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# Impact of wastewater treatment plants on microbiological contamination for evaluating the risks of wastewater reuse

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## Abstract

**Background:** Wastewater reuse represents a promising alternative source of water supply considering the water scarcity related to climate change. However, if not adequately treated, wastewater represents a source of microbiological health risk. The purpose of this work was to investigate the role of wastewater treatment on microbiological contamination by evaluating the possible risks associated with wastewater effluent reuse, taking into account new EU legislation (2020/741) on minimum requirements for water reuse. *E. coli* that produce Shiga toxins (STEC) and thermotolerant *Campylobacter* were monitored using an enrichment step associated with specific PCR, while *Salmonella* spp. and *Legionella* were detected with both cultural and molecular methods (PCR and q-PCR, respectively). Culture method was also used for the enumeration of different microbial indicators. The bacteria detection was compared in different wastewater plants with membrane bioreactor (MBR) system or with disinfection step with chlorine dioxide (ClO<sub>2</sub>). Moreover a comparison between molecular and culture methods was discussed.

**Results:** The results obtained showed good abatement performance for WWTPs equipped with MBR. The high concentrations of *E. coli* (range between 0.88 and 5.21 Log MPN/100 mL) and contamination by *Salmonella* spp. in effluent disinfected with ClO<sub>2</sub> (17% of samples) showed the need to control the quality of this effluent. In addition, despite the absence of *Legionella* spp. with the culture method required by EU regulation, high concentrations of *Legionella* spp. (range between 2 and 7 log GU/L) and the presence of *Leg. pneumophila* with qPCR (15% of samples) highlight the need to carry out further investigations for reuse associated with aerosol formation (e.g. spray irrigation in agriculture).

**Conclusions:** The results obtained underline that the MBR technology can be suitable for wastewater reuse applications allowing to achieve the requirement proposed by the new European legislation. More attention should be given to wastewater reuse of effluents treated with ClO<sub>2</sub>. The use of the molecular methods for pathogens detection in wastewater could allow a more precautionary risks estimation associated with reuse. The overall results highlight that an evaluation of the effectiveness of the wastewater treatments is required for the prevention of a possible risk to public health.

**Keywords:** Wastewater reuse, MBR, Disinfection, Legislation, Pathogenic bacteria, Indicator microorganisms, STEC, *Legionella pneumophila*, *Legionella* spp.

## Background

The availability of freshwater reservoirs is severely influenced by different anthropic activities such as climatic change and urban development [1]. In particular, climate change can affect water necessity for human needs

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in terms of quality and quantity, further exacerbating the situation of water scarcity that already characterises many world areas. Moreover, the growth of the world population will determine in the future the increase in the global demand for water to satisfy domestic, agricultural and industrial needs [2]; in addition, a greater number of urban settlements and human activities will lead to an increase in wastewater production. In this context, wastewater can become the main alternative source of water supply, providing a resource, the availability of which is not subjected to seasonality and does not require additional depletion of groundwater and surface water bodies. However, treated wastewater remains a minor source of water supply: at present, 0.59% of treated wastewater in the world and 2.4% in Europe are reused [3].

To ensure that wastewater reuse does not pose a threat to human health, the efficacy of wastewater treatment and the monitoring of effluent quality to prevent and limit the spread of diseases represent the main topics. Recently, European regulation (2020/741) on minimum requirements for water reuse has been adopted with the purpose of facilitating the safe reuse of treated urban wastewater for agricultural irrigation. This EU regulation proposes an approach based on minimum requirements in terms of reference indicators or pathogens and risk management when wastewater is reused for irrigation [4]. The reclaimed water quality requirements for agricultural irrigation according to new EU Regulation (2020/741) are reported in Table 1.

In fact, if not adequately treated, urban wastewater represents a source of microbiological health risk: its direct use in agriculture can lead to the production of contaminated food and to the spread of contamination in the environment through water leaching and runoff [5]. Moreover, the wastewater reuse can pose direct risks to workers or indirect risks to people living near application sites due to aerosolisation. It is known that numerous pathogenic microorganisms with various resistance capabilities towards environmental conditions and wastewater treatments can be transmitted through water reuse. Therefore, it is important to study pathogens that can represent a health risk in wastewater reuse.

Different studies have shown that pathogenic *Campylobacter* (*jejuni* and *coli*), *Salmonella* spp. and *E. coli* that produce Shiga toxins (STEC) were observed in treated wastewater [6], highlighting that irrigation with reused water could represent a source of contamination for fruit and vegetables. The Surveillance for Foodborne Diseases Outbreaks—United States annual reports from 2009 to 2015—reported *Salmonella* and STEC as two of the most common causes of large outbreaks frequently associated with vegetable consumption [7].

Additionally, in the EU, *Campylobacter* (*jejuni* and *coli*), *Salmonella* (mainly *S. Enteritidis* serovar) and STEC represent the most frequently reported causative agents of foodborne outbreaks in 2019; different cases of illness caused by *Salmonella* and STEC are associated with vegetables [8]. Moreover, the raw vegetables are a relevant source of risk for campylobacteriosis for consumers [5].

Wastewater reuse in agriculture may also represent a possible health risk associated with aerosol production. In fact, the irrigation method (e.g. spray irrigation) can affect the dispersion of aerosols containing pathogens such as *Legionella*, associated with occupational risk. Although it is known that wastewater could represent an important source of *Legionella*, only a few studies have focused on the occurrence of this bacterium in wastewater treatment systems [9, 10]. Several outbreaks of Legionnaires' disease have been associated with aerosol production in the biological step of wastewater treatment plants, and aerosols can spread great distances from the treatment site (up to 1.6 km) [10]. Both reuse for agricultural and landscape irrigation and for cooling purposes should be considered possible sources of aerosol generation, underlining the importance of *Legionella* monitoring in wastewater-treated effluents [11]. The occurrence of *Legionella* was reported in reclaimed wastewater utilised for different purposes (e.g. agricultural use and toilet flushing) in the USA, France and Australia [10].

To improve the microbiological quality of wastewater for reuse purposes, different approaches can be used: it is possible to introduce new technologies in the secondary step of wastewater treatment (e.g. membrane bioreactors, MBRs) or optimise the performance of the final disinfection step of the wastewater treatment plant (e.g. type and amount of disinfectant). The advantages related to MBR use are a small footprint and lower chemical requirements than conventional membrane systems. However, the main disadvantage is the energy consumption largely associated with membrane fouling [12, 13]. MBRs could be particularly suitable for wastewater reuse applications because this technology seems to show a greater ability to reduce some viruses (e.g. coliphage) or bacterial indicators (e.g. faecal coliforms) with respect to conventional activated sludge processes [13]. Despite these promising results, further investigation is deemed required to gain a more complete understanding, because different factors can influence the MBR performance (e.g. the nominal pore size, membrane integrity, development of a gel/cake layer on membrane surface, the adsorption of pathogens to suspended solids) [12, 13]. In this context, it is of particular interest to study the abatement against different bacterial pathogens in full-scale wastewater treatment plants.

