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Addressing Which Pathogens Are More Suitable to Ensure Water Reuse Safety—A Proof-of-Concept

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Abstract

Current regulations and legislation promote water reuse as a response to the global water crisis, while simultaneously highlighting the need for an urgent revision to ensure the protection of public health. Changes to these regulatory frameworks are generally supported by scientific evidence, particularly when defining the most appropriate water quality parameters to be monitored. In a previous study, our group proposed that Norovirus genogroups I and II should be further investigated as potential indicators for water quality monitoring, and, consequently, for assessing wastewater treatment efficiency. In the present study, we evaluated the presence of these enteric viruses, together with hepatitis A and hepatitis E viruses, using real-time PCR. Additionally, we assessed the occurrence of *Clostridioides difficile*—an undervalued pathogen that represents a major cause of healthcare-associated diarrhea, increasingly raising concern in community settings—alongside currently established microbiological parameters. Samples were collected from two stages of wastewater treatment and from water intended for reuse. Water quality was further evaluated using a bioluminescence-based bioassay. Overall, this proof-of-concept study supports the inclusion of Norovirus genogroup II and *Clostridioides difficile* as complementary microbial indicators for assessing the safety of water for reuse, particularly within the context of European regulatory standards.

Keywords: water reuse; enteric viruses; *Clostridioides difficile*; Microtox; public health



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1. Introduction

1.1. Study Rationale

The concept of proof-of-concept (POC) traditionally applied in technological and engineering contexts, is here adapted to the field of environmental microbiology and water reuse safety [1]. In this study, the POC aims to evaluate the scientific feasibility of using selected pathogens as indicators of microbiological risk in reclaimed water. The success of the POC is assessed based on microbiological performance criteria, including sensitivity

to wastewater treatment processes, representativeness of broader pathogen groups, and relevance for risk assessment and regulatory decision-making [1].

Testing methodological approaches prior to full-scale implementation allows the robustness and applicability of proposed strategies to be evaluated before broader adoption. Accordingly, this study seeks to identify pathogens suitable for use as complementary indicators of water reuse safety through an integrated proof-of-concept analysis combining microbiological, molecular, and biotoxicity approaches.

By demonstrating that the proposed analytical framework can identify pathogens that are both detectable and relevant to public health protection goals, this study establishes the feasibility of an indicator-selection strategy applicable to the monitoring of water intended for reuse.

1.2. Water for Reuse and Legal Framework

Water reuse refers to the intentional use of treated wastewater for a subsequent beneficial purpose. The terms “recycled water” and “reclaimed water” are frequently used interchangeably in the literature to describe treated wastewater that meets quality standards for its intended use [2,3]. In principle, any type of wastewater, originating from domestic, municipal or industrial sources may be considered for reuse [4], provided that appropriate treatment is applied.

As stated above, water reuse can involve various water sources, including municipal wastewater, discharges from industrial activities, rainwater, agricultural runoff, and natural watercourses [3]. These waters are subjected to treatment processes of varying complexity and requirements, depending on the intended final purpose. The treatment must ensure compliance with environmental protection requirements, preventing adverse impacts such as chemical or microbiological contamination of ecosystems [5]. Additionally, the quality of reused water must also meet specific public health standards to ensure that its use does not pose risks to the community exposed to it [6].

Following appropriate treatment, reclaimed water can be used for a wide range of applications, including street and urban space cleaning [7], mitigation of potable water shortages [8], agricultural fields and landscape areas irrigation [7,9], aquaculture [10], industrial cooling [7], and in energy production [11]. Although reclaimed water is typically not discharged to the natural environment, aquifer recharge can represent a relevant strategy for water resource preservation [12], despite being less commonly implemented.

Water reuse has emerged as a key strategy to address increasing water demand, combined with the impacts of climate change. As freshwater resources become progressively constrained, sustainable water management practices are essential to ensure long-term water availability, particularly in regions affected by water scarcity [13–15]. Water reuse is also recognized as an effective adaptation measure to cope with the effects of climate change, like the increasing frequency and intensity of droughts and periods of water scarcity [16], providing a resilient water source that helps communities and industries adapt to climate change by ensuring water availability during droughts and extreme weather events [13,17].

To ensure the safe and sustainable implementation of water reuse, national and international legal frameworks have been established. These regulations define quality standards and monitoring requirements and promote best practices designed to safeguard public health and environmental integrity [18].

At the European level, Regulation (EU) 2020/741, in force since 26 June 2023, established minimum requirements for the safe reuse of treated wastewater, primarily for agricultural irrigation [18]. The regulation sets harmonized water quality standards and monitoring obligations, to ensure safety and promoting water reuse to mitigate water scarcity and manage droughts effectively. These objectives are aligned with the United Na-

tions (UN) Sustainable Development Goals, particularly Goal 6: clean water and sanitation; and Goal 12: sustainable consumption and production [19].

In Portugal, Law-Decree No. 119/2019 establishes the legal framework for the production and utilization of water for reuse, obtained through wastewater treatment. This legislation permits the reuse of water from domestic, urban, and industrial wastewater treatment plants (WWTPs) for non-potable applications, including irrigation, landscaping, and industrial ones. It also defines quality parameters for different uses to ensure environmental and public health protection, promoting sustainable water management while preventing adverse health and environmental effects [20]. Additionally, Ordinance No. 266/2019 further specifies information and signage requirements for both producers and users of water for reuse, establishing labeling protocols to ensure clarity and compliance with safety standards [21].

The Portuguese Law-Decree No. 119/2019 defines five quality classes (A to E) based on the intended use and human exposure level, using *Escherichia coli* and helminth eggs as key microbiological indicators [20]. It also requires appropriate signage and public information, as detailed in Ordinance No. 266/2019, which specifies visual symbols and safety communication to be displayed at reuse sites [21]. At the European Union (EU) level, Regulation (EU) 2020/741 sets limits for *E. coli*, helminth eggs, and *Legionella* spp. and performance parameters such as turbidity and biochemical oxygen demand [18]. Together, these legal instruments provide a comprehensive framework to ensure safe and sustainable reclaimed water use.

Traditional fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, remain essential components of microbiological monitoring frameworks because they are well established, are relatively cost-effective and broadly representative of fecal contamination from human and animal sources [22]; however, they may not be completely adequate on their own.

Although Portuguese and European Union regulations establish minimum requirements for microbiological monitoring and good practices for the reuse of treated wastewater, these traditional indicators reflect regulatory compliance rather than the full spectrum of microbiological risks and may not fully reflect the presence of critical pathogens that pose a real risk to public health.

The approach proposed in this study explores the added value of also using complementary more resistant microorganisms, namely enteric viruses and *Clostridioides difficile*. These pathogens are not necessarily correlated to FIB alone and may reveal additional hazards, namely in specific water reuse scenarios. Due to their waterborne transmission, low infectious dose, and documented resistance to commonly applied disinfectants such as chlorine, noroviruses and *C. difficile* spores can persist in treated water and on environmental surfaces [22,23], which can serve as additional safety indicators. Consequently, their monitoring should be considered especially in high-risk applications, such as irrigation of gardens accessible to children or irrigation of fruits and vegetables that are consumed raw, where even small quantities of pathogens can pose a significant public health risk. In these contexts, complementing FIB with viral and *C. difficile* analyses can provide a more complete assessment of water safety, without intending to replace traditional indicators.

The present study aims to illustrate, through a proof-of-concept approach, that combining microbiological and molecular analyses with toxicity bioassays, to monitor these complementary pathogens and overall toxicity, can strengthen the safety of water reuse and provide the scientific evidence to support the revision and improvement of existing directives and national legal instruments. Although the study focuses on the Portuguese context, discussing compliance, identified gaps and methodology is relevant to other European

and international regulatory frameworks, contributing to safer and more comprehensive management of reclaimed water resources.

1.3. Highly Resistant Waterborne Pathogens

Accurate assessment of pathogen presence in water matrices and monitoring of water quality are key factors to decide the proper water distribution systems, choosing the best water treatment, and preventing outbreaks of waterborne diseases.

The World Health Organization (WHO) in 2017 has classified viruses as agents of moderate to high importance in human health [24], highlighting enteroviruses, Hepatitis A virus (HAV), Hepatitis E virus (HEV) and Noroviruses, as significant contributors to waterborne transmission via the fecal–oral route.

Noroviruses were previously known as Norwalk or Norwalk-like viruses because they were originally described in an outbreak of gastroenteritis at a school in Norwalk, Ohio, USA, in 1968. They are currently classified into 10 genogroups (GI–GX), with genogroups I, II and IV containing the main viruses associated with human gastroenteritis.

These viruses are highly contagious and spread very easily and quickly, with an incubation period ranging from 12 to 48 h [25]. Noroviruses are the main cause of sporadic or epidemic outbreaks of gastroenteritis in both adults and children, commonly in community settings such as schools, cruise ships, hospitals, and holiday camps. While norovirus infections can occur all year-round [25,26], they are more prevalent during colder months in the United States, from November to April [26], being thus called “winter vomiting disease”. Globally, noroviruses are responsible for approximately 20% of acute gastroenteritis cases [27].

