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Wastewater Treatment Plants Performance for Reuse: Evaluation of Bacterial and Viral Risks

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Abstract: Reusing reclaimed water is of paramount importance to achieve the 2030 Agenda for Sustainable Development Goals 6 and 13. In Europe, a recent Regulation set minimum requirements for water reuse in agriculture. However, some challenges remain considering microbial risks and their prevention. In this study, two urban wastewater treatment plants (WWTPs) were investigated from the perspective of reuse. A five-year investigation was performed on routine monitoring parameters collected under different weather conditions (wet/dry) and treatments (chlorination/non-chlorination) in inlet and outlet samples. Moreover, a three-month investigation focused on microbial parameters, including indicators, index pathogens (Human Adenovirus—HAdV, *Salmonella* spp.), and other viral pathogens (norovirus, enterovirus, and SARS-CoV-2). The long-term study revealed the compliance of both WWTPs for chemical parameters (organic substances and solids) in more than 90% of samples, whereas for *Escherichia coli*, the compliance ranged from 96.1% with chlorination under dry weather to 16.7% without chlorination in wet days. *E. coli* was positively associated with chemical oxygen demand (COD), which could be a promising and online measurable proxy of *E. coli*. The study on microbial performance demonstrated sound reliability in detecting *E. coli* as a suitable surrogate for *Salmonella* in chlorinated effluents, but neither bacterial nor viral indicators are able to represent HAdV. Although chlorination was able to remove most of the pathogens considered, the compliance with microbial indicators seems insufficient to represent viral water safety.

Keywords: reclaimed water; wastewater reuse; sewage; reclamation facility; Regulation 741/2020; index pathogen; Human Adenovirus; enteric virus; microbial risk; microbial indicators

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1. Introduction

Water resources are under pressure as a result of increasing water demand for the irrigation of crops, various water uses, and industrial processes, as well as climate change, which is responsible for persistent droughts that exacerbate water shortages. Such pressure on water causes a deterioration of the quality and quantity of freshwater resources, as a result of pollution, eutrophication and over-exploitation, or drought. In fact, the United Nations reports that half of the world's population is already experiencing severe water scarcity at least one month a year [1]. In Europe, it was estimated that water stress affected at least 30% of the population on average every year, while up to 70% of the European territory experiences seasonal water stress, mainly during the summer months. In the Mediterranean region and densely populated cities across the EU, water stress is a permanent, year-round issue [2]. Given the global dimension of water issues, they are addressed by the 2030 Agenda, in particular Sustainable Development Goal (SDG) number 6 "*Clean water and sanitation*", which is aimed at ensuring the availability and sustainable management of water and sanitation for all [3]. Specifically, water reuse practices are mentioned in

Target 6.3 [4], because they are sustainable, affordable, and scalable solutions to fight climate change by reducing the pressure on water resources and increasing water security and resilience [5]. Water reuse also produces benefits from economic and social points of view, reducing energy consumption, supplying water with nutrients for agriculture, and increasing employment [6–8]. In recent years, the growing relevance of the integrated use of waste has increased scientific interest worldwide in the assessment of quality and performance of wastewater treatment plants (WWTPs), specifically focused on the potential reuse of treated wastewater, e.g., [9–11].

On the other hand, in Europe, only a fraction (2–3%) of treated wastewater is really reused due to various types of barriers: economic regarding the high cost of treatment, regulatory regarding the different requirements among countries, technical regarding the difficulties in reaching the required depuration efficacy, and social, due to the low acceptability of reused water consumption [12]. The same problems have also been experienced by water-scarce countries across North Africa and the Middle East (e.g., Syrian Arab Republic, Egypt, and Israel), although the percentage of reused sewage effluents is higher (up to 70%) compared to Europe due to tackling extreme drought conditions [13]. Among these barriers, the health risks related to reused water contamination are considered prominent and have been assessed through epidemiological and risk assessment studies indicating microbial risks as the most evident ones, in particular for agricultural uses. In the city of Colorado Springs (CO, USA), urban reuse revealed a slight increase in gastrointestinal symptoms among people frequenting public parks irrigated with reused water compared to the use of a conventional water source [14]. Moreover, risk assessment studies reported an increase in gastrointestinal risk from the consumption of vegetables irrigated with treated sewage attributable to enteric viruses, i.e., human adenoviruses and noroviruses [15–17], and respiratory risk from the inhalation of Legionellae [18]. In fact, untreated sewage can contain human pathogens (viruses, bacteria, protozoa, and helminth eggs) in very high concentrations, i.e., in the order of 10^8–10^{10} per liter [19] and pathogen elimination is highly variable, depending on the treatment scheme and the resistance of the microbes. The WWTPs with conventional processes (e.g., settlement and activated sludge systems) are able to eliminate 20% to 80% of pathogenic enteric viruses [20–23], with even lower reduction efficiency in the case of (oo)cysts of pathogenic protozoa [24].

In recent years, Europe has been tackling reclaimed water reuse in the agricultural irrigation of crops (EU Regulation 2020/741 [25]) in order to overcome past regulatory issues regarding water quality standards that were too restrictive or inconsistent. The new regulation follows a fit-to-purpose approach; thus, the quality of reclaimed waters can be adjusted to the type of crop: (i) food crops consumed raw, (ii) processed food crops (i.e., cooked or industrially processed), and (iii) non-food crops (e.g., pastures and forage, fiber, ornamental, seeds, and energy). Specifically, reclaimed waters are divided into four water quality classes (A, B, C, and D) on the basis of *Escherichia coli*, biochemical oxygen demand (BOD_5), total suspended solids (TSSs), and turbidity (only for class A); then, other health-relevant monitoring parameters can be added after a risk assessment [26,27]. Although this approach represents an improvement compared to the previous European regulation, some concerns still remain regarding the choice of routine monitoring parameters for the verification (and surveillance) of reclaimed water compliance for reuse applications. Firstly, the removal efficiency of the parameters is considered only for validation monitoring (and only for class A uses), namely, when a system is initially constructed or rehabilitated; however, it can also provide useful insights into the causes of thresholds exceeding in the effluents, such as plant failures (e.g., low dosage of disinfectant) or fluctuation in quality and quantity of the entering sewages, attributable to precipitation (especially in WWTPs served by combined sewer networks) or variations in served populations (e.g., tourism) [28]. Secondly, the microbial requirements are based on *E. coli*, of which the determination relies on growth-based enumeration methods and does not provide a timely indication of performance; thus, a readily measured chemical proxy of the microbial levels could be useful for the monitoring of treatment efficacy and operational adjustments. Moreover,

E. coli is traditionally used as an indicator of bacterial pathogens (e.g., *Salmonella* spp.), but its reliability regarding the destruction of human viral pathogens is limited because viruses can be more resistant to treatment processes compared to indicators [24]. From a health perspective, the viral contamination of reclaimed waters is relevant since viruses exhibit low infectious doses and can persist in the external environment longer than other pathogens [29,30]. The great variety of pathogens present in wastewater does not allow us to consider all of them in a microbial risk assessment, and thus international guidelines recommend selecting appropriate reference pathogens (also frequently referred to as index pathogens), on the basis of their relevance to the exposure pathway, the representativeness of the likely pathogens from each microbial group (bacteria, protozoa, and viruses), and the availability of the dose–response relationship [31].

