to enhance the sensitivity, specificity and efficiency of detecting a wide range of viruses in aquatic environments.

One of these emerging techniques is droplet digital PCR (ddPCR), which allows for the absolute and precise quantification of viral genetic material in samples with low virus concentration. Consequently, standards for quantification are not required. ddPCR divides the sample into thousands of independent microdroplets, facilitating the detection of weak signals and

reducing the interference of inhibitors present in environmental samples (Li et al., 2018). However, this technique incurs higher costs compared to other methods and in samples such as wastewater, prior dilution of the sample is necessary due to its narrow quantification range (Farkas et al., 2020a).

Another notable technique is loop-mediated isothermal amplification (LAMP), which offers a quick and simple alternative to traditional PCR. LAMP selectively amplifies specific sequences of viral DNA or RNA at constant temperatures, eliminating the need for thermal cycling and simplifying the amplification and detection process (Tomita et al., 2008). Additionally, it is less sensitive to inhibitors compared to traditional PCR and can be multiplexed (Huang et al., 2018; Zhang et al., 2018).

The PCR technique based on clustered regularly interspaced short palindromic repeats (CRISPR-PCR) has emerged as a promising tool for the rapid and specific detection of viruses in environmental and food samples. By leveraging the ability of Cas proteins to recognize specific viral DNA sequences, CRISPR-PCR can detect and distinguish viruses with high precision and sensitivity. However, there are limitations to applying this technique, such as the lack of standardization, preventing cross-reactions and a high background signal caused by nonspecific activation of the CRISPR-Cas systems (reviewed by Yin et al., 2021).

Molecular techniques serve as valuable and sensitive tools for detecting the majority of genetic material present in a sample. However, these techniques cannot provide information about viral infectivity (Corpuz et al., 2020) (Figure 5). Alternative methods assessing the binding ability, capsid integrity, or nucleic acid integrity have been proposed as indirect measurements of viral infectivity. Saliva and porcine gastric mucin (PGM) contain multiple human histo-blood group antigens (HBGAs), which have been recognized as (co-)receptors for HuNoV (Tian et al., 2005). These binding assays have been used to evaluate the occurrence of potentially infectious viruses in influent and effluent wastewater samples (Figure 5), not only for HuNoV but also for RV and HAstV (Cuevas-Ferrando et al., 2022).

As an alternative, researchers have explored proxies for viral infectivity, mainly focusing on capsid-integrity approaches. These assays primarily use intercalating dyes like propidium monoazide (PMA) and platinum chloride (PtCl4). These compounds interact with the genomes of inactivated viruses that have compromised capsids or with free viral genomes, thereby inhibiting (RT-)qPCR amplification. Consequently, only viruses with intact capsids can be detected by (RT-)qPCR following a capsid integrity treatment, due to the inability to bind to the genomes of viruses with intact capsids (Canh et al., 2022) (Figure 5). The procedure involves pre-treating the concentrated sample with an intercalating dye (viability marker) that specifically binds to free and accessible genetic material from capsid-compromised viruses, preventing PCR amplification

(Fittipaldi et al., 2010). Viability PCR using PMAxx and PtCl4 has proven useful in estimating virus infectivity in viral suspensions subjected to certain inactivation/disinfection processes, such as thermal treatments, high-pressure and chlorine treatment (Canh et al., 2019; Randazzo et al., 2018; Sánchez et al., 2012). However, when applied to naturally-contaminated water samples, further improvements are needed, as PCR signals from inactivated viruses are still detected (Randazzo et al., 2018).

An additional analytical tool is full-length or long-range RT-PCR, which has been used to estimate genomic integrity as a proxy for viral infectivity. However, due to decreasing amplification efficiency with increasing fragment size, its robustness and sensitivity have not always been consistent (Pecson et al., 2011; Raymond et al., 2023)).

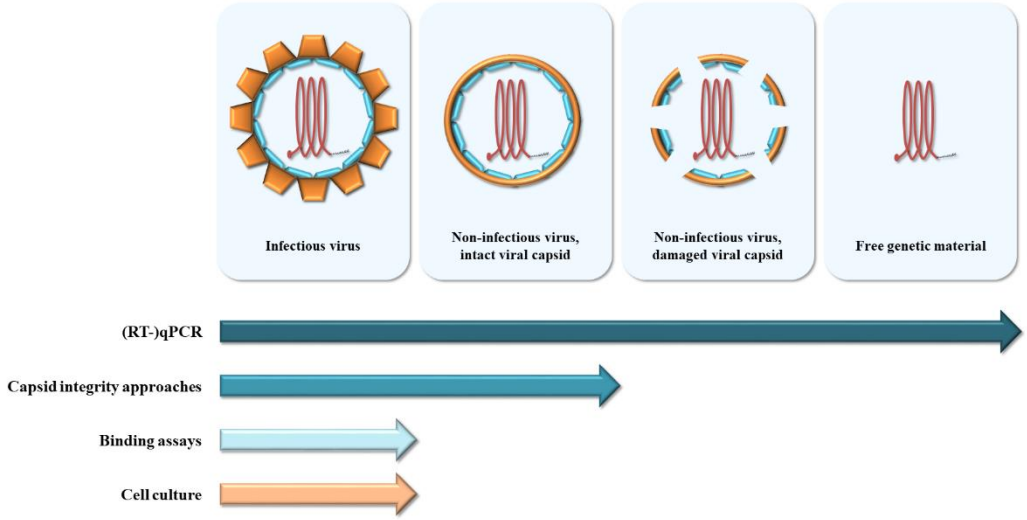

Figure 5. Methods for virus detection depending on viral infectivity/capsid integrity

1.4.3. Virus characterization by sequencing

Sequencing is a technique that allows to understand organism's genomes, meaning the precise order of nucleotide bases that make up its genetic material (De Salazar et al., 2022). This information helps in understanding from the evolution and spread of a particular virus, to its behaviour in the context of an epidemic outbreak or pandemic (Quer et al., 2022). This technology has undergone three generations of development. The 1st Generation Sequencing or Sanger sequencing employs enzymatic synthesis to generate the complementary DNA strand to the original one, being sequenced by adding marked dideoxynucleotides (Sanger et al., 1977). However, the high costs of this methodology made the routine use of this technique unfeasible for

large sequencing projects, which forced researchers to develop techniques that would reduce costs. This is how the second generation of sequencers emerged, offering higher performance at a lower cost by generating millions of DNA fragments in a single process, known as high-throughput sequencing. Different chemistries were developed, such as the pyrosequencing (nowadays discontinued), the sequencing by synthesis, or the sequencing by ligation (Satam et al., 2023). Lastly, the 3rd Generation Sequencing, also known as Single Molecule Sequencing, represents a significant advancement by allowing real-time sequencing without DNA fragmentation or clonal amplification. In this sequencing approach, polymerases are fixes in wells and is the DNA strand which passes through the enzyme. Two main techniques are employed in these sequencers: detection of an electric voltage shift and fluorophore caption (Goodwin et al., 2016). This third generation has some advantages over other sequencing types, being easy and fast and allowing the sequencing of long reads in real time, although the accuracy and depth of analysis are lower (De Salazar et al., 2022; Hu et al., 2021).

The use of sequencing techniques for the study of viruses in water and food has increased in the last years. Different approaches can be employed, such as the shotgun metagenomic studies or the genomic characterization by amplicon sequencing. In the case of metagenomics, the methodology allows the analysis of a wide number of viruses present in a single sample (Wooley et al., 2010). Despite its powerful resolution capacity, it has several issues that must be considered. For example, as the presence of viruses in environmental samples are lower than other microorganisms, the sample preparation its crucial to reduce the non-viral nucleic acids. Additionally, it requires deep bioinformatic analyses that not always can be automated and the data interpretation can be challenging due to miss-annotation of sequences or the low knowledge about environmental and food viromes (Nieuwenhuijse and Koopmans, 2017).

The genomic study of environmental viruses using amplicon sequencing is also one approach that allows the analysis and characterization of a specific virus in the analysed sample. In contrast with metagenomics, this approach needs a previous knowledge of the targeted genome, but allows the study of the complete variants of the targeted virus. This approach has been widely used during the SARS-CoV-2 pandemics, evidencing the usefulness of the technique to determine the presence of variants of concern in the population, as well as the ability to detect the introduction of new variants and the detection of unknown mutations (Crits-Christoph et al., 2021; Nemudryi et al., 2020; Pérez-Cataluña et al., 2022b).

1.5. Virus stability in Waters

Given the relevance of viruses in the water cycle, research has focused on their stability in water samples and the factors affecting their behaviour. This includes not only human enteric viruses but also other viruses that may be present in water. Thus, in recent years, significant attention has been given to evaluating virus decay in water. Traditionally, these evaluations involved contaminating a water sample with a known amount of virus and measuring the viral titter after storing the sample under specific conditions (Pinon and Vialette, 2019). However, this approach relied on viruses that could be cultured in cell lines and quantified through infectivity assays. This factor limited the range of viruses and strains that could be studied, such as HuNoV and wild-type strains of HAV and HEV (Estes et al., 2019; Fu et al., 2019; Kanda et al., 2020; Todt et al., 2020), for which in-vitro cultivation systems remain challenging, or viruses like SARS-CoV-2 and MPXV, which require BSL-3 laboratories (Hughes et al., 2017; Widera et al., 2021).

Virus detection through cell culture primarily relies on observing cytopathic effects (CPE), followed by quantification using plaque assays, the most probable number method, or tissue culture infectious dose (TCID50) with surrogate viruses or cell-adapted strains. Recently, significant progress has been made in developing systems capable of cultivating difficult-to-grow viruses, such as HuNV, using HIEs (Ettayebi et al., 2016), as well as alternative models like the zebrafish model and human salivary glands (Ghosh et al., 2022; Tan et al., 2023; Van Dycke et al., 2019).

However, it is crucial to acknowledge that certain limitations persist in conducting viral stability studies with wild-type viruses in cell culture. In response, researchers have turned to cultivable surrogates. Nevertheless, the appropriateness of each surrogate has been questioned and requires additional validation (Leland and Ginocchio, 2007).

Numerous studies have explored the stability of viruses in water, identifying various factors that influence their persistence and viability. From the perspective of the environment surrounding the virus and conditioning its stability, the following factors are identified: the type of matrix, temperature, sunlight, salinity, indigenous microbial population, organic matter, the presence of disinfectants such as chlorine, extreme pH levels and aeration (Garver et al., 2013; Rzezutka and Cook, 2004; Shahid et al., 2009; Wade et al., 2010; Yates et al., 1985).

Temperature may expedite the process of protein denaturation and nucleic acid breakdown (Yeager and O'Brien, 1979), but sunshine, especially UV radiation, can directly harm viruses (Flannery et al., 2013). Salinity weakens the protective barrier of viruses by raising osmotic pressure (Poulson et al., 2016), while organic matter affects the clumping together and

sticking of viruses to surfaces (Gassilloud and Gantzer, 2005). Nevertheless, when viruses successfully adhere to surfaces or particles, such as microplastics, their stability in water is enhanced (Moresco et al., 2022). The autochthonous microbial community in the sample has the ability to inactivate or outcompete viruses. Disinfectants like chlorine, substances with very high or low pH levels and copper function by oxidizing the viral components, hence diminishing their ability to cause infection (as discussed by Pinon and Vialette, 2019). Furthermore, the intrinsic properties of a virus, such as the presence of a lipid envelope, capsid constraints, or type of genome, greatly impact virus stability and resistance to environmental factors (De Paepe and Taddei, 2006). Enveloped viruses, in particular, tend to be more vulnerable to disinfectants and unfavourable environments (Howie et al., 2008).

While human enteric viruses exhibit high persistence in water, limited information is available for non-enteric viruses. Overall, studies have shown that human enteric viruses or their surrogates survive longer than enveloped viruses or their surrogates in different types of waters. A recent meta-analysis review on the decay rates of waterborne viruses concluded that these rates vary significantly between viruses and are influenced by factors such as temperature, light conditions and enumeration method (Boehm et al., 2019). Overall, studies indicated greater stability at 5°C compared to 25°C. For instance, in a human volunteer study, groundwater inoculated with HuNoV remained infectious for 61 days at room temperature, leading to illness (Seitz et al., 2011), while HAV can readily survive in water for several months. Studies with non-enteric viruses are limited; however, avian influenza viruses can persist for many weeks in water with low salinity and low temperatures (Brown et al., 2007; Weber and Stilianakis, 2008).

OBJECTIVES

2. OBJECTIVES

The main objective of this thesis was to detect and characterize viral pathogens present in waters through the use of molecular and cell culture methods in order to better understand their dynamics and behavior.

For this purpose, the following specific objectives were set:

- To apply wastewater-based epidemiology as a method to detect viruses circulating in the population.

- To compare and optimize methods for the concentration and detection of viral pathogens in water.

- To identify the presence of viruses at different stages of the urban water cycle and evaluate the effectiveness of urban water treatment plants.

- To study the stability of viruses in water by cell culture and viability PCR.

RESULTS

3. RESULTS

3.1. Urban wastewater-based epidemiology for multi-viral pathogen surveillance in the Valencian region, Spain

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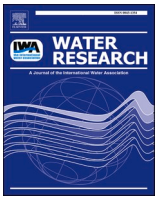

Urban wastewater-based epidemiology for multi-viral pathogen surveillance in the Valencian region, Spain

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ABSTRACT

Wastewater-based epidemiology (WBE) has lately arised as a promising tool for monitoring and tracking viral pathogens in communities. In this study, we analysed WBE's role as a multi-pathogen surveillance strategy to detect the presence of several viral illness causative agents. Thus, an epidemiological study was conducted from October 2021 to February 2023 to estimate the weekly levels of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Respiratory Syncytial virus (RSV), and Influenza A virus (IAV) in influent wastewater samples (n = 69). In parallel, a one-year study (October 2021 to October 2022) was performed to assess the presence of pathogenic human enteric viruses. Besides, monitoring of proposed viral fecal contamination indicators crAssphage and Pepper mild mottle virus (PMMoV) was also assessed, along with plaque counting of somatic coliphages. Genetic material of rotavirus (RV), human astrovirus (HAStV), and norovirus genogroup I (GI) and GII was found in almost all samples, while hepatitis A and E viruses (HAV and HEV) only tested positive in 3.77 % and 22.64 % of the samples, respectively. No seasonal patterns were overall found for enteric viruses, although RVs had a peak prevalence in the winter months. All samples tested positive for SARS-CoV-2 RNA, with a mean concentration of 5.43 log genome copies per liter (log GC/L). The tracking of the circulating SARS-CoV-2 variants of concern (VOCs) was performed by both duplex RT-qPCR and next generation sequencing (NGS). Both techniques reliably showed how the dominant VOC transitioned from Delta to Omicron during two weeks in Spain in December 2021. RSV and IAV viruses peaked in winter months with mean concentrations 6.40 and 4.10 log GC/L, respectively. Moreover, the three selected respiratory viruses strongly correlated with reported clinical data when normalised by wastewater physico-chemical parameters and presented weaker correlations when normalising sewage concentration levels with crAssphage or somatic coliphages titers. Finally, predictive models were generated for each respiratory virus, confirming high reliability on WBE data as an early-warning system and communities illness monitoring system. Overall, this study presents WBE as an optimal tool for multi-pathogen tracking reflecting viral circulation and diseases trends within a selected area, its value as a multi-pathogen early-warning tool stands out due to its public health interest.

1. Introduction

Advances in molecular and next-generation sequencing techniques have revolutionised the analysis of viral genomes from wastewater samples, offering real-time insights into the transmission of infectious diseases among populations (Guo et al., 2022; Gonçalves et al., 2022). In this context, Wastewater-based Epidemiology (WBE) has become a powerful tool for multi-pathogen surveillance, most notably for detecting and tracking COVID-19 and other emerging infectious diseases (Mandal et al., 2020).

Traditionally, WBE has been used to detect and track enteric viruses such as poliovirus (PV), noroviruses genogroup I (HuNoV GI) and GII

Abbreviation list: Wastewater-based Epidemiology, (WBE); Norovirus genogroup I, (HuNoV GI); Norovirus genogroup II, (HuNoV GII); Rotavirus, (RV); Astrovirus, (HAstV); Hepatitis A virus, (HAV); Hepatitis E virus, (HEV); Influenza virus, (IAV); Respiratory syncytial virus, (RSV); Porcine epidemic diarrhea virus, (PEDV); Pepper Mild Mottle Virus, (PMMoV); Valencian Region, (CV); Variant of Concern, (VOC); Mean standard error, (% MSE).

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(HuNoV GII), rotaviruses (RVs), human astroviruses (HAstVs), and hepatitis A (HAV) and E viruses (HEV), which are major causes of infectious diseases, such as poliomyelitis, gastroenteritis and hepatitis, and can have a significant impact on public health, particularly among vulnerable populations like young children and the elderly (Prevost et al., 2015; Hirose et al., 2016; Cuevas-Ferrando et al., 2022). As clinical surveillance for most human enteric viruses, influenced by epidemiological, administrative, and financial constraints is very restricted, WBE testing is for instance a well-established tool for poliovirus surveillance and outbreak response (WHO, 2003; Hovi et al., 2005; Ndiaye et al., 2014; Kim et al., 2016).

The virus responsible for COVID-19, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has profoundly impacted global health and economy and its early detection and tracking is crucial to control its spread and mitigate its effects. WBE has been proven effective in detecting the presence of SARS-CoV-2 in wastewater, with results often preceding clinical cases by several days to weeks (Randazzo et al., 2020; Ahmed et al., 2020; La Rosa et al., 2020; Medema et al., 2020; Wurtzer et al., 2020; Wu et al., 2020). Additionally, the ongoing spread and evolution of the virus has led to the appearance of several variants of interest and concern, including Alpha, Beta, Gamma, Kappa/Delta, and Omicron, which can impact the transmissibility, disease severity, and the effectiveness of treatments and vaccines to varying degrees (Choi et al., 2021; Tao et al., 2021). Studies have shown that the prevalence of these variants in wastewater is correlated with clinical data, making early detection and monitoring of local variant spread in wastewater a complementary approach to genomic epidemiology based on individual patient samples (Pérez-Cataluña et al., 2021; Bar-Or et al., 2021; Crits-Christoph et al., 2021; Izquierdo-Lara et al., 2021; La Rosa et al., 2021; Rios et al., 2021; Carcereny et al., 2021; Lee et al., 2022; Haver et al., 2023).

During the COVID-19 pandemic, containment measures significantly reduced the impact of other respiratory viruses, such as influenza virus (IAV) and respiratory syncytial virus (RSV) (Chow et al., 2023). However, after the easing of COVID-19 lockdown restrictions, an increase in influenza and RSV infections has been observed, earlier than in pre-COVID years (Toribio-Avedillo et al., 2023; Hughes et al., 2022; Wolfe et al., 2022). RSV is estimated to cause 33 million cases and 66,000–199,000 deaths in children under five years old worldwide (Hall et al., 2009; Borchers et al., 2013; Shi et al., 2017; Langedijk et al., 2022), while influenza is estimated to result in 3 to 5 million severe illnesses and 290,000 to 650,000 respiratory deaths each year (Iuliano et al., 2018). The shedding of respiratory viruses such as RSV and influenza A in faeces has not been well documented yet, leading to limited use of WBE estimates for these viruses. However, it has been found that these respiratory viruses can be present and detectable in wastewater as stool is not the only source of human contribution (sputum, body secretions, etc.) to wastewater (Ahmed et al., 2023; Koureas et al., 2023; Hellmer et al., 2014).

The primary objective of this study was to gain insights into the epidemiology of respiratory and enteric viruses, particularly in the context of the COVID-19 pandemic, in the Valencian region (Spain). We monitored the levels of viruses, fecal indicators and physico-chemical parameters in wastewater samples and results were confronted with available clinical data to establish accurate and robust estimates of prevalence from wastewater, with the final aim of demonstrating WBE to be a valuable tool for multi-pathogen surveillance and support public health authorities to gain a comprehensive understanding of the spread of infectious diseases.

2. Materials and methods

2.1. Sampling site and sample collection

Grab influent wastewater samples were collected weekly from October 2021 to February 2023 from a wastewater treatment plant (WWTP) located in the Valencian region of Spain serving a total population of 321,622 individuals according to the Government of the autonomous community of Valencia (GVA) (data from 2022). Prevalence of enteric viruses in influent wastewater samples was assessed over one year (October 2021 to October 2022, $n = 53$) whilst respiratory viruses presence was analysed over a longer period (October 2021 to February 2023, $n = 69$) to better evaluate the impact of COVID-19 lockdown restrictions on the selected respiratory diseases. Samples were grabbed early in the morning (7–12 a.m.) by collecting ~500 mL of wastewater in sterile HDPE plastic containers (Labbox Labware, Spain). Collected samples were transferred on ice to the laboratory, kept refrigerated at 4 °C, and concentrated within 24 h.

2.2. Viral concentration method and nucleic acid extraction

Wastewater samples were inoculated with porcine epidemic diarrhea virus (PEDV) strain CV777 as a coronavirus model process control. Two hundred milliliters of wastewater samples were concentrated with the aluminium-based adsorption-precipitation method (Randazzo et al., 2020; AAVV, 2018). In brief, 200 mL of sample were transferred into 250 mL PPCO centrifuge bottles (Thermo Fisher Scientific, Rochester, US) and artificially inoculated with PEDV. Then, pH was adjusted to 6.0 and $Al(OH)_3$ precipitate formed by adding 1 part 0.9 N $AlCl_3$ (Acros organics, Geel, Belgium) solution to 100 parts of sample. The pH was readjusted to 6.0 and sample mixed using an orbital shaker at 150 rpm for 15 min at room temperature. Then, viruses were concentrated by centrifugation at 1700 × *g* for 20 min and the pellet was resuspended in 10 mL of 3 % beef extract pH 7.4, transferred in 50 mL PPCO centrifuge tubes and shaken for 10 min at 150 rpm. Concentrate was recovered by centrifugation at 1900 × *g* for 30 min and pellet resuspended in 1 mL of PBS.

Nucleic acid extraction from wastewater concentrates was carried out using the Maxwell® RSC Instrument (Promega, Spain) with the Maxwell RSC Pure Food GMO and authentication kit (Promega) and the "Maxwell RSC Viral total Nucleic Acid" running program (Pérez-Cataluña et al., 2021).

2.3. SARS-CoV-2 detection and quantification

SARS-CoV-2 quantification was achieved by targeting the N1 region of the nucleocapsid gene. The One Step PrimeScript™ RT-PCR Kit (Perfect Real Time Takara Bio, USA) was used with N1 primers and conditions described by the Centers for Disease Control and Prevention (CDC, 2019).

The prevalence of SARS-CoV-2 Alpha, Delta, and Omicron variants (BA.1 and BA.2) was assessed using five different duplex TaqMan RT-qPCR procedures, with primers targeting the S and N genes - UK/Alpha (S:69/70del), Delta (S:157/158del), Omicron BA.1 (S:214insEPE, S:69/70del), and Omicron BA.2 (S:25/27del) - and a mastermix prepared using the One-Step PrimeScript RT-PCR Kit as previously (Carcereny et al., 2022, 2023).

Each RT-qPCR analysis included duplicate wells with undiluted RNA and a 10-fold dilution to check for inhibition, as well as corresponding negative controls (amplification and extraction) on the LightCycler 480 instrument (Roche Diagnostics, Germany). Standard curve used for SARS-CoV-2 genome quantitation was prepared using commercially available Twist Synthetic SARS-CoV-2 RNA Control (Control 2, MN908947.3) targeting N1. For variant-characteristic specific mutations quantification, Twist Synthetic SARS-CoV-2 RNA Controls 14 (B.1.1.7 lineage), 48 (B.1.1.529, BA.1 lineage), and 50 (B.1.1.529, BA.2 lineage) (Twist Bioscience HQ, USA) were used for Alpha and Omicron variants quantification, respectively. SARS-CoV-2 B1.617.2 (Delta) complete genome was used as Delta variant control (EPI_ISL_2,967,855, Vircell, Spain).

To estimate SARS-CoV-2 gene viral titers, Cq values ≤ 40 were converted into genomic copies per liter using the standard curve and

volumes tested. Inhibition was assessed by comparing average viral titers from duplicate wells of undiluted RNA and 10-fold diluted RNA. Inhibition was confirmed when the difference in average viral titers was $> 0.5 \log_{10}$, and viral titers were inferred from the 10-fold RNA dilution. The relative presence of each SARS-CoV-2 genomic variant was calculated according to Carcereny et al., 2023. Primers and probes, amplification conditions, and limits of quantification and detection for each genomic target detected in this work is available in the Table S3.

2.4. Detection and quantification of other respiratory, enteric, and faecal indicator viruses

Levels of HuNoV GI and GII, HAstV, RV, HAV and HEV were determined using the RNA UltraSense One-Step kit (Invitrogen, USA) as previously described by Cuevas-Ferrando et al. (2022). Detection of crAssphage was established using the qPCR Premix Ex Taq™ kit (Takara Bio Inc, USA) using primers and conditions described by Stachler et al. (2017). Viral detection of PEDV was performed by RT-qPCR using the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio Inc., USA) as described by Puente et al. (2020). Amplification of Pepper Mild Mottle Virus (PMMoV) was determined by using the PMMoV Fecal Indicator RT-qPCR Kit (Promega) following manufacturer's instructions. Viral detection of Influenza A virus was determined using primers from CDC (Research Use Only CDC Influenza SARS-CoV-2 "Flu SC2" Multiplex Assay Primers and Probes) and as described by CDC (protocol of realtime RTPCR for influenza A(H1N1)) by using PrimeScript RT-PCR kit (Takara Bio Inc.) (Sanghavi et al., 2012). RSV detection was performed as previously described (Sanghavi et al., 2012).

Different controls were used in all assays: negative process control consisting of PBS; whole process control to monitor the process efficiency of each sample (spiked with PEDV); and positive (reference material) and negative (RNase-free water) RT-qPCR controls.

Synthetic gBlock gene fragments (Integrated DNA Technologies, Inc., USA) comprising the PCR targeted regions of crAssphage, HuNoV GI and GII, HAstV, RV, HAV, HEV were used to prepare standard curves for quantification. For IAV and RSV quantification, Twist Synthetic InfluenzaV H1N1 RNA control (part number: 103,001) and purified RNA of RSV (Vircell, S.L) were used. PMMoV Fecal Indicator RT-qPCR Kit (Promega) provided PMMoV RNA for generating a standard curve.

2.5. SARS-CoV-2 sequencing

A monthly sample, selected for presenting RT-qPCR results for N1 with Ct<32, was used for SARS-CoV-2 genomic sequencing. Genomic amplification was performed using the primer scheme of the Artic protocol v3 for samples between October 2021 and January 2022, and v4 for the other months (https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019). Amplicon libraries were built following the Classic PCR tilling protocol and sequenced using the MinION Mk1C system (Oxford Nanopore Technologies, Oxford, UK) (Quick et al., 2020). After sequencing, basecalling was performed with guppy software (Oxford Nanopore) with the high accuracy algorithm using a cut-off value of 8 for base quality (Quick et al., 2020). Obtained reads were analysed with Freyja software (https://github.com/andersen-lab/Freyja) using default parameters.

2.6. Coliphage quantification

Five mL of sewage sample were filtered through sterile 0.45 μm pore syringe filters (Labbox Labware, S.L., Spain) to remove bacteria and fungus (Toribio-Avedillo et al., 2021; Nappier et al., 2006). Quantification was performed by using a commercial Bluephage Easy Kit for Enumeration of Somatic Coliphages (BLUEPHAGE S.L., Spain), following the manufacturer's instructions.

2.7. Data collection, normalisation, and statistical analyses

Clinical data for COVID-19, IAV, and RSV cases was obtained from the "Sentinel Surveillance of Acute Respiratory Infection in Primary Care and Hospitals" (SiVIRA) yearly reports, provided by the Microbiological surveillance network of the Valencian Region (MIVA network). SiVIRA-CV is the Acute Respiratory Infection (ARI) Surveillance System in primary care and hospitals in the Valencian Region (CV). In primary care, it is based on syndromic surveillance that provides information on the weekly incidence of ARI through the weekly cases of ARI diagnosed in primary care consultations, through the Ambulatory Information System (SIA) and the Epidemiological Surveillance Analysis (AVE) system. It also includes sentinel surveillance of a selection of patients with ARI, in which primary care family doctors and pediatricians record clinical-epidemiological data and collect a sample for virologic analysis of influenza, SARS-CoV-2 and RSV in the microbiology laboratories. In hospitals, severe ARI clinical processes correspond to hospitalised patients obtained through the ALUMBRA platform (Corporate Analysis Platform of the Regional Ministry of Health).

For Spearman's correlation performance, respiratory viruses levels (GC/L) were normalised by both fecal viral indicators' quantification data (crAssphage, PMMoV, and somatic coliphages) and physicochemical water samples properties (water inflow, chemical oxygen demand, biochemical oxygen demand, phosphorus, total nitrogen, solids in suspension, and electric conductivity). Physico-chemical parameters for each sample were provided by the WWTP. Spearman correlations were carried out between the unshifted time series (each clinic data with its time corresponding virus levels in wastewater) and shifting the data in time by up to 5 weeks before and after wastewater sampling dates.

Predictive models were implemented with the Random Forest algorithm using the R package *randomForest*, known for its intrinsic explainability, robustness to outliers, stability with new data, and adeptness in handling non-linear correlations (Liaw and Wiener, 2002; Keyel et al., 2019; Koureas et al., 2021; Singh et al., 2023; Marin-Ramirez et al., 2024). For each respiratory virus, the data were separated into two sets, "training" ($n = 40$) and "test" ($n = 29$), to fit the models and to have a "test" measure to compare results with. Ideally, the two sets should have exactly the same distribution of the data, so we found this separation to be sufficient. Thus, the same procedure was repeated five times so that the model metrics were the mean of the 5 training runs as a cross-validation method to avoid possible induced biases due to the random splits of the training and test data sets and to assess the performance of the method in a more robust way (Fig. S2).

3. Results

3.1. Occurrence and evolution of viruses detected in wastewater

Weekly influent wastewater samples from a WWTP located in the Valencian region (Spain) were processed by (RT)-qPCR to determine the occurrence and levels of respiratory viruses (SARS-CoV-2, RSV, and IAV), enteric viruses (HuNoVs GI and GII, HAstVs, RVs, HAV, and HEV), and proposed viral fecal indicators crAssphage and PMMoV (Fig. 1) (Table S1). Somatic coliphage quantification were assessed by plaque counting. The recoveries of PEDV, spiked as viral process control, ranged between 6.78 and 49.63 % (data not shown); thus, results of targeted viruses were validated according to Haramoto et al. (2018) and the criteria included in the ISO 15216–1:2017 (recovery of control virus ≥1 %).

Enteric viruses prevalence in wastewater was analysed over one year (October 2021 to October 2022, $n = 53$) (Fig. 1). The detection rates of HuNoV GI, HuNoV GII, HAstVs, and RVs were 98.11, 100, 100, and 92.45 %, respectively. In contrast, HAV and HEV were only detected in 3.77 % and 22.64 % of the samples, respectively. As for the mean concentrations from positive samples for each virus, the highest values were those of RV (7.95 log GC/L), HAstV (7.00 log GC/L), and HuNoV GII

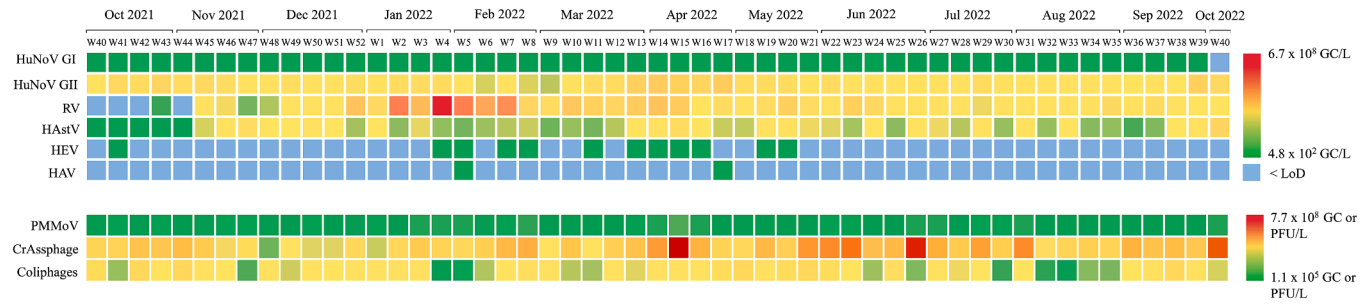


Fig. 1. Levels of enteric viruses and faecal viral indicators (GC/L or PFU/L) in wastewater samples from a single WWTP in the Valencian Region from October 2021 to October 2022. < LoD: Below limit of detection.