**Table 1** Classes of reclaimed water quality, permitted agricultural use, irrigation method and reclaimed water quality requirements for agricultural irrigation according to new EU Regulation (2020/741) [4]

Minimum reclaimed water quality class			
A	B	C	D
Crop category (*)	All food crops consumed raw where the edible part is in direct contact with reclaimed water and root crops consumed raw	Food crops consumed raw where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops and non-food crops including crops used to feed milk- or meat-producing animals	Food crops consumed raw where the edible part is produced above ground and is not in direct contact with reclaimed water, processed food crops and non-food crops including crops used to feed milk- or meat-producing animals
Irrigation method	All irrigation methods	All irrigation methods	Drip irrigation (**) or other irrigation method that avoids direct contact with the edible part of the crop
Indicative technology target	Secondary treatment, filtration and disinfection	Secondary treatment and disinfection	Secondary treatment and disinfection
Quality requirements			
E. coli (number/100 mL)	≤ 10	≤ 100	≤ 10,000
BOD <sub>5</sub> (mg/L)	≤ 10	In accordance with Directive 91/271/EEC (Annex I, Table 1)	
TSS (mg/L)	≤ 10	In accordance with Directive 91/271/EEC (Annex I, Table 1)	
Turbidity (NTU)	≤ 5	-	-
Other	<i>Legionella</i> spp.: < 1000 CFU/L where there is a risk of aerosolisation Intestinal nematodes (helminth eggs): ≤ 1 egg/L for irrigation of pastures or forage		

\*If the same type of irrigated crop falls under multiple categories of the table, the requirements of the most stringent category shall apply  
 \*\*Drip irrigation (also called trickle irrigation) is a micro-irrigation system capable of delivering water drops or tiny streams to the plants and involves dripping water onto the soil or directly under its surface at very low rates (2–20 L/h) from a system of small-diameter plastic pipes fitted with outlets called emitters or drippers  
 \*\*\*In the case of irrigation methods which imitate rain, special attention should be paid to the protection of the health of workers or bystanders. For this purpose, appropriate preventive measures shall be applied

For the tertiary treatment of wastewater before reuse, disinfection can be carried out with chemical substances (e.g. Cl, ClO<sub>2</sub>, PAA) or physical treatments (e.g. UV) [14]. Chlorine dioxide (ClO<sub>2</sub>) has several advantages: it is a better bactericide and virucide than sodium hypochlorite and chlorine, in relationship to its greater oxidative capacity [15]. However, physical and chemical characteristics of wastewater (temperature, pH, organic load) can influence the disinfecting capacity of ClO<sub>2</sub> and, consequently, the disinfectant dosage required and the contact time. Different studies comparing the removal of bacteria indicators with ClO<sub>2</sub> and other tertiary treatments showed that ClO<sub>2</sub> is more efficient than peracetic acid (PAA) and UV at high concentration (5 mg/L) [16]. At lower dosage (2 mg/L) ClO<sub>2</sub> and PAA presented similar abatement against bacterial and viral indicators [15].

The purpose of this study was to evaluate the role of wastewater treatment on microbiological contamination, investigating the possible risks associated with wastewater effluent reuse, with a view to new EU legislation on minimum requirements for water reuse. For this scope, pathogenic bacteria (*Salmonella*, STEC, thermotolerant *Campylobacter*, *Legionella*) and microbial indicators (total coliform, *E. coli*, enterococci and spores of *Clostridium perfringens*) were compared in different full-scale municipal wastewater plants equipped with MBR systems or disinfection step with ClO<sub>2</sub>. Moreover, a comparison between molecular and culture methods for *Salmonella* and *Legionella* was discussed.

## Materials and methods

### Sampling

Samples were collected from three Italian wastewater treatment plants (WWTP1, WWTP2, WWTP3) in seven sampling periods (from January to September 2020). WWTP1 (35,000 population equivalent) and WWTP2 (36,000 population equivalent) employ wastewater pre-treatment (screening, sand and oil removal), biological treatment and ultrafiltration with monolithic hollow fibre membranes (MBR) of polyvinylidene fluoride (PVDF) (nominal membrane pore size: 0.04 µm; trans membrane pressure—TMP (max): 60 kPa). WWTP3 (168,000 population equivalent) employs wastewater pre-treatment (screening, sand and oil removal), primary settling, oxidation, secondary settling, and disinfection with ClO<sub>2</sub> (dose applied 1.2 mg/L; contact time 1 h) before effluent discharging. The wastewater influent and the final effluent were sampled in WWTP1 and WWTP2, while in WWTP3, the effluent before and after the final disinfection treatment (final effluent) was monitored. The diagram of wastewater treatment plants and the sampling points is shown in Fig. 1.

