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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