International Journal of Food Microbiology 356 (2021) 109352

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

New standards at European Union level on water reuse for agricultural irrigation: Are the Spanish wastewater treatment plants ready to produce and distribute reclaimed water within the minimum quality requirements?

Pilar Truchado [a,*], Maria I. Gil [a], Cecilia López [b], Alberto Garre [c], Román F. López-Aragón [d], Karola Böhme [b], Ana Allende [a]

[a] Research Group on Microbiology and Quality of Fruit and Vegetables, Department of Food Science and Technology, CEBAS-CSIC, Campus Universitario de Espinardo, 25, 30100 Murcia, Spain
[b] Fundación Centro Tecnológico Agroalimentario de Lugo, CETAL, F.S.P., C/ Montirón, n° 154, 27002 Lugo, Spain
[c] Food Microbiology, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands
[d] Entidad Regional de Saneamiento y Depuración de Murcia (ESAMUR), Avda. Juan Carlos I, s/n. Ed. Torre Jemeca, 30009 Murcia, Spain

ARTICLE INFO

Keywords:
Irrigation water
Leafy greens
Reclaimed water
Wastewater treatment plants
Regulation
Pathogenic bacteria

ABSTRACT

The new European regulation on minimum quality requirements (MQR) for water reuse (EU, 2020/741) was launched in May 2020 and describes the directives for the use of reclaimed water for agricultural irrigation. This Regulation will be directly applicable in all Member States from 26 June 2023. Since its publication in 2020, concerns have raised about potential non-compliance situations in water reuse systems. The present study represents a case study where three different water reuse systems have been monitored to establish their compliance with the MQR. Each water reuse system includes a wastewater treatment plant (WWTP), a distribution/storage system and an end-user point, where water is used for irrigation of leafy greens. The selected water reuse systems allowed us to compare the efficacy of water treatments implemented in two WWTPs as well as the impact of three different irrigation systems (drip, furrow and overhead irrigation). The presence and concentration of indicator microorganisms (*Escherichia coli* and *C. perfringens* spores) as well as pathogenic bacteria (Shiga toxin-producing, *E. coli* (STEC), *E. coli* O157:H7, and *Salmonella* spp.) were monitored in different sampling points (influent and effluent of the WWTPs, water reservoirs located at the distribution system and the end-user point at the irrigation system as well as in the leafy greens during their growing cycle. Average levels of *E. coli* (0.73 ± 1.20 log cfu *E. coli*/100 mL) obtained at the point where the WWTP operator delivers reclaimed water to the next actor in the chain, defined in the European regulation as the 'point of compliance', were within the established MQR (<1 log cfu/100 mL) (EU, 2020/741). On the other hand, average levels of *E. coli* at the end-user point (1.0 ± 1.2 log cfu/100 mL) were below the recommended threshold (2 log cfu *E. coli*/100 mL) for irrigation water based on the guidance document on microbiological risks in fresh fruits and vegetables at primary production (EC, 2017/C_163/01). However, several outlier points were observed among the samples taken at the irrigation point, which were linked to a specific cross-contamination event within the distribution/storage system. Regarding pathogenic bacteria, water samples from the influent of the WWTPs showed a 100% prevalence, while only 5% of the effluent samples were positive for any of the monitored pathogenic bacteria. Obtained results indicate that reclaimed water produced in the selected water reuse system is suitable to be used as irrigation water. However, efforts are necessary not only in the establishment of advance disinfection treatments but also in the maintenance of the distribution/storage systems.

1. Introduction

Water scarcity is becoming a cause of concern across Europe (EU, 2020/741). Currently, one-third of the EU territory suffers from water stress all year round due to climate change. Agriculture uses about 70% of water withdrawals from freshwater resources, but this average

* Corresponding author.
E-mail address: ptruchado@cebas.csic.es (P. Truchado).

https://doi.org/10.1016/j.ijfoodmicro.2021.109352
Received 27 January 2021; Received in revised form 23 June 2021; Accepted 29 July 2021
Available online 31 July 2021

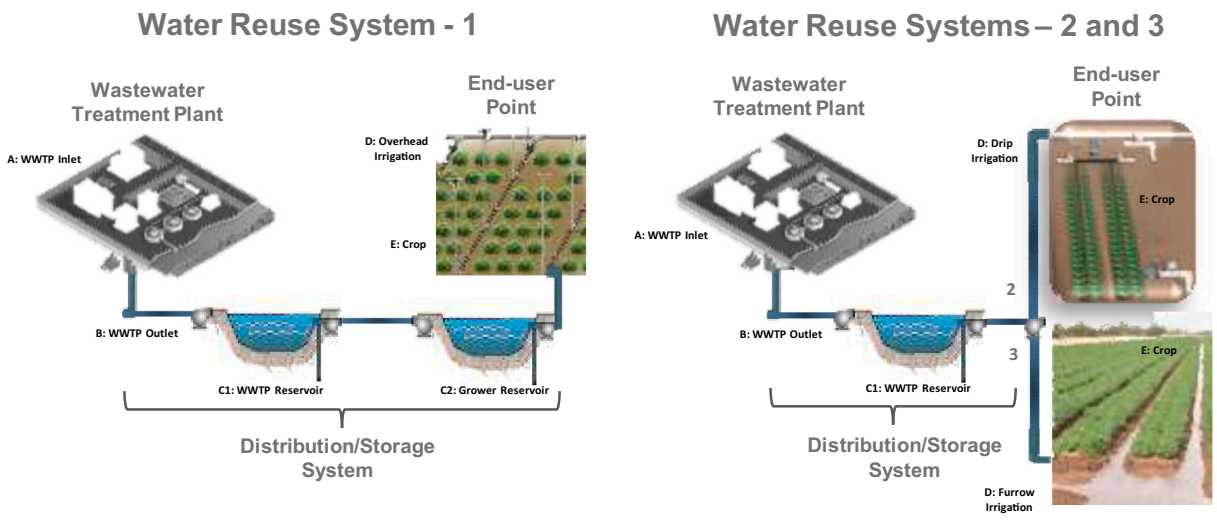

Fig. 1. Description of the water reuse systems included in the study. The water reuse system consisted in two WWTPs, one or two water reservoirs and an irrigation system. A total of 5/6 sampling points were included for each water reuse system.

represents even more in some areas. Water reuse is defined as the use of treated wastewater for beneficial use (Alcalde-Sanz and Gawlik, 2017). The European Commission promotes an integrated water management approach, in which treated wastewater from urban wastewater treatment plants (WWTPs) can represent an alternative water source to alleviate the demand for irrigation water. Thus, water reclamation and reuse for agriculture irrigation are priority innovation practices (EU, 2020/741). However, the potential use of reclaimed water has remained mostly unexploited in the EU (Sánchez-Cerdà et al., 2020). Different factors have been highlighted as potential drawbacks, including 1) water reuse cost; 2) a general public distrust related to human health risks, and 3) the lack of harmonization in the regulatory framework to manage health and environmental risks related to water reuse at the EU level (Alcalde-Sanz and Gawlik, 2017). Several EU Member States (MS), including Cyprus, Greece, France, Italy, Portugal, and Spain had previous standards on water reuse in place (EU, 2018). However, a common legal framework was necessary to avoid the lack of harmony among the existing standards of different MS, because it could create trade barriers across Europe for agricultural goods irrigated with reclaimed water and a perception among end-users that there are various safety levels for similar irrigation practices (Drewes et al., 2017). For example, the Spanish legislation on reclaimed water, which was in place since 2007 (Real Decreto 1620/2007, 2007), has been repealed by the new European regulation (EU, 2020/741), which include more stringent parameters. However, the new regulation includes additional barriers, which make the established thresholds more flexible. The report elaborated by the European Committee of the Regions' Commission for the Environment, Climate Change, and Energy named "Water Reuse—Legislative Framework in EU Regions" includes a comparison of different national legislations with the new European regulation on water reuse, highlighting the differences among the cover parameters and monitoring requirements (EU, 2018).

