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Correlation between *E. coli* levels and the presence of foodborne pathogens in surface irrigation water: Establishment of a sampling program

Pilar Truchado [a], Natalia Hernandez [a], Maria I. Gil [a], Renata Ivanek [b], Ana Allende [a, *]

[a] Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, Campus Universitario de Espinardo, 25, 30100, Murcia, Spain
[b] Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NW, USA

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ABSTRACT

To establish the association between microbial indicators and the presence of foodborne pathogens in irrigation water, *Escherichia coli* was enumerated using two quantification methods (plate counts and PMA-qPCR) and presence/absence of pathogenic microorganisms, including five strains from the Shiga toxigenic *E. coli* (O157:H7, O26, O103, O111 and O145) and *Salmonella* spp. were evaluated. The results confirmed that surface water can be considered a microbial hazard when used for irrigation. The levels of viable *E. coli* were very similar to those of cultivable *E. coli*, except for irrigation water obtained from water reservoirs. Comparison between the *E. coli* counts in samples positive and negative for the presence of pathogenic bacteria for the evaluated water sources identified *E. coli* level of 2.35 log cfu/100 mL as a cut-off able to correctly predict positive and negative samples with 93% sensitivity and 66% specificity, respectively. Thus, for the samples with levels of *E. coli* under 2.35 log cfu/100 mL (e.g., 2.24 log cfu/100 mL) there was a 90% probability that the samples were not contaminated with pathogenic microorganism in locations with similar prevalence. *E. coli* levels in irrigation water were affected by the ambient temperature confirming that water source and climate conditions should be taken into account by growers when designing a sampling program and the frequency of the monitoring to make a better and more efficient use of their resources.

1. Introduction

Agricultural water has been identified as one of the most important risk factors for fresh produce contamination with food borne pathogens during primary production (EFSA, 2014). Water sources used for irrigation are very diverse and usually characterized by different microbiological quality (Pachepsky et al., 2011). Classification of water sources based on their potential microbial contamination can be performed considering the origin of the water and the distribution systems. Based on this and the final uses of the water, reclaimed and surface water have been categorized as the riskiest water sources (Pachepsky et al., 2011). This is particularly relevant in water-scarce areas where intensive cropping is practiced and surface water is the main source of water for irrigation. This is the case in different areas in US (i.e. California) and the south of Europe (i.e. south east of Spain). However, in these areas, growers generally have access to different types of surface water, including water reservoirs, canals, and drainage diches. Information concerning the microbial quality of different surface water types is still scarce.

The microbiological quality of irrigation water is affected by environmental factors, of which weather conditions are the most important (Wilkes et al., 2009; Pachepsky et al., 2011; Holvoet et al., 2014). Recently published studies have indicated a potential association between irrigation water and the microbiological quality of fresh produce (Decol et al., 2017; Ceuppens et al., 2015). Establishment of microbial sampling programs for irrigation water has been purposed as a good strategy to reduce fresh produce contamination (Uyttendaele et al., 2015). In order to develop a sampling program, growers should be assisted in how the data should be collected including when, where and how to perform sampling. Sampling programs should be based on guidelines and legislation where microbial requirements are established such as the Codex Alimentarius, US Food and Drug Administration's Food

* Corresponding author.
E-mail address: aallende@cebas.csic.es (A. Allende).

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Safety Modernization Act (FSMA), as well as several guidelines on good agricultural practices (GAP) and quality assurance standards (Allende and Monaghan, 2015). Specifically, the new Produce Safety Rule established under FSMA has elaborated sampling programs based on data collection on *E. coli* as an indicator of fecal contamination and potential presence of pathogens (FDA, 2014). The rule requires growers to test and evaluate the microbial quality of their agricultural water by creating a water quality profile (WQP) that provides a characterization of risk based on two calculated values: geometric mean (GM) and the statistical threshold value (ST) FDA, 2014). Characterization of water source via a water quality profile would entail taking 20 samples over a two-year period as close to harvest as is practicable. One reason for this request might be the fact that several factors affect the microbiological quality of irrigation water including weather factors (i.e. precipitation), geographical location (i.e. presence of wild and domestic animals) and seasonality (i.e. water temperature) (Allende and Monaghan, 2015). If the *E. coli* levels exceed the GM and ST criteria, growers should discontinue use of that water source or apply a mitigation strategy to reduce the microbial levels before resuming the use of water. Sampling programs must therefore be designed to meet the specific objectives for which the data are collected and should determine the best sampling location, the sampling frequency and the details of the sampling procedures (Harter, 2008). Additionally, a direct correlation between the levels of any indicator and enteric pathogens in water are random, site-specific, or time specific (Ashbolt et al., 2001; Payment and Locas, 2011).