In this study, routine monitoring parameters and microbiological parameters including viral and bacterial pathogens were collected from two WWTPs with the aim of (i) understanding the role of external factors (weather conditions) and treatment (chlorination) on WWTP performance; (ii) evaluating the compliance of the current treatments with EU regulations for reuse, specifically looking for a readily measurable proxy to optimize the control of the treatment process; and (iii) investigating the suitability of traditional indicators of index pathogens and pathogenic viruses.

2. Materials and Methods

2.1. Selection of the WWTPs

Two medium-size urban WWTPs located in the Tuscany region coastal area (Italy) were selected for reuse purposes, given the availability of developing an adequate supportive infrastructural framework for water reuse (e.g., accumulation basins to store reclaimed water during periods in which it is not needed and distribution networks to transport them from the WWTP to the point of use). Both WWTPs receive household sewage through separate sewer network structures that are occasionally interested by the intrusion of parasitic rainwaters. Specifically, WWTP1 (approximately 50.000 population equivalent, P.E.) treats wastewater generated by the urban area of the city with a small portion originating from a hospital, whereas WWTP2 (approximately 35.000 P.E.) serves the coastal area of the same Municipality.

The treatment scheme in both WWTPs is based on activated sludge processes for carbon and nitrogen removal and the last stage of chlorine disinfection with sodium hypochlorite (NaClO) at 15% solution strength but with some differences in the concentration and dosage conditions. In WWTP1, the NaClO concentration was set at approximately 15–20 mL/m^3, considering a fixed flowrate, whereas in WWTP2, the NaClO concentration was set at 17 mL/m^3 and the dosage was adjusted based on the flow of wastewater to the plant. During the study period, chlorine disinfection was periodically applied as illustrated in Figures S1 and S2, separately for each WWTP.

2.2. Monitoring Scheme and Parameters

The WWTPs were studied using two levels of investigation (Figure 1):

(i) Long-term investigation on routinely collected parameters: routine parameters monitored by the sewage company for compliance with Italian Law on WWTP discharges [32] were gathered from a private sewage company database from 2018 to 2023 on a monthly basis. In particular, biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), and total suspended solids (TSSs) were determined at the entrance and exit of the WWTPs, while *E. coli* was determined only in the exit samples.

(ii) Specific short-term investigation on microbial parameters: parameters mentioned in the regulation for water reuse [25] were determined during a dedicated monitoring campaign, with weekly samples collected from July to September 2023 (hereafter fieldwork sampling). In particular, grab samples were collected at different stages of the sewage treatment process from each WWTP: at the entrance—untreated samples (16 samples, 2.5 L each); at the inlet of the chlorination units, after biological treatment—

secondary effluents (16 samples, 2.5 L each); and after chlorination—tertiary samples (16 samples, 7.5 L each). They included *E. coli* and intestinal enterococci as indicators for bacteria, somatic coliphages for viruses, and spores of sulfite-reducing clostridia for protozoa (Annex 1, Section 2, Reg. 2020/741). Moreover, *Salmonella* and Human Adenovirus (HAdV) were selected as index pathogens [33–35], and enterovirus, norovirus, and SARS-CoV-2 were also monitored.

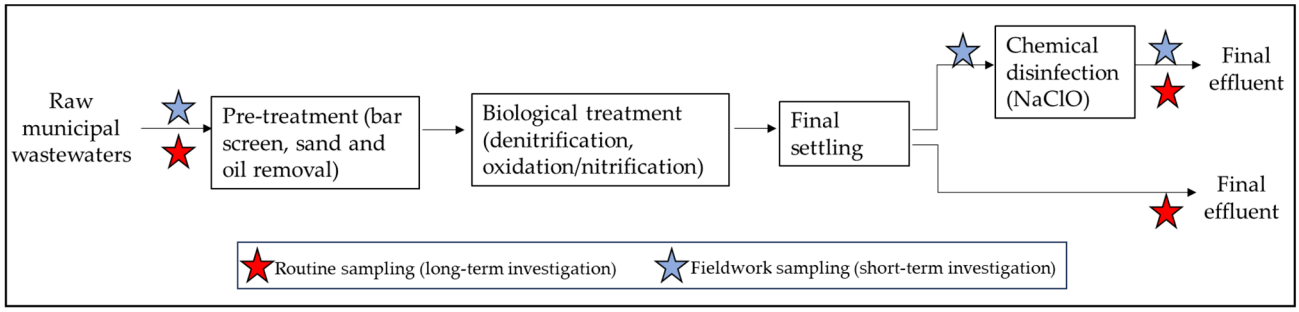


Figure 1. Scheme of the sampling strategy along the wastewater treatment stages.

2.3. Routinely Collected Parameters

BOD_5 (mg/L) and TSS (mg/L) were determined according to the accredited internal methods of the Acque SpA laboratory: BOD_5 was measured through chemiluminescence (MI-001 Rev2 2022) and TSS was determined using a gravimetric method with sediment collection via filtration on a 0.45 µm membrane filter (MI-002 Rev. 3 2021). COD (mg/L) was determined by photometric detection at 600 nm following the ISO 15705:2002 [36] protocol. *E. coli* (MPN/100 mL) were enumerated by UNI EN ISO 9308-2:2014 [37] as detailed in Section 2.4.2. A total of 579 and 297 pairs of entrance–exit samples were collected for WWTP1 and WWTP2, respectively, while *E. coli* concentrations were determined on a limited number of samples at the exit of the WWTPs (77 for WWTP1 and 123 for WWTP2), considering either chlorinated or non-chlorinated effluents (Figure 1).