Based on the new regulation, it is clear that WWTPs operators shall provide a certificate of compliance with the establish conditions to the end-users of the reclaimed water but it also mentions that other interested parties, beyond reclamation plant operators, are responsible for ensuring the quality requirements (EU, 2020/741). As already stated by Sánchez-Cerdà et al. (2020), the compliance point of the plant operators must be the point of delivery of the reclaimed water to the users. This means that the final user should take preventive actions to maintain the quality of the water. In an attempt to help growers to prevent microbial contamination of fruit and vegetables, the European Commission (EC) published in 2017 a guidance document to address the microbiological risks in fresh fruit and vegetables at primary production (EC, 2017/C_163/01). This guidance document includes the recommended microbiological threshold values and the frequency of monitoring for analysis of the water for indicators of faecal contamination (indicator *E. coli*) and the end-user point (where water is use for irrigation), considering the source and the intended use of agricultural water (e.g. irrigation system, fresh fruits and vegetables characteristics, intended use of fresh fruits and vegetables). The suggested values are based on scientific opinions previously published by the European Food Safety Agency (EFSA) (EFSA, 2014). This means that different thresholds apply at different points of the water reuse system, mostly because different approaches have been applied to the effluents and the irrigation water. In the European regulation, levels of *E. coli* lower than 1 log cfu/100 mL at the point of delivery of the reclaimed water to the users, aim to guarantee a reduction in the prevalence of pathogenic microorganisms in the reclaimed water in agreement with the tolerable burden of disease of 10^{-6} Disability Adjusted Life Years (DALYs) per person per year (pppy) (Alcalde-Sanz and Gawlik, 2017). However, the *E. coli* thresholds at the end-user point (where water is use as irrigation water) included in the guidance document of the EC are linked to the probability of finding a pathogen in the irrigation water based on the scientific evidences (EFSA, 2014).

This study represents a practical example of the current situation in the South East of Spain, known as the European orchard, which represents (in economic terms) 10% of the world production of fruit and vegetables and 12% at European level and where reclaimed water represents a valuable water source (FAOstat, 2019). Data obtained in this study were acquired through a 2-year systematic sampling of water obtained from two WWTPs used to irrigate commercial fields producing leafy greens. Analyses of bacterial indicators and pathogens were performed in 7 growing cycles. Interpretation of the data obtained was used to determine if current practices are compatible with the new European regulation.

2. Materials and methods

2.1. Experimental design

Three water reuse systems have been included in this study as it is illustrated in Fig. 1. Two different WWTPs with tertiary treatments located in Cartagena and Lorca (Murcia, Spain) were selected due to its location with respect to the crop fields, which allowed the use of the reclaimed water as irrigation water using different irrigation systems.

The WWTP located in the Cartagena area used ultraviolet-C light as tertiary treatment (WWTP_UV-C), while the WWTP located in the Lorca area used sodium hypochlorite as tertiary treatment (WWTP_NaClO). The point of delivery of the WWTPs was established after the tertiary treatment. Reclaimed water was then transferred to the water reservoir, as part of the distribution/storage system. The number of water reservoirs varied between the water reuse systems as it is illustrated in Fig. 1. Three different end-user points (commercial growing fields) were included in the water reuse systems, and irrigation water was applied using different irrigation systems including drip, furrow and overhead irrigation.

2.2. Meteorological parameters

For each location and growing cycle, weather parameters including solar radiation, relative humidity (RH), and temperature were acquired from the nearby climatic stations at 'Torrepacheco' (37° 44′ 51.81″ N, 0° 59′ 12.02″ W) and 'Lorca' (37° 30′ 13.86″ N, 1° 41′ 38.07″ W), located 10 km far from the WWTPs. The climatological database of Sistema de Informacion Agraria de Murcia (SIAM) was used (SIAM, 2019).

2.3. Water sample collection

Water sampling was performed in 4–5 different sampling points depending on the water reuse system as indicated in Fig. 1 (A: WWPT inlet; B: WWTP outlet; C1: water reservoir close to the WWTP, WWTP reservoir; C2: the water reservoir at the growing field, Grower reservoirs; D: irrigation system, Irrigation). A total of 470 water samples were obtained distributed as follows: 100 samples from A, 100 samples from B, 100 samples from C1, 75 samples from C2 and 95 samples from D. Samples were taken between 2 and 4 times along the 7 growing cycles of the leafy greens from September 2017 to March 2019. At each sampling point, 5 water samples (2 L each) were collected for microbial analyses in sterile polypropylene plastic bottles. Additionally, 10-L of water from B, C1, C2 and D were collected at each sampling day for pathogenic bacteria analyses. Water samples from the irrigation systems were collected while the leafy greens were irrigated. In the case of drip and furrow irrigation, water samples were collected at the point where the water enters the field. In the case of the overhead irrigation, water was collected directly from the sprinkler.

2.4. Leafy greens sample collection

The leafy greens planted in the commercial growing fields included in this study were whole head lettuces and baby spinach leaves. As in the case of water samples, a total of 100 leafy green samples were taken along the 7 growing cycles from September 2017 to March 2019. Each sampling day, 5 leafy green samples were manually collected using scissors and following a random zig-zag pattern to cover the entire growing area. Scissors were wiped with ethanol between samples. Each sample was stored in a sterile plastic bag and transported to the lab under refrigerated conditions.

2.5. Culturable E. coli enumeration

The level of culturable *E. coli* was performed in all water samples. Depending on the expected *E. coli* concentration, pour plating (1 mL), or membrane filtration (10 and 100 mL) was used. Samples were filtered through 0.45 μm membrane filters (Sartorius, Madrid, Spain) using a filter holder manifold (Millipore, Madrid, Spain). Chromocult coliform agar (Merck, Darmstadt, Germany) was used for membrane incubation and pour plating. Plates were incubated for 24 h at 37 °C before interpretation of the results. Dark blue-violet colonies were considered positives for *E. coli*.

Leafy green (25 g) samples were homogenized in 100 mL of sterile 0.2% buffered peptone water (BPW, Scharlab, Barcelona, Spain) to quantify culturable *E. coli*. The homogenate was serially diluted, and 1 mL aliquots were pour plated using Chromocult coliform agar (Merk). Incubation of the plates and interpretation of results were performed as explained before for water samples. The limit of detection for *E. coli* enumeration in the water samples obtained from the WWTP inlet was 3 log cfu/100 mL (1000 cfu/100 mL) while for the rest of the water samples (e.g. WWTP outlet, water reservoir and irrigation water), the limit of detection was 0 log cfu/100 mL (1 cfu/100 mL). In the case of leafy green samples, the limit of detection was 0.7 log cfu/g (5 cfu/g).

2.6. Detection of spores of C. perfringens spores

Clostridium perfringens spores were examined following the protocol established in the UNE-EN ISO 14189: 2017 standard, using Tryptose Sulfite Cyclosserine (TSC; Oxoid, Basingstoke, UK) agar and fluorescent supplement (TSCF; Oxoid) according to the manufacturer's instructions. To enumerate the spores, aliquots (100 mL) of water samples were heated at 60 °C ± 2° for 15 min. Afterward, samples were filtered through 0.45 μm membrane filters (Sartorius) using a filter holder manifold (Millipore). The filters were placed on the TSCF agar plate. The plates were anaerobically incubated under a CO_2 atmosphere (Anaero-Pack® system, Oxoid.) in anaerobic jars at 44 °C for 24 h. After incubation, plates were examined under a UV light lamp, and black or light brown colonies were counted as positive colonies when emitted fluorescence. Results are expressed in cfu/100 mL. In all water samples, the limit of detection for *C. perfringens* spores was 0 log cfu/100 mL (1 cfu/100 mL).

2.7. Detection of pathogenic microorganisms

In the case of water samples from the WWTP inlet, the presence of pathogenic microorganisms was detected using selective culture media (Truchado et al., 2018). Briefly, serial dilutions of water samples were cultured on CHROMAgar STEC, CHROMAgar O157:H7 and IBISA (Oxoid), respectively. Water samples from the WWTP inlet that show counts above the LOQ (1000 cfu/100 mL) for STEC, *E. coli* O157:H7 and *Salmonella* spp., were confirmed using selective media and latex confirmation. In the case of *Salmonella*, green colonies were picked from the IBISA plates and transferred to broth and confirmed as *Salmonella* via LATEX (Oxoid). In the case of STEC and *E. coli* O157, a single pink and mauve colony was picked from CHROMagar STEC and CHROMagar O157 plate, respectively, and confirmed by Latex Confirmation (Oxoid).