Noroviruses are highly resistant to unfavorable conditions in the environment, persisting at refrigeration temperatures ($\sim 4^{\circ}\text{C}$) with little inactivation, surviving freezing conditions (-18°C), and tolerating heat up to 60°C . Additionally, these viruses can resist chlorine concentrations up to 10 ppm, which exceeds the typical value found in drinking water (~ 2 ppm) [25,28–30]. This environmental persistence makes GI and GII noroviruses particularly suitable as indicators for assessing the microbiological safety of reused water. Unlike adenoviruses, which are ubiquitous and may persist for months, noroviruses more accurately reflect recent fecal contamination relevant to human health [22].

Their detection complements traditional fecal bacterial indicators such as *E. coli*, and provides additional insight into viral contamination not captured by FIB alone.

Hepatitis A is an acute liver infection caused by a small RNA virus, the hepatitis A virus (HAV), and is highly contagious. Six HAV genotypes have been identified, of which genotypes I, II, and III, subdivided into subtypes A and B, infect humans [31,32].

Some genotypes have geographical distribution patterns. Genotype I is most prevalent worldwide, with subtype IA being more frequent than IB, while subtype IIIA is more common in Central Asia. In areas of low endemicity such as the United States and Western Europe, subtype IA predominates, although all genotypes and subtypes have been reported [31].

Hepatitis E is an acute infection caused by the hepatitis E virus (HEV). Eight genotypes are known to be endemic in different regions of the world with distinct hosts. HEV-1 and HEV-2 infect only humans and are responsible for most human diseases caused by infection with this virus worldwide and have been associated with large waterborne outbreaks [33]. To date, HEV-5 and HEV-6 genotypes have only been reported in wild boars and are not associated with human infections. HEV-7 and HEV-8 genotypes have been detected in camels, and there is one reported case of human infection with HEV-7 [34].

Transmission of HEV is related to poor sanitary conditions [33,35]. Because HEV can infect both humans and animals, it represents a relevant zoonotic and environ-

mental pathogen, reinforcing the importance of One Health approaches in water reuse safety assessments.

Clostridioides difficile is an obligate anaerobic, spore-forming bacterium capable of long-term survival in environmental matrices and able to colonize the human gut. Its spores are able to survive extreme environmental conditions, which the vegetative form could not withstand. Spores are considered the infectious form of *C. difficile*, as they are highly resistant to heat, UV light, radiation and oxygenated environments, and can be found in soil, water or animals. Due to their resistance to common cleaning and disinfection practices, *C. difficile* spores can persist in the environment for months or even years, facilitating pathogen dissemination and long-term persistence, including survival throughout wastewater treatment processes [23,36]. This makes *C. difficile* a suitable indicator for assessing the efficacy of treatment processes against resistant microorganisms and, therefore, a bacterial pathogen of particular interest in this study.

When the normal intestinal microbiota is disrupted, often due to antibiotic use, symptoms are triggered [37]. The clinical presentation of *Clostridioides difficile* infection (CDI) varies with patient-related risk factors. Elderly patients, especially those with a history of institutionalization, and individuals experiencing recurrent infections with frequent antibiotic exposure are at higher risk of severe disease. Manifestations can span from mild diarrhea to pseudomembranous colitis, septic shock, and death [37,38]. Transmission occurs via the fecal–oral route, and CDI is commonly associated with healthcare settings. In Europe, CDI is the leading cause of healthcare-associated diarrhea, entailing an unreasonable burden for healthcare systems [39]. The mortality rate associated with CDI and its complications can range from 5% to 10% and go as high as 34% in hospitalized patients [38,40]. However, CDI incidence is rising in populations once considered low risk, with cases being increasingly reported in the community [36]. Due to its public health significance, several European countries have established surveillance systems to monitor CDI trends, antibiotic resistance, and infection control practices.

The pathogenicity of *C. difficile* is mainly due to the production of toxins A and B, and in some strains, a binary toxin (CDT), often linked to hypervirulent ribotypes [41,42].

Recent studies suggest an emerging prevalence of environmental ribotypes, more commonly linked to community-acquired CDI (CA-CDI), as opposed to hospital-associated hypervirulent ribotypes, showing a shift in the epidemiology of CDI [39,43,44]. However, knowledge about the environmental reservoirs outside the healthcare facilities is largely lacking, highlighting the need for further investigation into *C. difficile* persistence and transmission in wastewater and reclaimed water systems.

1.4. Bioassays

To complement pathogen-specific analyses, a rapid biotoxicity screening approach can be applied using the Microtox[®] assay to assess potential chemical toxicity, particularly in reclaimed water samples. This test employs the bioluminescent marine bacterium *Aliivibrio fischeri* to assess acute toxicity as an integrated biological response to complex chemical mixtures [45].

The term bioluminescence comes from the Greek word *bios* (life) and the Latin word *lumen* (light), literally meaning “living light” [46]. The Microtox[®] test, with *A. fischeri*, has been applied since 1979 and presents several advantages over other toxicity assays. Toxicity is determined by measuring changes in bacterial luminescence after exposure to a sample, as toxic substances or mixtures can impair cellular respiration and consequently reduce light emission.

The Microtox[®] assay was designed to function as a rapid screening tool for acute toxicity, requiring relatively small sample volumes and short exposure times compared

with other bioassays that use organisms such as algae, *Daphnia*, fish, plants, worms, mussels or oysters [47,48].

Barceló et al. [49] showed the successful application of the Microtox[®] assay to assess the ecotoxicity of treated wastewater samples prior to discharge, to ensure minimal environmental impact. In the particular case of treated urban and industrial wastewater effluents, biomonitoring using Microtox[®] revealed good results to assess contamination risk [49]. While this bioassay does not identify specific contaminants, it offers valuable complementary information on overall sample toxicity when integrated with microbiological and molecular analyses.

2. Materials and Methods

A total of 19 wastewater samples were analyzed, comprising eight samples of urban influent, eight samples of urban effluent, and three samples of water intended for reuse. Samples were collected in the Lisbon Metropolitan Area between February and June 2024. The reduced sampling window for water intended for reuse was associated with the seasonal irrigation demands of the irrigated spaces and scheduling defined by the Municipality of Lisbon. During the early months of 2024, irrigation using reclaimed water was carried out only on a sporadic basis, reflecting lower irrigation demand and non-systematic use. From May onwards, reclaimed water irrigation began to be applied in a regular and systematic manner, in accordance with municipal irrigation programs and irrigation needs. Consequently, sampling was deliberately concentrated between May and June to ensure that the collected samples were representative of the reclaimed water used under routine and consistent irrigation conditions, rather than during intermittent or exceptional applications.

This study combined molecular detection techniques, culture-based isolation methods, and toxicity bioassays to evaluate pathogen persistence and overall water safety.

2.1. RNA Extraction and Quantitative Real-Time PCR

RNA extraction from viral particles present in wastewater and reclaimed water samples was performed using a protocol optimized from Teixeira et al. [50]. Prior to filtration, 10 µL of Mengo virus (ceeramTOOLS[®], bioMérieux, Marcy-l'Étoile, France) was added to each sample as extraction control, and only samples with extraction efficiencies $\geq 1\%$ were considered valid, following ISO/TS 15216-1 [51] recommendations.

A volume of 25 mL of each sample was filtered through 0.45 µm pore-size nylon membranes, positively charged (Roche, Basel, Switzerland), and the filters were agitated in 10 mL of TGBE buffer (Tris-glycine-beef extract, pH 9.5) at 100 rpm for 1 h. After filter removal and pH adjustment to 7.0 ± 0.5 with HCl 0.1 N, samples were concentrated using an Amicon[®] Ultra-15 Centrifugal Filter Devices with a 100 kDa cut-off (Merck Millipore, Burlington, MA, USA) and centrifugated at $5000 \times g$ until a final volume of approximately 250–300 µL was obtained.

Prior to extraction, 500 µL of TRIzol[™] Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added to each concentrate. The mixture was incubated at 30 °C for 5 min in a thermomixer (Eppendorf, Hamburg, Germany) at 350 rpm, followed by the addition of 200 µL of chloroform (Merck, Darmstadt, Germany) and further agitation and incubation for 3 min under the same conditions. After centrifugation at $12,000 \times g$ for 10 min, the upper aqueous phase was carefully collected for RNA purification.

RNA purification was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The recovered aqueous phase (150 µL) was mixed with AVL buffer and carrier RNA, followed by ethanol addition. The mixture was loaded onto QIAamp Mini spin columns, washed with buffers AW1 and AW2,

and dried by centrifugation. RNA was eluted in two steps using 50 µL of AVE buffer each time and the eluate was stored at −80 °C until analysis.