(7.58 log GC/L), while the lowest values were for HuNoV GI (4.41 log GC/L), HAV (3.05 log GC/L) and HEV (3.64 log GC/L). Notably, the mean concentration of HuNoV GII was three logarithms higher than that of HuNoV s GI. In terms of distribution, a higher presence of rotavirus was observed in winter (Fig. 1).

As regards the viruses proposed as indicators of fecal contamination, crAssphage and PMMoV were detected in all samples from October 2021 to February 2023 ($n = 69$), while somatic coliphages were also present in all analysed samples from October 2021 to October 2022 ($n = 53$) (Fig. 1). Among the three proposed indicators, mean crAssphage concentration levels were the highest (8.09 log GC/L), being almost three logarithms higher than those of PMMoV (5.71 log GC/L). The plaque count of somatic coliphages yielded a mean concentration value of 7.28 log PFU/L.

In parallel to enteric viruses and viral fecal indicators monitoring, wastewater samples were tested for respiratory viruses over a longer period covering from October 2021 to February 2023 (Table S2). SARS-CoV-2 RNA was detected in all samples tested ($n = 69$) (Fig. S1and Fig. 4A. In contrast, RSV and IAV genetic material was only found in 39.13 % (27/69) and 31.88 % (20/69) of the wastewater samples analysed, respectively (Fig. S1, Figs. 5A, 6A). Regarding the monthly distribution of these viruses, the detection of SARS-CoV-2 in wastewater was fairly constant throughout the entire period analysed, while RSV was detected more in winter and IAV presented a very pronounced peak between March and April 2022. Quantification of SARS-CoV-2 targeted RNA yielded mean concentration levels of 5.43 log GC/L. When testing positive, IAV and RSV mean concentration values in wastewater samples were 6.42 log GC/L and 3.88 log GC/L, respectively.

3.2. *Detection of SARS-CoV-2 variants of concern by molecular and sequencing techniques*

All wastewater samples collected weekly from October 2021 to February 2023 were screened using duplex RT-qPCR to detect the prevalence of specific variants of concern (VOC), namely UK/Alpha (S:69/70del), Delta (S:157/158del), and Omicron (S:69/710del,

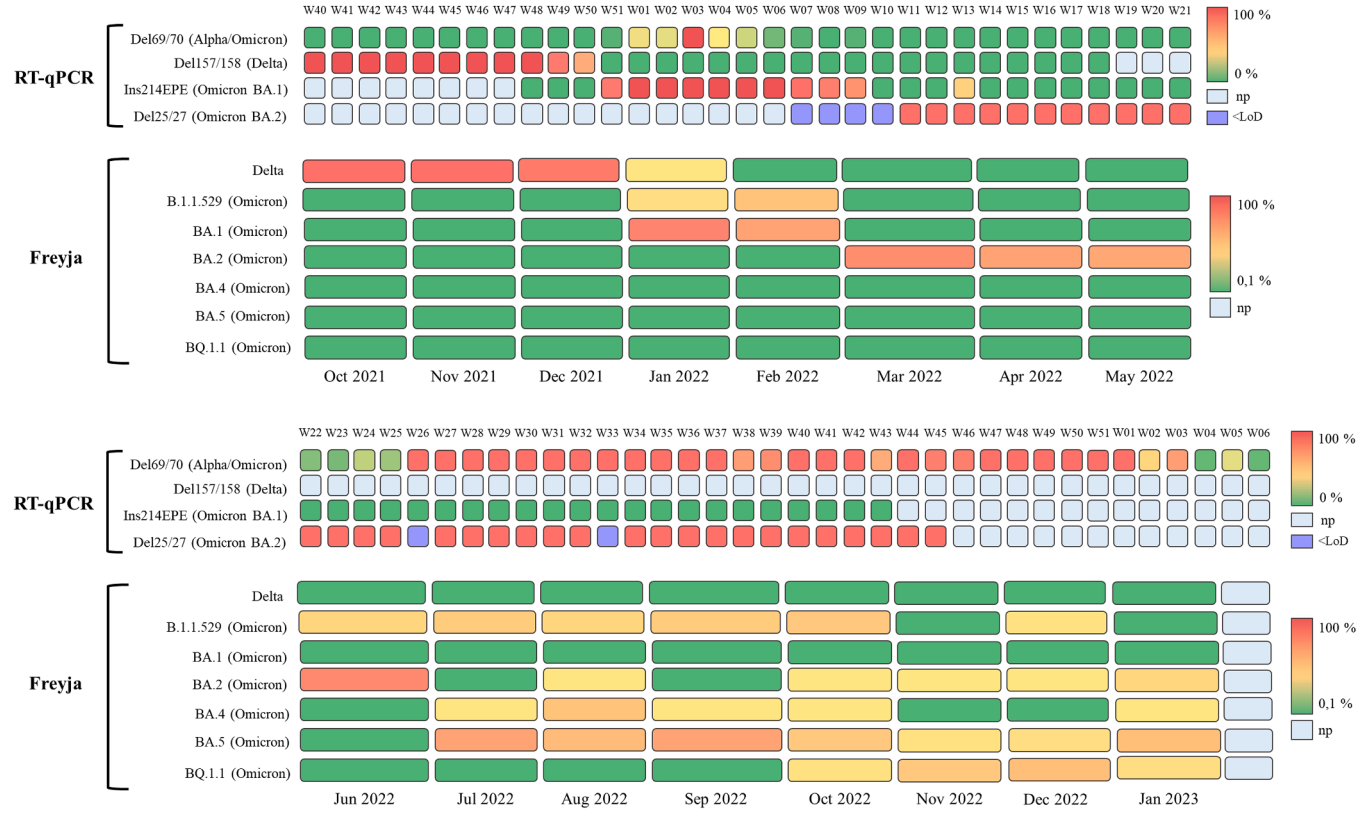


Fig. 2. Percentage of SARS-CoV-2 variants of concern in wastewater detected by duplex RT-qPCR and sequencing analysis with Freyja from October 2021 to February 2023. Np: not performed; < LoD: below the limit of detection.

S:214insEPE, and S:25/27del) (Fig. 2). In addition, these samples were sequenced with an Oxford Nanopore MinION system and analysed using Freyja software, a tool created to recover relative lineage abundances from mixed SARS-CoV-2 samples from a sequencing dataset (Fig. 2).

Duplex RT-qPCR results showed a 100 % prevalence of the Delta variant between weeks 40 and 48 of 2021. Then, between weeks 49 (W49) and 50 (W50, December) of 2021 a reduction in the prevalence of Delta was observed (W49: 90 %, W50: 50 %). Finally, in week 51, the imposition of the Omicron variant (S:214insEPE) was already observed, with a prevalence of 91 %. From week 12 of 2022, the S.25/27del characteristic of Omicron variant BA.2 was imposed. The S:69/70del, associated with Alpha VOC at that time, was not detected during the Delta VOC period but could be found again in the Omicron VOC majority period, as both Alpha and Omicron BA.1 share this mutation.

Freyja analysis included fewer samples as only one sample with sufficient genomic load for efficient sequencing was processed per month. Even so, the results were consistent with those obtained by duplex RT-qPCR. In week 48 of 2021 a slight decrease in the prevalence of the Delta variant was observed (from 100 % in previous weeks to 93.5 %) (Fig. 2) and in January 2022 a majoritarian prevalence of the Omicron variant was already observed, which extends throughout the following weeks of 2022. In line with duplex RT-qPCR results, Freyja analyses detected the emergence of Omicron BA.1 (Jan 2022 – Feb 2022), Omicron BA.2 (Mar 2022 – Jun 2022), and BA.5 (from Jul 2022) (Fig. 3). Also, the emergence of variant of concern BQ.1.1 on October 2022 could be efficiently detected by using Freyja software analyses (Fig. 3). Raw data regarding mutation prevalences can be found in supplementary table S4.

3.3. Correlation of levels of respiratory viruses in wastewaters with public health records and evaluation of normalising factors suitability

RNA quantification data of SARS-CoV-2, IAV, and RSV in wastewater were normalised with somatic coliphages quantification data, PMMoV, crAssphage, and various physico-chemical parameters (chemical and biological oxygen demand, total nitrogen, phosphorus, inflow, electrical conductivity) and Spearman's correlation was calculated with reported clinical cases of infections caused by the aforementioned respiratory viruses. Data from October 2021 to February 2023 were used. Moreover, Spearman's correlations were made with clinical case data reported the same day of sewage sampling (week delay 0) as well as from previous and subsequent weeks (week delay −5 to 5).

Regarding SARS-CoV-2 correlations, COVID-19 cases in Spain were no longer reported from April 2022 in patients below 65 years. Thus, three different correlation studies were performed: a) using all available data from October 2021 to February 2023; b) using only data prior to April 2022 (Fig. 4); or c) using data collected after April 2022. It was observed that in analyses a) and c) the correlations between SARS-CoV-2 RNA and reported clinical cases were neutral or very weak, so they were not included in this work due to its irrelevance. On the contrary, analysis b) (when all clinical cases during the pandemic were being reported) resulted in extraordinarily high ($r > 0.8$, p-value < 0.05) correlations (Fig. 4B). Furthermore, correlations were slightly stronger or equal when comparing virus quantification data in wastewater with clinical case data from the week following water sampling date (delay = 1) compared to data from the same week (delay = 0). Moreover, normalising the wastewater data with microbiological or physico-chemical parameters showed that the weakest correlations were obtained when adjusting RNA quantification data with coliphage counts ($r = 0.434$, p-value < 0.05, delay = 0) and crAssphage RNA levels ($r = 0.572$, p-value < 0.05, delay = 0). Notably, the strongest correlation was obtained by applying no delay and adjusting obtained virus genomic copies per litre with WWTP inflow (m^3/day) ($r = 0.866$, p-value < 0.05), even though, in general terms, correlations were slightly stronger when applying a one-week delay.

As for Influenza virus, strong Spearman's correlations ($r > 0.7$) were observed between IAV genomic copies in wastewater and reported clinical cases (Fig. 5B). Interestingly, the strongest correlations were observed when comparing virus quantification data in wastewater with clinical cases data from the week following water sampling (delay 1). The correlation of unadjusted IAV genomic copies per litre with clinical cases was $r = 0.799$ (p-value < 0.05, delay = 1). Additionally, after normalising the wastewater data with microbiological or physico-chemical parameters, it was found that the lowest correlations were

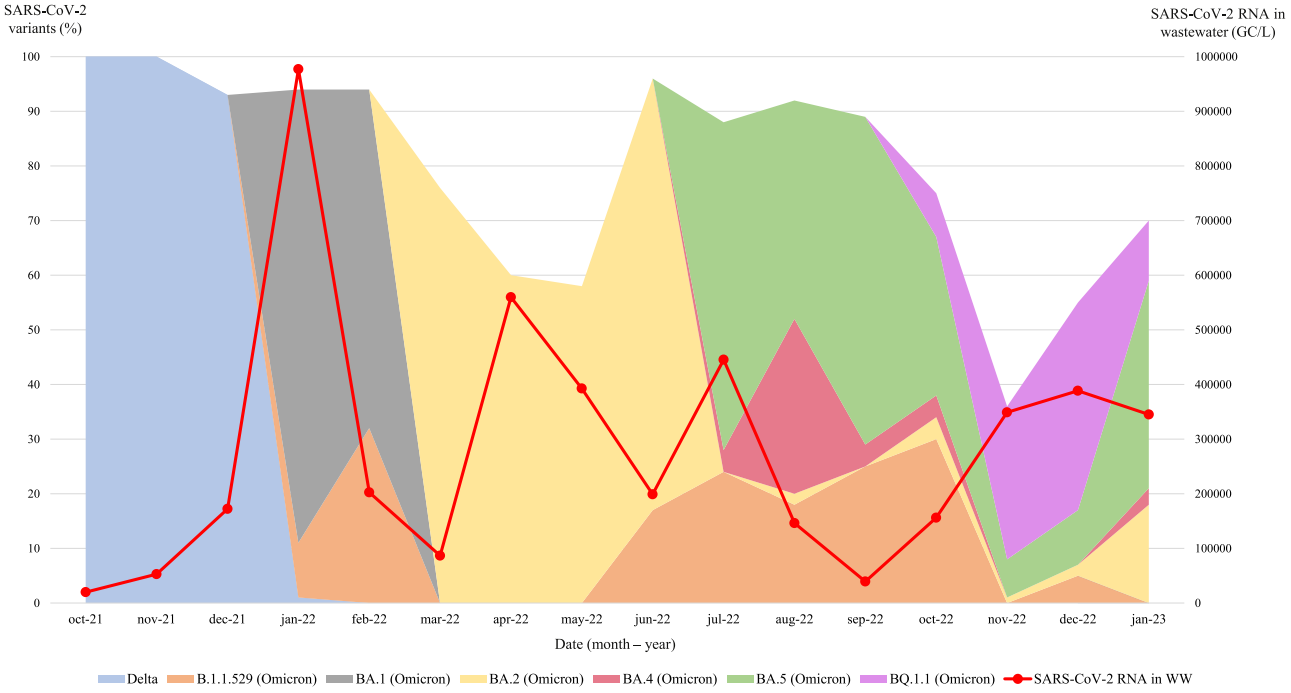


Fig. 3. RT-qPCR results for SARS-CoV-2 RNA levels in influent wastewater samples (red line) and percentage of variants of concern obtained via sequencing analysis with Freyja software from October 2021 to January 2023.

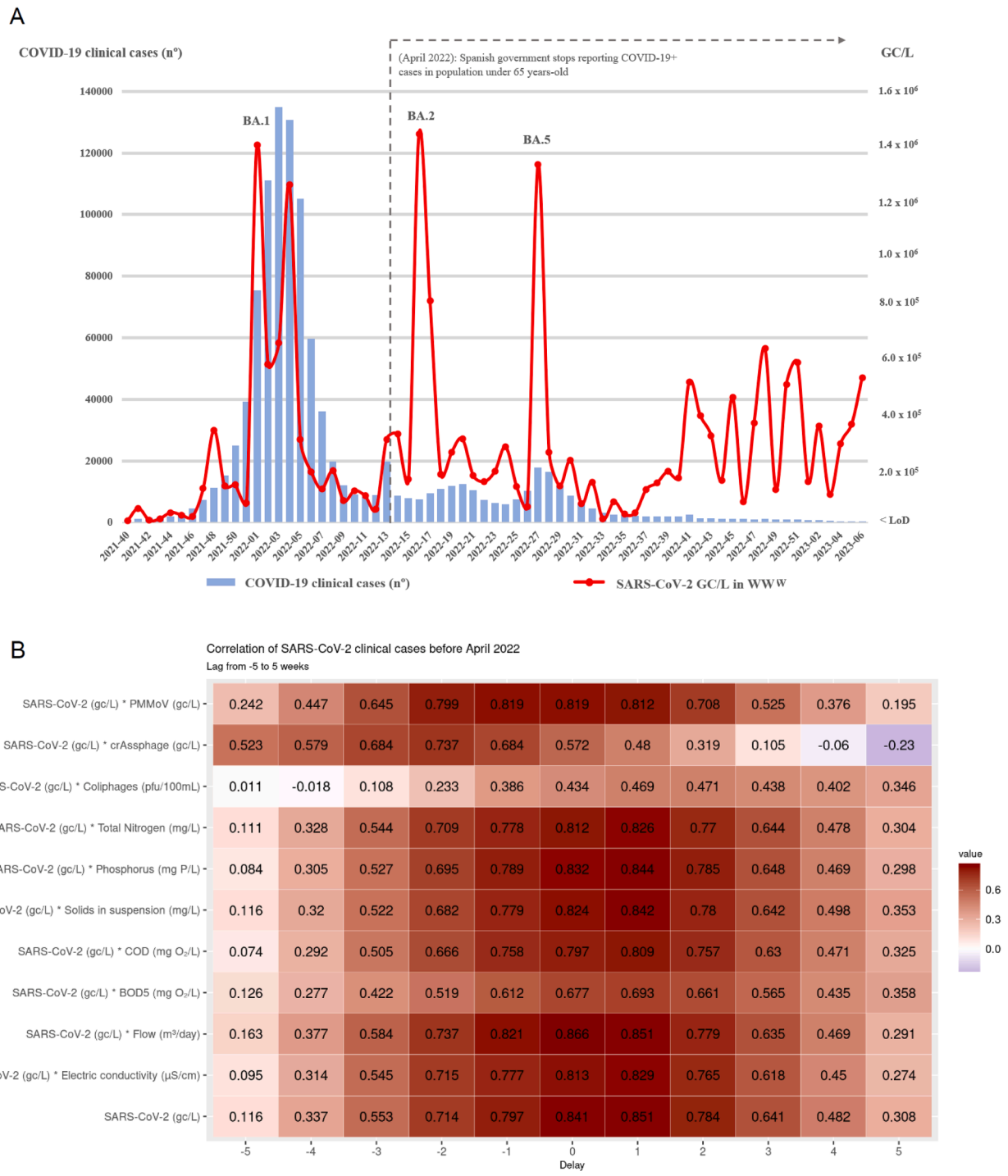


Fig. 4. A) Representation of COVID-19 clinical cases (blue) and SARS-CoV-2 RNA quantification data (GC/L) (red) in wastewaters; B) Spearman's Correlation analysis between COVID-19 clinical cases and SARS-CoV-2 RNA quantification data in wastewater covering the period before April 2022. < LoD: below the limit of detection.

obtained when adjusting water PFU/L of coliphages, while the strongest correlation ($r = 0.808$, p-value < 0.05) was obtained by applying a one-week delay and adjusting virus quantification data with WWTP inflow (m^3/day). Furthermore, in order to estimate the minimum viral concentration in sewage to detect clinical cases in the Valencian region, the relationship between the number of clinical cases on the week following sewage samples collection of IAV and the detection rate of its RNA in wastewater samples was analysed. Those weeks with less than 110 clinical cases reported resulted in 0 % positive wastewater samples for IAV RNA ($n = 33$). As the number of clinical cases increased to a range of 110 to 205 cases per week ($n = 12$), the proportion of positive wastewater samples for IAV RNA significantly increased to 44.5 %. Notably, when the clinical cases surpassed 205 ($n = 22$), all wastewater samples tested positive for IAV RNA, indicating a 100 % detection rate.

In the case of RSV, moderate correlations ($r > 0.6$, p-value < 0.05) were observed between RSV quantification data in wastewater and reported clinical cases without applying any delay to the latter (Fig. 6B). The correlation between unadjusted RSV GC/L and clinical cases was $r = 0.668$ (p-value < 0.05). Notably, the most robust correlations were observed when comparing virus quantification data in wastewater with

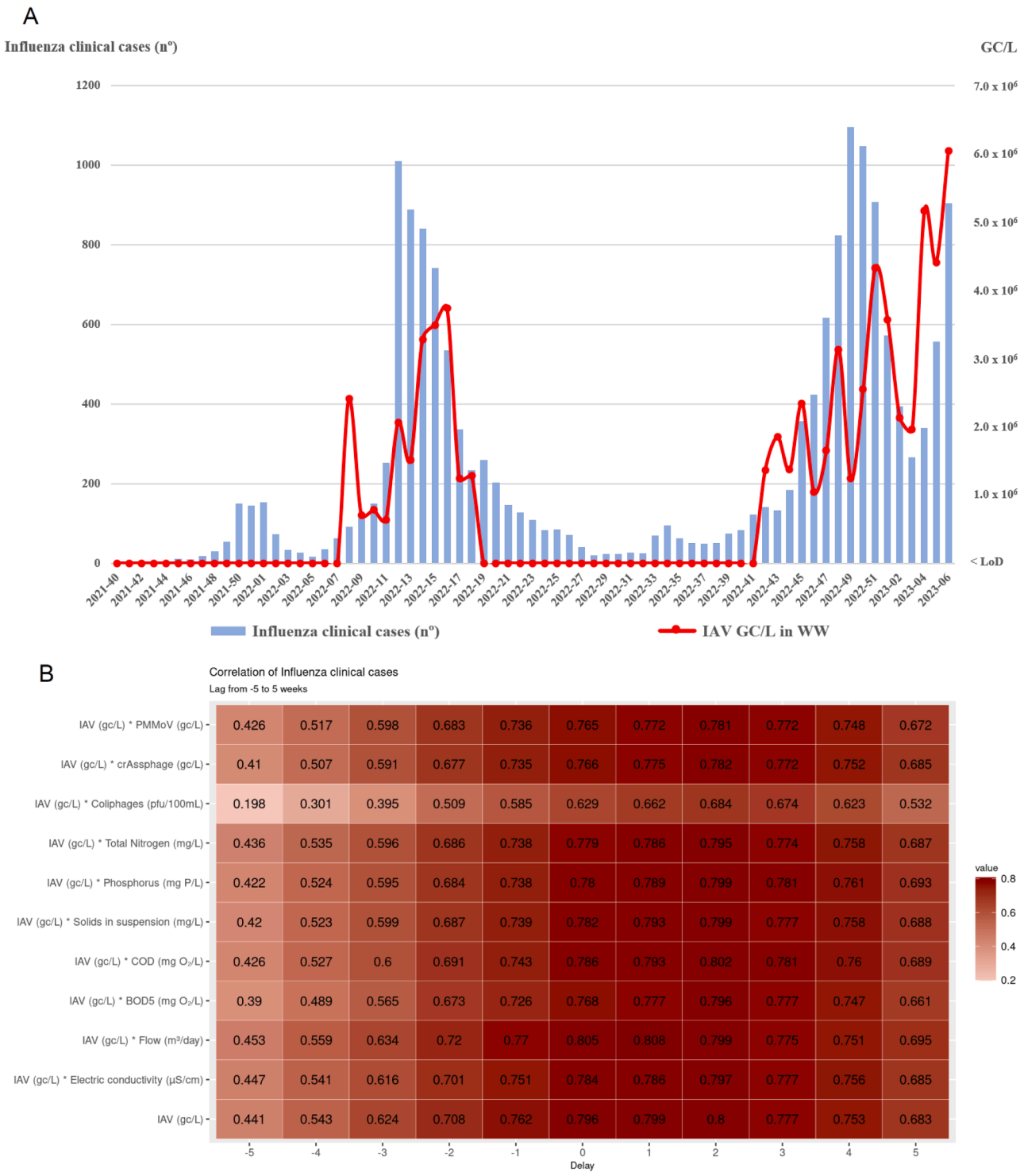


Fig. 5. A) Representation of reported Influenza virus positive clinical cases (blue) and IAV RNA quantification data (GC/L) (red) in wastewaters; B) Spearman's correlation analysis between Influenza virus positive clinical cases and IAV RNA quantification data from October 2021 to February 2023. < LoD: below the limit of detection.

clinical case data collected three weeks after water sampling (delay 3). Similar to the other analysed respiratory viruses, the weakest correlation was observed when adjusting water GC/L with the coliphages PFU/L data ($r = 0.53$, delay = 0) and the strongest correlation was obtained when applying a one-week delay on raw clinical cases data ($r = 0.706$, p-value < 0.05) and adjusting RSV RNA quantification data with the WWTP inflow (m^3/day) ($r = 0.705$, p-value < 0.05). Moreover, the minimum number of clinical cases required for RSV RNA to be detected in wastewater samples collected from the Valencian Region of Spain was estimated regarding clinical cases data of the third week following wastewater sample collection (which yielded the strongest correlations). When the weekly clinical cases were 40 or less ($n = 37$), only two positive wastewater samples for RSV RNA were observed, indicating a 5.4 % detection rate. Subsequently, as the number of clinical cases increased to a range of 40 to 210 per week ($n = 18$), half of the wastewater samples tested positive for RSV RNA, accounting for 50 %. Finally, when the

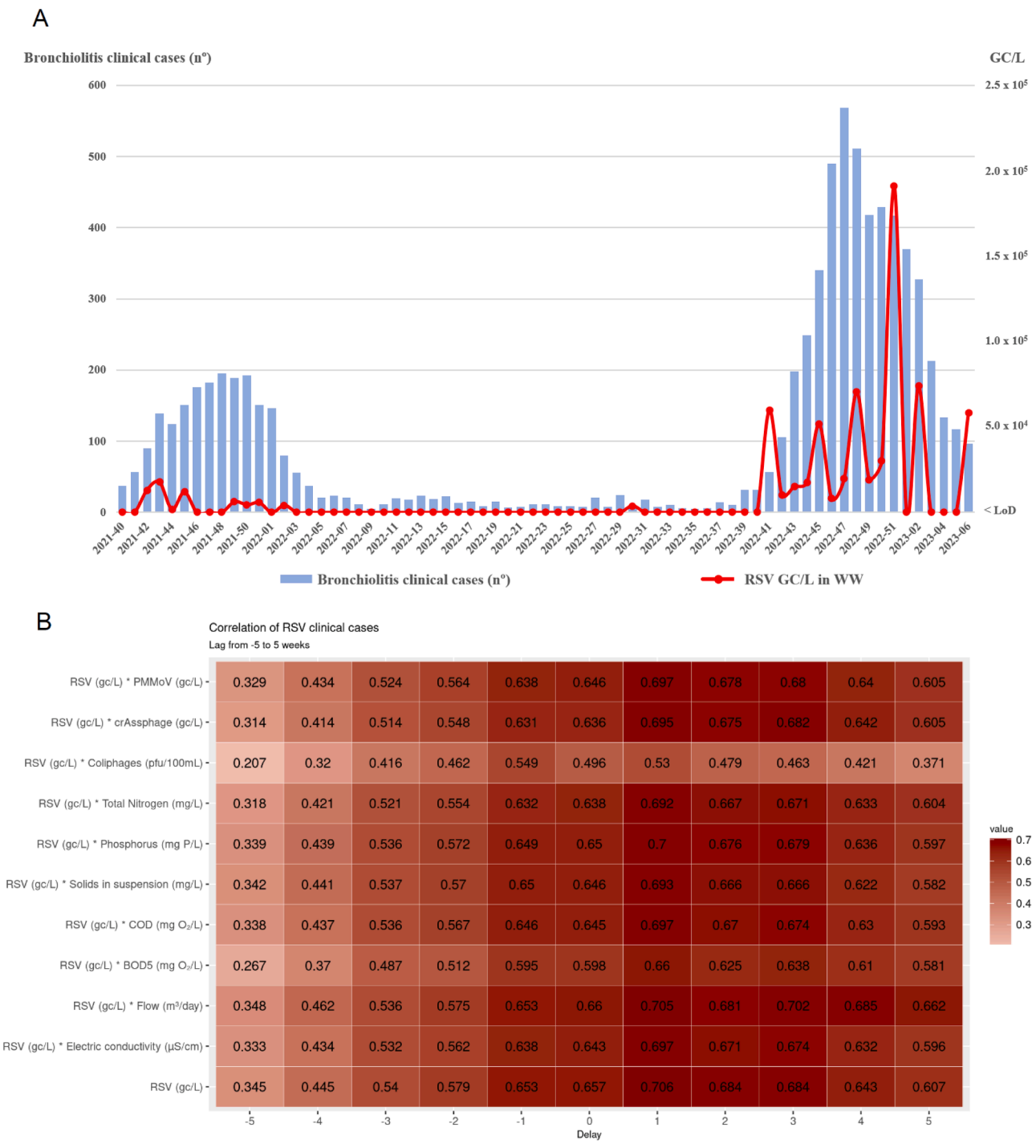


Fig. 6. A) Representation of reported bronchiolitis clinical cases caused by respiratory syncytial virus (RSV) (blue) and RSV RNA quantification data (GC/L) (red) in wastewaters; B) Spearman's correlation analysis between reported RSV positive clinical cases and RSV RNA GC/L in wastewaters from October 2021 to February 2023. < LoD: below the limit of detection.

number of clinical cases surpassed 210 (n = 11), all the wastewater samples tested positive for RSV RNA, indicating a 100 % detection rate.

3.4. Mathematical approximation for respiratory viral illnesses' cases prediction based on virus quantification data in sewage waters

In order to analyse the robustness of the correlations obtained in the previous section and to rule out the spurious nature of these correlations, a model for predicting clinical cases from the quantification of the genetic material of each virus in wastewater was performed by the Random Forest algorithm. For each respiratory virus, predictive models were performed. In the case of RSV and IAV, the clinical data were five-times randomly separated into two sets, "training" (n = 40) and "test" (n = 29), to fit the Random Forest models (RF1-RF5), while for SARS-CoV-2 clinical data were separated in "training" and "test" with a n = 12 each because of the limited data. Five different Random Forest models were run for each virus depending on data set selection: A) complete: with all the available data, both the standardised concentrations and the standardisation factors themselves; B) standardised: only including as variables the virus concentration values standardised according to

biological or physicochemical factors; C) standardised with delay: including the data with the delay that offered the best correlation with the clinical cases; D) standardised concentrations with and without delay; E) standardised concentrations together with the standardisation factors and with and without applying delay.

For SARS-CoV-2, the model with the least error in its estimates (mean error of 18,629.9 clinical cases) was the one that included the normalised virus concentrations and the number of clinical cases from the same week and the week after the wastewater sampling. It should be noted that because the model had to be modelled with few data (due to underreporting of all positive clinical cases), the model has a low reliability (Fig. 7).

In the case of IAV, the model that gave the least error in its estimates (mean error of 108.5 clinical cases) was the one that included the normalised virus concentrations and the number of clinical cases in the week after the wastewater samples were taken (Fig. 8). Furthermore, it was observed that the most relevant factor when introducing error in the model was the one composed of the IAV quantification data corrected for flow rate; modifying or removing this parameter increases the error in the model predictions by 4 %.

With regard to RSV, the model that gave the least error in its estimates (mean error of 52.3 clinical cases) was the one that included the values of all the normalised virus concentrations, the values of the normalisation factors themselves, and the number of clinical cases in the same week and in the third week after wastewater sampling (Fig. 9). In addition, the most important factors introducing error into the composite model were the unnormalised RSV GC/L values themselves, followed by normalisation with crAssphage and flow rate (Fig. 9). Finally, even if the models demonstrate the ability to predict clinical cases, they may not be fully functional given the limited dataset size, but serve well to perform an exploratory analysis of all the data variables. It would be necessary to collect more data from more years and with more frequency to have highly-reliable predictive models, but this study demonstrates its viability as well as describing which variables would be the most interesting when implementing these models in subsequent works.

4. Discussion

In this study, we have undertaken a multifaceted analysis centered around three key objectives. Initially, the prevalence and temporal patterns of human pathogenic enteric and respiratory viruses within wastewater samples were assessed. Subsequently, duplex RT-qPCR and Freyja sequencing approaches were performed to identify SARS-CoV-2 Variants of Concern. Finally, the alignment of our findings with public health records was explored, while also evaluating diverse indicators for the normalisation of data. These interrelated investigations collectively contribute to a comprehensive understanding of viral dynamics in wastewater, their implications for public health, and the methodological nuances essential for accurate data interpretation.

4.1. Occurrence and trends of human pathogenic viruses detected in wastewater

Overall, the results obtained for the prevalence of enteric viruses in wastewater are in line with existing literature (Cuevas-Ferrando et al., 2022; Kitajima et al., 2014; La Rosa et al., 2010; Haramoto et al., 2018). Prevalence of 100 % or nearly 100 % was observed for RVs, HAstVs and HuNoV GI and GII. Notably, the mean concentration of HuNoV GI was three logs GC/L lower than that of the GII genotype. HAV and HEV, on the other hand, were only detected in 3.77 % and 22.64 % of the samples analysed, respectively, with average concentrations of ~3 logs GC/L. In general, these results agree with those described in Corpuz et al., 2020 review paper but show lower HAV detection rates than those recently described in Argentina (Fantilli et al., 2023). However, the HuNoV GI concentrations obtained in this work (~4 logs GC/L) are much lower than those included in the aforementioned review (9 logs GC/L), and would be more in line with the results of other published works (Farkas et al., 2018).