For the detection of non-O157:H7 Shiga-toxigenic *E. coli* (STEC), *E. coli* O157:H7 and *Salmonella* spp. in the water samples from WWTP outlet, WWTP reservoir, grower reservoirs and irrigation, confirmation was performed using molecular methods. In this case, 10-L samples were filtered through Modified Moore Swabs (MMS) as previously described (Sbodio et al., 2013). The MMS were transferred aseptically into sterile stomacher plastic bags and transported to the lab in refrigerated conditions. Once in the lab, 200 mL of 20 g/L BPW (Scharlab) was added to the bags, and samples were incubated for 24 h at 37 °C for pre-enrichment. After incubation, pre-enriched samples were supplemented with 30% glycerol and maintained at −20 °C until the analyses were performed. For the leafy green analyses, the homogenate previously described was supplemented with 125 mL of 20 g/L BPW (Scharlab). Samples were homogenized by massaging the stomacher bags by hand. Afterward, bags were incubated for 24 h at 37 °C for pre-enrichment. Pre-enriched samples were supplemented with 30% glycerol and maintained at −20 °C until further analysis. For STEC and *E. coli* O157:H7, 1 mL of these pre-enriched samples was enriched in 9 mL of brain heart infusion (BHI; Scharlab) broth for 4 h at 37 °C to resuscitate injured cells. After that, 1 mL of BHI was transferred into 9 mL of Modified Buffered Peptone Water (20 g/L) supplemented with pyruvate (mBPWp; Scharlab) and incubated for 24 h at 42 °C. For *Salmonella*, 1 mL of these pre-enriched samples was enriched in 9 mL of trypticase soy broth (TSBn; Scharlab) and incubated for 4 h at 37 °C. One mL aliquot

Table 1
Spearman rank correlations between *E. coli* level and weather parameters in each sampling site in different sampling points of the water reuse system.

		Inlet	WWTP outlet	WWTP reservoir	Grower reservoir	Irrigation	Crop
Spray							
Temperature (°C)	Coefficient	−0.275	−0.136	0.080	−0.045	0.221	−00126*
	Sig	0.241	0.689	0.754	0.894	0.395	0.788
Solar radiation (W/m^2)	Coefficient	−0.148	−0.136	0.264	−0.464	−0.306	0.198
	Sig	0.533	0.689	0.289	0.151	0.232	0.670
Relative humidity	Coefficient	−0.006	0.391	−0.339	−0.127	−0.226**	0.432
	Sig	0.981	0.235	0.168	0.709	0.384	0.333

* Significant correlations at $P < 0.05$.
** Significant correlations at $P < 0.01$.

was transferred into 9 mL of tetrathionate broth base, Hajna supplemented with 1 mL of iodine solution (TT; Scharlab), and incubated overnight at 44 °C. RT-PCR analyses were performed to detect the presence of STEC, *E. coli* O157:H7, and *Salmonella* spp. in water (n = 370) and leafy green samples (n = 100) using the *Salmonella*-STEC GeneDisc Pack in a Genedisc Cycler (Pall Corporation, Port Washington, USA) following manufacturer instructions. The limit of detection was estimated using DNA from *E. coli* O157:H7 (CECT 4782) and *Salmonella* (CECT 4625). Strains were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain). The LOD was determined based on the concentration that demonstrated a positive result at a particular dilution. The LOD values for each PCR product were 10.3 cells/μL and 182 cells/μL for pathogenic *E. coli* and *Salmonella,* respectively. The positive samples detected by GeneDisc were cultured and confirmed in selective culture media, as previously described (Truchado et al., 2018).

2.8. Confirmation of presumptive isolates obtained from cultured-based methods

A conventional polymerase chain reaction (PCR) method was performed for the confirmation of *E. coli* O157:H7 and STEC isolates, using a ProFlex™ PCR System (Applied Biosystems® thermal cycler). Strains were tested by PCR with specific primers for confirming the presence of stx1, stx2, eae, O157-rfbE and fliC-H7 genes (Table 1). The primer sequences and main PCR conditions are listed in Table 1. For each PCR, a positive (reference *E. coli* O157:H7, ATCC 43895) and a negative (sterile distilled water) controls were included. Template DNA for PCR was prepared by boiling method as described elsewhere (Blanco et al., 2003; Blanco et al., 2004). The PCR products were run on 2% agarose gels (SeaKem LE agarose, Lonza) in Tris–borate–EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) at 130 V for 20–40 min and visualized on a UV transilluminator (Gel Documentation System, Bio-Rad) after staining with GelRed™ Nucleic Acid Gel Stain (Biotium).

The isolated *Salmonella* strains were submitted to identification by MALDI-TOF mass spectrometry, using the Biotyper platform (Bruker Daltonics, Germany). For that the protocol recommended by the manufacture has been applied. Briefly, isolated colonies grown on tryptic soy agar have been spotted onto a target plate and overlayed by 1 μL of 70% formic acid. After drying, sample spots were overlaid with 1 μL of 10 mg/mL of α-cyano-4-hydroxycinnamic acid (HCCA) solution (Freiwald and Sauer, 2009). Mass spectral analysis has been carried out using a Microflex LT mass spectrometer and FlexControl V3.0 software (Bruker Daltonics, Germany). For external calibration 1 μL of a Bacterial Test Standard (*Escherichia coli* DH5α) (Bruker Daltonics, Germany) was spotted onto the same target plate. Spectra were achieved in the mass range between 2000 and 20 000 Da, applying a laser frequency of 60 Hz, an acceleration voltage of 20 kV and extraction delay time of 120 ns. Two colonies have been analyzed for every sample and identification has been carried out in duplicate by the MALDI BioTyper™ software (Bruker Daltonics, Germany). For correct species identification, confidence threshold scores above 2 are considered as high confidence identification (Sauer et al., 2008).

2.9. Logistic regression

Bacterial cell concentration (log cfu/100 mL) and prevalence (number of positive samples / total samples × 100) data were modelled using logistic regression. The presence/absence of each pathogen (STEC, *E. coli* O157:H7, and *Salmonella*) was examined as a dependent variable and the concentration of *E. coli* (log cfu/100 mL) as an independent variable. The logistic regression model is shown in Eq. (1), where p stands for the probability of the pathogen of interest being present, x is the concentration of *E. coli* (in log cfu/100 mL) and β_0 and β_1 are the intercept and the slope coefficients.

$$\text{logit}p = log\frac{p}{p-1} = \beta_0 + \beta_1 x \quad (1)$$

Due to identifiability issues for the *E. coli* model, the models were fitted using Bayesian regression with slightly regularizing priors (normal distributions with mean 0 and standard deviation 10 for both the intercept and the slope of the logistic regression model). The models were fitted using the Hamiltonian Monte Carlo sampler Stan (Carpenter et al., 2017) using the interface implemented in the Rstan package (Stan Development Team, 2019) for R version 3.6.3 (R Core Team, 2020).

2.10. Statistical analysis

E. coli in water and leafy green samples were $\log_{10}$ transformed and expressed as log cfu/100 mL or gram, respectively. Calculation and graphical representation of the median and interquartile range (IQR) of microbial counts were performed using Sigma Plot 13 Systat Software, Inc. (Addilink Software Scientific, Barcelona, Spain). Samples with the observed zero count in the enumeration analysis were substituted with the LOQ (1 cfu/100 mL) before $\log_{10}$ transformation to calculate the average levels of microorganisms in the water samples. For calculation and graphical presentation of the median IQR all microbial counts have been taken into consideration, including samples with the observed zero count, which was substituted with the LOQ. The parameter log (N/N0) was used to evaluate the reduction level, where N = number of bacteria after the disinfection treatment in WWTP and N_0 = the number of bacteria before the disinfection treatment. IBM SPSS statistics 25 was used for statistical analysis.

