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Project Title
Demonstration of practical, effective and environmentally sustainable agricultural water treatments to achieve compliance with microbiological criteria

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Objectives

1. *Microbial and physicochemical characterization of different water sources commercially use to grow leafy greens in Spain*
2. *Pilot plant studies to establish effective dose thresholds of stabilized ClO_2 in different water sources*
    a. *Required doses for minimal compliance with microbiological criteria established in recommended guidelines of good agricultural practices as well as current legislation*
    b. *Required doses for removal of E. coli to drinking water standards*
    c. *Required dose for removal of foodborne pathogens such as Salmonella spp. and VTEC*
3. *Practical demonstration of the stabilized ClO_2 stability under field conditions*
4. *Evaluation of the impact of repeated ClO_2 treatments at the highest selected doses for pathogen removal on the soil microbial community*

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FINAL REPORT

Abstract

Growers should be aware of the risk associated with agricultural water, and the best mitigation option to remove pathogens if needed. Water disinfection is one of the most recommended intervention strategies for irrigation water. The main purpose of this project was to demonstrate a practical, effective, and environmentally sustainable water disinfection treatment. A stable solution of chlorine dioxide (ClO_2) was used to disinfect irrigation water of different water sources and qualities to ensure compliance with the established microbial limits, particularly fecal indicator bacteria such as *E. coli*. Optimal operational conditions for ClO_2 were established at a pilot scale, and two mathematical models (ClO_2 residual and *E. coli* inactivation) were generated. Initial *E. coli* load and UV absorbance at 254 nm of the irrigation water can predict the initial ClO_2 concentration that growers should use to reduce the initial *E. coli* load below the established limits. Demonstration of practical, effective, and environmentally sustainable agricultural water disinfection treatment was also performed at commercial production fields (open field and greenhouse trials). The impact of continuous application of irrigation water disinfected with the stable solution of ClO_2 on the natural microbiota of soil and plants was also evaluated. In both studies (open field and greenhouse trials), the selected ClO_2 concentrations were effective in reducing the *E. coli* load of irrigation water below the established microbial limits (FSMA). Results obtained demonstrated that when a continuous ClO_2 treatment was applied to disinfect irrigation water, the water bacterial community was affected, changing the abundance and diversity. However, irrigation water disinfected with ClO_2 generally slightly affected the bacterial community of baby spinach and soil. The main changes were observed in the abundance of *Pseudomonadaceae* and *Enterobacteriaceae*, which were decreased in baby spinach treated with ClO_2 compared with the control. This is remarkable because a large proportion of foodborne and pathogenic bacteria associated with fresh produce belong to these families. The use of a stable solution of ClO_2 may be a suitable intervention strategy to maintain the microbiological quality of irrigation water without affecting the abundance and diversity of the soil and plant microbiota.

1. Background

Leafy greens present challenges for maintaining food safety, as they have been identified as one of the five top groups of risk for food/pathogen associated commodities (Anderson et al., 2011; EFSA, 2013). Irrigation water has been recognized as a risk factor for fecal contamination of fresh produce (Park et al., 2013). Increasing evidence of produce contamination from irrigation water and the scarcity of water resources leave little doubt about the need to pay more attention to the fate and transport of pathogens in irrigation water (Allende and Monaghan, 2015).

Recommended guidelines indicate that remedial actions should be taken when microbial test results are higher than normal, or indicate an upward trend (LGMA, 2012). Water disinfection is one of the most recommended intervention strategies for irrigation water if the microbial quality is out of the recommended range (EFSA, 2013). The most commonly used water disinfection agent is sodium hypochlorite. However, concerns have risen in the last years regarding the absence of water treatment and the excessive use of potentially toxic chemicals to treat irrigation water. Thus, the selection of environmentally friendly technology to reduce, control or eliminate efficiently the risk of microbial pathogen contamination in irrigation water has been considered a priority. One of the postulated alternatives to sodium hypochlorite is the use of stabilized chlorine dioxide (ClO_2), which possesses higher oxidation capacity and lower formation of halogenated by-products (Lopez-Galvez et al., 2010).

E. coli is an established fecal marker organism, and its presence provides evidence of an increased likelihood of potential contamination by ecologically closely related pathogens. Molecular *E. coli* by qPCR-based *E. coli* assay has been identified as a good indicator for bacterial pathogen contamination when compared to cultivable *E. coli* (Ferguson et al., 2013).

Growers should be assisted in determining the risk associated with the agricultural water and if a mitigation option to remove pathogens is needed. **Sustainable and eco-friendly technologies can be appropriate to use in the growing fields to ensure compliance with the established microbial criteria, particularly fecal indicator bacteria such as *E. coli*. Within this regard, we propose the use of stabilized ClO_2 as a suitable disinfection treatment for irrigation water.** The establishment of optimal operational conditions of the proposed system based on the different properties of irrigation water obtained from different water sources will provide very valuable information, which may be integrated in guidelines of good water management practices.

This project has the aim of demonstrating the suitability of an eco-friendly disinfection technology to treat agricultural water on the basis of fecal indicator and pathogenic bacteria. The efficacy of a stable solution of ClO_2 to eliminate microorganisms will give very valuable data for growers in order to establish the dose thresholds needed to treat different water sources, characterized by different physicochemical parameters. This information will be valuable to set good water management practices.

2. Research Methods

2.1. Microbial and physicochemical characterization of different water sources used commercially for leafy greens

Experimental design and sample collection. Microbial collection by systematic sampling of agricultural water was performed on three different types of water sources, including: 1) water from drainage ditches bordering farmlands and urban areas, 2) water reservoirs, and 3) canal water from a river transfer. These water sources were selected because they are the three main water sources used for crop irrigation in the southeast region of Spain, the area with the highest production of leafy greens in Europe and in which climate and environmental conditions are close to those in Yuma (Arizona) in winter and Salinas (California) in spring-summer. Water samples were collected in duplicate weekly from January until December 2015 (n=226). Two liters of each type of water were collected in sterile polypropylene bottles. Water temperature in each water source was measured. Additionally, at each sampling point, 10 L of each water source was passed through a modified Moore swab (MMS) by means of a peristaltic pump, as previously described by Sbodio et al. (2013).

Cultivable and molecular-based *E. coli* quantification. Cultivation-based enumeration of *E. coli* in irrigation water was performed according to ISO 9308-1:2014. Samples of 100 mL were filtered through 0.45-µm pore size nitrocellulose membranes (Sartorius, Madrid, Spain). Membranes were aseptically removed from the filter base and placed on Chromocult agar. Plates were incubated at 37°C for 24 h. Molecular quantification of *E. coli* in the water was performed as previously described in Truchado et al. (2016). Briefly, two samples (250 ml) were further pooled in one 500-mL sample (n=113) at each sampling point; this pooled sample was centrifuged at 4000 rpm for 20 min. The pellets were activated with PMA and held at -20 ºC until genomic DNA extraction was performed.

Physicochemical analysis. The pH was measured using a pH and redox multimeter (Crison, Barcelona, Spain). Chemical oxygen demand (COD) was determined by the standard photometric method using the Spectroquant NOVA 60 photometer. Alkalinity (meq $CaCO_3$/L) was determined by acid titration using hydrochloric acid 0.1 N (Panreac, Barcelona, Spain). Turbidity was tested using

the Turbiquant 3000R turbidimeter (Merck, Madrid, Spain). For measurement of water absorbance at 254 nm (UV254), water was first filtrated and then measured by a UV-VIS spectrophotometer; quartz cuvettes with a 1-cm path length were used.

Detection and confirmation of pathogenic microorganisms. The modified Moore swabs previously obtained after filtering 10 L of water were placed in a stomacher bag containing 200 mL of 40% BPW and then incubated at 37 °C for 18–20 h. Pre-enriched samples were supplemented with 30% glycerol and maintained at −20 °C until further analysis. One mL of these pre-enriched samples was enriched in 9 mL of BHI for 4 h at 35 °C to resuscitate injured cells. For *E. coli* O157:H7 detection, 1 mL of BHI was then transferred into 9 mL of trypticase soy broth, supplemented with novobiocin at 20 mg/L (TSBn; Scharlau Chemie, Spain) and incubated overnight at 37 ºC. In the cases of Shiga toxin–producing *E. coli* (STEC), 1 mL of enrichment in BHI was then transferred in 9 mL of tryptone phosphate broth (TP; Oxoid, UK) and incubated overnight at 42 °C. For *Salmonella,* 1 mL of enrichment in BHI was then transferred in 9 mL of tetrathionate broth base, Hajna, supplemented with 1 mL of iodine solution (TT; Scharlau Chemie) and incubated overnight at 44 °C. One mL of each overnight enrichment sample was transferred to a microcentrifuge tube and centrifuged at 9000 ×*g* for 10 min. DNA extraction of the pellet was performed using a NucleoSpin Tissue kit (Macherey-Nagel, Germany) following manufacturer instruction. Presence or absence of *E. coli* O157:H7, STEC, and *Salmonella* spp. in water samples was performed using a PCR system (Applied Biosystems, Madrid, Spain). To detect the presence of *Salmonella*, specific primers targeting genes *invA* were used. In the cases of *E. coli* O157:H7 and STEC, a conventional multiplex PCR assay, targeting the virulence factors *stx1*, *stx2*, *eae* and *ehxA*, plus the O157:H7 specific +93 *uidA* single nucleotide polymorphism, was performed as previously described (Son et al., 2014). When the five bands in the multiplex PCR were amplified, this sample was considered as presumptive *E. coli* O157:H7 (Son et al., 2014). If *stx1* or/and *stx2* as well as *eae* genes were positive bands, we considered this sample as a presumptive STEC (EFSA, 2013). The presumptive positive samples detected by PCR were cultured and confirmed in selective culture media. Briefly, positive enrichments samples were streaked onto IBISA (Oxoid), CHROMagar O157, and CHROMagar STEC media (DRG International, USA) for *Salmonella*, *E. coli* O157:H7, and STEC, respectively. Green colonies were picked from IBISA plate broth and confirmed as *Salmonella* via LATEX (Oxoid). In the case of *E. coli* O157, a single mauve colony was picked from CHROMagar O157 plates and confirmed by LATEX.