Regarding the distribution of enteric viruses, only a slight increase in the concentration of RVs was noted during the winter months. As in other studies, despite the reported seasonality of infections caused by this type of viruses, specially RVs (which are more common in winter),

	Data set				
	A	B	C	D	E
Training	6559.9	6691.9	6,462.7	5,617.3	6,844.4
Test	23,367.9	21,344.0	21,701.0	18,629.8	20,453.5
Training %OM	0.191	0.195	0.188	0.164	0.2
Test %OM	0.681	0.622	0.633	0.543	0.596

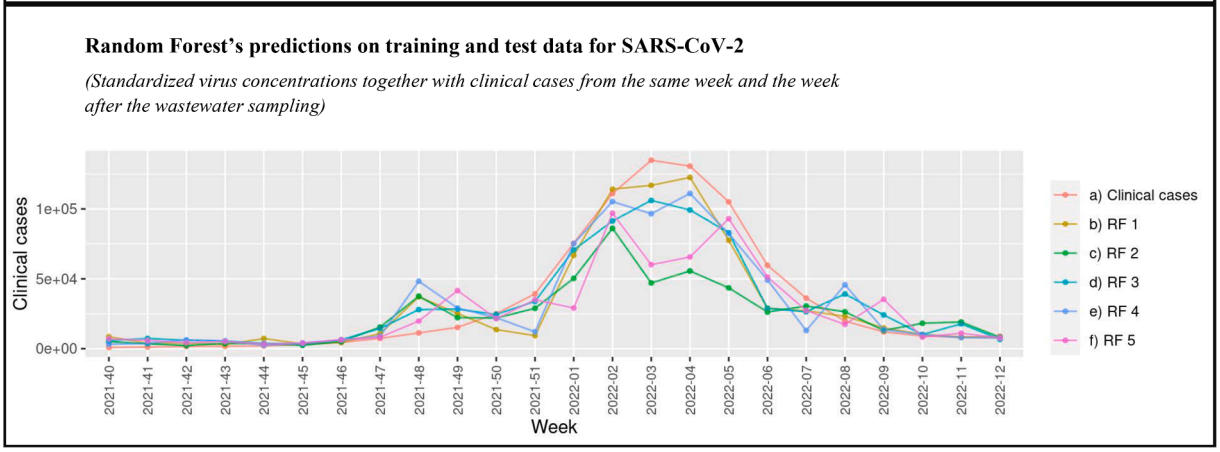
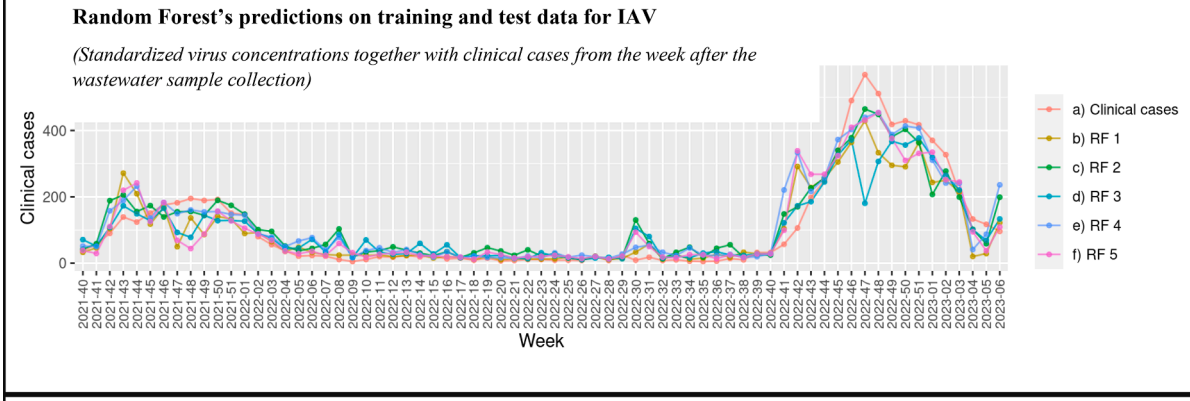


Fig. 7. Top: mean absolute error and its percentage over the mean of clinical cases for the "A-E" data sets; Bottom: Five Random Forest's predictions on training and test data for D) data set (standardised virus concentrations taking into account the number of clinical cases from the same week and the week after the wastewater sampling) for SARS-CoV-2 versus reported "Clinical cases" in the Valencian Region.

	Data set				
	A	B	C	D	E
Training	51.9	67.0	64.2	58.2	49.5
Test	125.6	130.4	108.5	110.6	110.9
Training %OM	0.215	0.277	0,266	0.241	0.205
Test %OM	0.52	0.54	0.449	0.458	0.459



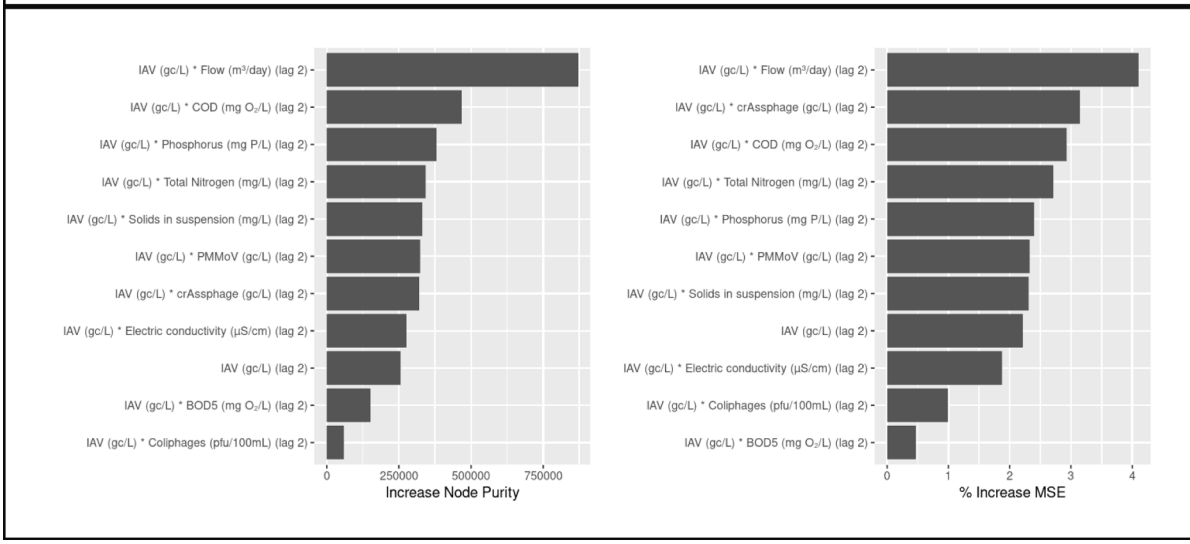


Fig. 8. Top: mean absolute error and its percentage over the mean of clinical cases for the "A-E" data sets; Middle: Random Forest's predictions on training and test data for C) data set (standardised virus quantification data and delayed clinical cases: including the data with the clinical cases from the week following wastewater sample collection) for IAV. Bottom: Importance of factors affecting Random Forest's predictions expressed as Increase in node purity and percentage increase on mean standard error (% MSE).

no marked seasonality was detected for any of them (Farkas et al., 2018; Barril et al., 2015). Elevated concentrations of RV were previously observed in Spain (Silva-Sales et al., 2020). RVs are recognized for causing illnesses in children below 5 years old, particularly in neonates up to 2 years old (WHO, 2013). Consequently, the majority of viruses are retained in diapers and do not enter the sewage system. This suggests that the identified RVs could potentially be affecting older children and adults, likely asymptomatic, and thus highly increasing the virus circulation (SantisoBellón et al., 2020). Also, the continuous detection of enteric viruses throughout the year highlights the intricate nature of their transmission and it is unlikely that weather is the sole cause for the widespread occurrence of enteric virus diseases during the winter season. Multiple studies suggest that factors such as the stability and ability of the virus to persist, alternative modes of transmission, the interaction between the virus and host, and changes in human behavior and susceptibility (including hygiene practices, birth rates, and the likelihood of exposure to the environment) could all contribute to the seasonal trend of enteric virus illnesses (Jagai et al., 2012; Atchison et al., 2009; Pitzer et al., 2011). Furthermore, it should be considered that the Mediterranean climate is characterised by less marked seasons, and with the current climate change scenario, the cold period is much shorter.

For respiratory viruses, IAV and RSV were detected at mean concentrations of 4 and 6 logs GC/L, respectively. These mean concentrations were similar to those reported in the US, Canada, and Australia for the same time period for IAV, and were at least one log higher for RSV (Toribio-Avedillo et al., 2023; Hughes et al., 2022; Wolfe et al., 2022; Ando et al., 2023; Mercier et al., 2022). In the case of RSV, peak concentrations concentrated in the winter months, while IAV had a moderate peak in winter and a much more pronounced peak in spring, as recently reported (Wolfe et al., 2022).

Regarding the prevalence and quantification data of SARS-CoV-2 in wastewater, results in this work are in concordance with many others

	Data set				
	A	B	C	D	E
Training	27.7	50.8	44.8	31.3	22.7
Test	69.1	76.9	62.9	53.3	52.3
Training %OM	0.253	0.464	0.41	0.286	0.208
Test %OM	0.632	0.702	0.575	0.487	0.478

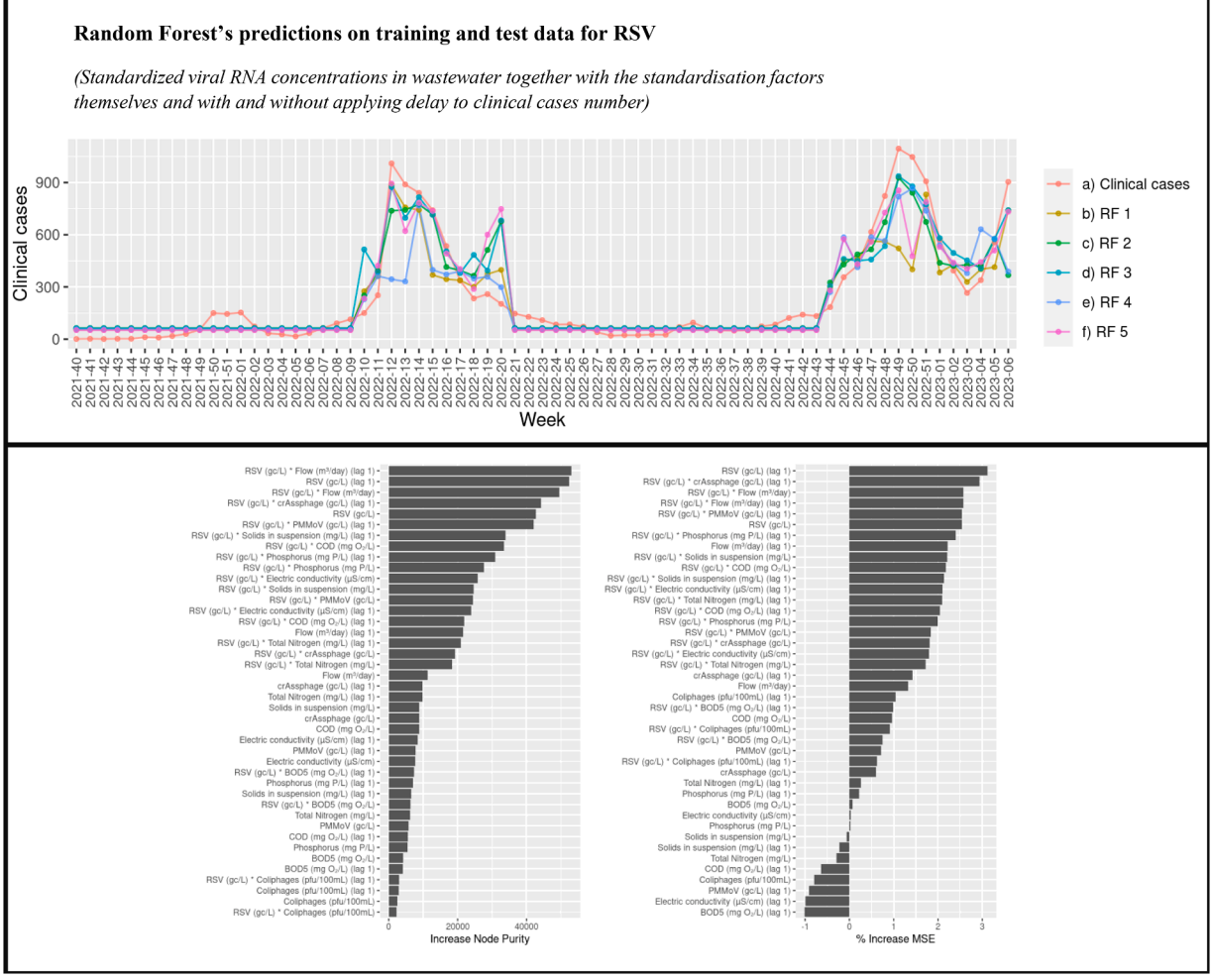


Fig. 9. Top: mean absolute error and its percentage over the mean of clinical cases for the "A-E" data sets; Middle: Random Forest's predictions on training and test data for E) data set for RSV (standardised viral RNA concentrations in wastewater together with the standardisation factors themselves and with and without applying delay to clinical cases number. Bottom: Importance of factors affecting Random Forest's predictions expressed as Increase in node purity and percentage increase on mean standard error (% MSE).

WBE-related works covering different Spanish localities, showing a prevalence of 100 % and an average concentration of 5 logs GC/L throughout the whole sampling period (López-Peñalver et al., 2023; Trigo-Tasende et al., 2023; Mattei et al., 2023).

Finally, the prevalence of crAssphage and PMMoV was assessed due to its proposed role as fecal viral indicator along with the detection of somatic coliphages (Honap et al., 2020; Farkas et al., 2019; Bivins et al., 2020). All three viral indicators were detected in 100 % of the analysed samples, with mean concentrations of 8.09, 5.71, and 7.28 log GC or PFU per liter for crAssphage, PMMoV, and somatic coliphages, respectively. CrAssphage and PMMoV concentrations were in line with existing data (Bivins et al., 2020; Sabar et al., 2022).

Finally, it is essential to consider that the detected concentrations of viruses in wastewater may be influenced by whole-process method variability, fecal shedding that may vary among different variants, heavy rainfall diluting raw wastewater, and population movements (Dhakar et al., 2022; Bivins et al., 2021).

4.2. *Detection of SARS-CoV-2 variants of concern by duplex RT-qPCR and sequencing*

Various global initiatives have emerged to detect SARS-CoV-2 in wastewater during the pandemic, utilising a range of analytical workflows primarily based on RT-qPCR following genetic material preparation and enrichment (Randazzo et al., 2020; Ahmed et al., 2020; La Rosa et al., 2020; Medema et al., 2020; Wurtzer et al., 2020; Wu et al., 2020). As the pandemic progressed, new variants with specific mutations have continuously appeared, some of which have caused worldwide infections.

In addition to RT-qPCR, NGS-approaches using genome sequencing can be used to detect viral mutations, particularly those found in SARS-CoV-2 VOCs and has been recommended as standard procedure for

variant monitoring (Pérez-Cataluña et al., 2022; Crits-Christoph et al., 2021; Prasek et al., 2023).

In the present study, comparison of the lineage designation using duplex RT-qPCR VOC assays to genome sequencing with Oxford Nanopore MinION system and Freyja analysis software showed that the duplex RT-qPCR assays achieved significantly quicker results while maintaining appropriate clinical sensitivity and specificity (EC, 2021). Moreover, RT-qPCR assays allowed the characterisation of samples with low viral loads that would not be possible to process by genome sequencing. However, it should be noted that as the number of new SARS-CoV-2 variants increases, it becomes more difficult to find discriminatory mutations between them, making it difficult to develop new duplex RT-qPCR protocols for the identification of VOCs.

Although the detection of new SARS-CoV-2 variants in wastewater using sequencing methods typically needs higher concentrations of viral particles than PCR-based approaches, identification of samples with Cts over 30 cycles was efficiently performed (Fig. S1 and Fig. 4A). As other studies reported, in mid-December 2021 the Omicron variant overtook the Delta variant, which had been in the majority during the months of October and November 2021 (Pérez-Cataluña et al., 2022; Bar-Or et al., 2021; Crits-Christoph et al., 2021; Izquierdo-Lara et al., 2021; La Rosa et al., 2021; Nemudryi et al., 2020; Rios et al., 2021; Carcereny et al., 2021). The transition from Delta to Omicron occurred rapidly in 2–3 weeks in the month of December (weeks 49 to 51 of 2021), as shown by results obtained by both duplex RT-qPCR and by Freyja software from sequencing data. This Omicron emergence dates fully match the first reports of Omicron clinical cases in the Valencian Region of Spain (week 50 of 2021) (Fig. S3). Interestingly, according to the Spanish Health Ministry, the transition from Delta to Omicron variant in the Valencian Region of Spain took much longer to become clear when looking at clinical case data (6 to 8 weeks) with respect to this study based on wastewater's metagenomic or RT-qPCR data (2–3 weeks) for the same geographical area (Spanish Government, 2022). Also, as stated by the Spanish Health Ministry, Omicron BA.2 and BA.5 variants reached almost 100 % prevalence from the 14th and 28th weeks of 2022, respectively (Spanish Government, 2022). This observation fully matches the two peaks observed in SARS-CoV-2 RNA GC/L in wastewaters from the WWTP analysed in this study by duplex RT-qPCR and Freyja software analyses, which correspond to weeks 16th (April) and 28th (July) of 2022. Thus, the three peaks in SARS-CoV-2 RNA quantification data in wastewater samples (Figs. 3 and 4) correspond to Omicron BA.1, BA.2, and BA.5 variants imposition in the Spanish city of Valencia.

4.3. Correlation with public health records and comparison of indicators for data normalisation

Correlation with clinical data is another key component of WBE whereas measured viral concentrations in wastewater and reported clinical cases of disease should be established, strengthening the proposed methodology. The establishment of these correlations can serve as a validation for a prediction model that accounts for the factors discussed above, providing evidence for the notion that changes of viral concentrations in wastewater will indicate changes in viral disease cases in humans. When infectious agents are linked to non-specific symptoms or asymptomatic patterns, simultaneous monitoring of a wide range of respiratory viruses may be able to shed light on the dynamics of respiratory disease circulation in communities and improve the ability of Public Health authorities to combat seasonal human pathogenic viruses (Toribio-Avedillo et al., 2023; Ahmed et al., 2023; Guido et al., 2016; Britton et al., 2018).

In this context, it is important to analyse the reemergence of respiratory diseases such as the caused by IAV and RSV during the COVID-19 post-pandemic period. As other studies have reported before, the high sanitary restrictions and mask use during the pandemic months caused the prevalence of IAV and RSV to fall drastically among the population (Ando et al., 2023; Eden et al., 2022; Feng et al., 2021). However, once the strict containment measures were lifted, a large increase in cases of disease caused by these two viruses was observed (Ando et al., 2023; Eden et al., 2022; Feng et al., 2021; Emborg et al., 2022; Tempia et al., 2021; Fourgeaud et al., 2021; Boehm et al., 2023). In this sense, RSV and IAV monitoring in wastewater has recently been carried out in the USA, Canada, Spain, and Australia, and the results showed reasonable correlations with clinical cases and outbreaks (Toribio-Avedillo et al., 2023; Hughes et al., 2022; Wolfe et al., 2022; Ahmed et al., 2023; Ando et al., 2023). The RSV and IAV data included in the current research, possess significant potential for informing and shaping public health strategies in response to seasonal respiratory pathogens. This encompasses the dissemination of targeted messages during cold seasons to raise awareness about associated symptoms, the implementation of preventive measures like mask-wearing for vulnerable population, offering guidance to healthcare practitioners for efficient diagnostic triage, and optimising the allocation of resources.

The clinical cases pattern in this study, as in previous studies, nicely matched the viral RNA prevalence pattern in wastewater samples (Toribio-Avedillo et al., 2023; Ahmed et al., 2023). For both IAV and RSV, we found statistically significant associations between the reported confirmed cases and the corresponding quantification data in wastewater data (Figs. 5 and 6). This demonstrates the value of wastewater surveillance for monitoring infectious diseases in the community and supports their future application for monitoring additional respiratory viruses. Our experience is in line with what has been seen in other localities for IAV and RSV (Toribio-Avedillo et al., 2023; Hughes et al., 2022; Wolfe et al., 2022; Ahmed et al., 2023; Ando et al., 2023; Mercier et al., 2022).

As demonstrated by the correlation of respiratory virus in wastewater samples with clinical data, WBE can offer representative samples of the community by collecting population health data. However, sewage flow and faecal load in wastewater are known to vary significantly from day to day (Bertels et al., 2022). Two viruses suggested as indicators of fecal contamination, PMMoV and crAssphage, were quantified from wastewater samples to normalise the SARS-CoV-2, IAV, and RSV concentrations along with somatic coliphage plaque counting (Figs. 4–6). Besides, wastewater physico-chemical parameters data was also used for respiratory virus's concentration normalisation (Figs. 4–6). This was done to account for the many factors capable of changing estimated fecal load over time and potential dilution due to rain. Although viral shedding could be a relevant variable when conducting these type of studies, several publications regarding viral shedding have been published with very variable data that seems to not accurately describe the real shedding rates (Chan et al., 2011; Hughes et al., 2022). Besides, respiratory viruses can also reach sewage from other sources distinct to stool (mucus and saliva). Thus, the viral shedding of each of the analysed viruses has not been taken into account as a variable in drawing conclusions.

According to obtained Spearman's correlations, the association between the SARS-CoV-2, RSV, and IAV titers and clinical case numbers was mainly improved by the physico-chemical parameters-based normalisation (Figs. 4–6). It should also be noted that there was an important improvement in resulting correlations strength when applying a delay on clinical cases data, as there exists a logical frame of time between an individual getting infected and its search for sanitary aid. Interestingly, PMMoV-based normalisation did not significantly alter the correlation, and finally both crAssphage-based and somatic coliphage-based normalisations led to much weaker correlations (Figs. 5–7). These results differ from some works stating crAssphage or PMMoV loads are proper normalising parameters (Wu et al., 2020; Wilder et al., 2021; D'Aoust et al., 2021), but are in line with several of other studies which are reporting that these approaches might not be as consistent as other literature suggests (Gerrity et al., 2021; Jafferali et al., 2021; Langeveld et al., 2023). For example, Ai et al. (2021) states that crAssphage-based normalisation of SARS-CoV-2 titers in

wastewater samples led to much weaker correlation with clinical cases while PMMoV slightly but not significantly improved that correlation. On the other hand, physico-chemical parameters seem to better adjust to the role of normalising factor. In particular, normalising GC/L values by WWTP inflow parameter, which is lately being proposed as the optimal normalising factor (Langeveld et al., 2023), showed outstanding improvements on obtained correlations with clinical data. Interestingly, the fact that virus quantification data in wastewater can be normalised with physico-chemical parameters makes the analysis process cheaper, since, unlike in the case of normalising via biological indicators, a PCR or viral plaque counting step is needless. Besides, our results reflected other physico-chemical parameters (total nitrogen, phosphorus, solids in suspension, electrical conductivity, COD, and BOD5) to also be robust parameters for data normalising, while normalisation based on PMMoV genome concentration suggested a viral rebound in February 2022 that was not observed on the incidence curve and presented lower correlations. Finally, Random Forest results demonstrated that the algorithm can be effectively applied to respiratory virus GC/L in wastewater data to determine realistic clinical trends of respiratory illness in a given community (Figs. 7–9). The results obtained in the clinical case prediction models using Random Forest reinforced the conclusions obtained from the Spearman's correlations: the inflow to the WWTPs seems to be the normalising factor that best fits the predictive model. In addition, the existence of a delay between the onset of infections in the population (translated into increased virus load in wastewater) and the appearance of clinical cases should be taken into account, since people do not go to health centers immediately post-infection. Despite the low reliability, the models demonstrated the existence of shared information between the collected data and the clinical cases, supporting the previous results of the Spearman's correlations. This opens the way to the possibility of the implementation of predictive models as an early warning system. More data is required to train such models, which was not possible to collect to the extent of this text.

5. Conclusions

Overall, this work reinforces the potential of wastewater-based epidemiology as a multi-pathogen monitoring tool. This study shows that it is possible to reliably monitor the confluence of respiratory and enteric viruses and the diseases they cause in the same geographical area, with a great interest in public health. Finally, this work also highlights the high normalising capacity of fecal load normalisation by wastewater physico-chemical parameters, specially WWTP inflow, while raising doubts about the appropriateness of normalising data by quantification of crAssphage, PMMoV, or somatic coliphages.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT (https://chat.openai.com/) in order to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Inés Girón-Guzmán: Writing – review & editing, Methodology. **Enric Cuevas-Ferrando:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Regino Barranquero:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Azahara Díaz-Reolid:** Writing – review & editing, Methodology. **Pablo Puchades-Colera:** Writing – review & editing, Methodology. **Irene Falcó:** . **Alba Pérez-Cataluña:** Writing – review & editing, Methodology, Formal analysis. **Gloria Sánchez:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121463.

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3.2. Spanish wastewater reveals the current spread of Monkeypox virus

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Spanish wastewater reveals the current spread of Monkeypox virus

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SUMMARY

Besides nasopharyngeal swabs, monkeypox virus (MPXV) DNA has been detected in a variety of samples such as saliva, semen, urine and fecal samples. Using the environmental surveillance network previously developed in Spain for the routine wastewater surveillance of SARS-CoV-2 (VATar COVID-19), we have analyzed the presence of MPXV DNA in wastewater from different areas of Spain. Samples (n = 312) from 24 different wastewater treatment plants were obtained between May 9 (week 19 of 2022) and August 4 (week 31 of 2022). Following concentration of viral particles by a validated aluminum adsorption-precipitation method, a qPCR procedure allowed us to detect MPXV DNA in 56 wastewater samples collected from May 16 to August 4, 2022, with values ranging between 2.2×10^3 to 8.7×10^4 genome copies (gc)/L. This study shows that MPXV DNA can be reproducibly detected by qPCR in longitudinal samples collected from different Spanish wastewater treatment plants. According to data from the National Epidemiological Surveillance Network (RENAVE) in Spain a total of 6,119 cases have been confirmed as of August 19, 2022. However, and based on the wastewater data, the reported clinical cases seem to be underestimated and asymptomatic infections may be more frequent than expected.

1. Introduction

In early May 2022, a multi-country outbreak of Monkeypox virus (MPXV) started in non-endemic regions, and on 23 July WHO declared a Public Health emergency of international concern (WHO, 2022). In Europe, a total of 13,911 cases of MPX have been reported up to 19 August 2022, with Spain accounting for 6,119 cases, the second highest number of Monkeypox (MPX) cases worldwide, being present in most regions of the country (Spanish Ministry of Health, 2022).

Symptoms developed include the appearance of rash, fever, fatigue, muscle pain, vomiting, diarrhea, chills, sore throat or headache, and the hospitalization rate is around 8–13% (European Centre for Disease Prevention and Control (ECDC), 2022; Thornhill et al., 2022). Sadly, two deaths linked to this outbreak have occurred in Spain due to complications associated with encephalitis (Aguilera-Alonso et al., 2022). It is assumed that transmission occurs after close contact with skin lesions of an infected person, as well as through contact with respiratory droplets and fomites, and that infection is symptomatic in all patients (McCollum and Damon, 2014). However, antibodies have been found in exposed asymptomatic individuals, which can be linked to subclinical infections (Wilson et al., 2014), and positive MPXV PCR results from anal samples in asymptomatic men who have sex with men (MSM) have also been documented (Baetselier et al., 2022; Ferré et al., 2022). The virus is also excreted in fluids, and its detection in saliva, semen, urine and feces has been reported (Peiró-Mestres et al., 2022; Hernaez et al., 2023). This implies that routine wastewater surveillance can be applied as a tool for early detection of the disease expansion as very recently reported following a model-based theoretical evaluation (Chen and Bibby, 2022). According with this model, wastewater-based epidemiology (WBE) can detect on average 7 MPX cases out of 100,000 people. Currently, various

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studies detected MPXV DNA in wastewater worldwide (de Jonge et al., 2022; la Rosa et al., 2023; Wolfe et al., 2022), highlighting again wastewater analysis as a non-invasive tool for monitoring the status and trend of an emerging infection. The aim of the present study was to trace the community circulation of the MPXV from potentially symptomatic, asymptomatic, or presymptomatic individuals using the previous established Spanish National SARS-CoV-2 Wastewater Surveillance Network (VATar COVID-19).

2. Material and methods

2.1. Sample concentration and DNA extraction

Grab sewage samples were weekly collected from 24 Spanish wastewater treatment plants (WWTPs) (Fig. 1C) between May 9 (week 19 of 2022) and August 4 (week 31 of 2022) and kept at 4 °C until analysis. Concentration of viral fraction was performed by a previously validated method for SARS-CoV-2 using an aluminum-based adsorption precipitation procedure (Pérez-Cataluña et al., 2021). In order to evaluate the analytical performance of this concentration method, 200 ml of grab sewage samples ($n = 4$) that previously tested negative for MPXV were inoculated with 10^7 PFU of inactivated MPXV. MPXV suspension was obtained by infecting a clinical MPXV specimen obtained from a patient pustule in BSC-1 cells. MPXV stock was inactivated in the BSL-3 laboratory (Molecular Biology Center Severo Ochoa, CBM, Madrid) by limited cross-linking with a combination of psoralen (4–9-amino-methyl-Trioxsalen; Sigma) and long-wave UV light, as previously described for other poxviruses (Tsung et al., 1996). Briefly, virus stock

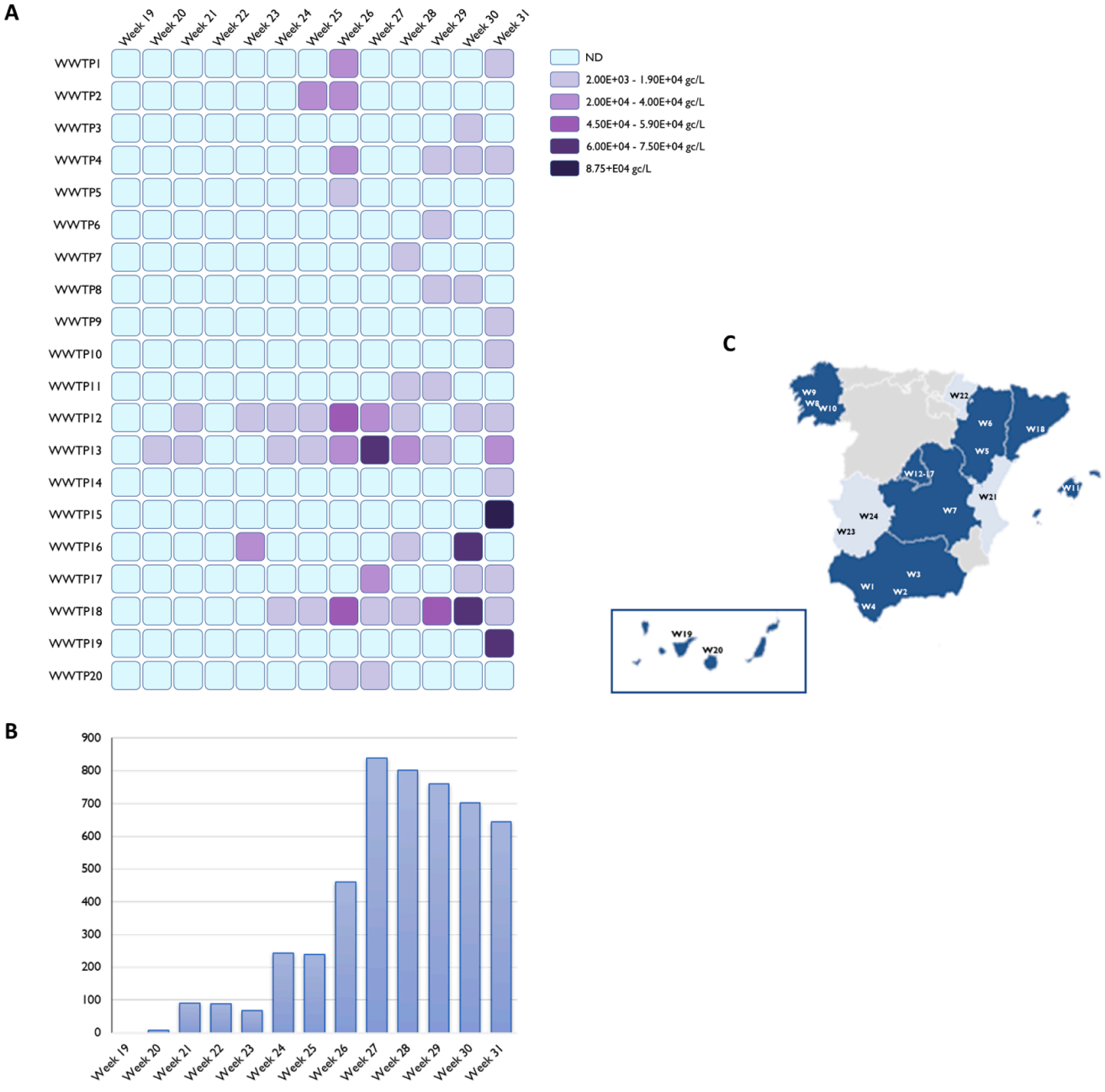

Fig. 1. (A) Evolution of MPXV DNA prevalence over time, as measured by qPCR in wastewater samples from 20 wastewater treatment plants with positive detection (B) Number of cases of monkeypox per week (Spanish Ministry of Health) (C) Geographical localization of wastewater treatment plants, dark blue (Autonomous Community with positive detection in the analyzed wastewater samples), light blue (no detection in wastewater samples) and gray (regions not covered in the study). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was incubated with 2 μg/ml psoralen for 10 min at room-temperature followed by 10 min irradiation with long-wave ultra-violet light (365 nm). Virus inactivation was further confirmed by two consecutive plaque assays in BSC-1 cells to discard virus-induced effects.

