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Suitability of chlorine dioxide as a tertiary treatment for municipal wastewater and use of reclaimed water for overhead irrigation of baby lettuce

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ABSTRACT

Reclaimed wastewater used for agricultural irrigation should meet specific microbiological standards in order to prevent microbial contamination of the irrigated produce. The objective of this study was to evaluate the suitability of chlorine dioxide (ClO_2) for the disinfection of secondary-treated municipal wastewater and its subsequent use for overhead irrigation in greenhouse production of baby lettuce. The impact of reclaimed water tertiary-treated with ClO_2 on *E. coli* concentration, the presence of pathogenic bacteria, and the occurrence of chlorates as disinfection by-products was evaluated in water and in baby lettuce. *E. coli* was quantified using both conventional plating methods and a quantitative real time PCR (qPCR) method with propidium monoazide (PMA) pre-treatment to differentiate between viable and non-viable bacteria. Population density of cultivable *E. coli* was significantly lower ($p < 0.05$) in reclaimed water, tertiary-treated using ClO_2 (ClO_2W), when compared with secondary-treated municipal wastewater (SW). However, no significant differences in viable but non-cultivable (VBNC) *E. coli* loads were observed between treatments when quantifying using the PMA-qPCR method. These results could indicate that ClO_2 treatment of water did not kill the bacteria but it induced bacteria to enter a VBNC state. The proportion of samples positive for the presence of pathogenic bacteria was lower in ClO_2W (1/8) compared with SW (7/8). Significantly lower *E. coli* counts ($p < 0.05$) were detected in plants irrigated with ClO_2W compared with those irrigated with SW. Relationship between higher *E. coli* counts and the presence of pathogens was observed when lettuce samples were analyzed by PMA-qPCR (Mann-Whitney *U* Test, $p < 0.05$). Baby lettuce irrigated with ClO_2W showed a significantly higher concentration of chlorates than lettuce irrigated with SW. The quantification of viable bacteria using molecular methods suggests that the efficacy of ClO_2 could be overestimated when conventional plating quantification methods are used. Additionally, the accumulation of chlorates in the tissue should be considered as it represents an adverse effect of this disinfection treatment.

1. Introduction

Water reclamation and reuse for irrigation in agriculture are priority innovation practices. Water reuse is indicated by the European Commission (EC) as an important topic for the circular sustainable economy, a regenerative system in which resource input and waste, emission, and energy leakage are minimized. Currently, reclaimed water is mostly used in agricultural irrigation, especially in semi-arid and arid regions to overcome water scarcity (Becerra-Castro et al., 2015). Limited awareness of potential benefits among targeted end-users, and lack of a supportive and coherent framework for water reuse are major reasons for reducing this practice in the EU (EC, 2017a). Wastewater usually contains pathogenic microorganisms, many of which are able to survive in the environment and be transmitted to humans through fresh produce (EPA, 2004; López-Gálvez, Gil, Pedrero-Salcedo, Alarcón, & Allende, 2016a; Steele & Odumeru, 2004; Uyttendaele et al., 2015). Although reclamation treatments can improve the microbiological quality of water, the effluents of wastewater treatment plants can be vehicle of microbiological and chemical hazards that can affect the safety of irrigated vegetables (Pérez-Sautu

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et al., 2012). In fact, the microbiological safety of irrigation water is one of the most important factors to be considered in the production of leafy greens (Allende & Monaghan, 2015; Decol et al., 2017; Uyttendaele et al., 2015).

Chemical disinfection treatments are often used as tertiary treatments for the reclamation of municipal wastewater. Among the chemical disinfectants, chlorine is one of the most commonly used biocides for wastewater disinfection and irrigation water treatment. However, chlorine is highly reactive with organic matter and causes the generation of organo-halogenated disinfection by-products (e.g. trihalomethanes and haloacetic acids) (Ayyildiz, Ileri, & Sanik, 2009; Nikolaou & Lekkas, 2001; Rodriguez & Serodes, 2001). Chlorine dioxide (ClO_2) has been defined as a potential alternative to chlorine for disinfection of agricultural water. The main advantage of ClO_2 over chlorine is the formation of fewer types and lesser amounts of organo-halogenated by-products (López-Gálvez et al., 2010; Van Haute et al., 2015, 2017; Veschetti et al., 2003). However, the use of ClO_2 can lead to the presence of other DBPs such as chlorites (ClO_2^-) and chlorates (ClO_3^-) in the treated water via disproportionation reactions (AHDB, 2016). ClO_2 shows a higher oxidation capacity and bactericidal capability compared to chlorine (Hassenberg, Geyer, Mauerer, Praeger, & Herppich, 2017). Bactericidal effectiveness of ClO_2 depends on several factors, including disinfectant dose, contact time, water temperature, pH, and organic load (Junli, Li, Nanqi, Fang, & Juli, 1997; Ayyildiz et al., 2009).

Sprinkler irrigation is the most utilized irrigation system for the commercial growth of baby leaves (Oron, 2002; Pachepsky, Shelton, McLain, Patel, & Mandrell, 2011). Current Spanish and US legislation classify reclaimed water based on specific microbiological standards in different categories, each one for specific crops and irrigation system (Real Decreto 1620/2007, 2007). For example, only reclaimed water with less than 2 log CFU *E. coli*/100 mL can be applied in direct contact with the edible part of the crop using overhead irrigation.

The method of irrigation can influence how effectively pathogens, present in irrigation water, are transmitted to plants. Drip or surface irrigation can minimize contact of crops with contaminants present in irrigation water, compared with sprinkler irrigation, because the edible portions of plants are not in direct contact with water (Steele & Odumeru, 2004).