2.2. Pilot plant studies to establish effective dose thresholds of stabilized ClO_2 in different water sources

Modelling approach. Inactivation experiments were performed and models for the prediction of *E. coli* inactivation and residual chlorine dioxide (ClO_2) were developed. Three types of surface irrigation water were used to obtain residual ClO_2 data: 1) water from drainage ditches bordering farmlands and urban areas (drainage ditch), 2) water reservoirs (reservoir) and 3) canal water from a river transfer (canal). To obtain *E. coli* inactivation data, only drainage ditch and canal water were used because of the very low concentration of *E. coli* naturally present in reservoir water (<1 log cfu/100 mL). Water samples were analyzed regarding physicochemical characteristics and *E. coli* concentration, as previously described. ClO_2 solution was prepared the day before its use according to manufacturer instructions (Servicios Técnicos de Canarias, Las Palmas de Gran Canaria, Spain), and kept at 4 °C. Concentration of ClO_2 in the solution was measured before its use by the DPD method using a photometer. Samples (250 mL) were treated in agitation at 20 °C protected from light and aeration. Applied ClO_2 concentrations ranged from 0.1 to 2.5 mg/L and contact time was 1 min. Samples for measurement of residual ClO_2 and *E. coli* concentration were taken during the time. ClO_2 measurement was done by the DPD method. In *E. coli* inactivation samples, residual

ClO_2 was quenched using sodium thiosulfate. Data obtained were used for the development of residual ClO_2 and *E. coli* inactivation models by linear regression. SPSS statistics 23 and Microsoft Excel 2010 were used for the analyses. Data were divided into a calibration set and a validation set. The stepwise linear regression with backwards elimination was applied to the data. Selection of the most suitable model was based on the coefficient of determination (R^2) and the ratio of prediction to deviation (RPD) values.

Dose selection. To select suitable doses for pathogenic bacteria inactivation in irrigation water (canal and drainage ditch), inactivation experiments in water inoculated (≈10^5 cfu/100 mL) with *Salmonella* spp. and *E. coli* O157:H7 were performed using the same conditions and procedures described for the *E. coli* experiments. ClO_2 doses ranging from 0.25 to 2 mg/L were tested, and samples were taken after 1, 3, and 5 minutes of contact time. Size of the surviving population was assessed by surface plating in CT-SMAC for *E. coli* O157:H7 and Salmonella-Shigella agar for *Salmonella* spp. Also, an enrichment of the samples to recover damaged cells was performed. For the enrichment, samples were mixed with buffered peptone water and incubated at 37 °C for 24 h. After incubation, enrichments were plated in selective media as previously described.

2.3. Practical demonstration of the ClO_2 stability under field conditions

Two different types of experiments were performed to test ClO_2 efficacy under field conditions: 1) open field, and 2) commercial greenhouse studies. Two repetitions for each type of experiment were performed.

Open field study: Irrigation water obtained from a water reservoir (reservoir) was used for cultivation of baby spinach. Water from sprinklers and plants of two agricultural plots (treatment and control plots of 0.5 and 0.8 hectares, respectively) were analyzed. For the treatment plot, irrigation water was continuously treated with a stable solution of ClO_2 AGRI DIS® (Servicios Técnicos de Canarias, Las Palmas de Gran Canaria, Spain). Concentrated ClO_2 solution (≈6000 mg/L) was diluted using irrigation water in an opaque plastic tank (1000 L) according to manufacturer instructions. For the treatment plot, the diluted ClO_2 solution was dosed into the irrigation water using a programmable Venturi system suction unit. Concentrations of ClO_2 were adjusted to achieve <1 mg/L at the sprinklers. ClO_2 concentration was measured by chronoamperometry (Palintest, Gateshead, UK) in control and treatment sprinklers during irrigation. Each sampling day, five water samples (2.5 L each) from different sprinklers were taken from each of the two experimental fields. In the case of ClO_2 treated water, sodium thiosulfate was added to the bottles to quench disinfectant residuals. Water was analyzed for the concentration of culturable and viable generic *E. coli* and for the occurrence of bacterial pathogens (*Salmonella* and pathogenic *E. coli*), as previously described. Physicochemical parameters, such as water absorbance at 254 nm (UV254), were also determined as mentioned above. Plant sampling was performed manually under aseptic conditions. Each sampling day, five samples of baby spinach leaves (100 g) were taken at five different points in each plot and then transported, under refrigeration, to the lab. Analyses were performed on the same harvest day. Plants were analyzed for presence of *Enterobacteriaceae* in four samplings performed weekly during the last month before harvest, using the protocol described in López-Gálvez et al. (2010). Experiments were performed in two growing seasons (Nov–Dec 2015, and Feb–Mar 2016).

Commercial greenhouse study: Effluent from the secondary treatment of a wastewater treatment plant (secondary water) was used for sprinkler irrigation of baby leaf lettuce. Plants were cultivated in trays, with peat as the substrate. The impact of irrigation using secondary water and secondary water treated with ClO_2 was examined. ClO_2 preparation, application, and measurement were performed as explained for the open field experiment. In this case, the target was the reduction of *E. coli* to levels below 2 log cfu/100 mL in irrigation water. Water samples were taken twice per week

during the growth cycle, while plant samples were taken five times along the last two weeks of the growth cycle. Water samples were analyzed for culturable *E. coli*, pathogenic bacteria, and physicochemical characteristics (pH, temperature, ORP, and UV254) following the described protocols. Plant samples were analyzed for culturable *E. coli* and pathogenic bacteria. In lettuce samples, culturable *E. coli* was analyzed as previously described; for pathogenic bacteria, lettuce samples were submitted to a non-selective enrichment in buffered peptone water and then the protocol continued as for water samples.

2.4. Evaluation of the impact of repeated ClO_2 treatments at the highest selected doses for pathogen removal on the soil and plant microbial communities

The impact of repeated ClO_2 treatments on the soil and plant microbial communities was carried out on samples taken in the experiments performed in the open field where baby spinach were grown. The experimental design was the same as previously mentioned.

Sampling**.** Baby spinach was sampled at the commercial stage (12–15 cm long, measured from the petiole) from plots irrigated with untreated and treated water. The experimental set up was the same as that used for open field experiments (section 2.3). Samples of 100 g each were hand harvested, by using scissors to cut from the base of the petioles, and stored in sterile plastic bags. Soil samples (25 g) were taken at the surface (no more than 3-cm depth) around each sampled plant. Soil and fresh produce samples were taken from five different positions distributed homogeneously in each sampling field. Irrigation water samples were taken as previously indicated.

DNA extraction*.* Baby spinach leaves (60 g) were sonicated with 240 ml of 0.2% sterile buffered peptone water (BPW; Scharlau Chemie, Barcelona, Spain) supplemented with 1% of Tween-80 (Sigma Aldrich, St Louis, MO, USA).). For soil, 3-g samples were stomached with 150 mL of BPW for 1 min. Sonicated spinach and stomached soil were centrifuged at 3000 ×*g* for 10 min and the pellet obtained was stored at -20 °C for genomic DNA extraction. Genomic DNA was extracted from pellets using the FastDNA® SPIN kit for soil and the FastPrep® 24-Instrument (MPBiomedicals, Germany), according to the manufacturer's indications. The quality and concentration of DNA extracts were determined by spectrophotometric measurement at 260/280 nm and 260/230 nm using a NanoDrop®ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For water samples, the methodology described previously (section 2.1) was used.

Illumina metagenomic sequencing*.* The V3-V4 hypervariabe region of the 16S rRNA gene was amplified using primers 534R and 8F with Illumina overhang adapters. The library performed as described previously (Camarinha-Silva et al., 2014). Libraries were sequenced in a MiSeq platform (Illumina). Paired-end raw sequences were matched using mothur (Schloss et al., 2009) and subsequently filtered as described previously (Camarinha-Silva et al., 2014). Briefly, reads were clustered allowing two mismatches using mothur. The data set was then filtered to consider only those phylotypes that were present in at least one sample at a relative abundance >0.1% or in all samples at a relative abundance >0.001%. Samples were re-sampled to the minimum sequencing depth using the package phyloseq from R-studio program. A total of 1,446,171 reads were obtained, and grouped into 803 phylotypes.

Statistical analyses*.* Microbiological and physicochemical data were statistically analyzed using IBM SPSS Statistics 19. Except when stated otherwise, *P* values below 0.05 were considered statistically significant. Shapiro-Wilk test was performed to assess the normality of the data ($P > 0.05$). Mann Whitney U and Kruskal–Wallis tests were used to examine the significant differences between *E. coli* levels and the presence/absence of pathogens. The Pearson´s correlation coefficient (*r* value) was calculated to determine the correlation between *E. coli* levels and

physicochemical parameters. The significance was determined at the 95% confidence level. In the case of Illumina metagenomic sequencing data, all statistical analyses were performed in R-studio program and IBM SPSS Statistics 19. Within each sample, total number of species, Fisher's diversity, Shannon, Simpson and inverse Simpson indices were calculated to assess the alpha diversity. Pielou's index was used as indicator of evenness in the community. Differences in alpha diversity and evenness measures between treatments were compared using Mann Whitney U and Kruskal–Wallis tests. Bray-Curtis indices were used to construct dissimilarity matrices of the communities. Beta diversity of the community was determined and nonmetric multidimensional scaling (NMDS) was employed to visualize the differences among samples, using the vegan package in R (Oksanen et al., 2007). Dissimilarity analyses of bacterial community structures in samples from different treatment were calculated using the function Adonis (PERMANOVA) and ANOSIM. Differences in relative abundances of bacterial genera were compared using Mann Whitney U and Kruskal–Wallis tests.

Outcomes and Accomplishments

2.1. Microbial and physicochemical characterization of different water sources used commercially to grow leafy greens in Spain

Microbial characterization. *E. coli* levels of the tested water sources were significantly different using both cultivable and PMA-qPCR techniques (**Fig. 1**). Propidium monoazide (PMA) was used to distinguish between dead and live *E. coli* cells. The highest *E. coli* values where obtained from drainage ditches (median 4.03 log cfu/100 mL, IQR 3.79 – 4.51) followed by surface water obtained from canals (median 2.87 log cfu/100mL, IQR 2.66 – 2.97). These levels were above the *E. coli* limits included in current legislation (i.e., FSMA) and the *E. coli* levels recommended by most GAP guidelines (≤2 log/100 mL). Surface water obtained from reservoirs showed the lowest *E. coli* levels (median 1.08 log cfu/100 mL, IQR 0.71–1.27) which, in most cases, were below 2 log/100 mL (**Fig. 1A**). When the PMA-qPCR assay was used to quantify the *E. coli* levels, the same tendency observed using culture methods was also obtained. Furthermore, *E. coli* levels quantified using the PMA-qPCR assay were similar to those obtained using culture methods, except for water samples from reservoirs (**Fig. 1**). *E. coli* levels in water reservoirs quantified using PMA-qPCR were higher than those reported by culture methods, leading to an underestimation of the current *E. coli* load. These differences could be explained because, in most of the cases, water reservoirs are frequently treated with chemical agents to avoid algae development, which might have an impact on the viability of *E. coli* cells. In these cases, the PMA-qPCR technique may be a more suitable method for the accurate estimation of *E. coli* loads when compared with traditional cultivation methods. Based on the results obtained, clear differences were observed among the different types of surface water, indicating that water stored in reservoirs could represent the lowest risk, but caution should be applied because the levels of *E. coli* can vary significantly depending on several factors, including geographical location, weather conditions, and season.