Nucleic acids extraction (100 μl) of the concentrated samples (300 μl) was performed by the Maxwell® RSC Instrument (Promega) using the Maxwell RSC Pure Food GMO and authentication kit (Promega) and the "Maxwell RSC Viral total Nucleic Acid" program.

2.2. MPXV real-time PCR assays

The MPXV West Africa (G2R_WA) assay (Li et al., 2010) was applied to quantify MPXV DNA using the qPCR Premix Ex Taq™ kit (Takara Bio Inc). Additionally, a subset of samples (Table S1) was tested for MPXV DNA using the MPXV generic (G2R_G) assay (Li et al., 2010). Both assays targeted the TNF receptor gene. Undiluted and ten-fold diluted DNA (2.5 μl) was tested in duplicate. Positive control consisted in the nucleic acid material extracted from a cell culture infected with a clinical MPXV specimen obtained from a patient pustule. For each qPCR, serial dilutions of standard curves were run in quintuplicates and the numbers of estimated genome copies were calculated (Table S2). Each run included negative controls (nuclease-free water and negative extraction controls). Depending on the laboratory, reactions were carried out in the QuantStudio™ 3 and QuantStudio™ 5 Real-Time PCR Systems (ThermoFisher Sci.). For each specific target, Cq values $\leq$ 40 were converted into genome copies (gc) per liter using the corresponding standard curve and volumes tested. Occurrence of inhibition was estimated by comparing average viral titers obtained from duplicate wells tested on undiluted DNA with duplicate wells tested on 10-fold diluted DNA. Inhibition was ascertained when difference in average viral titers was higher than 0.5 $\log_{10}$, and if that occurred, viral titers were inferred from the 10-fold DNA dilution.

2.3. Clinical epidemiological data

The number of declared active cases per week for the different Autonomous communities was obtained from the Spanish Ministry of Health (Spanish Ministry of Health, 2022).

3. Results

3.1. Estimated levels of Monkeypox virus DNA in wastewater samples

Here, we report the first detection of MPXV DNA in wastewater samples from different regions of Spain. 56 out of 312 samples showed positive results for MPXV DNA, corresponding to samples collected from week 20 to week 31 of 2022. Cycle threshold values ranged between 39.98 and 34.5, corresponding to values from 2.2×10^3 to 8.7×10^4 estimated gc per liter (Fig. 1A). The aluminum-based adsorption-precipitation method was tested by spiking negative wastewater samples with inactivated MPXV suspension. On average, MPXV was recovered at ranges of 31.5 ± 15.9% and 45.5 ± 25.7% in the undiluted and ten-fold diluted samples, respectively, thus validating the results (Fig. 2).

First detection of MPXV DNA in wastewater samples occurred in WWTP13 from the city of Madrid in week 20 of 2022 (Figs. 1A and 3), with positive detection using two different assays (Table S1). On that week, Madrid reported the first suspected cases of MPX which represented the first cases of MPX in Spain accounting for one of the largest outbreaks reported outside Africa (Martínez et al., 2022). Later on, several cases were reported in Madrid before the outbreak declaration on 17 May, most of them attending the same sauna in the city of Madrid or with travel history to Maspalomas Gay Pride festival that took place on 5–15 May in Gran Canaria.

In week 21 of 2022, MPXV DNA was detected in the nearest WWTPs of Madrid city (WWTP12 and WWTP13) and in WWTP20 of Gran Canaria (Canary Islands) (Fig. 1, Table S1). Interestingly, we consistently detected MPXV DNA in samples collected from week 23 of 2022 in WWTP12 and WWTP13 from Madrid, when only 275 cumulative cases were declared in the entire region (Martínez et al., 2022). Our data also showed percentages of WWTPs with MPXV DNA detection increased progressively, up to 15% by week 24 of 2022 and 40% by week 26 of 2022 (Figs. 1A and 3). In Barcelona, the second largest Spanish city, first detection occurred in week 24 of 2022 in WWTP18 with the first peak observed in week 26 of 2022 when 130 cumulative cases were detected in Catalonia (Fig. 3, Table S3). Intermittent detection (negative results after previous qPCR detection) was reported from some WWTPs where the number of confirmed clinical cases was low (Figs. 1 and 3). The regions of Murcia, Asturias, Cantabria, Basque country and Castilla Leon (Fig. 1C) were under reported as none WWTP were analyzed in this study. Furthermore, all weekly samples collected from Valencia (WWTP21), Extremadura (WWTP23 and WWTP24), and Navarra (WWTP22) regions tested negative for the presence of MPXV DNA (Fig. 3), with a total number of clinical cases of 331, 21 and 13 as August 9, respectively (Spanish Ministry of Health, 2022).

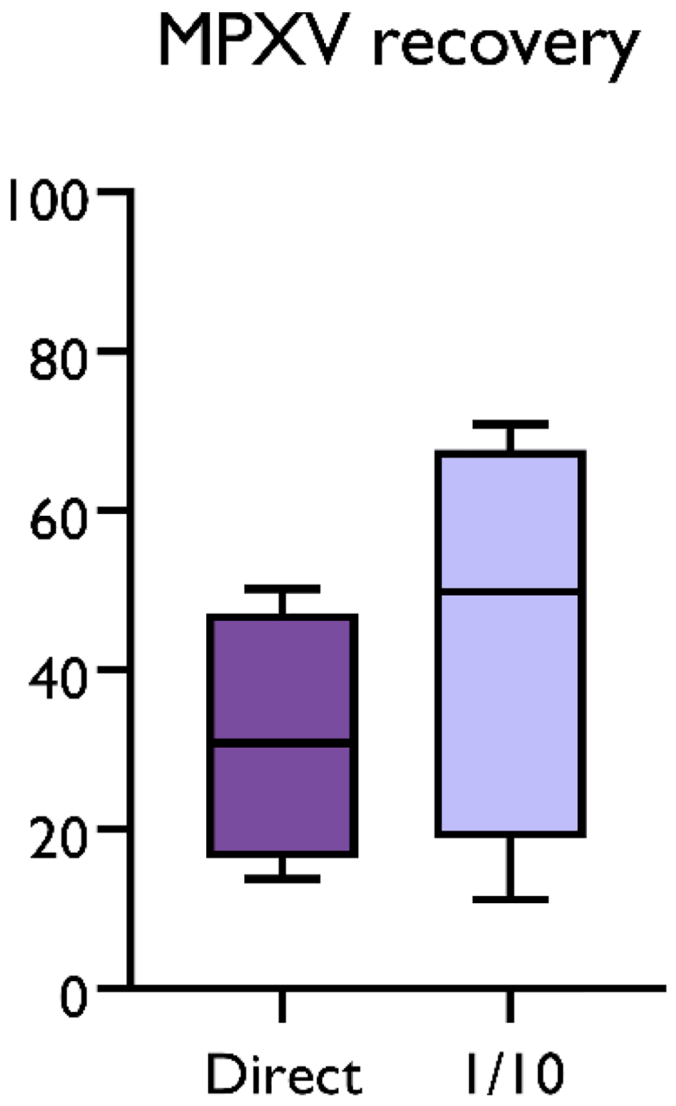

Fig. 2. Monkeypox virus recovery (%) in wastewater samples using the aluminum-based adsorption-precipitation method. MPXV detection was performed using the West Africa (G2R_WA) assay (Li et al., 2010) of undiluted and ten-fold diluted DNA.

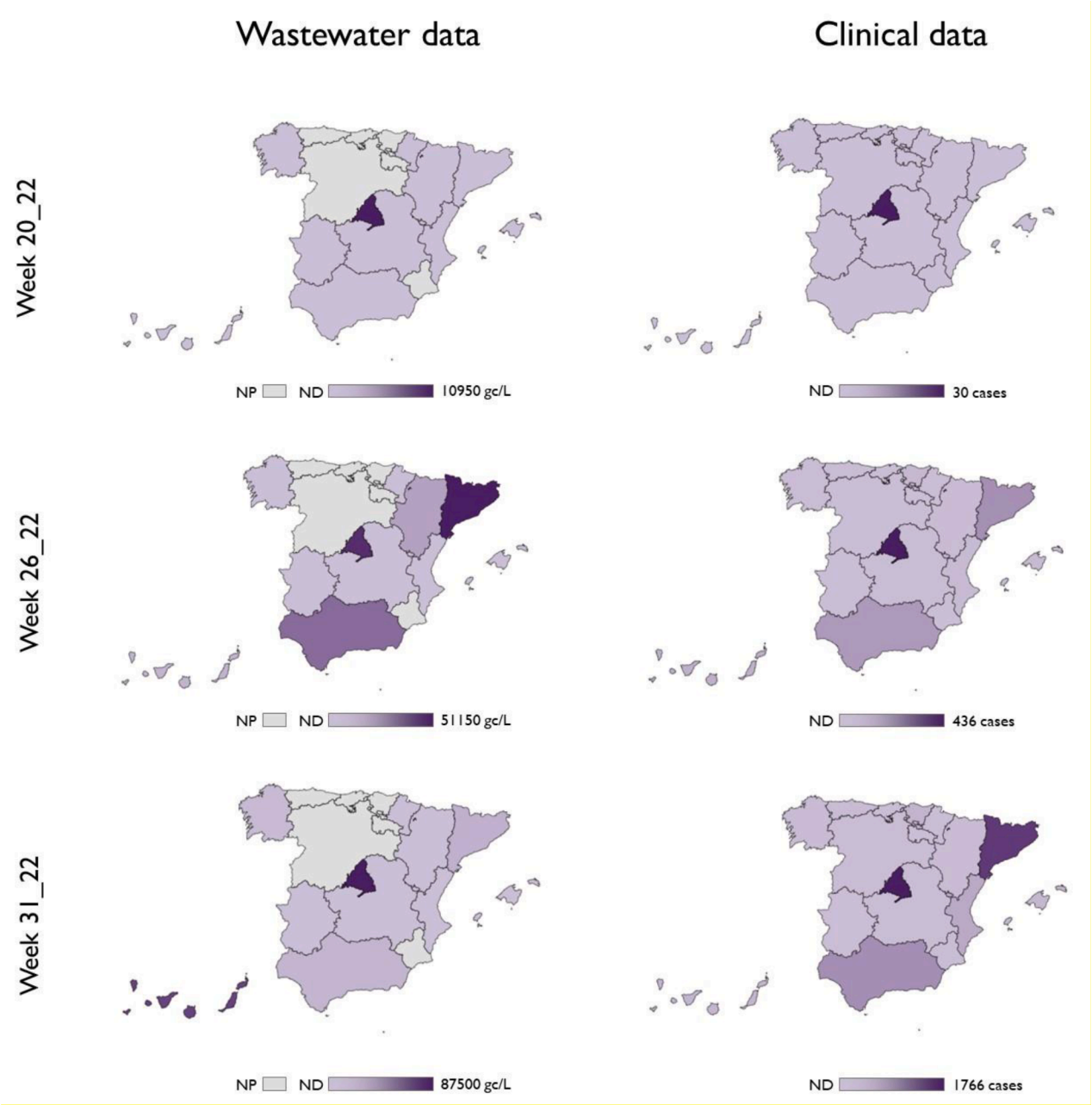

Fig. 3. Comparison of MPXV DNA estimates from wastewater testing (left panels) and confirmed cases of monkeypox by Autonomous Community reported by the Health Ministry authorities (right panels). For wastewater samples, highest level within the same Autonomous Community are depicted. ND: No MPXV detection (light purple); NP: regions without WWTP analyzed in the study (gray). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The COVID-19 pandemic has demonstrated that WBE is a cost-effective tool to anticipate the circulation of SARS-CoV-2 in a community and to closely track its incidence, evolution and geographic spread (Bivins et al., 2020). WBE has been implemented worldwide and most of the countries are ready to perform this monitoring as a routing basis for other emerging pathogens likely to be found in wastewater, due to their presence in feces and/or urine. In Spain, the National WBE Network, VATar COVID-19, has been successfully used to determine the extent of the COVID-19 disease along the country (Carcereny et al., 2021).

The increasing number of MPXV cases around the world continue to pose challenges to control its transmission with a total number of 41,358 cases as of 19 Aug 2022 (CDC, 2022). This underscores the urgent needs for simple and cost-effective tools to facilitate early detection, evolution and spatial distribution of cases. DNA of MPXV has been detected in urine and feces from symptomatic individuals (Antinori et al., 2022; Peiró-Mestres et al., 2022), and although limited data are available, viral shedding has been observed in stool in 63% of patients (Cts values from 17.8 to 31.4) and in urine in 56% (Cts values from 19.1 to 40.0) (Peiró-Mestres et al., 2022). It is not known whether MPXV present in stool and urine is infectious. Altogether, these findings warned the interest of assessing the presence of MPXV DNA in sewage samples (Chen and Bibby, 2022). In the current study, a qPCR assay designed for the West African clade (Li et al., 2010) was applied on wastewater samples collected from week 19 to week 31 of 2022, showing that MPXV DNA can be reproducibly detected by qPCR in longitudinal samples collected from several Spanish WWTPs. First detection of MPXV DNA was

retrieved in a single sample from WWTP13 collected on May 17 (Week 20 of 2022) using the specific qPCR assay and confirmed by the MPXV generic assay, providing the earliest piece of evidence that the virus was circulating in the community of Madrid. Interestingly, we consistently detected MPXV DNA in samples collected in WWTP18 (Barcelona) since week 23 of 2022, when only 39 cumulative cases were declared in the entire Autonomous Community of Catalonia. In line, MPXV DNA was also detected in week 21 of 2022 on wastewater samples collected from Schiphol Airport and in different Dutch WWTPs from week 22 of 2022 onwards (de Jonge et al., 2022).

The viral concentration method used in this study has been validated for SARS-CoV-2 detection and quantification (Pérez-Cataluña et al., 2021) and it seems promising for MPXV monitoring in wastewater, too. However, in contrast to what has been reported for SARS-CoV-2 (Bivins et al., 2020; Medema et al., 2020; Randazzo et al., 2020), anticipation of clinical cases has not been observed for MPXV, for which the first wastewater detection occurred at the same time that MPX cases were declared (Fig. 3). This could be due to several factors, including differences in shedding levels and kinetics, proportion of asymptomatic cases, diagnosis of the disease and fast identification of cases, environmental factors affecting virus stability, a much larger scale of transmission of SARS-CoV-2 in the community, and low performance of the method to concentrate MPXV. This latter was further rule out as the performance characteristics of the methodology was carried out for MPXV (Fig. 2) showing similar mean recoveries compared with SARS-CoV-2 and its surrogates (Pérez-Cataluña et al., 2021). Moreover, it is important to highlight that wastewater positive samples have been found in areas with very low reported disease prevalence. For instance, in Castilla la Mancha, a region located at the middle-south of Spain, MPXV was detected in sewage with only 42 clinical cases being reported, indicating that probably, a higher number of people may be affected. As previously discussed by other authors, stigma and discrimination may be limiting the awareness or willingness of at-risk people to have their symptoms evaluated. In these situations, WBE may be even more useful, because the anonymous pooled samples can evidence the contributions of a community without divulging individual identities (Nelson, 2022).

5. Conclusions

Using an environmental surveillance tool previously developed for SARS-CoV-2, we have been able to detect MPXV DNA in wastewater samples from different regions of Spain when communicated clinical cases in that region were only incipient. We also found that the wastewater viral DNA detection increased rapidly and anticipated the subsequent ascent in the number of declared cases showing, once again, that WBE is a sensitive and cost-effective strategy for the surveillance of emerging viral threats. In those cases where stigma and blame might undermine the capacity to effectively respond during outbreaks, i.e., driving people away from health services, the implementation of WBE may represent a most valuable tool.

Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.119621.

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3.3. Evaluation of two different concentration methods for surveillance of human viruses in sewage and their effects on SARS-CoV-2 sequencing

Inés Girón-Guzmán, Azahara Díaz-Reolid, Enric Cuevas-Ferrando, Irene Falcó, Pablo Cano-Jiménez, Iñaki Comas, Alba Pérez-Cataluña, Gloria Sánchez

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Evaluation of two different concentration methods for surveillance of human viruses in sewage and their effects on SARS-CoV-2 sequencing

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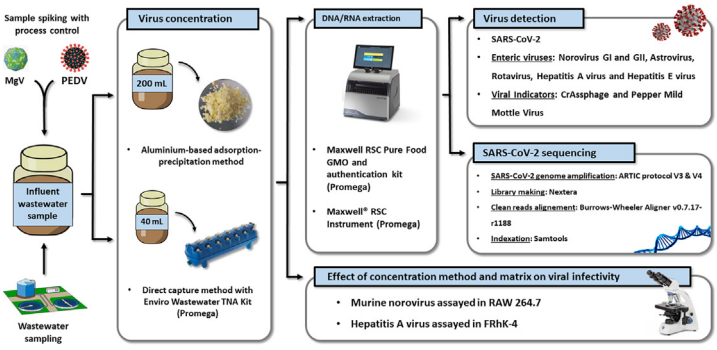
HIGHLIGHTS

- Concentration methods are critical for virus surveillance in sewage.
- Direct capture system (TNA) produces better results in terms of RT-qPCR sensitivity.
- TNA system combined with Artic v4 yields the best SARS-CoV-2 sequencing results.
- Aluminum precipitation would be recommended for infectivity assays.

GRAPHICAL ABSTRACT



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ABSTRACT

During the current COVID-19 pandemic, wastewater-based epidemiology (WBE) emerged as a reliable strategy both as a surveillance method and a way to provide an overview of the SARS-CoV-2 variants circulating among the population. Our objective was to compare two different concentration methods, a well-established aluminum-based procedure (AP) and the commercially available Maxwell® RSC Enviro Wastewater TNA Kit (TNA) for human enteric virus, viral indicators and SARS-CoV-2 surveillance. Additionally, both concentration methods were analyzed for their impact on viral infectivity, and nucleic acids obtained from each method were also evaluated by massive sequencing for SARS-CoV-2. The percentage of SARS-CoV-2 positive samples using the AP method accounted to 100 %, 83.3 %, and 33.3 % depending on the target region while 100 % positivity for these same three target regions was reported using the TNA procedure.

The concentrations of norovirus GI, norovirus GII and HEV using the TNA method were significantly greater than for the AP method while no differences were reported for rotavirus, astrovirus, crAssphage and PMMoV. Furthermore, TNA kit in combination with the Artic v4 primer scheme yields the best SARS-CoV-2 sequencing results. Regarding impact on infectivity, the concentration method used by the TNA kit showed near-complete lysis of viruses. Our results suggest that although the performance of the TNA kit was higher than that of the aluminum procedure, both methods are suitable for the analysis of enveloped and non-enveloped viruses in wastewater by molecular methods.

1. Introduction

Over the last two years, molecular analysis of SARS-CoV-2 in wastewater samples, has become very popular due to the potential for epidemiological

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surveillance using wastewater collected from wastewater treatment plants (WWTP), sewers or even aircrafts (Ahmed et al., 2022; Davó et al., 2021; Polo et al., 2020). However, the analysis of wastewater for virus surveillance is not new and had been used long before for epidemiological tracking of human enteric viruses such as poliovirus, norovirus, enterovirus, rotavirus, adenovirus and hepatitis A and E viruses (Asghar et al., 2014; Cuevas-Ferrando et al., 2020; Hellmér et al., 2014; Miura et al., 2016; Prevost et al., 2015; Santiso-Bellón et al., 2020).

Human enteric viruses pose one of the highest microbiological risks of water-borne infections (Wyn-Jones and Sellwood, 2001). Due to their excretion in feces, these viruses reach wastewater treatment systems and can contaminate other water sources into which they are discharged. The application of RT-qPCR is currently used as a gold standard method to provide information about levels of these pathogens in wastewater as well as in effluents (Haramoto et al., 2020). However, the low presence of viruses in wastewater in relation to other organisms and the complexity and variability of wastewater samples make viral concentration and nucleic acid extraction methods critical for these types of analyses (Haramoto et al., 2020).

During the current COVID-19 pandemic, several studies have compared different procedures for SARS-CoV-2 detection by RT-qPCR or digital PCR (Rusiñol et al., 2020b; Torii et al., 2022). Moreover, high-throughput sequencing techniques have been used for the analysis of SARS-CoV-2 genomes in wastewaters, evidencing their usefulness in detecting the linage introduction in a population, as well as in profiling new outbreaks, and tracking viral strains (Crits-Christoph et al., 2021; Izquierdo-Lara et al., 2021; Nemudryi et al., 2020; Pérez-Cataluña et al., 2022). Nevertheless, considering the potential of WBE for current and future treats, a broader comparison is needed, not only to establish methods for viral detection and quantification but also to characterize these using high-throughput sequencing techniques.

Therefore, the aim of this study was to evaluate the performance of two concentration methods for the detection of enteric viruses, viral fecal indicators, and SARS-CoV-2. Additionally, nucleic acids obtained from each concentration method were evaluated by targeted sequencing in terms of coverage across the SARS-CoV-2 genome.

2. Materials and methods

2.1. Viral concentration methods

Grab wastewater samples, collected from 6 different WWTPs on August 2021, were inoculated with 100 μL of porcine epidemic diarrhea virus (PEDV) strain CV777 as a coronavirus model and mengovirus (MgV) vMC0 (CECT 100000) as a non-enveloped counterpart. Two hundred milliliters of wastewater samples ($n = 6$) were concentrated through a previously validated aluminum-based adsorption-precipitation method (hereafter referred to as AP) (Pérez-Cataluña et al., 2021; Randazzo et al., 2019). In parallel, 40 mL of wastewater samples ($n = 6$) were processed with the vacuum concentration system using by Enviro Wastewater TNA Kit (Promega Corp., Spain) following the manufacturer's protocol (hereafter referred to as TNA). In brief, 0.5 mL of protease solution was added to 40 mL of wastewater, and samples were incubated statically for 30 min at room temperature (RT) and centrifuged at 3000 $\times g$ for 10 min. Then, in duplicate, 20 mL of the supernatant was transferred to a clean tube and 5.5 mL of Binding Buffer 1, 0.5 mL of Binding Buffer 2, and 24 mL of isopropanol were added. The mixture was passed through a PureYield™ Midi Binding Column (Promega) using a VacMan® Vacuum Manifold (Promega). Five milliliters of Inhibitor Removal Wash (complemented with 40 % isopropanol as specified by the manufacturer's protocol) followed by 20 mL of RNA Wash Solution (complemented with 63 % ethanol 95 % as specified by the manufacturer's protocol) were passed through the column. Finally, the concentrated sample was eluted in 500 μL of nuclease-free water for nucleic acid extraction.

2.2. RNA extraction and virus quantification

Viral extraction from wastewater concentrates, obtained by the AP and the concentration procedure of the TNA kit, was carried out using the Maxwell® RSC Instrument (Promega) with the Maxwell RSC Pure Food GMO and authentication kit (Promega) and the "Maxwell RSC Viral total Nucleic Acid" running program.

Samples concentrated using the AP method were processed as described previously by Pérez-Cataluña et al. (2021). Samples concentrated by the TNA method were subjected to nucleic acid extraction using 500 μL of the eluate. This eluate was mixed with 150 μL of Binding Buffer 1 and 50 μL of Binding Buffer 2, both provided with the TNA kit, vortexed, and incorporated into a Maxwell RSC Cartridge (Promega).

Viral detection of SARS-CoV-2, PEDV, and MgV was performed by RT-qPCR using the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio Inc., USA). SARS-CoV-2 detection was achieved by targeting the N1 region of the nucleocapsid gene and the IP4 region of the RNA-dependent RNA polymerase gene (Institut Pasteur, 2020). For N1, two RT-qPCR assays were tested; the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) was used with N1 primers and conditions described by (CDC, 2020) (hereafter referred to as N1-CDC); and the duplex RT-qPCR kit detection Wastewater SARS-CoV-2 RT-qPCR System (Promega) for SARS-CoV-2 and pepper mild mottle virus (PMMoV) (hereafter referred to as N1-Dup). Membrane gene (M) specific primers were used for PEDV detection as described by Puente et al. (2020). For MgV, detection was carried out using primers and probe described in (ISO 15216-1:2017). Reaction mixes, thermal cycling conditions, and sequences for primers and probes are listed in Pérez-Cataluña et al. (2021).

Levels of norovirus GI and GII, human astrovirus (HAstV), rotavirus (RV), hepatitis A virus (HAV) and hepatitis E viruses (HEV) were determined using the RNA UltraSense One-Step kit (Invitrogen, USA) as previously described (Randazzo et al., 2019).

Occurrence of crAssphage was established using the qPCR Premix Ex Taq™ kit (Takara Bio Inc.) using primers and conditions described by (Stachler et al., 2017).

Different controls were used in all assays: negative process control consisting of PBS; whole process control to monitor the process efficiency of each sample (spiked MgV and PEDV); and positive (reference material) and negative (RNase-free water) RT-qPCR controls. Moreover, undiluted and ten-fold diluted nucleic acid were tested in duplicate to check for inhibitors for all the targeted viruses.

Standard curves were determined according to the Public Health England (PHE) Reference Materials for Microbiology for norovirus GI (batch number 0122-17), norovirus GII (batch number 0247-17) and HAV (batch number 0261-2017) and reported as genomic copies (gc), while standard curves for RV, MgV, and HAstV were generated by amplifying ten-fold serial dilutions of viral suspensions in quintuplicates and calculating the number of PCR units (PCRU). Standard DNA material for crAssphage standard curve generation relied on a customized gBlock gene fragment (Integrated DNA Technologies, Coralville, IA) containing target sequence for CPQ_064 crAssphage primers set (Stachler et al., 2017).

Commercially available Twist Synthetic SARS-CoV-2 RNA Control (Control 2, MN908947.3) was used to prepare standard curves for SARS-CoV-2 quantification.

2.3. Effect of concentration procedure on viral infectivity

To accomplish this, 500 mL of a grab wastewater sample was inoculated with Murine norovirus (MNV-1, kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) and HAV strain HM-175/18f (ATCC VR-1402). In parallel, 500 mL of PBS was also artificially inoculated with both viruses. Two hundred milliliters of wastewater samples ($n = 2$) or PBS ($n = 2$) were concentrated through the AP procedure while 40 mL of wastewater samples ($n = 2$) or PBS ($n = 2$) were processed with the TNA Kit as described above. Then, concentrated samples were ten-fold diluted, and infectious viruses quantified using the Spearman-Karber

method on confluent RAW 264.7 (ATCC TIB-71) and FRhK-4 (ATCC CRL_1688) monolayers for MNV and HAV, respectively (Falcó et al., 2018).

2.4. SARS-CoV-2 sequencing

Genomic sequencing of SARS-CoV-2 present in wastewater samples was carried out following ARTIC protocol versions 3 and 4, as version 4 was released during the study in response to the realization that some V3 primers were located in regions with key mutations. Sequencing libraries were generated using the Nextera Flex kit (Illumina, CA, USA) and sequenced on Illumina MiSeq platform by paired-end reads (2 × 200) (Pérez-Cataluña et al., 2022). Adaptors and nucleotides below Q30 Phred score were cleaned by using *cutadapt* software (Martin, 2011) and *reformat.sh* from bbmap (sourceforge.net/projects/bbmap/), respectively. Obtained clean reads were aligned to the genome of SARS-CoV-2 isolate Wuhan-Hu-1 (MN908947.3) using the Burrows-Wheeler Aligner v0.7.17-r1188 with default parameters (Li and Durbin, 2009) and indexed by samtools (Li et al., 2009). Genomic coverage for each sample was calculated using nucleotide positions with at least 20× depth.

2.5. Statistical analyses

Normal distribution was evaluated with Shapiro-Wilk tests. Significance of the differences in viral detection by RT-qPCR was evaluated using Student's *t*-test for normally distributed data (i.e. norovirus GI and GII, rotavirus, PMMoV, crAssphage, and MgV) and Mann-Whitney-Wilcoxon or Dunn's tests with adjusted *p*-values with the Holm method for not normally distributed data (i.e. PEDV, HAstrV, HEV, SARS-CoV-2).

The statistical analysis of differences in logarithmic reductions after cell culture assays was carried out by the post-hoc Tukey's method (p-value < 0.05) to compare and determine the difference among different concentration procedures.

The statistically significant differences in the results obtained after the genomic analysis (i.e. percentage of reads identified as SARS-CoV-2, percentage of SARS-CoV-2 genome coverage, and mean values of genomic depth) were calculated by pairwise comparisons using Student's *t*-test for the percentage values of SARS-CoV-2 reads and genomic coverage, and with the Mann-Whitney-Wilcoxon test for mean depth values. Differences were considered significant when the p-value was <0.05. All the statistical analyses were made with R Statistical Software (version 3.6.3).

3. Results

3.1. Comparison of the aluminum-based adsorption precipitation method (AP) and the direct capture method (TNA) for viral detection and recovery

Wastewater samples were processed using both the AP method (initial sample volume 200 mL) and the TNA Kit (initial sample volume 40 mL) for their ability to concentrate SARS-CoV-2, human enteric viruses, and viral fecal indicators from wastewater samples.

Wastewater samples were analyzed by targeting two different SARS-CoV-2 genomic fragments (N1 and IP4) to evaluate the sensitivity of each concentration method. The percentage of positive samples using the AP method was 100 %, 83.3 %, and 33.3 %, for N1-Dup, N1-CDC and IP4, respectively, while 100 % positivity for the three targets was reported using the TNA procedure (Fig. 1, Sup. Table S1). Significative differences (p-value = 0.02) were found between SARS-CoV-2 levels targeting IP4 in samples concentrated using the AP method and targeting N1-Dup concentrated by the TNA method (Fig. 1) while no differences were retrieved targeting N1-CDC.

The AP and TNA methods were also evaluated for their relative consistency in quantifying human enteric viruses (Fig. 2) and viral indicators (Fig. 3). HAV was not detected in any sample regardless of the method used (Sup. Table S1). The concentrations of norovirus GI (6.16 ± 0.73 $\log_{10}$ gc/L), norovirus GII (6.88 ± 0.43 $\log_{10}$ gc/L), and HEV (3.87 ± 0.49 $\log_{10}$ gc/L) using the TNA method were significantly greater (p-values of 0.042, 0.007, and 0.036, respectively) than using the AP method (Fig. 2).

No significant differences were found for RV and HAstrV levels. Furthermore, using the AP method, the percentage of positive samples were 50 % and 33.3 %, for HAstrV and HEV respectively, compared to 66.6 % and 100 % of positivity using the TNA method (Fig. 2, Sup. Table S1). Viral indicators showed mean values of 7.82 ± 0.36 $\log_{10}$ gc/L and 9.55 ± 0.25 with the AP method, and 8.32 ± 0.22 $\log_{10}$ gc/L and 9.45 ± 0.21 $\log_{10}$ gc/L with the TNA method, for PMMoV and crAssphage, respectively (Fig. 3). Neither of the two viruses showed significant differences in terms of their detection using the two methods.

Regarding the process controls recoveries, mean values for PEDV were 141.20 % ± 36.03 % and 38.57 ± 5.22 % for the AP and TNA methods, respectively. For MgV, these values were 6.82 ± 4.80 % in the AP method and 33.68 ± 11.62 % in the TNA method. Statistically significant differences were found for MgV (p-value = 0.001) and PEDV (p-value = 0.0008) recoveries, showing higher recoveries with the AP method for PEDV and with the TNA method for MgV (Fig. 3).

3.2. Comparison of the aluminum-based adsorption precipitation method and direct capture method for virus viability

Table 1 shows the infectious viruses recovered after concentration of PBS and sewage samples using both approaches. In samples concentrated using the AP procedure, levels of infectious MNV and HAV were not reduced (p > 0.05). The TNA procedure did not retrieve infectious MNV in PBS and wastewater samples while HAV concentration was statistically (p < 0.05) reduced by 2.5 and 2.1 log in PBS and wastewater samples respectively.

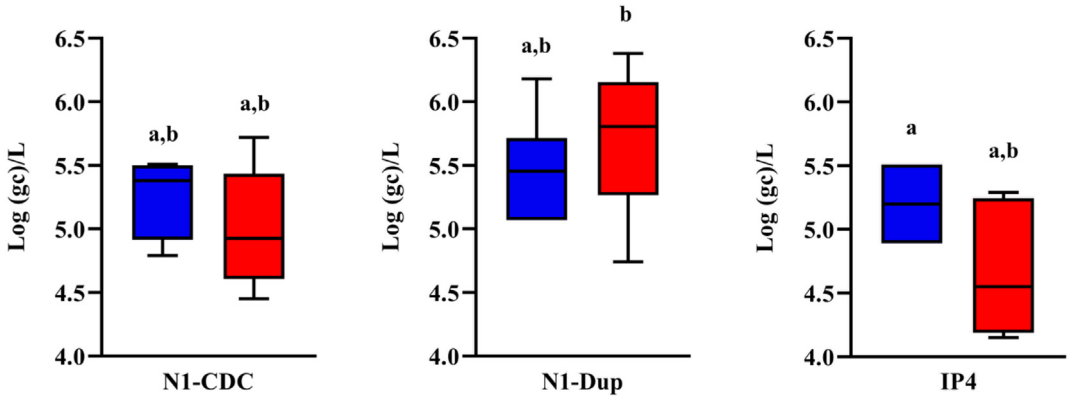

Fig. 1. Levels ($\log_{10}$ gc/L) for three genetic SARS-CoV-2 targets in analyzed wastewaters (n = 6) using the aluminum-based adsorption-precipitation (AP, blue boxes) and the Enviro Wastewater TNA Kit (TNA, red boxes). Different letters denote significant differences (p-value < 0.05) with non-parametric Dunn's test.