Based on these factors, the aim of present study was to evaluate the suitability of ClO_2 for the reduction of the microbiological contamination present in secondary-treated wastewater used for overhead irrigation of commercially grown baby lettuce. The effect of ClO_2 on the concentration of the fecal indicator *E. coli*, and on the presence of the bacterial pathogens Shiga-toxigenic *Escherichia coli* (STEC) and *Salmonella* spp. were assessed. These pathogenic microorganisms were selected as the most relevant foodborne pathogens on leafy greens (Ahmed, Richardson, Sidhu, & Toze, 2012; Decol et al., 2017; EFSA and ECDC, 2015; Ferguson et al., 2012). Additionally, the potential occurrence of chlorates in water and in the irrigated plants was evaluated.

2. Materials and methods

2.1. Experimental setup

Ten day-old Red oak leaf lettuce plants obtained from a local nursery (Semilleros Jimenado S.A., Torre Pacheco, Spain) were grown in a greenhouse located next to the wastewater treatment plant (WWTP) (Murcia, Spain) (37°47′48″ N, 0°57′33″ W). Data acquisition including climatological data and the irrigation system's layout have been described in previous studies (López-Gálvez, Allende, Pedrero-Salcedo, Alarcon, & Gil, 2014). In the present study, two types of water were used for overhead irrigation system. The first water type was secondary effluent from the WWTP (SW) obtained by the treatment of municipal wastewater. Secondary treatment consisted in activated sludge systems followed by coagulation-flocculation for the removal of suspended solids, colloids and organic matter present in wastewater (López-Gálvez et al., 2016b; Renault et al., 2009). The second irrigation water type consisted of secondary effluent from the WWTP treated with chlorine dioxide (ClO_2W). The plants were grown on trays with peat as substrate. A total of eight lettuce trays with 294 plants each were used for each irrigation water type.

Two preliminary tests were carried out in order to adjust the ClO_2 doses and the experimental conditions for lettuce growth. For the selection of the optimum disinfectant doses, the reduction of *E. coli* in the water and the phytotoxic effects in the plants were taken into account. During November and December 2016, a final experiment that lasted 21 days was performed using the optimum conditions previously established. In the final experiment, minimum and maximum temperatures inside the greenhouse were 14.4 °C and 28.3 °C, respectively, with an average of 17.2 °C. The relative humidity (RH) in the greenhouse ranged from 52.6% to 91.6% with an average of 76.8%. The day length during this period was approximately 10 h. The approximate total amount of irrigation water applied throughout the experiment was 1.6 m^3 per treatment. During the growing cycle, the lettuce plants were irrigated once a day for 5–15 min.

During the experiments, the concentration of culturable and presumptive viable *E. coli*, as well as the presence of pathogenic bacteria were assessed in water and lettuce samples. Additionally, residual ClO_2 concentration and other physicochemical parameters of the irrigation water such as pH, temperature, oxidation reduction potential (ORP), absorbance at 254 nm (UV254), and the concentration of chlorates (ClO_3^-) were analyzed.

2.2. Preparation, measurement, and application of chlorine dioxide

The company Servicios Técnicos de Canarias (STC S.L.U., Las Palmas de Gran Canaria, Spain) provided reagents and instructions for the preparation of the stable chlorine dioxide solution AGRI DIS® (ClO_2). A concentrated ClO_2 solution (7000 mg/L) was prepared weekly and kept in an opaque plastic jerry can at ambient temperature. Chronoamperometric measurement of ClO_2 concentration was performed using the equipment ChlordioXense® (Palintest, Gateshead, United Kingdom). A diluted ClO_2 solution (100–300 mg/L ClO_2) was prepared daily just before starting the irrigation using the concentrated ClO_2 solution and tap water. The diluted ClO_2 solution was pumped directly to the pipes using a peristaltic pump. Pipe length and contact time from the ClO_2 application point to the sprinklers were ≈50 m and ≈6 min, respectively. The ClO_2 doses applied were selected based on the results of the preliminary trials.

2.3. Sample collection

Water sampling was performed every 2–5 days over the lettuce growing cycle. Irrigation water was sampled during the irrigation of lettuce from the sprinkler emitters located at the end of the irrigation lines. Each sampling day, three to five 2-L water samples were collected using sterile plastic jars. For microbiological analysis, in order to quench residual ClO_2, 5 mL of sodium thiosulfate (17.5 g/L) were added to the ClO_2W samples. A total of n = 74 water samples (38 SW, and 36 ClO_2W samples) were collected in eight different sampling days along the lettuce growing cycle. For pathogenic bacteria analysis, one 10-L sample per treatment was collected each sampling day (n = 16). Additionally, for the measurement of chlorates, three samples (45 mL) per treatment (n = 74) were taken each sampling day.

Lettuce sampling was performed 5 times during the last two weeks of the lettuce growing cycle. Samples were taken every 2–5 days, and the last sampling was done at the end of the cycle. Each sampling day, three samples (60 g each) per treatment were aseptically taken and were transported to the lab in refrigerated conditions. A total of n = 30 lettuce samples were collected (15 samples per treatment) in five different sampling days. Lettuce samples were always taken before irrigation.

2.4. E. coli analysis

Culturable *E. coli* quantification was carried out on water and lettuce samples. For water samples, depending on the expected *E. coli* concentration, pour plating (1 mL) and/or membrane filtration (10 and 100 mL) were 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*. For lettuce samples, 25 g were homogenized in 100 mL of sterile 0.1% buffered peptone water (BPW, Scharlab, Barcelona, Spain) for the quantification of culturable *E. coli*. The homogenate was serially diluted and 1 mL aliquots were pour plated using Chromocult coliform agar. Incubation of the plates and interpretation of results were performed as explained before for water samples.

