Research Article

Received: 12 April 2017 Revised: 4 September 2017 Accepted article published: 24 November 2017 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.8794

Demonstration tests of irrigation water disinfection with chlorine dioxide in open field cultivation of baby spinach

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Abstract

BACKGROUND: Treatments for the disinfection of irrigation water have to be evaluated by demonstration tests carried out under commercial settings taking into account not only their antimicrobial activity but also the potential phytotoxic effects on the crop. The consequences of the treatment of irrigation water with chlorine dioxide (ClO_2) used for sprinkler irrigation of baby spinach in two commercial agricultural fields was assessed.

RESULTS: Residual ClO_2 levels at the sprinklers in the treated field were always below 1 mg L^{-1}. ClO_2 treatment provoked limited but statistically significant reductions in culturable *Escherichia coli* counts (0.2–0.3 log reductions), but not in the viable *E. coli* counts in water, suggesting the presence of viable but non-culturable cells (VBNC). Although disinfected irrigation water did not have an impact on the microbial loads of *Enterobacteriaceae* nor on the quality characteristics of baby spinach, it caused the accumulation of chlorates (up to 0.99 mg kg^{-1} in plants) and the reduction of the photosynthetic efficiency of baby spinach.

CONCLUSION: Low concentrations of ClO_2 are effective in reducing the culturable *E. coli* present in irrigation water but it might induce the VBNC state. Presence of disinfection by-products and their accumulation in the crop must be considered to adjust doses in order to avoid crop damage and chemical safety risks.

Keywords: food safety; disinfection by-products; fresh produce; irrigation water; pathogen; food quality

INTRODUCTION

Fresh produce can be linked to foodborne outbreaks of pathogens.[1,2] Irrigation water is one of the vectors involved in the contamination of produce with human pathogenic microorganisms.[3,4] Control of plant disease, algae and biofilms in the irrigation systems are the primary reasons for growers adopting water-treatment technologies.[5] However, these technologies could also reduce risks linked to the occurrence of human pathogenic microorganisms in irrigation water.[6–8]

One of the technologies that can be used for irrigation water disinfection is ClO_2. In drip irrigated fields, ClO_2 can be dosed to avoid clogging of irrigation lines, achieving at the same time a beneficial effect in reducing faecal indicators.[9] Some studies have reported good efficacy of ClO_2 for *Escherichia coli* inactivation in irrigation water.[10,11] For greenhouses and nurseries, a maximum ClO_2 concentration of 0.25 mg L^{-1} at water outlets is recommended to avoid phytotoxic effects.[12] When assessing the efficacy of disinfection methods, the presence of viable but non-culturable (VBNC) cells after treatment should be taken into account to determine bactericide or bacteriostatic effect. One of the technologies that can be used to study the presence of VBNC cells is real-time PCR on propidium monoazide (PMA)-treated samples.[13]

ClO_2 does not form organohalogen by-products, but ClO_2 decomposition produces chlorate and chlorite ions.[14] Chlorate ions have been shown to be harmful for erythrocytes and for mammalian cells.[15,16] Chlorate cannot be used as a pesticide in the European Union in order to avoid the presence of chlorates in food.[17] The disinfection of irrigation water with ClO_2 could lead to the presence of chlorates in water and in the irrigated plants.[18]

Apart from the efficacy for water disinfection, potential antimicrobial and phytotoxic effects of the disinfection treatment on the crop must be assessed.[19] Parameters like microbial load (*Enterobacteriaceae*), size, colour, photosynthetic efficiency, and presence of disinfection by-products should be evaluated.

The objective of the present study was to test the suitability of a stable and highly concentrated solution of ClO_2 (AGRI DIS®) for the disinfection of irrigation water in commercial agricultural fields of baby spinach, taking into account the effects on the quality characteristics of the irrigation water and the crop.

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MATERIALS AND METHODS

Experimental set-up

Two commercial agricultural fields (0.5 and 0.8 ha) located in Pozo de la Higuera (Almería, Spain) used for cultivation of baby spinach (*Spinacea oleracea* L.) were used in the experiments. Water used for irrigation was surface water stored in a reservoir close to the growing fields. Water and plants from the two agricultural fields were analysed during the crop's growth cycle. Two repetitions were performed, one in October–December 2015 and another in February–March 2016. One plot was used as a control plot (irrigated with untreated water) in one repetition and in the other it was the treated plot (ClO_2-treated irrigation water) to avoid differences in the type of soil, permeability and slopes that could affect the results. For the untreated and treated irrigation water, samples were taken from the sprinklers. *E. coli* (viable and culturable), presence of pathogenic bacteria, chlorate content and absorbance at 254 nm were analysed five to six times during the growing cycle. For baby spinach, plant samples were taken during the last 3 weeks before harvest for analysis of size, colour, photosynthetic efficiency and occurrence of *Enterobacteriaceae*.