Quantitative RT-Real-Time PCR (RT-qPCR) was performed for the specific detection of NoV GI, NoV GII, HAV and HEV viruses. An initial screening using Mengo virus detection and quantification was performed to confirm the efficiency of RNA extraction and also the absence of inhibitors using 10-fold dilutions of each sample. RT-qPCR was performed using NoV GI, NoV GII, HAV and HEV commercial kits (ceeramTOOLS[®], bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's specifications.

2.2. *Clostridioides difficile* Isolation

For every wastewater sample, two 0.45 µm pore cellulose membranes (EZ-Pak[®] Merck, Darmstadt, Germany) were obtained by filtering 25 mL of the water matrix through each membrane. To isolate *C. difficile*, two distinct methods were employed: one membrane of each filtered sample was either placed in 10 mL of *C. difficile* enrichment broth (BIOGERM[®], Maia, Portugal) or directly inoculated onto ChromID[®] *C. difficile* agar (bioMérieux, Marcy l'Etoile, France). Both media were incubated anaerobically using the Anoxomat[®] III Anaerobic Jar System (Anoxomat, Mart, The Netherlands) at 37 °C. The enrichment broth was incubated for seven days, with atmosphere renewal every 48 h, while the plates were incubated for 48–72 h. The isolation and culture methods employed were adapted from Chisholm et al. [23].

Plates from influent samples, where filters were directly inoculated, revealed too numerous to count. To obtain isolated colonies, ~10 µL of biomass from influent plates was suspended in 0.5 mL of *Brucella* broth and a loop full of the dilution was streaked onto two new ChromID[®] *C. difficile* agar (bioMérieux, Marcy l'Etoile, France), using the four-quadrant streak method. For effluent and water for reuse ChromID[®] *C. difficile* agar plates, suspected *C. difficile* colonies were selected from the inoculated filters and directly transferred to a new ChromID[®] *C. difficile* agar. All plates were incubated in anaerobic conditions at 37 °C, for 48 h. For enrichment broth samples (from influent, effluent and water for reuse), an alcohol shock treatment was performed by mixing 2 mL of the enrichment mixture with 2 mL of absolute ethanol and incubating at room temperature for 1 h. The suspension was then centrifuged (3500 rpm, 10 min), the supernatant was discarded, and the pellet plated onto a ChromID[®] *C. difficile* agar plate using the four-quadrant streak method. Plates were incubated anaerobically at 37 °C for 48 h. From each wastewater sample plate, at least five suspected *C. difficile* colonies were selected and identified by MALDI-TOF (VITEK[®] MS, bioMérieux, Marcy-l'Étoile, France). After identification, each colony was subcultured onto *Brucella* blood agar supplemented with hemin and vitamin K1 (BD BBL[™], Heidelberg, Germany) and incubated for 24 h, at 37 °C, under anaerobic conditions.

Toxin Gene Profiling, PCR Ribotyping and Real-Time PCR Detection of *Clostridioides difficile*

For typing purposes, DNA of each *C. difficile* isolate recovered was extracted using the NucliSENS[®] easyMAG[®] automated system (bioMérieux, Marcy-l'Étoile, France), according to manufacturer's instructions. For toxin profile determination, a multiplex PCR was used, targeting the toxin-encoding genes *tcdA* (toxin A), *tcdB* (toxin B) and *cdtA/cdtB* (binary toxin), as well as glutamate dehydrogenase encoding gene (*gluD*) for species confirmation [52,53].

Ribotypes were determined by PCR ribotyping, with the profile being generated by capillary gel electrophoresis, according to Fawley et al. [54], and resorting to the Webribo database (<https://webribo.ages.at/> accessed on 1 March 2024) or the European Union Reference Laboratory.

A *C. difficile* strain of RT027 (*tcdA*+/*tcdB*+/*cdtA*/*cdtB*+) was used for quality control in both techniques. For DNA extraction, 25 mL of each water sample was filtered using a polycarbonate membrane with a 0.45 µm pore size, mounted on a sterilized filtration apparatus connected to an air pump. Two commercial kits were used: the Aquadien Kit (Bio-Rad, Hercules, CA, USA) and the DNeasy PowerWater Kit (Qiagen, Hilden, Germany), following the manufacturers' instructions.

In the Aquadien Kit protocol, the folded filter membrane was placed into a 1.5 mL microtube containing 1 mL of R1 solution, vortexed and incubated at 95 °C for 15 min. After a second vortex, the membrane was removed, and the tube was then left to settle. For purification, 500 µL of the supernatant was transferred to the spin column. For elution, 100 µL of R2 solution was added to the column. The final eluate was stored at −20 °C until analysis (Bio-Rad, CA, USA).

For the DNeasy PowerWater Kit, the folded membrane was placed into a PowerWater Bead Pro tube, to which 1 mL of pre-warmed Solution PW1 was added. The sample was vortexed for 20 s, incubated at 65 °C for 10 min, then vortexed at maximum speed for an additional 10 min. The supernatant was collected to a clean tube and followed the additional extraction and purification steps. Elution was done using 100 µL of Solution EB, and purified DNA was stored at −20 °C until further use.

Real-time PCR was performed from the total DNA extracted from WW samples, using the RIDA® GENE *Clostridium difficile* kit, targeting the bacterium and toxins A and B (R-Biopharma AG, Darmstadt, Germany), according to the manufacturer's instructions.

The analytical sensitivity of the RIDA® GENE *Clostridium difficile* & Toxin A/B was determined with <5 DNA copies.

2.3. Microtox®-Based Toxicity Evaluation

Toxicity tests using bacterium *Aliivibrio fischeri* (NRRL B-11177) were carried out in accordance with the recommendations of ISO Standard 11348-3:2007, Standard methods (8050 B) [55,56] and Microtox® FX Analyser—User Manual (Modern Water Inc, Modern Water Inc., York, UK). Samples were analyzed within 48 h of collection and stored at 4 °C until testing.

First, the freeze-dried bacteria (preserved at −20 °C) were reactivated by hydration with a 2% sodium chloride solution. The test began with the reading of luminescence intensity at time zero ("0"), expressing the absence of exposure to potential toxicants. Luminescence intensity was then read 15 min after exposure to the sample under analysis.

A negative control was included in each test sequence only by exposing *A. fischeri* to a 2% saline solution. A positive control was also included by exposing the bacteria to two known toxic substances: potassium dichromate (K₂Cr₂O₇) (Merck, Darmstadt, Germany) and zinc sulphate heptahydrate (ZnSO₄·7H₂O) (Merck, Darmstadt, Germany), according to ISO 11348-3:2007 [56].

The toxic effect was expressed as the percentage inhibition of luminescence relative to the control. A reduction in light emission indicates interference with bacterial respiration, reflecting the presence of toxic substances in the sample.

3. Results

3.1. Sampling Overview

Wastewater (WW) samples were collected from both the influent (entry point) and effluent (exit point) of a wastewater treatment plant (WWTP) located in the Lisbon Metropolitan Area. Sampling was conducted between February and June 2024 across eight sampling events.

During the first five sampling events, influent and treated effluent samples were collected, in the final three sampling events, samples from influent, effluent, and water intended for reuse were obtained. Water for reuse underwent an additional tertiary treatment step and disinfection, consisting of either ultrafiltration with sodium hypochlorite addition or microfiltration with ultraviolet (UV) disinfection, depending on the treatment line in operation. In total, 19 WW samples (identified from CD1 to CD19) were collected and analyzed: eight influent, eight effluent, and three water for reuse samples. Table 1 summarizes the characteristics of the WW samples collected for this study.

Table 1. Overview of the wastewater samples, treatment level and the effluent's destination.

Sampling Date	Sample ID Code	Sample Type	WWTP Treatment Level	Destination of the Effluent
8 February 2024	CD1	Influent	Pretreatment	Tagus River Estuary
	CD2	Effluent	Pretreatment; primary; secondary	
8 March 2024	CD3	Influent	Pretreatment	
	CD4	Effluent	Pretreatment; primary; secondary	
26 March 2024	CD5	Influent	Pretreatment	
	CD6	Effluent	Pretreatment; primary; secondary	
11 April 2024	CD7	Influent	Pretreatment	
	CD8	Effluent	Pretreatment; primary; secondary	
24 April 2024	CD9	Influent	Pretreatment	
	CD10	Effluent	Pretreatment; primary; secondary	
9 May 2024	CD11	Influent	Pretreatment	Reuse
	CD12	Effluent	Pretreatment; primary; secondary	
	CD13	Water for reuse	Pretreatment; primary; secondary; tertiary and disinfection *	
23 May 2024	CD14	Influent	Pretreatment	Tagus River Estuary
	CD15	Effluent	Pretreatment; primary; secondary	
	CD16	Water for reuse	Pretreatment; primary; secondary; tertiary and disinfection *	
7 June 2024	CD17	Influent	Pretreatment	Tagus River Estuary
	CD18	Effluent	Pretreatment; primary; secondary	
	CD19	Water for reuse	Pretreatment; primary; secondary; tertiary and disinfection *	

* Tertiary treatment and disinfection consist of either ultrafiltration with sodium hypochlorite addition or microfiltration with UV disinfection, depending on the treatment line.