Traditionally, cultivation techniques have been commonly used to enumerate *E. coli* in irrigation water (Holvoet et al., 2014; Castro-Ibáñez et al., 2015). However, this methodology may not represent the most suitable method for predicting the presence of bacterial pathogens. Ferguson et al. (2013) reported that a qPCR-based *E. coli* assay was the best method for predicting the presence of bacterial pathogens in water samples. It could be attributed to the presence of viable but non cultivable (VBNC) bacteria in environmental samples. Unlike normal cells that are culturable on suitable media and develop into colonies, VBNC cells are living cells that have lost the ability to grow on routine media (Li et al., 2014). In a later study, Truchado et al. (2016a) have demonstrated that both cultivable and VBNC *E. coli* are accurate predictors of the presence of pathogenic bacteria.

It can be hypothesized that environmental factors might have a different impact in different irrigation water sources, affecting survival of fecal indicator bacteria and pathogenic microorganisms. This information, however, is critical to establish the water quality profile of a water source. Therefore, the aims of this study were (i) to establish the association between microbial indicators and the presence of foodborne pathogens in three types of surface water, and (ii) to give insights on how a water sampling program should be established.

2. Material and methods

2.1. Experimental design

Microbial collection by systematic sampling of irrigation water was performed at three different water sources including: 1) water from drainage ditches bordering farmlands and urban areas, 2) water reservoirs and 3) canal water from a river transfer. All these water sources correspond to surface water and have been selected because they are the three main water sources used for crop irrigation in the South East area of Spain. At certain times of the year, these water sources can be mixed with reclaimed water coming from wastewater treatment plants and in the case of water from drainage ditches bordering farmlands and urban areas, some old houses located near-by these water sources may not have a wastewater discharge system in place, representing a potential source of contamination.

2.2. Sample collection

Three representative locations of the three selected water sources were selected and sampled from January until December 2015. Water samples were weekly-collected in duplicate (n = 226). Two samples of 2 L each were collected using sterile polypropylene plastic bottles. Water temperature was measured in each water source. Samples were transported to the laboratory (30 min approx.) and stored at 4 °C for 16 h maximum before processing. Additionally, at each sampling point, 10 L of water was passed through a Modified Moore Swabs (MMS) by means of a peristaltic pump as previously described by Sbodio et al. (2013).

2.3. Meteorological parameters

For each location and sampling day, weather data for ambient temperature and precipitation were obtained during the sampling period from the nearby climatic stations at 'La Alberca' (37° 56′ 24, 24" N, 1° 8′ 4, 99" W), 'Torre Pacheco' (37° 44′ 51,81" N, 0° 59′ 12,02" W) and 'Ulea' (38° 11′ 28, 96" N, 1° 15′ 28, 53" W), located within 10 km of the water sources. The climatological database of Sistema de Informacion Agraria de Murcia (SIAM) was used (SIAM, 2015).

2.4. Cultivation and molecular-based E. coli quantification in irrigation water samples

Culture-based enumeration of *E. coli* was performed as previously described in Castro-Ibáñez et al., 2015. The detection limit was 1 CFU/100 mL. The molecular quantification of *E. coli* was performed following the combined use of propidium monoazide (PMA; Biotium Inc. USA) and quantitative polymerase chain reaction (PMA-qPCR) as previously described in Truchado et al. (2016b). Briefly, at each sampling point two subsamples of 250 mL were taken from each 2 L and further pooled into one sample (n = 113). This pool (500 mL) was centrifuged at 4000 rpm for 20 min. The pellets were activated with PMA and kept at -20 °C until the genomic DNA extraction was performed. and the supernatant was removed. The pellets were resuspended in 1 mL of sterile water with 10 μM of PMA. Samples were incubated in the dark during 5 min at RT with shaking to allow reagent penetration. Then, samples were exposed to a 500-W halogen light (GE lightning, Cleveland, USA for 5 min) light for 5 min. After PMA activation, the samples were pelleted by centrifugation at 9000*g* for 10 min and kept at -20 °C until the genomic DNA extraction was performed. Genomic DNA was extracted using the Master Pure TM Complete DNA and RNA purification kit (Epicenter, Madison, USA) following the manufacturer's instructions. PMA-qPCR procedure were identical to that describes in Truchado et al. (2016a).