The prevalence of enteric foodborne pathogens in the different types of irrigation water are shown in **Table 1**. Among the tested water samples, 73% (84/113) were PCR positive for at least one bacterial pathogen (e.g., *Salmonella* spp., *E. coli* O157:H7, STEC). However, only 49% (55/113) were confirmed by selective media and subsequent latex agglutination test (when applicable). Based on confirmed results, *Salmonella* was present in 43% of samples (49/113), followed by *E. coli* O157:H7 (12%; 14/113), and non-O157:H7 STEC strains (11%; 12/113). Significant differences were observed among the different water sources. The highest prevalence of foodborne pathogens was found in surface water obtained from drainage ditches (70%; 30/43) and canals (70%; 21/30), followed by water samples obtained from the reservoirs (10%; 4/40). Based on the results obtained,

the use of water from drainage ditches and canals represents a high risk of produce contamination with foodborne pathogens.

Correlation between indicator and pathogenic microorganisms. To determine the potential correlation between *E. coli* levels in irrigation water and the prevalence of enteric foodborne pathogens, Mann Whitney U and Kruskal-Wallis tests were used. Our results showed that *E. coli* levels higher than approximately 1.5 log cfu/100 mL were associated with a higher probability of presence of foodborne pathogens (**Fig. 2**).

Additionally, the suitability of two *E. coli* quantification techniques (plate counts and PMA-qPCR) and their ability to detect the presence of pathogenic bacteria were also examined. In this case, we only considered data from water reservoirs where *E. coli* levels were significantly different when using both quantification techniques. The results obtained did not detect significant differences between the two enumeration methods and the correlation with the presence/absence of pathogens (**Fig. 3**). However, the significance values found between levels of *E. coli* of those samples positive and negative and the presence of foodborne pathogens were lower in the PMA-qPCR cases ($P < 0.164$) than in those with the plate count method ($P < 0.445$). Therefore, based on the results obtained, *E. coli* levels using PMA-qPCR quantification technique might be a more accurate indicator to predict the presence of pathogenic bacteria in this type of irrigation water in which chemicals were used to avoid algae development.

Physicochemical characterization. The physicochemical parameters of the water samples were monitored from January to December. The objective was to identify physicochemical parameters highly correlated with the microbial load of the irrigation water. Several physicochemical parameters, such as organic load (monitored as chemical oxygen demand, COD), pH, conductivity, alkalinity, turbidity, total soluble solid content and UV254, were monitored weekly. Only UV254 results showed a pattern similar to the load of *E. coli* present in the irrigation water samples (**Fig. 4**).

2.2. Pilot plant studies to establish effective dose thresholds of stabilized ClO_2 in different water sources

The first objective was to select the most relevant parameters related with the disinfectant demand of the irrigation water. Thus, the relationship between different physicochemical parameters (COD, turbidity, pH, ORP, UV254, and conductivity) and the initial ClO_2 concentration of irrigation water was established by linear regression. UV254 showed the strongest relationship with ClO_2 demand of water (R^2=0.63). As a consequence, this parameter was selected for residual ClO_2 modelling. UV254 ranged between 0.02 and 0.11 (cm^{-1}) in the water samples used for residual ClO_2 tests. Regarding contact time, it was observed that beyond 1 min of contact time the concentration of ClO_2 did not change significantly. As a consequence, for the model development, only data obtained with a contact time of 1 min were used. Equation (1) shows the residual ClO_2 model obtained by stepwise linear regression. None of the terms initially included were removed by the regression procedure. The model had an adjusted R^2 of 0.93 and an RPD of 4.06.

$$C_t = 0.33 + 0.79 \cdot C_0 - 15.70 \cdot UV254 + 0.19 \cdot {C_0}^2 + 107.99 \cdot UV254^2 - 5.00 \cdot C_0 \cdot UV254 \quad \text{eq. (1)}$$

Where Ct is the concentration of ClO_2 after treatment (mg/L), C_0 is the initial ClO_2 concentration (mg/L), and UV254 is the UV absorbance of filtered irrigation water measured at 254 nm (cm^{-1}).

Figure 5 shows a good fit of calibration data used to develop the model with the data predicted by the model (**Fig. 5A**), and also a good fit of validation data to model predictions (**Fig. 5B**). Validation had an adjusted R^2 of 0.94 and an RPD of 2.87. The RPD >2 in calibration and validation indicates good fit and good predictions of the model. The residual ClO_2 model would be useful when aiming

for a specific residual concentration. For example, to avoid phytotoxic effects of an excess of disinfectant in contact with crops, the recommended residual ClO_2 concentration for continuous use in nurseries and greenhouses is 0.25 mg/L (WEAH, 2016).

UV254 ranged between 0.01 and 0.09 (cm^{-1}) in the water samples used for *E. coli* inactivation tests. *E. coli* concentration did not change when contact time was prolonged beyond 1 min, and therefore only data obtained with a 1-min contact time were used for model development. Initial *E. coli* concentrations ranged between 2.4 and 4.2 log cfu/100 mL for canal water, and between 2.8 and 5.3 log cfu/100 mL for drainage ditch water. The limit of detection for *E. coli* concentration in water was 0 log cfu/100 mL (1 cfu/100 mL). Reductions in canal water ranged between 0.0 to 2.7 log cfu/100 mL, while they ranged between 0.0 and 3.6 log cfu/100 mL in drainage ditch water.

The most suitable model for *E. coli* inactivation in irrigation water had the following expression (equation (3)):

$$\log\left(\frac{Nt}{N_0}\right) = 0.69 - 0.82 \cdot C_0 - 54.87 \cdot UV254 + 0.63 \cdot {C_0}^2 - 29.81 \cdot UV254 \cdot C_0 - 0.24 \cdot {N_0}^2 + 25.27 \cdot N_0 \cdot UV254 \quad \text{eq. (3)}$$

Where Nt is the concentration of *E. coli* after treatment (log cfu/100 mL), N_0 is the initial concentration of *E. coli* (log cfu/100 mL), C_0 is the initial ClO_2 concentration (mg/L), and UV254 is the UV absorbance of filtered irrigation water measured at 254 nm (cm^{-1}).

The adjusted R^2 value of the *E. coli* inactivation model was 0.77, while RPD was 1.92. **Figure 6** shows the fit of the coupling of predicted and observed data to the perfect-fit line. Note that too large reductions are predicted by the model when low reductions (<0.5 log) are observed. These cases correspond to trials in which low concentrations of disinfectant were used in water with high UV254 (drainage ditch water). This trend was confirmed by additional data obtained from experiments in which low disinfectant concentrations (0.1 mg/L) were used to treat drainage ditch water. Fitting of validation data to model predictions (**Fig. 7**) gave an R^2 value of 0.55 and an RPD of 1.75. The RPD value in calibration and validation (2>RPD>1.5) indicates fair fit and fair predictions of the model; RPD values <1.5 would indicate poor predictions. The predictive model for *E. coli* inactivation could help growers in the selection of appropriate doses to comply with microbiological criteria established in guidelines and legislation. For example, the Food Safety Modernization Act (FDA, 2011) establishes criteria of geometric mean <126 cfu *E. coli*/100 mL and statistical threshold <410 cfu *E. coli*/100 mL for agricultural water directly applied to growing produce. On the other hand, the legislation in Spain on reuse of reclaimed water states a maximum concentration of 10^2–10^3 cfu *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 for the number of *E. coli* (M) = 1000 cfu/100 mL, number of sample units where the *E. coli* count may be between m and M (c = 3) when there is direct contact of water with produce that will be consumed raw (Real Decreto 1620/2007, 2007).

Regarding pathogenic bacteria inactivation, results obtained by direct plating varied depending on the microorganism and on the water type. Initial ClO_2 doses of 0.25 and 0.5 mg/L were needed to bring *E. coli* O157:H7 levels below the limit of detection in 1 min in canal and drainage ditch water, respectively. In the case of *Salmonella* spp., an initial dose of 1 mg/L was needed in both types of irrigation water to obtain the same results. Enrichment results showed recovery of damaged cells in the case of *Salmonella* spp. For this microorganism, many samples that were negative after direct plating were positive after enrichment. This means that higher ClO_2 doses would be needed for complete inactivation. In the case of *E. coli* O157:H7 there was no recovery of damaged cells, as samples that were negative after direct plating were not positive after enrichment.

2.3. Practical demonstration of the ClO_2 stability under field conditions

Open field study. Detected ClO_2 concentrations were always <1 mg/L in both experiments. During the first trial, control of ClO_2 concentration was performed approximately once a week. However, fluctuations in concentration were detected, and there were important information gaps using this measurement schedule (**Fig. 8A**). To improve the control and the establishment of lower residual concentrations, ClO_2 measurements were performed approximately three times per week in the second trial. In this way, a better control of ClO_2 concentration was achieved, and lower and more constant residual ClO_2 was maintained (**Fig. 8B**). Mean initial concentration of ClO_2 injected at the irrigation head was 0.42±0.16 mg/L and 0.26±0.11 mg/L in the first and second trials, respectively. A mean concentration of 0.32±0.25 mg/L and 0.07±0.04 mg/L was measured in sprinklers from the treatment plot during the first and the second experiments, respectively. Measurements performed on the sprinklers of the untreated field always gave results below the limit of detection (<0.02 mg/L).