Removal efficiencies were calculated for organic compounds and solids using Equation (1) [38].

$$Removal\ efficiency\ (\%) = \frac{(Ci - Ce) \times 100}{Ci} \quad (1)$$

where *Ci* is the concentration at the inlet of the WWTP (sewage entrance) and *Ce* is the concentration at the exit of the WWTP (final effluent).

2.4. Microbiological Parameters during Fieldwork

After collection, samples were transferred to the laboratory and kept refrigerated at 4 °C until the analysis within 24 h for bacteria and spores and 48 h for viruses [39].

2.4.1. Somatic Coliphages

Somatic coliphages were enumerated using Bluephage® Enumeration of Somatic Coliphages Easy Kit for 1–10 mL. Briefly, the sample was diluted to 1:1000 (untreated sewage) in sterile deionized water or undiluted (secondary and tertiary effluents), and then 1 mL was analyzed according to the UNI EN ISO 10705-2:2001 [40] procedure (double agar-layer method) on *E. coli* strain WG5, and the results were expressed as Plaque-Forming Units per liter (PFU/L).

2.4.2. Bacterial Indicators

Bacterial indicators were detected using IDEXX Defined Substrate Technology® for total coliform/*E. coli* (Colilert-18, UNI EN ISO 9308-2:2014 [37]) and for intestinal enterococci (Enterolert-E, AFNOR IDX 33/04-02/15 [41]), using the Quanti-Tray enumeration procedure. Briefly, the sample was diluted to 1:100,000 (untreated sewages) in sterile deionized

water or undiluted (secondary and tertiary effluents), and then 1 mL was analyzed according to the manufacturer's instructions, and the results were expressed as Most Probable Number per 100 mL (MPN/100 mL).

2.4.3. Spores of Sulfite-Reducing Clostridia

Spores of sulfite-reducing clostridia were determined using the standardized culture-based method using the membrane filtration approach, following the procedure described in the Italian Standard APAT-IRSA/CNR 7060:2003 [42]. Briefly, the sample was heat-treated in a water bath (75 °C for 15 min), diluted to 1:100 in sterile deionized water, and 100 mL was filtered through a 0.45 µm membrane filter (Frisenette Aps, Energivej, Denmark) and the filter was placed onto a Sulphite Polymyxin Sulphadiazine (SPS) solid agar medium, which had been covered with molten SPS to create anaerobic conditions. After incubation in an anaerobic jar at 36 °C for 48 h, black colonies were visually counted, and the results were expressed as colony-forming units per liter (CFU/L).

2.4.4. *Salmonella* spp.

Salmonella spp. was determined using a qualitative, culture-based assay following the procedure described in the Italian Standard APAT-IRSA/CNR 7080:2003 [43]. Briefly, a 1 L sample was concentrated via membrane filtration on a 0.45 µm filter, which was then placed in Buffered Peptone Water for pre-enrichment (36 °C for 24 h). Then, further enrichment was performed in Rappaport Vassiliadis broth (42 °C for 24 + 24 h). Finally, a loopful of the 24 h (and 48 h) broth culture was subcultured onto xylose lysine desoxycholate agar. After incubation at 36 °C for 24 h, black colonies were confirmed for *Salmonella* using a carbohydrate fermentation assay onto Kliger Iron Agar (and Lysine Iron Agar) and serologically tested using latex agglutination. The results were expressed as presence/absence in a liter.

2.4.5. Human Viruses

Sample preparation and concentration. Wastewater samples (1 L for untreated samples and secondary effluents, 5 L for tertiary effluents) were passed through 142 mm diameter cellulose nitrate membrane filters with an 8 µm pore size (Sartorius, Goettingen, Germany) with a peristaltic pump to remove solid debris. The membrane was thereafter eluted utilizing 30 mL of 3% beef extract at pH 9, and the supernatant was added to the prefiltered sample. Then, the sample was concentrated via ultrafiltration with polyethersulfone (PES) membranes with a 10 kDa molecular cutoff using two Vivaflow200® cassettes (Sartorius, Stenehouse, UK) in parallel to obtain an active filtration membrane area of 400 cm^2. At the end of the process, the final volume of the sample was 50 mL [44].

Biomolecular analysis. The nucleic acids were extracted from 200 µL (for DNA) and 140 µL (for RNA) of the eluant fluids using QIAmp Viral DNA and RNA kits (Qiagen, Hilden, Germany), respectively, according to the manufacturer's protocols. Then, the extracted genome was purified using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA). The samples were tested for HAdV, norovirus ggII (NoV ggII), enterovirus, and SARS-CoV-2, using primers, probes, and the thermal protocol listed in Table S1. Reactions were performed in duplicate using the Taq Man Universal Master Mix (Applied Biosystems—Thermo Fisher Scientific, Warrington, UK) and AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems—Thermo Fisher Scientific, Austin, TX, US) for HAdV and RNA viruses, respectively, and the CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories, Inc., Singapore). For viral quantification, standard curves were developed for each viral target via serial dilution of a synthetic dsDNA (from 10^1 GC/µL to 10^5 GC/µL) and they were considered acceptable when they had a slope between −3.1 and −3.6 and an R^2 equal to or greater than 0.98 [45,46]. The (RT)-qPCR data were adjusted according to the volume used in each step of the process (i.e., concentration, extraction), and the results were expressed as GC/L.

2.5. Meteorological Data

Meteorological data were extracted in the same study period from the public database of the Regional Hydrological Service (SIR; https://www.sir.toscana.it/, accessed on 12 January 2024), using site-specific monitoring stations for each WWTP (TOS01000544 for WWTP1 and TOS01005251 for WWTP2). For this study, wet weather is defined as total precipitation greater than 1 mm in the previous 24 h and dry weather is defined as a non-wet day (Copernicus Climate Change Service; https://climate.copernicus.eu/ESOTC, accessed on 12 January 2024). Based on these criteria, wet weather represented 22.5% (130/579) and 18.2% (54/297) of the study period for WWTP1 and WWTP2, respectively. During fieldwork, only one sampling date was performed during wet weather (29 August 2023).

2.6. Statistical Analysis

The water quality of the final effluent was categorized according to the European regulation, which classifies reclaimed water into 4 classes from A to D in descending order of water quality [25]. In the present paper, we considered BOD_5, TSS, and *E. coli* with the threshold values (named minimum requirements [25]) reported in Table 1.