### Microbial indicators

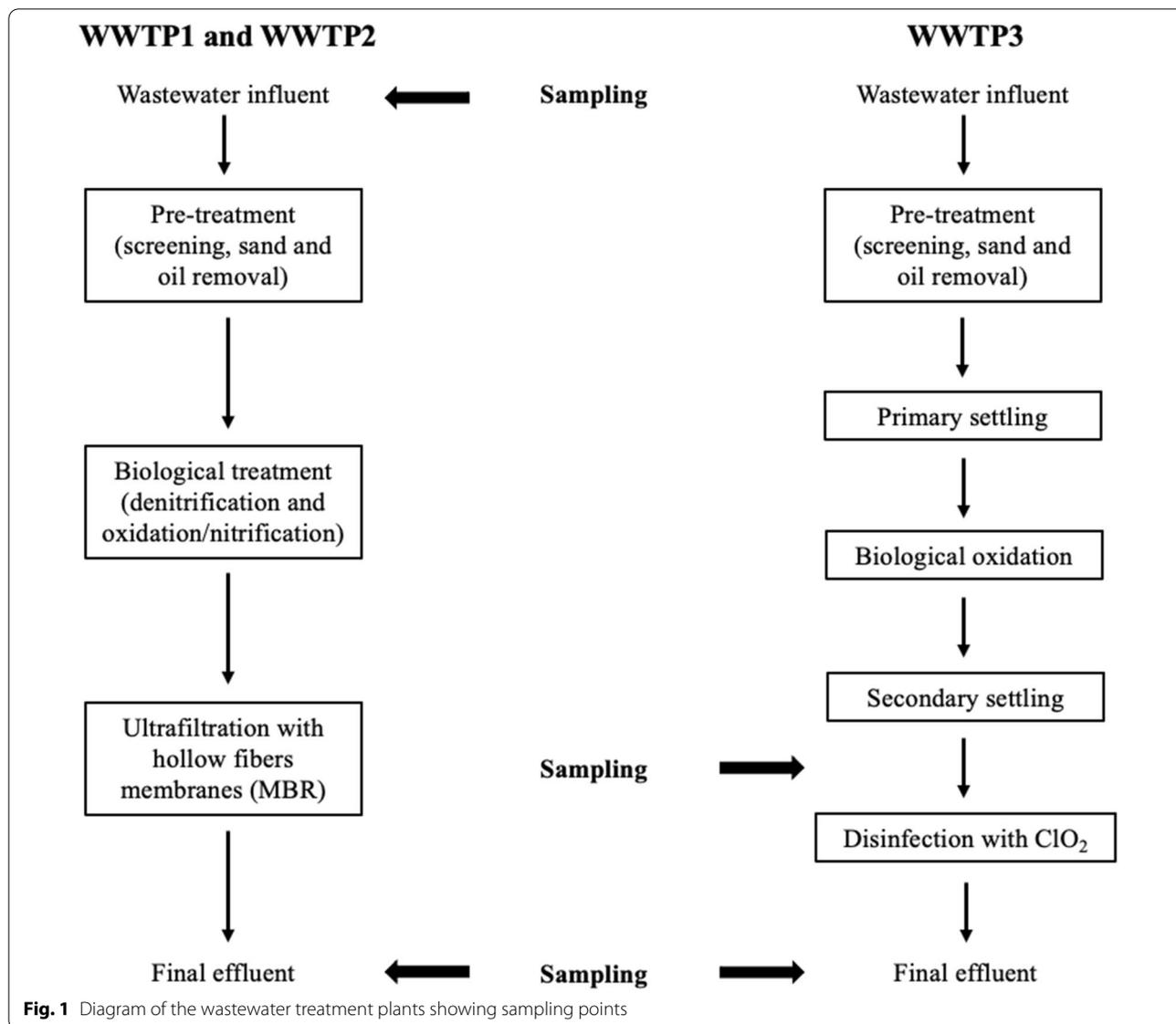
All samples were analysed for total coliforms, enterococci, *E. coli* and *Clostridium perfringens* spores counts. Quanti-Tray™ 2000 (IDEXX Laboratories, Milan, Italy) was utilised for the quantification of coliforms, enterococci and *E. coli*, and the results were expressed as Log MPN/100 mL. The enumeration of *C. perfringens* spores was performed using a membrane filtration method according to ISO 14189:2013 [17], and the results were reported as Log CFU/100 mL. All wastewater samples were transported refrigerated and processed within 24 h of collection.

### Pathogens

The presence/absence of STEC, *Salmonella* spp. and pathogenic *Campylobacter* (*jejuni* and *coli*) in wastewater samples was carried out using a PCR method with a previously reported protocol [7]. Briefly, samples (influent = 100 mL; effluent = 1 L) were concentrated by filtration and enriched in specific media. DNA extraction and purification was carried out with the DNeasy PowerSoil Kit (Qiagen). PCR was conducted as reported in Bonetta et al. [7, 18]. Positive controls were prepared spiking an influent sample, for each sampling period, with high concentration (~10<sup>6</sup> CFU) of *E. coli* O157:H7 (NCTC129), *S. typhimurium* (ATCC14028) and *Cam. jejuni* (ATCC33291).

*Legionella* detection (spp. and *pneumophila*) was carried out using a quantitative molecular PCR method as previously described [19]. Briefly, 20 mL of influent and 250 mL of different effluents were concentrated by filtration; DNA was extracted from filter using the DNeasy PowerWater Kit (Qiagen) and quantified using commercial kits (iQ-Check Quanti *Legionella* spp. and *Leg. pneumophila*—BioRad). Each sample was tested in duplicate and the results were reported as the genome units (GUs) per litre of sample. The detection limit was 2500 GU/L for influent samples and 200 GU/L for effluent or pre-disinfected samples. The quantification limit was 20,000 GU/L for influent samples and 1600 GU/L for effluent or pre-disinfected samples.

Both *Salmonella* spp. and *Legionella* spp. were monitored with culture method in parallel with molecular detection. The culture method described in Bonetta et al. [7] was used for *Salmonella* spp. and results were expressed as presence/absence. The isolation and identification of *Legionella* was performed following ISO 11731:2017 [20]. Briefly, the effluents (concentrated by centrifugation) and the influents were analysed untreated or pretreated with heat (50 °C for 30 min) and acid (pH 2.2 for 5 min) to reduce the growth of interfering microflora, serially diluted, plated on GVPC agar and



incubated for 7–10 days at 37 °C. Suspected colonies were randomly selected for subculture on  $\alpha$ -BCYE agar and  $\alpha$ -BCYE agar without L-cysteine. Confirmed colonies were identified using an agglutination test (*Legionella* latex test; Oxoid). All wastewater samples were transported refrigerated and processed within 24 h of collection. The results were reported as CFU/L; the detection limits were  $2 \times 10^3$  and  $10^2$  CFU/L for influents and effluents, respectively. *S. typhimurium* (ATCC14028) and *Leg. pneumophila* (ATCC 33152) strains were used as positive control.

#### Physical and chemical analyses

Chemical oxygen demand (COD) was determined using the sealed tube method [21]; biochemical oxygen demand

(BOD) was determined by dilution and seeding with suppression of nitrification [22]; and total suspended solids (TSS) was collected by filtration on membrane and gravimetric analysis was performed after filter drying [23]. These parameters were not available for pre-disinfected samples in the WWTP3.

#### Statistical analyses

The presence/absence of pathogens and concentration of bacterial indicators and *Legionella* (Log conversion) were statistically analysed (IBM SPSS Statistics version 26.0 for Windows).

