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Suitability of different *Escherichia coli* enumeration techniques to assess the microbial quality of different irrigation water sources

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ABSTRACT

The use of fecal indicators such as *Escherichia coli* has been proposed as a potential tool to characterize microbial contamination of irrigation water. Recently, not only the type of microbial indicator but also the methodologies used for enumeration have been called into question. The goal of this study was to assess the microbial quality of different water sources for irrigation of zucchini plants by using *E. coli* as an indicator of fecal contamination and the occurrence of foodborne pathogens. Three water sources were evaluated including reclaimed secondary treated water (RW-2), reclaimed tertiary UV-C treated water (RW-3) and surface water (SW). The suitability of two *E. coli* quantification techniques (plate count and qPCR) was examined for irrigation water and fresh produce. *E. coli* levels using qPCR assay were significantly higher than that obtained by plate count in all samples of irrigation water and fresh produce. The microbial quality of water samples from RW-2 was well predicted by qPCR, as the presence of foodborne pathogens were positively correlated with high *E. coli* levels. However, differences in the water characteristics influenced the suitability of qPCR as a tool to predict potential contamination in irrigation water. No significant differences were obtained between the number of cells of *E. coli* from RW-2 and RW-3, probably due to the fact that qPCR assay cannot distinguish between viable and dead cells. These results indicated that the selection of the most suitable technique for enumeration of indicator microorganisms able to predict potential presence of fecal contamination might be influenced by the water characteristics.

1. Introduction

Irrigation water is well recognized as one of the main risk factors for contamination with pathogenic microorganism of fresh produce during the primary production (EFSA, 2014). Different water sources can be used for primary production depending on water availability and quality. Surface water seems to be the most predominant water source for irrigation in many countries (Uyttendaele et al., 2015; Allende and Monaghan, 2015). However, reclaimed water is being increasingly used in arid and semi-arid zones around the world due to scarcity of other water sources (Pachepsky et al., 2011). Among all types of water, surface and reclaimed water have been classified as the most risky irrigation water sources (Ceuppens et al., 2015). To promote produce safety, periodical water tests and monitoring of water sources have been recommended as preventive measures for most of the Good Agricultural Practices (GAP) guidelines.

The investigation of microbial indicators has been proposed as a good strategy to characterize microbial contamination in water mostly due to the low prevalence of foodborne pathogens as well as the high cost and time consumption of pathogen detection (Ferguson et al., 2012). Currently, generic *Escherichia coli* seem to be the best indicator bacteria able to identify fecal contamination when compared with the rest of microbial indicators for irrigation water (Uyttendaele et al., 2015; Ceuppens et al., 2015). Research focused on systematic longitudinal samplings demonstrated that presence of *E. coli* provides evidence of an increased likelihood of potential contamination by ecologically closely related pathogens such as pathogenic *E. coli* and *Salmonella* (Ogden et al., 2001; Harwood et al., 2005; Wilkes et al., 2009; EFSA, 2014; Holvoet et al., 2015; Ceuppens et al., 2014, 2015; Castro-Ibáñez et al., 2015a, 2015b). However, other studies did not find a good correlation between levels of *E. coli* and prevalence of pathogenic microorganisms, questioning the value of *E. coli* as a good indicator

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microorganism (Benjamin et al., 2013; Pachepsky et al., 2014; Orlofsky et al., 2016). The reported differences between previously published research papers could be due to discrepancies on the sampling size and enumeration techniques.

Currently, traditional culture techniques are commonly used to enumerate *E. coli* loads in environmental samples, including irrigation water. However, developed qPCR methods are able to detect and enumerate *E. coli* in environmental samples and have been shown to be more sensitive, reproducible and faster than plate counts (Ahmed et al., 2012; Mendes Silva and Domingues, 2015; Truchado et al., 2016). Ferguson et al. (2012) reported that a qPCR-based *E. coli* assay was the best indicator for bacterial pathogens in water samples. Based on these reports, there is a need to determine the most suitable techniques for quantification of *E. coli* and its ability to distinguish the microbial quality of different irrigation water sources.

The goal of this study was to determine the most suitable techniques to quantify generic *E. coli* in irrigation water and fresh produce samples and their ability to predict the occurrence of foodborne pathogens. Two selected enumeration methods, plate count and qPCR, were subsequently used to evaluate the microbial quality of different irrigation water sources based on their *E. coli* levels and foodborne pathogen prevalence.