Concentration of culturable *E. coli* in sprinkler water in the first and the second test is shown in **Figure 9**. Culturable *E. coli* concentration (mean±SD) in the first test was 0.4±0.4 log cfu/100 mL in control water and 0.2±0.3 log cfu/100 mL in treated water; in the second test, concentrations were 0.5±0.2 log cfu/100 mL in control water and 0.15±0.13 log cfu/100 mL in treated water. The difference between control and treated water for culturable *E. coli* was statistically significant in both tests ($P < 0.05$). The concentration of viable *E. coli* in sprinkler water was also examined to determine if the use of quantification techniques based on cultivation could overestimate the efficacy of the disinfection treatment (**Fig. 10**). Concentration of viable *E. coli* in the first trial was 1.51±0.26 log cfu/100 mL in control water and 1.5±0.3 log cfu/100 mL in treated water; in the second trial, concentrations were 2.1±0.4 log cells/100 mL in control water and 2.2±0.6 log cells/100 mL in treated water. The difference between control and treated water for viable *E. coli* was not statistically significant in both trials ($P > 0.05$). Real-time PCR results of samples from treated and untreated water from the first (n=10) and the second experiment (n=12) were all negative for the presence of *Salmonella* spp. and pathogenic *E. coli*. Mean±SD of UV254 in control water was 0.02±0.00 cm^{-1} in the first trial and 0.03±0.00 cm^{-1} in the second trial. Taking into account data from both tests, a significant negative correlation between UV254 of untreated water and residual ClO_2 at the sprinklers was found ($P < 0.01$; Pearson's correlation coefficient = −0.54). *Enterobacteriaceae* counts in baby spinach were between 3 and 5 log cfu/g in both experiments (**Fig. 11, Fig. 12**), without significant differences between control and treated plants.

Commercial greenhouse study. During the greenhouse study, initial ClO_2 doses in irrigation water ranged between 3.3 and 9.8 mg/L, while the residual ClO_2 concentration measured at the sprinkler ranged between <0.02 and 0.3 mg/L. Mean concentration of culturable *E. coli* was 3.6±0.8 and 2.0±0.9 log cfu/100 mL in untreated and treated secondary water, respectively. Seven water samples (n=16) were positive for the presence of pathogenic bacteria, six of them corresponding to untreated water and one to treated water. *E. coli* O157:H7 was present in all the water samples positive for pathogens. One of the positive untreated water samples was also positive for *Salmonella* spp. ORP and pH were higher in treated water compared with untreated water. Mean culturable *E. coli* concentration was 1.2±0.3 and 0.8±0.1 log cfu/g in plants irrigated with untreated water and ClO_2 treated water, respectively. Only one sample of lettuce (n=30) irrigated with untreated water was positive for the presence of pathogenic bacteria (*E. coli* O157:H7).

2.4. Impact of repeated ClO_2 treatments on the soil, plant and water microbial communities

Changes in the bacterial communities of irrigation water, baby spinach and soil. In terms of bacterial diversity, the species richness, the alpha-diversity index and evenness for the different agronomic habitats (soil, baby spinach and irrigation water) were calculated. These indices did not

show significant differences (Mann Whitney test, $P < 0.01$) between the control and samples treated with ClO_2, suggesting a similar range of diversity between treatments (**Table 2**). Nonmetric multidimensional scaling (NMDS) plots using Bray-Curtis distance, based on the matrix of operational taxonomic units (OTUs), were performed to evaluate the structure of bacterial communities from the different agronomic habitats, control and treated with stabilized ClO_2 (**Fig. 13**). NMDS plots for baby spinach revealed that control and treated bacterial communities clustered together (**Fig. 13B**). In contrast, bacterial communities from control and treated soil and water were separated (**Fig. 13A**, **Fig. 13C**). However, the dissimilarity analysis of bacterial community structures from irrigation water, plants and soil of control and treated samples revealed that disinfection with ClO_2 contributed significantly to the differences on OTU abundance only in the irrigation water (**Table 3**). Therefore, these results suggested that ClO_2 treatments only impacted the bacterial community structure of irrigation water.

Bacterial community composition of soil, baby spinach and irrigation water. The composition and distribution of sequences from bacterial communities present in the different habitats (soil, baby spinach and irrigation water) from control and treated samples (treated with ClO_2) were studied. To gain insight into the microbial changes elicited by disinfection treatments, control and treated samples were compared.

The dominant phyla associated with all soil samples were *Actinobacteria* (43 and 40%; average for control and treated samples, respectively), followed by *Proteobacteria* (28 and 28%) and *Firmicutes (*22 and 24%*)*. *Actinobacteria, Bacilli, Alphaproteobacterias* and *Gammaproteobacterias* were the more predominant classes in all samples (**Fig. 14**). Further analyses of data at family or genus levels revealed the presence of 23 major families (abundance >1% in at least one sample) and 58 genera (abundance >0.5% in at least one sample) in the soil samples. Among them, the four most abundantly represented families were *Bacillaceae* (13.5 and 14.3%), *Nocardioidaceae* (9.6 and 10.1%), *Micrococcaceae* (9.7 and 9.8%), and *Streptomycetaceae* (7.2 and 7.4%) as well as the members of genera *Bacillus* (10.0 and 9.9%), *Streptomyces* (6.6 and 6.7%), *Arthrobacter* (6.1 and 6.0%), and *Nocardioides* (5.9 and 6.0%). To determine the continuous impact of ClO_2 treatment on the changes in the composition of bacterial community of soil, untreated and treated soil samples were compared. Based on the results obtained, no significant differences in the diversity at phylum and class levels were observed between treatments, except for *Betaproteobacteria.* In treated soil, ClO_2 treatment reduced (by 1.6 fold) the relative abundance of *Betaproteobacteria* compared with the control. At lower taxonomic levels**,** the relative abundances of the phylotypes belonging to two families and two genera were significantly reduced by the treatment (**Table 4**)*.* The relative abundance of *Limnobacter* and *Pontibacter* significantly decreased in treated samples compared with the control. Therefore, changes in the relative abundance of the most dominant members from phylum, class, family and genus levels were generally small in the soil irrigated with treated water.

In baby spinach, the most dominant phyla were *Proteobacteria*, *Actinobacteria* and *Firmicutes,* which accounted for more than 88% of all the sequences. *Actinobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacilli* and *Betaproteobacteria* were the bacterial families with highest relative abundance in all baby spinach samples (**Fig. 14**). Therefore, comparison between treatments did not show significant differences at phyla and class level. However, when the major families and genera (those with average abundance >0.5% in at least one samples) were compared, significant differences were observed between control and treated baby spinach. The results showed that ClO_2 treatment significantly reduced the relative abundance of two families and four genera in the treated baby spinach compared with the control (**Table 4**). In general, those genera were not the most predominant in the bacterial community of baby spinach, except for *Pseudomonas*. Based on these results, ClO_2 treatments significantly reduced the relative abundance of *Pseudomonas* as well as other genera of lower abundance such as *Erwinia*, *Enterobacter* and *Tolumonas.*

In the case of all irrigation water samples, the bacterial community was dominated by *Proteobacteria* (41.5 and 39.6%, control and treated) and *Actinobacteria* (16.7 and 14.7%), followed by *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Verrumicrobia*. *Actinobacteria* and *Alpha*-, *Beta*-, and *Gammaproteobacterias* were the most predominant classes (**Fig. 14**). There were no significant differences in the relative abundance of these taxonomic groups of bacteria between control (untreated) and irrigation water treated with ClO_2. However, we observed that the relative abundance of *Verrumicrobia* and *Synergitetes* was significantly reduced in the treated samples compared with the control. Similar results were detected in their members, *Verrucomicrobiae* and *Synergistia*, respectively (**Fig. 14**). Regarding the core microbiota at genus level, 73 major genera (abundance >0.5% in at least one sample) were identified in the irrigation water samples, with 17 of them showing significant differences between treatments. Among them, ClO_2 treatment significantly reduced the relative abundance of phylotypes belonging to different genera, but it increased the relative abundance of a small proportion of irrigation water bacteria (**Table 4**). One of the most predominant genera, *Dethiosulfovibrio*, decreased in irrigation water treated with the stabilized ClO_2.

Summary of Findings and Recommendations

The results obtained confirm that *E. coli* load is very variable depending on the source of irrigation water. Surface water has been classified as the irrigation water type at "most risk" and this study confirms that statement. However, among the different types of surface water that growers might have available, water from drainage ditches should be avoided. Additionally, it was confirmed that levels of *E. coli* higher than 1.5–2.0 log cfu/100 mL were closely related to a higher probability of the occurrence of pathogens. It should be taken into account that samples positive for the presence of pathogenic bacteria were occasionally isolated from water samples with *E. coli* levels below 1.5 log cfu/100 mL. Plate count techniques seem to be a suitable quantification method, as no significant differences were observed between this classical method and the molecular PMA-qPCR technique. In cases where chemicals might be used for disinfection or to prevent algae formation, however, the plate count techniques might underestimate the *E. coli* population in irrigation water.

The mathematical model based on initial *E. coli* load of the irrigation water and the physicochemical parameter UV254 can be a useful tool to predict the initial ClO_2 concentration that growers can use to reduce the initial *E. coli* load below the established limits.

In both studies (open field and commercial greenhouse) the selected ClO_2 concentrations were efficient to reduce the *E. coli* load in irrigation water below the established microbial limits (FSMA). In the open field study, the microbiological quality of the irrigation water was very good, and the need for the disinfection treatment was very limited. However, when irrigation water of poor microbiological quality was used, as in the commercial greenhouse study, the selected treatment significantly reduced the *E. coli* load in the water. The comparison of two quantification methods (PMA-qPCR and plate count techniques) revealed that the slight differences observed between the *E. coli* loads in untreated and treated irrigation water disappeared. Therefore the applied disinfection treatment may not to be efficient enough to kill the bacterial cells but it may inactivate them.

The abundance and diversity of the water microbiota were impacted by disinfection treatment with ClO_2. However, disinfection with ClO_2 in irrigation water slightly affected the bacterial community of baby spinach and soil, as only small changes were detected at lower taxonomic levels. Abundance of *Pseudomonadaceae* and *Enterobacteriaceae* decreased in baby spinach treated with ClO_2 compared with the control. This finding is remarkable because a large proportion of foodborne and pathogenic bacteria associated with fresh produce belong to these families. Therefore, stabilized ClO_2 could be defined as an eco-friendly disinfection technology.

APPENDICES

Publications derived from the project:

Truchado, P., Gil, M.I., Kostic, T., Allende, A. 2016. Optimization and validation of a PMA qPCR method for *Escherichia coli* quantification in primary production. Food Control, 62, 150-156.

Truchado, P., Lopez-Galvez, F., Gil, M.I., Pedrero-Salcedo, F., Alarcon, J.J., Allende, A. 2016. Suitability of different *Escherichia coli* enumeration techniques to assess the microbial quality of different irrigation water sources. Food Control, 58, 29-35.

Truchado, P., Gil, M.I., Hernandez, N., Allende, A. 2017. Relationship between cultivable and VBNC *E. coli* levels and presence of pathogens in surface irrigation water and adequacy of current microbiological targets for agricultural water. Frontiers in Microbiology, *Submitted*.