Molecular quantification of *E. coli* in irrigation water and baby lettuce was performed following the combined use of propidium monoazide (PMA, Biotium Inc, Hayward, CA, USA) and quantitative polymerase chain reaction (q-PCR) (PMA-qPCR) as previously described in Truchado et al. (2016) with some modifications. Three water samples (200 mL) per treatment (SW and ClO_2W) and sampling day were centrifuged at 3000 g for 20 min. In the case of lettuce samples, three samples (25 g each) were homogenized in 100 mL of sterile 0.1% BPW using a Stomacher at low speed for 1 min. The homogenate of each sample was centrifuged at 3000 g for 10 min. Then, each obtained pellet was activated with PMA (20 µM) and kept at −20 °C until the DNA extraction was performed. Master Pure TM Complete DNA and RNA purification kit (Epicenter, Madison, USA) following the manufacturer's instructions was used. For molecular quantification, primers and probes for detecting genes of *E. coli* 23S rRNA as well as the PMA-qPCR procedure were identical to those previously described (Truchado et al., 2016). Standard curves were made using known concentrations of genomic DNA isolated from *E. coli* CECT 515T. The *E. coli* concentration in the stock solution was verified by plating on PCA.

2.5. Detection of pathogenic microorganisms

For the detection of Shiga-toxigenic *E. coli* (STEC), *E. coli* O157:H7 and *Salmonella* spp. in water samples, 10-L samples were filtered at the greenhouse through Modified Moore Swabs (MMS) as previously described (Sbodio, Maeda, López-Velasco, & Suslow, 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 buffered peptone water (Scharlab, Barcelona, Spain) was added to the bags that were incubated for 24 h at 37 °C for enrichment. After incubation, a volume of 7 mL was transferred into 15 mL centrifuge tubes and stored at −20 °C with 30% glycerol until the analyses were performed.

For the lettuce samples analyses, the homogenate described in paragraph 2.3.1 was supplemented with 125 mL of BPW (40 g/L) and homogenized by massaging by hand the stomacher bags. Afterwards, bags were incubated for 24 h at 37 °C for enrichment, and then a volume of 7 mL was transferred into 15 mL centrifuge tubes and stored at −20 °C with 30% glycerol until the analyses were performed.

Aliquots of 1 mL from each frozen sample were added to 9 mL of selective enrichment broth specific for the target pathogens. Modified Buffered Peptone Water (20 g/L) supplemented with sodium pyruvate (Scharlau, Barcelona, Spain; mBPWp) incubated for 24 h at 42 °C was used for STEC and *E. coli* O157:H7. Tetrathionate Broth (TT; Scharlau) incubated for 24 h at 37 °C was used for *Salmonella*. Prevalence of pathogenic microorganisms in water (n = 16) and lettuce samples (n = 30) were performed using the Salmonella-STEC GeneDisc Pack in a Genedisc Cycler multiplex PCR (Pall® Corporation, WA, USA) following manufacturer instructions. Confirmation of presumptive positive samples was performed by isolation in selective culture media. For STEC, CHROMagar STEC (CHROMagar, Paris, France) incubated for 24 h at 37 °C was used. For *E. coli* O157:H7, two culture media were used: CT-SMAC (Scharlab, Barcelona, Spain) and CHROMagar O157 (CHROMagar, Paris, France), followed by further confirmation using a latex test (Oxoid, Basingstoke, UK). For *Salmonella*, the IBISA method (AES Chemunex, Bruz, France) was used, followed by further confirmation using a latex test (Oxoid, Basingstoke, UK).

2.6. Physicochemical analysis of irrigation water

Temperature, pH and ORP were measured using a multimeter (pH and redox 26, Crison, Barcelona, Spain). For measuring UV254, water was filtered through 0.45 µm syringe nylon filters (Fisherbrand-Fisher Scientific, Waltham, USA), and a UV-VIS spectrophotometer (Jasco V-630, Tokyo, Japan) and quartz cuvettes with a path length of 1 cm (Hellma, Müllheim, Germany) were used.

2.7. Presence of chlorates in irrigation water and in lettuce

Chlorates (ClO_3^-) content in water and lettuce was analyzed by LC-MS as described in Gil, Marín, Andujar, and Allende (2016), using an analytical standard of chlorate (RTC, ICS-004-100, Fluka, Sigma-Aldrich, Spain) for quantification. Areas of the peaks detected by MS were used for the quantification of chlorates. Results were expressed in mg/L and in mg/kg for water and lettuce samples, respectively.

2.8. Statistical analysis

Counts derived from microbiological analyses were log transformed and entered in an Excel spreadsheet (Microsoft Excel, 2016). Results were compiled and graphs were made using Sigma Plot 11.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). SPSS statistics 21 (IBM, Armonk, NY, USA) was used for statistical analysis at a significance level of 5% ($p = 0.05$). The Kolmogorov–Smirnov test and Levene's test were used to assess normality and equality of variance, respectively. When data was not following a normal distribution, non-parametric tests were applied. Mann–Whitney U and Kruskal–Wallis tests were used to determine the difference between the raw data of the indicators and the presence of pathogens. The Pearson's correlation coefficient was calculated ($p < 0.01$) to assess links between physicochemical characteristics of wastewater (SW and ClO_2W).