Table 1. Threshold values used in this paper for minimum requirements for water quality, according to EU regulation [25].

Parameters	Water Quality Classes			
	A	**B**	**C**	**D**
BOD_5 (mg/L)	10	25		
TSS (mg/L)	10	35		
E. coli (MPN/100 mL)	10	100	1000	10,000

E. coli data have been dichotomized according to the threshold value for the class D minimum requirement, and the chi-square statistic was used to understand if the frequency of compliant samples was influenced by chlorination.

For the purpose of further statistical analysis, the data distributions of *E. coli*, COD, BOD_5, and TSS in the outlet samples, as well as the removal efficiency of the above-mentioned physico-chemical parameters, were checked for normality using the Shapiro–Wilk normality test and the Skewness statistic, separately for each WWTP. Such a normality test revealed departures from normality (Section 3.1) for all the tested data distributions. Thus, the effect of Log_{10}-transformation of *E. coli* data was also examined and was able to achieve similarity to a normal distribution, thus supporting the use of the parametric analytical methods [47] described below.

A Two-way Analysis of Variance (ANOVA) was performed to understand the influence of weather conditions (dry/wet) and chlorination (yes/no) on Log_{10}*E. coli* in the exit samples, separately for each WWTP (the usage of a parametric test was consistent with the normal distribution of this variable, Section 3.1). The results are graphically illustrated using interaction plots (R software, package *ggpubr*).

For chemical parameters, removal efficiencies were calculated as reported in Equation (1) (Section 2.3) and were expressed as average values, and the variability of the data was reported as the 10th and 90th percentiles of the distribution of the data [38]. The Mann–Whitney U test was performed to understand the differences in removal efficiency of physico-chemical parameters (COD, BOD_5, and TSS) according to weather conditions, i.e., dry or wet days.

The Spearman correlation coefficient (ρ) was used to investigate the association between *E. coli* at the exit of each WWTP and the values of the chemical parameters (COD, BOD_5, and TSS) in the same samples. The values of the Spearman coefficient were represented by a correlation matrix (R software, package *corrplot*).

In the fieldwork, the removal of each microbial parameter was expressed as a logarithmic reduction, considering the microbial concentrations of water entering the WWTP (untreated sewage) and those at discharge (secondary or tertiary effluents), following a well-established approach for evaluating waterborne microorganism reductions using common water treatment technologies [48]. Such an approach is also adopted by the Regulation on reuse for assessing performance targets for the entire treatment chain during the microbial validation monitoring of the WWTP producing class A waters (Annex I, Section 2 of the EU Regulation [25]). The log-reduction data were examined using general descriptive statistics, namely the median and interquartile range (IQR), considering the first and third quartiles. The Fisher exact statistic was used to investigate the association between index pathogens and indicators in the exit samples: for bacteria, *Salmonella* presence/absence was tested with *E. coli* dichotomized according to the threshold value for class D, while for viruses, HAdV presence/absence was tested with somatic coliphages data, which were dichotomized according to their median value (owing to the lack of a regulatory threshold).

All statistical analyses and graphical representations were performed using R v. 4.3.2.

3. Results

3.1. Long-Term WWTP Performance According to the EU Regulation 2020/741

Normality tests revealed that the data distribution of *E. coli*, COD, BOD_5, and TSS in the final effluents and the removal efficiency of the physico-chemical parameters were not normally distributed, with $p < 0.05$ for Shapiro–Wilk tests and skewness statistic less than −1 (ranging from −11.50 to −2.41) or greater than +1 (ranging from +2.18 to +7.58). On the other hand, Log_{10}*E. coli* concentrations in the final effluents showed similarity to a normal distribution, with skewness statistics of −1.05 and −0.45 for WWTP1 and WWTP2, respectively.

The frequency of exit samples compliant with requirements for all water quality classes was high for physico-chemical parameters, e.g., more than 97% of samples were compliant for BOD_5 and TSS classes B, C, and D requirements, regardless of the chlorination treatment (Tables 2 and 3 for WWTP1 and WWTP2, respectively). On the other hand, as expected, compliance with *E. coli* requirements differed markedly between chlorinated and non-chlorinated effluents: WWTP1 met the threshold value for class D in 36.4% of disinfected effluents and 18.2% of non-disinfected ones, and this difference was greater and statistically significant in WWTP2, with 94.6% (53/56) compliance in chlorinated versus 20.9% (14/67) in not chlorinated (chi-square, $p < 0.0001$). Similar results were also obtained for class C (85.7%, 48/56 of compliance in chlorinated samples), class B (71.4%, 40/56), and class A (46.4%, 26/56), while non-chlorinated effluents exhibited compliance of less than 5% (3/67). Regarding the role of weather on compliance, in both WWTPs, exceedances of *E. coli* thresholds for reuse occurred mainly in wet conditions, either in chlorinated or non-chlorinated effluents. As an example, the class D requirement was met in 42.3% of chlorinated samples and 22.2% of non-chlorinated samples in dry weather conditions in WWTP1, but these percentages were reduced to 21.4% and 0% in wet weather. Similarly, in WWTP2, chlorinated and non-chlorinated effluents were compliant in 96.1% and 21.9% of samples, respectively, in dry conditions, which decreased to 80.0% and 16.7% during wet weather (Tables 2 and 3).

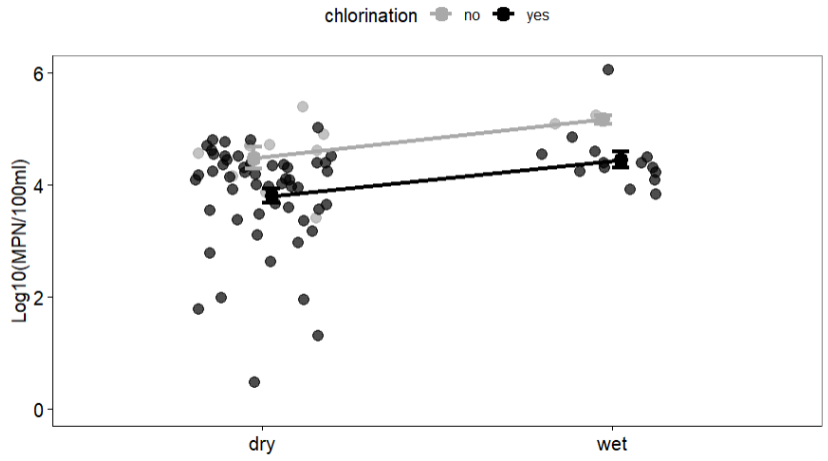
Figure 2 shows the effects of chlorination and meteorological variables on *E. coli* levels in the outlet samples: the concentrations of *E. coli* in chlorinated effluents were significantly lower than in non-chlorinated ones, especially in dry conditions, in both WWTPs (see Table S2 for the results of the two-way ANOVA).