ClO_2 preparation, dosing and monitoring

For the disinfection treatment, irrigation water was continuously treated with the ClO_2 solution AGRI DIS® (Servicios Técnicos de Canarias, Las Palmas de Gran Canaria, Spain). Treatment of the irrigation water was prepared *in situ* at the irrigation head according to manufacturer's instructions (Servicios Técnicos de Canarias). The concentrated ClO_2 solution (~6000 mg L^{-1}) was diluted using irrigation water in a 1000 L opaque plastic tank. For the treatment plot, the diluted ClO_2 solution was dosed into the irrigation water using a programmable Venturi system suction unit (INTA Crop Technology S.L., Águilas, Spain) with the aim of achieving a concentration of ClO_2 below 1 mg L^{-1} at the sprinkler. Measurements of ClO_2 were carried out by chronoamperometry analysis (Chlordioxense; Palintest, Gateshead, UK) during irrigation at the sprinklers in the control and treatment water samples. Chronoamperometry involves applying a fixed voltage to a working electrode and recording the resulting current–time dependence. The magnitude of the current is directly proportional to the concentration of chlorine dioxide in the test sample.

Water sampling and analyses

For microbiological analyses, each sampling day, five water samples (2.5 L) from five different sprinklers homogenously distributed in each field were taken into aseptic polyethylene bottles (2.7 L) (Deltalab, Barcelona, Spain) and transported to the laboratory in refrigerated conditions. In the case of ClO_2 treated water, sodium thiosulfate (Sigma–Aldrich, Darmstadt, Germany) was added to the bottles to quench disinfectant residuals. Water was analysed for the concentration of culturable and viable generic *E. coli*, and for the occurrence of bacterial pathogens (*Salmonella* and pathogenic *E. coli*). For culturable *E. coli*, analyses were performed as in López-Gálvez *et al.*,[20] samples were plated in Chromocult coliform agar (Merck, Darmstadt, Germany) and plates were incubated for 24 h at 37 °C before interpretation. Dark blue–violet colonies were considered positives for *E. coli*. For viable *E. coli*, real-time-PCR of PMA treated samples was performed following the protocol of Truchado *et al.* with some modifications.[13] Three water samples (500 mL) per treatment and sampling day, were vacuum filtered through sterile cellulose nitrate filters (0.45 µm). Filters were placed in Falcon tubes (50 mL) containing phosphate buffered saline + Tween 80 (20 mL) (1 mL L^{-1}), Sigma–Aldrich, Saint Louis, MO, USA) and shaken in a vortex for 1 min. After that, the filters were discarded and the tubes centrifuged for 10 min at 3000 *g*. Then, the pellet was re-suspended in sterile distilled water (1 mL) and transferred to clear transparent microtubes (2 mL). From this point, the protocol was identical to that described in Truchado *et al.*[13] A concentration of PMA of 10 µmol L^{-1}, and the MasterPure™ complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) were used. PCR was performed using the primers and probe included in Table 1. A region of the 23S rRNA gene was targeted. In the case of bacterial pathogens, two samples (one per treatment) were taken each sampling day. Samples (10 L) were filtered through Modified Moore Swabs prepared as explained in Sbodio *et al.*[21] Five-litre sprayer pumps (Geolia, Lille, France) were used to pump the water through the swabs in the field. Modified Moore Swabs were then transported to the laboratory in refrigerated conditions. Swabs were then placed in sterile stomacher bags, covered with buffered peptone water (20 g L^{-1}) and massaged by hand for 1 min before incubating for 24 h at 37 °C. After incubation, 7 mL of enrichment were transferred to sterile tubes and mixed with glycerol (3 mL). Tubes were kept at −20 °C until DNA extraction was performed. For DNA extraction and real-time-PCR detection, the Genedisc system and protocols were used (Pall; Port Washington, NY, USA) as described in López-Gálvez *et al.*[20] Fifty microlitres of each enriched sample were used to extract and purify the bacterial DNA using a commercial extraction kit (Extraction Pack Food; Pall). Commercially available GeneDisc® plates were used for the screening in parallel of specific gene sequences of human pathogenic verotoxin producing *E. coli* virulence factors (stx1, stx2, eae), *E. coli* O157:H7 (rfbEO157 and fliCH7) and *Salmonella* spp. specific genes (iroB), while also including inhibition control and negative control.

Table 1. Primers and probe used for real-time PCR

Primer name	Sequence (5′–3′)	Concentration (nM)
23F	GGTAGAGCACTGTTTTGGCA	800
23R	TGTCTCCCGTGATAACTTTCTC	800
23P	(FAM)TCATCCCGACTTACCAACCCG(TAMRA)	160

For the assessment of chlorates in treated and untreated water, five samples (14 mL each) were taken in Falcon tubes (15 mL) each sampling day, transported under refrigeration to the laboratory and frozen at −20 °C until analysis. Chlorate content of water samples was analysed by LC–MS as explained in Gil *et al.* and expressed as mg L^{-1}.[22]

Two samples of treated and untreated water were taken each day of sampling for measurement of absorbance at 254 nm (UV254). Measurements were performed as described in Van Haute *et al.*[23]

Plant sampling and analysis

Plant sampling was performed manually in aseptic conditions, using disinfected scissors and clean gloves. Five samples of baby spinach (250 g) were taken from five different locations, distributed in the corners and in the middle of each plot following a zig-zag pattern. Samples were transported in refrigerated conditions to the laboratory.