López-Gálvez, F., Sampers, I., Gil, M.I., Allende, A., 2017. Modelling of generic *E. coli* inactivation by chlorine dioxide in irrigation water. Food Control, *Submitted*.

López-Gálvez, F., Meireless, A., Truchado, P., Gil, M.I., Allende, A. 2017. Demonstration of practical, effective and environmentally sustainable agricultural water disinfection based on chlorine dioxide in open field experiments. Postharvest Biology and Technology, *Submitted*.

Truchado, P., Moreno, M., Gil, M.I., Allende, A. 2017. Characterization of microbial communities on field grown baby spinach irrigated with untreated and treated water using chlorine dioxide. Frontiers in Microbiology, *In preparation*.

Truchado, P., Moreno, M., Gil, M.I., Allende, A. 2017. Changes in soil and baby spinach main bacteria genera as influenced by agricultural practices during a growing season on commercial open fields. Food Microbiology, *In preparation.*

Presentations derived from the project:

Lopez-Galvez, F., Truchado, P., Gil, M.I., Allende, A. 2016. Demonstration of practical, effective and environmentally sustainable agricultural water treatments to achieve compliance with microbiological criteria. Oral presentation at CPS Produce Research Symposium, Seattle, June 28-29, 2016.

Truchado, P., Lopez-Galvez, F., Gil, M.I., Allende, A. 2015. Demonstration of practical, effective and environmentally sustainable agricultural water treatments to achieve compliance with microbiological criteria. Oral presentation at CPS Produce Research Symposium, Atlanta, Georgia, June 23-24, 2015.

Budget Summary

	2015				
	March	**June**	**September**	**December**	**TOTAL**
SUPPLIES AND MATERIALS	5,300.60	5,208.70	5,939.00	5,359.60	
TRAVEL AND	343.30	657.10	4,610.00	4,462.20	
OTHERS	0	0	712.78	0	
PAYROLL WAGES AND WITHHOLDINGS	0	3,220.00	5,609.60	10,508.70	
TOTAL	5,643.90	9,085.00	16,871.40	20,330.50	**51,930.80**
OVERHEAD COSTS	0	161.00	280.50	525.40	**966.90**
	2016				
	March	**June**	**September**	**December**	**TOTAL**
SUPPLIES AND MATERIALS	8,267.20	23,995.03	25,031.50	48,607.60	
TRAVEL AND	358.60	3,123.70	7,727.60	469.20	
OTHERS	266.00	779.60	320.30	4,058.60	
PAYROLL WAGES AND WITHHOLDINGS	14,647.30	26,109.80	20,313.10	17,440.80	
TOTAL	23,539.20	54,008.20	53,392.50	70,136.80	**201,076.70**
OVERHEAD COSTS	732.30	1,305.50	1,015.60	872.00	**3,925.40**
TOTAL DIRECT COSTS					**253,007.50**
TOTAL INDIRECT COSTS					**4,892.30**
TOTAL					**257,899.80**

Tables and Figures

Table 1. Prevalence of pathogenic microorganisms in different irrigation water sources.

Sample type*	***E. coli* O157:H7**		**Non-O157:H7 STEC**		***Salmonella***	
Irrigation water	**PCR**	**Confirmed**	**PCR***	**Confirmed**	**PCR**	**Confirmed**
Water reservoirs	4/40	4/4	14/40	1/14	3/40	2/3
Drainage ditches	14/43	6/14	28/43	7/28	33/43	26/33
Canal	9/30	4/9	28/30	4/28	24/30	21/24

*****Positive samples to *stx1* and/or *stx2* and *eae.*

Table 2. Species richness (total species), diversity (Shannon, Fisher's alpha, Simpson, and inverse Simpson indices) and evenness (Pielou's index) in different habitats (soil, baby spinach and irrigation water) for control and samples treated with stabilized ClO_2.

Index		**Soil (n = 10)** Mean ± SEM	**Plants (n = 20)** Mean ± SEM	**Irrigation Water (n = 20)** Mean ± SEM
Total species	Control	613 (609 ± 633)	556 (490 ± 607)	523 (514 ± 557)
	Treatment	599 (587 ± 613)	576 (470 ± 6.21)	558 (534 ± 572)
Shannon	Control	4.67 (4.61 ± 4.78)	3.05 (1.05 ± 4.46)	4.11 (3.97 ± 4.19)
	Treatment	4.73 (4.56 ± 4.78)	2.86 (1.50 ± 0.43)	4.18 (4.05 ± 4.39)
Fisher	Control	8.70 (8.57 ± 8.76)	8.26 (6.80 ± 9.16)	7.75 (7.50 ± 8.17)
	Treatment	8.85 (8.70 ± 9.01)	8.14 (6.74 ± 9.05)	8.02 (7.77 ± 8.54)
Simpson	Control	0.97 (0.97 ± 0.95)	0.75 (0.42 ± 0.94)	0.95 (0.94 ± 0.90)
	Treatment	0.97 (0.96 ± 0.98)	0.69 (0.42 ± 0.93)	0.96 (0.95 ± 0.97)
Inverse Simpson	Control	34.01 (33.25 ± 41.32)	6.52 (1.74 ± 19.53)	21.25 (18.03 ± 27.56)
	Treatment	39.48 (32.32 ± 42.47)	13.03 (2.11 ± 16.15)	25.01 (21.27 ± 26.81)
Pielou	Control	0.73 (0.72 ± 0.75)	0.48 (0.24 ± 0.70)	0.65 (0.64 ± 0.69)
	Treatment	0.73 (0.71 ± 0.74)	0.45 (0.24 ± 067)	0.66 (0.64 ± 0.69)

Table 3. Bacteria genera that showed significant differences ($P < 0.05$) in their relative abundances between control and treated sample in the different environmental samples (soil, baby spinach and irrigation water).

Sample	Taxonomy*							
	Phylum	**Class**	**Order**	**Family**	**Genera**	***P***	**Control**	**Treatment**
Soil	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Limnobacter*	0.009	0.64±0.17	0.20±0.11
	Bacteroidetes	*Sphingobacteriia*	*Sphingobacteriales*	*Flexibacteraceae*	*Pontibacter*	0.016	0.78±0.12	0.45±0.15
Baby spinach	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.019	7.96±4.58	2.92±0.31
	Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Erwinia*	0.004	0.36±0.03	0.06±0.06
	Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter*	0.023	0.37±0.05	0.10±0.13
	Proteobacteria	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Tolumonas*	0.043	0.30±0.32	0.09±0.07
Irrigation water	*Actinobacteria*	*Acidimicrobiia*	*Acidimicrobiales*	*Acidimicrobiaceae*	*Acidimicrobium*	0.043	0.30±0.13	0.18±0.10
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Cellulomonadaceae*	*Demequina*	0.029	2.50±1.50	4.03±0.55
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*	*Kribbella*	0.007	0.94±0.35	0.58±0.14
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Mycobacteriaceae*	*Mycobacterium*	0.000	1.33±0.59	0.33±0.19
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	0.002	0.33±0.13	0.17±0.04
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Pseudonocardiaceae*	*Saccharopolyspora*	0.000	4.40±1.04	1.57±0.57
	Actinobacteria	*Nitriliruptoria*	*Euzebyales*	*Euzebyaceae*	*Euzebya*	0.000	0.51±0.14	0.25±0.04
	Bacteroidetes	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*	*Chitinophaga*	0.000	0.27±0.13	0.62±0.21
	Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	0.009	1.44±0.35	1.89±0.33
	Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Cryomorphaceae*	*Fluviicola*	0.000	0.37±0.22	0.95±0.25
	Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	0.290	0.54±1.38	2.26±0.87
	Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	0.004	0.28±0.05	0.41±0.10
	Planctomycetes	*Brocadiae*	*Brocadiales*	*Brocadiaceae*	*Candidatus Scalindua*	0.007	1.01±0.68	1.93±0.55
	Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*	*Acidiphilium*	0.000	1.14±0.51	2.80±0.64
	Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Kaistobacter*	0.004	0.15±0.30	0.05±0.07
	Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*	0.000	0.85±0.29	0.38±0.09
	Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter*	0.003	1.40±0.25	0.91±0.30
	Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingobium*	0.002	0.45±0.63	0.10±0.02
	Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	0.000	1.61±0.14	0.56±0.03
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Polaromonas*	0.002	0.21±0.15	0.48±0.16
	Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Polynucleobacter*	0.043	0.31±0.11	0.44±0.01
	Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Campylobacter*	0.000	3.97±0.81	13.34±5.96
	Proteobacteria	*Gammaproteobacteria*	*Legionellales*	*Legionellaceae*	*Legionella*	0.000	0.39±0.10	0.24±0.04
	Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Luteibacter*	0.002	3.17±0.97	2.00±0.37
	Synergistetes	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*Dethiosulfovibrio*	0.002	5.20±1.99	2.58±0.88
	Tenericutes	*Mollicutes*	*Mycoplasmatales*	*Mycoplasmataceae*	*Mycoplasma*	0.001	0.90±0.18	1.67±0.41
	Verrucomicrobia	*Spartobacteria*	*Chthoniobacterales*		*Candidatus Xiphinematobacter*	0.002	1.42±0.27	0.95±0.20

*Only genera greater and equal to 0.5% and differed with a *P*-value less than 0.05 (Mann Whitney) are shown.

Table 4. Dissimilarity analysis of bacterial communities structured in soil, baby spinach and irrigation water.