3. Results and discussion

3.1. ClO_2 treatment

In our study, the initial ClO_2 concentration applied for the treatment of the secondary wastewater ranged between 3.3 and 9.2 mg/L. These initial ClO_2 doses were necessary to reduce the levels of *E. coli* of reclaimed water below 2 log CFU/100 mL, which is the threshold recommended for irrigation water in the guidance addressing microbiological risks of fresh fruits and vegetables at primary production (EC, 2017b). Fig. 1 shows the initial and residual ClO_2 levels in the ClO_2W samples taken the days in which water and lettuce samples were taken for microbiological and physicochemical analysis. The contact time between ClO_2 and reclaimed water in the irrigation distribution system was approximately 6 min. In all the cases, the ClO_2 residual in wastewater as measured in the irrigation emitter was less than 1 mg/L (< 0.02–0.33 mg/L) to avoid any damage of phytotoxicity to the plants (WEAH, 2016).

Absorbance at 254 nm (UV254) was measured as an indicator of the organic matter content of the irrigation water distribution system. Values of UV254 for SW ranged between 0.05 and 1.36 cm^{-1}. Based on previous studies the increasing order of reactivity of some disinfectants commonly used in the treatment of wastewater with organic matter is:

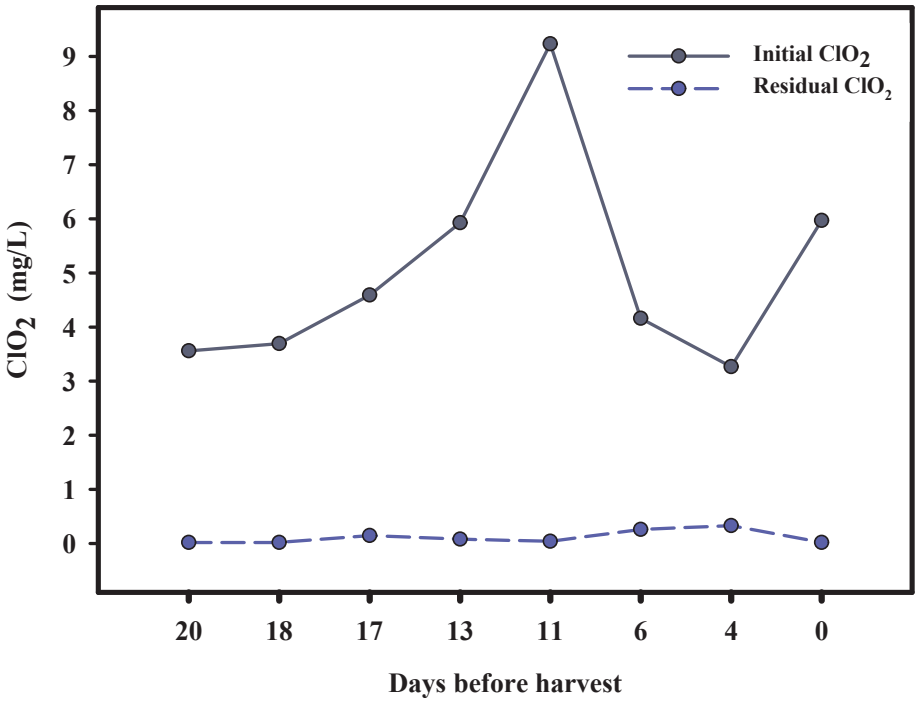

Fig. 1. Initial and residual concentrations of chlorine dioxide (ClO_2) in treated water from the secondary effluent of a wastewater treatment plant (ClO_2W) used for irrigation of lettuce cultivated in a greenhouse. The figure shows only the data obtained in days when lettuce and water samples for microbiological and physicochemical analyses were taken.

peracetic acid < ClO_2 < chlorine < ozone (Hassenberg et al., 2017; Van Haute et al., 2017; Veschetti et al., 2003). Although ClO_2 is less reactive with organic matter than chlorine (Rodriguez & Serodes, 2001; Van Haute et al., 2015), the concentration of ClO_2 is affected by the presence of organic matter. In the present study, the concentration of organic matter in the water influenced the residual ClO_2 concentration. For example, when lower concentrations of organic matter were observed (UV254 = 0.05 cm^{-1} at day 4 and 0.08 cm^{-1} at day 6), higher ClO_2 residuals were detected (0.26 and 0.33 mg/L) (Fig. 1). A significant ($p < 0.01$) negative correlation of −0,508 was found between residual ClO_2 concentration and UV254. Similar results have been described in other studies (Hassenberg et al., 2017; Praeger, Herppich, & Hassenberg, 2016; Tomás-Callejas et al., 2012; Van Haute et al., 2017).