Except when stated otherwise, P values below 0.05 were considered statistically significant. The Kolmogorov-Smirnov test and Levene's test were used to assess normality and equality of variance, respectively. When normality could be assumed, an ANOVA test was carried out, and when significant differences were observed, the Tukey's HSD (Honestly Significant Difference) test was applied. When data did not follow a normal distribution, Man-Whitney and Krustal-Wallis tests were used. The Spearman correlation coefficient was calculated between *E. coli* loads and weather parameters, including temperature (°C), solar radiation (W/m^2), and relative humidity (%).

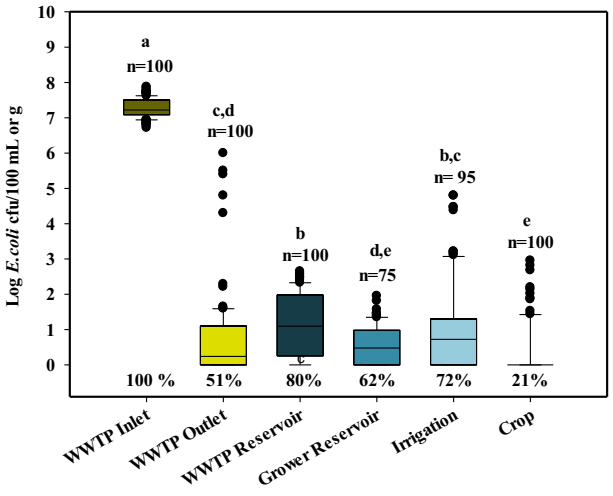

Fig. 2. Levels of *E. coli* (log cfu/100 mL) in each sampling site through the water reuse system. Box plots show median concentrations (log cfu/100 mL or g) with the 25th and 75th percentile values of *E. coli*. The percentage indicates the presence of *E. coli* in each sample. Box plots labelled with different letters show significant differences at $P < 0.05$.

3. Results and discussion

3.1. The fate of E. coli in water reuse system and irrigated leafy greens with reclaimed water

Prevalence and levels of *E. coli* in water and leafy green samples at each sampling point are shown in Fig. 2. Average levels of *E. coli* in the WWTP inlet samples were 7.3 ± 0.3 log cfu/100 mL. These high concentrations are within the same range as the data reported in previous studies on sewage (Barrios-Hernández et al., 2019; Chahal et al., 2016; Servais et al., 2007). Reclamation treatments (primary, secondary and tertiary treatments) applied in the WWTPs included in this study significantly reduced *E. coli* loads in reclaimed water samples. Average *E. coli* levels of all the samples from the WWTP effluents were 0.73 ± 1.20 log cfu/100 mL (Fig. 2). The *E. coli* levels observed in the effluent samples were below the MQR established for this indicator bacteria (<10 cfu/100 mL) in the European regulation at the 'point of compliance', where a reclamation facility operator delivers reclaimed water to the next actor in the chain (EU, 2020/741). Therefore, the reclaimed water generated in the WWTPs included in this study complied with the MQR included in the new European regulation for water reuse in agricultural irrigation (EU, 2020/741). Several studies on the use of UV-C and NaClO as tertiary treatments reported similar reductions (Bischoff et al., 2013; Makuwa et al., 2020). Fig. 2 shows several outliers points (above 4 log cfu/100 mL) at the WWTP effluent samples which have been linked with specific failures of the tertiary treatments in one of the WWTPs.

Average *E. coli* levels were 1.2 ± 0.9 log cfu/100 mL and 0.4 ± 0.5 log cfu/100 mL in WWTP and grower's water reservoirs, respectively (Fig. 2). It was observed that the prevalence of *E. coli* was higher in water samples obtained from the water reservoirs when compared to the WWTP outlet (Fig. 2). The increase in the prevalence as well as in the *E. coli* levels from the WWTP effluent to the water reservoirs could be explained by different hypothesis. Based on the conclusions raised by Jjemba et al. (2014), monitoring reclaimed water immediately after treatment does not provide a true representation of quality at the point of use because storage and distribution cause deterioration in water quality. Deterioration of water quality during storage in reservoirs and the distribution network is a major challenge for the industry mostly due to the presence of high bacterial levels in distribution pipes, poor microbial quality in storage facilities due to sediment or biofilm accumulation and animal intrusion (Jjemba et al., 2014). In this study, the distribution/storage systems consist in open canals and reservoirs, which might contribute to the deterioration of the water quality.

On the other hand, some authors have described that abiotic factors such as temperature and solar radiation could play a significant role in the microbial quality of water stored in reservoirs (Lebaron et al., 2015). In the present study, there was no correlation between climatic data, including temperature (°C), solar radiation (W/m^2) and relative humidity (%) during the growing cycle of leafy greens and the *E. coli* levels in water reservoirs (Table 1). In agreement with these results, a previous study conducted in the Region of Murcia (Spain) did not show a significant correlation between *E. coli* loads in water samples from a water reservoir and the climatic conditions (temperature and solar radiation) (Truchado et al., 2018). Contrarily, other researchers found a correlation between weather parameters and *E. coli* levels in agricultural water samples (Francy et al., 2013; Weller et al., 2020). Belias et al., 2020 evaluated the impact of weather from three different climatic regions (California, New York, and Spain) on the die-off rate of *E. coli* and *Salmonella* on spinach and lettuce under field conditions over 2 years. They found that an increase in relative humidity was associated with a decrease in microbial die-off. All these results indicate that the direct comparison of climatic factors with *E. coli* count is very complex and may rely on other factors such as geographical location, water source, time of sampling, and method of bacteria identification (Rodrigues et al., 2020).

At the end-user point, where reclaimed water was used for irrigation of commercial crops, levels of *E. coli* (1.0 ± 1.2 log cfu/100 mL) were higher than in the effluent water samples. In agreement with these results, a recently published study showed that prevalence and levels *E. coli* in reclaimed water used as irrigation water were 83% and 2.51 ± 0.80 log cfu/100 mL, respectively (Rusiñol et al., 2020). Based on the

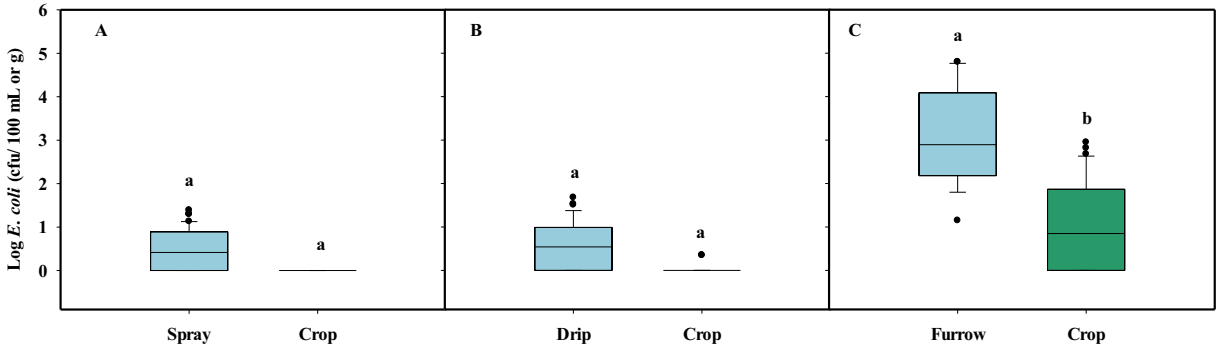

Fig. 3. Levels of *E. coli* (log cfu/100 mL) in the different irrigation systems and leafy greens irrigated with reclaimed water A) overhead, B) drip and C) furrow. Box plots show median concentrations (log cfu/ 100 mL or g) with the 25th and 75th percentile values of *E. coli*. Box plots labelled with different letters indicate significant differences at $P < 0.05$.

Table 2
Pathogen microorganisms in each sampling site.

Samples type	Non-O157:H7 STEC			*E. coli* O157:H7			*Salmonella*		
	Positive[a]	Confirmed[b]	Prevalence[c]	Positive	Confirmed[b]	Prevalence	Positive	Confirmed[d]	Prevalence
WWTP inlet	100/100	–	100	100/100	–	100	100/100	–	100
WWTP outlet	2/100	0/2	0	0/100	–	0	5/100	5/5	5
WWTP reservoir	1/100	0/1	0	0/100	–	0	1/100	1/1	1
Grower reservoir	1/75	0/1	0	0/75	–	0	0/75	–	0
Irrigation	14/95	0/14	0	0/95	–	0	0/95	–	0
Crop	3/100	0/3	0	0/95	–	0	0/95	–	0

[a] Positive samples by Genedisc and selective media.
[b] Positive samples by gene: *vt*1, *vt*2, and *eae*.
[c] Prevalence of confirmed positive in samples (%).
[d] Confirmed by MALDITOF.