2. Materials and methods

2.1. Experimental design

Zucchini plants (*Cucurbita pepo* L.) were grown under hydroponic system (Coconut fiber; Pelemix, Alhama de Murcia, Spain) in a greenhouse located next to the wastewater treatment plant (WWTP) (Roldán-Balsicas, Murcia, Spain) from December 2014 until March 2015. Irrigation water obtained from the WWTP was subjected to a secondary treatment, using an extended aeration system (reactor + clarifier system), followed by a tertiary treatment with an ultraviolet-C (UV-C) disinfection system. Three types of water were used for irrigation: surface water (SW), reclaimed water obtained from the WWTP after a secondary treatment (RW-2) and reclaimed water obtained from the WWTP after a tertiary treatment (RW-3). Surface water and reclaimed water from the secondary treatment were obtained as previously described (Lopez-Galvez et al., 2014). Briefly, Reclaimed water RW-2 was obtained after a secondary treatment using an extended aeration system (reactor + clarifier system). Reclaimed water RW-3 was obtained from the tertiary treatment plant after treating RW-2, including coagulation-flocculation and complementary lamellar clarification, followed by filtration on an open sand bed filter and disinfection using a UV-C light treatment. The UV disinfection system is based on two lamp modules (8 lamps each module). Lamps are a 254 nm high intensity amalgam (SLR32143 HP, WEDECO, USA). All types of irrigation water were supplemented with fertilizer solutions as needed based on commercial practices for hydroponic production of zucchini plants. Three replicates of 15 plants (n = 45) were grown per treatment.

2.2. Sample collection

Water samples were collected in duplicate twice per week between January–March 2015 (n = 108). One liter of each type of water was collected using sterile polypropylene plastic bottles. Samples were transported to the laboratory (30 min) and stored at 4 °C for 16 h maximum before processing. Additionally, each sampling day, 10 L of each water source was passed through Modified Moore Swabs (MMS) by means of a peristaltic pump as previously described by Sbodio et al. (2013).

Zucchini samples (n = 135) were taken weekly during the harvest period January–March 2015 that corresponded to 9 weeks. Each sampling day, 5 zucchini samples of similar maturity stage were harvested from plants irrigated with each type of irrigation water. Zucchini samples were randomly picked from the plants and aseptically transferred into sterile bags.

2.3. Cultivation and molecular-based E. coli quantification

Cultivation-based enumeration of *E. coli* in irrigation water (n = 108) and zucchini (n = 135) samples was performed as previously described in Lopez-Galvez et al. (2014).

For *E. coli* qPCR quantification in each type of water, two samples (250 mL) were further pooled in one sample (n = 54) at each sampling day. This pool (500 mL) was filtered through 0.45 μm pore size nitrocellulose membranes. The filters were kept at −80 °C until the genomic DNA extraction was performed. For zucchini, 3 samples per treatment and sampling day (n = 81) were taken. Buffered peptone water (BPW, AES Chemunex, Marcy l'Etoile, France) was used to homogenize the zucchini samples. The homogenate was centrifuged at 3000 *g* for 10 min and the obtained pellet kept at −80 °C until the genomic DNA extraction was performed.

2.4. DNA extraction

Bacterial community from irrigation water was recovered from membrane filters as follows. Each membrane was washed off with 20 mL of a 0.01% PBS-Tween80 solution. The obtained bacterial cell suspension was pelleted by centrifugation at 3070 *g* during 10 min. DNA extraction of the pellet was performed using a commercial kit as previously described (Truchado et al., 2016). In the case of zucchini, genomic DNA from the previously obtained pellet was isolated using E.Z.N.A.® Genomic DNA Isolation Kits (Omega Bio-Tek, Norcross, USA).

2.5. qPCR procedure

Quantitative PCR was performed using an ABI 7500 Sequence Detection System (ABI, Applied Biosystems, Madrid, Spain). Primers and probes for detecting genes of *E. coli* 23S rRNA as well as the applied cycling parameters were as previously described (Knappett et al., 2011). Amplification and detection were carried out in 96-well plates using KAPA PROBE FAST Universal qPCR Master mix kit (KapaBiosystems, Massachusetts, USA). Each reaction was run in triplicate containing 5 μL of DNA template. A non-template control (NTC) was included. Standard curves were made using known concentrations of genomic DNA isolated from *E. coli* CECT 5945. The *E. coli* concentration in the stock solution was verified by plating on plate count agar (PCA; Oxoid, Hampshire, UK).