3.2. Changes in microbiological quality of irrigation water by chlorine dioxide

In our study, when SW and ClO_2W samples were analyzed by conventional plate count methods, significant differences ($p < 0.05$) in *E. coli* counts were observed (Fig. 2A). *E. coli* counts of SW and ClO_2W samples ranged between 2.00 and 4.76 log CFU/100 mL (IQR = 3.32–3.82) and between 0.70 and 3.49 log CFU/100 mL (IQR = 1.59–2.18), respectively. Considering the mean counts of SW and ClO_2W, it was possible to calculate a mean reduction of 2.21 log CFU/100 mL. However, when *E. coli* levels were quantified using the PMA-qPCR method, the average difference in *E. coli* enumerations between SW and ClO_2W was 1.07 logarithmic units, and no significant differences were detected ($p > 0.05$) (Fig. 2B). *E. coli* levels in SW and ClO_2W samples as determined by PMA-qPCR were 3.17–6.27 log cells/100 mL (IQR 4.19–5.10) and 2.71 to 5.46 log cells/100 mL (IQR 3.66–4.54), respectively (Fig. 2B). In agreement with our results, some studies have reported that the levels of *E. coli* cells quantified by PMA-qPCR assay in different water samples such as drinking water, wastewater, irrigation water and seawater were higher than those obtained by cultivation based techniques (Gensberger et al., 2014; Li et al., 2014; López-Gálvez, Gil, Meireles, Truchado, & Allende, 2018a; Truchado et al., 2016; Van Frankenhuyzen, Trevors, Flemming, Lee, & Habash, 2013). PMA is a DNA photoreactive binding dye, which allows the differentiation between viable and dead cells, avoiding an overestimation of results by qPCR (Gensberger et al., 2014; Truchado et al., 2016). Therefore, the differences observed between PMA-qPCR and plate counts may be due to the presence of viable but not cultivable (VBNC) bacteria. The results obtained could indicate a bacteriostatic action of ClO_2, which can induce the entrance of *E. coli* cells into a VBNC state. Oliver, Dagher, and Linden (2005) reported that the VBNC stage can be induced when microorganisms are exposed to chemical disinfectants. The presence of VBNC bacteria in the reclaimed wastewater due to water reclamation processes has been demonstrated previously (Kibbee & Örmeci, 2017; Lin et at., 2016; Zhang, Ye, Lin, Lv, & Yu, 2015). Recently, Kibbee and Örmeci (2017) have evaluated the levels of *E. coli* present in secondary wastewater effluent after chlorine disinfection, showing that high numbers of VBNC *E. coli* survive chlorination. Therefore, the use of conventional plate counting methods might lead to an overestimation of the efficacy of the water disinfection treatments (Zhang et al., 2015). It should be taken into account that the only culture method used in the present study to detect injured *E. coli* cells after treatment was based in the use of a selective culture medium and incubation for 24 h at 37 °C.

Several studies have shown that different factors may influence the bactericidal effect of ClO_2 (Ayyildiz et al., 2009; Junli et al., 1997). Some of the most important are temperature, pH, and presence of organic matter. The temperature of water can strongly influence the microbial inactivation capacity of ClO_2. Barbeau, Desjardins, Mysore, and Prevost (2005) observed that 0.25 mg/L of free ClO_2 were sufficient to inactivate 99% of *E. coli* in water after 16 s at 30 °C. However, when the temperature of water was 5 °C, the same reduction was reached only after 110 s. Junli et al. (1997) reported that 10 °C increase of the water temperature doubles the inactivation power of ClO_2 against microorganisms. Although these studies reported the influence of temperature on the microbiological inactivation capacity of ClO_2, this influence was not observed in the present study. The reductions of *E. coli* observed in reclaimed wastewater demonstrated no correlation with water temperature ($p > 0.01$) and this may be due to the narrow range of temperature variation in the irrigation water (15.2–19.3 °C) (Table 1).

Globally, water demand is predicted to increase significantly over the coming decades. According to WWAP (2017), more than 70% of the water that is consumed all over the world is used for agricultural

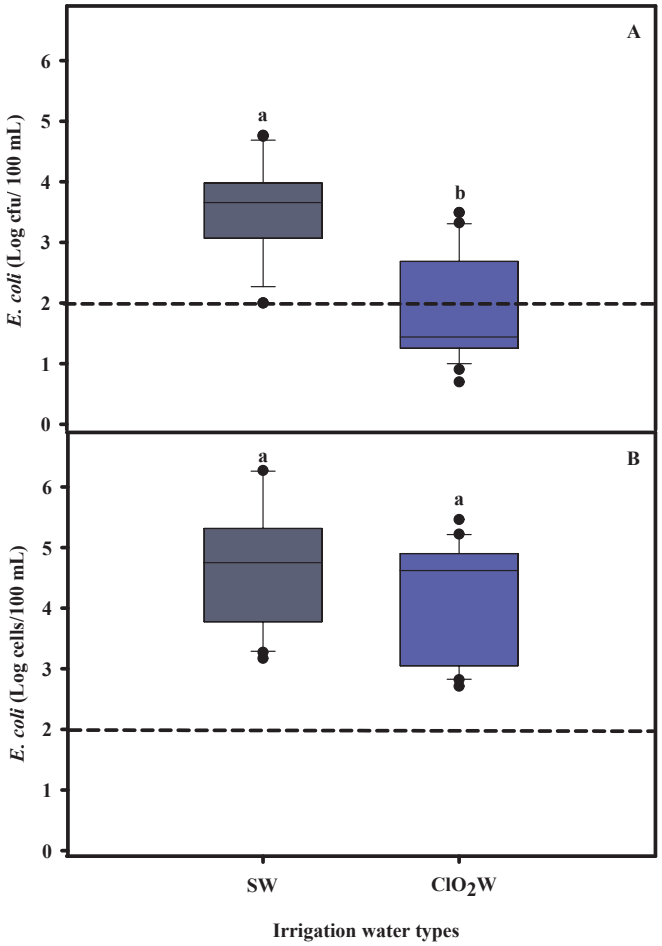

Fig. 2. Boxplot representing *E. coli* counts (log CFU/100 mL) of water from the secondary effluent of a wastewater treatment plant untreated (SW) and ClO_2 treated (ClO_2W) used for irrigation of lettuce cultivated in a greenhouse. (A) Counts obtained by conventional plate count method. (B) Counts obtained by PMA–qPCR molecular quantification method. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Different letters indicate significant differences ($p < 0.05$).

Table 1
pH, temperature, and oxidation reduction potential (ORP) of secondary effluent of a wastewater treatment plant untreated (SW) and ClO_2 treated (ClO_2W) for irrigation of baby lettuce cultivated in a greenhouse.