3.2. Enteric Viruses Found in Wastewater Detection

The results obtained with the RT-qPCR screening for enteric viruses showed that, with the exception of Norovirus GI and GII, the treatments applied prior to effluent release are effective in removing enteric viruses. Norovirus GI was detected in one effluent sample from February and Norovirus GII remained detectable in effluent from the two samples collected in April and June. In contrast, the tertiary treatment applied to produce water for reuse successfully eliminated all the viruses analyzed. These results are displayed in Table 2.

Table 2. Summary of the enteric viruses's presence/absence, as well as *Clostridioides difficile* detection, by culture and real-time PCR (16S-rDNA; *tcdA/tcdB*), frequency (in number of isolates per sample) and toxin genetic profile, in the wastewater samples and respective water for reuse.

Sample Number	Type of Sample	Sampling Date	Norovirus GI	Norovirus GII	Hepatitis A Virus	Hepatitis E Virus	<i>Clostridioides difficile</i>		
							Culture (No. of Isolates Per Sample)	Toxin Genetic Profile *	Real-Time PCR (16S-rDNA; <i>tcdA/tcdB</i>)
CD1	Influent	8 February 2024	Positive	Negative	Positive	Negative	Positive (16)	Toxigenic	Positive; non-toxigenic
CD3	Influent	8 March 2024	Negative	Negative	Positive	Negative	Positive (20)	Toxigenic and non-toxigenic	Positive; non-toxigenic
CD5	Influent	26 March 2024	Positive	Positive	Negative	Positive	Positive (15)	Toxigenic and non-toxigenic	Sample inhibited
CD7	Influent	11 April 2024	Negative	Positive	Negative	Negative	Positive (12)	Toxigenic and non-toxigenic	Positive; non-toxigenic
CD9	Influent	24 April 2024	Positive	Positive	Negative	Negative	Positive (12)	Toxigenic and non-toxigenic	Positive; non-toxigenic
CD12	Influent	9 May 2024	Negative	Negative	Negative	Negative	Positive (10)	Toxigenic and non-toxigenic	Positive; non-toxigenic
CD15	Influent	23 May 2024	Negative	Negative	Negative	Negative	Positive (7)	Toxigenic and non-toxigenic	Positive; non-toxigenic
CD18	Influent	6 June 2024	Positive	Positive	Positive	Positive	Positive (7)	Toxigenic and non-toxigenic	Positive; non-toxigenic
CD2	Effluent	8 February 2024	Positive	Negative	Negative	Negative	Positive (10)	Toxigenic and non-toxigenic	Negative
CD4	Effluent	8 March 2024	Negative	Negative	Negative	Negative	Positive (5)	Non-toxigenic	Negative
CD6	Effluent	26 March 2024	Negative	Negative	Negative	Negative	Positive (5)	Toxigenic	Negative
CD8	Effluent	11 April 2024	Negative	Negative	Negative	Negative	Positive (2)	Toxigenic	Negative
CD10	Effluent	24 April 2024	Negative	Positive	Negative	Negative	Positive (10)	Toxigenic and non-toxigenic	Negative
CD13	Effluent	9 May 2024	Negative	Negative	Negative	Negative	Negative	-	Negative
CD16	Effluent	23 May 2024	Negative	Negative	Negative	Negative	Positive (5)	Toxigenic	Negative
CD19	Effluent	6 June 2024	Negative	Positive	Negative	Negative	Positive (4)	Toxigenic and non-toxigenic	Negative
CD11	Water for reuse	9 May 2024	Negative	Negative	Negative	Negative	Negative	-	Negative
CD14	Water for reuse	23 May 2024	Negative	Negative	Negative	Negative	Negative	-	Negative
CD17	Water for reuse	6 June 2024	Negative	Negative	Negative	Negative	Negative	-	Negative

Positive results are highlighted in blue. * Toxigenic strains were positive for genes encoding toxin A (*tcdA*), toxin B (*tcdB*), and/or binary toxin (*cdtA/cdtB*); non-toxigenic strains lacked toxin genes.

3.3. *Clostridioides difficile*

3.3.1. *Clostridioides difficile* Cultures

C. difficile was detected in 15 of the 19 samples (78.9%) of the wastewater samples analyzed. All influent samples tested positive, whereas only one effluent and all three water for reuse samples were negative.

Table 2 summarizes the WW sample characteristics and *C. difficile* isolation results. A total of 181 isolates were cultured, 140 of which were confirmed as *C. difficile*, with recovery rates being 100.0% for influent samples (99 isolates) and 87.5% for effluent samples (41 isolates). The remaining isolates were identified as *Clostridium butyricum* or *Lactobacillus mucosae*.

3.3.2. *Clostridioides difficile* Strains—Toxin Genetic Profile and PCR-Ribotyping

Among the 140 *C. difficile* isolates recovered from wastewater, 74.0% were toxigenic. Of these, 88.3% were positive for toxins A and B (*tcdA*+/ *tcdB*+), while 11.7% harbored genes encoding toxins A, B, and the binary toxin (*tcdA*+/ *tcdB*+/*cdtA*/B+). The remaining 26.0% of the isolates were non-toxigenic. PCR-ribotyping revealed 33 distinct ribotype profiles. Among influent isolates, the most prevalent toxigenic ribotypes were the toxigenic (positive for toxins A and B) RT014/020 and RT106 (15.2% each), followed by the hypervirulent (positive for toxins A, B and binary) RT078/126 (12.1%), and both the toxigenic RT011 and the non-toxigenic RT009 (7.1% each). In the effluent, the most common ribotypes included the toxigenic RT430 (24.4%), the non-toxigenic types RT010 (17.1%) and RT009 (14.6%), and the toxigenic RT103 (12.2%). Representative PCR-ribotyping profiles are shown in Figure S1.

Overall, toxigenic strains predominated in both influent (76.7%) and effluent (65.9%) samples. Six ribotypes, three toxigenic (toxA/toxB positive), RT103, RT081, and RT104, and three non-toxigenic, RT009, RT010 and RT084, were detected in both sample types. Surprisingly, five PCR-ribotype profiles (all toxigenic, positive for toxins A and B) were found exclusively in effluent samples: RT154, RT430, RT651 and two distinct unknown RTs.

Within the framework of the national *C. difficile* infection surveillance program, and according to the European surveillance of *Clostridioides (Clostridium) difficile* infections protocol [57], human fecal samples previously tested as positive for CDI are sent to the National Reference Laboratory to generate microbiological data, including molecular characterization and antimicrobial susceptibility testing data. Between September 2023 and May 2024, a total of 384 *C. difficile* isolates were recovered from these clinical samples.

The most common toxin profile (75.5%) identified among the 384 clinical isolates was A+B+/binary−. Non-toxigenic strains were also detected in clinical samples, indicating possible co-colonization of the gut by both toxigenic and non-toxigenic strains. A total of 31 toxigenic PCR-ribotype profiles were shared between clinical and wastewater isolates. The five most prevalent RTs in clinical isolates were also abundant in WW, particularly influent, namely: RT106, RT014/020, RT078/126, RT012, and RT013. Regarding non-toxigenic RTs, five were common between clinical and wastewater samples: RT009, RT084, RT031, RT039 and RT010. The distribution of ribotypes found in influent and effluent samples is illustrated in Figure S2.

3.3.3. *Clostridioides difficile* Diversity According to the Isolation Method

Both isolation methods applied successfully recovered *C. difficile*. Direct plating of membrane filters onto selective agar yielded higher positivity rates in both influent (100.0%) and effluent (75.0%) samples, but resulted in lower ribotype diversity. In contrast, the enrichment broth method showed lower positivity rates (62.5% in influent samples and 25.0% in effluent samples), but recovered with a greater diversity of ribotypes. Specifically, direct plating yielded 80 *C. difficile* isolates from influent samples, corresponding to 27 distinct ribotypes, and 23 isolates from effluent samples, corresponding to seven ribotypes. In comparison, the enrichment broth method recovered 19 isolates representing eight ribotypes from influent samples and 18 isolates representing six ribotypes from effluent samples.

3.3.4. Real-Time PCR Detection of Toxigenic *Clostridioides difficile*

The RIDA[®]GENE *Clostridium difficile* multiplex real-time PCR assay detects both *C. difficile* (16S-rDNA) and *C. difficile* toxin genes A (*tcdA*)/B (*tcdB*). *C. difficile* was detected in seven (87.5%) of the eight influent samples, with cycle threshold (Ct) values ranging from 28.9 to 31.58, with one (12.5%) sample being inhibited, and no toxigenic strains being detected. In contrast, *C. difficile* DNA was not detected in any of the eight effluent samples, nor in the three water for reuse samples. A comparison of the PCR results with culture-based results is summarized in Table 2: PCR failed to detect seven out of eight culture-positive effluent samples; all four culture-negative samples (one effluent and three water for reuse) were also PCR-negative. In addition, PCR could not detect the toxin genes in any of the positive influent samples.