The limit of detection and the recovery of the molecular assay (including sample preparation and qPCR analysis) were estimated using a three-strain cocktail of *Escherichia coli* (CECT 434, 471, and 515) in logarithmic growth phase obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain). Serial dilutions were prepared and 1 mL of the concentration range of 10^5–10^0 CFU was spiked in 100 mL of sterile water obtained from one of the water sources used in the study (water from a canal) and submitted to complete experimental procedure (centrifugation, (PMA treatment), DNA extraction and qPCR). The selection of the water was based on the physico-chemical properties of the water. In this case,

the water with the highest turbidity (36.28 NTU) was selected because a previous study associated turbidity with potential interferences with the photoactivation step when PMA was using, reducing the efficiency of the treatment (van Frankenhuyzen et al., 2011). Bacteria levels were quantified by plating and (PMA)-qPCR. Analysis was performed in triplicates and extraction blanks were also included. The limited of detection of experimental procedure was estimated in 77.8 ± 6.0 bacterial cells per 100 mL, and the average of recovery was 85 ± 3.9%.

2.5. Pathogenic microorganisms

2.5.1. Pre-enrichment

The Modified Moore Swabs (MMS) obtained after filtering 10 L of water were placed in a stomacher bag containing buffer peptone water (200 mL of 40% BPW; Scharlau Chemie, Spain) and 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 brain heart infusion (BHI; Scharlau Chemie) broth for 4 h at 35 °C to resuscitate injured cells. For *E. coli* O157:H7 detection, 1 mL of BHI was transferred into 9 mL of trypticase soy broth, supplemented with novobiocin (20 mg/L) (TSBn; Scharlau Chemie) and incubated overnight at 37 °C. In the case of Shiga toxin-producing *E. coli* (STEC), 1 mL of enrichment in BHI was then transferred into 9 mL of tryptone phosphate broth (TP; Oxoid, UK) and incubated overnight at 42 °C. For *Salmonella,* 1 mL of enrichment in BHI was transferred into 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 centrifuged at 9000 *g* for 10 min. DNA extraction of the pellet was performed using a NucleoSpin Tissue (Macherey-Nagel, Germany) following manufacture instruction.

2.5.2. Detection and confirmation of pathogenic microorganisms

To detect presence of *E. coli* O157:H7, STEC and *Salmonella* spp. in water samples a PCR System (Applied Biosystems, Spain) was used. For *E. coli* O157:H7 and STEC, a conventional multiplex PCR assay, targeting the five virulence factors *stx1*, *stx2*, *eae* and *ehxA*, plus the O157:H7 specific +93 *uidA* single nucleotide polymorphism, was performed (Son et al., 2014). The limit of detection, according the sensitivity of PCR Multiplex was estimated using DNA from *Escherichia coli* O157:H7 (CECT 4782). DNA was serially diluted to produce five average concentrations ranging between 1.05 ± 0.01 cells/μL and 1050 ± 95.46 cell/μL. The LOD was determined based on the concentration that demonstrated a positive result at a particular dilution. The LOD values for each PCR product were 10.5 ± 0.09 cells regardless of whether the amplifications were run individually or in the multiplex format. To detect the presence of *Salmonella*, specific primers targeting genes *invA*, were tested (Al-Moghazy et al., 2014). PCR products were separated by electrophoresis in 2% (w/V) agarose gels (Pronadisa, Spain) at 85 V for 60 min. Gels were stained with 1 μg/mL Red dye (Biotium Inc. USA) and visualized on a UV transilluminator (Bio-Rad, Spain). When the five bands in the multiplex PCR were amplified, the 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, the sample was considered as a presumptive STEC. The presumptive positive samples detected by PCR were cultured and confirmed in selective culture media as previously described (Holvoet et al., 2014; Castro-Ibáñez et al., 2015) with some modifications. Briefly, positive enrichment 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 colony was 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 plate and confirmed by Latex Confirmation (Oxoid).