Length, area and colour of baby spinach were measured as quality characteristics of the leaves to detect any potential phytotoxic effects of the disinfection treatment. Length and area

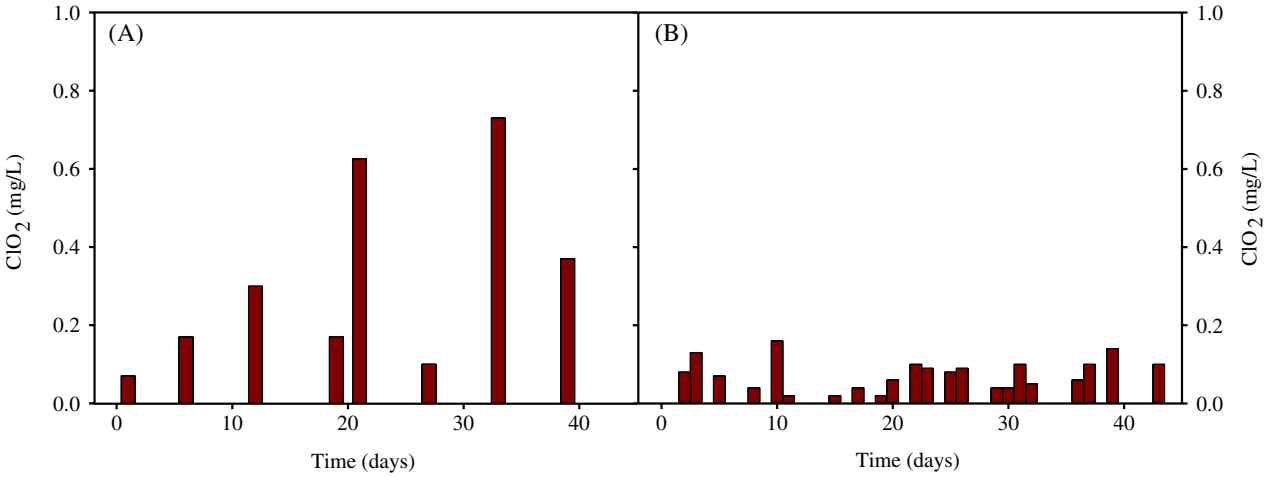

Figure 1. Concentration of ClO_2 (mg L^{-1}) in irrigation water from the sprinklers at the treated field. (A) First test (eight sampling days); (B) second test (22 sampling days).

of spinach leaves were measured by digital image analysis. In the first test, leaf colour (L^*, a^* and b^*) was determined on the adaxial side of the leaves using a compact tristimulus colorimeter (CR-300; Minolta, Ramsey, NJ, USA) with an 8 mm diameter viewing aperture. In the second test, colour was also measured by digital image analysis. Leaf images were taken using a digital camera (Canon 70D) mounted on the top of a photography box of 100 cm × 60 cm × 60 cm (height × length × width) with matt white translucent walls and black mat ground. The light was provided by one LED panel of 60 × 60 cm of 42 W and 6400 K mounted on the top of the box. The image acquisition was conducted in a darkroom maintained at room temperature. Acquired picture files were saved in RAW format (5472 × 3648 pixels) to obtain the sRGB colour space. Images were transformed to HSB (hue, saturation, brightness) colour space and measured using the ImageJ 1.50i image processing software (NIH, Bethesda, MD, USA). Fifteen leaves from each sampling point were analysed each sampling day.

Fluorescence of chlorophyll was measured with a portable non-modulated fluorimeter Handy PEA (Plant Efficiency Analyser; Hansatech Instruments, Kings Lynn, UK) as described by Živčák *et al.* with modifications.[24] Leaves were harvested and maintained for 30 min into black polyethylene plastic bags to avoid light exposure. After dark-incubation of leaves a single strong 1 s light pulse (3000 µmol m^{-2} s^{-1}) was applied on the surface of the leaves and the minimum and the maximum fluorescence yields were monitored (one measure per leaf). The maximum quantum yield of photosystem II (PSII) (Fv/Fm) was analysed each sampling day in 100 leaves, 20 leaves per each location in the field. Chlorate content in plant samples was analysed by LC–MS as explained in Gil *et al.*[22] *Enterobacteriaceae* in baby spinach were analysed using the protocol described in López-Gálvez *et al.*[25] utilising Violet Red Bile Dextrose Agar (VRBD) (Scharlab, Barcelona, Spain) incubated at 37 °C for 24 h.