Days before harvest	SW			ClO_2W		
	pH	Temperature (°C)	ORP (mV)	pH	Temperature (°C)	ORP (mV)
20	6.43	19.3	472	6.36	19.1	477
18	6.34	16.7	482	6.38	16.7	493
13	6.31	19.2	463	6.57	20.0	460
11	7.60	–	–	7.75	–	–
6	8.13	16.5	431	7.95	16.9	432
4	8.01	15.2	514	7.89	15.3	791
0	7.58	16.9	450	7.51	17.0	698

irrigation. Therefore, there is a big potential for the application of reclaimed water for irrigation (WHO, 2006). To evaluate the microbiological suitability of reclaimed municipal wastewater for agricultural irrigation, the recommendations and microbiological limits described in guidelines and regulations should be used. For example, World Health Organization (WHO, 2006) recommends that wastewater used for irrigation of agricultural crops likely to be eaten raw should have a level of fecal coliforms $\leq 10^3$ CFU/100 mL. In the United States, the Food and Drug Administration (FDA) established a limit of 23 CFU/100 mL for *E. coli* concentration in agricultural irrigation water when there is a direct contact with the produce (Sugano et al., 2016). In Italy, the limit for *E. coli* concentration in treated wastewater used for agricultural irrigation is 10 CFU/100 mL (Decreto Ministeriale, 2003). Spanish legislation establishes in reclaimed water in direct contact with produce consumed raw a maximum of 10^2–10^3 CFU of *E. coli* per 100 mL in irrigation water (number of sample units (n) = 10, threshold value for the number of *E. coli* (m) = 100 CFU/100 mL, maximum value (M) = 1000 CFU/100 mL, number of sample units where the *E. coli* count may be between m and M (c) = 3) (Real Decreto 1620/2007, 2007).

In the present study, *E. coli* concentration was above the 2 log/100 mL threshold in all SW samples analyzed. On the other hand, due to the treatment with ClO_2, 69.4% of ClO_2W samples (25 out of 36) were in accordance with the Spanish legislation for *E. coli* (Real Decreto 1620/2007, 2007) based on conventional plate counting techniques. Therefore, even after ClO_2 treatment, 30.6% of the ClO_2W samples were not acceptable for being used in overhead irrigation of raw consumed leafy green vegetables according to the Spanish legislation.

3.3. Microbiological characteristics baby lettuce irrigated with reclaimed water disinfected with chlorine dioxide

Culturable *E. coli* counts in baby lettuce irrigated with SW and ClO_2W ranged between < 0.70 and 2.90 log CFU/g (IQR 0.69–1.30) and between < 0.70 and 1.40 log CFU/g (IQR 0.69–0.69), respectively, throughout the sampling period (Fig. 3A). Significant differences ($p < 0.05$) in culturable *E. coli* counts were observed between baby lettuce irrigated with SW and ClO_2W. These differences could be explained by the higher microbial contamination of SW that caused a higher contamination over the entire growing period. Similar results were demonstrated by Makkaew, Miller, Cromar, and Fallowfield (2016) and Amahmid, Asmama, and Bouhoum (1999) investigating the influence of different levels of *E. coli* contamination present in wastewater stabilization ponds in South Australia and retention of *E. coli* contamination when compared with the other types of lettuces. In our study, we used the variety Red Oak Leaf, whose morphology/topography may have contributed to the retention of *E. coli*.

E. coli enumerations using PMA-qPCR rendered counts in SW and ClO_2W irrigated lettuce of 2.17–3.83 log cells/g (IQR 2.83–3.25) and 2.18 to 3.31 log cells/g (IQR 2.55–2.98), respectively (Fig. 3B). PMA-qPCR analysis resulted in log counts around 2 logarithmic units higher than those from the same samples analyzed by the plating method in agreement with previous findings (Truchado et al., 2016). As mentioned before for water samples, the differences observed between plate count and PMA-qPCR methods could be due to the presence of VBNC *E. coli* by the biocide and the stress induced by the environmental conditions. The phyllosphere is a hostile habitat for microorganisms due to nutrient limitation, shifts in temperature, and solar radiation exposure, which can induce the VBNC state of the bacteria (Wilson & Lindow, 2000). This phenomenon should be considered with caution because bacteria can persist for long periods of time in this state and they could retain their virulent potential (Dinu & Bach, 2011).

3.4. Correlation between E. coli levels and presence/absence of pathogens

The presence of pathogens was detected by a multiplex PCR in 9 out of 16 samples of all irrigation water samples (56.2%). Among the positive samples, 8 samples were confirmed using selective media and latex agglutination tests. Seven out of eight corresponded to SW (1 *Salmonella* and 6 STEC) while only one sample of ClO_2W was positive for STEC. For the lettuce samples, the presence of pathogens was detected in 4 out of 33 samples (13.33%), by a multiplex PCR. Only 1 lettuce sample irrigated with SW was confirmed for the presence of