	Adonis		**Anosim**	
	F	***P***	***R***	***P***
Soil	1.866	0.07	0.196	0.08
Baby spinach	0.934	0.31	0.008	0.29
Irrigation water	5.881	**0.01**	0.476	**0.01**

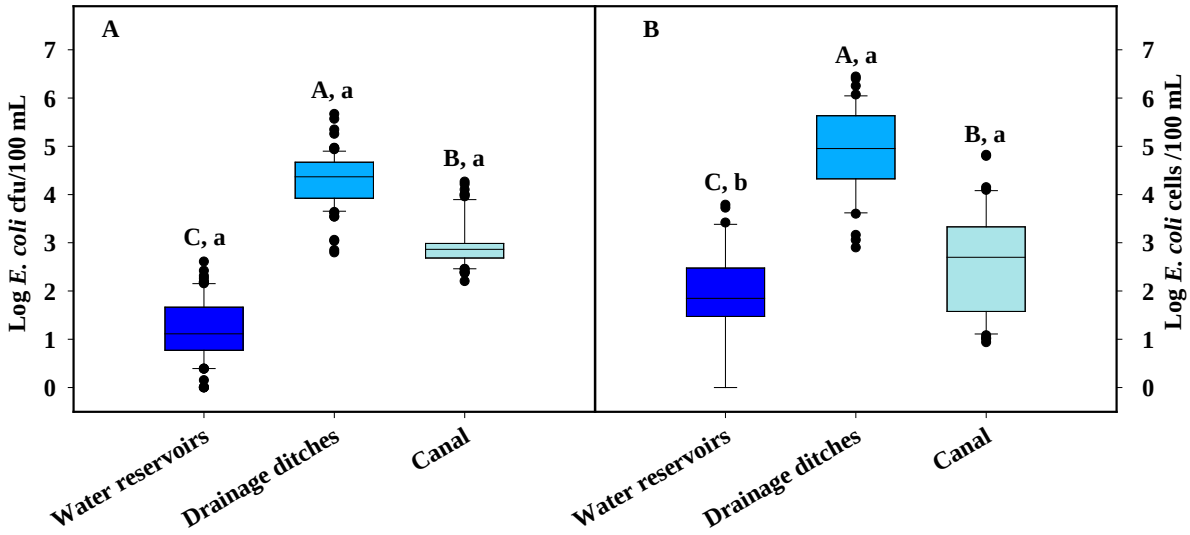

Figure 1. Box plots representing cultivable (plate count) (**A**) and molecular (PMA-qPCR) (**B**) *E. coli* counts (log cfu or cells/100 mL) in the different types of irrigation water sources.

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, whiskers showing the greatest values excluding outliers, and dots representing outliers (defined as values more than 3/2 times the corresponding quartile). Bars labelled with different uppercase letters indicate a significant difference among water sources at $P < 0.05$. Different lowercase letters indicate a significant difference between molecular (PMA-qPCR) and cultivable (plate count) methods at $P < 0.05$.

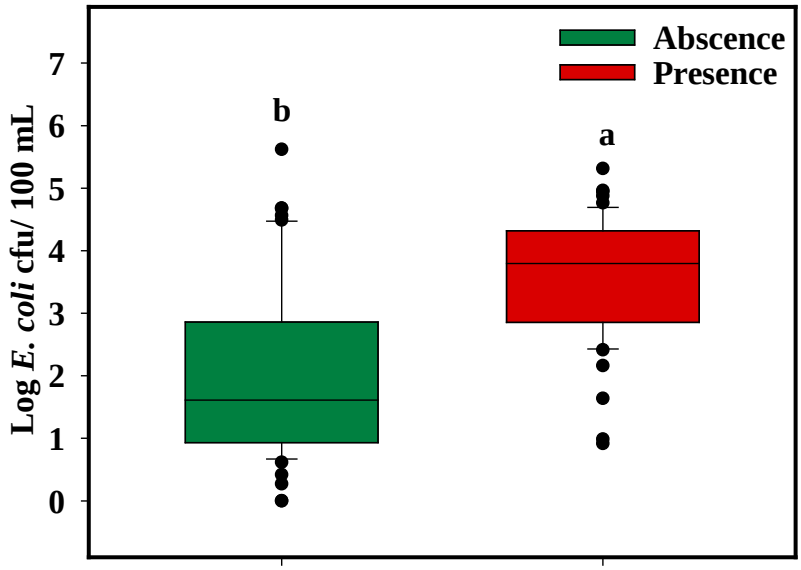

Figure 2. Box plots representing *E. coli* levels *(*log cfu/100 mL*)* in water samples, separated into those with absence or presence of foodborne pathogenic bacteria.
Box plots labelled with different letters are significantly different ($P < 0.05$).

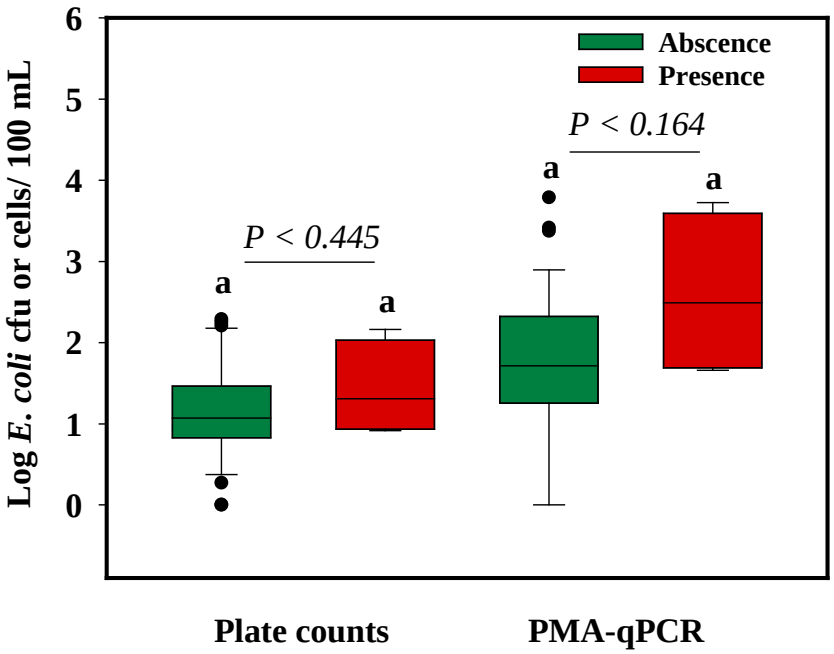

Figure 3. Box plots representing *E. coli* levels (log cfu/100 mL) in water reservoirs quantified by plate counts and PMA-qPCR, separated into those with absence or presence of foodborne pathogenic bacteria. Box plots labelled with different letters are significantly different ($P < 0.05$).

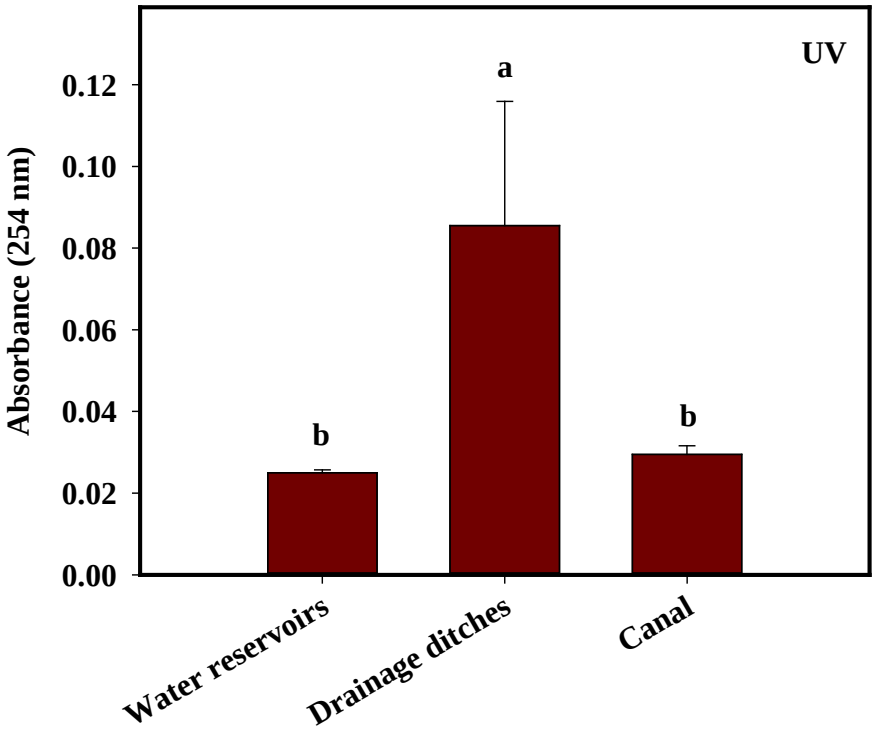

Figure 4. Physicochemical characterization of different irrigation water sources by absorbance at UV254. Bars labelled with different letters indicate significant differences at $P < 0.05$.

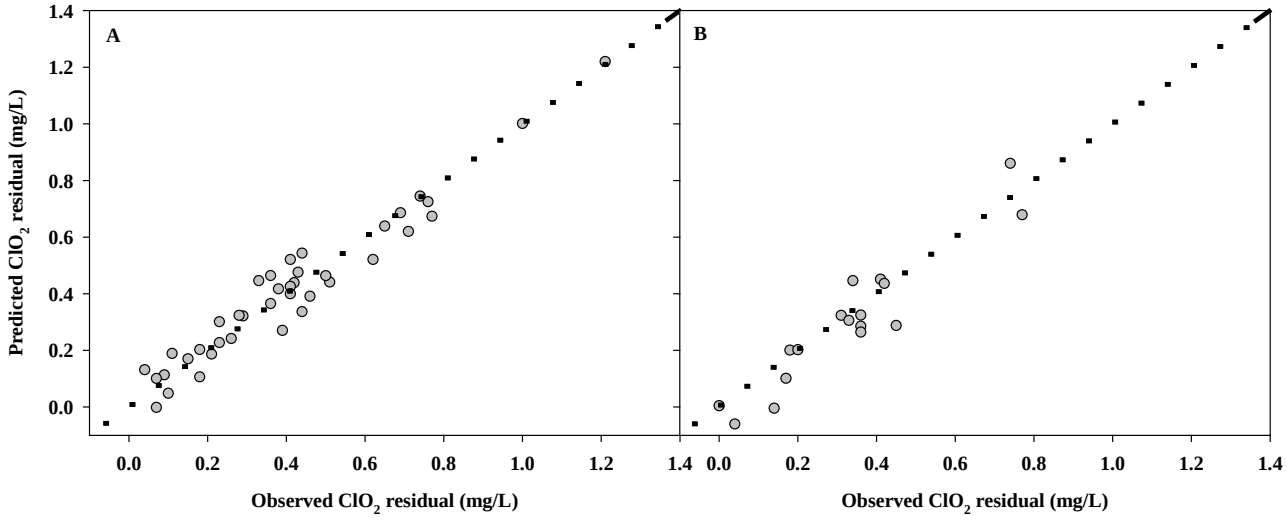

Figure 5. Scatter plots of observed data used to build the residual ClO_2 model vs data predicted by the model (A), and of observed validation data vs data predicted by the model (B).