The normal distribution was evaluated using Shapiro–Wilk test. To analyse the differences in microbial contamination between the influent and effluent samples,

the effluents collected in the pre- and post-disinfection step and influent samples of WWTP1 and WWTP2 Student's *t*-test was applied. ANOVA and Tukey's post hoc analysis were carried out to study the effect on microbial contamination of the different treatments performed in the three WWTPs. To evaluate the relationship between concentration of indicator bacteria and the occurrence (presence/absence) of *Salmonella* spp., STEC and *Campylobacter* (*jejuni* and *coli*) in wastewater samples binary logistic regression was carried out. Spearman's correlation was used to evaluate the association between the microbiological and physicochemical parameters in each wastewater plant. Significance was evaluated within 95% confidence intervals ( $p \leq 0.05$ ).

## Results and discussion

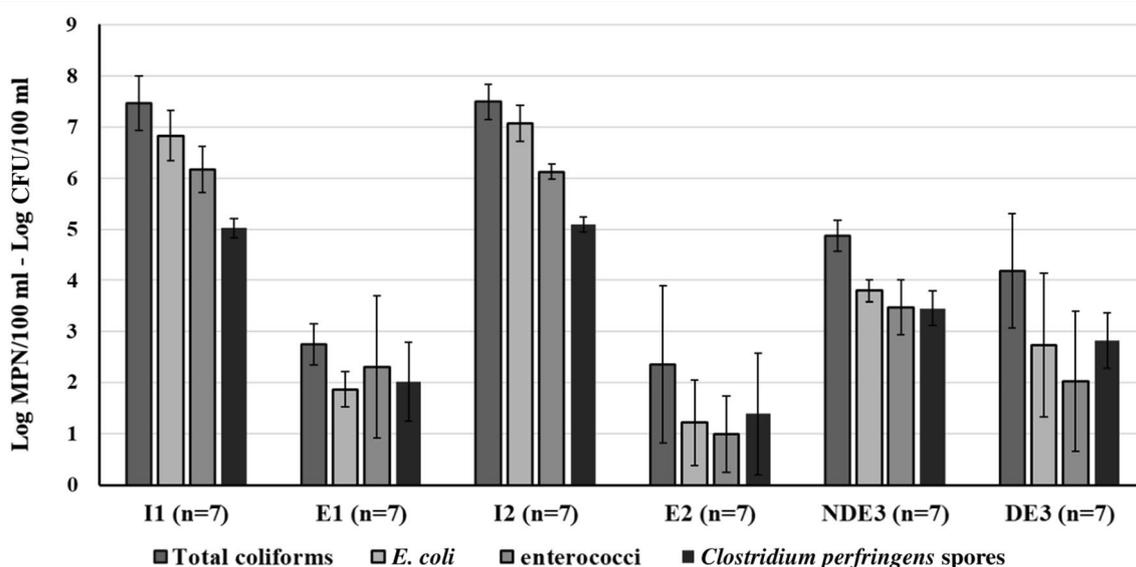
### Microbial indicators

The results of the microbial indicator concentrations in all samples investigated are reported in Fig. 2. As observed, the concentrations of *E. coli*, total coliforms, enterococci and spores of *C. perfringens* were similar in the influent samples collected from WWTP1 and WWTP2 (*t*-test,  $p > 0.05$ ), highlighting that the microbiological characteristics of the two influents are analogous. By comparing the indicator concentrations in the effluents of the three plants, in general, the counts were higher in WWTP3 than in the other two plants. However, this difference was statistically significant only between WWTP3 and

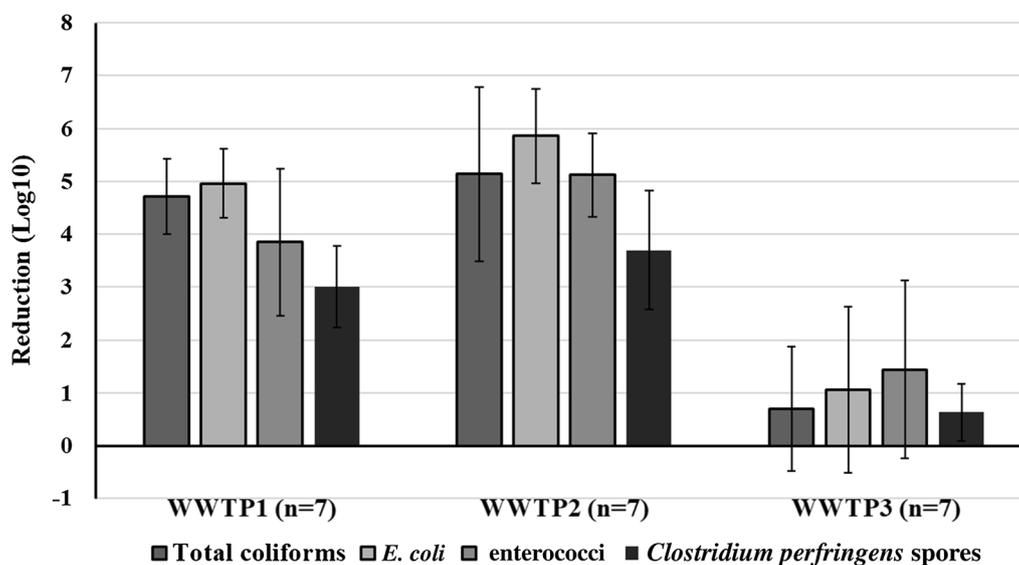
WWTP2 for all parameters, with the exception of the enterococci count (ANOVA,  $p < 0.05$ ).

In both WWTP1 and WWTP2, a statistically significant reduction (*t*-test,  $P < 0.001$ ) of all the microbiological parameters investigated was revealed in the effluent with respect to the influent, with a range of abatement of 3–5 Log and 3.5–6 Log for WWTP1 and WWTP2, respectively (Fig. 3). These results showed good abatement performance for both wastewater treatments in which MBRs were used, especially for WWTP2, although no statistically significant differences were observed between the effluents of WWTP1 and WWTP2. This behaviour could be related to the fact that the two WWTPs had MBR with the same characteristics (e.g. kind of membrane, membrane pore size, membrane material, TMP). Bolzonella and collaborators [24] also reported similar abatement values by MBR. However, other studies highlighted a higher efficiency of this treatment on domestic and industrial wastewaters, allowing reduction values up to 7 log for coliforms and *E. coli* and 5 log for *C. perfringens* [25, 26]. Moreover, a further improvement of the microbial reduction was observed by adding a disinfection step with hypochlorite and/or UV, reaching a total reduction of *E. coli* and enterococci and a further abatement (~1Log) of total coliforms [27, 28].