Statistical analysis

IBM SPSS statistics 23 was used for statistical analyses. The Shapiro–Wilk test was performed to assess the normality of the data. When normality could be assumed, *t*-tests were performed to compare the two treatments. When data did not follow a normal distribution, the Mann–Whitney *U* test was applied. In samples with culturable *E. coli* counts below the detection limit (1 CFU 100 mL^{-1}), a value of 0 Log CFU 100 mL^{-1} was used for statistical analysis. Pearson's correlation coefficient was calculated to evaluate correlation between data.

RESULTS

Monitoring the disinfection of irrigation water with ClO_2

Figure 1 shows the concentration of ClO_2 in water from the sprinklers in the treated field during the first (Fig. 2A) and the second tests (Fig. 2B). Detected ClO_2 concentration was always lower than 1 mg L^{-1} in both tests. During the first test, control of ClO_2 concentration was performed once a week approximately. However, fluctuations in concentration were detected using this measurement schedule (Fig. 2A). To improve control and maintain lower residuals, in the second test measurements were performed three times per week approximately. In this way, a better control of ClO_2 concentration was achieved, and lower and more constant ClO_2 residuals were maintained (Fig. 2B). Mean initial ClO_2 concentration injected at the irrigation head was 0.42 ± 0.16 and 0.26 ± 0.11 in the first and in the second tests, respectively. A mean concentration of 0.32 ± 0.25 mg L^{-1} and 0.07 ± 0.04 mg L^{-1} was measured in the sprinkler water from the treatment field during the first and the second tests, respectively. Measurement performed in the sprinklers of the untreated field always gave a concentration of ClO_2 below the limit of detection (<0.02 mg L^{-1}).

Impact of ClO_2 on the microbial and physico-chemical characteristics and disinfection by products of irrigation water

Concentration of culturable *E. coli* in sprinkler water in the first and the second tests is shown in Fig. 2. Mean ± SD of culturable *E. coli* in the first test was 0.41 ± 0.41 Log CFU 100 mL^{-1} in control water ($n = 25$) and 0.19 ± 0.33 Log CFU 100 mL^{-1} in ClO_2 treated water ($n = 25$). Concentration of culturable *E. coli* in the second test was 0.49 ± 0.25 Log CFU 100 mL^{-1} in control water ($n = 30$) and 0.15 ± 0.13 Log CFU 100 mL^{-1} in ClO_2 treated water ($n = 30$). The difference between control and treated water was statistically significant in both tests ($P < 0.05$).

Figure 3 shows the concentration of viable but non-culturable (VBNC) *E. coli* in sprinkler water in the first and the second tests. Mean ± SD of viable *E. coli* in the first test was 1.51 ± 0.26 Log

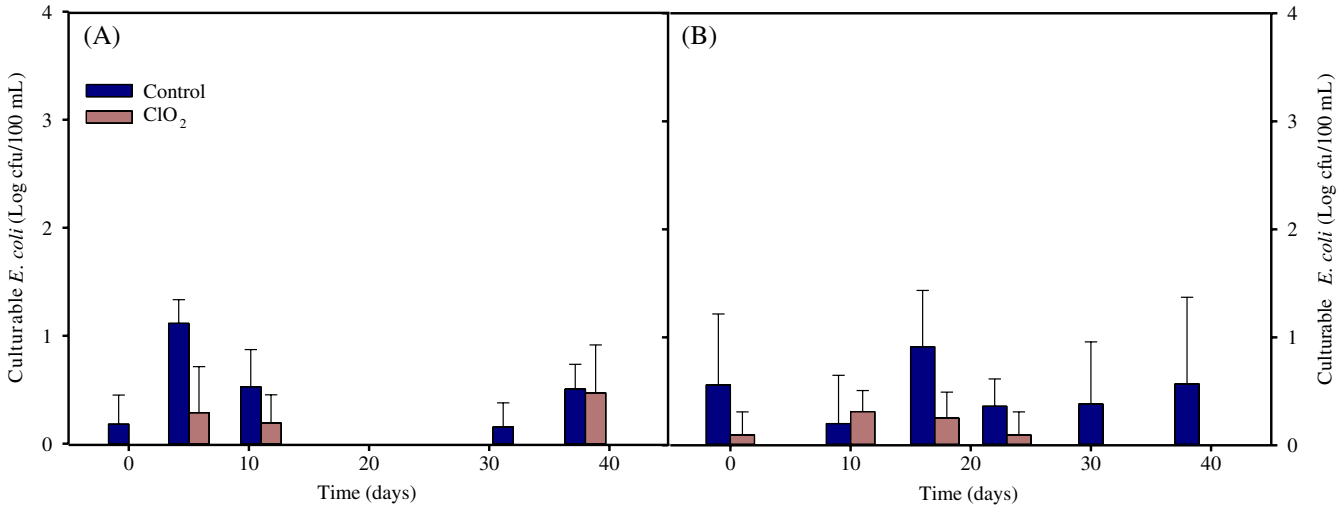

Figure 2. Culturable *E. coli* (Log CFU 100 mL^{-1}) in irrigation water from the sprinklers at control and ClO_2 treated fields. (A) First test (five sampling days); (B) second test (six sampling days).