2.6. Statistical analysis

Calculation and graphical representation of the median and interquartile range (IQR) of microbial counts were performed using Sigma Plot 12.5 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). *E. coli* levels, evaluated by plate count and PMA-qPCR assay, were $\log_{10}$ transformed. Two samples that had observed zero counts in the culture data and one sample in the PMA-qPCR data were left censored. The two samples with observed zero counts were substituted with 1 CFU/100 mL (limit of detection) before $\log_{10}$ transformation. It was considered that this censoring approach had minimal effect on the results considering the relatively low limit of detection and that just 2 samples were subjected to it. The single sample with the observed zero count in the PMA-qPCR analysis was substituted with 1 cell/100 mL before $\log_{10}$ transformation. Because the detection limit was 77.8 ± 6 cells/100 mL, this approach to censoring had a small effect on the summery statistics by affecting the lowest point in the observed range, which was considered acceptable. IBM SPSS Statistics 19 and R software were used for statistical analyses. 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 association between *E. coli* levels and presence of pathogens. The Spearman correlation coefficient (*r* value) was calculated to determine the correlation between *E. coli* levels and water temperature. For the proportion of confirmed positive samples of a particular water type, a 95% confidence interval (CI) was calculated using the Wilson score method (Newcombe, 1998). A classification tree analysis was used to determine the cut-off level of *E. coli* with the smallest misclassification error (based on the "1 - standard error" rule) in predicting the presence of pathogenic microorganisms in the sample; the analysis used Gini index as a measure of node impurity and 5-fold cross-validation. The identified cut-off was used to summarize samples into a confusion matrix, from which sensitivity, specificity, and positive and negative predictive values could be computed.

3. Results and discussion

3.1. Microbial quality of surface irrigation water

The three tested types of water sources had significantly different levels of *E. coli* established through culture-based and culture-independent methods (Fig. 1). 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/100 mL IQR 2.66–2.97). These levels were above the *E. coli* limits included in the current legislation (i.e. FSMA) and those recommended by most of the GAP guidelines (≤ 2 log/100 mL) for irrigation water (MAPAMA, 2015). Surface water obtained from water 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). Based on the results obtained, water from water reservoirs could represent the lowest risk. However, available literature suggests caution mostly because the levels of *E. coli* can vary significantly depending on several factors including geographical location and weather conditions (Forslund et al., 2012; Jones et al., 2014; Castro-Ibáñez et al., 2015).

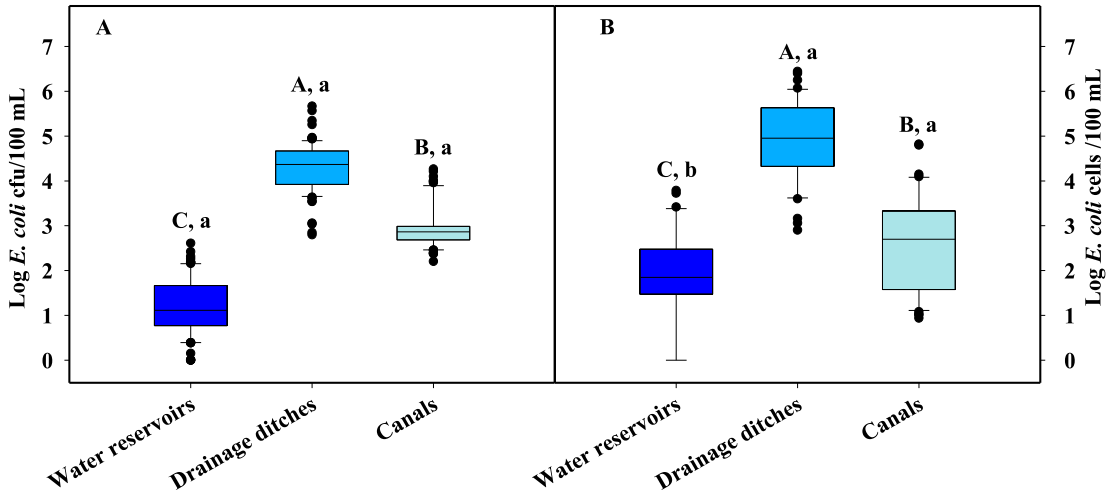

Fig. 1. Boxplots representing cultivable (plate count) (**A**) and molecular (PMA-qPCR) (**B**) *E. coli* counts (log cfu or cells/100 mL) in the different sources of surface irrigation water. In a boxplot, the bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box represents the median, whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). Bars labelled with different upper case letters indicate significant difference among water sources at $P < 0.05$. Lower case letters indicate significant difference between molecular (PMA-qPCR) and cultivable (plate count) methods at $P < 0.05$ for the same water source.