Overall, enteric viruses and *C. difficile* were frequently detected in influent samples, but largely removed after treatment, with no detection in water for reuse samples.

3.4. Assessment of Acute Toxicity in Wastewater

The Microtox[®] bioassay is widely used as a screening methodology to assess the acute toxicity of water pollutants in different matrices. It allows results to be obtained in minutes in a simple manner and with a small sample volume, without the use of animals [45,58]. The test measures the reduction in light emission by the *Aliivibrio fischeri* marine bacteria when

exposed to toxic substances, which causes the interruption of their respiratory processes, resulting in a reduction in the light emitted [59].

In each sample sequence, a negative control was analyzed, which expresses the intrinsic loss of cell viability over time. The percentage inhibition of the samples calculated has this value into account and subtracts it. The average luminescence intensity value recorded for the negative control was 5,316,795 relative light units (RLU).

As a positive control, two standards with different toxicities were tested: one with higher toxicity, zinc sulphate (ZnSO_4), and another less toxic, potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$). The positive control that presented higher toxicity recorded an average luminescence intensity value of 116,847 RLU. The response sensitivity of *A. fischeri* bacteria was demonstrated at different two toxicity levels. The two dose–response curves were prepared with zinc sulphate for a working range of 3–10 mg/L that presented % effect values between 67.0 and 95.0%. Potassium dichromate was prepared for a working range of 16–129 mg/L and displayed % effect values ranging from 30.0% to 60.0%.

It was possible to determine the amount of toxic substance that induced the unviability of 50% of bacteria (EC50), with zinc sulphate displaying an $\text{EC}_{50} = 2.09$ mg/L and $\text{EC}_{50} = 77.7$ mg/L for exposure to potassium dichromate.

According to Baran et al. [60] the results can be grouped as follows: 11 samples produced a loss of luminescence of less than 20.0% and are therefore considered non-toxic; 1 sample produced a loss of luminescence of between 20.0% and 50.0%, indicating slight toxicity.

Our results showed that only one influent sample was considered slightly toxic (CD18), although all samples from influent tested show inhibitions between 5.0 and 20.0%. However, all the effluent and water for reuse samples analyzed did not present any inhibition. The results obtained for the 12 samples analyzed are displayed in Figure 1.

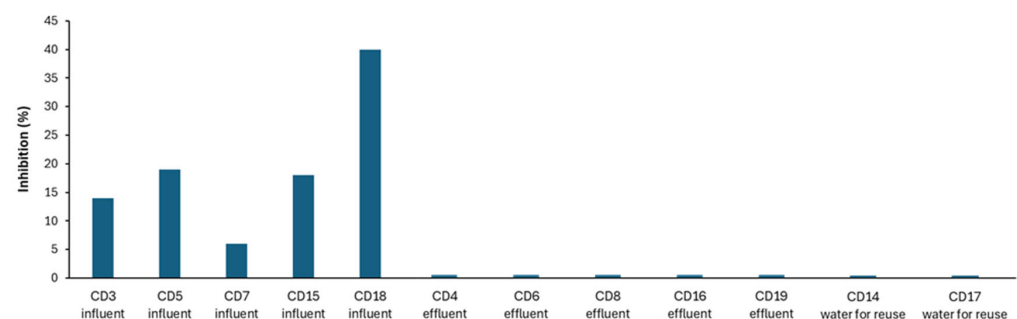


Figure 1. Luminescence inhibition (%) of *Aliivibrio fischeri* after 15 min of exposure for influent, effluent and water for reuse samples.

4. Discussion

4.1. Enteric Viruses and *C. difficile* vs. Current Microbiological Monitoring Approaches

Multiple field studies and systematic reviews have reported weak, inconsistent, or context-dependent correlations between traditional FIBs (*E. coli* and enterococci) and human enteric viruses in wastewater and reclaimed water. This pattern has been observed across different treatment stages, including raw influent, secondary effluent, and disinfected effluents, and across diverse reuse scenarios [61–63].

These inconsistencies reflect fundamental biological and environmental differences between bacterial indicators and viral pathogens, including differences in persistence, resistance to treatment processes and sources of contamination between viruses and bacteria. For instance, *E. coli* and enterococci are typically removed more efficiently during biological treatment and disinfection, whereas enteric viruses such as noroviruses and adenoviruses

tend to persist for longer periods and exhibit removal behaviors more similar to viral indicators such as coliphages [62].

Meta-analyses have demonstrated that virus-to-FIB ratios vary considerably depending on viral genotype, geographic region, season, and treatment extent. This variability suggests that the use of static conversion factors or bacterial surrogates can lead to under or overestimation of viral risk in quantitative microbial risk assessments [61].

Recent work by Cuevas-Ferrando et al. [64] further highlights the limitations of surrogate indicators by demonstrating that, although human-associated crAssphage correlates strongly with fecal contamination, it performs poorly as a marker of viral infectivity or persistence. This finding emphasizes that even viral indicators have inherent limitations. Therefore, many authors recommend virus-specific monitoring or multi-marker approaches (e.g., somatic and F+ coliphages, pepper mild mottle virus [PMMoV], crAssphage, or direct viral assays) to improve the accuracy of water reuse risk assessments [61–64].

In addition to our findings, recent European environmental surveillance studies provide compelling evidence of the widespread circulation and persistence of enteric viruses in wastewater systems and related waters. For example, Veneri et al. [65] detected human adenovirus genomes in over 93.0% of 168 untreated wastewater samples collected across Italy. The combined application of quantitative methods (RT-qPCR) and high-resolution sequencing (Nanopore) demonstrated that traditional monitoring approaches may underestimate both the presence and genetic diversity of adenoviruses. Together with our proof-of-concept results on norovirus GI/GII and *C. difficile* in reclaimed water, these findings support the inclusion of pathogen-specific viral and spore-forming bacterial markers to complement traditional indicators, and enable a more comprehensive assessment of microbiological safety in water reuse settings. Overall, our observations align with the growing body of evidence indicating that traditional bacterial indicators alone are insufficient to assess the persistence or removal of highly resistant pathogens. Integrating targeted molecular detection methods with toxicity screening, provides a more robust and informative framework for water reuse safety evaluation.

Community-associated *Clostridioides difficile* infection (CA-CDI) arises from exposure to *C. difficile* reservoirs or sources within the community, rather than the typical hospital-associated settings. While the impact of the environment on *C. difficile* epidemiology has been recognized globally, available data remains limited, including in Portugal. Hence, and due to the changing epidemiology, integrating wastewater surveillance into CDI surveillance could significantly enhance public health efforts by providing early detection, outbreak tracking, and a more comprehensive understanding of CDI epidemiology [66,67]. This approach aligns with the One Health paradigm, emphasizing the interconnectedness of human, animal, and environmental reservoirs.

In the present study, we provide insight into *C. difficile* surveillance in municipal wastewater treatment plants (WWTPs) in Portugal by assessing its presence in untreated and treated wastewater, characterizing circulating strains, and evaluating the overlap between environmental and human strains.

C. difficile was detected in all influent samples, while detection rates were lower in effluent samples and absent in water for reuse samples. The absence of *C. difficile* in reclaimed water is likely attributable to the application of tertiary treatment processes, including microfiltration or ultrafiltration combined with disinfection using sodium hypochlorite or UV radiation. These findings indicate that tertiary treatment was effective in removing *C. difficile* spores suggesting that reclaimed water intended for irrigation or public space cleaning is unlikely to pose a significant public health risk. Nevertheless, the limited number of samples analyzed ($n = 3$) warrants cautious interpretation and highlights the need for further studies with expanded sampling. Nevertheless, our results are consistent with

previous studies, reporting effective removal of *C. difficile* following advanced treatment processes [23].

Regarding the isolation methodology, although direct inoculation of filters onto selective media was used, it was expected that the inclusion of a prior enrichment step would improve the detection of *C. difficile* present at low concentrations in environmental samples. Contrary to theoretical expectations, direct plating yielded higher positivity rates for both influent and effluent samples. This finding aligns with those of Cizek et al. [67], who reported *C. difficile* recovery from five samples using direct plating compared to only one sample following enrichment. Nevertheless, the enrichment resulted in greater ribotype diversity among isolates from both influent and effluent samples.