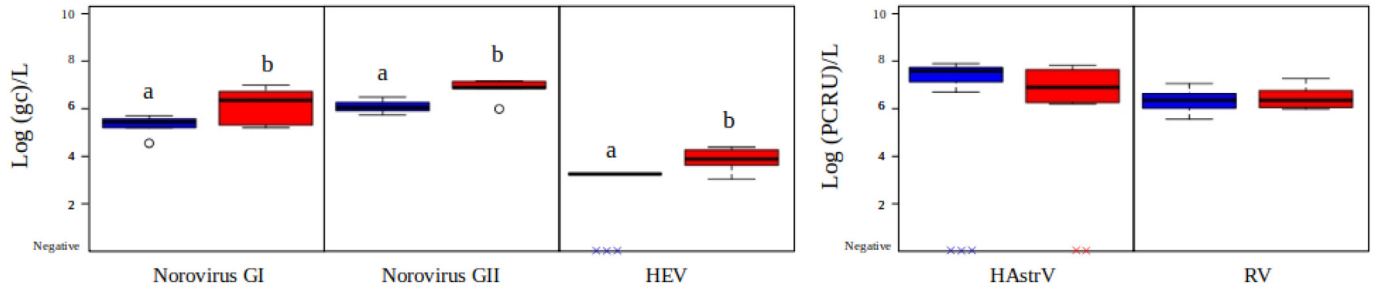

Fig. 2. Levels (log_{10} gc/L for Norovirus GI and GII, and HEV; log PCRU/L for HAstrV and RV) of human enteric viruses in wastewaters ($n = 6$) using the aluminum-based adsorption-precipitation (AP, blue boxes) and the Enviro Wastewater TNA Kit (TNA, red boxes). Different letters denote significant differences (p-value < 0.05) for each virus levels between each concentration method with Student's t-test (norovirus GI and norovirus GII) and Wilcoxon test (HEV). Crosses at the bottom represent negative samples.

3.3. SARS-CoV-2 sequencing

Six grab wastewater samples were concentrated with both concentration methods and nucleic acids were extracted as described above. Additionally, two primer schemes (i.e. Artic V3 and V4, https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019) were used. Fig. 4 shows the results obtained after bioinformatics analyses regarding percentage of viral reads classified as SARS-CoV-2, the

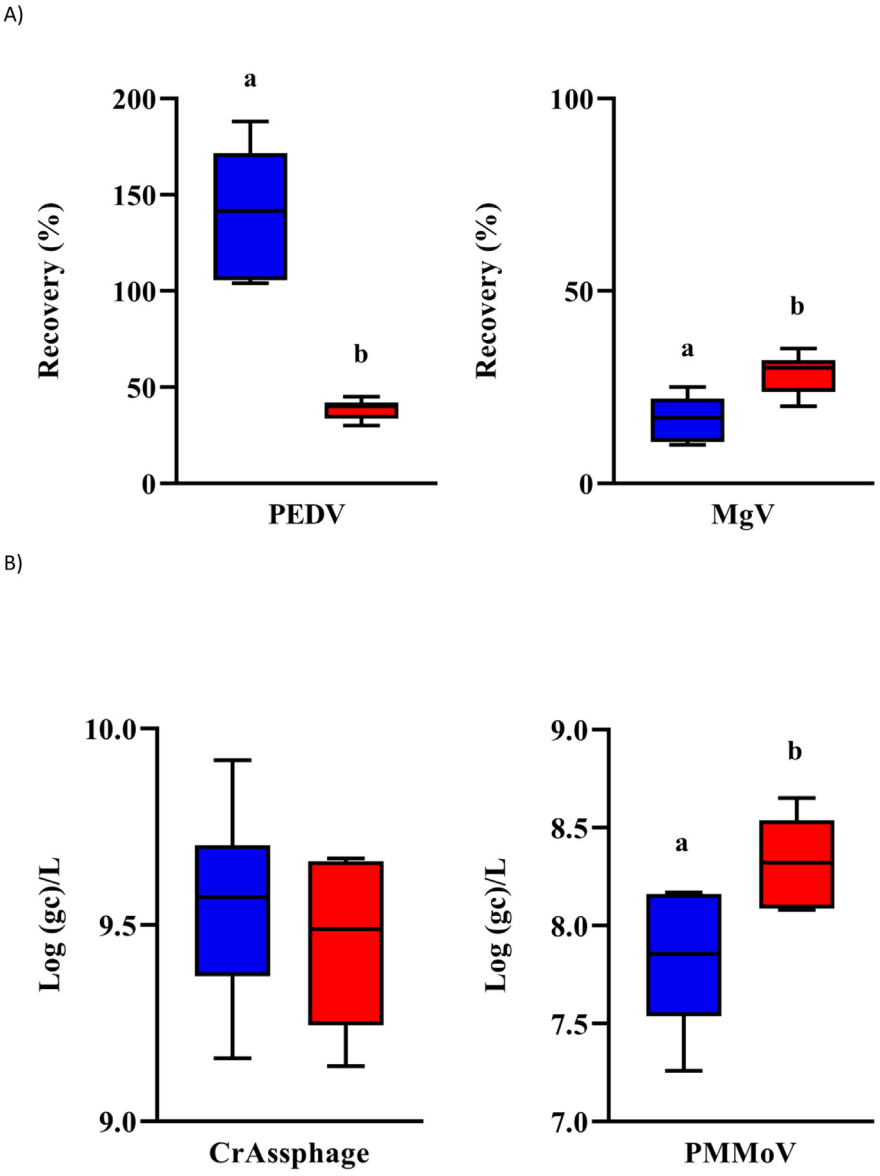

Fig. 3. A) Percentages of PEDV and MgV recoveries, and B) Levels (log_{10} gc/L) of viral indicators PMMoV and crAssphage, in wastewaters ($n = 6$) using the aluminum-based adsorption-precipitation (AP, blue boxes) and the Enviro Wastewater TNA Kit (TNA, red boxes). Different letters denote significant differences (p-value < 0.05) for each virus levels between each concentration method with t-test (MgV and PMMoV) and Wilcoxon test (PEDV).

Table 1
Mean values of murine norovirus (MNV) and hepatitis A virus (HAV) titers (log $TCID_{50}$/mL) and logarithmic reductions obtained for PBS and wastewater samples concentrated using the aluminum-based adsorption-precipitation (AP) and the Enviro Wastewater TNA Kit (TNA). Different letters denote significant differences between treatments.

Concentration method	Sample	MNV		HAV	
		Titer (log $TCID_{50}$/mL)	Log reduction	Titer (log $TCID_{50}$/mL)	Log reduction
	PBS	6.76 ± 0.07 [a]	–	6.04 ± 0.21 [a]	–
AP	PBS	6.64 ± 0.24 [a]	0.13	5.95 ± 0.27 [a]	0.09
	Wastewater	7.14 ± 0.07 [b]	−0.38	5.45 ± 0.10 [b]	0.59
TNA	PBS	<1.15 [c]	>5.61	3.57 ± 0.00 [d]	2.47
	Wastewater	<1.15 [c]	>5.61	3.95 ± 0.00 [c]	2.09

percentage of genome of SARS-CoV-2 covered, and the mean values of coverage depth. The mean percentage of reads identified as SARS-CoV-2 ranged from 20.5 ± 15.0 % in AP-V4 to 55.1 ± 26.7 % in TNA-V4. Statistical analyses showed significative differences in the percentages of SARS-CoV-2 reads between the AP method amplified with the primer scheme V4 and the TNA method (*p*-values of 0.03 for TNA-V3 and 0.008 for TNA-V4), with the reads being lower when the AP-V4 method was used. Regarding the percentage of genome coverage, samples processed with the TNA method and amplified with the V4 primer scheme showed higher genome coverages (83.7 ± 15.5 %) and significant differences (p-value = 0.02) compared with the other methods, with the exception of the TNA method with V3 primer scheme (61.4 ± 26.8 %) which did not show significant differences with respect to the results obtained with TNA-V4 (Figs. 4 and 5). Mean depth values were higher with method TNA-V4 (mean values 727.2 ± 367.8) which showed slight significative differences (p-value = 0.04) with method AP-V3 (318.2 ± 70.7). However, variability was higher in TNA-V4 than in the other analyzed methods.

4. Discussion

Wastewater-based epidemiology has proven to be an effective and useful tool for virus surveillance and outbreak detection, both for enteric viruses and for viruses that can be excreted in feces and urine (Asghar et al., 2014; Cuevas-Ferrando et al., 2020; Hellmér et al., 2014; Miura et al., 2016; Polo et al., 2020; Prevost et al., 2015; Santiso-Bellón et al., 2020). However, the detection of viruses in wastewater entails a previous step of sample concentration due to the low proportion of viruses compared to other microorganisms in these types of samples. Different concentration procedures have already been described and compared; however so far there is no standardized protocol for human enteric virus and SARS-CoV-2 detection (Rusiñol et al., 2020a; Torii et al., 2022). In this study, two different methods for wastewater concentration were evaluated for the detection of human enteric viruses, and viral indicators. Moreover, the performance of these procedures for SARS-CoV-2 detection and characterization by sequencing was evaluated using two different primer schemes.

The aluminum-based adsorption precipitation method (AP) has been used for the detection of enteric viruses in wastewater so far (Cashdollar and Wymer, 2013; Ikner et al., 2012). Furthermore, this method has also been validated for SARS-CoV-2 detection (Pérez-Cataluña et al., 2021) and it is currently used as a reference method in the Spanish COVID-19 wastewater surveillance project for the detection of SARS-CoV-2 and its variants (VATar COVID-19) (Carcereny et al., 2021). On the other hand, the TNA method has been recently validated for SARS-CoV-2 and viral fecal indicators, but no data about its feasibility for enteric virus detection has been published (Jiang et al., 2022; Mondal et al., 2021).

Even though the number of samples analyzed was limited, our results showed differences in viral recoveries of process control viruses (i.e. PEDV and MgV). In the case of PEDV, used as a model of enveloped viruses, the AP method showed higher recovery rates than the TNA method. However, the percentage of SARS-CoV-2 positive samples using the TNA method performed better (Fig. 1, Sup. Table S1). Regarding recovery rates of MgV, used as a model on non-enveloped viruses, higher recoveries were obtained when the TNA method was used. These recovery rates (mean 33.7 %) were similar to the ones obtained by Borgmästars et al. (2021) for MgV and human enteric viruses (Norovirus GI and GII, and HAV) with skimmed milk flocculation (SMF). However, with the SMF technique, 10 L were used for sample concentration, while with the TNA method only 40 mL were processed, simplifying the whole procedure. Moreover, enteric viruses (with the exception of RV and HAstrV) and PMMoV were detected more frequently when the TNA method was used, reinforcing the suitability of the TNA method in the detection of non-enveloped viruses.

Cell culture assays were carried out to evaluate the potential viability/infectivity of the viral particles present in the sewage after both concentration methods were applied. Our results showed that the AP concentration method is more successful for this purpose than TNA, which reported no infectious titers for MNV or infectivity loss of >2 log for HAV after being concentrated. This result could be due to the presence of alcohols (isopropanol and ethanol) in the TNA kit composition affecting viral infectivity. As expected, HAV was more resistant to the alcohols present in the TNA kits. Therefore, with regards to viral infectivity in sewage samples (Cuevas-Ferrando et al., 2021), the present results showed that the concentration methods applied need to be carefully validated.

Due to the limitations that classical techniques used in virus detection sometimes present, such as PCR or cell culture techniques, the use of massive sequencing technologies for the study of viruses in the environment is currently on the rise. For this reason, the European Union urges researchers to analyze SARS-CoV-2 in wastewater using these techniques. However, few studies have analyzed the concentration effects in genome sequencing. Thus, the effect of the two concentration methods as well as

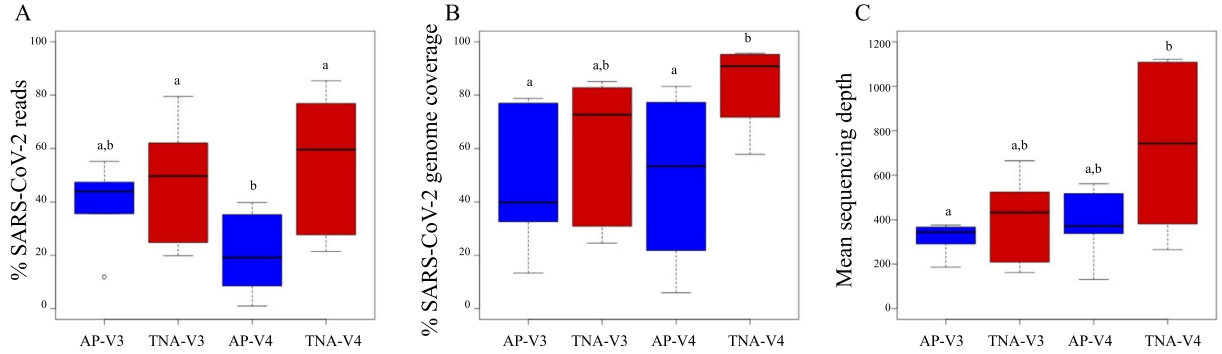

Fig. 4. Values obtained between the different concentrations methods tested in the study of the percentage of SARS-CoV-2 reads (A), SARS-CoV-2 genome coverage (B), and mean genome depth above 20× (C) after amplicon-based sequencing of SARS-CoV-2 with Artic primer scheme version 3 (V3) and 4 (V4). For each analysis (n = 6), boxes with the same letter show differences not statistically significant (p-value < 0.05).

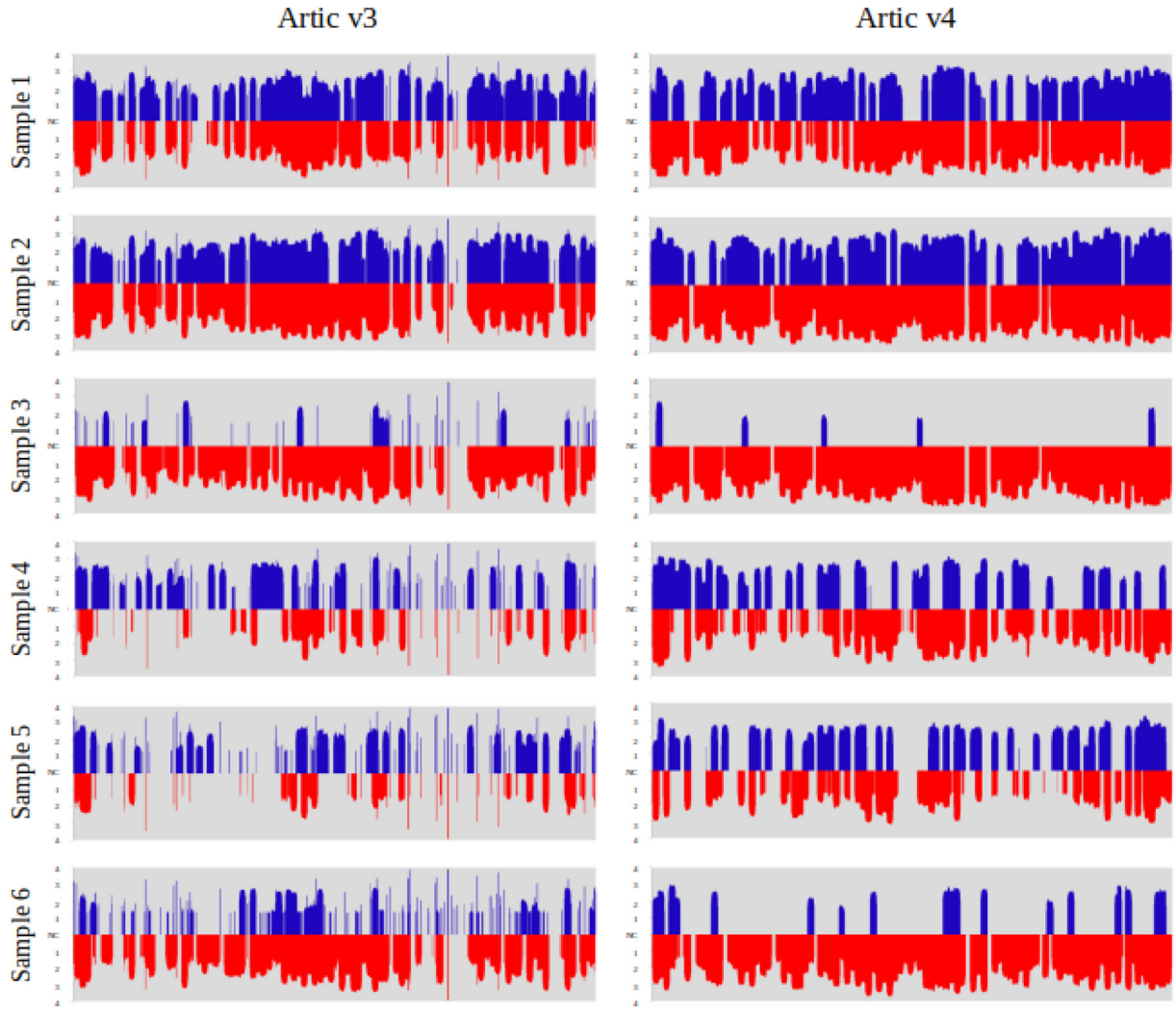

Fig. 5. X-axis: genome coverage of the SARS-CoV-2 reference genome MN908947.3 (only nucleotides with depth higher to 20×) in logarithmic scale (max 4 log) for each sample. Y-axis: logarithm of the depth (>20×) for each nucleotide position of the SARS-CoV-2 genome. NC, not covered. Blue, aluminum-based adsorption-precipitation; Red, Enviro Wastewater TNA Kit.

the primer scheme effect in SARS-CoV-2 genome sequencing was also evaluated. Regarding the percentage of genome coverage, samples processed with the TNA method showed higher genome coverages than with the other studied method. Similar results were obtained for genome coverage in the study performed by Kevill et al. (2022), although the authors did not find significative differences between the methods tested in their study. Values obtained with TNA-V4 regarding genome coverage were higher than the ones reported by Izquierdo-Lara et al. (2021) who showed average values of the percentage of SARS-CoV-2 genome of 51.3 ± 14.7 %. However, these authors performed an ultracentrifugation method for sample concentration that can produce lower virus recoveries, which would also affect sequencing (Hmaïed et al., 2016; Izquierdo-Lara et al., 2021; Prado et al., 2021; Ye et al., 2012). These results suggested that the use of the TNA method combined with the amplification of SARS-CoV-2 genomes using the Artic primer scheme V4 would give better results than with the other methods; although a high intravariability between samples can be produced.

5. Conclusions

WBE has proven to be an effective tool in epidemiological surveillance. However, the different methods used for the analysis of wastewater samples may produce differences in the results obtained. In this work, two sample concentration methods for virus analysis using molecular and cell culture techniques were compared alongside the two most commonly used primer schemes for SARS-CoV-2 genomic sequencing. Our results showed concentration methods are critical for the surveillance of human enteric viruses and SARS-CoV-2. In this sense, the use of the concentration system through the TNA system produces better results in terms of sensitivity and SARS-CoV-2 coverage sequencing. However, this technique completely reduces virus viability, indicating that methods such as aluminum precipitation would be recommended if these samples are to be tested on cell culture. Furthermore, concentration by the TNA method in combination with the Artic v4 primer scheme yields better sequencing results on sewage samples. Our results provide new information on the effects of the methods used for WBE studies, allowing us to improve this tool for use in epidemiology.

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CRediT authorship contribution statement

Inés Girón-Guzmán: Investigation, formal analysis, writing, and reviewing. Azahara Díaz-Reholid: Investigation, formal analysis, writing, and reviewing. Enric Cuevas-Ferrando: Investigation, formal analysis, visualization, writing, and reviewing. Irene Falcó: Investigation, formal analysis, writing, and reviewing. Pablo Cano-Jiménez: Investigations and formal analysis. Iñaki Comas: Supervision, funding acquisition, writing,

and reviewing. Alba Pérez-Cataluña: Investigation, formal analysis, visualization, writing, and reviewing. Gloria Sánchez: Conceptualization, supervision, funding acquisition, writing, and reviewing.

Data availability

Data will be made available on request.

Declaration of competing interest

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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3.4. Sewage reclamation process as multifactorial public health risk concern: a longitudinal study

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Under Review

Sewage reclamation process as multifactorial public health risk concern: a longitudinal study.

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Abstract

This year-long research analysed emerging risks in influent, effluent wastewaters and biosolids from six wastewater treatment plants in Spain's Valencian Region. Specifically, it focused on human enteric and respiratory viruses, bacterial and viral faecal contamination indicators, extended spectrum beta-lactamases-producing *Escherichia coli* and antibiotic resistance genes. Additionally, particles and microplastics in biosolid and wastewater samples were assessed. Human enteric viruses were prevalent in influent wastewater, with limited post-treatment reduction. Wastewater treatment effectively eliminated respiratory viruses, except for low levels of SARS-CoV-2 in effluent and biosolid samples, suggesting minimal public health risk. Antibiotic resistance genes and microplastics were persistently found in effluent and biosolids, thus indicating treatment inefficiencies and potential environmental dissemination. This multifaced research sheds light on diverse contaminants present after water reclamation, emphasizing the interconnectedness of human, animal, and environmental health in wastewater management. It underscores the need for a One Health approach to address the United Nations Sustainable Development Goals.

1. Introduction

Water is a fundamental resource for human life, being also essential for crops and livestock production. However, the increasing global population and limited freshwater resources pose significant challenges to meeting the demands of various sectors, including agriculture. Water reuse has emerged as a sustainable solution to preserve freshwater resources and reduce environmental pressure. Reclaimed water, also known as recycled water or effluent from wastewater treatment plants (WWTPs), refers to the treated wastewater that undergoes a series of physical, chemical, and biological processes to remove contaminants and pathogens. The reclaimed water is then suitable for non-potable uses, such as irrigation, industrial processes and groundwater recharge according to national regulations[1].

Water reuse has become increasingly important in agriculture due to the limited freshwater resources and the growing demand for food production. Agriculture accounts for approximately 70 % of global freshwater withdrawals and the water demand for crops and livestock is projected to increase in the coming decades[2]. Reclaimed water offers a sustainable solution to reduce the demand for freshwater resources and ensure the availability of water for irrigation, while reducing the discharge of treated wastewater into the environment and the cost of water supply. However, water reuse also poses several challenges, particularly in terms of microbiological and chemical safety. Reclaimed water may contain a variety of contaminants, including bacteria, viruses, protozoa, and emerging pollutants, such as microplastics (MPs), antibiotic resistant genes (ARGs) and pharmaceuticals[3].

In particular, human enteric viruses are responsible for causing viral gastroenteritis, hepatitis, and various illnesses primarily transmitted through the faecal-oral route[4]. The spread of these viruses is primarily linked to person-to-person contact and the consumption of contaminated food and water. Enteric viruses are excreted in substantial quantities, up to 10^{13} particles per gram of stool, by both symptomatic and asymptomatic individuals[5,6]. Major causative agents of waterborne viral gastroenteritis and hepatitis outbreaks worldwide include rotaviruses (RVs), norovirus genogroups I (HuNoV GI) and II (HuNoV GII), hepatitis A and E viruses (HAV and HEV), and

human astroviruses[5] (HAstVs). In this context, and related to microbiological risks dissemination, a new European regulation (EC, 2020/741) on minimum quality criteria (MQR) for water reuse is in place since June 2023, outlining the guidelines for the use of reclaimed water for agricultural irrigation[7]. However, questions have arisen concerning potential non-compliance scenarios in European water reuse systems[8-12]. According to EC 2020/741 regulation, validation monitoring needs to assess whether the performance targets reductions are met. Monitoring of pathogen elimination in the water reclamation process is necessary to assess the suitability of reclaimed water in its secondary uses. In this respect, the WHO has suggested that another problem to be tackled in the framework of "One Health" is the rise of antibiotic resistance (AR)[13]. AR is frequent in places where antibiotics are employed, but antibiotic resistant bacteria (ARB) and ARGs are also widely prevalent in water environments[14,15]. According to several reports, surface water and reclaimed wastewater used for irrigation are significant sources of ARBs and ARGs[16]. Due to inadequate removal of ARGs, which are crucial in the growth of extremely unfavourable drug-resistant superbugs, reuse of WWTP effluents may be harmful to human health[17].

On the other hand, plastic pollution is currently one of the most important environmental problems that humanity must face. The exponential growth of plastic production since 1950s (up to 368 million of tons were produced in 2019) and the massive use of plastics, together with insufficient/inadequate waste management/disposal strategies, are the main causes of the global presence of plastics in every environmental compartment[18]. The European Commission has recently published an amending Annex to Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) as regards synthetic polymer microparticles, where the intentional use of microplastics in commercial products is prohibited[19].

Current research is showing that one of the main concerns about plastics, apart from the fact that they persist in the environment for an extremely long time, is their constant fragmentation into even smaller particles called microplastics (MPs, 1 μm – 5 mm) or nanoplastics (< 1 μm), depending on their final dimensions, though they are also released as such[20].

MPs are emerging global threats as they can end up in our body through water and food ingestion or by air inhalation[21]. The larger MPs can cause mechanical damage to the intestinal epithelium, while the smaller particles can cross the epithelial barrier[22] and end up in the lung[23], colon[24], placenta[25], and even blood[26].

MPs can transport pathogens over long distances, due to their ability to harbor biofilms on the surface, which can lead to the spread of pathogenic viruses and bacteria to new areas where they were not previously found[27]. Another of the main risks associated with MPs is that plastic materials include approximately 4% by weight of additives[28], some of them declared as possible human carcinogens and most of them considered endocrine disruptors[29]. In addition, MPs also contain traces of persistent organic pollutants (COPs), such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides[22].

It is important to highlight that depending on the performance of WWTPs high amounts of pathogens, MPs and ARGs can be released on a daily basis into rivers, lakes, and oceans[9,14,30]. On the other hand, the sludge generated as well as the effluent water from the WWTPs are generally used in agriculture as a fertilizer and for irrigation respectively, and, therefore, the presence of emerging contaminants in these biosolids and reclaimed waters can favour the propagation of plastic particles, emerging pathogens, and ARGs through agricultural soils which could reach cultivated vegetables and ultimately the human body through the trophic chain.

In overall terms, understanding the distinct risk factors involved in the water reclamation process is critical to ensuring the safety of water reuse in agriculture and other sectors, and the analysis of the water reclamation process can serve as an important risk assessment tool. Moreover, by analysing wastewater, we gain valuable insights into the collective health of a community, as it contains traces of chemical pollutants, pathogens, and biomarkers from human and animal sources. Thus, monitoring wastewater helps identifying trends in the prevalence of diseases, antibiotic resistance patterns, zoonotic pathogens, and exposure to environmental pollutants as microplastics, providing early warning and valuable data for public health interventions. This integration of environmental, human, and animal health data underscores the significance of

wastewater analysis in promoting a comprehensive and proactive “One Health” approach to public health and the well-being of both the planet and its inhabitants.

2. Results

Incidence of human enteric viruses, respiratory viruses and viral faecal indicators in influent and effluent wastewater samples.

The presence of human enteric viruses, including HuNoV GI, HuNoV GII, HAstV, HAV, HEV, and RV, was analysed, along with novel viral faecal contamination indicators pepper mild mottle virus (PMMoV), crAssphage and somatic coliphages in influent, effluent and biosolid samples from six different WWTPs in the Valencian region of Spain (Figures 1 and 2).

In influent wastewater samples, the mean highest levels of viruses were observed for RV (8.55 log GC/L), followed by HuNoV GII (7.80 log GC/L) and HAstV (7.72 log GC/L). The lowest concentration levels were detected for HuNoV GI (4.46 log GC/L), HEV (4.13 log GC/L), and HAV (3.47 log GC/L) (Figure 2). HAV was only detected in 4 out of 72 influent wastewater samples (Figure 1). PMMoV and crAssphage were detected in all influent samples, with mean levels of 5.95 log GC/L and 8.44 log GC/L, respectively.

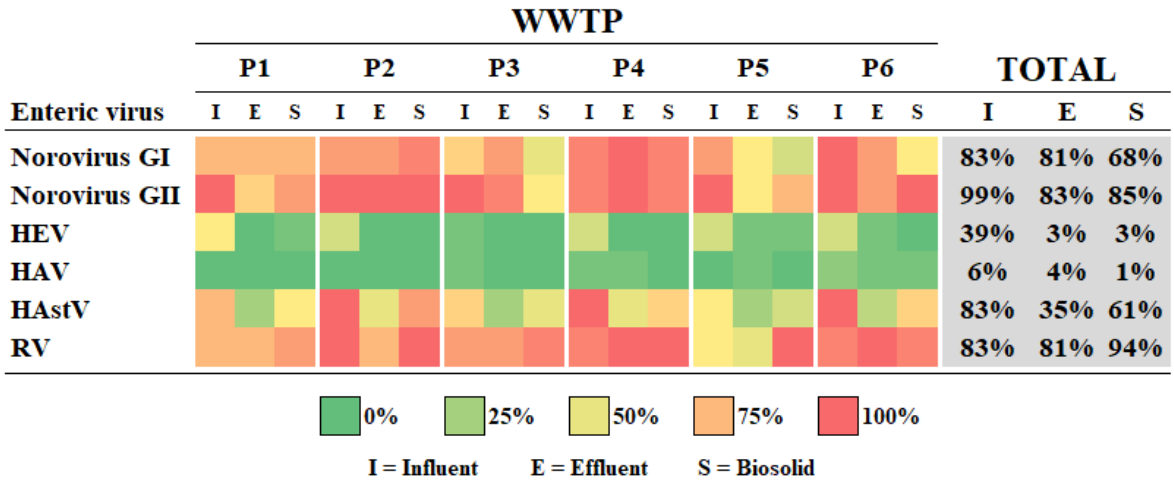

Figure 1. Prevalence of human enteric viruses (%) in influent (I), effluent (E), and biosolid (S) samples collected from six different WWTPs (P1-P6).

In the effluent wastewater samples, the titres of all viruses decreased after the water reclamation process. HuNoV GI, HuNoV GII, HAstV, and RV showed mean concentrations titers of 3.51, 6.25, 6.35, and 7.69 Log GC/L when detected, respectively (Figure 2). On the contrary, HEV was not detected in any of the effluent samples. In the case of faecal viral indicators, PMMoV (4.72 Log GC/L) and crAssphage (6.23 Log GC/L) were present in all effluent samples. The highest reduction in virus levels were observed for HEV, with a reduction of 4 Log GC/L, even though the vast majority of viruses' reduction levels were below 2 Logs GC/L (Figure S1). Interestingly, viable coliphages were found at levels of 4.73 Log plaque forming units (PFU)/100 mL in effluent waters, with a mean reduction of 1.83 Log PFU/100 mL compared to the influent waters (6.54 Log PFU/100 mL) when testing positive.

As for biosolid samples, HuNoV GI, HuNoV GII, HAstV, and RV showed the highest mean concentrations, with titers ranging from 5.37 (HuNoV GI) to 7.27 Log GC/L (RV) when detected (Figure 2). HAV and HEV rendered lower mean concentrations of 3.24 and 3.91 Log GC/L, respectively. Besides, proposed viral faecal indicators yielded mean concentrations levels of 7.06 Log GC/L for crAssphage, 4.85 Log GC/L for PMMoV. and 5.63 Log PFU/100 ml for somatic coliphages.