Levels of *E. coli* showed similar trends when measured by the PMA-qPCR assay and the culture-based approach. Several studies have reported that the use of culture-based methods may lead to an underestimation of the *E. coli* levels in water samples when compared to the PMA-qPCR technique (Gensberger et al., 2014; Truchado et al., 2016b). The discrepancy between the two enumerations techniques have been attributed to the presence of viable but non cultivable (VBNC) bacteria. Irrigation water can be exposed to many environmental stresses and treatments that can induce cultivable bacteria into a VBNC state (Heim et al., 2002). A portion of these VBNC may be resuscitated back to cultivable cells during water storage and distribution system, thereby presenting a potential risk (Lin et al., 2016). In the present study, *E. coli* levels quantified using the PMA-qPCR assay were similar to those obtained using the culture-based methods. As previously described, the loss in cultivability was most likely due to cell death rather than an inability to form colonies on standard laboratory media. An exception was observed in the case of water samples obtained from water reservoirs (Fig. 1). In this case significant differences were observed between the two quantification techniques (plate count and PMA-qPCR), which could be explained by the addition of chemical agents to the water reservoirs to avoid algae development, which might have an impact on the viability of *E. coli* cells (Gil et al., 2015). Therefore, the true *E. coli* levels in water reservoir seemed to be higher than those reported by culture methods. In cases like this, the PMA-qPCR technique may be a more suitable method for the accurate estimation of *E. coli* loads when compared to traditional cultivation-based method.

3.2. Association between microbial indicators and the presence of foodborne pathogens in surface irrigation water

The prevalences 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) of samples were confirmed by selective media and subsequent latex agglutination test (where applicable). Similar results have been shown in previous studies screening water samples, which are probably due to the presence of a wide range of native competing microbiota on selective agar (Delbeke et al., 2015). Based on the 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). In agreement with the obtained results, several microbiological surveys have also reported a high *Salmonella* spp. prevalence in irrigation water (Micallef et al., 2012). In fact, surface water has been reported as a major reservoir for *Salmonella* spp. (Micallef et al., 2012; Jones et al., 2014). *E. coli* O157:H7 and non-O157:H7 STEC strains have also been isolated from surface water used for irrigation (Johnson et al., 2003). Wright (1989) reported that a higher prevalence of *Salmonella* when compared to that of *E. coli* O157:H7 and non-O157:H7 STEC may be explained by better *Salmonella* spp. persistence in surface water sources when compared with persistence of other pathogenic enteric bacteria.

Significant differences were observed among the prevalences of the tested foodborne pathogens in the different water sources. The highest prevalence of foodborne pathogens was found in surface

Table 1
Pathogen microorganisms in different water sources.

Samples type	*E. coli* O157:H7			Non-O157:H7 STEC			*Salmonella*		
	PCR	Confirmed	Prevalence[a]	PCR[b]	Confirmed	Prevalence	PCR	Confirmed	Prevalence
Water reservoirs	4/40	4/4	0.1 (0.04, 0.23)	14/40	1/14	0.03 (0.00, 0.13)	3/40	2/3	0.05 (0.01, 0.17)
Drainage ditches	14/43	6/14	0.14 (0.07, 0.27)	28/43	7/28	0.16 (0.08, 0.30)	33/43	26/33	0.60 (0.46, 0.74)
Canal	9/30	4/9	0.13 (0.05, 0.30)	28/30	4/28	0.13 (0.05, 0.30)	24/30	21/24	0.70 (0.52, 0.83)

[a] Prevalence of confirmed positive among tested samples (95% confidence interval).
[b] Positive samples to *stx1* and/or *stx2* and *eae*.

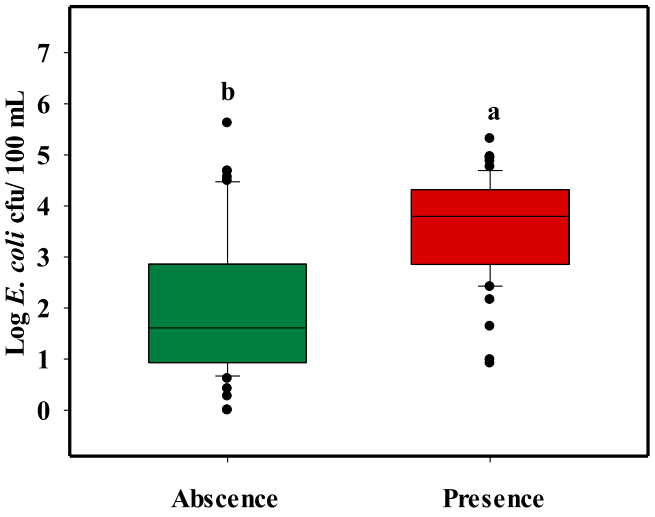

Fig. 2. Boxplots representing *E. coli levels* (log cfu/100 mL) in all surface water samples separated into those with absence or presence of foodborne pathogenic bacteria. Box-plots labelled with different letters indicate significant difference at $P < 0.05$.