The combined use of both isolation strategies therefore represents a methodological advantage, as instances where one method failed to recover *C. difficile* were often compensated by successful isolation using the alternative approach. Moreover, the marked discrepancy between molecular and culture-based detection underscores that molecular assays performed on DNA extracted directly from wastewater samples may have limited sensitivity. However, real-time PCR remains a valuable confirmatory tool for presumptive *C. difficile* colonies isolated from food, animal, and wastewater samples, as shown by Rivas et al. [68]. Both culture-based and molecular methods were applied to thoroughly assess microbial contamination. Culture-based approaches detect viable and potentially infectious organisms but may underestimate viable yet non-culturable pathogens, whereas molecular methods, such as PCR and RT-qPCR, offer high analytical sensitivity for non-culturable organisms, but cannot distinguish between infectious and inactivated microorganisms. The combined application of both approaches therefore strengthens their complementarity, and enhances reliability and interpretability of surveillance data. Collectively, these results highlight the need to optimize isolation protocols and to establish standardized methodologies for *C. difficile* detection in environmental studies.

Even though hypervirulent ribotypes were detected in influent samples, a marked reduction in ribotype diversity was observed at the effluent stage. This finding suggests that wastewater treatment processes are effective in decreasing the prevalence of *C. difficile*, although not totally eliminating it. Both non-toxigenic and toxigenic strains of *C. difficile* were identified in effluent samples, indicating that viable organisms can be released into the environment. Although environmental *C. difficile* strains do not necessarily represent an immediate risk to public health, its presence highlights the importance implementing integrated, risk-based monitoring to ensure the safe use of reclaimed water for irrigation and public space cleaning. Notably, most of the ribotypes recovered from effluent samples were toxigenic and have been associated with clinical cases of CDI in humans, reinforcing the hypothesis of bidirectional transmission between community and healthcare settings.

A high prevalence of non-toxigenic strains was also observed. Although these strains lack toxin-encoding genes, they may still play a substantial role in CDI epidemiology. Non-toxigenic strains often harbor antibiotic resistance genes acquired by horizontal gene transfer (HGT), either from other *C. difficile* strains or from co-existing bacterial species within the human gut or complex microbial communities such as those found in WWTPs. Consequently, these strains may constitute reservoirs of mobile genetic elements (MGEs) associated with antimicrobial resistance, which can subsequently be transferred to toxigenic strains, as demonstrated by Brouwer et al. [69]. Furthermore, selective pressures such as residual antibiotics in WWTPs provide favorable conditions for these genetic exchanges [23]. These dynamics highlight the potential contribution of wastewater effluents to the environmental dissemination of *C. difficile* strains of clinical relevance, and to the emergence of CA-CDI.

Study limitations should be acknowledged. The sample size ($n = 19$) and limited temporal coverage constrain statistical generalization. Future research aims to expand seasonal and geographic sampling, incorporate infectivity-based methods, and further evaluate the applicability of pathogen-specific indicators like Norovirus GI/GII and *C. difficile* for routine water reuse monitoring.

4.2. Usefulness of Bioassays in Water for Reuse

The Microtox® bioassay proved to be a valuable complementary tool for the rapid assessment of acute toxicity in wastewater and reclaimed water samples. Its short response time, low sample volume requirements, and ability to capture the combined effects of individual substances or complex chemical mixtures makes it particularly suitable as a screening method in water reuse monitoring programs.

In this study, Microtox® results indicated the absence of acute toxicity in effluent and reclaimed water samples, supporting the effectiveness of treatment processes in reducing chemical toxicity alongside microbiological hazards. When integrated with microbiological and molecular analyses, bioassays contribute to a more holistic evaluation of reclaimed water quality and potential risks associated with its reuse.

4.3. Compliance with Current and Future Legislation

To comply with Regulation (EU) 2020/741, Member States are required to assess the feasibility of water reuse within river basin management plans and implement adequate treatment processes that do not compromise ecological flow or public health. The regulation also allows for Member States' derogations under specific conditions, particularly for tertiary treatment requirements when wastewater is reused exclusively for agricultural irrigation [18].

Article 15 of the revised Urban Wastewater Treatment Directive [70] further reinforces the promotion of water reuse, particularly in water-stressed regions. Member States should promote the reuse of treated wastewater while ensuring environmental and health safety. Urban wastewater discharges from treatment plants serving 1000 population equivalents or more must be regulated through prior authorizations and periodic reviews conducted at least every ten years. Additionally, urban wastewater infrastructure must be adapted to accommodate increased wastewater loads [18,70]. Within this evolving regulatory landscape, the limitations of relying solely on traditional bacterial indicators becomes increasingly evident.

Our findings, together with previous work demonstrate that fecal indicator bacteria counts, namely *E. coli*, show poor correlation with more resistant pathogens, including enteric viruses and *Clostridioides difficile*, in effluent wastewater [22]. This study provides additional evidence supporting the integration of pathogen-specific monitoring approaches that combine microbiological, molecular, and ecotoxicological indicators to enhance risk-based water reuse assessments. Such integrated framework is essential for ensuring EU regulatory compliances while effectively safeguarding public health.

5. Conclusions

Regulations for water reuse are closely linked to public health protection and environmental sustainability. Contemporary regulatory frameworks increasingly incorporate a "One Health" perspective recognizing the interdependence of human, animal, and environmental health, and ensuring that compliance with these regulations prevents waterborne diseases, protects ecosystems, and enhances resilience against water scarcity.

Water reuse plays a crucial role in addressing water scarcity, improving resource efficiency, and promoting environmental sustainability. Through national and European

regulations, safe and effective reuse is encouraged, ensuring that treated wastewater can be used without compromising public health. As environmental challenges continue to evolve, the adaptation and enforcement of these regulations will be crucial to secure a sustainable water future. This study adds practical value to existing water reuse regulatory frameworks by providing evidence that can support their risk-based implementation. International and regional regulatory frameworks for water reuse, including those issued by the World Health Organization [71] and the EU Regulation 2020/741 [18], are supported by a risk-based approach to water reuse, where minimum microbiological requirements may be complemented by additional monitoring, when necessary, to ensure public health safeguard. Our results show that Norovirus (GI and GII) and *Clostridioides difficile* can be detected in treated wastewaters that comply with current bacterial standards, highlighting their potential role as complementary indicators within existing monitoring schemes. These findings support the inclusion of complementary microbial indicators within water reuse risk management plans, strengthening pathogen surveillance, and complementing existing regulatory requirements. Through this work, we aim to contribute to the following: (1) The creation of stronger evidence highlighting the need to screen for enteric viruses in treated wastewaters intended for reuse, as a fundamental step to safeguard public health. (2) The development of an integrated approach for monitoring water resources, enabling the implementation of appropriate mitigation measures when necessary.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms14020430/s1>, Figure S1: Representative PCR-ribotyping profiles of *Clostridioides difficile* ribotypes (RT) 078/126, 014/020 and 009, obtained from isolates recovered from wastewater samples. The *x*-axis indicates fragment size (base pairs), while the *y*-axis depicts relative fluorescence units (RFU), representing the intensity of emitted fluorescence; Figure S2: Distribution and diversity of ribotypes identified amongst the 140 *Clostridioides difficile* isolates from wastewater samples, collected between February and June 2024, according to sample type, in influent (*n* = 99) and in treated effluent (*n* = 41). Ribotypes identified with “⊙” have not been found in clinical samples in this study; while “●” integrate the top six most prevalent clinical ribotypes, “*” refers to non-toxigenic (although RT011 is not marked, this toxin genetic profile was found in one isolate). “Unknown” refers to profiles that were not identified by Webribo.

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References

1. Kendig, C.E. What Is Proof of Concept Research and How Does It Generate Epistemic and Ethical Categories for Future Scientific Practice? *Sci. Eng. Ethics* **2016**, *22*, 735–753. [CrossRef] [PubMed]
2. McClaran, N.; Behe, B.K.; Huddleston, P.; Fernandez, R.T. Recycled or Reclaimed? The Effect of Terminology on Water Reuse Perceptions. *J. Environ. Manag.* **2020**, *261*, 110144. [CrossRef] [PubMed]