2.6. Pathogenic microorganisms

Presence or absence of *E. coli* O157:H7, STEC (Shiga toxin-producing *E. coli*) and *Salmonella* spp. were determined in water and zucchini samples. As previously mentioned, two water samples taken at each sampling day were pooled in one sample and a total of 54 samples were analyzed for presence of pathogenic bacteria. Similarly, the five zucchini samples taken per treatment and each sampling point were polled in one sample and a total of 27 samples were analyzed. The MMS previously obtained after filtering 10 L of water were placed in a stomacher bag containing 200 mL of 40% BPW and incubated at 37 °C for 18–20 h. For zucchini samples, about 160 mL of the BPW homogenate obtained as previously described were incubated at 37 °C for 18 ± 2 h. Enriched samples were supplemented with 30% glycerol and maintained at −20 °C

until further analysis. Prevalence of pathogenic microorganisms in water and zucchini samples was performed using a Genedisc Cycler multiplex PCR (Pall® Corporation, WA, USA) (Castro-Ibáñez et al., 2015b). The presumptive positive samples detected by the multiplex PCR were cultured and confirmed in selective culture media (Holvoet et al., 2015; Castro-Ibáñez et al., 2015b). When needed, additional confirmation of presumptive *Salmonella* spp. colonies was performed using a PCR System (Applied Biosystems, Madrid, Spain) (Al-Moghazy et al., 2014).

2.7. 16S rRNA gene sequencing analysis of pathogenic strains

Suspected positive strains of *Salmonella*, *E. coli* O157:H7 and STEC obtained from the multiplex PCR and culture isolation were further confirmed using 16S rRNA sequencing analysis (Truchado et al., 2015). All sequences were subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the closest known taxon and aligned using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). To determine the taxonomy of each suspected *E. coli* strain, a 99% or higher match between the sequence obtained (~800 bp) and the GenBank data was considered to represent identity.

2.8. 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, determined by plate count and qPCR assay, were $\log_{10}$ transformed. IBM SPSS Statistics 19 was used for statistical analysis. 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 data obtained using plate count and qPCR assay. The significance was determined at the 95% confidence level.

3. Results

3.1. Suitability of different E. coli quantification techniques to predict fecal contamination

In all water samples, counts of cultivable *E. coli* were significantly lower than the levels of the qPCR *E. coli* (Fig. 1A). In the case of zucchini, *E. coli* levels obtained by using the plate count method were always below the detection limit (≤0.7 log cfu/g) while qPCR *E. coli* levels were always above the detection limit (≤0.8 cells cfu/g) with a median count of 2.64 log cells/g (IQR 2.46–3.05) (Fig. 1B). A strong correlation was observed between both enumeration methods ($r = 0.72$; $P < 0.01$) ($n = 81$) (Fig. 2). In order to determine the potential correlation between *E. coli* levels and the prevalence of enteric foodborne pathogens, a multiplex PCR was performed (Table 1). Among the tested water samples, 24.1% was positive for at least one bacterial pathogen, including 6 samples for *Salmonella* spp., 4 samples for *E. coli* O157:H7 and 3 samples for non-O157:H7 STEC strains. In the case of zucchini, only one sample was positive for *Salmonella* spp. by multiplex PCR. When confirmation of the presumptive positive samples was performed, none of the multiplex PCR positives for *Salmonella* spp. was confirmed by culture media or *inv* gene amplification by PCR assay. In the case of *E. coli* O157:H7, only one presumptive positive sample was confirmed by culture media and latex agglutination test. To further characterize the isolated strain, 16S rRNA gene sequencing analysis was carried out. The sequencing results showed resemblance to *E. coli* O157:H7 strain WS4202 (99% similarity). Presumptive positives of non-O157:H7 STEC strains were isolated by selective media and further characterized by 16S rRNA gene sequencing analysis. Sequence similarity analysis was listed in Table 2. Two isolated strains were similar to STEC O104:H4 and another strain were similar to STEC O78 (99% similarity).

In order to determine the most suitable *E. coli* quantification method, the levels of *E. coli* obtained by plate count and qPCR in positive and negative samples for the presence of pathogenic bacteria were compared. Based on the obtained results, positives samples for pathogenic bacteria showed significantly higher *E. coli* levels than samples negative for the presence of foodborne pathogens when both enumeration methods were used (Fig. 3). This indicates that both enumeration techniques were able to predict potential presence of fecal contamination. However, the differences found between the levels of *E. coli* of those samples positive and negative to the presence of foodborne pathogen were less significant in the case of qPCR ($P < 0.01$) than plate count method ($P < 0.001$).