water obtained from drainage ditches and canals (70%, 30/43 and 70%, 21/30, respectively) followed by water reservoirs (10%, 4/40). The low presence of pathogens in water reservoirs might be due to the addition of potassium permanganate (2 mg/L $KMnO_4$), as a strategy to control algae development (Castro-Ibáñez et al., 2015). Potassium permanganate has been described as a strong oxidizing agent highly toxic for bacteria (Tucker and Boyd, 1977). Conversely, the high prevalence of foodborne pathogens in surface water obtained from drainage ditches and canals might be the result of direct discharge of waste from agricultural runoff and/or untreated human sewage. Wild-life, livestock and humans have been identified as the major sources of water contamination with *Salmonella* and *E. coli,* and could be a valid explanation for the high prevalence observed in this study (Navarro-Gonzalez et al., 2012). Based on the present results, the use of irrigation water obtained from representative drainage ditches and canals represents a high risk of produce contamination with foodborne pathogens. As previously indicated, at certain times of the year, these water sources can be mixed with reclaimed water coming from wastewater treatment plants. Additionally, in the case of water from drainage ditches bordering farmlands and urban areas, some old houses located near-by these water sources may not have a wastewater discharge system in place, representing a potential source of contamination. All this can contribute to the high contamination found in these water samples. Recently, several studies have identified *E. coli* concentrations as a good indicator of the presence of foodborne pathogen in irrigation water (Payment and Locas, 2011; Holvoet et al., 2014; Truchado et al., 2016b). In order to establish the potential association between *E. coli* levels in irrigation water and the

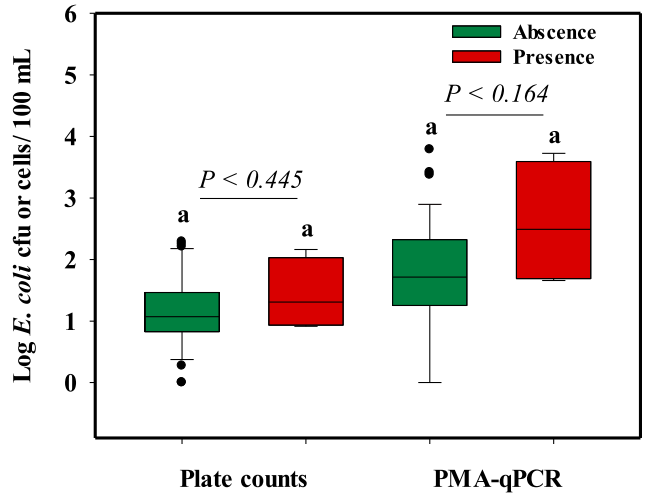

Fig. 3. Boxplots representing *E. coli levels* (log cfu/100 mL) in water reservoirs quantified by plate counts and PMA-qPCR separated into those with absence or presence of foodborne pathogenic bacteria. Box-plots labelled with different letters indicate significant difference at $P < 0.05$ for the same enumeration technique.