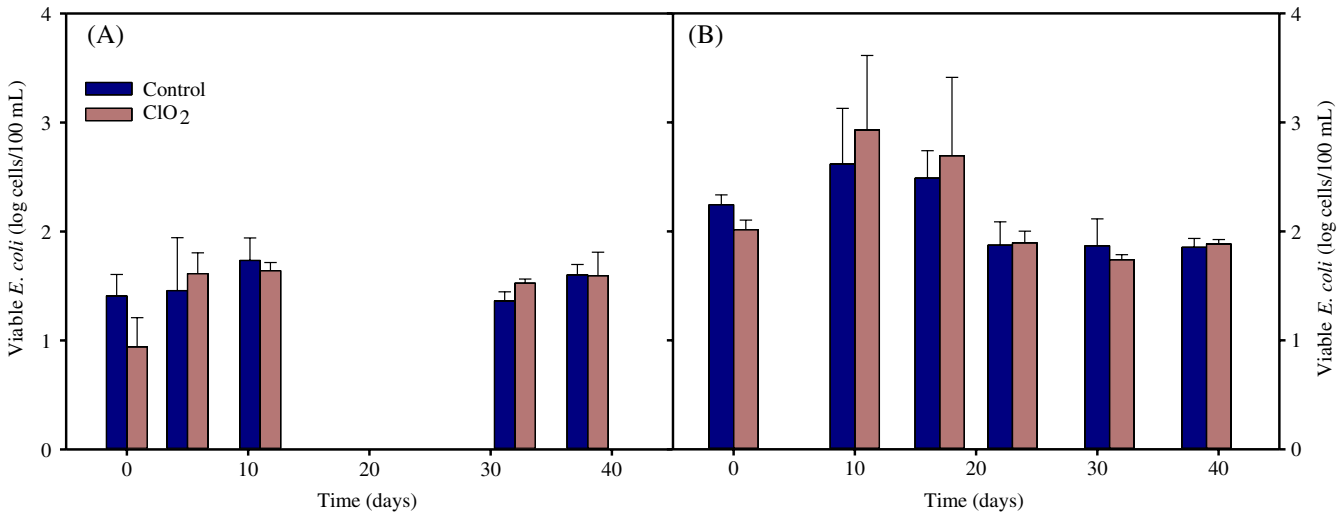

Figure 3. Viable *E. coli* (Log cells 100 mL^{-1}) in irrigation water from the sprinklers at control and ClO_2 treated fields. (A) first test (five sampling days); (B) second test (six sampling days).

cells 100 mL^{-1} in control water ($n = 15$) and 1.46 ± 0.31 Log cells 100 mL^{-1} in treated water ($n = 15$). Concentration of VBNC *E. coli* in the second test was 2.14 ± 0.39 Log cells 100 mL^{-1} in control water ($n = 18$) and 2.21 ± 0.59 Log cells 100 mL^{-1} in treated water ($n = 18$). The difference between control and treated water was not statistically significant in both tests ($P > 0.05$).

Real-time PCR results of samples for the presence of *Salmonella* spp. and pathogenic *E. coli* in treated and untreated water from the first ($n = 10$) and the second tests ($n = 12$) were all negative.

When absorbance of UV was measured at 254 nm (UV254) it was observed that mean ± SD was 0.02 ± 0.00 cm^{-1} in control water and 0.04 ± 0.01 cm^{-1} in treated water in the first test while it was 0.03 ± 0.00 cm^{-1} in control water and 0.04 ± 0.00 cm^{-1} in treated water in the second test. The difference between control and treated water was statistically significant in both tests ($P < 0.05$). Taking into account data from both tests, a significant negative correlation between UV254 of untreated water and residual ClO_2 in treated water at the sprinklers was found ($P < 0.01$; Pearson's correlation coefficient = −0.54).

Content of chlorates (mean ± SD) in the first test was 0.04 ± 0.05 mg L^{-1} in control water and 2.46 ± 2.46 mg L^{-1} in treated water. In the second test, the content was 0.01 ± 0.01 mg L^{-1} in control water and 0.46 ± 0.31 mg L^{-1} in treated water. The difference between control and treated water was statistically significant in both tests ($P < 0.05$). No correlation between UV254 of untreated water and chlorate content in treated water was found ($P > 0.05$). On the other hand, a significant positive correlation was detected between residual ClO_2 concentration in the sprinkler water and chlorate content ($P < 0.01$; Pearson's correlation coefficient = 0.59).