3.2. Suitability of different E. coli enumeration methods to assess microbial quality of irrigation water sources

Three different types of irrigation water were compared to assess if there were differences in microbial quality. Independently

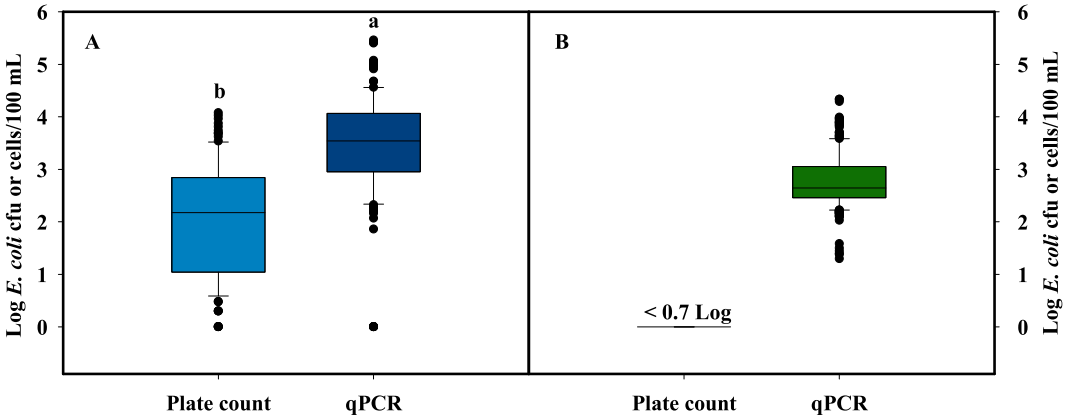

Fig. 1. Boxplots representing cultivable (plate count) and molecular (qPCR) *E. coli* counts in irrigation water (log cfu or cells/100 mL) (A) and zucchini (log cfu or cells/g) (B). Box-plot with different letters indicates significant difference at $P < 0.05$.

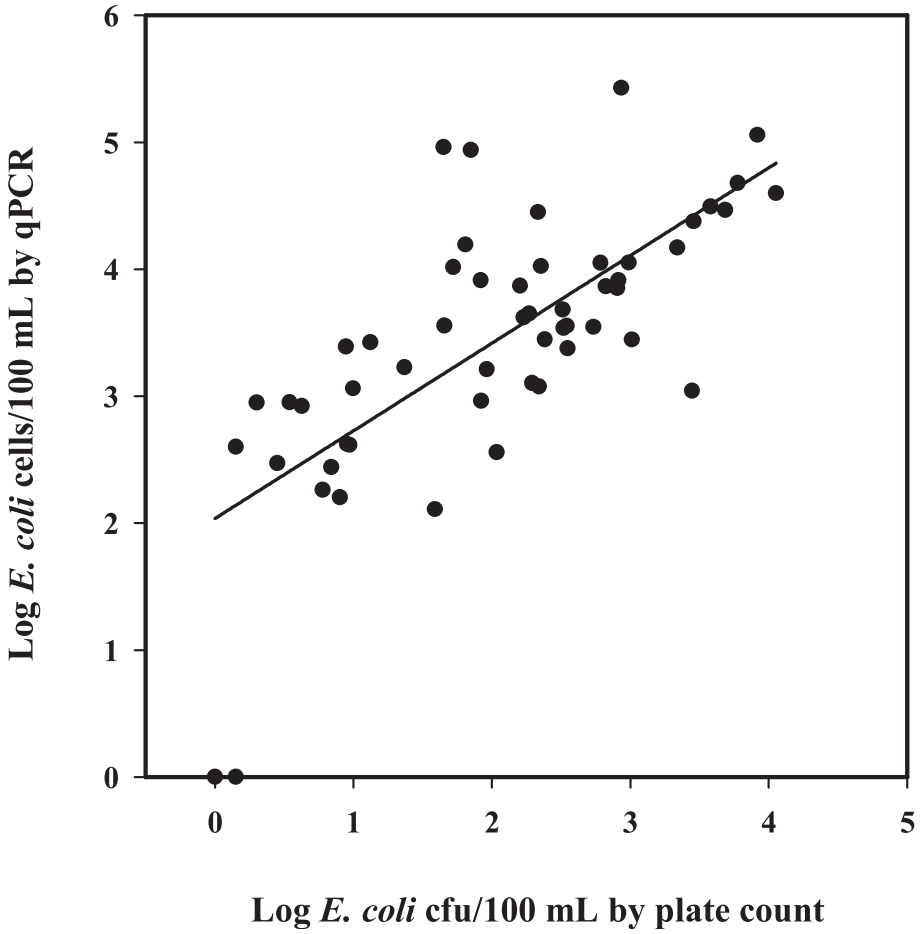

Fig. 2. Scatter plot of cultivable *E. coli* vs molecular *E. coli* levels in different types of irrigation water (n = 81).

Table 1
Pathogen microorganisms in different water sources and in zucchini irrigated with different water sources.