Table 2. Compliance with minimum requirements for WWTP1. The results are expressed as percentages and refer to the values in the exit samples.

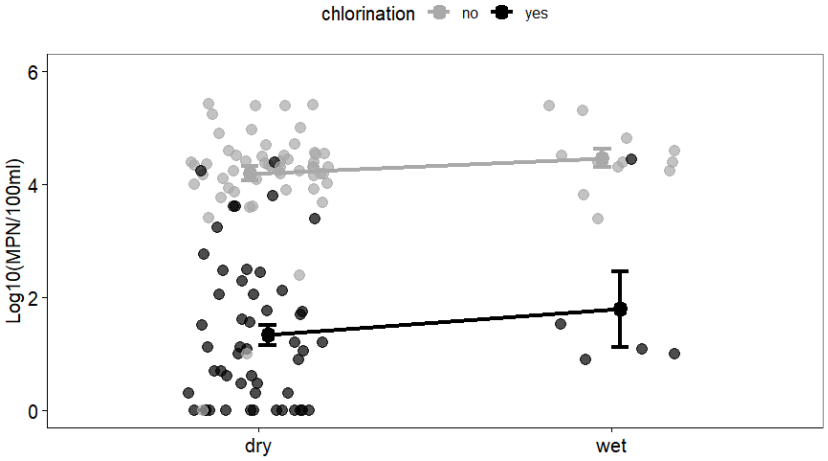
Parameter	Effluent Treatment	Weather Condition	n. obs (n)	Compliance with Minimum Requirements (%)			
				Class A	Class B	Class C	Class D
BOD_5	Chlorinated	Dry	104	92.3		100	
		Wet	34	88.2		97.1	
		Total	**138**	**91.3**		**99.3**	
	Not chlorinated	Dry	345	92.5		99.7	
		Wet	96	92.7		100	
		Total	**441**	**92.5**		**99.8**	
TSS	Chlorinated	Dry	104	89.4		97.1	
		Wet	34	82.4		97.1	
		Total	**138**	**87.7**		**97.1**	
	Not chlorinated	Dry	345	92.2		99.4	
		Wet	96	91.7		100	
		Total	**441**	**92.1**		**99.5**	
E. coli	Chlorinated	Dry	52	1.9	9.6	15.4	42.3
		Wet	14	0.0	0.0	0.0	21.4
		Total	**66**	**1.5**	**7.6**	**12.1**	**36.4**
	Not chlorinated	Dry	9	0.0	0.0	0.0	22.2
		Wet	2	0.0	0.0	0.0	0.0
		Total	**11**	**0.0**	**0.0**	**0.0**	**18.2**

Table 3. Compliance with minimum requirements for WWTP2. The results are expressed as percentages and refer to the values in the exit samples.

Parameter	Effluent Treatment	Weather Condition	n. obs (n)	Compliance with Minimum Requirements (%)			
				Class A	Class B	Class C	Class D
BOD_5	Chlorinated	Dry	64	98.4		100	
		Wet	9	100		100	
		Total	**73**	**98.6**		**100**	
	Not chlorinated	Dry	179	95.0		99.4	
		Wet	45	97.8		100	
		Total	**224**	**95.5**		**99.6**	
TSS	Chlorinated	Dry	64	87.5		100	
		Wet	9	77.8		100	
		Total	**73**	**86.3**		**100**	
	Not chlorinated	Dry	179	88.8		99.4	
		Wet	45	97.8		100	
		Total	**224**	**90.6**		**99.5**	
E. coli	Chlorinated	Dry	51	47.1	70.6	86.3	96.1
		Wet	5	40.0	80.0	80.0	80.0
		Total	**56**	**46.4**	**71.4**	**85.7**	**94.6**
	Not chlorinated	Dry	55	3.6	3.6	5.5	21.9
		Wet	12	0.0	0.0	0.0	16.7
		Total	**67**	**3.0**	**3.0**	**4.5**	**20.9**



(**a**)

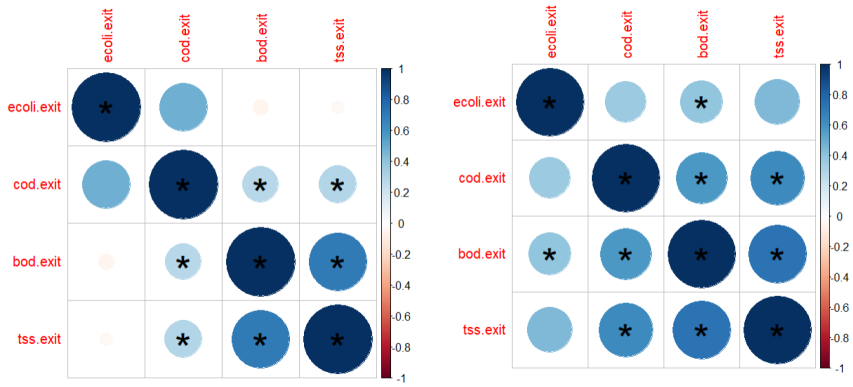


(**b**)

Figure 2. Interaction plot showing Log_{10} *E. coli* data in the exit effluents under different weather conditions (dry, wet) and chlorination treatment (yes, no), separately for each WWTP: (**a**) WWTP1, (**b**) WWTP2. Error bars represent mean ± standard deviation of data distribution for each group.

The long-term analysis of the removal efficiency of organic substances and solids parameters showed high performances for both WWTPs, with average values greater than 90%, and the influence of weather conditions. In fact, removal efficiency values were lower during wet weather than in dry conditions, and this was statistically significant for WWTP1 (Table 4).