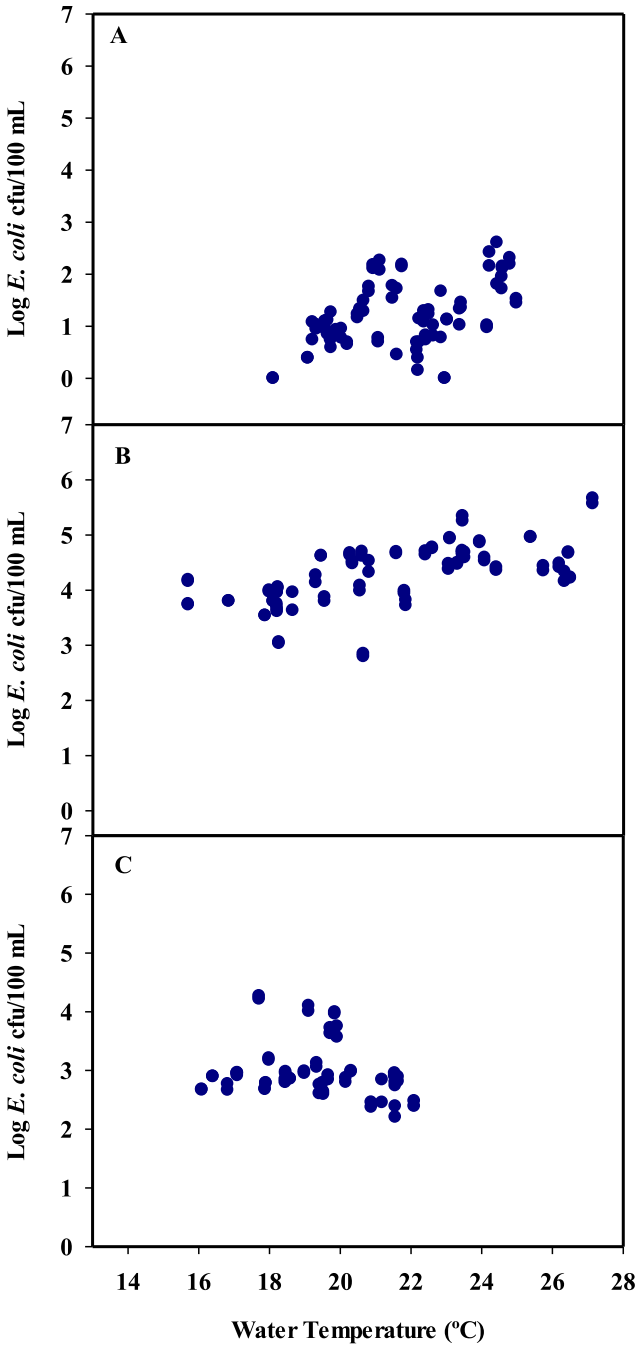

Fig. 4. Scatter plots showing the relationship between water temperature (°C) and viable *E. coli* in the different sources of surface irrigation water. Water reservoirs (A), water drainage ditches (B), water canal (C).

presence of enteric foodborne pathogens, Mann Whitney U and Kruskal-Wallis tests were used. Our results are in agreement with previously published studies showing that *E. coli* levels higher than 2.0–2.5 log cfu/100 mL were associated with a higher prevalence of foodborne pathogens (Fig. 2) (Ceuppens et al., 2015). In heavily polluted waters such as untreated waste waters, a better correlation between indicator organisms and pathogens is generally observed, but with other water qualities and environments this correlation can become erratic and biologically improbable (Payment and Locas, 2011).

Specifically, for the evaluated water sources, the *E. coli* level of 2.35 log cfu/100 mL was identified as a cut-off with the smallest misclassification error in the classification tree analysis. This cut-off was able to correctly predict positive and negative samples with 93% sensitivity and 66% specificity, respectively. The positive and negative predictive values were 74% and 90% respectively. Thus, for the samples with levels of *E. coli* under 2.35 log cfu/100 mL (i.e., 2.24 cfu/100 mL) there was a 90% probability that the samples were not contaminated with pathogenic microorganism. On the other hand, almost three quarters of samples contaminated with *E. coli* at levels above 2.24 cfu/100 mL were also contaminated with pathogenic microorganisms. A different cut-off would have a different predictive ability. For example, lowering the cut-off to 2.10 log cfu/100 mL slightly increased the sensitivity (95%) and the negative predictive value (91%) but at a cost of a decrease in the specificity (54%) and positive predictive value (68%); thus as expected, a lower cut-off would increase the probability of detecting water contaminated with pathogens but the number of false positives would increase as well.

Some authors have proposed the quantification of VBNC *E. coli* as a more suitable indicator of the presence of enteric pathogens (Ferguson et al., 2013). To validate this hypothesis, we evaluated the correlation between the *E. coli* levels from both plate count and PMA-qPCR methods and the presence of pathogenic bacteria in the water reservoirs over the study period of one year (Fig. 3). The results showed no significant differences between the two quantification techniques (plate count and PMA-qPCR), and thus did not corroborate the proposed hypothesis.