3. US EPA. Basic Information About Water Reuse. Available online: <https://www.epa.gov/waterreuse/basic-information-about-water-reuse> (accessed on 20 January 2026).
4. Water Reuse Europe. Water Reuse Europe (WRE)—Front Page Water Reuse Europe. Available online: <https://www.water-reuse-europe.org/> (accessed on 27 January 2026).
5. Ong, C.N. Water Reuse, Emerging Contaminants and Public Health: State-of-the-Art Analysis. *Int. J. Water Resour. Dev.* **2016**, *32*, 514–525. [CrossRef]
6. Nappier, S.P.; Soller, J.A.; Eftim, S.E. Potable Water Reuse: What Are the Microbiological Risks? *Curr. Environ. Health Rep.* **2018**, *5*, 283–292. [CrossRef]
7. National Research Council (U.S.). *Water Reuse: Potential for Expanding the Nation's Water Supply Through Reuse of Municipal Wastewater*; National Academies Press: Washington, DC, USA, 2012; p. 13303. [CrossRef]
8. Fono, L.J.; Kolodziej, E.P.; Sedlak, D.L. Attenuation of Wastewater-Derived Contaminants in an Effluent-Dominated River. *Environ. Sci. Technol.* **2006**, *40*, 7257–7262. [CrossRef]
9. Mishra, S.; Kumar, R.; Kumar, M. Use of Treated Sewage or Wastewater as an Irrigation Water for Agricultural Purposes—Environmental, Health, and Economic Impacts. *Total Environ. Res. Themes* **2023**, *6*, 100051. [CrossRef]
10. Zaibel, I.; Arnon, S.; Zilberg, D. Treated Municipal Wastewater as a Water Source for Sustainable Aquaculture: A Review. *Rev. Aquac.* **2022**, *14*, 362–377. [CrossRef]
11. Frijns, J.; Hofman, J.; Nederlof, M. The Potential of (Waste) Water as Energy Carrier. *Energy Convers. Manag.* **2013**, *65*, 357–363. [CrossRef]
12. Chakir, Z.; Lekhlif, B.; Sinan, M.; El Maki, A.A. Wastewater Reuse through Soil Aquifer Treatment: Regulations and Guideline for Feasibility Assessment. *Desalin. Water Treat.* **2023**, *304*, 60–80. [CrossRef]
13. Tortajada, C. Contributions of Recycled Wastewater to Clean Water and Sanitation Sustainable Development Goals. *NPJ Clean. Water* **2020**, *3*, 22. [CrossRef]
14. Hristov, J.; Barreiro-Hurle, J.; Salputra, G.; Blanco, M.; Witzke, P. Reuse of Treated Water in European Agriculture: Potential to Address Water Scarcity Under Climate Change. *Agric. Water Manag.* **2021**, *251*, 106872. [CrossRef] [PubMed]
15. Rich, D.; Andiroglu, E.; Gallo, K.; Ramanathan, S. A Review of Water Reuse Applications and Effluent Standards in Response to Water Scarcity. *Water Secur.* **2023**, *20*, 100154. [CrossRef]
16. Água para Reutilização (ApR) | Agência Portuguesa do Ambiente. Available online: <https://apambiente.pt/agua/agua-para-reutilizacao-apr> (accessed on 20 January 2026).
17. Giakoumis, T.; Vaghela, C.; Voulvoulis, N. The Role of Water Reuse in the Circular Economy. In *Advances in Chemical Pollution, Environmental Management and Protection*; Elsevier: Amsterdam, The Netherlands, 2020; Volume 5, pp. 227–252. [CrossRef]
18. European Union. *Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on Minimum Requirements for Water Reuse (Text with EEA Relevance)*; European Union: Brussels, Belgium, 2020; Volume 177, Available online: <http://data.europa.eu/eli/reg/2020/741/oj> (accessed on 20 January 2026).
19. THE 17 GOALS | Sustainable Development. Available online: <https://sdgs.un.org/goals> (accessed on 27 January 2026).
20. Decreto-Lei n. 119/2019 | DR. Available online: <https://diariodarepublica.pt/dr/detalhe/decreto-lei/119-2019-124097549> (accessed on 20 January 2026).
21. Portaria n. 266/2019 | DR. Available online: <https://diariodarepublica.pt/dr/detalhe/portaria/266-2019-124171144> (accessed on 20 January 2026).
22. Teixeira, P.; Salvador, D.; Brandão, J.; Ahmed, W.; Sadowsky, M.J.; Valério, E. Environmental and Adaptive Changes Necessitate a Paradigm Shift for Indicators of Fecal Contamination. *Microbiol. Spectr.* **2020**, *8*, 10–1128. [CrossRef]
23. Chisholm, J.M.; Putsathit, P.; Riley, T.V.; Lim, S.-C. Spore-Forming *Clostridium* (*Clostridioides*) *difficile* in Wastewater Treatment Plants in Western Australia. *Microbiol. Spectr.* **2023**, *11*, e0358222. [CrossRef]
24. World Health Organization (WHO). *Guidelines for Drinking-Water Quality, Fourth Edition Incorporating the First Addendum*; World Health Organization: Geneva, Switzerland, 2017.
25. Norovirus Infection. Available online: <https://www.ecdc.europa.eu/en/norovirus-infection> (accessed on 27 January 2026).
26. Norovirus. Available online: <https://www.cdc.gov/norovirus/index.html> (accessed on 27 January 2026).
27. Liao, Y.; Hong, X.; Wu, A.; Jiang, Y.; Liang, Y.; Gao, J.; Xue, L.; Kou, X. Global Prevalence of Norovirus in Cases of Acute Gastroenteritis from 1997 to 2021: An Updated Systematic Review and Meta-Analysis. *Microb. Pathog.* **2021**, *161*, 105259. [CrossRef]
28. Lee, S.J.; Si, J.; Yun, H.S.; Ko, G. Effect of Temperature and Relative Humidity on the Survival of Foodborne Viruses During Food Storage. *Appl. Environ. Microbiol.* **2015**, *81*, 2075–2081. [CrossRef]
29. Barclay, L.; Park, G.W.; Vega, E.; Hall, A.; Parashar, U.; Vinjé, J.; Lopman, B. Infection Control for Norovirus. *Clin. Microbiol. Infect.* **2014**, *20*, 731–740. [CrossRef]
30. Ong, C.W. Norovirus: A Challenging Pathogen. *Healthc. Infect.* **2013**, *18*, 133–142. [CrossRef]