In the WWTP3, the mean reduction of microbial indicators range from 0.63 to 1.44 with values >1 Log for *E. coli* and Enterococci (mean concentration of  $\text{ClO}_2$ : 1.2 mg/L) (Fig. 3). Similar efficiency of the disinfection step with  $\text{ClO}_2$  was observed by De Luca and



**Fig. 2** Mean concentration ( $\pm$  standard deviation) (log CFU/100 mL or Log MPN/100 mL) of microbial indicators in WWTP samples (I: influent; E: effluent; NDE: pre-disinfected effluent; DE: post-disinfected effluent; 1: WWTP1; 2: WWTP2; 3: WWTP3)



**Fig. 3** Mean reduction ( $\pm$  standard deviation) (Log CFU/100 mL or Log MPN/100 mL) of microbial indicators in the WWTPs

collaborators in a WWTP that operated with a  $\text{ClO}_2$  dose of 1.5 mg/L. In the same study a better performance was obtained with 2 mg/L of  $\text{ClO}_2$  applied dose [15]. Moreover, other studies reported higher values of microbial abatement related to final disinfection with  $\text{ClO}_2$  (range 2–5 Log for total coliforms and *E. coli*). However these results were obtained combining the  $\text{ClO}_2$  disinfection step with a pre-treatment phase (e.g. filtration or adding flocculant agent) [16] or using higher doses of  $\text{ClO}_2$  [29].

In the current Italian legislation for wastewater reuse, the regulatory reference limit for *E. coli* concentration is 10 CFU/100 mL (for at least 80% of samples) [30]. No samples of the effluent collected in the WWTP1 comply with this limit, underlining that, in these conditions, this treated wastewater could not be reused for irrigation, industrial or civil purposes (Additional file 1: Fig. S1). Forty-three percent of effluent samples (3/7) of WWTP2 presented values below this limit, highlighting a greater potential of the effluent of this WWTP for reuse. Since both WWTPs are equipped with MBR technology, generally suitable in the case of wastewater reuse, a thorough and careful analysis of the process conditions and the filtration efficiency of the membranes would allow us to identify useful changes to obtain a higher microbiological quality of the wastewater. In addition to membrane characteristics other factors that can affect pathogen removal by membranes should be considered and managed. In fact the development of a gel/cake layer on membrane surface, due to the accumulation of biomaterials, can modify the pathogens removal. The adsorption of pathogens to suspended solids, which depends on the

characteristics of the influent water (e.g. pH, concentration of  $\text{NH}_3$  or NaCl), can also affect membranes removal capacity. Membrane integrity is another important factor, because irregularities due to physical and chemical damage can result in a loss of pathogen removal efficiency [12, 13]. Moreover, as reported in other studies [27, 28], the application of an adequate final disinfection of the effluent could contribute to a further microbial reduction. However, this possibility should be carefully evaluated from an economic perspective, considering that the same MBR systems already involve higher energy and management costs than traditional activated sludge treatment.

The analysis of the results obtained in the samples from WWTP3 effluents demonstrate that only one sample complied within the limit value for *E. coli*, while the others showed concentrations higher than 2 log MPN/100 mL and up to 5 log MPN/100 mL, highlighting the need for a more efficient tertiary treatment (e.g. higher  $\text{ClO}_2$  dose) or, in some cases, the requirement to secondary treatment improvement (e.g. MBR technologies).

The new EU Regulation [4] for wastewater reuse establishes different limit values for *E. coli* concentration according to the specific use (Table 1). Referring to this regulation, the effluent of all WWTPs, except a sample of WWTP3, complied with both the limit values for “industrial, energy and seeded crop use” ( $\leq 10,000$  CFU/100 mL, class D) and for “drip irrigation of food crops consumed raw (with no direct contact of edible part of vegetable with reclaimed water), processed food crops and non-food crops” ( $\leq 1000$  CFU/100 mL,

class C). Moreover, 71% (5/7), 86% (6/7) and 14% (1/6) of all effluents analysed showed *E. coli* values lower than 100 CFU/100 mL (Class B), a limit established for water reused for the same food crops previously reported but “using all irrigation method”. Only a few samples complied with the limit prescribed for the irrigation of all food crops consumed raw (with direct contact of edible part of vegetable with reclaimed water) and root crops consumed raw ( $\leq 10$  CFU/100 mL, class A).

### Pathogens

Table 2 shows the results of *Salmonella* spp., STEC and *Campylobacter* (*jejuni* and *coli*) detection carried out in the three wastewater plants monitored. The presence of *Salmonella* spp. was reported in 100% (7/7) and 86% (6/7) of WWTP1 influents using molecular and cultural methods, respectively. In the WWTP2 *Salmonella* spp. was detected in 71% (5/7) of influent samples with the molecular method, while a sole sample (14%) was positive with the culture method. Additionally, Cataldo and collaborators [31] reported a similar percentage of contamination in the influent samples (from 100 to 25%). Fifty percent of pre-disinfected samples (3/6) collected in the WWTP3 were contaminated by *Salmonella* spp. with both methods. These results highlight a greater sensitivity of the molecular method over the culture method. This is probably related to the difficulty of isolating *Salmonella* on culture media due to the presence of interfering microflora, as previously reported [32] or to the use of an enrichment step for the molecular method that can aid the recovery of injured, stressed or lag-phase bacterial cells [33]. Moreover, *Salmonella* spp. was not detected in the effluents of WWTP1 and WWTP2 using either method, highlighting the efficacy of MBR in *Salmonella* abatement. In contrast, in 17% (1/6) of the WWTP3 disinfected samples, *Salmonella* spp. contamination was observed with both methods underlining that the disinfection step, although showing a reduction in positive samples, was not able to remove the contamination completely. No relationship between *Salmonella* spp. presence and microbial indicators was observed with binary logistic regression ( $p > 0.05$ ); this finding suggests that the microbial parameters analysed are probably not reliable indicators of the presence of this pathogenic bacterium [6, 34].

Italian legislation for wastewater reuse in agriculture requires the absence of *Salmonella* spp. in 100% of effluent analysed with the culture method. All effluents of WWTP1 and WWTP2 complied with this limit, and therefore, on the basis of these parameters and excluding high counts for *E. coli*, they could potentially be utilised for reuse purposes. Additionally, in the case of the WWTP3, considering the presence of *Salmonella* spp.

in one sample in addition to the high counts of *E. coli*, further investigations on the efficiency of the disinfection treatment is necessary to improve microbial reduction and obtain the necessary requirements for the reuse of wastewater.

However, considering *Salmonella* spp. contamination, it is important to emphasise that in the new EU regulation (2020/741) on minimum requirements for water reuse [4], this parameter was deleted from the water quality requirements (Table 1); therefore, with reference to this regulation, the WWTP3 effluent could also be reused in agriculture.