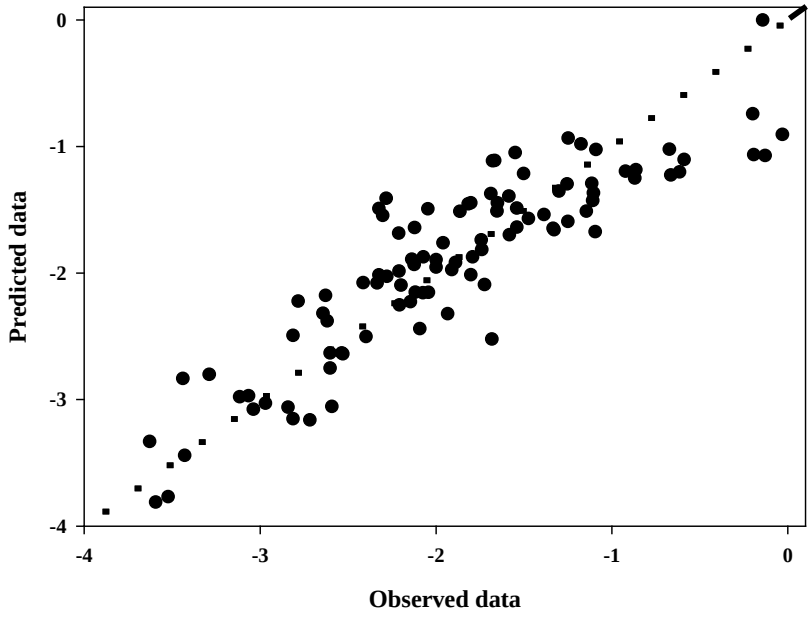

Figure 6. Scatter plot of observed *E. coli* reduction data (log cfu/100 mL) used to build the inactivation model vs reduction data predicted by the model.

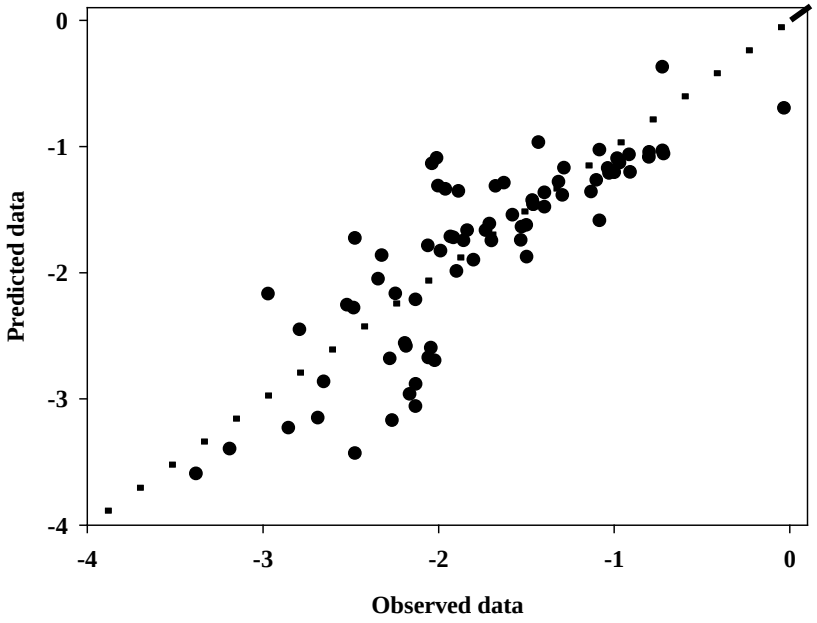

Figure 7. Scatter plot of observed validation data vs *E. coli* inactivation model predictions (log cfu/100 mL *E. coli* reductions).

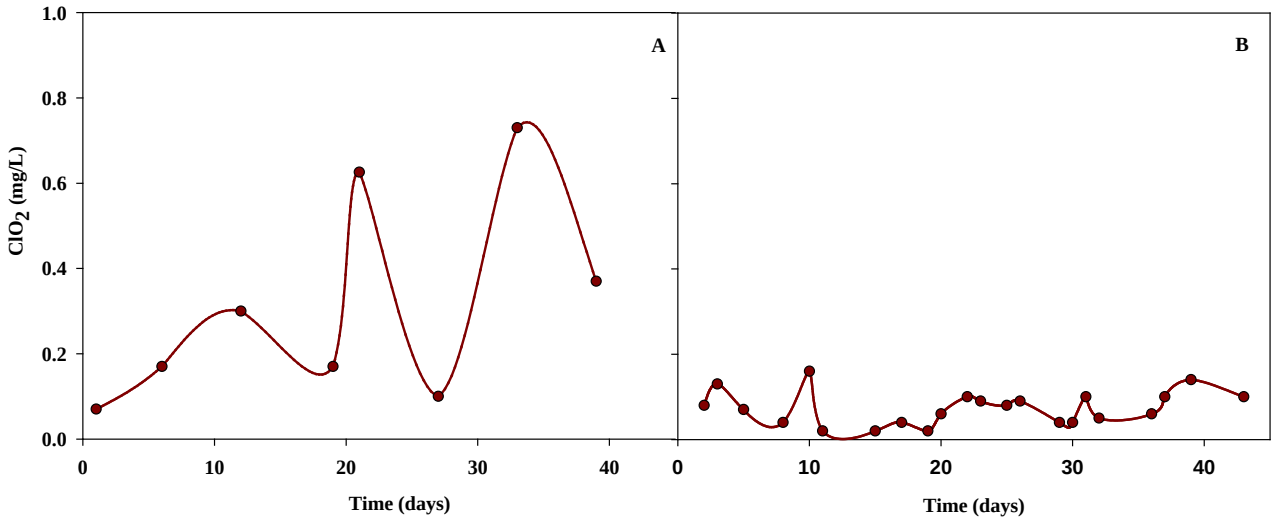

Figure 8. ClO_2 concentration (mg/L) in sprinkler water from the treated field. A: First test; B: second test.

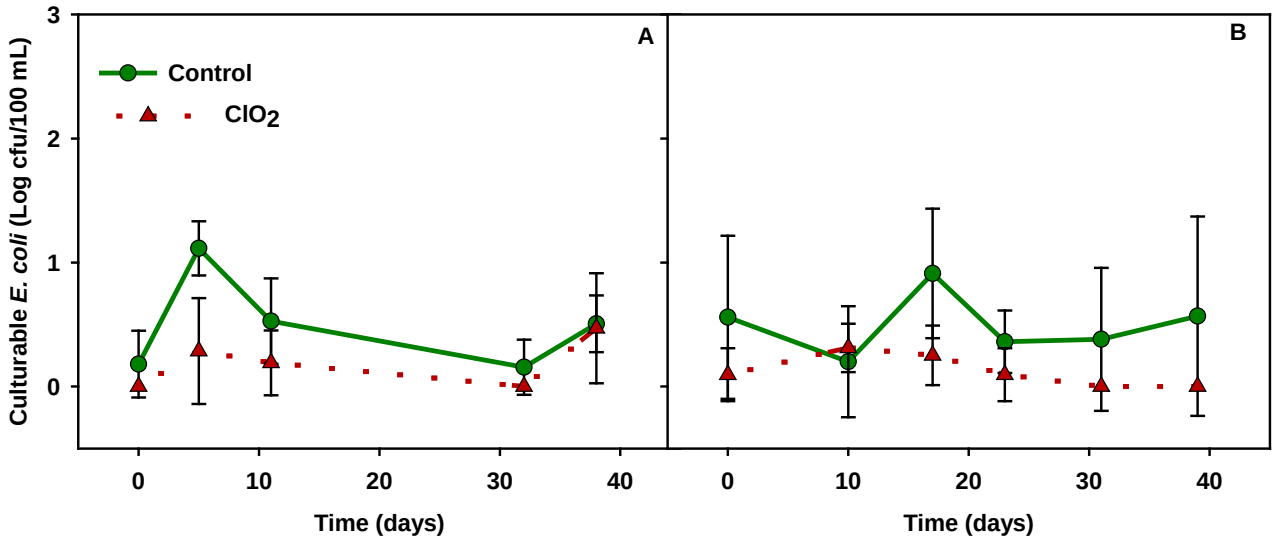

Figure 9. Culturable *E. coli* (log cfu/100 mL) in sprinkler water from control and ClO_2 treated fields. A: first test; B: second test.

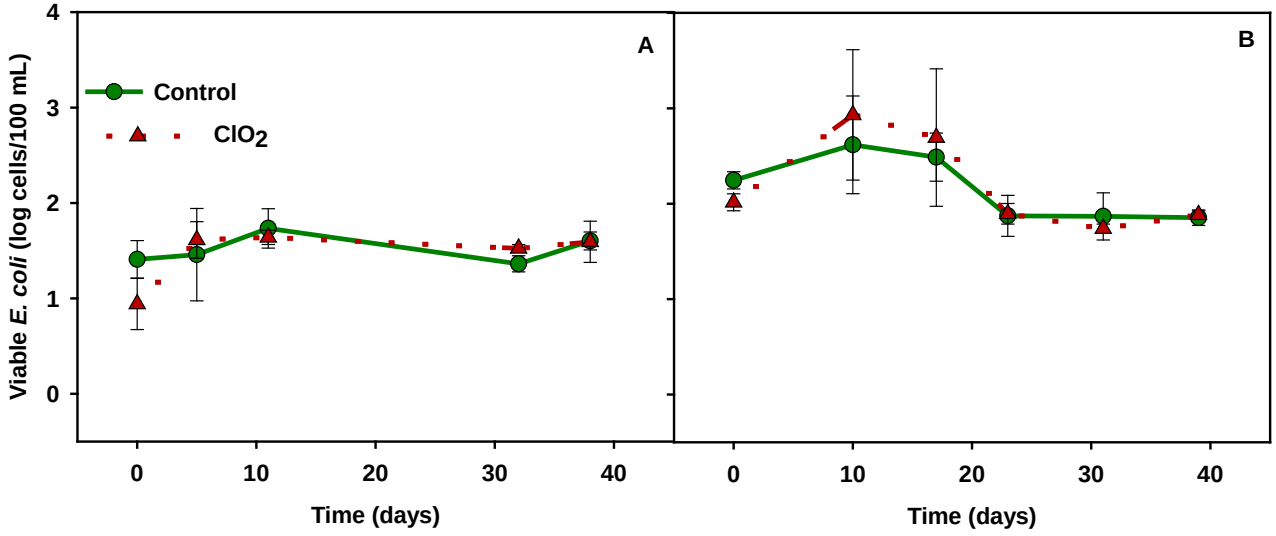

Figure 10. Viable *E. coli* (log cells/100 mL) in sprinkler water from control and ClO_2 treated fields. A: first test; B: second test.