3.3. Establishment of a sampling program

In the present study, the microbiological quality of irrigation water from three sources of surface water was evaluated over a period of one year and data were compiled and juxtaposed to weather factors. Results showed that there was no correlation between *E. coli* levels in irrigation water and UV radiation nor rainfall (data not shown). However, it should be noted that this result could have been affected by the low rainfall during the period of the study. In agreement with the results obtained, other studies did not find a positive correlation between *E. coli* and precipitation (Jones et al., 2014; Castro-Ibáñez et al., 2015). A positive relationship was observed between water temperature (°C) and *E. coli* levels depending on the water source (Pearson rank, $P < 0.01$) (Fig. 4). Water samples from drainage ditches ($r = 0.608$, $P < 0.01$) (Fig. 4A), and water reservoirs ($r = 0.453$, $P < 0.01$) (Fig. 4B), showed a moderate but significant correlation between water temperature and *E. coli* levels. This positive correlation between *E. coli* and outside temperature in irrigation water was previously reported by others authors (Holvoet et al., 2014). Additionally, a correlation was observed between water temperature and outside temperature ($r = 0.739$, $P < 0.01$). These results suggest that fecal indicator concentrations may increase as the temperature increase, which can be associated with a higher probability of finding foodborne pathogens. This data could be of interest for growers, as they could modify their sampling programs accordingly, investing more resources in monitoring of irrigation water during those months of higher temperatures.

On the other hand, there was no correlation between water temperature and *E. coli* levels in water samples taken from the canal (Fig. 4C). One reason for this finding can be the location selected for sampling the canal. Along the canal, there were discharges from a nearby wastewater treatment plant (WWTP). Having a continuous discharge from the WWTP, the microbiological quality of the water was more homogeneous and independent of the outside temperature. Therefore, the selection of the sampling location could probably buffer the impact of water temperature on the *E. coli* levels in this water source. Regarding the use of reclaimed water for irrigation of fresh produce, Spanish legislation specifies permissible *E. coli* levels according to crop and mode of water application (Real Decreto 1620/2007, 2007). In those cases where a direct contact of reclaimed water with produce occurs and the product is consumed as raw, the maximum authorized level for *E. coli* is 10^2 cfu/100 mL. In the present study, levels of *E. coli* were, in most of the cases, above 10^2 cfu/100 mL, which restricts its use to those cases in which the irrigation water does not come into direct contact with the edible part of the product.

Table 2
Microbial Water Quality Profile (MWQP) in different water sources.

Water sources	GM[a] (log CFU or MPN *E. coli*/100 mL)	STV[b] (log CFU or MPN *E. coli*/100 mL)
1. Water reservoirs		
Produce Safety Rule Criteria	2.10	2.61
Your MWQP Results	1.20	2.06
Deviation from Criteria	−0.90	−0.55
Does your Water meet PSR criteria?	Yes	Yes
2. Drainage ditches		
Produce Safety Rule Criteria	2.10	2.61
Your MWQP Results	4.29	4.99
Deviation from Criteria	2.19	2.38
Does your Water meet PSR criteria?	No	No
3. Canal		
Produce Safety Rule Criteria	2.10	2.61
Your MWQP Results	2.27	3.91
Deviation from Criteria	0.17	1.30
Does your Water meet PSR criteria?	No	No

[a] GM; Geometric Mean.
[b] STV; Statistical Threshold Value.

There are some applications that can be used by growers to evaluate the microbial water quality profile (MWQP) of the irrigation water source to avoid the complexity of establishing the GM and STV values. For that purpose we selected an Excel spreadsheet developed by Western Center for Produce Safety at University of California, Davis (WCFS, 2016). Data obtained from this excel tool showed that only irrigation water stored in reservoirs complied with the FSMA established microbiological *E. coli* levels (Table 2). A tool like this is easy to use and gives very important information to growers, who based on the results obtained might decide to implement mitigation options that allow microbial die-off before harvest.

4. Conclusions

- The microbiological quality of surface irrigation water, in terms of *E. coli* levels, depends on several factors including water source, climate and in some cases, on the quantification methodology selected to evaluate the indicator microorganisms.
- Surface water has been confirmed as a risky water source for irrigation, but significant differences were observed among the different types of surface water evaluated in this study. This information should be taken into account to avoid grouping together all the surface water sources when assurance schemes are elaborated.
- Based on the results obtained, *E. coli* levels higher than 2.35 log cfu/100 mL were able to predictive the presence of pathogens using cultivable techniques. Caution should be taken into account because samples positive for the presence of pathogenic bacteria were occasionally isolated from water samples with *E. coli* levels below 1.0 log cfu/100 mL.
- Both quantification techniques (plate count and PMA-qPCR) can be used to evaluate the concentration of *E. coli* in surface water, except to water sources where chemical are used to avoid the algae formation. In this case, the use of conventional cultivation techniques might lead to an underestimation of the *E. coli* population present in irrigation water.

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