Samples type[a]	*E. coli* O157:H7		Non-O157:H7 STEC		*Salmonella*	
Water	Genedisc®	Confirmed	Genedisc®	Confirmed	Genedisc®	Confirmed
RW-2	3/18	1/3	3/18	3/3	4/18	0/4
RW-3	0/18		0/18		2/18	0/2
SW	1/18	0/1	0/18		0/18	
Zucchini	Genedisc®	Confirmed	Genedisc®	Confirmed	Genedisc®	Confirmed
RW-2	0/9		0/9		0/9	
RW-3	0/9		0/9		1/9	0/1
SW	0/9		0/9		0/9	

[a] RW-2: secondary reclaimed water; RW-3: tertiary reclaimed water; SW: surface water.

Table 2
Sequencing of 16S rRNA gene from suspected *E. coli* strains isolated from water samples.

Samples[a]	Closest relative microorganisms	% identity	Accession number
RW-2-1	*Escherichia coli* O157:H7 strain WS4202	99	CP012802
RW-2-2	*Escherichia coli* O104:H4 strain C227-11	99	CP011331
RW-2-3	*Escherichia coli* O104:H4 strain C227-11	99	CP011331
RW-2-4	*Escherichia coli* O78 strain ACN002	99	CP007491.1

[a] RW-2: secondary reclaimed water; RW-3: tertiary reclaimed water; SW: surface water.

of the enumeration method, SW showed the lowest *E. coli* levels, which agreed with the expected results (Fig. 4). When *E. coli* levels were quantified by plate count significant differences were found among the water sources tested (Fig. 4A). However, when the qPCR *E. coli* was used, no significant differences were obtained between RW-2 and RW-3 (Fig. 4B). Results on water samples positive for the presence of pathogenic bacteria showed that only secondary RW-2 samples were positive after confirmation (Table 1). When samples

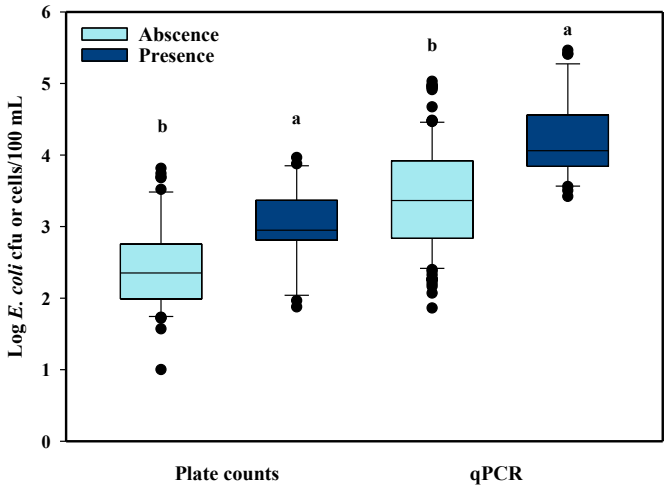

Fig. 3. Boxplots representing cultivable (plate count) and molecular (qPCR) *E. coli* counts in irrigation water (log cfu or cells/100 mL). *E. coli* samples separated into those with absence or presence of foodborne pathogenic bacteria. Box-plots labeled with different letters indicate significant difference at $P < 0.05$.

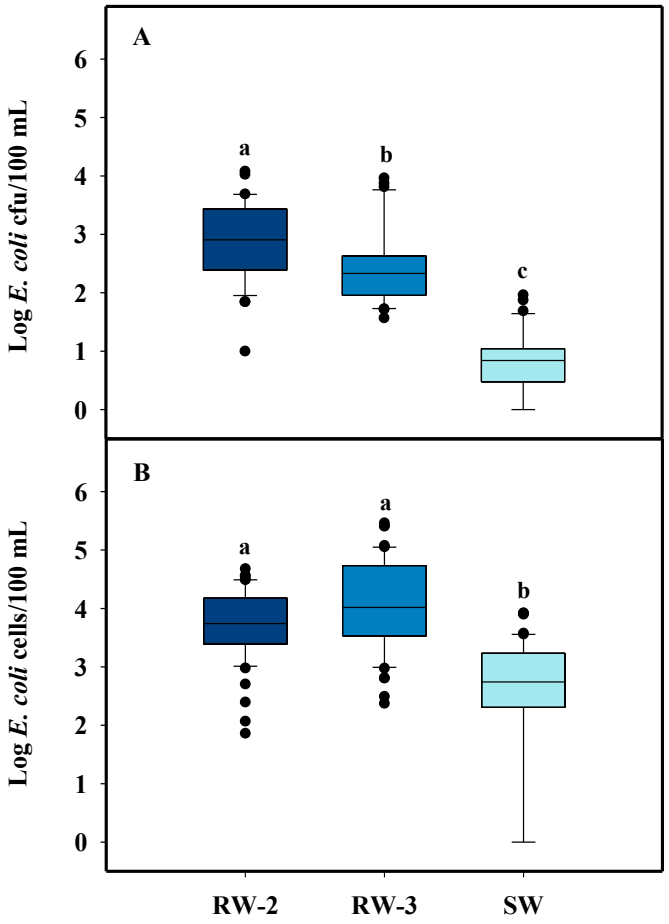

Fig. 4. Boxplots representing cultivable (plate count) (A) and molecular (qPCR) (B) *E. coli* levels (log cfu or cells/100 mL) in secondary reclaimed water (RW-2), tertiary reclaimed water (RW-3) and surface water (SW). Box-plots labeled with different letters indicate significant difference at P < 0.05.