Commission guidance document to address the microbiological risks in fresh fruit and vegetables at primary production (EC, 2017/C_163/01), the MQR for irrigation water at the end-user point, where reclaimed water is used for agricultural irrigation, should be below 2 log cfu/100 mL if irrigation water is in direct contact with the edible part of the crop and it is consumed raw. Levels of *E. coli* levels of about 2 log cfu/100 mL have been associated with a low probability of finding pathogenic microorganisms in irrigation water (Ceuppens et al., 2015). Truchado et al. (2018) reported that *E. coli* levels higher than 2.35 log cfu/100 mL in irrigation water were able to predictive the presence of pathogens using cultivable techniques. Fig. 2 shows several outlier points (>2 log cfu/100 mL) in the water samples taken at the irrigation point. These outline points have been linked to a specific cross-contamination event within the distribution/storage system associated to the presence of cattle close to the open canals used for the distribution of the water. This source of cross-contamination have been already highlighted as a critical point because distribution canals are open to the environment, and contamination from adjacent farms or wild animals could impact the microbiological quality of the water (Mogren et al., 2018). On average, leafy green samples showed very low level of *E. coli* (0.3 ± 0.7 cfu/g). However, if we only focused on the *E. coli* levels of those samples with counts above the LOQ (0.7 log cfu/g), which represent 21% of the total samples, *E. coli* levels were 1.4 ± 0.8 log cfu/g. The observed levels were within the microbiological limits establish for the European legislation for ready-to-eat fruits and vegetables (EC, 2073/2005). Similar levels of *E. coli* were detected by Tombini-Decol et al. (2019) in baby lettuce after irrigation with reclaimed water treated with ClO_2.

The evaluated water reuse systems included three different irrigation systems. The data obtained in the irrigated crop when different irrigation systems were used showed that when high levels of *E. coli* are present in the irrigation water, the use of an irrigation system that allows the contact between the water and the edible part of the crop increases the risk of contamination. This has been the case of the furrow irrigation system (Fig. 3). Previous studies have already demonstrated that furrow irrigation increases the likelihood of direct contact between water contaminated with pathogens and the edible parts of crops (Balkhair, 2016; Song et al., 2006).

The results obtained showed that the reclaimed water generated in the water reuse systems included in this study represents a good alternative source for irrigation. Despite the potential cross-contamination of the reclaimed water during distribution and storage, the levels of *E. coli* in the reclaimed water, irrigation water and the leafy greens were within the MQR. Therefore, efforts should be focused on i) the effectiveness of the reclamation treatments and ii) the whole water reuse system, including the storage, distribution, and endpoint practices.

The prevalence of enteric foodborne pathogens in water and leafy green samples are shown in Table 2. All water samples from the WWTP inlet were positive for pathogenic bacteria, including STEC, *E. coli* O157:H7, and *Salmonella*. These results were confirmed by selective media and subsequent latex agglutination tests (where applicable). In the rest of the water samples (WWTP effluents, water from reservoir and irrigation water) as well as in the leafy greens, results confirmed by conventional PCR, STEC and *E. coli* O157:H7 were not found present in any reclaimed water samples or leafy greens. The prevalence of *Salmonella* was 5% (5/100) in the reclaimed water and only in 1% (1/100) of samples taken from the water reservoirs. Recently, Sharma et al. (2020) also observed a lower prevalence of *Salmonella* recovered from reclaimed water than in other irrigation sources such as pond or river.

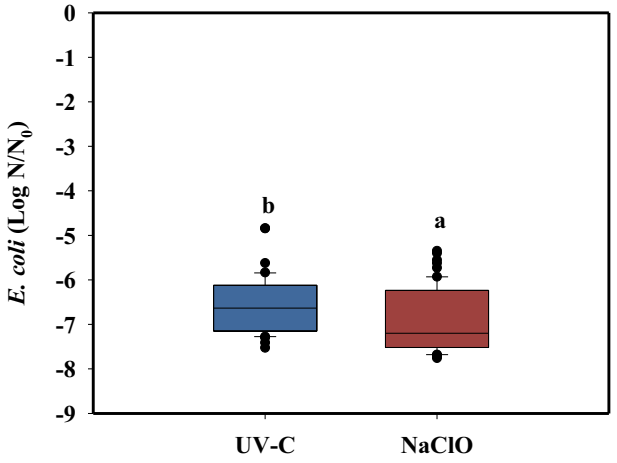

Fig. 4. Reduction of *E. coli* levels after UV-C and chlorine disinfection treatments. The 0 log (N/N0) indicates no reduction of *E. coli* in water. Box plots show median with the 25th and 75th percentile values of reduction. Box plots labelled with different letters indicate significant differences among treatments at $P < 0.05$.

In the case of STEC, water and leafy green samples positive after the GeneDisc tests and selective media were not confirmed by conventional PCR when the gene *vt1, vt2* and *eae* were analyzed. One possible reason could be the spontaneous loss of *vtx* genes from the isolate after the first sub-cultivation step (Joris et al., 2011). This fact is a limitation of the present study. To avoid false-negative results, more colonies were needed to be isolated and analyzed for the presence of *vtx* genes.

3.2. Efficacy of the tertiary treatment

UV-C light and chlorination are widely used and well-characterized disinfection processes in WWTP. In the present study, the efficacy of these tertiary treatments were compared. For that purpose, in each WWTP between 2017 and 2019, *E. coli* reductions between the WWTP influent, as the initial point, and the WWTP effluent (reclaimed water) as the final point were calculated. Results showed that UV-C and chlorine disinfection treatments significantly reduced the levels of *E. coli* in reclaimed water (Fig. 4). However, *E. coli* inactivation was slightly different between UV-C and chlorine, achieving 6.6 and 7.5 log/100 reductions, respectively. Several studies have already demonstrated that

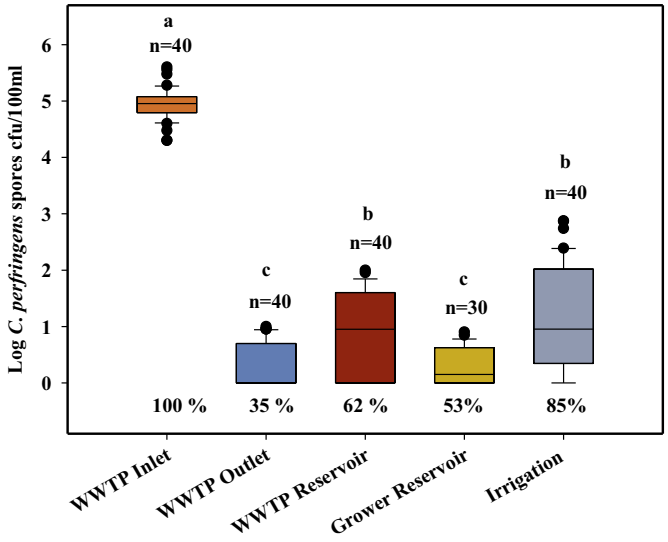

Fig. 5. Levels of *C. perfringens* spores (log cfu/100 mL) in each sampling site of the water reuse system Boxplots show median concentrations (log cfu/100 mL or g) with the 25th and 75th percentile values of *C. perfringens* spores. Percentage indicates the presence of *C. perfringens* spores in each sample. Box plots labelled with different letters indicate significant differences at $P < 0.05$.