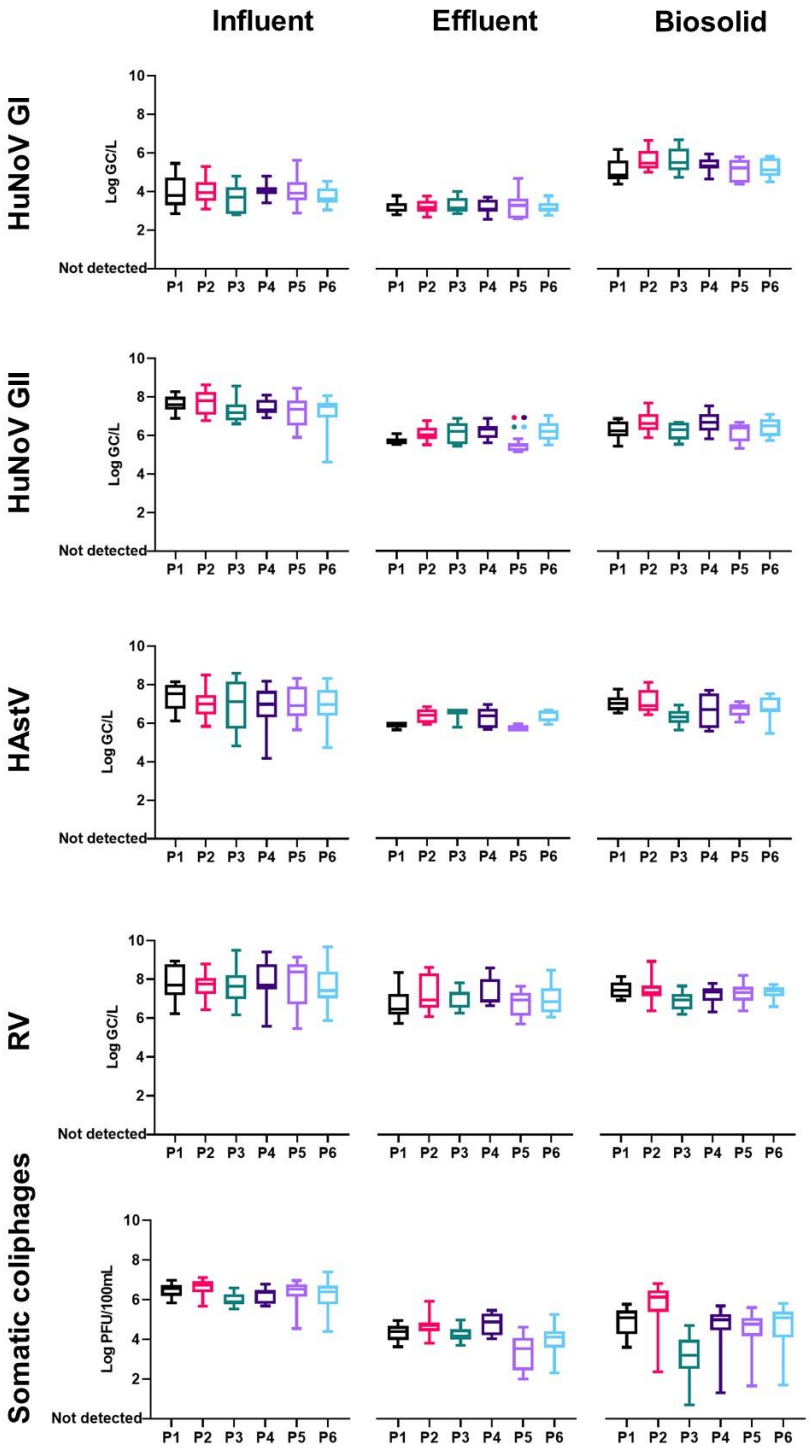

Figure 2. Mean concentrations of human enteric viruses (when detected) and somatic coliphages in influent wastewater, effluent wastewater, and biosolid samples in each of the six WWTPs analysed (P1 - P6). Coloured circles above a box indicate significant differences between that box and the box with that same colour ($p < 0.05$). GC: genome copies; PFU: plate forming units, RV: rotavirus; HuNoV: human norovirus, HAstV: human astrovirus.

Regarding respiratory viruses, respiratory syncytial virus (RSV) showed a remarkable seasonality, with almost all positive samples being collected on November and December 2022 (Figure 3). Influenza A virus (IAV) was intermittently detected over the year, with the most noteworthy peaks taking place in spring and winter (Figure 3). Finally, SARS-CoV-2 was present in 99% and 32% of the influent and effluent samples, respectively. When testing positive, mean concentration values for RSV, IAV, and SARS-CoV-2 were 4.57, 6.20, and 5.27 Log GC/L, respectively. Notably, any of the analysed effluent wastewater samples tested positive for either RSV or IAV.

Regarding biosolid samples, SARS-CoV-2 was found positive in the 71% of the samples at mean concentration of 4.44 Log GC/L, while RSV and IAV only tested positive in three biosolid samples.

In general, no significant differences were found among the six different WWTPs analysed neither for enteric nor respiratory viruses.

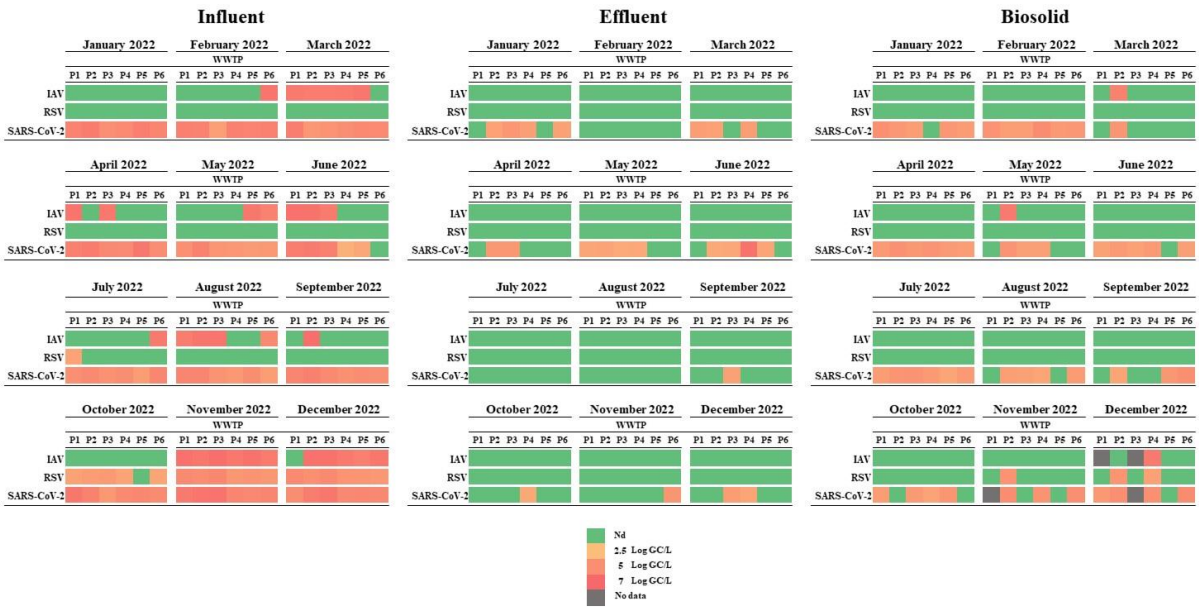

Figure 3. Concentration (in Log GC/L) of RSV, IAV, and SARS-CoV-2 in influent, effluent, and biosolid samples collected over a one-year period in six different WWTPs (P1-P6). Nd: not detected. GC: genome copies; RSV: respiratory syncytial virus; IA: Influenza A virus

Quantification of *Escherichia coli*, Extended Spectrum Beta-Lactamases-producing *E. coli* and ARGs in wastewater and biosolids samples.

In influent wastewater samples, the mean concentration of *E. coli* and ESBL-*E. coli* were 7.08 Log CFU/100 mL and 6.19 Log CFU/100 mL, respectively (Figure 4). After the wastewater treatment process, the mean concentrations of *E. coli*, and ESBL-*E. coli* in the effluent wastewater samples were significantly reduced, with mean concentrations of 5.43 Log CFU/100 mL, and 4.76 Log CFU/100 mL, respectively.

Regarding biosolid samples, the mean concentration of *E. coli* was 5.64 Log PFU/100 mL, while ESBL-*E. coli* yielded a mean concentration of 4.89 Log CFU/100 mL.

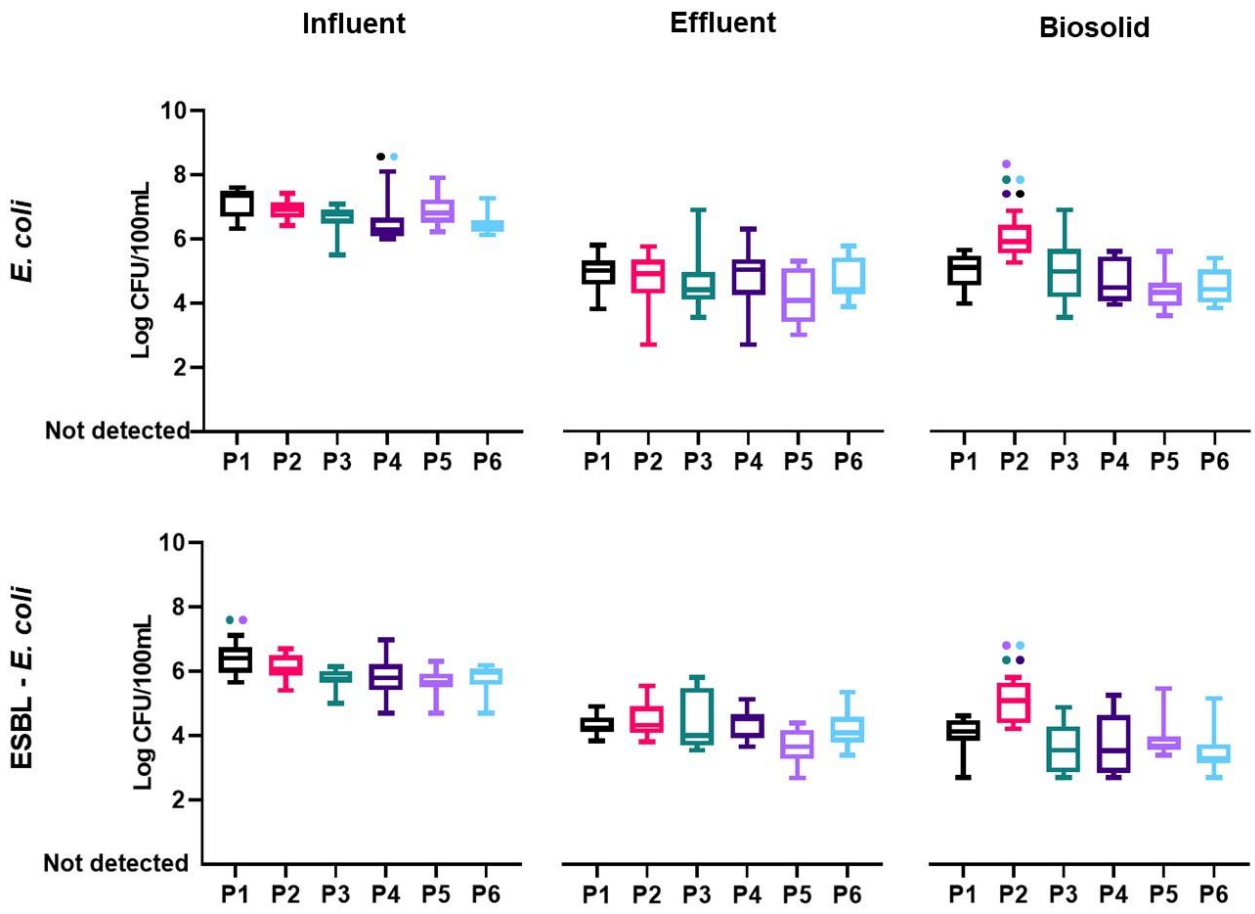

Figure 4. Levels of *E. coli* and ESBL-*E. coli* in influent, effluent, and biosolid samples in each of the six WWTPs analysed (P1-P6). Coloured circles above a box indicate significant differences between that box and the box with that same colour ($p < 0.05$). CFU: colony forming unit.

Furthermore, a deeper analysis on the ARGs present in effluent and biosolids samples was performed due to the high levels of ESBL-*E. coli* in biosolids and the observed low performance of the water reclamation process (less than 2 log reduction; Figures 2 and 4). ARGs including tetPB_3, tetA_1, and qacA_1 were not detected in effluent wastewater and biosolids. ARG sul1_1, sul2_1, pbp2b, bla_{CTX-M}, cmlA_2, nimE, and ermB were detected in effluent samples at mean concentrations of 9.20, 8.78, 8.57, 8.42, 8.31, 8,24, and 8.39 Log GC/100 mL, respectively (Figure 5).

ARGs were identified in biosolids, with the following values: 9.87, 9.25, 8.58, 8.42, 8.50, 8.64, 8.28 Log GC/100 mL for sul1_1, sul2_1, pbp2b, bla_{CTX-M}, cmlA_2, ermB, and ermA, respectively. Notably, nimE was not found in any of analysed biosolids.

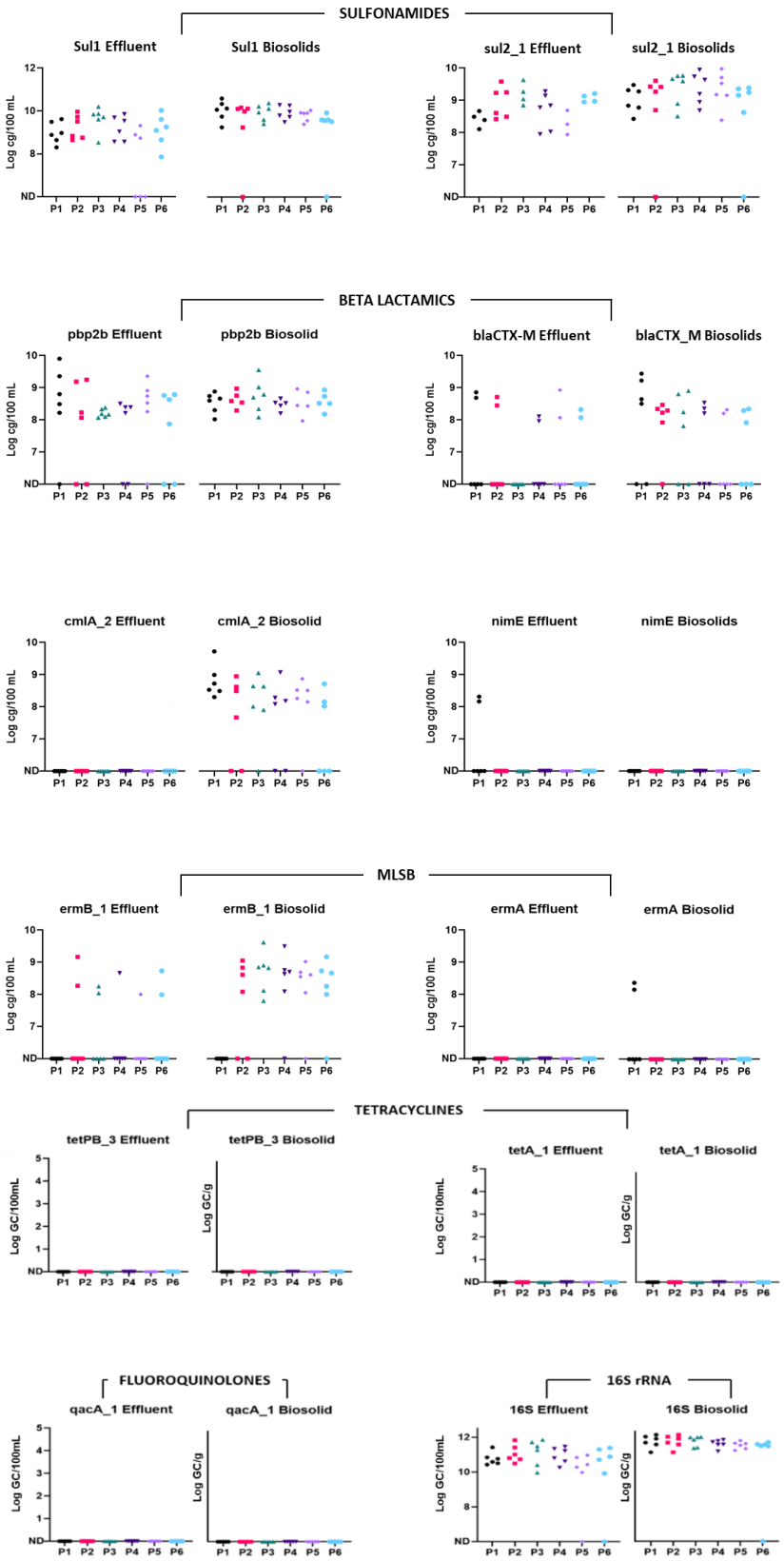

Figure 5. Levels of different ARGs in effluent wastewaters (in Log GC/100 mL) and biosolids (in Log GC/g) samples for each of the six WWTPs analysed (P1-P6). ND: Not detected. MLSB: Macrolide-lincosamide-streptogramin B group antibiotics; GC: genome copies.

Quantification of particles and microplastics present in biosolids and reclaimed water samples.

The presence of solid particles and microplastics was bi-monthly analysed in both influent and effluent wastewater samples. In general, a great reduction in both the number of particles between 1 µm and 5 mm or (T)-P and particles larger than 300 µm or (S)-P was observed after the wastewater treatment process (Figure 6). Although there was not a clear effect derived from seasonality, WWTPs were slightly less efficient in removing (T)-P in January and March.

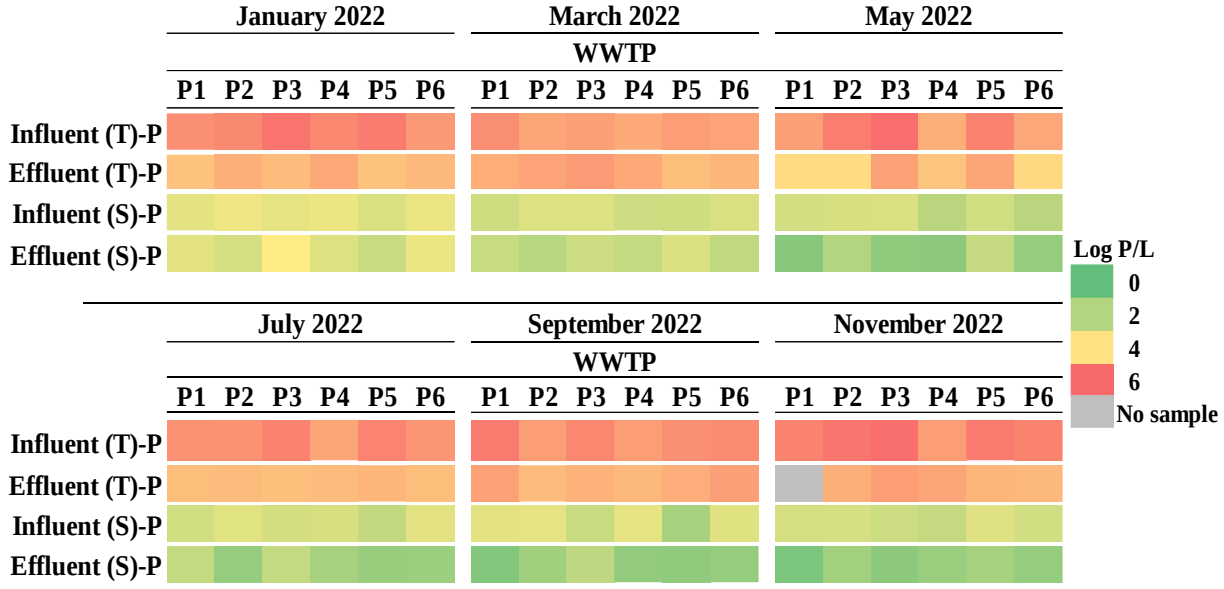

Figure 6. Concentration (log P/L) of total particles (T)-P and sieved particles (> 300 µm, (S)-P) in influent and effluent wastewater samples in even months over a one-year period in six different WWTPs (P1-P6).

The efficiency of each WWTPs regarding the reduction of (T)-P and (S)-P particles was determined considering the average number of particles in the influent and effluent wastewater samples (Figure 7). At the WWTP level, the calculated efficiency in (T)-P reduction was approximately 84, 68, 69, 46, 80 and 71%, for the different WWTPs (P1-P6) samples analysed. Notably, the efficiency in removing (S)-P was higher than in removing (T)-P, with the most

noteworthy reduction taking place for P2 and P6 wastewater samples (91 and 93% approximately and respectively), while the lowest efficiency in (T)-P reduction was approximately 40% for P5.

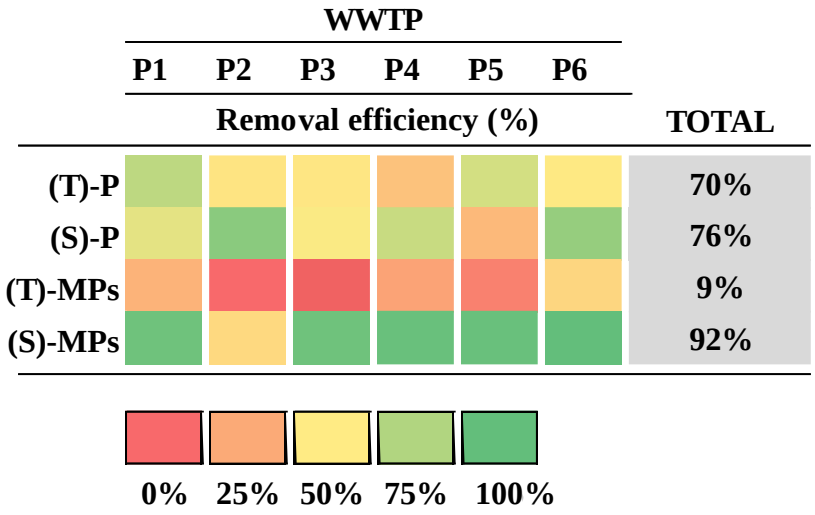

Figure 7. Removal efficiency (%) of all solid particles (P) and microplastics (MPs) between influent and effluent samples collected from six different WWTPs (P1-P6) after both pre-treatment protocols (T) and (S).

Once (T)-P and (S)-P particles were quantified, all samples were spectroscopically characterized in order to identify the presence of MPs derived from synthetic polymer particles, fibres, and films. In general terms, the highest reduction was observed in (S)-MPs as compared to (T)-MPs, thus suggesting the lower efficiency of wastewater treatments in removing microplastics smaller than 300 μm (Figure 7). It should be highlighted that the efficiency of WWTPs for removing MPs of smaller particle size or (T)-MPs was lower than for removing all solid particles or (T)-P, being 59% the highest (T)-MPs efficiency (sample P6). In general, a higher efficiency in reducing (S)-MPs was observed (around 98-100%) in all samples, except in P2 (77%) (Figure 7).

Considering the pre-treatment (T), the annual average MPs concentration in influent samples was around 1816 MPs/L which was slightly reduced in effluent samples (1724 MPs/L). In contrast, the annual average concentration of (S)-MPs (larger than 300μm) in influent samples was 198 MPs/L and it was significantly reduced in effluent wastewater samples until 11 MPs/L in average (Figure 8).

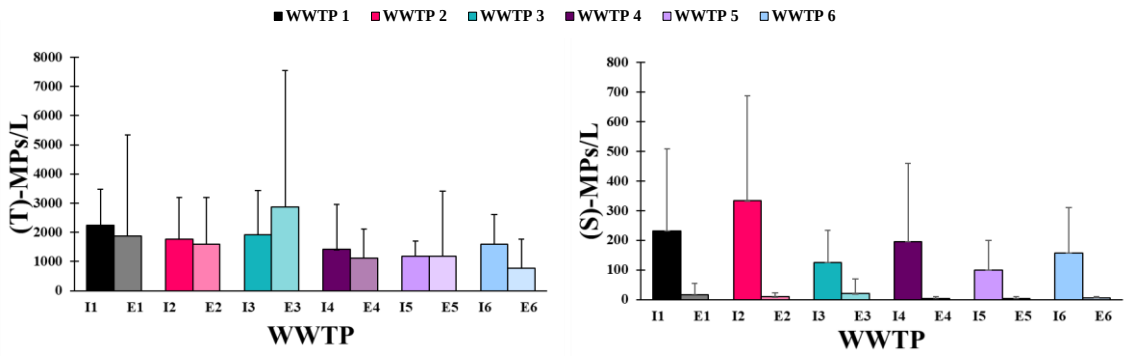

Figure 8. Annual average concentration of microplastics (MPs) in influent (I) and effluent (E) after (T) (Panel A and (S) (Panel B) protocols collected from six different WWTPs.

The annual average percentage of MPs respect to all solid particles in influent and effluent wastewater samples and biosolids was also determined and the results (Figure S2). It is worth mentioning that, regarding the particles larger than 300 μm, the MPs/all solid particles ratio in biosolid samples was similar to the MPs/all solid particles ratio in influent wastewater samples, reaching values up to 35 in some of the WWTPs (Figure S2).

In all the analysed biosolid samples a significant number of (S)-P was also detected, and no significant effects due to seasonality were found (Figure 9). The average highest concentration of (S)-MPs was 122 MPs/g and 99 MPs/g for P1 and P2, respectively. In contrast, the lowest level of MPs was detected for P3 (23 MPs/g) (Figure S3).

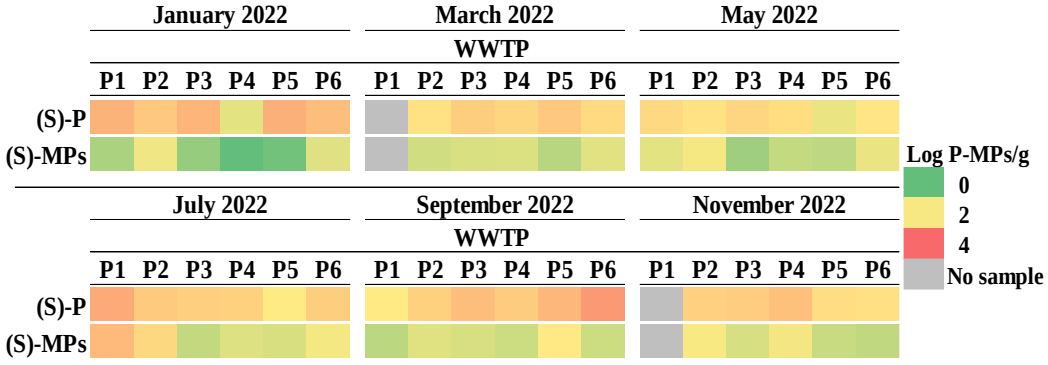

Figure 9. Concentration (in log/g) of (S)-P and (S)-MPs in biosolids in even months over a one-year period in six different WWTPs (P1-P6).

Analysing the morphology and type of MPs identified in the WWTPs samples may help to understand the origin of water pollution. As depicted in Figure 10, the majority of MPs existing in influent wastewater samples had the shape of fragments (~ 86 %), percentage that was further increased in effluent wastewater samples. The percentage of particles identified as films was negligible both in influent or effluent samples. Most of the MPs found in influent samples were between 0-100 µm (~ 61 %) in size, percentage that was increased in effluents (up to 73 %), and a small fraction of MPs (~ 3-5 %) were larger than 300 µm in size, in agreement with the results commented above (Figure 8). The composition of the MPs was dominated by common polymers, whereas the PS, PA, PVC, and PET were greatly decreased in effluent samples (Figure 10). It is worth mentioning that the distribution of polymer type was quite different when comparing wastewater and biosolids samples. PE was dominant in all samples, accounting for 56, 46 and 57 % of the total MPs, for wastewater (T)-MPs and (S)-MPs, and for biosolids (S)-MPs, respectively (Figure S4). The amount of PA was more than two-fold higher in (T)-MPs samples from wastewater than in (S)-MPs from biosolids (31% vs. 12%, respectively). PET represented around 21-28% of the (S)-MPs in wastewater and biosolid samples. Other polymers such as PS, polytetrafluoroethylene PTFE, PVC and PS were detected in lower amounts.

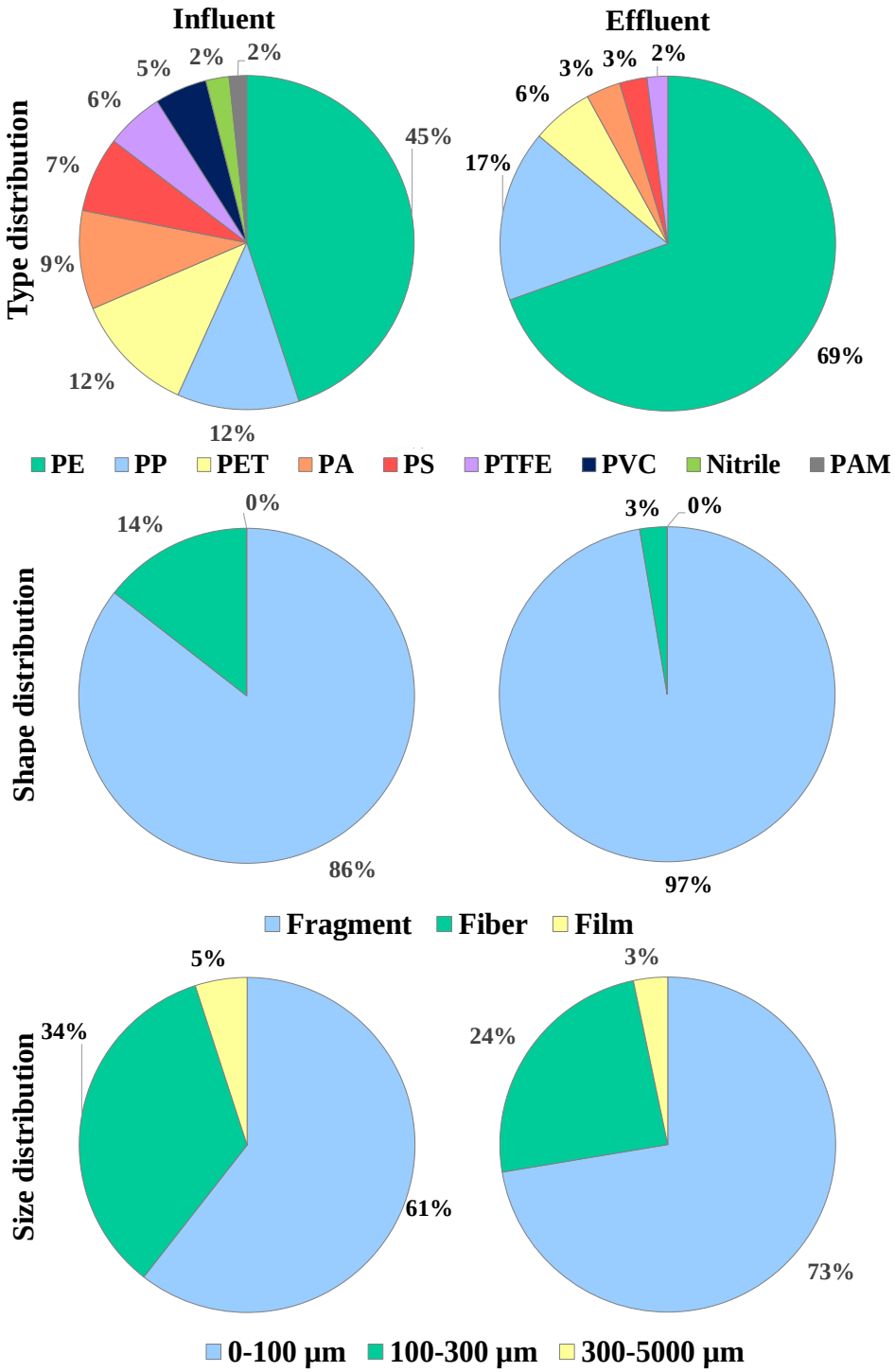

Figure 10. Morphological distribution of type, size, and shape of (T)-MPs in influent and effluent wastewater samples from six different WWTPs (P1-P6). PE: polyethylene; PET: polyethylene terephthalate; PA: polyamide; PP: polypropylene; PS: polystyrene; PVC: polyvinyl chloride; PTFE: polytetrafluoroethylene; PAM: polyacrylamide.

3. Discussion

Reuse of effluent wastewater and biosolids in agriculture is essential to face the increasing demand of water and agricultural products in combination with global warming and water scarcity[31]. Effluent wastewater and biosolids, however, are sources of emerging contaminants of concern such as viral pathogens, antibiotic resistance genes and microplastics. The reuse of water and the release of reclaimed water into the environment may compromise public health due to the combination of several risk factors. In recent years, several publications have pointed out the low efficiency of WWTPs in removing viral pathogens[9]. While decay rates of human enteric viruses in effluents wastewater samples are frequently studied, very few studies have reported the incidence of respiratory viruses, MPs and ARGs in effluent wastewaters and biosolids, with potential of being used in agriculture.

The present study investigated the presence of human enteric viruses, including HuNoV GI and GII, HAstV, HEV, and RV, as well as ARBs, ARGs, MPs and two novel viral faecal contamination indicators (PMMoV and crAssphage) in influent, effluent and biosolids samples. Consistent with findings from earlier research, influent wastewater samples exhibited elevated concentrations of human enteric viruses, MPs and ARBs[14,32] (Figures 1, 2, 4, 6, and 8).