of RW-2 were evaluated, it was observed that only when qPCR was used positives samples for pathogenic bacteria showed

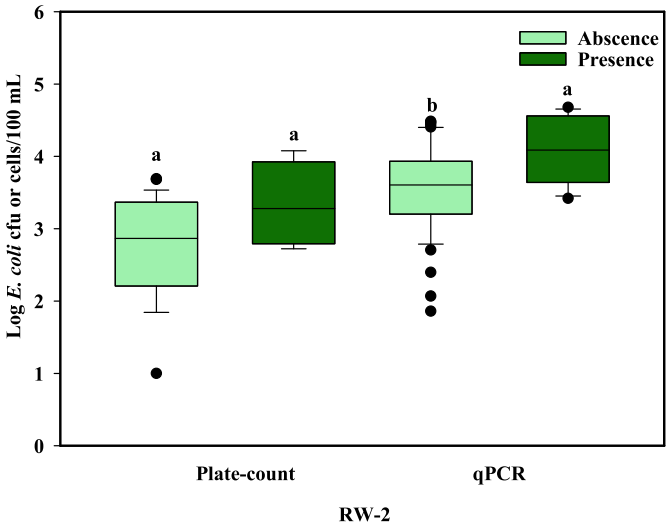

Fig. 5. Boxplots representing cultivable (plate count) and molecular (qPCR) *E. coli* levels (log cfu or cells/100 mL) in secondary reclaimed water (RW-2). *E. coli* samples separated into those with absence or presence of foodborne pathogenic bacteria. Box-plots labeled with different letters indicate significant difference at P < 0.05.

significantly higher *E. coli* levels than samples negative for the presence of foodborne pathogens (Fig. 5).

4. Discussion

The use of *E. coli* as an indicator of fecal contamination has been proposed as a good strategy to characterize microbial contamination of irrigation water (Park et al., 2013; Castro-Ibáñez et al., 2015b; Ceuppens et al., 2015). Recently, it has been reported that a qPCR-based *E. coli* assay was the best indicator for bacterial pathogens in water samples (Ferguson et al., 2012). *E. coli* levels quantified by qPCR assay in different samples such as rainwater and municipal sewage sludge were always higher than those obtained using cultivation-based techniques (Ahmed et al., 2012; van Frankenhuyzen et al., 2013). In our study, qPCR *E. coli* counts of irrigation water samples were always around 1.3 log units higher than those obtained by traditional plate counts. As previously described, a strong correlation between *E. coli* levels was obtained using both enumeration techniques (Gonzalez and Noble, 2014; Ahmed et al., 2012). In the case of zucchini samples, *E. coli* levels quantified by plate count were always below the detection limits while positive samples (median 2.64 log cells/g, IQR 2.46–3.05) were detected by qPCR. This discrepancy observed between plate count and qPCR methods in water and zucchini samples could be due to the presence of viable but not cultivable (VBNC) bacteria. The presence of VBNC bacteria in irrigation water and plant tissue has been previously demonstrated (Juhna et al., 2007; Moyle et al., 2013).

In most of the studies, enumeration of fecal indicators such as *E. coli* has been traditionally performed using cultivation-based assays. However, other enumeration techniques such as qPCR, have been reported as suitable to predict the potential presence of enteric foodborne pathogens in environmental samples (i.e. groundwater) (Ferguson et al., 2012; Truchado et al., 2016). Results obtained in the current study showed that high concentrations of *E. coli* were well correlated with the presence of pathogens in irrigation water including reclaimed water treated with secondary and tertiary processes when both plate count and qPCR were used. In groundwater samples, Ferguson et al. (2012) found a better correlation between fecal indicator determined by molecular

techniques and the presence of pathogens than by using MPN.