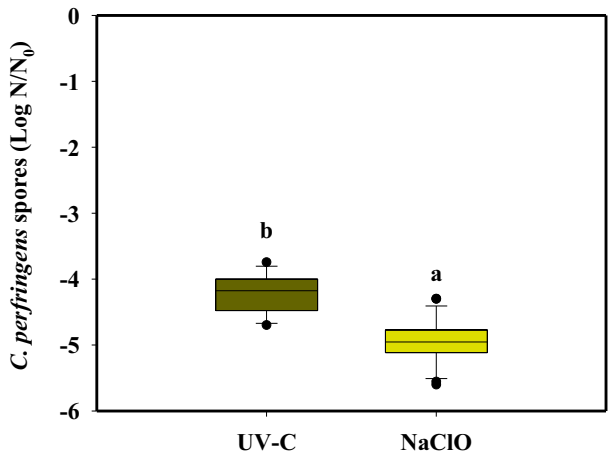

Fig. 6. Reduction of *C. perfringens* spores after UV-C and chlorine disinfection treatments. The 0 log (N/N0) indicates no reduction of *C. perfringens* spores in water. Box plots show median with the 25th and 75th percentile values of reduction. Box plots labelled with different upper case letters indicate significant differences between treatments at $P < 0.05$. Lower case letters indicate significant differences between *C. perfringens* spores at $P < 0.05$ for the same treatment.

both disinfection treatments are efficient in reducing the levels of *E. coli* in the effluent water (Bischoff et al., 2013; Collivignarelli et al., 2018; Owoseni et al., 2017). UV-C light seems to be less effective for *E. coli* than chlorine in agreement with our results (Collivignarelli et al., 2018). However, there should be taking into account that the efficacy of disinfection treatments depends on the doses applied and the physicochemical characteristics of the wastewater (e.g. turbidity, total suspended solids, particle size, and chemical and biochemical oxygen demands) (Hassaballah et al., 2020; Mezzanotte et al., 2007). In general, the inactivation values obtained in this study demonstrated that both tertiary treatments reduced *E. coli* levels ≥5 log cfu/100 mL as stipulated in the validation of the MQR included in the new European regulation (EU, 2020/741).

3.3. Fate of C. perfringens spores in water reuse system

C. perfringens spores are commonly used as an indicator microorganism for parasites because of their similar size and also because they are widely distributed in sewage (Stelma Jr, 2018). However, as *C. perfringens* spores are more resistant to commonly use tertiary treatments (e.g. chlorine and UV-C light), more intense treatments are needed to reduce their levels compared with the ones on parasites (Momba et al., 2019). The new regulation indicates the use *C. perfringens* spores as an indicator for protozoa in water reclamation (EU 2020/7041). Results showed that all water samples obtained at the inlet of the WWTP were positive for the presence of spores showing average levels of about 5 log cfu/100 mL (Fig. 5). Several studies have reported similar *C. perfringens* spores' values in WWTP influent samples (Nasser et al., 2017; Thwaites et al., 2018). After tertiary treatments, average levels of *C. perfringens spores* detected were 0.26 ± 0.4 log cfu/100 mL, and only 35% (14/40) of the samples were detected above of the detection limit (0 log cfu/100 mL) for this indicator microorganisms. Previous studies have also demonstrated similar levels of *C. perfringens spores* in water upon disinfection (Gomila et al., 2008; Park et al., 2016; Rubiano et al., 2012). Remarkably, the presence and levels of *C. perfringens* spores significantly increased in those samples taken from the water reservoirs and the endpoint of use (Fig. 5). As mentioned for other pathogenic bacteria, this fact may be due to potential cross-contamination due to the presence of animals or soil runoff (Harris et al., 2018).

Table 3
Model parameters of the logistic regression model for each pathogen.

Pathogenic microorganism	Intercept	Slope (1/log cfu/100 mL)
Non-O157:H7 STEC	−13.63 ± 2.78	2.56 ± 0.48
E. coli O157:H7	−23.39 ± 5.51	3.98 ± 0.86
Salmonella	−6.05 ± 0.58	1.27 ± 0.12

The MQR included in the current European regulation indicates that at least a minimal reduction of 4 log/100 mL is needed to validate a reclamation technology to produce class A water (CE, 2020). To validate the efficacy of the reclamation treatments applied in the WWTPs included in the present study, the use of UV-C and chlorine as tertiary treatments were compared. Both tertiary treatments reduced the levels of *C. perfringens* spores within the limits established in the current regulation (CE, 2020) (Fig. 6). However, chlorine treatment was more effective in reducing *C. perfringens* spores that UV-C light. In agreement with our results, Nasser et al. (2006) reported that *C. perfringens* spores were more sensitive to chlorine than to UV-C irradiation. However, Nasser et al. (2017) did not find significant differences in the levels of *C. perfringens* spores after disinfection of reclaimed water by UV-C (3.6 log) or chlorine (3.3 log). As previously indicated for *E. coli,* the obtained reductions for the levels of *C. perfringens* spores depend on several factors such as the physicochemical quality of the wastewater, the applied dose, and also the sensitivity of the target microorganism to the treatment.

3.4. Logistic regression

Out of 570 observations, the presence/absence test for pathogenic STEC was positive in 100 samples (18%) for *E. coli* O157:H7 and 106 (19%) for *Salmonella*. Table 3 provides the model parameters of the logistic regression model fitted for each microorganism. The model

Table 4
Confusion matrices of the logistic regression models for each pathogen.

Pathogenic microorganism	False positives	False negatives
Non-O157:H7 STEC	3 (0.5%)	2 (0.4%)
E. coli O157:H7	1 (0.2%)	0 (0%)
Salmonella	6 (1%)	6 (1%)

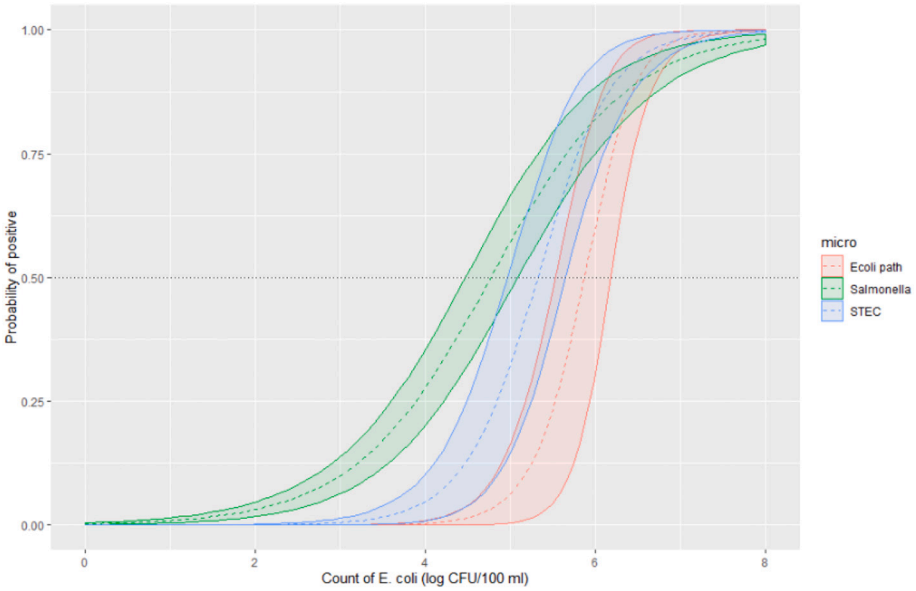

Fig. 7. Correlation between pathogenic bacteria and *E. coli* in all the samples (n = 570).

predictions had a good agreement with the data, as shown by the low number of false-positives and false-negatives (Table 4). Even in the model with the poorest accuracy (the one for *Salmonella*) the proportion of false-positives and false-negatives was lower than the 1%. Hence, we conclude the concentration of *E. coli* is a good predictor of the presence of the pathogenic species studied in our samples based on the logistic regression model.

Fig. 7 shows the predicted probability of having a positive result in the presence/absence test for each microorganism as a function of the concentration of *E. coli*. These curves can be used as basis to define a threshold concentration of *E. coli* as a predictor of the presence of a pathogenic microorganism in the sample. Using a probability of 0.5 (usually used in logistic regression), results in a threshold of 5.3 log cfu/100 mL of *E. coli* for the presence of STEC, 5.8 log cfu/100 mL for the presence of pathogenic *E. coli* and 4.8 log cfu/100 mL for the presence of *Salmonella*. These threshold values are higher than those previously described for irrigation water and fresh products following a similar approach (Truchado et al., 2018). This could be attributed to the fact that our data is divided in two large clusters: samples with a very high concentration of *E. coli* and samples with very low concentration. This property of the data makes it hard to estimate a threshold concentration of the indicator microorganism, and is common in data that was not generated in a laboratory but was obtained in actual processing conditions. Nevertheless, it must be highlighted that the goal of this model is to predict the presence of pathogenic microorganisms in the water samples of the water reuse systems studied. It is true that there is still uncertainty regarding the concentration of *E. coli* that can be used as a generic threshold in water samples. However, none of the samples analyzed had a concentration close to that threshold. Therefore, this source of uncertainty is not relevant for our case study, as illustrated by the high predictive power of our model (Table 4).