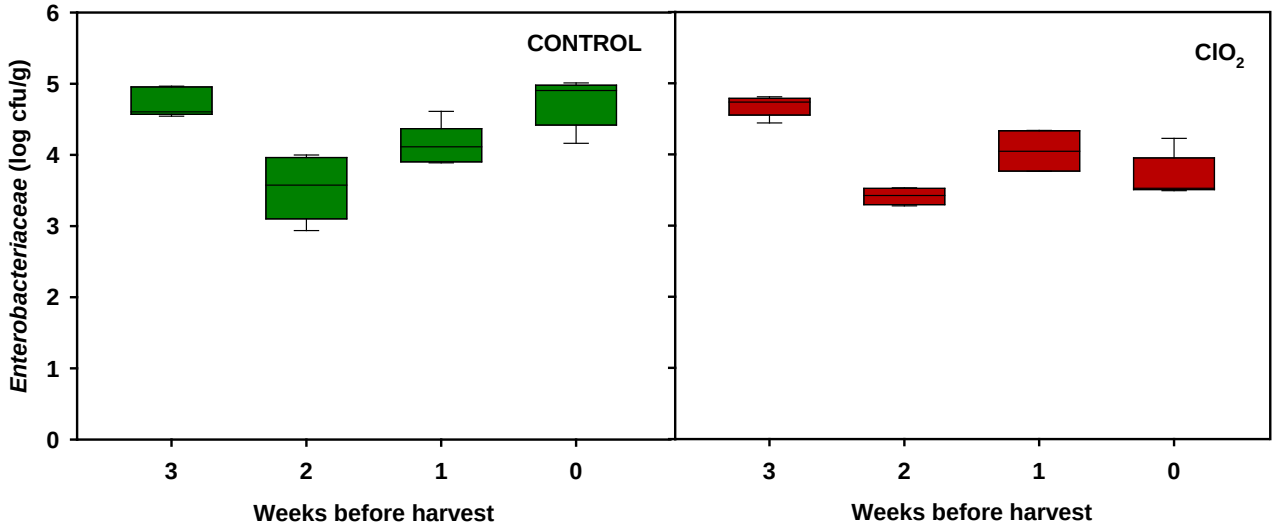

Figure 11. *Enterobacteriaceae* in baby spinach during the first trial.

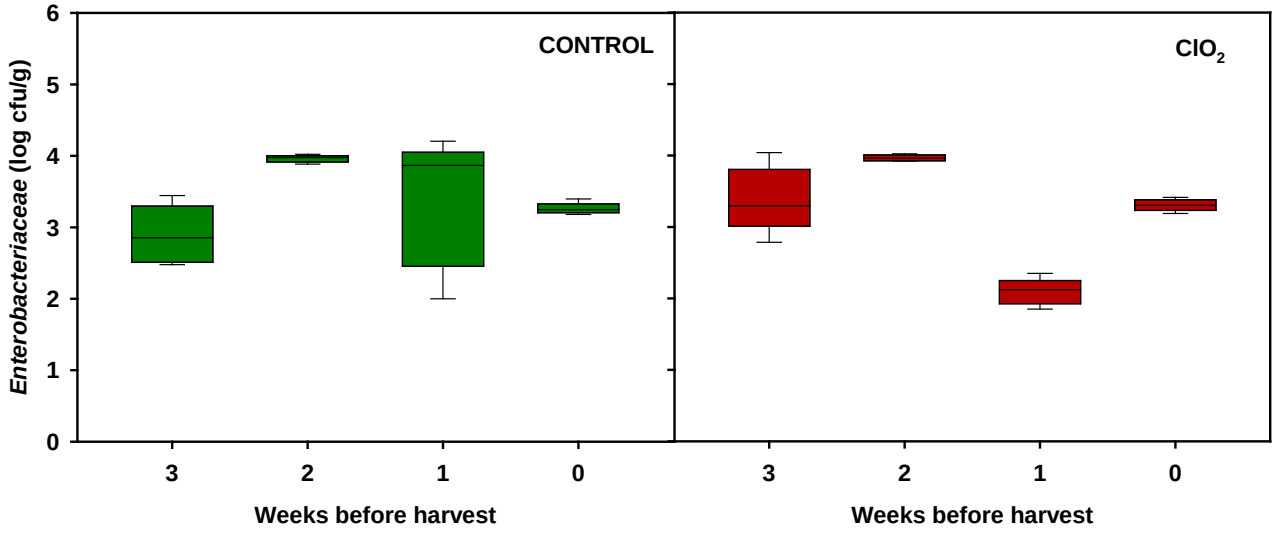

Figure 12. *Enterobacteriaceae* in baby spinach during the second trial.

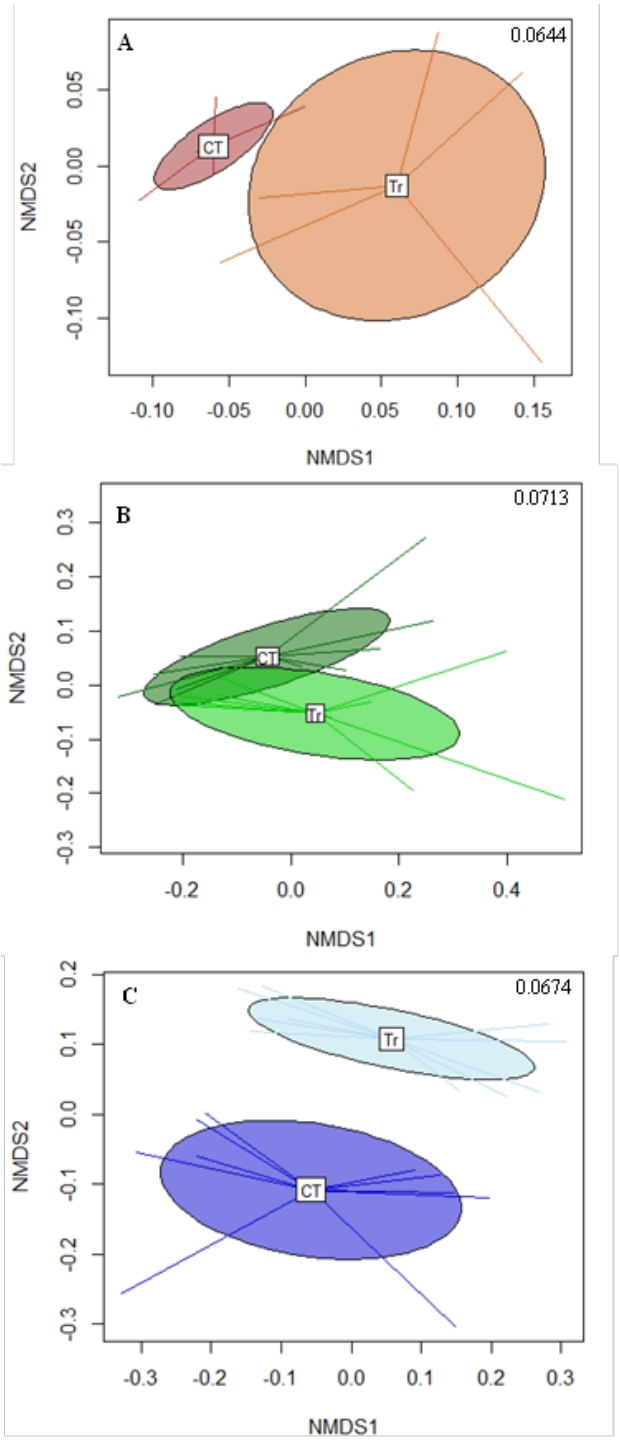

Figure 13. Nonmetric multidimensional scaling **(**NMDS) based on Bray–Curtis distance from OTU abundance, comparing bacterial communities of control and treated samples recovered from different habitats: soil (**A**), baby spinach (**B**), and irrigation water (**C**) samples.

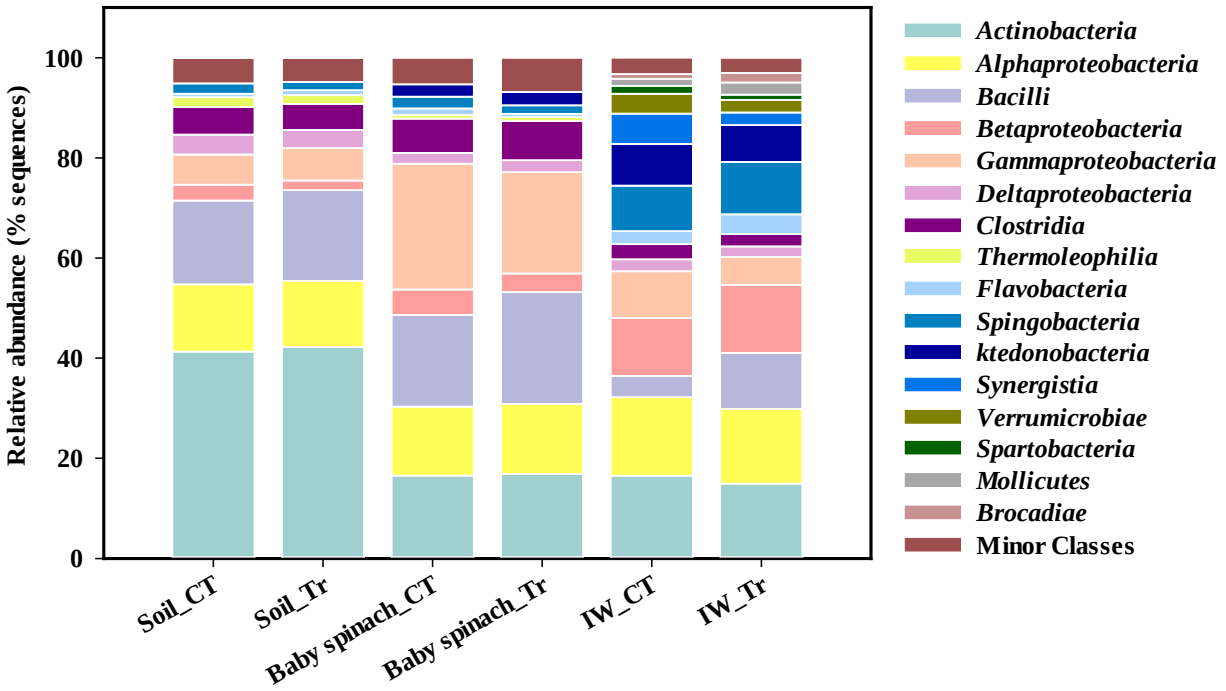

Figure 14. Composition of bacterial communities in control (CT) and treated (Tr) irrigation water (IW) and in associated soil and baby spinach samples. Bacterial communities are averages of 5 individual samples. Shown are classes that comprised at least 1% of the sequences in at least one sample of a given habitat.

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