When the suitability of different enumeration methods to classify the microbial quality of different irrigation water sources was carried out, it was observed that the cultivable-based method was able to differentiate the microbial quality among the water sources. However, using qPCR *E. coli* enumeration, no significant differences were obtained between the two recycled water types, RW-2 and RW-3. One hypothesis could be that qPCR assay as such, cannot distinguish between viable and dead cells, leading to an overestimation of *E. coli* levels in RW-3. Tertiary recycled water implied an additional treatment based on coagulation-flocculation, lamellar clarification, sand filtration and UV-C light. The UV-C treatment might be able to reduce viable cells but not the genetic marker densities in the water. Different attempts have been performed trying to avoid false-positive results when qPCR is used. The most widespread strategy is the use of DNA-dyes such as PMA, which allow the differentiation between viable and dead cells (Truchado et al., 2016). This technique is based on the ability of PMA to penetrate into the dead cells with compromised membrane integrity and in turn inhibit DNA amplification by PCR following light-induce cross-linking (Nocker and Camper, 2009; Varma et al., 2009; Fittipaldi et al., 2012). However, UV-C treatments rely upon DNA break-up of the bacterial cells without compromising membrane integrity (Li et al., 2014). Samples positive for the presence of foodborne pathogens were only found in RW-2. When *E. coli* levels of RW-2 were enumerated using both plate count and qPCR and correlated with the presence/absence of pathogens, levels of qPCR *E. coli* were significantly higher in samples positive for the presence of pathogenic microorganism while no significant differences were detected by the plate count enumeration technique.

5. Conclusions

Results confirm the positive correlation between high concentration of generic *E. coli*, as indicator bacteria of fecal contamination and the likelihood of finding pathogens in irrigation water. Cultivable and qPCR *E. coli* enumeration techniques seem to be accurate methodologies for enumeration of *E. coli* as an indicator to predict the presence of pathogenic bacteria in different water sources. However, the suitability of the different techniques to predict potential fecal contamination might be affected by the characteristics of the irrigation water. qPCR *E. coli* enumeration method seems to be a better technique when poor quality water is evaluated. However, the results obtained were based in a sample size of n = 108 and further studies should be performed using higher sample size to confirm the suitability of both *E. coli* enumeration methods in other water sources.

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References

Ahmed, W., Richardson, K., Sidhu, J.P.S., Toze, S., 2012. *Escherichia coli* and *Enterococcus* spp. in rainwater tank samples: comparison of culture-based methods and 23S rRNA gene quantitative PCR assays. Environ. Sci. Technol. 46, 11370–11376.

Al-Moghazy, A., Boveri, S., Pulvirenti, A., 2014. Microbial safety in pistachios and pistachio containing products. Food Control 36, 88–93.

Allende, A., Monaghan, J.M., 2015. Irrigation water quality for leafy crops: a perspective of risks and potential solutions. Int. J. Environ. Health Res. 12, 7457–7477.

Benjamin, L., Atwill, E.R., Jay-Russell, M., Cooley, M., Carychao, D., Gorski, L., Mandrell, R.E., 2013. Occurrence of generic *Escherichia coli, E. coli* O157 and *Salmonella* spp. in water and sediment from leafy green produce farms and streams on the Central California coast. Int. J. Food Microbiol. 165, 65–76.

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Allende, A., 2015a. Microbial safety considerations of flooding in primary production of leafy greens: a case study. Food Res. Int. 68, 62–69.

Castro-Ibáñez, I., Gil, M.I., Tudela, J.A., Ivanek, R., Allende, A., 2015b. Assessment of microbial risk factors and impact of meteorological conditions during production of baby spinach in the Southeast of Spain. Food Microbiol. 49, 173–181.

Ceuppens, S., Hessel, C.T., de Quadros Rodrigues, R., Bartz, S., Tondo, E.C., Uyttendaele, M., 2014. Microbiological quality and safety assessment of lettuce production in Brazil. Int. J. Food Microbiol. 181, 67–76.

Ceuppens, S., Johannessen, G.S., Allende, A., Tondo, E.C., El-Tahan, F., Sampers, I., Jacxsens, L., Uyttendaele, M., 2015. Risk factors for *Salmonella*, shiga toxin-producing *Escherichia coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries. Int. J. Environ. Health Res. 12, 9809–9831.

EFSA Panel on Biological Hazards (BIOHAZ), 2014. Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and norovirus in leafy greens eaten raw as salads). EFSA J. 11, 3600. Available at: www.efsa.europa.eu/efsajournal (Accessed January 2016).

Ferguson, A.S., Layton, A.C., Mailloux, B.J., Culligan, P.J., Williams, D.E., Smartt, A.E., Sayler, G.S., Feighery, J., McKay, L., Knappett, P.S.K., Alexandrova, E., Arbit, T., Emch, M., Escamilla, V., Ahmed, K.M., Alam, M.J., Streatfield, P.K., Yunus, M., van Geen, A., 2012. Comparison of fecal indicators with pathogenic rotavirus in groundwater. Sci. Total Environ. 431, 314–322.

Fittipaldi, M., Nocker, A., Codony, F., 2012. Progress in understanding preferential detection live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods 91, 276–289.