Impact of ClO_2 on the microbial and quality characteristics, and the disinfection by-products of baby spinach

Some quality characteristics of baby spinach such as length, area, colour and photosynthetic efficacy were determined in baby spinach at three different sampling points (3, 2 and 1 weeks before harvest) and also at harvest where commercial maturity was reached. Those parameters measured at harvest are included in

Table 2. Length, area, colour parameters and photosynthetic efficiency of control and ClO_2 irrigated baby spinach in the first and second tests at harvest

Test	Treatment	Length (cm)	Area (cm^2)	L^*	a^*	b^*	Hue	Chroma	Photosynthetic efficiency (Fv/Fm)
1	Control	12.2 ± 0.4^{a}	$28.4 \pm 1.^{8a}$	40.0 ± 0.9^{NS}	-15.1 ± 0.7^{b}	21.3 ± 1.4^{a}	125.4 ± 0.6^{b}	26.2 ± 1.5^{a}	0.76 ± 0.05^{NS}
	ClO_2	10.8 ± 0.5^{b}	24.0 ± 1.4^{b}	39.6 ± 0.8^{NS}	-14.2 ± 0.8^{a}	19.7 ± 1.4^{b}	125.9 ± 0.6^{a}	24.2 ± 1.6^{b}	0.74 ± 0.07^{NS}
2	Control	7.9 ± 0.9^{B}	13.5 ± 2.9^{B}	57.9 ± 1.6^{B}	-14.2 ± 0.8^{A}	32.0 ± 3.3^{A}	116.5 ± 0.7^{A}	30.8 ± 1.8^{B}	0.71 ± 0.07^{NS}
	ClO_2	8.9 ± 1.4^{A}	17.5 ± 4.3^{A}	61.5 ± 2.1^{A}	-15.9 ± 1.2^{B}	27.4 ± 1.6^{B}	117.3 ± 0.3^{B}	35.7 ± 3.5^{A}	0.69 ± 0.07^{NS}

Values are mean ± SD.
Different lowercase letters in the same column indicate significant differences in test 1.
Different uppercase letters in the same column indicate significant differences in test 2.
NS, non-significant.
Mean value for colour (L^*, a^*, b^*) was calculated based on the measurements performed in 25 leaves per treatment, sampled from five different locations in the field.
Mean value for photosynthetic efficiency was calculated based on the measurements performed in 50 leaves per treatment, sampled from five different locations in the field.Mean value for length and area was calculated based on the measurements performed in 30 leaves per treatment, sampled from five different locations in the field.

Table 2; however, data corresponding to the other samplings were also considered for statistical significance. Taking into account data from all the sampling days, in the first test, leaves from control plants were significantly longer and larger than leaves from treated plants ($P < 0.05$). In the second test, there were no significant differences in length except at harvest as leaves from treated plants were longer and larger than leaves from control plants (Table 2).

Colour parameters at harvest in both tests are included in Table 2. In the first test, taking into account data from all samplings, in treated plants L^*, b^*, and chroma parameters were significantly lower, while a^* and hue were significantly higher ($P < 0.05$). The same differences were observed with data at harvest, with the exception of L^* that was not different between treatments (Table 2). On the other hand, in the second test, taking into account data from all the samplings, there were no differences in L^*, a^* and hue, but b^* and chroma were significantly higher in treated plants. At harvest, colour parameters in the second test were opposite to the first test. L^*, hue and chroma were significantly higher, while a^* and b^* were significantly lower in treated plants (Table 2).

In both tests, taking into account data from all samplings, photosynthetic efficiency measured as the ratio Fv/Fm was significantly higher in control plants compared with treated plants ($P < 0.05$). In the first test, mean ± SD of Fv/Fm ratio was 0.76 ± 0.05 and 0.74 ± 0.07 in control and treated plants, respectively. In the second test, mean ± SD of Fv/Fm ratio was 0.71 ± 0.07 and 0.69 ± 0.07 in control and treated plants, respectively. However, there was no difference in photosynthetic efficiency measured at harvest between treatments in both tests (Table 2).

Content of chlorates in baby spinach was significantly higher in plants irrigated with treated water ($P < 0.01$) in both tests. In the first test, mean ± SD of chlorates was 0.00 ± 0.01 and 0.49 ± 0.20 mg kg^{-1} for control and treated plants, respectively. In the second test, mean ± SD of chlorates was 0.05 ± 0.10 and 0.99 ± 0.40 mg kg^{-1} for control and treated plants, respectively.