As reported in Table 2, *E. coli* O157:H7, Shiga-like toxin (I and II) and *Campylobacter* (*jejuni* and *coli*) were never found in the samples analysed (influent, effluent, pre- and post-disinfection samples). Since the spread of these microorganisms in wastewater influents can reflect the local epidemiological situation and the presence of contamination sources (e.g. livestock production, food industries and slaughterhouses) [35], the absence of these bacteria could be related to a low circulation of these pathogens in the local population. The absence of STEC and pathogenic *Campylobacter* (*jejuni* and *coli*) in wastewater samples was also reported in other studies [6, 32, 36].

The presence of *Legionella pneumophila* sg1 was only observed with the culture method in a sole sample of the WWTP1 influent (6.3 Log CFU/L), and in the same sampling, the effluent was contaminated by *Legionella* spp. (3 Log CFU/L). Mosteo et al. [37] also showed low contamination by *Legionella* spp. in wastewater samples using culture method. Additionally, molecular analysis with qPCR reported the occurrence of *Legionella* spp. in all samples (influent, effluent, pre- and post-disinfection samples) collected in the three plants (Fig. 4) with a range of concentrations between 5.1 and 7.2 Log GU/L. *Leg. pneumophila* was detected in 86% and 57% of the WWTP1 and WWTP2 influents, respectively, and in all pre-disinfected samples of WWTP3. In contrast, among effluent samples, only some of the WWTP3 samples were contaminated by *Leg. pneumophila* (50% of disinfected samples). The statistical analyses confirmed the higher values of *Legionella* spp. and *Leg. pneumophila* concentrations in effluents of WWTP3 with respect to WWTP1 and WWTP2 (ANOVA,  $p < 0.05$ ). It is important to highlight that the *Leg. pneumophila* concentrations monitored in all samples were very low or lower than the limit of quantification (LOQ), as reported in other studies [38].

For WWTP1 and WWTP2, a significant reduction in *Legionella* spp. and *Leg. pneumophila* contamination was obtained with MBR wastewater treatment (range 1.8–2 Log for *Legionella* spp. and 2.2–3 Log for *Leg. pneumophila*) ( $t$ -test,  $p < 0.001$ ). In contrast, this trend was

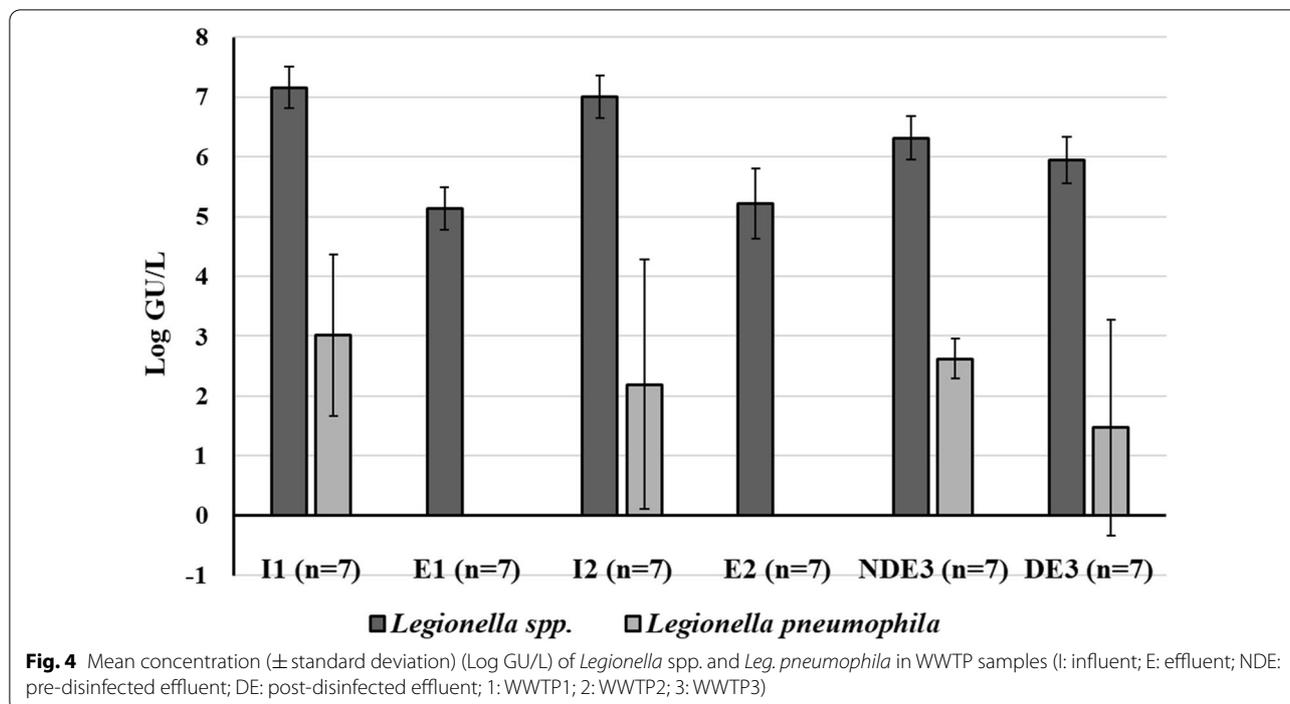
**Table 2** Detection of *Salmonella* spp., *E. coli* O157:H7, *E. coli* virulence genes and *Campylobacter* (spp., *C. coli*, and *C. jejuni*) in full-scale WWTPs