Gonzalez, R.A., Noble, R.T., 2014. Comparisons of statistical models to predict fecal indicator bacteria concentrations enumerated by qPCR- and culture- based, methods. Water Res. 48, 296–305.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., Rose, J.B., 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Environ. Microbiol. 71, 3163–3170.

Holvoet, K., Sampers, I., Seynnaeve, M., Jacxsens, L., Uyttendaele, M., 2015. Agricultural and management practices and bacterial contamination in greenhouse versus open field lettuce production. Int. J. Environ. Health Res. 12, 32–63.

Juhna, T., Birzniece, D., Rubulis, J., 2007. Effect of phosphorous on survival of *Escherichia coli* in drinking water biofilms. Appl. Environ. Microbiol. 73, 3755–3758.

Knappett, P.S., Layton, A., McKay, L.D., Williams, D., Mailloux, B.J., Huq, M.R., Alam, M.J., Ahmed, K.M., Akita, Y., Serre, M.L., Sayler, G.S., van Geen, A., 2011. Efficacy of hollow-fiber ultrafiltration for microbial sampling in groundwater. Ground Water 49, 53–65.

Li, D., Tong, T., Zeng, S., Lin, Y., Wu, S., He, M., 2014. Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (PMA-qPCR). J. Environ. Sci. China 26, 299–306.

Lopez-Galvez, F., Allende, A., Pedrero-Salcedo, F., Alarcon, J.J., Gil, M.I., 2014. Safety assessment of greenhouse hydroponic tomatoes irrigated with reclaimed and surface water. Int. J. Food Microbiol. 191, 97–102.

Mendes Silva, D., Domingues, L., 2015. On the track for an efficient detection of *Escherichia coli* in water: a review on PCR-based methods (review). Ecotoxicol. Environ. Saf. 113, 400–411.

Moyle, A.L., Harris, L.J., Marco, M.L., 2013. Assessments of total and viable *Escherichia coli* O157:H7 on field and laboratory grown lettuce. PLoS One 8, e70643.

Nocker, A., Camper, A., 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiol. Lett. 291, 137–142.

Ogden, L.D., Fenlon, D.R., Vinten, A.J., Lewis, D., 2001. The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. Int. J. Food Microbiol. 66, 111–117.

Orlofsky, E., Bernstein, N., Sacks, M., Vonshak, A., Benami, M., Kundu, A., Maki, M., Smith, W., Wuertz, S., Shapiro, K., Gillor, O., 2016. Comparable levels of microbial contamination in soil and on tomato crops after drip irrigation with treated wastewater or potable water. Agric. Ecosyst. Environ. 215, 140–150.

Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., Mandrell, R.E., 2011. Irrigation waters as a source of pathogenic microorganisms in produce: a review. In: Donald, L.S. (Ed.), Advances in Agronomy, vol. 113. Academic Press, pp. 73–138.

Pachepsky, Y., Shelton, D., Dorner, S., Whelan, G., 2014. Can *E. coli* or thermotolerant coliform concentrations predict pathogen presence or prevalence in irrigation waters? Crit. Rev. Microbiol. 1–10.

Park, S., Navratil, S., Gregory, A., Bauer, A., Srinath, I., Jun, M., Szonyi, B., Nightingale, K., Anciso, J., Ivanek, R., 2013. Generic *Escherichia coli* contamination of spinach at the preharvest level: the role of farm management and environmental factors. Appl. Environ. Microbiol. 79, 4347–4358.

Sbodio, A., Maeda, S., Lopez-Velasco, G., Suslow, T.V., 2013. Modified Moore swab optimization and validation in capturing *E. coli* O157:H7 and *Salmonella enterica* in large volume field samples of irrigation water. Food Res. Int. 51, 654–662.

Truchado, P., Van den Abbeele, P., Rivière, A., Possemiers, S., De Vuyst, L., Van de

Wiele, T., 2015. *Bifidobacterium longum* D2 enhances microbial degradation of long-chain arabinoxylans in an in vitro model of the proximal colon. Benef. Microbes 6, 849–860.
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.
Uyttendaele, M., Jaykus, L.-A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Rao Jasti, P., 2015. Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. Compr. Rev. Food Sci. Food Saf. 14, 336–356.
van Frankenhuyzen, J.K., Trevors, J.T., Flemming, C.A., Lee, H., Habash, M.B., 2013. Optimization, validation and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*. J. Ind. Microbiol. Biot. 40, 1251–1261.
Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., Haugland, R., 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res. 43, 4790–4801.
Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E., Lapen, D.R., 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium oocysts*, *Giardia cysts*, and hydrological indices for surface waters within an agricultural landscape. Water Res. 43, 2209–2223.