4. Conclusions

Three water reuse system from an intensive production area of Spain have been monitored to determine their compliance with the current European legislation. The microbiological quality of the reclaimed water generated in the WWTPs included in the current study complied with the MQR (<10 cfu/100 mL) at the "point of compliance", where the WWTPs delivers the reclaimed water to the next actor in the chain (EU, 2020/741). On the other hand, the microbiological quality of the water at the end-user point, where the water is used for irrigation, was also within the thresholds established in the Commission guidance document to address the microbiological risks in fresh fruit and vegetables at primary production (EC, 2017/C_163/01). The distribution/storage systems included in the water reuse system affect the microbiological quality of the water. A punctual cross-contamination of reclaimed water was observed during the distribution system, which increase the levels of *E. coli* (>4 log cfu/100 mL). It is remarkable that pathogenic bacteria were only detected in 1.3% of the samples (6/470). On the other hand, the reductions of *E. coli* and *C. perfringens* spores in chlorinated treated effluents were higher than those treated with UV-C light treatment. This study shows that the current technologies implemented in the WWTPs produced reclaimed water within the MQR established. Regarding the potential correlation between *E. coli* and pathogenic bacteria, results indicate a good correlation between *E. coli* concentrations and pathogenic bacteria found in samples, indicating that *E. coli* can be used as an indicator for the presence of pathogenic bacteria.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109352.

Declaration of competing interest

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

Acknowledgements

The authors are thankful for the financial support from ESAMUR (Project 20180705), Center for Produce Safety, Ministerio de Ciencia e Innovación (Project PID2019-104931RB-I00) and CSIC (Intramural 201670E056). Support provided by the Fundación Séneca (19900/GERM/15) was also appreciated. P. Truchado is holding a Ramón y Cajal contract from the Ministerio de Ciencia e Innovación. The technical assistance of Macarena Moreno, Jenifer Cascales, Silvia Andújar and Natalia Hernández was very much appreciated.

References

Alcalde-Sanz, L., Gawlik, B.M., 2017. Minimum quality requirements for water reuse in agricultural irrigation and aquifer recharge — towards a water reuse regulatory