Following the water reclamation process, the concentrations of all analysed viruses decreased in the effluent samples. However, it is worth noting that the reductions for HuNoV GI, HuNoV GII, HAstV, and RV (when detected in effluent) were below 2 Logs, suggesting the persistence of these viruses to a relevant extent after being exposed to either UV or chlorination treatments. Only HEV was not detected in any of the analysed effluent samples thus resulting in higher reductions (> 4 Log GC). The reductions observed for human enteric viruses along the year substantially differ from current European legislation (Regulation (EU) 2020/741, 2020) on water reuse, which indicates the need for ≥ 6 Log decreases on the presence of these pathogens[7]. Even though enteric viruses' presence detected by RT-qPCR in this study might not correspond with infectious particles, several publications have pointed out the presence of infectious enteric viruses in reclaimed waters by capsid-integrity or cell culture approaches[8-11,33].

Owing to the microbiological risk that the presence of enteric viruses in these waters could entail, this study also aimed to assess the levels of somatic coliphages and *E. coli* in influent and effluent wastewater samples, as well as biosolid samples. Coliphages have been found in locations where faecal contamination

is present[34,35], and numerous studies have suggested utilizing coliphages as markers for enteric viruses' presence[34-39]. Following the water treatment process, reductions of 1.83 Log PFU and 1.65 Log CFU were observed for somatic coliphages and *E. coli*, respectively. These reductions, which are far from those stipulated by the legislation EU 2020/741, 2020, highlight the low performance of the WWTPs in decreasing the microbial load and mitigating the potential risks associated with these pathogens (pathogenicity and antibiotic resistance transmission)[7]. For somatic coliphages and *E. coli*, obtained counts in biosolids were similar to those obtained in effluent wastewater samples, pointing out the risk of using biosolids without any further treatment in agriculture. Besides, in recent years, both crAssphage and PMMoV have been proposed as viral indicators of faecal contamination in water bodies and as a virus model to assess the performance of WWTPs[40-46]. Regarding effluent samples, the mean concentration of crAssphage detected in reclaimed waters was 6.25 Log GC/L, which consistently matches the reported mean concentrations of 6.5 Log GC/L in high income countries as reviewed by Adnan et al. (2022)[47]. PMMoV concentrations in effluent wastewater samples are in line with existing bibliography, which reports mean concentration values of ~ 4 Log GC/L[48-50]. Notably, obtained mean concentrations of PMMoV in untreated wastewaters (5.95 Log GC/L) are slightly under-average when compared with previously reported data, as the common concentration values of PMMoV published in influent wastewater samples range from 6 to 10 Log GC/L[48-54]. Interestingly, to our knowledge, this study includes the first report on PMMoV levels in biosolid samples which may also pose a risk for the dissemination of this plant pathogen.

As for respiratory viruses, SARS-CoV-2 and IAV were detected at mean titres similar to those reported in the US, Canada, Australia, and other regions in Spain covering the same time period, while RSV levels were at least one Log GC/L over the reported in the aforementioned studies[55-60]. In recent years, the possibility of transmission of various respiratory viruses through food and water consumption has been discussed[61]. The absence of RSV and IAV in all effluent samples analysed in this study indicates an almost non-existent risk of transmissibility caused by ineffective water treatment. Nevertheless, the high presence of SARS-CoV-2 in effluent samples, together with the presence of these respiratory viruses in several of the analysed biosolids samples and the lack of studies regarding non-respiratory routes of transmission, warrant the need for further studies to assess public health risks.

Recently, a new proposal by The Urban Wastewater Treatment Directive (UWWTD), requested that member states should monitor antibiotic resistance at WWTPs serving over 100,000 individuals[62]. As this monitoring has been proposed to be performed for both influent and effluent wastewater samples, it should tackle both environmental transmission risks arising from WWTPs and provide insights into resistance patterns within specific regional areas.

In this study, ESBL-*E. coli* levels in influent samples were very high, with 6.63 Log CFU /100 mL on average, with no statistical differences among the different WWTPs and along the year. When analysing the reclamation treatment applied by the WWTPs, only mean reductions of 1.43 Log were observed for ESBL-*E. coli*, with 4.30 Log counts on average in effluent samples, which surpass by 3 Logs the levels reported in other studies, suggesting the important role of effluent water in the dissemination of ARB in the food chain if used for irrigation and the need to improve water reclamation processes[14,63,64]. Similarly, the high levels of ESBL-*E. coli* in biosolids, suggest the need for further treatments before application in agriculture.

As well as resistant bacteria, the spread of ARGs needs to be addressed worldwide[13]. Thus, it is important to understand and mitigate their occurrence in different ecological systems. This study has shown the prevalence of 11 different ARGs belonging to 7 of the most widely used antibiotic groups in effluent water and biosolids[65]. Our study revealed that sulfonamide ARGs (sul1 and sul2) were the genes with higher concentrations in effluents and biosolid samples. In line with previous studies, levels of sulfonamide resistance genes in effluent samples were higher than macrolide, tetracycline, and quinolone resistance genes[65,66]. Furthermore, sulfonamide gene levels were higher in biosolids than effluents (Figure 5) as in the Mao et al. 2015 aforementioned study, highlighting the risk of biosolids as carriers of ARGs[65]. Levels of $bla_{CTX\text{-}M}$, ARG that confer resistance to beta-lactamase, were 4 Log higher than levels of viable ESBL-*E. coli*, which could be explained by the longer persistence of DNA[67], the presence of extracellular genetic material with bacterial surfaces, colloids, and bacteriophages, which shields it from nucleases[68-71]. This fact supports the idea that the dissemination of ARGs is not only carried out by viable bacteria but

also by being found free in the environment or carried by other microorganisms such as bacteriophages[72].

ARGs profiles were comparable in effluents and biosolids despite gene concentration differences except for cmlA_2 and ermB_1. The cmlA_2 gene, which confer resistance to phenicol, was not found in any effluent samples indicating that environmental conditions, microbial populations, or the presence of contaminants in water treatment facilities may have impacted effluents but not biosolids. In March–May 2022, the ermB_1 gene was only detected in effluent samples, whereas the ermA gene, conferring resistance to macrolide-lincosamide-streptogramin B group antibiotic, was only detected in biosolid samples collected in January, consistent with previously reported data, whereas erm genes were only detected in biosolids[73]. Cold stress, which is linked with low temperatures, may increase horizontal gene transfer of ARGs, explaining this fluctuation along the year[74]. The significant presence of the ARGs and ESBL-*E. coli* supports assertions that land application of biosolids may disseminate ARGs to soil bacteria and demonstrate their potential introduction to food products via both irrigation and amendment[75].

The wide distribution of MPs present in wastewater sources undoubtedly brings about environmental pollution and risk. Therefore, removing MPs before they reach environmental water courses is highly recommended. In this sense, WWTPs play an important role in hindering MPs from entering water environments[76]. As observed in this work, the concentration of MPs in wastewater decreased in effluent samples as compared to influent samples, being the water treatment more efficient in removing higher size particles. The number of MPs found in the different samples agreed with those reported in the literature. Previous works investigated the abundance of MPs in urban WWTPs, with ranges of 0.28 to 3.14×10^4 particles/L in the influent, which significant differed from 0.01 to 2.97×10^2 particles/L in the effluent[77]. However, they did not refer to the removal efficiency depending on the particle size. In this work, a higher efficiency in reducing MPs (between 77-100%) of higher particle size (S)-MPs has been observed, which was similar to the 88–94 % efficiency of municipal WWTPs previously reported[78]. However, this value was significantly reduced for MPs with smaller particle size (S)-MPs and presented a great

variability depending on the WWTP studied (4-59%). Deng et al. (2023) reported that the removal efficiency of MPs in a petrochemical WWTPs reached ~ 92% and highlighted that the primary treatment removed most of the MPs[79] (87.5%). Talvitie et al. (2015) also stated that the primary treatment could remove most of the MPs, although they did not refer to their particle size[80]. They reported that the major part of the fibers can be removed already in primary sedimentation process, which agreed with the lower proportion of fibers (as compared to fragments) found in these samples.

Concerning the type of polymers detected, there is a higher prevalence of PE, PET, PS and PA, as it has been previously reported for drinking water and petrochemical and urban WWTPs[79,81-83]. Furthermore, WWTPs were more efficient in removing polymers with higher density such as PA and PET, probably during the density separation step, favouring a significant reduction of these polymers in the effluent wastewater. Furthermore, the size of more than 90% of microplastic particles detected in WWTPs ranged between 1 and 300 μm and fragments were found to be the most prevalent shape of microplastics, in agreement with other works[84].

Within this context, MPs release into the environment through sludge and effluent wastewater can also pose another risk, since MPs can accumulate/transport harmful pollutants, posing concerns about their role in treatment resistance and disease spread[85]. Bacteria and viruses have been reported to adsorb onto MPs, forming plastispheres[86]. Pathogenic bacteria, including those harmful to humans and fish, have also been found in communities of MPs[87-89]. Regarding viruses, the primary interaction with MPs involves electrostatic adhesion, increasing the risk of waterborne viral transmission. These viral or bacterial plastispheres not only resist UV treatment but can also promote infections, as shown for polystyrene MPs, which have been observed to facilitate IAV infection of host cells[89,90]. Additionally, the persistence of pathogen-carrying MPs in aquatic environments raises concerns about reverse zoonosis, where these plastispheres might be ingested by aquatic organisms, potentially endangering human populations through the food chain[100]. In summary, MPs can act as carriers for pathogenic bacteria and viruses in municipal sewage, intensifying concerns about public health and the environment.

Overall, the findings of this research underscore the potential threats to public health associated with the reuse and release of reclaimed water, particularly concerning microbiological pathogens and environmental pollutants like microplastics, as well as the release of emerging contaminants into the environment and food chain through the use of biosolids in agriculture. These risk factors, including the persistence of enteric viruses, the inadequate reduction of microbial load and antibiotic resistance genes, and the prevalent presence of microplastics, emphasize the need for a holistic approach in addressing health concerns. Integrating these insights from wastewater analysis as well as human epidemic respiratory viruses monitoring into the broader One Health framework is crucial for devising effective policies, improving water treatment processes, and safeguarding both human and ecosystem health in a sustainable manner.

4. Materials and methods

Water concentration method and nucleic acid extraction for viruses and ARGs

Grab influent (n = 72) and effluent (n = 72) wastewater samples were collected along with dehydrated biosolid samples (n = 72) from 6 different WWTPs over a one-year period (January 2022 – December 2022). Samples were grabbed early in the morning (8 am) by collecting ~500 mL of wastewater in sterile HDPE plastic containers (Labbox Labware, Spain). Collected samples were transferred on ice to the laboratory, kept refrigerated at 4°C, and concentrated within 24 h. Samples were artificially contaminated with 10^6 PCR units (PCRU) of porcine epidemic diarrhea virus (PEDV) strain CV777, serving as a coronavirus model. Additionally, 10^6 PCRU of mengovirus (MgV) vMC_0 (CECT 100000) were used as a non-enveloped counterpart for recovery efficiency assessment. Effluent wastewater samples were concentrated through a previously validated aluminium-based adsorption-precipitation method[11,91]. Alternatively, 40 mL of influent wastewater samples were processed with the Enviro Wastewater TNA Kit (Promega Corp., Spain) vacuum concentration system following the manufacturer's instructions[92]. For biosolid samples, 0.1g of biosolid were resuspended in 900 µL PBS for nucleic acid extraction prior to PCR analyses.

Nucleic acid extraction from influent and effluent wastewater concentrates and biosolid suspensions was performed by using the Maxwell® RSC Instrument (Promega, Spain) with the Maxwell RSC Pure Food GMO for viral and ARG extraction. Specific programs, namely 'Maxwell RSC Viral Total Nucleic Acid' and 'PureFood GMO and Authentication,' were employed for viral and ARG extractions, respectively.

Virus detection and quantification

The detection of process control viruses, PEDV and MgV, was carried out through RT-qPCR using the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio Inc., USA) as detailed elsewhere[93]. Levels of HuNoV GI and GII, HAstV, RV, HAV and HEV were determined using the RNA UltraSense One-Step kit (Invitrogen, USA), following previously described procedures[9,11]. The occurrence of crAssphage was established using the qPCR Premix Ex Taq™ kit (Takara Bio Inc)[94]. PMMoV detection was determined using the PMMoV Fecal Indicator RT-qPCR Kit (Promega, Spain) following the manufacturer's instructions. SARS-CoV-2 detection was performed by targeting the N1 region of the nucleocapsid gene. The One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) was used with N1 primers and conditions described by CDC[95]. IAV detection followed the protocol described by CDC (2009) using primers from CDC (2020) and the One Step PrimeScript™ RT-PCR Kit (Perfect Real Time)[96].

Different controls were used in all assays: negative process control consisting of PBS; whole process control to monitor the process efficiency of each sample (spiked with PEDV and MgV); and positive (targeted gene reference material) and negative (RNase-free water) RT-qPCR controls. The recoveries of PEDV and MgV, spiked as enveloped and non-enveloped viral process controls, respectively, ranged between 6.31 and 59.65 % (data not included). The validation of results for targeted viruses adhered the criteria specified in ISO 15216-1:2017, where a recovery of the process control of ≥1% is required[97].

Commercially available gBlock synthetic gene fragments (Integrated DNA Technologies, Inc., USA) of HuNoVs GI and GII, HAstV, RV, HAV, HEV, and crAssphage were used to prepare standard curves for quantification. For IAV and RSV quantification, Twist Synthetic InfluenzaV

H1N1 RNA control (Twist BioScience, South San Francisco, CA, USA), and purified RNA of RSV (Vircell, S.L., Spain) were used. The PMMoV Fecal Indicator RT-qPCR Kit (Promega) provided PMMoV RNA for generating a standard curve. A table, featuring primers, probes, PCR conditions, limit of quantification (LOQ/L), and limit of detection (LOD/L) for all targeted viruses in this work is available in the Supplementary materials (Table S1).

Quantification of viable somatic coliphages, *E. coli*, and Extended Spectrum Beta-Lactamases producing *E. coli*.

Somatic coliphages were determined from wastewater samples filtered through sterile filters (0.45 μm pore) by using a commercial Bluephage Easy Kit for Enumeration of Somatic Coliphages (Bluephage S.L., Spain), following manufacturer's instructions. For biosolid samples, 1g of biosolid was resuspended in 100 mL PBS for both somatic coliphages and *E. coli* enumeration.

For all water and biosolid samples, *E. coli* and Extended Spectrum Beta-Lactamases producing *E. coli* (ESBL-*E. coli*) enumeration was assessed by using selective culture media Chromocult coliform agar (Merck, Darmstadt, Germany) and CHROMagar ESBL (CHROMagar, Paris, France), respectively. Spread plating (0.1 mL) or membrane filtration (200 mL) was used depending on the anticipated bacterial concentration. Influent wastewater samples were diluted serially, and 0.1 mL aliquots were spread-plated. Effluent samples were filtered through a 0.45 μm cellulose nitrate membrane filter (Sartorius, Madrid, Spain). Following incubation at 37 ºC for 24 hours, results were interpreted, with. dark blue-violet colonies considered positive for *E. coli* and dark pink-reddish colonies considered positive for ESBL-*E. coli*. The analysis was performed in duplicate, and the results were expressed as CFU/100 mL. The detection limit (LOD) for *E. coli* and ESBL-*E. coli* counts in the influent and biosolid samples was 2.0 Log CFU/100 mL (100 CFU/100 mL), while in the effluents, the LOD was 0 Log CFU/100 mL (1 CFU/100 mL).

Detection and quantification of antimicrobial resistance genes in effluent waters and biosolids

In this study, 11 ARGs that confer resistance to Sulfonamides (sul1, sul2_1), beta-lactamase (pbp2b, bla_{CTX-M}), phenicols (cmlA_2), nitroimidazoles (nimE), MLSB (ermB_1, ermA), tetracyclines (tetPB_3, tetA_1) and fluoroquinolones (qacA_1), were only detected in effluent waters and biosolids. The 16S rRNA gene was used as positive control for qPCR measurement. Quantification of the 12 selected genes was performed by high-throughput quantitative PCR (HT-qPCR) using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). qPCR cycling conditions and processing of raw data were described elsewhere[98-100]. Each DNA sample was analysed in duplicate. Data processing and analysis were performed by using a python-based script by Resistomap Oy (Helsinki, Finland)[101,102].

Digestion of organic material and isolation of MPs

Initial steps consisted on optimizing the protocol for the removal of organic material and the isolation of the maximum number of MPs from wastewater and biosolid samples. Different volumes of water, amounts of biosolids and digestion strategies for organic biomass removal were tested to remove the greatest amount of organic material without compromising the integrity of the MPs. Avoiding filter clogging was a requirement during the methodology development, to facilitate further identification of MPs. To reduce the risk of external contamination by MPs, laboratory consumables made of glass were used, the reagents were purified by filtering through a 0.2 µm pore size nitrocellulose filter (Whatman, Maidstone, UK), 100% cotton lab aprons were used, samples were processed in a laminar flow cabinet, the beakers were covered with a watch glass, disposable nitrile gloves were used and, before and after using the material, all used materials were rinsed thoroughly with deionized water. In order to assure that the isolation of MPs was effective and external contamination did not occur, a negative control (NC) was included every month and a positive control (PC) was carried out every 3 months. The positive control was made with fluorescent polystyrene microspheres (Invitrogen, Waltham, USA) of 1 µm in

diameter. Specifically, a solution of 1000 beads/20 µL was prepared and 20 µL of this solution was incorporated before the pre-treatment and, the number of remaining microbeads after the digestion protocol was determined to calculate the percentage of recovery. The average value of particle recovery was 93.9 %.

Two different pre-treatment protocols were finally defined:

1) Sieved > 300 µm or (S): With this pre-treatment, all solid particles (including MPs) larger than 300 µm were isolated from 2 L of wastewater or 5 g of biosolid samples after sieving, oxidative digestion, and filtration steps.

2) Total Particles or (T): With this pre-treatment all solid particles (including MPs) with a size between 1 µm and 5 mm were isolated from a 10 mL aliquot of wastewater after oxidative digestion, density separation, and filtration steps.

Through protocol (S), a larger and more representative amount of wastewater was treated, but particles smaller than 300 µm were lost. In the other hand, protocol (T) allowed the analysis of particles down to 1 µm in size, but the amount of analysed wastewater was much smaller to avoid filter clogging.

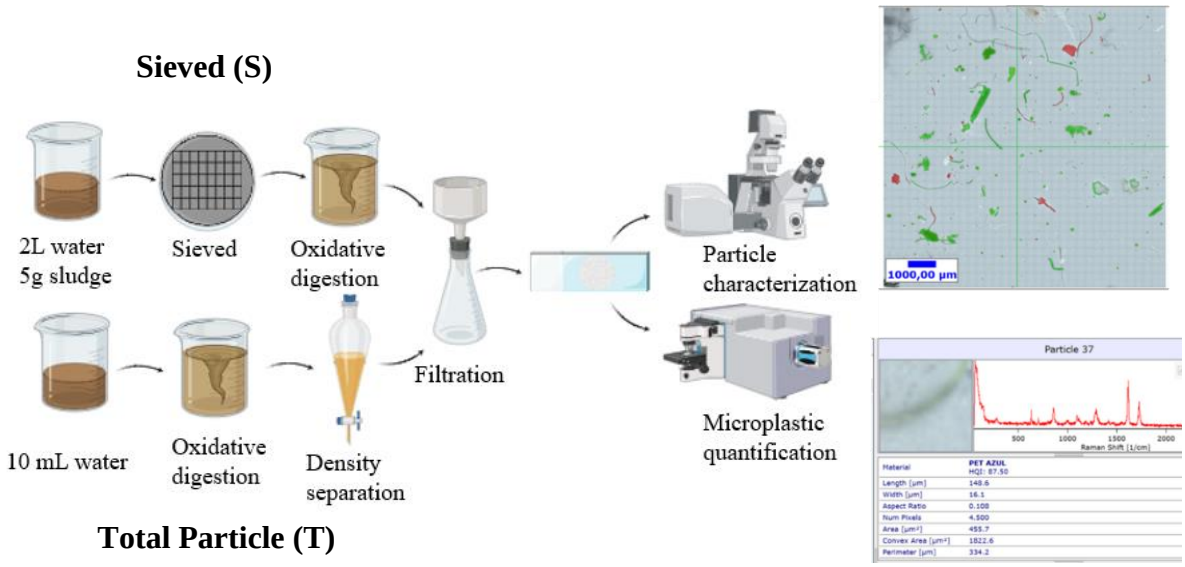

Figure 11. Scheme summary of the methodology used for the isolation, quantification and identification of MPs.

In both protocols (S) and (T), oxidative digestion was performed to remove organic material, adapting the method described by the National Oceanic and Atmospheric Administration (NOAA)[103].

In the case of the Sieved 300 µm or (S) protocol (Figure 11), 2L of wastewater or 5 g of biosolids were treated. The 5 g of biosolids were previously dispersed in 100 mL of ultrapure MilliQ water by applying stirring and heat during 30 minutes at 30 ºC. The wastewater or biosolid dispersion were subsequently poured through a 300 µm mesh stainless steel sieve. The retained particles were collected by washing with MilliQ water into a beaker and digested by adding an equivalent volume of NaClO (14%, VWR chemical, USA). After heating at 75 ºC for 3 h under stirring, the sample was sieved again to remove the disaggregated smallest particles. The particles retained on the sieve were collected by washing with MilliQ water on a 0.8 µm pore size nitrocellulose filter (Whatman, USA). The filter was protected from external contamination between a microscope glass slide and a glass cover, and finally dried at 40ºC for 24 h in a convection oven.

In the case of the Total Particles or (T) protocol, an oxidative digestion (Fenton reaction) was performed on a 10 mL wastewater sample by adding 20 mL of a H_2O_2 (30%, Sigma- Aldrich, USA) solution and 20 mL of a 0.05 M Fe (II) solution prepared by mixing $FeSO_4$ (Sigma-Aldrich, USA), H_2SO_4 (96%, PanReac AppliChem, ITW Reagents, USA) and deionized water. The sample was then heated at 75°C for 30 min under stirring. The digestion step was repeated if any remaining organic material was visually. Thereafter, a density separation was performed after adding NaCl (99.5%, Sigma- Aldrich, USA) until saturation. Subsequently, the sample was left to sediment for 30 min in a separatory funnel and the supernatant was filtered through a 0.8 µm pore size nitrocellulose filter (Whatman, USA) under vacuum. The filter was also protected between glass slide and coverslip and dried at 40ºC for 24 hours.

Characterization of particles present in biosolid and wastewater samples.

Filters obtained after pre-treatment protocols (S) and (T) were photographed using an EVOCAM II macrophotography equipment (Vision engineering, Woking, UK) and the ViPlus software (2018, Vision Engineering). Two partially overlapping 2MPx color photos were taken for each filter, always at 20x magnification, with half of the filter appearing in each photo. These images were fused by digital stitching techniques using the mosaic J command of the FIJI software (ImageJ 1.49q Software, National Institutes of Health, USA). Each image showed a 25*15mm field of view. The pixel size was 13.3 microns, obtaining an image to calibrate in each photo session to have a precise external calibration data. A rough quantification was performed, and all particles, including MPs, were characterized using the Nis Elements BR 3.2 software (Nikon corporation, Japan). To achieve this, a macro of programmed actions was designed in which, firstly, the pixel size was calibrated in the complete image of the filter, then a matrix-iterative detection tool for particles less bright than the filter was applied, which facilitated a binary segmentation by brightness levels and achieve the selection of the particles of each filter in an automated way, only in the filtration zone. Finally, the data of all the particles were exported to obtain the count and the different morphological values of numerous parameters and perform the statistical calculations.

For the characterization, the particles were classified into 3 size ranges of 1-100 µm, 100-300 µm and 300-5000 µm. The particles were also classified according to their circularity, calculated from the measured perimeter and area of each particle according to Equation 1, in 3 ranges: 0-0.4, 0.4-0.8 and 0.8-1. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon. Particles with a circularity less than 0.4 were considered as fibers.

$$Circularity = 4\pi\left(\frac{area}{perimeter^2}\right) \quad (1)$$

In addition, the efficiency of WWTPs in removing particles was calculated according to the following equation:

$$Efficiency = \frac{\text{influent} - \text{effluent}}{\text{influent}} \times 100 \quad (2)$$

Where: Efficiency = particle removal efficiency (%); influent = number of particles detected at the WWTP influent; effluent = number of particles detected at the WWTP effluent.

Quantification of microplastics present in biosolid and wastewater samples.

Quantification, identification and characterization of MPs was carried out only on samples from the odd months. The analysis was performed using an automated Raman microscope Alpha300 apyron (Witec, Ulm, Germany). First, each filter was mapped by acquiring a total of 1089 images, which after reconstruction represented a 27% of the filter area or 1 cm^2. The present particles were detected and selected by performing image analysis using the ParticleScout 6.0 software in automatic mode.

After particle selection, analysis on each particle by Raman spectroscopy and subsequent identification were carried out. The optimal conditions for Raman spectra acquisition were as follows: 785 nm laser which facilitates to identify fluorescent particles, 300 lines/mm diffraction grating opening, spectral range between 0 and 3000 cm^{-1}, 10 accumulations, 0.2 second acquisition time, and 40 mW laser power. The spectrum of each particle was registered and compared with an in-house build spectral library of polymers. The reference polymer materials included in the spectral library were polyethylene (PE), polyethylene terephthalate (PET), polyamide (PA), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polytetrafluoroethylene (PTFE), polyacrylamide (PAM), Polyarylsulfones (PSU), Polymethylmethacrylate (PMMA), nitrile rubber (NBR), Cellophane and Melamine. Particles that had a 75% or better match (HQI) between the sample and reference spectra were identified as composed of the same material or of a similar chemical nature. In addition, a visual inspection

was carried out and the spectrum acquisition was repeated on the particles where a clear identification was not initially possible. Three rules were considered to discriminate between plastics and non-plastics and to prioritize the particles to be analysed: i) the object must not show cellular or natural organic structures; ii) the fibre thickness must be uniform along the entire length; iii) the colour of the particles must be clear and homogeneous[104]. The MPs already identified were classified based on material type, size, morphology, and area.

Statistical analysis

Results were statistically analysed and significance of differences was determined on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. In all cases, a value of $p < 0.05$ (confidence interval 95%) was deemed significant.

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Declaration of competing interest

All authors declare no financial or non-financial competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT (https://chat.openai.com/) in order to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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3.5. Survival of viruses in water microcosms

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In preparation

Survival of viruses in water microcosms

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Abstract

Human enteric viruses, along with emerging viruses such as SARS-CoV-2, influenza virus, and mpox virus, are frequently detected in sewage. While human enteric viruses exhibit high persistence in water, limited information is available for non-enteric viruses. This study evaluates the stability of hepatitis A virus (HAV), murine norovirus (MNV), influenza A virus H3N2 (IAV), human coronavirus (HCoV) 229E, and Vaccinia virus in reference water (RW), effluent wastewater (EW), and drinking water (DW) stored at 4 and 25 ºC. The decay of infectious viruses was analyzed using a monophasic decay model, generally showing that human enteric viruses demonstrate remarkable persistence in water samples. MNV infectivity experienced a significant decrease after 3 weeks at 25 ºC, while at 4 ºC, MNV infectivity only decreased after 12 weeks. Gradually decay of HAV infectivity was reported at 25 ºC, whereas at 4 ºC, infectious viruses were even recovered after 14 weeks. HCoV-229E, IAV, and VACV were completely inactivated in DW and EW at 25ºC between 1 and 3 weeks, with longer stability observed at 4 ºC. Additionally, the decay of infectivity of IAV H3N2, HCoV-229E, and vaccinia virus was assessed in parallel using (RT)-qPCR to determine genome persistence and viability PCR to determine intact viral capsid persistence on both EW and DW samples. Overall, our findings suggest that viability PCR is not suitable for tracking virus decay in water under real environmental conditions.

1. Introduction

In recent years, the intersection of virology, environmental science, and public health has grown in importance as the global community is dealing with the complex problem of viral infections originating from environmental reservoirs. Faecal contamination of water supplies has been historically recognised as a risk for human health: water can provide a vehicle for pathogen spread, creating the conditions for outbreaks or sporadic cases of infection. Several types of viruses, including enteric and respiratory viruses, are often detected in water environments and human enteric viruses are deemed to be responsible for a considerable proportion of waterborne diseases (Hamza and Bibby, 2019; Haramoto et al., 2018; WHO, 2017).

Human enteric viruses, including but not limited to human noroviruses (NoV), rotaviruses (RV), and sapovirus, are globally recognized as significant etiological agents. These pathogens commonly replicate in the gastrointestinal tract and are released into the environment via faeces. Additionally, hepatitis A and E viruses are also excreted in stools. In contrast, respiratory viruses, such as influenza viruses and coronaviruses, primarily target the respiratory tract and spread into the environment via respiratory secretions and aerosols. However, they can also be excreted in urine or stools. Likewise, emerging viruses such as monkeypox virus (MPXV), the zoonotic pathogen that caused the global outbreak in 2022, are excreted through secretions and released into sewage (Altindis et al., 2022; Nakhaie et al., 2023).

Water scarcity, exacerbated by drought and shortages, is a pressing global issue, underscoring the importance of water reuse. Despite effluent wastewater and sludge being utilized worldwide (Corpuz et al., 2020), a significant portion is informally or inadequately treated, posing health and environmental risks (Mateo-Sagasta et al., 2015). Properly managing these resources can improve food production, water resilience, and promote a circular economy (Regitano et al., 2022). However, water reuse poses challenges related to chemical and microbiological safety. Studies frequently detect human enteric viruses in treated wastewater (Haramoto et al., 2018), but the extent of viral contamination remains insufficiently explored. Furthermore, the discharge of untreated or inadequately treated wastewater into natural water systems poses a substantial risk, facilitating the entry of both enteric and respiratory viruses into aquatic environments (Medema et al., 2020). Thus, the complex web of sources and transmission routes highlights the multifaceted nature of viral contamination in water and underscores the need for a holistic approach to mitigate associated risks. Addressing this issue requires comprehensive strategies to mitigate the risks of viral contamination in water. Human enteric viruses, but also respiratory viruses, can persist in diverse environmental matrices, including water sources. This persistence, combined with their potential for human infection, raises questions regarding the mechanisms by which they enter water sources, potentially contaminate food, and ultimately endanger human health.

Understanding the factors affecting the stability of viruses in water is crucial for assessing transmission risks. Recent research highlights the influence of environmental conditions, virus characteristics, and biological interactions (Bibby and Peccia, 2013). Factors such as temperature, pH, salinity, and the presence of protective particles impact viral survival (Chen et al., 2021; Rusiñol and Girones, 2019; Ye et al., 2016). Microbial communities in water bodies can also affect viral fate. UV radiation and flow dynamics play roles in viral inactivation and dispersion. Efficient wastewater treatment is vital to prevent viral contamination (Sims and Kasprzyk-Hordern, 2020 Haramoto et al., 2018). These findings emphasize the need for a comprehensive understanding to mitigate public health risks.

Recent research underscores the pivotal role of water as a potential vehicle for viral transmission, and understanding the factors that influence viral stability and persistence in aquatic environments is paramount for public health. This knowledge can inform more targeted and effective strategies for water treatment, sanitation, and food safety, ensuring the protection of communities worldwide. Traditionally, cell culture-based methods have been used to investigate virus persistence in the environment. However, since virus detection in water primarily relies on molecular methods, researchers have investigated proxies for viral infectivity using molecular approaches, with a predominant focus on methods assessing capsid integrity. These assays use intercalating dyes like propidium monoazide (PMA) and platinum chloride ($PtCl_4$) that interact with the genomes of inactivated viruses with compromised capsids or with free viral genomes, inhibiting (RT-)qPCR amplification. Consequently, only viruses with intact capsids are detected by (RT-)qPCR following a capsid integrity treatment (Canh et al., 2022).

This study aims to investigate the stability of enteric viruses in water microcosms, as well as the persistence of other viruses or their surrogates commonly found in sewage, using cell-culture methods. To assess the inactivation rates of viruses in water, our approach also includes experiments analyzing the decay of (RT)-qPCR to determine genome persistence and viability PCR to determine intact viral capsid persistence.