The correlation matrix for *E. coli* and physico-chemical parameters (COD, BOD_5, and TSS) in the chlorinated effluents is reported separately for each WWTP in Figure 3. In WWTP1, the *E. coli* concentration was positively correlated with the COD level ($\rho = 0.48$, $p = 0.18$), while the associations with the other parameters were negligible. In WWTPs, positive associations were observed between *E. coli* and all the other parameters, namely COD ($\rho = 0.37$, $p = 0.13$), BOD_5 ($\rho = 0.40$, $p < 0.05$), and TSS ($\rho = 0.45$, $p = 0.12$). Interestingly, the association between *E. coli* and COD was maintained in both WWTPs.



(**a**) (**b**)

Figure 3. Spearman correlation matrix for *E. coli*, COD, BOD_5, and TSS in the chlorinated effluents, separately for each WWTP: (**a**) WWTP1 (66 data for each variable) and (**b**) WWTP2 (56 data for each variable). Asterisk represents the statistical significance (p) at 0.05 level.

Table 4. Removal efficiency for physico-chemical parameters, according to WWTP and weather conditions. p-values refers to Mann–Whitney U test between dry and wet conditions (significant p-values are in bold).

Parameter	WWTP Type	Weather Condition	n. obs	Removal Efficiency % Average (10th–90th)	Mann-Whitney U (p-Value)
COD	WWTP1	Dry	449	93.1 (88.2–98.6)	**<0.0001**
		Wet	130	87.8 (77.7–100)	
		Total	579	91.9 (85.5–98.7)	-
	WWTP2	Dry	243	93.3 (86.0–100)	0.9185
		Wet	53	92.6 (84.7–100)	
		Total	296	93.2 (85.6–100)	-
BOD_5	WWTP1	Dry	449	97.7 (93.5–100)	**<0.01**
		Wet	130	94.6 (85.2–100)	
		Total	579	97.0 (92.3–100)	-
	WWTP2	Dry	243	98.3 (94.4–100)	0.9451
		Wet	53	96.2 (92.6–100)	
		Total	296	97.9 (94.2–100)	-
TSS	WWTP1	Dry	448	96.6 (90.2–100)	**<0.01**
		Wet	130	92.6 (79.9–100)	
		Total	578	95.7 (88.6–100)	-
	WWTP2	Dry	242	96.3 (88.7–100)	0.9596
		Wet	53	96.8 (91.2–100)	
		Total	295	96.4 (89.2–100)	-

3.2. Specific Short-Term Investigation on Microbiological Performance

3.2.1. Microbial Indicators

Microbial removal at different stages of the treatment process is reported separately for each WWTP in Table 4 (details on descriptive statistics of microbial concentrations at the entrance and secondary and tertiary effluents are provided in Table S3, separately for each microbial parameter and WWTP).

Bacterial indicator levels at the entrance were slightly higher in WWTP2 compared to WWTP1. In fact, median *E. coli* concentrations were 1.43×10^8 MPN/L (IQR = 1.12×10^8 MPN/L − 1.73×10^8 MPN/L) in WWTP2 and 6.68×10^7 MPN/L (IQR = 5.47×10^7 MPN/L − 6.87×10^7 MPN/L), and the values observed for enterococci were 1.53×10^7 MPN/L (IQR = 1.38×10^7 MPN/L − 1.77×10^7 MPN/L) and 1.16×10^7 MPN/L (IQR = 8.17×10^6 MPN/L − 1.31×10^7 MPN/L), respectively. Overall, the median log-removal of bacterial indicators attributable to the entire treatment process ranged from 3.42 to 4.13 (Table 5).

Table 5. Bacterial and viral indicators removal at different stages of the treatment process. Results are expressed as median and interquartile range of the logarithmic removal efficiency (log-removal) of eight sampling dates.

Microbial Parameter	WWTP Type	Log-Removal between Entrance and Secondary Treatment	Log-Removal between Secondary and Tertiary Treatment	Log-Removal of the Entire Treatment Process (Entrance Sewage–Tertiary Effluent)
E. coli	WWTP1	2.49 (2.33–2.89)	0.30 (0.05–1.45)	3.46 (2.54–4.86)
	WWTP2	2.84 (2.50–3.08)	0.97 (0.20–2.34)	4.13 (3.30–5.31)
Intestinal enterococci	WWTP1	2.63 (2.32–2.82)	0.70 (0.02–0.99)	3.54 (3.06–3.82)
	WWTP2	2.58 (2.32–2.95)	0.56 (0.1–1.56)	3.42 (2.93–4.40)
Somatic coliphages	WWTP1	2.43 (1.86–3.09)	0.84 (0.36–0.99)	3.50 (2.64–4.01)
	WWTP2	2.51 (1.88–2.90)	0.99 (0.36–1.47)	3.24 (2.30–4.19)
Clostridia spores	WWTP1	2.49 (2.04–2.76)	0.51 (0.23–0.97)	3.09 (2.71–3.58)
	WWTP2	0.96 (0.79–1.16)	0.27 (0.12–0.59)	1.24 (1.03–1.46)

Somatic coliphages at the entrance of WWTP2 (1.05×10^7 PFU/L, IQR = 7.00×10^6 PFU/L − 1.35×10^7 PFU/L) were similar to those observed for WWTP1 (9.00×10^6 PFU/L, IQR = 5.73×10^6 PFU/L − 1.60×10^7 PFU/L), and the removal attributable to the entire treatment process was around 3.5 log in both WWTPs (Table 5).

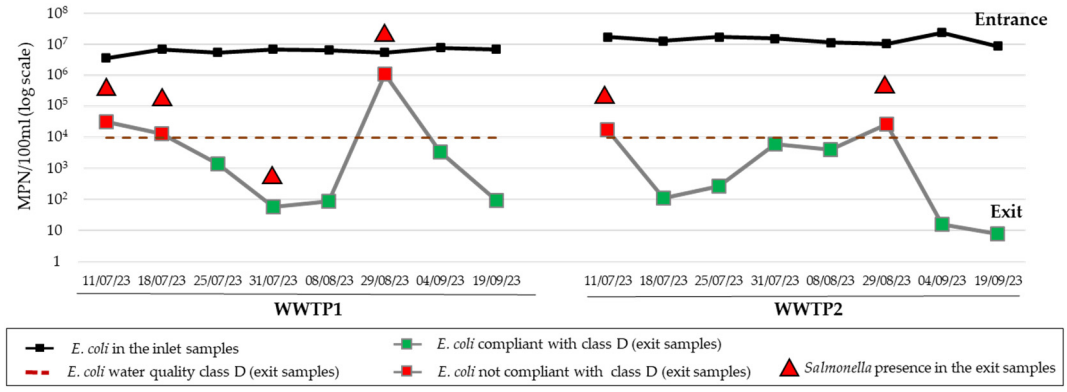
Both WWTPs also showed similar entrance concentrations for spores of sulfite-reducing clostridia: 9.25×10^4 CFU/L (IQR = 4.55×10^4 CFU/L − 1.24×10^5 CFU/L) for WWTP2 and 1.10×10^5 CFU/L (IQR = 7.00×10^4 CFU/L − 1.80×10^5 CFU/L) for WWTP1; nevertheless, the median removal was markedly different between WWTPs, and WWTP1 was more efficient (Table 5).