instrument at EU level. In: EUR 28962 EN. Publications Office of the European Union, Luxembourg. https://doi.org/10.2760/887727. PUBSY No. 109291.
Balkhair, K.S., 2016. Microbial contamination of vegetable crop and soil profile in arid regions under controlled application of domestic wastewater. Saudi J. Biol. Sci. 23, S83–S92.
Barrios-Hernández, M.L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2019. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. Water Res. 6, 100040.
Belias, A.M., Sbodio, A., Truchado, P., Weller, D., Pinzon, J., Skots, M., Allende, A., Munther, D., Suslow, T., Wiedmann, M., Ivanek, R., 2020. Effect of weather on the die-off of *Escherichia coli* and attenuated *Salmonella enterica* serovar *Typhimurium* on preharvest leafy greens following irrigation with contaminated water. Appl. Environ. Microbiol. 86 (17), e00899.
Bischoff, J., Fan, H., Cornel, P., Wagner, M., Ma, L.M., 2013. Disinfection of treated wastewater as an essential purification step for safe urban reuse: a comparative pilot study of UV- and ClO_2-disinfection systems for urban reuse applications in China. J. Water Reuse Desal. 3, 325–335.
Blanco, J.E., Blanco, M., Alonso, M.P., Mora, A., Dahbi, G., Coira, M.A., Blanco, J., 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J. Clin. Microbiol. 42, 311–319.
Blanco, M., Blanco, J.E., Mora, A., Rey, J., Alonso, J.M., Hermoso, M., Hermoso, J., Alonso, M.P., Dahbi, G., González, E.A., Bernárdez, M.I., Blanco, J., 2003. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. J. Clin. Microbiol. 41, 1351–1356.
Carpenter, B., Gelman, A., Hoffman, M.D., Lee, D., Goodrich, B., Betancourt, M., Brubaker, M., Guo, J., Li, P., Riddell, A., 2017. Stan: a probabilistic programming language. J. Stat. Softw. 76, 1–32.
Ceuppens, S., Johannessen, G.S., Allende, A., Tondo, E.C., El-Tahan, F., Sampers, I., Jacxsens, L., Uyttendaele, M., 2015. Risk factors for *Salmonella*, Shiga toxin-producing *Escherichia coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries. Int. J. Environ. Res. Public Health 12, 9809–9831.
Chahal, C., van den Akker, B., Young, F., Franco, C., Blackbeard, J., Monis, P., 2016. Pathogen and particle associations in wastewater: significance and implications for treatment and disinfection processes. Adv. Appl. Microbiol. 97, 63–119.
Collivignarelli, M.C., Abbà, A., Benigna, I., Sorlini, S., Torretta, V., 2018. Overview of the main disinfection processes for wastewater and drinking water treatment plants. Sustainability. 10, 86.
Drewes, J.E., Hübner, U., Zhiteneva, V., Karakurt, S., 2017. Characterization of unplanned water reuse in the EU. https://ec.europa.eu/environment/water/pdf/Report.
EC, 2017. European Commission Notice No. 2017/C 163/01. Guidance document on addressing microbiological risks in fresh fruit and vegetables at primary production through good hygiene. (available at https://eur-lex. europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52017XC0523%2803%29).
EFSA Panel on Biological Hazards (BIOHAZ), 2014. Scientific opinion on the riskposed by pathogens in food of non-animal origin. Part 2 (*Salmonell*a and Norovirus in leafy greens eaten raw as salads). EFSA J. 2 (3), 3600.
EU, 2018. The European Committee of the Regions, Commission for the Environment, Climate Change and Energy (CoR-ENVE). Water reuse — legislative framework in EU regions. 2018. Available at: https://cor.europa.eu/en/engage/studies/Documents/Water-reuse.pdf.
EU, 2020. Regulation (EU) No 2020/741 of the European Parliament and of the Council of May 25 2020 on minimum requirements for water reuse. Off. J. Eur. Union L 177 (63), 32–55. /741.
European Commission (EC) 2073/, 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ L 338, 1–26, 22.12.2005.
FAOstat, . FAOstat. http://www.fao.org/faostat/es/#home, 2019 (2019).
Francy, D.S., Stelzer, E.A., Duris, J.W., Brady, A.M., Harrison, J.H., Johnson, H.E., Ware, M.W., 2013. Predictive models for *Escherichia coli* concentrations at inland lake beaches and relationship of model variables to pathogen detection. Appl. Environ. Microbiol. 79, 1676–1688.
Freiwald, A., Sauer, S., 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. Nat. Protoc. 4, 732–742.
Gomila, M., Solis, J.J., David, Z., Ramon, C., Lalucat, J., 2008. Comparative reductions of bacterial indicators, bacteriophage-infecting enteric bacteria and enteroviruses in wastewater tertiary treatments by lagooning and UV-radiation. Water Sci. Technol. 58, 2223–2233.
Harris, C.S., Tertuliano, M., Rajeev, S., Vellidis, G., Levy, K., 2018. Impact of storm runoff on *Salmonella* and *Escherichia coli* prevalence in irrigation ponds of fresh produce farms in southern Georgia. J. Appl. Microbiol. 124, 910–921.
Hassaballah, A.H., Bhatt, T., Nyitrai, J., Dai, N., Sassoubre, L., 2020. Inactivation of *E. coli*, *Enterococcus* spp., somatic coliphage, and *Cryptosporidium parvum* in wastewater by peracetic acid (PAA), sodium hypochlorite, and combined PAA-ultraviolet disinfection. Environ Sci. Wat. Res. Tec. 6, 197–209.
Jjemba, P.K., Johnson, W., Bukhari, Z., LeChevallier, M.W., 2014. Review of the leading challenges in maintaining reclaimed water quality during storage and distribution. J. Water Reuse, Desal. 4 (4), 209.
Joris, M.A., Verstraete, K., De Reu, K., De Zutter, L., 2011. Loss of vtx genes after the first subcultivation step of verocytotoxigenic *Escherichia coli* O157 and non-O157 during isolation from naturally contaminated recal samples. Toxins 3, 672–677.
Lebaron, P., Cournoyer, B., Lemarchand, K., Nazaret, S., Servais, P., 2015. Environmental and human pathogenic microorganisms. In: Bertrand, J.C., Caumette, P., Lebaron, P., Matheron, R., Normand, P., Sime-Ngando, T. (Eds.), Environmental Microbiology: Fundamentals and Applications. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-9118-2_15.
Makuwa, S., Tlou, M., Fosso-Kankeu, E., Green, E., 2020. Evaluation of fecal coliform prevalence and physicochemical indicators in the effluent from a wastewater treatment plant in the north-west province, South Africa. Int. J. Environ. Res. Public Health 17, 6381.
Mezzanotte, V., Antonelli, M., Citterio, S., Nurizzo, C., 2007. Wastewater disinfection alternatives: chlorine, ozone, peracetic acid, and UV light. Water Environ. Res. 79, 2373–2379.
Mogren, L., Windstam, S., Boqvist, S., Vågsholm, I., Söderqvist, K., Rosberg, A.K., Lindén, J., Mulaosmanovic, E., Karlsson, M., Uhlig, E., Håkansson, Å., Alsanius, B., 2018. The hurdle approach—a holistic concept for controlling food safety risks associated with pathogenic bacterial contamination of leafy green vegetables. A review. Front. Microbiol. 9, 1965.
Momba, M., Edbon, J., Kamika, I., Verbyla, M., 2019. Using indicators to assess microbial treatment and disinfection efficacy. In: Rose, J.B., Jiménez-Cisneros, B. (Eds.), Global Water Pathogen Project. http://www.waterpathogens.org (A. Farnleitner, and A. Blanch (eds) Part 2 Indicators and Microbial Source Tracking Markers).
Nasser, A.M., Paulman, H., Sela, O., Ktaitzer, T., Cikurel, H., Zuckerman, I., Meir, A., Aharoni, A., Adin, A., 2006. UV disinfection of wastewater effluents for unrestricted irrigation. Water Sci. Technol. 54, 83–88.
Nasser, A.M., Benisti, N.L., Ofer, N., Hovers, S., Nitzan, Y., 2017. Comparative reduction of Giardia cysts, F+ coliphages, sulphite reducing clostridia and fecal coliforms by wastewater treatment processes. J. Environ. Sci. Health A, 52144–52148.
Owoseni, M.C., Olaniran, A.O., Okoh, A.I., 2017. Chlorine tolerance and inactivation of *Escherichia coli* recovered from wastewater treatment plants in the Eastern Cape, South Africa. Appl. Sci. 7, 810.
Park, E., Mancl, K.M., Tuovinen, O.H., Bisesi, M.S., Lee, J., 2016. Ensuring safe reuse of residential wastewater: reduction of microbes and genes using peat biofilter and batch chlorination in an on-site treatment system. J. Appl. Microbiol. 121, 1777–1788.
R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://www.R-project.org/.
Real Decreto 1620/2007, 2007. Real Decreto 1620/2007 de 7 de diciembre, por el que se establece el régimen jurídico de la reutilización de las aguas depuradas. BOE, p. 294 (08.12.07).
Rodrigues, C., da Silva, A.L.B.R., Dunn, L.L., 2020. Factors impacting the prevalence of foodborne pathogens in agricultural water sources in the Southeastern United States. Water. 12 (1), 51. https://doi.org/10.3390/w12010051.
Rubiano, M.E., Agulló-Barceló, M., Casas-Mangas, R., Jofre, J., Lucena, F., 2012. Assessing the effects of tertiary treated wastewater reuse on a Mediterranean river (Llobregat, NE Spain): pathogens and indicators. Environ. Sci. Pollut. Res. Int. 19, 1026–1032.
Rusiñol, M., Hundesa, A., Cárdenas-Youngs, Y., Fernández-Bravo, A., Pérez-Cataluña, A., Moreno-Mesonero, L., Moreno, Y., Calvo, M., Alonso, J.L., Figueras, M.J., Araujo, R., Bofill-Mas, S., Girones, R., 2020. Microbiological contamination of conventional and reclaimed irrigation water: evaluation and management measures. Sci. Total Environ. 710, 136298.
Sánchez-Cerdà, C., Salgot, M., Folch, M., 2020. Reuse of reclaimed water: what is the direction of its evolution from a European perspective? In: Verlicchi, P. (Ed.), Advances in Chemical Pollution, Environmental Management and Protection, vol. 5. Elsevier, pp. 1–64.
Sauer, S., Freiwald, A., Maier, T., Kube, M., Reinhardt, R., Kostrzewa, M., Geider, K., 2008. Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS One 3 (7), e2843. https://doi.org/10.1371/journal.pone.0002843.
Sbodio, A., Maeda, S., Lopez-Velasco, G., Suslow, T.V., 2013. Modified Moore swab optimization and validation in capturing *E. coli* O157:H7 and *Salmonella enterica* in large volume field samples of irrigation water. Food Res. Int. 51, 654–662.
Servais, P., Garcia-Armisen, T., George, I., Billen, G., 2007. Fecal bacteria in the rivers of the seine drainage network: source, fate and modeling. Sci. Tot. Environ. 375, 152–216.
Sharma, M., Handy, E.T., East, C.L., Kim, S., Jiang, C., Callahan, M.T., Allard, S.M., Micallef, S., Craighead, S., Anderson-Coughlin, B., Gartley, S., Vanore, A., Kniel, K. E., Haymaker, J., Duncan, R., Foust, D., White, C., Taabodi, M., Hashem, F., Parveen, S., May, E., Bui, A., Craddock, H., Kulkarni, P., Murray, R.T., Sapkota, A.R., 2020. Prevalence of *Salmonella* and *Listeria monocytogenes* in non-traditional irrigation waters in the Mid-Atlantic United States is affected by water type, season, and recovery method. PLoS One 15 (3), e0229365.
SIAM, 2019. Sistema de Información Agraria de Murcia. In: Informes agrometeorológicos. http://siam.imida.es/apex/f?p=101:46:4204976461560039.
Song, I., Stine, S.W., Choi, C., Gerba, C.P., 2006. Comparison of crop contamination by microorganisms during subsurface drip and furrow irrigation. J. Environ. Eng. 132, 1243–1248.
Stan Development Team, 2019. RStan: the R interface to Stan. http://mc-stan.org/.
Stelma Jr., G.N., 2018. Use of bacterial spores in monitoring water quality and treatment. J. Water Health 16491–16500.
Thwaites, B.J., Short, M.D., Stuetz, R.M., Reeve, P.J., Alvarez-Gaitan, J.P., Dinesh, N., van den Akker, B., 2018. Comparing the performance of aerobic granular sludge versus conventional activated sludge for microbial log removal and effluent quality: implications for water reuse. Water Res. 145, 442–452.
Tombini-Decol, L., López-Gálvez, F., Truchado, P., Tondo, E.C., Gil, M.I., Allende, A., 2019. Suitability of chlorine dioxide as a tertiary treatment for municipal

wastewater and use of reclaimed water for overhead irrigation of baby lettuce. Food Control 96, 186–193.
Truchado, P., Hernandez, N., Gil, M.I., Ivanek, R., Allende, A., 2018. Correlation between *E. coli* levels and the presence of foodborne pathogens in surface irrigation water: establishment of a sampling program. Water Res. 128, 226–233.
Weller, D., Brassill, N., Rock, C., Ivanek, R., Mudrak, E., Roof, S., Ganda, E., Wiedmann, M., 2020. Complex interactions between weather, and microbial and physicochemical water quality impact the likelihood of detecting foodborne pathogens in agricultural water. Front. Microbiol. 11, 134.