Plant	Sample	Sampling	<i>Salmonella</i> invA	<i>E. coli</i> O157:H7					<i>Campylobacter</i>			<i>Salmonella</i> Culture method
				O157	H7	Intimin	SLT-I	SLT-II	Genus	<i>C. jejuni</i>	<i>C. coli</i>	
WWTP 1	I	1	+	-	-	-	-	-	-	-	-	+
	E	1	-	-	+	-	-	-	-	-	-	-
	I	2	+	-	+	-	-	-	-	-	-	+
	E	2	-	-	-	-	-	-	-	-	-	-
	I	3	+	-	+	-	-	-	-	-	-	+
	E	3	-	-	-	-	-	-	-	-	-	-
	I	4	+	-	+	-	-	-	-	-	-	+
	E	4	-	-	-	-	-	-	-	-	-	-
	I	5	+	-	-	-	-	-	-	-	-	+
	E	5	-	-	-	-	-	-	-	-	-	-
	I	6	+	-	-	-	-	-	-	-	-	-
	E	6	-	-	-	-	-	-	-	-	-	-
	I	7	+	-	-	-	-	-	-	-	-	+
	E	7	-	-	-	-	-	-	-	-	-	-
WWTP 2	I	1	+	-	+	-	-	-	-	-	-	+
	E	1	-	-	+	-	-	-	-	-	-	-
	I	2	+	-	+	-	-	-	-	-	-	-
	E	2	-	-	+	-	-	-	-	-	-	-
	I	3	-	-	+	-	-	-	-	-	-	-
	E	3	-	-	-	-	-	-	-	-	-	-
	I	4	+	-	-	-	-	-	-	-	-	-
	E	4	-	-	-	-	-	-	-	-	-	-
	I	5	+	-	-	-	-	-	-	-	-	-
	E	5	-	-	-	-	-	-	-	-	-	-
	I	6	+	-	+	-	-	-	-	-	-	-
	E	6	-	-	+	-	-	-	-	-	-	-
	I	7	-	-	-	-	-	-	-	-	-	-
	E	7	-	-	-	-	-	-	-	-	-	-
WWTP 3	NDE	2	-	-	+	-	-	-	-	-	-	-
	DE	2	-	-	+	-	-	-	-	-	-	-
	NDE	3	-	-	+	-	-	-	-	-	-	-
	DE	3	-	-	-	-	-	-	-	-	-	-
	NDE	4	+	-	-	-	-	-	-	-	-	+
	DE	4	-	-	-	-	-	-	-	-	-	-
	NDE	5	-	-	+	-	-	-	-	-	-	-
	DE	5	-	-	-	-	-	-	-	-	-	-
	NDE	6	+	-	-	-	-	-	-	-	-	+
	DE	6	+	-	-	-	-	-	-	-	-	+
	NDE	7	+	-	-	-	-	-	-	-	-	+
	DE	7	-	-	-	-	-	-	-	-	-	-

I influent, E effluent, NDE pre-disinfected effluent, DE post-disinfected effluent, + positive, - negative

not encountered in WWTP3, showing that the reduction obtained with the disinfection step with ClO<sub>2</sub> was not relevant. It is important to note that in different studies conducted in WWTPs using different biological

processes (including chlorination treatment), the abundance of *Legionella* was similar or higher in treated wastewater than in influent even at concentrations of chlorine higher (2–3 mg/L) than those used in this study.



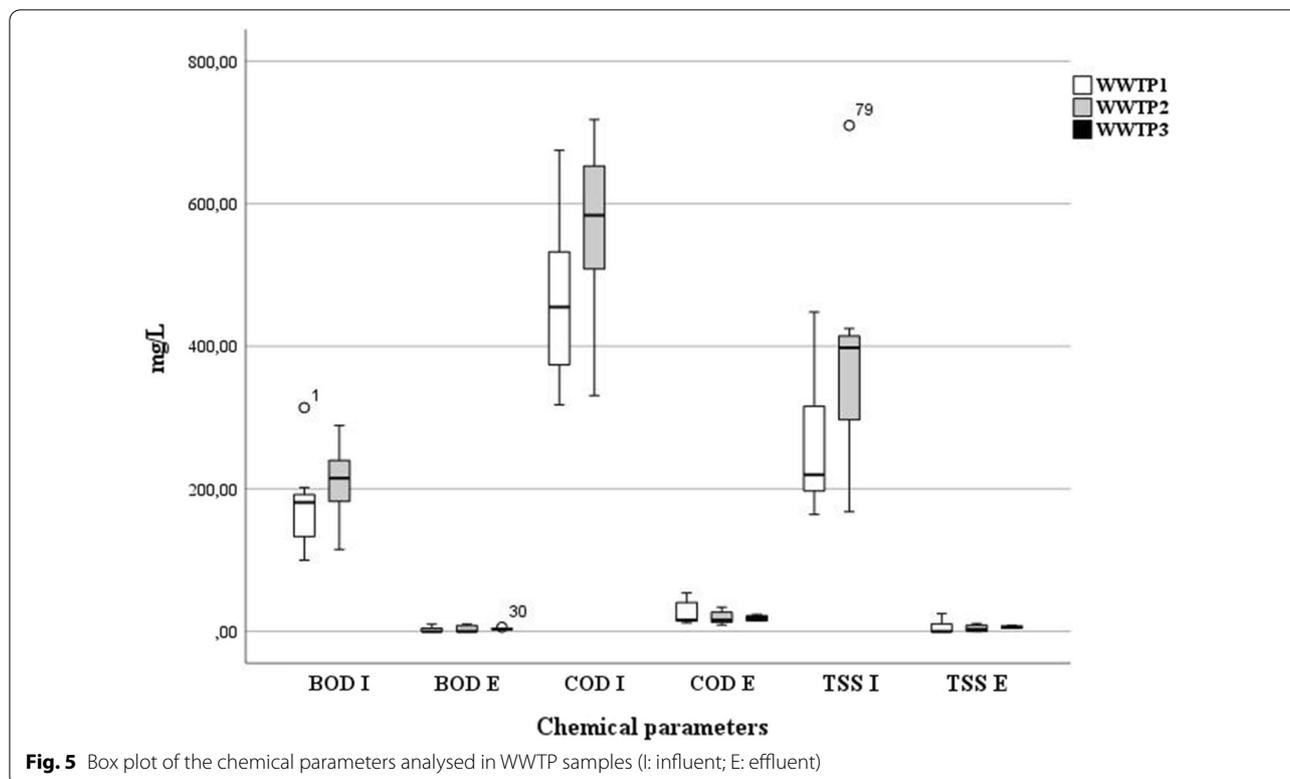
This fact confirms that *Legionella* can survive within the different steps of wastewater treatment because this bacterium is able to grow within protozoa or biofilms [10, 39]. Then, the significant reduction obtained in WWTP1 and WWTP2 investigated in this study highlights that the MBR treatment and the operational parameters used allow to control of *Legionella* spp. contamination, as was also observed for *Salmonella* spp.