Considering microbial reduction, log-removal was mostly attributable to the secondary treatment for all the analyzed microbial parameters, whereas disinfection was able to reduce the microbial concentrations by no more than 1 log (Table 5).

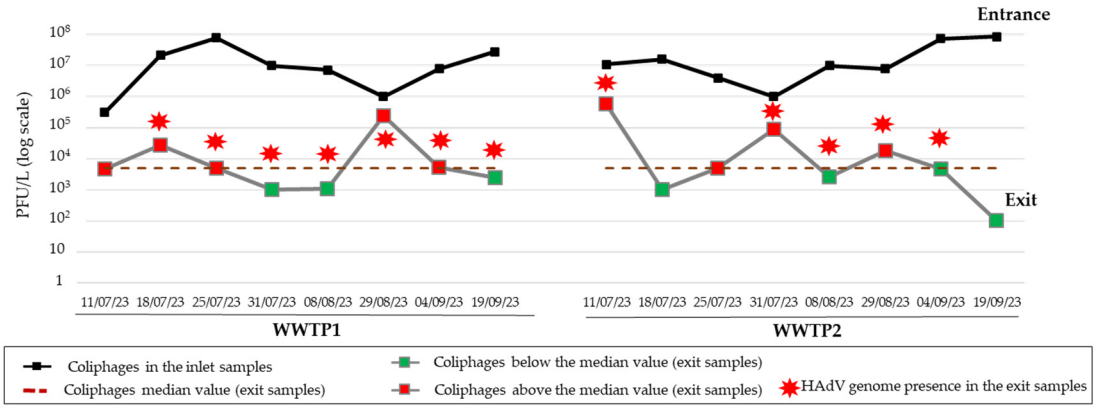
Interestingly, in both WWTPs, lower removal values were observed on the only sampling date with wet weather (29 August 2023); as an example, WWTP1 showed 0.69, 0.91, and 0.62 log-removal for *E. coli*, intestinal enterococci, and somatic coliphages, respectively.

3.2.2. Relationship between Index Pathogens and Microbial Indicators

The relationship between index pathogens and their microbial indicators is depicted in Figure 4, separately for *Salmonella* and *E. coli* (Figure 4a) and HAdV and somatic coliphages (Figure 4b). Regarding bacteria, *E. coli* values at the exit were dichotomized according to water quality for class D reuse (1.0×10^4 MPN/100mL) and they were related to *Salmonella* presence in the same samples, showing a significant association between *Salmonella* absence at the exit and *E. coli* compliance for class D (Fisher exact test, $p < 0.01$). Regarding viruses, somatic coliphages were dichotomized according to the median values of the distribution data at the exit of the WWTPs (4.9×10^3 PFU/L), given the lack of threshold requirements for viral indicators. In this case, the association between HAdV absence and coliphages compliance was not significant (Fisher exact test, $p = 0.4$).



(**a**)



(**b**)

Figure 4. Relationship between index pathogens and microbial indicators in the exit samples: (**a**) *Salmonella* and *E. coli*, (**b**) Human Adenovirus (HAdV) and somatic coliphages.

3.2.3. Pathogens

The HAdV genome was present in 100% of the entrance samples and at the exit in 100% of samples for WWTP1 and in 62.5% of samples for WWTP2. In fact, HAdV removal was higher in WWTP2 with a median log-reduction of 2.3 (IQR = 1.72–3.6), compared to WWTP1 (1.3, IQR = 1.02–1.5). In contrast to what was observed for microbial indicators, most of the HAdV abatement was attributable to the disinfection process: 1.5 (IQR = 0.8–2.7) in WWTP2 and 1.0 (IQR = 0.7–1.4) in WWTP1. Considering WWTPs data as a whole, the other pathogens were present at the entrance with different frequencies: 75.0% (12/16) for *Salmonella*, 93.8% (15/16) for enterovirus and NoV ggII, and 68.8% (11/16) for SARS-CoV-2. Their frequencies after the secondary treatment were 62.5% (10/16) for *Salmonella*, 19% (3/16) for enterovirus and NoV ggII, and 0% for SARS-CoV-2. After chlorination, only *Salmonella* (38%, 6/16) and NoV ggII (6%, 1/16) were present, especially during the wet weather event.

4. Discussion

Using recycled wastewater for urban and irrigational purposes represents a valuable strategy to tackle the quality and quantity deterioration of fresh and groundwater resources, as specifically committed by the 2030 Agenda for Sustainable Development in SDG 6. Water reuse is a long-established practice in states suffering from water scarcity: experiences of potable or irrigational reuse date back to 1968 in Namibia (Windhoeck) and the 1950s in Israel, respectively [49,50]. However, in Europe, changes in climatic conditions such as increases in seasonal rainfall or severe drought events are also contributing significantly to the strain on the availability of surface and groundwater bodies [26]. For this reason,

Europe recently applied, in June 2023, a water reuse Regulation that set uniform minimum water quality requirements for the safe reuse of treated urban sewages in agricultural irrigation; this will also possibly be extended to other purposes, including industrial, recreational, and environmental applications, during the next evaluation scheduled in June 2028 [25]. However, reuse practices can pose a threat to human health if the effectiveness of the treatment process is not adequately controlled. In particular, microbial pathogens in reclaimed water could be responsible for water-borne disease outbreaks (e.g., gastroenteritis) and other acute effects; nevertheless, microbial parameters are not suitable for operational monitoring because their determination is time-consuming, thus hampering the application of corrective actions in a timely manner [51].