Box-plots on *Enterobacteriaceae* counts of baby spinach are shown in Fig. 4A and B for the first and second test, respectively. In both tests, there were no significant differences in the concentration of *Enterobacteriaceae* in baby spinach between control and treated plants. In the first test, mean ± SD of

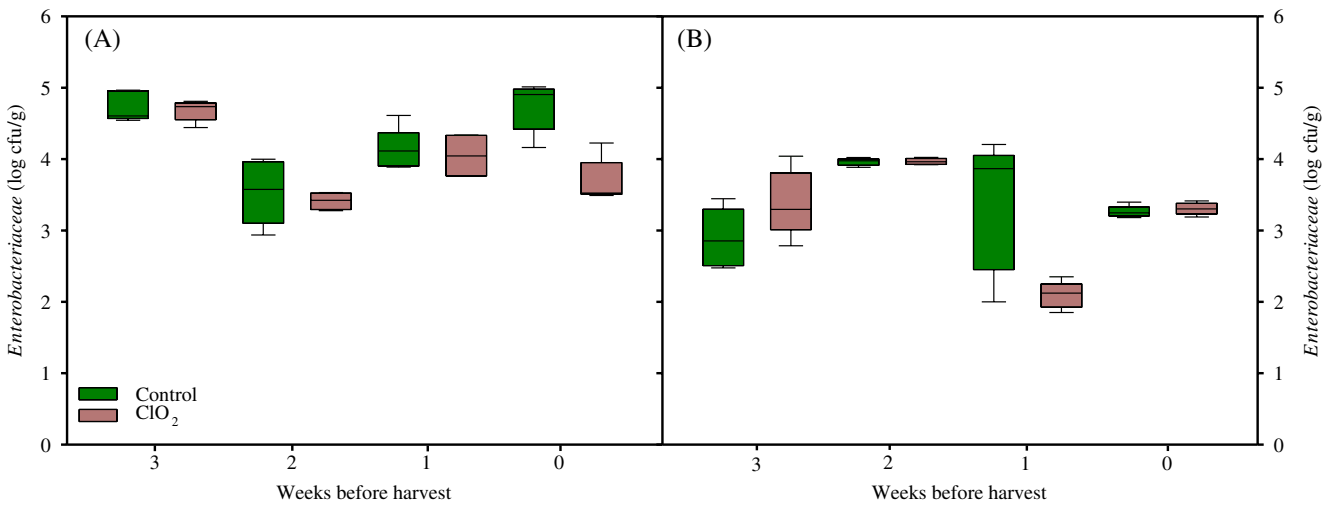

Figure 4. Boxplot representing *Enterobacteriaceae* counts of baby spinach irrigated with control and ClO_2 treated irrigation water during the last 3 weeks before harvest. (A) First test; (B) second test. Bottom and top of the boxes represent the 25th and 75th percentiles. The box-plot whiskers represent the minimum and maximum values.

Enterobacteriaceae was 4.3 ± 0.6 and 3.9 ± 0.5 Log CFU g^{-1} for control and treated plants, respectively. In the second test, mean ± SD of *Enterobacteriaceae* was 3.4 ± 0.6 and 3.2 ± 0.7 mg kg^{-1} for control and treated plants, respectively.

DISCUSSION

Irrigation water with good physico-chemical quality, like the one used in the present study, has a very low disinfectant demand. This circumstance would avoid the accumulation of by-products in water and plants. However, other goals of irrigation water treatment, such as inactivation of plant pathogens could require higher doses.[9,26] Chlorine dioxide can be used at 1–3 mg L^{-1} as initial concentration (residual 1 mg L^{-1}) in open-field drip irrigation to prevent obstruction of irrigation lines.[9]

The presence of organic matter in water is important for the performance of oxidant disinfection treatments. Measurement of UV254 renders information on the abundance of different organic compounds (lignin, tannin, humic substances, aromatic compounds) present in water samples.[27] In other studies, UV254 levels measured in surface water were similar to those detected in our study.[28,29] The higher absorbance of treated water samples would be explained by the presence of oxidants (e.g. chlorate, chlorite).[27]

Regarding the effect of ClO_2 treatment in reducing culturable *E. coli* present in irrigation water, most authors obtained significant reductions. Killinger *et al.*[11] reported significant reduction of generic *E. coli* in irrigation water treated with ClO_2, although effects on the presence of generic *E. coli* and pathogenic bacteria in produce were not evident. Chang[10] reported higher efficacy of ClO_2 compared with peroxyacetic acid in the inactivation of generic *E. coli* in surface irrigation water. Reitz *et al.*[9] observed substantial reductions in *E. coli* numbers when ClO_2 (1 and 3 mg L^{-1}) was applied to surface water in irrigation lines. Cueva[30] observed a reduction in the concentrations of indicator microorganisms (including *E. coli*) in irrigation water after ClO_2 treatment. In our study, the mean log count was lower in treated samples in the five samplings from the first experiment and in five out of six samplings in the second experiment. Furthermore, *E. coli* was always detected in control samples, while it was below the limit of detection in four out of the 10 samplings performed during the study. Taking into account the low initial *E. coli* levels in untreated irrigation water (<1–100 CFU 100 mL^{-1}) from the commercial settings, the reductions obtained after the ClO_2 treatment are considered significant. Low ClO_2 doses were sufficient to provoke a reduction in culturable *E. coli* presence in water. In contrast, Ivey and Miller[31] a reported lack of efficacy of ClO_2 (residual 1 mg L^{-1}) for the inactivation of low levels of *E. coli* (0.6–2.0 Log MPN 100 mL^{-1}) in irrigation water from a pond and a ditch.