As reported above, lower *Legionella* contamination was observed with culture than with molecular methods in the monitored samples. This difference can be attributed to several limitations of the culture method, including the presence of abundant and/or competitive flora, the pre-treatment conditions that could inhibit *Legionella* growth, and the inability of this technique to detect viable but non-culturable forms (VBNCs) [10]. In fact, in wastewater samples investigated high level of interfering microflora was observed; the plates reading of untreated samples was difficult and heat or acid pre-treatment of samples was often used. The underestimation of *Legionella* occurrence with the culture method in wastewater samples has been reported in other studies [9, 40]. On the other hand, the qPCR can instead overestimate health risks since it can also detect dead or damaged microorganisms and this deserves particular attention especially for samples that are subjected to treatments. Other aspects should be considered for the application of the molecular method in wastewater samples: despite the rapidity in the response times of the analysis, the cost

of materials and equipment are high; the PCR inhibitors can be present in the samples, then appropriate internal controls should be used; moreover, there is currently non consensus on how qPCR results should be translated into the quantitative limits based on culture reported in legislation. In fact, despite its disadvantages, the culture method actually represents the gold standard to quantify viable and culturable *Legionella*. Additionally, in the new EU regulation for wastewater reuse, *Legionella* detection with the culture method was added as a water quality parameter (*Legionella* spp.: <1000 CFU/L) when a risk of aerosol formation is supposed (Table 1). Referring to this limit, all effluents analysed in this study comply with EU new regulations; however, the high concentration of *Legionella* spp. and the presence of *Leg. pneumophila* in the WWTP samples analysed with qPCR underlines the need to carry out further investigation when the reuse of these effluents in agriculture is purposed.

#### Physical and chemical analyses

The physicochemical characteristics of the wastewater samples are shown in Fig. 5 (Additional file 1: Table S1). All values measured for BOD and almost all for TSS comply with the limits reported in the new EU regulation for reclaimed water quality class A, except for two effluents that have the TSS values required for reclaimed water quality class B (Table 1). A significant correlation was observed between microbiological parameters (total coliforms, *E. coli*, enterococci, *C. perfringens* spores,



**Fig. 5** Box plot of the chemical parameters analysed in WWTP samples (I: influent; E: effluent)

*Legionella* spp. and *Leg. pneumophila*) and physicochemical characteristics for WWTP1 and WWTP2 (Spearman correlation coefficient) (Additional file 1: Table S2), highlighting the possibility to use these parameters to support the wastewater quality evaluation and to evaluate the risk of *Legionella* occurrence in wastewater. It is possible to hypothesise that the physicochemical parameters monitored (TSS, BOD and COD) could be used as surrogates of microbiological indicators fate because the good correlation between parameters allows a good prediction of wastewater quality considering the reuse purpose. A positive correlation between *Legionella* load and COD was previously reported by Caicedo et al. [9]. The relationship between the removal of indicators and the removal of physicochemical parameters was found by Mailler et al. [41] in different wastewater samples monitored in Paris.

In contrast, no relationship was observed between the microbiological indicators and the physicochemical parameters of the WWTP3 samples, probably due to the limited number of analysed samples.

This study has various strengths: (a) it was performed in full-scale wastewater treatment plants supplied with a promising technology for the production of wastewater that can be reused in agriculture (MBR technology) or with a technology frequently used for wastewater

disinfection ( $\text{ClO}_2$ ); (b) different indicators and pathogens were investigated at the same time to evaluate the possible microbiological risk associated to the reuse purpose; (c) cultural and molecular methods were used for *Salmonella* spp. and *Legionella* to compare the results evaluating the usefulness in the monitoring programmes.

This study has also some limits: (a) the study have considered 3 WWTPs with specific characteristics (e.g. MBR pore size,  $\text{ClO}_2$  dose), then it could be interesting to carry out further investigation considering WWTPs equipped with others MBR system or using a disinfection step improved (e.g. higher  $\text{ClO}_2$  dose, adding of a pre-treatment) to achieve the requirement for wastewater reuse purpose; (b) the study mainly regarded bacterial contamination, but it could be interesting keeping virological risk associated to the wastewater reuse; (c) in order to study the risk associated to wastewater reuse the microbiological quality of agricultural products cultivated using treated wastewater could be also considered; (d) the use of qPCR with photoactivatable DNA intercalators (e.g. EMA or PMA) could allow to distinguish viable from dead cells improving the exposure risk evaluation to *Legionella*.

## Conclusions

The results obtained underline that the MBR technology can be suitable for wastewater reuse applications allowing to achieve the requirement proposed by the new European legislation. The slight difference between the performance of WWTP1 and WWTP2 confirms the importance of studying the optimal operative conditions of the process. More attention should be given to wastewater reuse of effluents treated with ClO<sub>2</sub>. An improved tertiary treatment (e.g. higher ClO<sub>2</sub> dose) could increase the performance.

The comparison between the results obtained with molecular and culture methods confirmed the greater sensitivity of the molecular method mainly associated to the presence of interfering microflora in wastewater samples. Although it is well known that qPCR for *Legionella* detection can overestimate the contamination level, its use could allow a more precautionary risks estimation. This is particularly important considering that aerosol formation from wastewater contaminated by *Legionella* could represent an occupational health risk associated with reuse.

The overall results highlight the importance to evaluate the effectiveness of wastewater treatments with the purpose of agricultural reuse. In this context, the improvement of the treatment system, when necessary, is crucial for the prevention of a possible risk to public health. This activity is also important considering the approach to risk management reported in the EU regulation that comprises assessing risks in a proactive way. That is in order to guarantee that wastewater reuse is safely and managed with aim to prevent the risks for the environment, human or animal health.

## Abbreviations

STEC: Shiga toxin-producing *E. coli*; MBR: Membrane bioreactor; EU: European Union; qPCR: Quantitative polymerase chain reaction; WWTP: Wastewater treatment plant; MPN: Most probable number; ISO: International Organization for Standardization; CFU: Colony forming unit; GU: Genome unit; COD: Chemical oxygen demand; BOD: Biochemical oxygen demand; TSS: Total suspended solids; ANOVA: Analysis of variance; VBNC: Viable but not culturable.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12302-022-00597-0>.

**Additional file 1: Figure S1.** *E. coli* concentration (Log MPN/100 mL) in WWTP effluent (E: effluent; DE: post-disinfected effluent; 1: WWTP1; 2: WWTP2; 3: WWTP3. **Table S1.** Results of physical and chemical analyses in the different WWTP samples. **Table S2.** Results of Spearman's correlation between microbiological parameters and physicochemical parameters in the full-scale WWTPs.

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## Authors' contributions

SIB: conceptualisation, methodology, investigation, formal analysis, writing—original draft; CP: conceptualisation, investigation, writing—review and editing; EG, LR: investigation, data curation; SaB: conceptualisation, investigation, formal analysis, writing—original draft; EC: conceptualisation, methodology, writing—review and editing, project administration, funding acquisition. All authors read and approved the final manuscript.

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## Availability of data and materials

The data sets used in this study are available from the corresponding author on reasonable request.

## Declarations

### Ethic approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

There are no conflicts to declare.

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