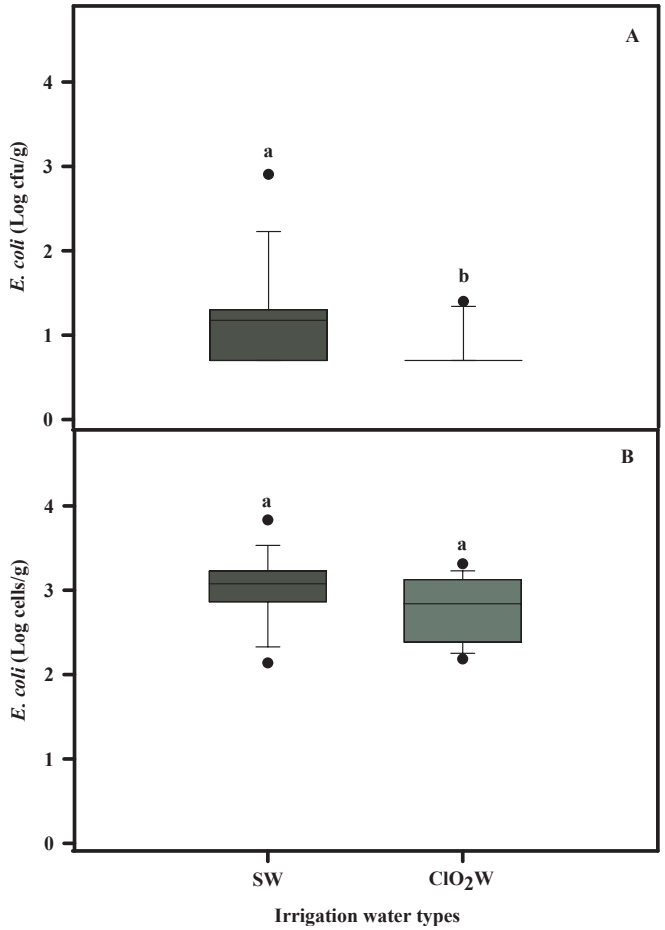

Fig. 3. Boxplot representing *E. coli* counts (log CFU/g) of baby lettuce irrigated with water from the secondary effluent of a wastewater treatment plant untreated (SW) and ClO_2 treated (ClO_2W) and cultivated in a greenhouse. (A) Counts obtained by conventional plate count method. (B) Counts obtained by PMA–qPCR molecular quantification method. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Different letters indicate significant differences ($p < 0.05$).

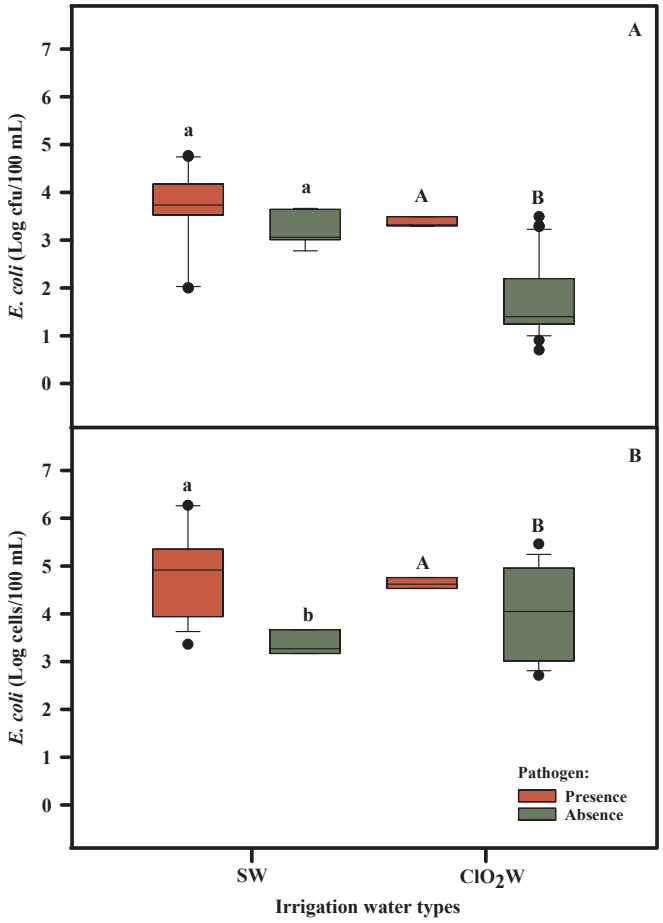

Fig. 4. Boxplot representing *E. coli* counts (log CFU/100 mL) in the subset of water samples with either absence or presence of pathogens in the water from the secondary effluent of a wastewater treatment plant untreated (SW) and ClO_2 treated (ClO_2W) used for irrigation of lettuce cultivated in a greenhouse. (A) *E. coli* counts obtained by conventional plate counting method. (B) *E. coli* counts obtained by PMA–qPCR quantification method. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box represents the median. Different letters indicate significant differences ($p < 0.05$).

STEC using selective media and latex agglutination tests. *E. coli* O157:H7 was not confirmed in any water or lettuce sample (Table 2).

Fig. 4 shows the relationship between the *E. coli* levels enumerated using plate count and PMA-qPCR methods and the presence/absence of pathogenic bacteria in irrigation water samples. For both quantification methods (plate count and PMA-qPCR) positive samples for pathogenic bacteria showed significantly higher *E. coli* levels than samples negative for the presence of pathogenic bacteria (Mann-Whitney *U* Test, $p < 0.05$) (Fig. 4B). The only exception was for SW when *E. coli* was enumerated using conventional plating methods, which could be due to the high levels of *E. coli* found in samples both positive and negative for pathogenic bacteria. Corroborating these findings, Ferguson et al. (2012) and Truchado et al. (2016) reported that molecular techniques are suitable techniques to predict the potential presence of pathogenic bacteria in groundwater, and secondary reclaimed water, respectively.

Other studies also found correlation between *E. coli* levels and the presence of STEC and *Salmonella* in agricultural settings (Ceuppens et al., 2014; Holvoet, Sampers, Seynnaeve, & Uyttendaele, 2014; López-Gálvez et al., 2014; Park et al., 2014; Truchado, Hernandez, Gil, Ivanek, & Allende, 2018). These findings support the hypothesis that considers *E. coli* as a good microbial indicator of fecal contamination and the association with enteric pathogens.