Treatment of samples with the DNA-binding dye PMA allows the distinction between viable and non-viable bacteria when integrity of membrane is used as viability criteria.[32] This method can be used to discriminate viability after the application of antimicrobial treatments.[33] In our study, a method optimised for irrigation water was used.[13] When tests were performed to compare the results of quantitative PCR analysis with and without PMA treatment, lower quantities of *E. coli* were measured when using PMA, confirming the effect of the PMA pre-treatment. In accordance with other studies, levels of *E. coli* detected with the PMA-PCR method were higher than those detected by plate culture.[13] This could have been caused by the presence of a sub-population of VBNC cells within the total *E. coli* population, even in untreated control samples. The *E. coli* counts shown in this study correspond to the native population of *E. coli* in the irrigation water, and not to inoculated laboratory-grown cells. Therefore, such a native population would have been exposed to environmental stresses that can promote the VBNC state in bacterial cells. In contrast with culturable *E. coli*, viable *E. coli* did not show a difference between treated and untreated water. This fact indicates that ClO_2 treatment could have had a bacteriostatic action, causing the entrance of *E. coli* cells into a VBNC state.

Difference between methods for the detection of indicator microorganisms is critical, particularly as legislation and guidelines of irrigation water are based on culturable *E. coli* measurements.[34,35] In accordance with our results, low prevalence of pathogenic bacteria in irrigation water in the same area (south-east of Spain) has been reported previously.[36]

Pre-harvest contamination of produce with chlorates should be avoided in order to prevent the presence of disinfection by-products in the food. Irrigation water disinfected with chlorine-based agents can be a potential source for chlorate residues in food.[37] In the European Union, there is a maximum residue limit for chlorate of 0.01 mg kg^{-1} in food.[38] A increase in the maximum residue level for chlorates in baby leaf crops to 0.06 mg kg^{-1} is being evaluated by the European Commission.[18] Korn *et al.*[39] highlighted non-purgeable organic carbon and UV254 as important parameters to predict chlorate and chlorite formation when treating lake and river water with ClO_2. In our study, due to the fact that the irrigation water had a low organic matter content, the ClO_2 dose was probably the main cause for chlorate presence. Accordingly, when higher ClO_2 doses were applied (first test), higher levels of chlorates were detected in water. In the present demonstration tests, low doses of ClO_2 were applied. However, chlorates accumulated in baby spinach irrigated with ClO_2 water, reaching levels above the current limit and those proposed by the European Commission.[18]

Plant damage and delay in the growth are both phytotoxic effects that have been previously reported when using high ClO_2 concentrations for the control of waterborne microbes in irrigation water.[19] Doses applied in the present study were not high, allowing the continuous application of disinfected water without causing major differences in leaf size. Lower ratio Fv/Fm of baby spinach irrigated with ClO_2 could indicate that the disinfection treatment caused some abiotic stress.[40] However, no changes in the Fv/Fm ratio of different ornamental plants overhead irrigated with chlorine treated irrigation water (up to 20 mg L^{-1}) have been reported.[41] This fact could be due to the stronger oxidising activity of ClO_2 or the longer application times (>40 days) of our demonstration tests. Regarding colour, the only difference observed in both tests at harvest was the higher b^* value in control plants, indicating a more yellowish colour. When taking into account data from all the samplings, there was no a clear tendency regarding phytotoxicity of ClO_2 on leaf colour. Regarding *Enterobacteriaceae* counts, there were no differences between control and treated baby spinach and counts were similar to those reported for other leafy greens.[42] Oliveira *et al.*[42] reported a concentration of *Enterobacteriaceae* of 3.8 ± 1.5 Log CFU g^{-1} in conventionally grown lettuce while Cardamone *et al.*[43] described *Enterobacteriaceae* levels between 2 and 6 Log CFU g^{-1} in unprocessed leafy vegetables.

When irrigation water has good physico-chemical quality, low doses of ClO_2 may be used to improve microbial safety without causing important phytotoxic effects to the crop. However, even when using low doses of ClO_2 for irrigation water disinfection, chlorates can be present in irrigation water and accumulate in the

crop. The decomposition of ClO_2 to chlorate and chloride ions is very slow but it can be accelerated in concentrated solutions.[44] Furthermore, regarding improvement of the safety of irrigation water, attention must be paid to issues like the induction of VBNC state in target microorganisms.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support from the Center for Produce Safety Grant Agreement (Project 2015-374) and the MINECO (Project AGL2016-75878-R). Support provided by the Fundación Séneca (19900/GERM/15) is also appreciated. The authors are indebted to the company Servicios Técnicos de Canarias SLU (www.clodostechnology.com) for their material and technical support during the study. AGRI DIS® is a registered product of the CLODOS technology® from Servicios Técnicos de Canarias SLU. F. López-Gálvez is indebted to CSIC and ESF (JAE-Doc-2011 contract co-funded by the European Social Fund).

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