2. Material and methods

2.1. Virus and cell lines

For human enteric viruses or their surrogates, murine norovirus (MNV-1, kindly provided by Prof. H.W. Virgin, Washington University School of Medicine, USA) and HAV, HM-175/18f strain, (ATCC VR-1402) were propagated and assayed in RAW 264.7 (ATCC TIB-71) and FRhK-4 cells (ATCC CRL-1688), respectively. Influenza A virus H3N2 (ATTCC VR-1680),

human coronavirus (HCoV) 229E (ATCC VR-740) and Vaccinia vAA6, as monkeypox virus surrogate (kindly provided by Dr. A. Alcamí, Molecular Biology Center of CSIC, Spain), were propagated in MDCK (ATCC CCL-34), Huh-7 (kindly provided by Dr. Ron Geller, I^2SysBio, Spain) and HeLa (ATCC CCL-2) cell lines, respectively. Cell lines were grown according to ATCC recommendations. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$/mL) in 96-well microtiter plates with eight wells per dilution and 20 µL of inoculum per well using the Spearman-Karber method (Falcó et al., 2018).

2.2. Sampling

Throughout this study, 3 types of water were used, i) Reference water (RW), prepared with Milli-Q® 7 mM NaCl water, used as a control, ii) Drinking water (DW) from chlorinated municipal tap water, collected in March 2023, and iii) Effluent wastewater (EW), which was obtained from a wastewater treatment plant (WWTP) in Valencia (Spain) in July 2022. All samples were aseptically collected and stored under refrigerated conditions, transported within 24 h to the laboratory, and stored at 4 ◦C until analysis. Temperature and pH were measured using a Fisher Scientific TM accumet TM AE150 (Thermo Fisher Scientific). Absorbance and conductivity were analyzed using the Cary 60 UV-Vis spectrophotometer (Agilent) and the EcoScan Hand-held Series conductometer (Eutech Instruments), respectively (Table S1).

2.3. Water microcosmos experiments

Each microcosm consisted of 50 mL of RW, DW and EW conditioned at 4 and 25 ºC. MNV-1, HAV, Influenza H3N2, HCoV-229E and Vaccinia viruses (ca. about 5-6 log $TCID_{50}$/mL) were artificially inoculated and stored at 4 and 25 ºC in the dark. Aliquots were collected at different intervals (0h, 24h, 1 week and every 2 weeks until week 14) in triplicate. Each aliquot was filtered through 0.22 µm filters, previously blocked with 300 µl of 10% fetal bovine serum (FBS) solution and centrifuged for 1 min at 1200 rpm. Ten-fold dilutions of each water sample were inoculated into confluent monolayers of the corresponding cell lines in 96-well plates. Infectious viruses were then enumerated by cell culture assays as described above. The decay of virus titers was calculated as $\log_{10}(N_x/N_0)$, where N_0 is the infectious virus titer for samples at the initial times and N_x is the infectious virus titer for each of the storage times.

2.4. Performance of viability PCR on water microcosmos experiments

Propidium monoazide (PMAxx™, Biotium) and platinum (IV) chloride ($PtCl_4$; Acros Organics, NJ, US) were evaluated as viability markers. PMAxx was dissolved in water at 4 mM, and $PtCl_4$ was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to 1.0 M and further diluted in nuclease-free water to 50 mM. Both markers were stored at − 20 ◦C protected from light.

Viability pre-treatments were performed in DNA LoBind tubes (Eppendorf Iberica). The performance of PMAxx™ (100 µM) and $PtCl_4$ (1000 µM) was evaluated on water microcosmos experiments. For a selected subset of experiments, i.e. EW and DW stored at ºC; aliquots were pre-treated with viability markers as detailed. A double photoactivation of PMAxx was performed by a two-cycle step including 10 min of dark incubation in an orbital shaker (150 rpm) at room temperature (RT) followed by 15 min blue LED light exposure in a photo-activation system (Led-Active Blue, GenIUL). Alternatively, 30 min incubation at RT in an orbital shaker (150 rpm) was used for viability treatments with $PtCl_4$. Following the viability treatment, viral nucleic acids were immediately purified as described hereafter. A control consisting of virus suspension without a viability marker was included in each assay.

2.5. Viral nucleic acid extraction and amplification

Viral nucleic acids were extracted using the Maxwell® RSC 16 instrument and Maxwell RSC Pure Food GMO and authentication kit (Promega, Spain) and detected by (RT)-qPCR for Influenza H3N2, HCoV-229E and Vaccinia virus (Table S2). Each (RT)-qPCR assay was performed in duplicate and included nuclease-free water as negative control. Ten-fold dilutions were consistently tested to check (RT)-qPCR inhibition due to viability marker residues or inhibitory substances from the sample. Amplification curves showing Ct values < 40 were converted into genome copies (gc) per liter using the corresponding standard curve and volumes tested. HCoV-229E quantification curves were generated by amplifying serial end-point tenfold dilutions of viral suspensions in quintuplicates and calculating the numbers of PCR units (PCRU).

2.6. Statistical analyses

All data were compiled from three independent experiments with at least two technical replicates for each variable. Data are presented as median ± SD. Significant differences in median cycle threshold (Ct) were determined by using either one- or two-way(s) ANOVA followed by Dunnett's multiple comparisons test on GraphPad Prism version 8.02 (GraphPad Software, US). Differences in means were considered significant when the p was<0.05.

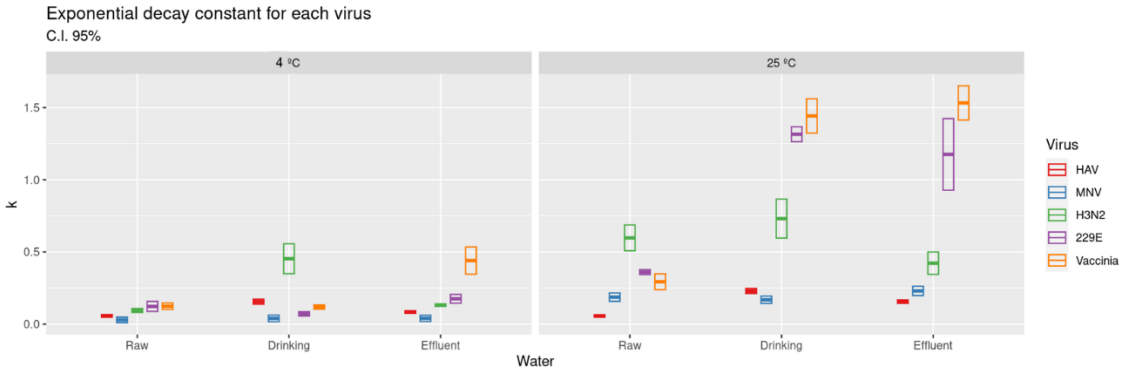
The decay of infectious viruses was analyzed by using a monophasic decay model, which assumes first-order decay. The equation used to calculate the decay rates was:
$-\ln(Nt/N0)=kt$ where Nt is the virus titer at time t, N0 is the virus titer at time 0, k is the decay rate, and t is the time in days. Reduction rates ($\log_{10}$ $TCID_{50}$/day), R^2 values and p values were calculated.

3. Results and discussion

3.1. Enteric virus persistence assessed by cell culture

A recent meta-analysis review on the decay rates of waterborne viruses concluded that the decay rates (k values) varied significantly between types, as well as with factors such as temperature, light condition and enumeration method (Boehm et al., 2019). As anticipated, human enteric viruses or their surrogates survive longer than respiratory viruses in the types of water and temperatures evaluated (Figure 1). The infectivity of HAV showed a consistent decrease over 56 days (8 weeks) at 25 ºC, at which point HAV levels were below the limit of detection in drinking water and effluent water samples, while longer persistence was recorded in the reference water (Figure 2). The counterparts at 4 ºC showed similar trends on HAV decay for reference water and drinking water (Figure 1), while longer stability was recorded in effluent water, with infectious HAV was recovered after 98 days (14 weeks). Overall, not much information was available regarding the decay of HAV. However, consistent with our findings, the review study by Boehm et al. (2019) indicated that depending on the type of water, greater stability was observed at 5°C compared to 25°C.

The decay rates of infectious HAV were 0.056, 0.228, and 0.155 log/day for reference water, drinking water and effluent water at 25ºC, respectively. Retrieved decay rates were 0.057, 0.155, and 0.083 in the same water matrices but stored at 4 ºC (Figure 1).



##	Virus	Temperature	Water	k	2.5%	97.5%	R2
## 1	HAV	4 °C	Raw	0.05710981	0.048455249	0.06576437	0.8626532
## 2	HAV	4 °C	Drinking	0.15594920	0.138746300	0.17315210	0.9386133
## 3	HAV	4 °C	Effluent	0.08333480	0.074938785	0.09173081	0.9342536
## 4	HAV	25 °C	Raw	0.05649621	0.049023116	0.06396930	0.8918187
## 5	HAV	25 °C	Drinking	0.22880710	0.211375061	0.24623914	0.9740140
## 6	HAV	25 °C	Effluent	0.15587693	0.144024440	0.16772943	0.9656443
## 7	MNV	4 °C	Raw	0.02899917	0.009504864	0.04849347	0.2419559
## 8	MNV	4 °C	Drinking	0.03944234	0.016491604	0.06239307	0.2987419
## 9	MNV	4 °C	Effluent	0.03992181	0.018317352	0.06152628	0.3299901
## 10	MNV	25 °C	Raw	0.18697565	0.157896863	0.21605443	0.8999499
## 11	MNV	25 °C	Drinking	0.16860986	0.143083266	0.19413645	0.9046905
## 12	MNV	25 °C	Effluent	0.23011451	0.198061810	0.26216721	0.9181236
## 13	H3N2	4 °C	Raw	0.09433477	0.080684624	0.10798491	0.8858637
## 14	H3N2	4 °C	Drinking	0.45296004	0.349452633	0.55646744	0.8939971
## 15	H3N2	4 °C	Effluent	0.13095937	0.122475048	0.13944369	0.9717240
## 16	H3N2	25 °C	Raw	0.59732447	0.507861535	0.68678740	0.9360935
## 17	H3N2	25 °C	Drinking	0.73090381	0.595818939	0.86598867	0.9280205
## 18	H3N2	25 °C	Effluent	0.42205753	0.344505672	0.49960940	0.9068201
## 19	229E	4 °C	Raw	0.12268619	0.088232612	0.15713977	0.7339513
## 20	229E	4 °C	Drinking	0.07121777	0.056073320	0.08636223	0.7823113
## 21	229E	4 °C	Effluent	0.17514823	0.144842891	0.20545357	0.8790375
## 22	229E	25 °C	Raw	0.35970513	0.342588716	0.37682155	0.9931560
## 23	229E	25 °C	Drinking	1.31521159	1.263678813	1.36674436	0.9976957
## 24	229E	25 °C	Effluent	1.17513766	0.927112731	1.42316259	0.9371925
## 25	Vaccinia	4 °C	Raw	0.12401476	0.101795246	0.14623426	0.8528543
## 26	Vaccinia	4 °C	Drinking	0.11803550	0.103425659	0.13264534	0.9239232
## 27	Vaccinia	4 °C	Effluent	0.44009069	0.345905328	0.53427605	0.8776600
## 28	Vaccinia	25 °C	Raw	0.29359268	0.238996502	0.34818885	0.8833400
## 29	Vaccinia	25 °C	Drinking	1.44182133	1.322994864	1.56064779	0.9898852
## 30	Vaccinia	25 °C	Effluent	1.53203263	1.413655815	1.65040944	0.9910981

Figure 1. K values for each virus in different water matrices, reference, drinking and effluent separated by temperature.

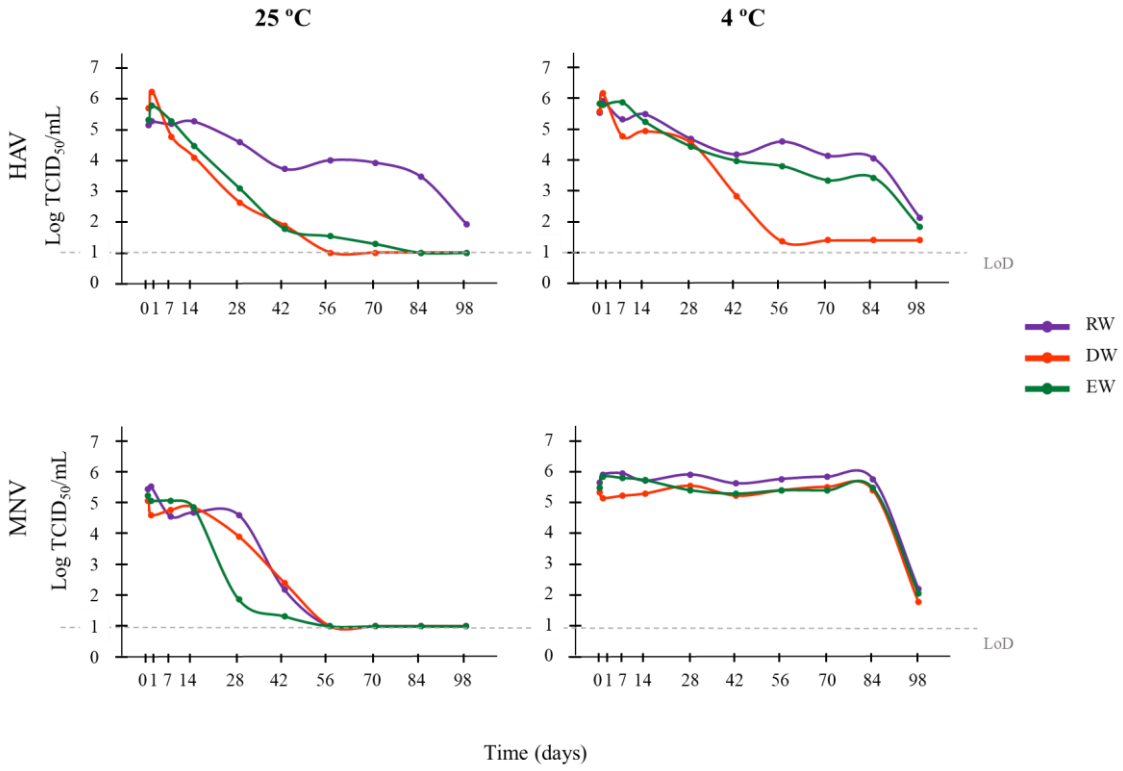


Figure 2. Decay of hepatitis A virus (HAV), and murine norovirus (MNV) at 25◦C or 4 ◦C in reference water (purple), drinking water (orange) and effluent water (green). Dashed lines indicate the limit of detection of the assay.

Studies on human norovirus have been hindered by the challenges associated with their replication in cell culture systems. However, in the last decade, new developments, such as the use of the human intestinal enteroids, HIE system, zebrafish model, and human salivary glands, have emerged (Dycke et al., 2019; Ettayebi et al., 2016; Ghosh et al., 2022). Consequently, much of the current information regarding the persistence and stability of norovirus in environmental samples relies on norovirus surrogates. The levels of MNV at 25 ºC remained stable over the first 28 days in both reference water and drinking water. However, in effluent water, more than a 2-log decay was reported between week 4 and 6. Conversely, the counterpart stored at 4 ºC maintained infectivity until week 12, without significant decay (Figure 2).

The decay rates of infectious MNV were 0.029, 0.039, and 0.040 log/day for reference water, drinking water and effluent water at 4 ºC, respectively (Figure 1), with no significant difference between the k-values for different water types. Retrieved decay rates were 0.187, 0.168, and 0.230 in the same water matrices but stored at 25 ºC. Similar decay rates for MNV have previously been reported for reference water and surface water incubated at 25 ºC, resulting in 0.02 and 0.16 Log/day−1 respectively (Bae and Schwab, 2008). Very recently, the decay of human norovirus

was established using the HIE model in surface water, tap water, and ultrapure water incubated at 18−22 °C. The decay rates were found to be 0.11, 0.08, and 0.10, respectively (Shaffer et al., 2022).

3.2. Non-enteric virus persistence assessed by cell culture

In addition to human enteric viruses, other viruses such as SARS-CoV-2, monkepox virus (MPXV), and influenza viruses have been detected in various types of environmental waters. This has sparked concerns and debate about the potential transmission of these viruses through contaminated water. The waterborne route of transmission is traditionally not considered relevant for respiratory viruses such as influenza and coronaviruses. However, the emergence of IAV H5N1 and SARS-CoV-2 as perceived pandemic threats has indeed changed this scenario.

In this study, model viruses that can be handled in a BSL-2 lab were used. Influenza H3N2 was employed as a model for highly pathogenic influenza A virus (IAV), HCoV-229E as a model for SARS-CoV-2, and vaccinia virus (VACV) as a model for MPVX. Among the three studied viruses, IAV exhibited the highest stability at 25°C (Figure 3), although complete inactivation was observed after 28 days in both reference and drinking water. In effluent water, no infectious viruses were detected at day 14 at either temperature. However, it is noteworthy that IAV was detected after 84 days in effluent water and after 98 days in reference water at 4°C. This aligns with previous findings, which indicated that avian influenza viruses can persist for many weeks in water with low salinity and low temperatures (Brown et al., 2007; Weber and Stilianakis, 2008). The decay rates of infectious IAV H3N2 were 0.597, 0.730, and 0.422 log/day for reference water, drinking water and effluent water at 25ºC, respectively. Retrieved decay rates were 0.094, 0.452, and 0.130 in the same water matrices but stored at 4 ºC (Figure 1).

The infectivity of HCoV-229E exhibited a rapid decrease by day 7 at 25 ºC, reaching levels below the limit of detection in both drinking water and effluent water samples, while a longer persistence was reported in the reference water. The decay rates were 0.359, 1.315 and 1.175 in reference water, drinking water and effluent water, respectively. Moreover, longer stability was observed at 4 °C, with decay rates of 0.122, 0.071, and 0.175 in the same types of water. The stability of SARS-CoV-2 was assessed in river water and seawater (Sala-Comorera et al., 2021), revealing decay rates of 0.61 and 1.07 at 4 ºC and 1.01 and 2.02 at 20°C, respectively. Consistent with our findings, coronavirus stability was found to be dependent on temperature and matrix conditions.

At 25 ºC, VACV infectivity followed a similar pattern to that of HCoV-229E, with decay rates of 0.293, 1.441 and 1.532 in reference water, drinking water and effluent water, respectively. In

contrast, at 4 ºC, these rates were 0.124, 0.118, and 0.440, detecting infectious viruses after 56 days. In previous studies infectious vaccinia virus have been recorded even after 144 days in storm waters (Essbauer et al., 2007).

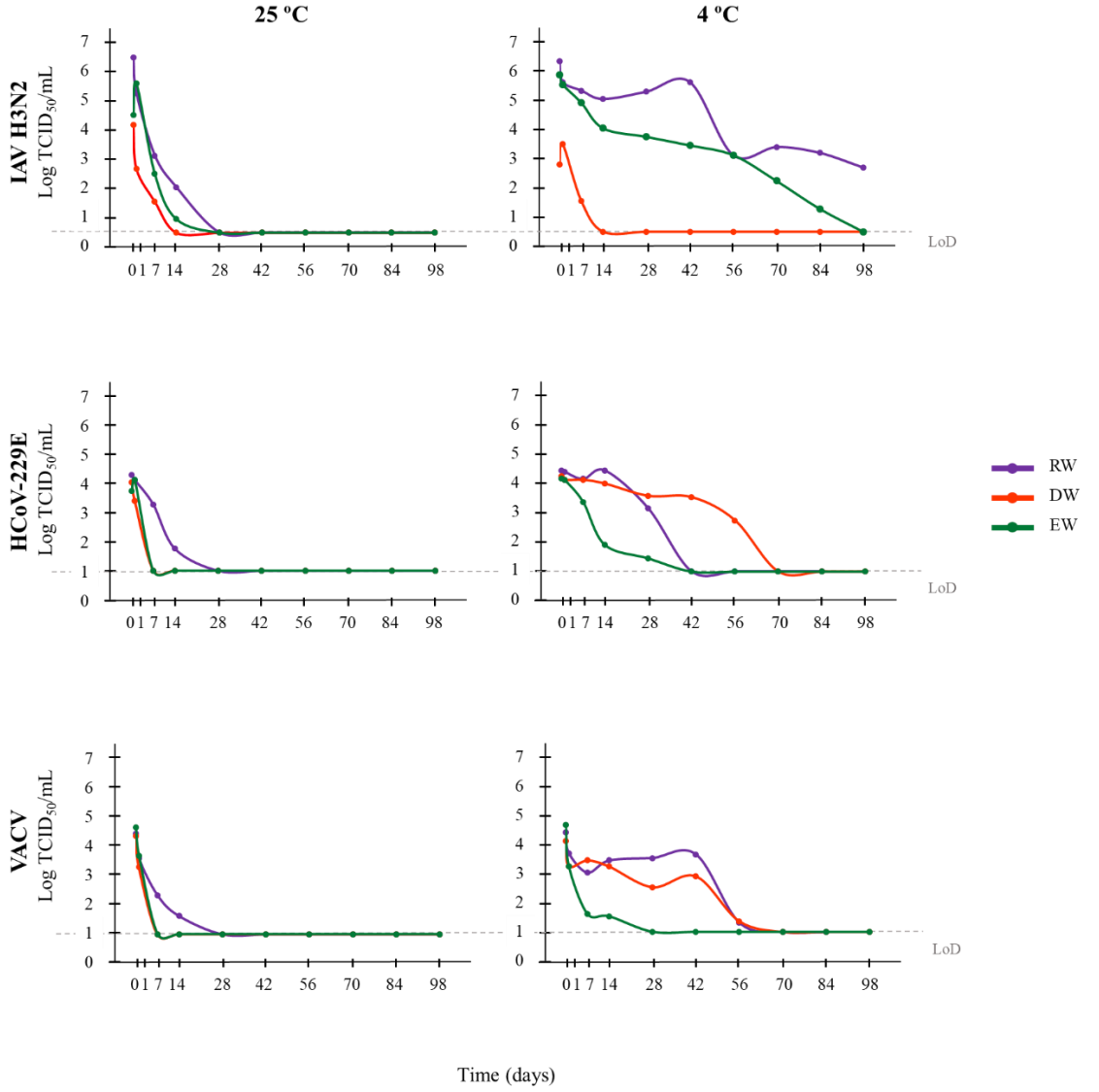


Figure 3. Decay of influenza virus H3N2 (IAV H3N2), human coronavirus 229E (HCoV-229E) and vaccinia virus (VACV) in water microcosmos stored at 25 ºC or 4 ºC in reference water (purple), drinking water (orange) and effluent water (green).

3.3. Virus decay measure by cell culture and viability PCR

SARS-CoV-2, MPXV, and influenza viruses have been detected in various environmental waters using molecular techniques, which do not provide information about the infectivity status of the viruses (Dumke et al., 2022; Sharkey et al., 2023). Due to the complexity and time-consuming

nature of isolating viruses from environmental samples, viability PCR using intercalating dyes has been widely employed over the last decade, not only for human enteric viruses but also for other viruses (Cuevas-Ferrando et al., 2022; Farkas et al., 2020). In this study, we assessed the infectivity of IAV H3N2, HCoV-229E and VACV by cell culture and in parallel by (RT)-qPCR (to determine genome persistence) and viability PCR (to determine intact viral capsid persistence) on drinking water and effluent water samples.
When assessing virus infectivity by cell culture, the infectivity of IAV H3N2 showed a rapid decline, falling below detectable levels in both drinking and effluent water samples by day 28 (Figure 3). However, viral genomes, whether detected by RT-qPCR alone or after viability pretreatments, were consistently found even after day 84 (Figure 4), showing less than a 3-log reduction over the storage period. This indicates that mild inactivation processes (which do not translate into viral capsid damage) are not discriminated by viability PCR, as previously described for other viruses (Randazzo et al., 2018).

Regarding HCoV-229E, the overall stability of RNA, even after viability PCR, was consistently higher than that observed in cell culture assays. This highlights a concern for risk assessment analysis: the detection of viruses in environmental waters using molecular methods may not accurately reflect infectivity. Nevertheless, in effluent waters, pretreatment with $PtCl_4$ was able to completely eliminate the RT-qPCR signal by day 56, achieving a 4-log reduction compared to RT-qPCR alone. PMAxx showed lower performance, as it only completely removed the signal by day 84, while some RNA was still detected by RT-qPCR alone.

Finally, the stability of infectious VACV or its DNA followed the same pattern as HCoV-229E and IAV in drinking water. There was a rapid decline in infectious viruses as measured by cell culture, while the DNA remained very stable even after 98 days of storage. The viability dye pretreatments resulted in less than a one-log reduction compared to qPCR results (Figure 3). In effluent waters, as observed for HCoV-229E RNA, performance of viability PCR was better that we observed in drinking water samples.

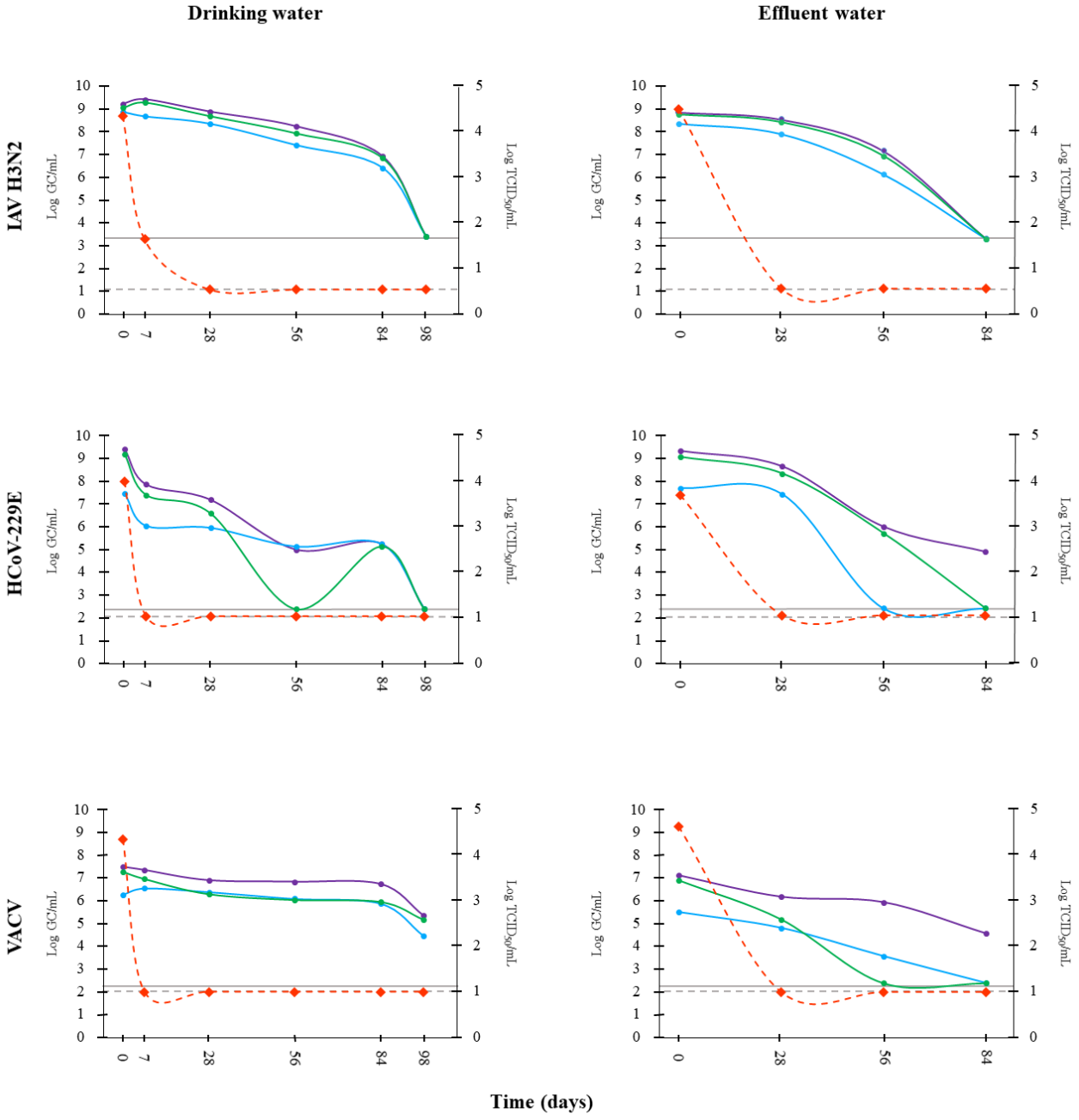


Figure 4. Decay of influenza virus H3N2 (IAV H3N2), human coronavirus 229E (HCoV-229E) and vaccinia virus (VACV) in water microcosmos stored at 25◦C in drinking water (DW) and effluent water (EW) measured as infectious viruses by cell-culture (dashed orange line) and genome detection by (RT)-qPCR (purple), PMAxx-viability PCR (green) and PtCl4-viability PCR (blue).

Conclusions

Due to the challenges in propagating viruses in cell cultures, which are considered the gold standard for assessing infectivity, their detection has traditionally relied on molecular methods that do not distinguish between infectious and non-infectious viruses, thereby limiting comprehensive risk assessment. As anticipated, human enteric viruses or their surrogates survive longer than enveloped viruses or their surrogates in the types of water and temperatures evaluated. At 4°C, based on decay rates, overall stability was ranked as MNV > HAV > IAV H3N2 > HCoV-229E > VACV. Infectious HAV and MNV were recovered after 84 and 98 days, respectively, depending on the type of water. Overall, at 25°C, HAV was the most stable virus, with infectious viruses recovered in reference water after 98 days. Furthermore, virus decay rates increased in effluent and drinking water, likely due to the presence of microbiota.

Finally, although viability PCR is suitable for monitoring virus inactivation under extreme conditions, our results indicate it is not suitable for monitoring virus decay in water under real environmental conditions. Results obtained solely by RT-qPCR, or even after viability pretreatments, were not suitable for assessing virus infectivity in waters.

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CONCLUSIONS

4. CONCLUSIONS

- This work reinforces the potential of wastewater-based epidemiology as a surveillance tool for multiple pathogens. The results demonstrate the effective monitoring of various respiratory viruses (SARS-CoV-2, RSV, and IAV) circulating within a given population.

- The use of predictive models enables the correlation of viral concentrations in wastewater with the number of reported clinical cases. In the analyzed WWTP, viruses can be detected in wastewater samples when there are at least 40 and 110 clinical cases reported for RSV and influenza A, respectively.

- Using the physicochemical parameter of wastewater inflow (m^3/day) as a normalization factor for virus concentrations yields more effective results than those obtained through the use of viral indicator.

- The methodologies developed during the SARS-CoV-2 pandemic, along with the established epidemiological surveillance network, enable rapid adaptation of procedures and swift responses to outbreaks of new pathogens, as exemplified by the MPXV outbreak in 2022.

- The use of WBE for epidemiological surveillance of MPXV is not effective as an early warning tool since it does not predict the emergence of cases. However, it does enable the monitoring of the disease within the population.

- The concentration and detection methods used for viruses in wastewater play a critical role in the development of epidemiological surveillance strategies. They significantly influence the results obtained and may lead to underestimation of positive samples for certain pathogens, potentially biasing the results obtained by predictive models.

- The evaluated direct capture method demonstrates enhanced sensitivity in detecting SARS-CoV-2, HuNoV, and HEV through RT-qPCR, as well as superior performance in genomic sequencing of SARS-CoV-2.

- The wastewater reclamation systems employed in the studied wastewater treatment plants fail to achieve the logarithmic reductions established by European regulation EU 2020/741. Consequently, the reclaimed water does not meet the necessary standards for agricultural use.

- The significant presence of enteric viruses in the biosolids generated by WWTPs highlights the potential health risks associated with their use as fertilizer.

- At refrigeration temperature, enteric viruses exhibit greater stability compared to influenza virus, respiratory coronavirus, and vaccinia virus. Furthermore, at room temperature, HAV proves to be the most stable, with viruses retaining infectious capacity for up to 98 days.

- The inactivation rates of the analyzed viruses are faster in both reclaimed water and in drinking water.

- Results obtained only by RT-qPCR, or even after viability pretreatments with intercalating agents, are not sufficient for assessing the infectivity of influenza virus, respiratory coronavirus, and vaccinia virus in waters.

ANNEX

5. ANNEX

ANNEX 1.

Viruses and the cell lines, in which each of them propagate, used in this thesis.

Virus	Cell Line	
	Name	**Origin**
Murine Norovirus (MNV)	RAW 264.7	Macrophage cells from male mouse tumour
Hepatitis A virus (HAV)	FRhK-4	Epithelial-like cells from monkey kidney
Rotavirus (RV)	MA-104	Epithelial cells from African green monkey kidney
Mengovirus (MgV)	HeLa	Epithelial cells isolated from human cervix
Human coronavirus 229-E (HCoV-229E)	MRC-5	Fibroblasts from human lung tissue
	HuH-7	Hepatocyte-derived carcinoma cells from human liver
Porcine Epidemic Diarrhea virus (PEDV)	Vero	Epithelial cells from African green monkey kidney
Influenza virus H3N2 (IAV H3N2)	MDCK	Cells from dog kidney
Vaccinia virus (VACV)	HeLa	Epithelial cell isolated from human cervix

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6. BIBLIOGRAPHY

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