31. Disease Information About Hepatitis A. Available online: <https://www.ecdc.europa.eu/en/hepatitis-A/facts> (accessed on 27 January 2026).
32. Hepatitis A. Available online: <https://www.cdc.gov/hepatitis-a/index.html> (accessed on 27 January 2026).
33. Hepatitis E. Available online: <https://www.ecdc.europa.eu/en/hepatitis-e> (accessed on 27 January 2026).
34. Dudášová, K.; Pavlová, A.; Kočíková, B.; Urda Dolinská, M.; Šalamúnová, S.; Molnár, L.; Kottferová, L.; Jacková, A. Molecular Detection and Genetic Typing of Hepatitis E Virus in Wild Animals from Slovakia. *Food Environ. Virol.* **2025**, *17*, 42. [CrossRef]
35. Hepatitis E Basics. Hepatitis E. Available online: <https://www.cdc.gov/hepatitis-e/about/index.html> (accessed on 27 January 2026).
36. Khun, P.A.; Phi, L.D.; Bui, H.T.T.; Bui, N.T.; Vu, Q.T.H.; Trinh, L.D.; Collins, D.A.; Riley, T.V. Environmental Contamination with *Clostridioides (Clostridium) difficile* in Vietnam. *J. Appl. Microbiol.* **2023**, *134*, lxad118. [CrossRef]
37. Oleastro, M.; Coelho, M.; Gião, M.; Coutinho, S.; Mota, S.; Santos, A.; Rodrigues, J.; Faria, D. Outbreak of *Clostridium difficile* PCR Ribotype 027—The Recent Experience of a Regional Hospital. *BMC Infect. Dis.* **2014**, *14*, 209. [CrossRef] [PubMed]
38. Crispino, P. Clustering Disease of *Clostridioides difficile* Infection: Implication for the Management in Internal Medicine. *Microbiol. Res.* **2023**, *14*, 1376–1397. [CrossRef]
39. Nazareth, C.; Leitão, I.; Reis, E.; Inácio, H.; Martins, F.; Ramalheira, E.; Cunha, F.; Santos, C.; Lino, S.; Moreira, H.; et al. Epidemiology of *Clostridioides difficile* Infection in Portugal: A Retrospective, Observational Study of Hospitalized Patients. *Acta Médica Port.* **2022**, *35*, 270–278. [CrossRef] [PubMed]
40. Morado, F.; Nanda, N. A Review of Therapies for *Clostridioides difficile* Infection. *Antibiotics* **2024**, *14*, 17. [CrossRef]
41. Buddle, J.E.; Fagan, R.P. Pathogenicity and Virulence of *Clostridioides difficile*. *Virulence* **2023**, *14*, 2150452. [CrossRef]
42. Hunt, J.J.; Ballard, J.D. Variations in Virulence and Molecular Biology among Emerging Strains of *Clostridium difficile*. *Microbiol. Mol. Biol. Rev.* **2013**, *77*, 567–581. [CrossRef]
43. Alves, F.; Castro, R.; Pinto, M.; Nunes, A.; Pomba, C.; Oliveira, M.; Silveira, L.; Gomes, J.P.; Oleastro, M. Molecular Epidemiology of *Clostridioides difficile* in Companion Animals: Genetic Overlap with Human Strains and Public Health Concerns. *Front. Public Health* **2023**, *10*, 1070258. [CrossRef]
44. Viprey, V.F.; Davis, G.L.; Benson, A.D.; Ewin, D.; Spittal, W.; Vernon, J.J.; Rupnik, M.; Banz, A.; Allantaz, F.; Cleuziat, P.; et al. A Point-Prevalence Study on Community and Inpatient *Clostridioides difficile* Infections (CDI): Results from Combatting Bacterial Resistance in Europe CDI (COMBACTE-CDI), July to November 2018. *Eurosurveillance* **2022**, *27*, 2100704. [CrossRef]
45. Domínguez, C.M.; Ventura, P.; Checa-Fernández, A.; Santos, A. Comprehensive Study of Acute Toxicity Using Microtox® Bioassay in Soils Contaminated by Lindane Wastes. *Sci. Total Environ.* **2023**, *856*, 159146. [CrossRef]
46. Kulkarni, V.S.; Kulkarni, B.S. Isolation of Bioluminescent Bacteria and Their Application in Toxicity Testing of Chromium in Water. *Int. J. Curr. Microbiol. App. Sci.* **2015**, *4*, 23–32.
47. Guzzella, L.; Bartone, C.; Ross, P.; Tartari, G.; Muntau, H. Toxicity Identification Evaluation of Lake Orta (Northern Italy) Sediments Using the Microtox System. *Ecotoxicol. Environ. Saf.* **1996**, *35*, 231–235. [CrossRef] [PubMed]
48. Araújo, C.V.M.; Nascimento, R.B.; Oliveira, C.A.; Strotmann, U.J.; da Silva, E.M. The Use of Microtox® to Assess Toxicity Removal of Industrial Effluents from the Industrial District of Camaçari (BA, Brazil). *Chemosphere* **2005**, *58*, 1277–1281. [CrossRef] [PubMed]
49. Barceló, D.; Žonja, B.; Ginebreda, A. Toxicity Tests in Wastewater and Drinking Water Treatment Processes: A Complementary Assessment Tool to be on Your Radar. *J. Environ. Chem. Eng.* **2020**, *8*, 104262. [CrossRef]
50. Teixeira, P.; Costa, S.; Brown, B.; Silva, S.; Rodrigues, R.; Valério, E. Quantitative PCR Detection of Enteric Viruses in Wastewater and Environmental Water Sources by the Lisbon Municipality: A Case Study. *Water* **2020**, *12*, 544. [CrossRef]
51. ISO 15216-1:2017(en); Microbiology of the Food Chain—Horizontal Method for Determination of Hepatitis A Virus and Norovirus Using Real-Time RT-PCR—Part 1: Method for Quantification. ISO (International Organization for Standardization): Geneva, Switzerland, 2017. Available online: <https://www.iso.org/obp/ui/en/#iso:std:iso:15216-1:ed-1:v1:en> (accessed on 26 January 2026).
52. Paltansing, S.; van den Berg, R.J.; Guseinova, R.A.; Visser, C.E.; van der Vorm, E.R.; Kuijper, E.J. Characteristics and Incidence of *Clostridium difficile*—Associated Disease in The Netherlands, 2005. *Clin. Microbiol. Infect.* **2007**, *13*, 1058–1064. [CrossRef]
53. Persson, S.; Torpdahl, M.; Olsen, K.E.P. New Multiplex PCR Method for the Detection of *Clostridium difficile* Toxin A (*tcdA*) and Toxin B (*tcdB*) and the Binary Toxin (*cdtA/cdtB*) Genes Applied to a Danish Strain Collection. *Clin. Microbiol. Infect.* **2008**, *14*, 1057–1064. [CrossRef]
54. Fawley, W.N.; Knetsch, C.W.; MacCannell, D.R.; Harmanus, C.; Du, T.; Mulvey, M.R.; Paulick, A.; Anderson, L.; Kuijper, E.J.; Wilcox, M.H. Development and Validation of an Internationally-Standardized, High-Resolution Capillary Gel-Based Electrophoresis PCR-Ribotyping Protocol for *Clostridium difficile*. *PLoS ONE* **2015**, *10*, e0118150. [CrossRef]
55. 8050 Bacterial Bioluminescence. In *Standard Methods for the Examination of Water and Wastewater; Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2017. [CrossRef]
56. ISO 11348-3:2007(en); Water Quality—Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio Fischeri* (Luminescent Bacteria Test)—Part 3: Method Using Freeze-Dried Bacteria. ISO (International Organization for Standardization): Geneva, Switzerland, 2007. Available online: <https://www.iso.org/obp/ui/en/#iso:std:iso:11348-3:ed-2:v1:en> (accessed on 26 January 2026).

57. Strategies and Guidelines for *Clostridioides difficile* Infections. Available online: <https://www.ecdc.europa.eu/en/clostridium-difficile-infections/facts> (accessed on 20 January 2026).
58. Park, G.S.; Lee, S.H.; Park, S.Y.; Yoon, S.J.; Lee, S.M.; Chung, C.S.; Hong, G.-H.; Kim, S.H. Ecotoxicological Evaluation of Sewage Sludge Using Bioluminescent Marine Bacteria and Rotifer. *Ocean Sci. J.* **2005**, *40*, 91–100. [CrossRef]
59. Hernando, M.D.; De Vettori, S.; Martínez Bueno, M.J.; Fernández-Alba, A.R. Toxicity Evaluation with *Vibrio fischeri* Test of Organic Chemicals Used in Aquaculture. *Chemosphere* **2007**, *68*, 724–730. [CrossRef]
60. Baran, A.; Tarnawski, M.; Koniarz, T.; Szara, M. Content of Nutrients, Trace Elements, and Ecotoxicity of Sediment Cores from Rożnów Reservoir (Southern Poland). *Environ. Geochem. Health* **2019**, *41*, 2929–2948. [CrossRef]
61. Hamadieh, Z.; Hamilton, K.A.; Silverman, A.I. Systematic Review of the Relative Concentrations of Noroviruses and Fecal Indicator Bacteria in Wastewater: Considerations for Use in Quantitative Microbial Risk Assessment. *J. Water Health* **2021**, *19*, 918–932. [CrossRef] [PubMed]
62. Worley-Morse, T.; Mann, M.; Khunjar, W.; Olabode, L.; Gonzalez, R. Evaluating the Fate of Bacterial Indicators, Viral Indicators, and Viruses in Water Resource Recovery Facilities. *Water Environ. Res.* **2019**, *91*, 830–842. [CrossRef] [PubMed]
63. Bailey, E.S.; Hopkins, M.; Casanova, L.; Sobsey, M.D. Evaluating Fecal Indicator and Pathogen Relationships in Sewage Impacted Surface Waters to Blend with Reclaimed Water for Potable Reuse in North Carolina. *Pathogens* **2021**, *10*, 1603. [CrossRef] [PubMed]
64. Cuevas-Ferrando, E.; Pérez-Cataluña, A.; Falcó, I.; Randazzo, W.; Sánchez, G. Monitoring Human Viral Pathogens Reveals Potential Hazard for Treated Wastewater Discharge or Reuse. *Front. Microbiol.* **2022**, *13*, 836193. [CrossRef]
65. Veneri, C.; Bonanno Ferraro, G.; Congiu, D.; Franco, A.; Brandtner, D.; Mancini, P.; Iaconelli, M.; Network, T.S.; Lucentini, L.; Suffredini, E.; et al. Wastewater-Based Surveillance of Human Adenoviruses in Italy: Quantification by Digital PCR and Molecular Typing via Nanopore Amplicon Sequencing. *Viruses* **2025**, *17*, 791. [CrossRef]
66. Baghani, A.; Alimohammadi, M.; Aliramezani, A.; Talebi, M.; Mesdaghinia, A.; Douraghi, M. Isolation and Characterization of a Multidrug-Resistant *Clostridioides difficile* Toxinotype V from Municipal Wastewater Treatment Plant. *J. Environ. Health Sci. Eng.* **2020**, *18*, 1281–1288. [CrossRef]
67. Cizek, A.; Masarikova, M.; Mares, J.; Brajerova, M.; Krutova, M. Detection of Plasmid-Mediated Resistance to Metronidazole in *Clostridioides difficile* from River Water. *Microbiol. Spectr.* **2022**, *10*, e0080622. [CrossRef]
68. Rivas, L.; Dupont, P.-Y.; Gilpin, B.J.; Cornelius, A.J. Isolation and Characterization of *Clostridium difficile* from a Small Survey of Wastewater, Food and Animals in New Zealand. *Lett. Appl. Microbiol.* **2020**, *70*, 29–35. [CrossRef]
69. Brouwer, M.S.M.; Roberts, A.P.; Hussain, H.; Williams, R.J.; Allan, E.; Mullany, P. Horizontal Gene Transfer Converts Non-Toxicogenic *Clostridium difficile* Strains into Toxin Producers. *Nat. Commun.* **2013**, *4*, 2601. [CrossRef]
70. Directive-EU-2024/3019-EN-EUR-Lex. Available online: <https://eur-lex.europa.eu/eli/dir/2024/3019/oj/eng> (accessed on 20 January 2026).
71. WHO. *Guidelines for the Safe Use of Wastewater, Excreta and Greywater, Volume 1: Policy and Regulatory Aspects*; World Health Organization: Albany, NY, USA, 2006.

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