Table 2
Presence and absence of pathogen microorganisms in water samples and baby lettuce irrigated with secondary effluent of a wastewater treatment plant untreated (SW) and ClO_2 treated (ClO_2W) for irrigation during cultivation in a greenhouse.

Samples type	*Salmonella*		STEC		*E. coli* O157:H7	
Water	Genedisc®	Confirmed	Genedisc®	Confirmed	Genedisc®	Confirmed
SW	5/8	1/5	7/8	6/7	6/8	0/6
ClO_2W	1/8	0/1	1/8	1/1	1/8	0/1
Lettuce	Genedisc®	Confirmed	Genedisc®	Confirmed	Genedisc®	Confirmed
SW	2/15	0/2	2/15	1/2	1/15	0/1
ClO_2W	2/15	0/2	1/15	0/1	1/15	0/1

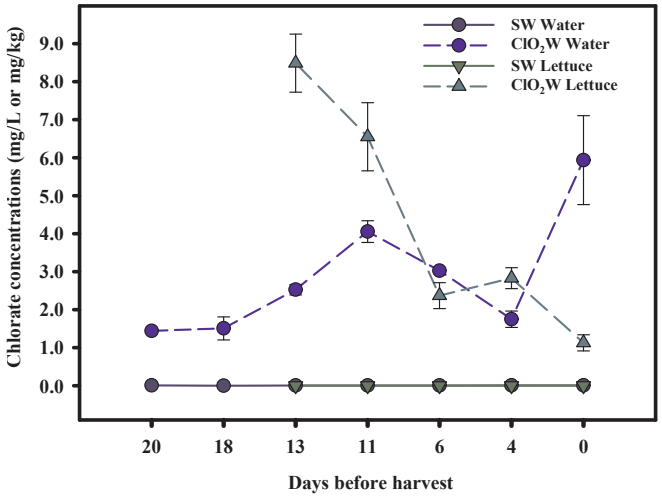

Fig. 5. Chlorate concentration in water samples (mg/L) and baby lettuce (mg/kg) irrigated with secondary effluent of a wastewater treatment plant untreated (SW) and ClO_2 treated (ClO_2W) for irrigation during cultivation in a greenhouse.

3.5. Occurrence of chlorates in chlorine dioxide treated reclaimed water and irrigated baby lettuce

ClO_2 has recently been used as an alternative to chlorine as it does not lead to the formation of chlorinated DBPs after reaction with organic matter. However, chlorite and chlorate ions (ClO_2^-, ClO_3^-) can be formed due to disproportionation reactions, and they are the main DBPs from ClO_2 that may have a significant human health risk.

When irrigation water samples were analyzed for the presence of ClO_3^-, SW showed very low levels (0.00–0.01 mg/L), while the concentration in ClO_2W ranged between 1.44 and 5.94 mg/L (Fig. 5). In other studies, lower chlorates concentrations were detected in irrigation water treated with disinfectants (López-Gálvez et al., 2018a, 2018b; Nitsopoulos, Glaumer, & Friedle, 2014). However, in those studies, water with a much better microbiological and physicochemical quality was used and, therefore, lower disinfectant concentration was needed for the treatment. In our study, due to the characteristics of the treated water, a high initial concentration of ClO_2 was needed to achieve the desired microbiological reduction (Fig. 1). The concentration of ClO_3^- showed a significant positive correlation ($p < 0.01$) of 0.65 with the initial ClO_2 concentration.

According to Korn, Andrews, and Escobar (2002), lower concentration of ClO_2 and lower levels of organic matter can reduce the presence of chlorates in treated water. In the present study, the high amount of organic matter present in the water influenced the chlorate concentration due to the higher disinfection capacity needed.

In baby lettuce irrigated with ClO_2W, concentration of ClO_3^- ranged between 1.13 and 8.49 mg/kg (Fig. 5). These levels are much higher than the maximum residue limit for chlorate allowed in the European Union in food (0.01 mg/kg; EC, 2005), although these levels are currently being revised (EC, 2014). In a previous study from our group (López-Gálvez et al., 2018a), lower chlorate concentrations were detected in baby spinach cultivated in open field and irrigated by overhead irrigation with ClO_2-treated surface water. However, much lower disinfectant concentrations were used compared with the present study, leading to lower chlorate concentration in irrigation water and in the crop. Other studies performed using irrigation water treated with chlorine-based disinfectants reported lower chlorate concentration in the crop compared with our study, probably due to the lower concentrations of disinfectants applied (López-Gálvez et al., 2018b; Nitsopoulos et al., 2014).

4. Conclusions

ClO_2 treatment reduced culturable *E. coli* concentration and the prevalence of pathogenic bacteria in the water used for irrigation. However, when viable bacteria was enumerated by molecular techniques combined with the use of PMA, no significant differences were observed between untreated and ClO_2 treated water, indicating a potential bacteriostatic action of ClO_2, which can induce the entrance of *E. coli* cells into a VBNC state. Baby lettuce irrigated with ClO_2 treated water beared lower concentrations of culturable *E. coli* than the plants irrigated with the untreated secondary effluent of the WWTP. However, as in the case of the water samples, the differences between treatments were not significant when the enumeration of *E. coli* was performed by PMA-qPCR. Detection of pathogens in lettuce was almost null, and, as a consequence, the potential effect of ClO_2 on the occurrence of pathogens in the lettuce plants could not be detected. The results obtained suggest that the use of plate count methods to estimate the efficacy of disinfection methods could lead to an overestimation of the microbial reductions. In any case, the accumulation of chlorates in the crop as a consequence of the ClO_2 treatment exceeded the maximum recommended limits of chlorates (0.01 mg/kg), making this treatment unsuitable to be applied under the conditions evaluated in the present study.

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