In our long-term investigation, we observed that WWTPs were efficient at removing chemical targets; in fact, considering pooled data, the threshold values of BOD_5 and TSS for class A were met in 90% or more of the samples, as requested by the EU Regulation for reuse [25]. However, when data were considered separately according to weather conditions, the compliance under wet conditions with the chemical requirements was reduced as a consequence of a general worsening of the treatment process. Similarly, the WWTPs experienced occasional failure in *E. coli* compliance especially during rainfall events. For such microbial parameters, the number of wet days was limited (<20 observations for each WWTP), but the same trend has been observed in both WWTPs, thus suggesting the reliability of the results. In fact, this phenomenon may be attributable to parasitic rainwater in sewer network structures, which could be responsible for an increase in the levels of microbial contamination in the entering waters as well as an increase in the flowrate, which can reduce the retention time and, consequently, the effectiveness of the treatments [52–54]. Moreover, *E. coli* levels in the outlet samples were also influenced by chlorine disinfection, which was more efficient in WWTP2 compared to WWTP1. This aspect can be justified by different dosage conditions: in WWTP2, the dosage was adjusted by flowrate, making the disinfection process more efficient and prone to fluctuating influent wastewater volume in a certain period of time, as in the case of precipitation.

Since such variation in water quality can happen rapidly, an easy-to-measure proxy for the occurrence of microbial parameters would be useful to control the treatment process, such as the chemical disinfectant dosage. As an example, Bonetta et al. (2022) [11] reported that various bacterial indicators, including *E. coli*, were positively associated with BOD_5, COD, and TSS considering sewage at various stages of the treatment process. Similarly, Foschi et al. (2021) [55] found positive associations between *E. coli* and COD, turbidity, and TSS at the inlet of the disinfection unit, and Rocher et al. (2021) [56] found that *E. coli* and intestinal enterococci after disinfection correlated with the initial bacterial concentration but also with TSS and COD in the disinfected effluents. Our results partially confirm these findings; in fact, we found that *E. coli* in the chlorinated samples was positively correlated with COD in both WWTPs under investigation, and the association was also significant regarding TSS and BOD_5 in the outlet samples for WWTP2. These results indicate that *E. coli* levels correlate with the degradation of water quality parameters in terms of both organic substances (COD and BOD_5) and suspended solids (TSS); however, only COD was associated with *E. coli* in both WWTPs, thus suggesting such a chemical parameter as a promising proxy, given the short analysis time. This aspect deserves further investigation and a dedicated site-specific data collection campaign to increase the sample size in order to develop a predictive model to estimate *E. coli* concentrations using chemical proxies.

The short-term investigation revealed the validity of *E. coli* as a monitoring parameter for bacterial pathogens in the outlet (and chlorinated) samples. In particular, we identified a significant association between the presence of *Salmonella* and the exceeding of the *E. coli* threshold for class D water quality, thus confirming a robust relationship between the loss of bacterial pathogens and *E. coli* removal and inactivation during treatment. Nevertheless, such an association was not revealed when HAdV was considered as an index pathogen, not even considering somatic coliphages as viral indicators of HAdV; thus, the compliance with microbial indicators is not enough to represent the viral safety of reclaimed waters [57].

However, chlorination allowed the removal of most of the enteric pathogens (*Salmonella*, norovirus, and enterovirus), reduced HAdV from 1.0 to 1.5 log, and produced log-removal values for microbial indicators in line with those reported in the international literature, namely 2.0–6.0 for *E. coli*, 1.0–2.0 for *C. perfringes*, and 0.0–2.5 for bacteriophages [58]. Nevertheless, as in the long-term study, a rainfall event was also responsible during this investigation for the loss of control of both WWTPs, resulting in the exceeding of *E. coli* values for the class D threshold and the detection of *Salmonella*, NoV, and HAdV in the outlet samples. However, the results of the microbiological investigation, especially those regarding the viral risk, need to be confirmed by increasing the monitoring frequency so as to cover different environmental and meteorological conditions, as the small sample size of this fieldwork represents a limitation of the short-term investigation.

5. Conclusions

In this study, we performed a two-level investigation on urban WWTPs from the perspective of reusing their effluents in order to consider both routine monitoring parameters and microbial parameters, including indicators and pathogens. In fact, a complete characterization of the performance of WWTPs is fundamental for potential reuse practices, especially to identify the hazards and situations that could be responsible for the loss of control of the water reuse system, such as rainfall events. A multi-barrier approach that includes chlorine-based disinfection was needed to obtain at least water quality class D according to EU regulations for *E. coli* and achieve the removal of most of the enteric pathogens. Nevertheless, the compliance with microbial indicators was not high enough to exclude the presence of viral contamination; in fact, although the treatments reduced somatic coliphages, a similar reduction was not evident for HAdV.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16101399/s1, Table S1: Oligonucleotide primers and probes used for the viral detection by (RT)qPCR. Target regions are also reported for each viral parameter. References are reported in the footnotes; Table S2: Effect of two independent variables, water treatment and weather conditions, on *E. coli* levels in the exit samples (dependent variable), separately for each WWTP. *E. coli* concentrations are reported as geometric mean ± standard deviation; Table S3: Bacterial and viral indicator concentrations at different stages of the treatment process. Results are reported as median and interquartile range (first and third quartiles) and refer to eight sampling dates; Figure S1: Compliance of WWTP1 with EU minimum requirements for BOD_5, TSS, and *E. coli* (data of *E. coli* have been Log_{10}-transformed). Information on rainfall and chlorination is also reported for the entire monitoring period in the bottom box. Precipitation corresponds to accumulated rainfall (mm) in the 24 prior to the sampling dates, according to Copernicus definition of wet days (https://climate.copernicus.eu/ESOTC; accessed on 12 January 2024); Figure S2: Compliance of WWTP2 with EU minimum requirements for BOD_5, TSS, and *E. coli* (data of *E. coli* have been Log_{10}-transformed). Information on rainfall and chlorination is also reported for the entire monitoring period in the bottom box. Precipitation corresponds to accumulated rainfall (mm) in the 24 h prior to the sampling dates, according to Copernicus definition of wet days (https://climate.copernicus.eu/ESOTC; accessed on 12 January 2024).

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Data Availability Statement: Meteorological data are available from publicly archived datasets of the Regional Hydrological Service of the Tuscany Region (SIR; https://www.sir.toscana.it/, accessed on 12 January 2024). Data on wastewater treatment plants are available upon request due to privacy restrictions.

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Conflicts of Interest: R.S., A.L., F.M., and S.L. are employees of the sewerage company (Acque S.p.A.) involved in the project research. I.F., G.L., A.P., M.V., and A.C. declare no conflicts of interest.

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