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Monitoring generic *Escherichia coli* in reclaimed and surface water used in hydroponically cultivated greenhouse peppers and the influence of fertilizer solutions

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ABSTRACT

Systematic monitoring of indicator microorganisms, such as *Escherichia coli*, can help to identify potential risk factors for faecal contamination in the agricultural environment. In this study, levels of *E. coli* in irrigation water (both reclaimed and surface water), water sprayed in humidifiers to regulate ambient humidity, and pepper fruits were assessed in a commercial greenhouse of hydroponically cultivated crops. Additionally, the role of fertilizer solutions as a potential vector of contamination was investigated. Lab-scale studies were also performed to evaluate the influence of fertilizer solutions on the growth/survival of *E. coli* in irrigation water. As expected, higher levels of *E. coli* were detected in reclaimed water compared with surface water. No link between *E. coli* prevalence in irrigation water and presence in fruit could be established. Regarding the fertilizer solutions, *E. coli* was detected more frequently and in higher levels in the fertilizer solution richer in micronutrients. Low concentrations of *E. coli* were also present in pulverized water sprayed inside the greenhouse to control humidity. In lab-scale experiments, *E. coli* showed potential for surviving but not for growing in most fertilizer solutions and irrigation water. Fertilizer solution of HNO_3, was the only solution in which no *E. coli* were able to survive in the irrigation head and a rapid inactivation was observed in lab-scale tests. These results suggest that there is a low risk of contamination in this agricultural system despite the combination of higher risk irrigation water sources (reclaimed and surface water) and the hydroponic growing system. Nevertheless, special care should be taken regarding the microbiological quality of the agricultural solutions in direct contact with the edible parts of the crop.

1. Introduction

Reclaimed water is used in agriculture in arid and semi-arid regions of the world as a way to overcome water scarcity (Becerra-Castro, Lopes, Vaz-Moreira, Silva, Manaia, 2015). However, there are concerns related to the microbiological quality of reclaimed water and its possible consequences on food safety (Olivieri, Seto, Cooper, Cahn, Colford, 2014). Even though irrigation water contact with edible plant parts is avoided, survival of pathogenic bacteria in agricultural substrates and potential internalization through the roots are relevant issues to be taken into account in agricultural use of reclaimed water (Bernstein, 2011).

Monitoring sampling programs are usually based on the detection of specific pathogens. However, there is an increased interest in using microbial indicators to characterize microbial contamination in the environment of primary production to overcome current limitations associated with the pathogen detection such as low prevalence and high cost (Mukherjee, Speh, & Diez-Gonzalez, 2007; Park, Navratil, Gregory, Anciso, & Ivanek, 2013). Nonetheless, reliability of indicator microorganisms can be affected by different factors such as climatic conditions or the use of agrochemicals (Pachepsky, Shelton, McLain, Patel, & Mandrell, 2011; Castro-Ibáñez, Gil, Tudela, Ivanek, & Allende, 2015).

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Hydroponic cultivation in greenhouses is supposed to reduce the number of vectors that can contaminate the crops with pathogenic bacteria (Orozco, Rico-Romero, & Escartín, 2008a). However, there are still different vectors (e.g. irrigation water) that can be the entry point of pathogenic bacteria in this agricultural system (Orozco et al., 2008b). Once in the environment, pathogenic microorganisms are very difficult to eliminate (Allende & Monaghan, 2015). Stanghellini and Rasmussen (1994) described that peat, water source and insects are potential vectors for the contamination of nutrient solution in hydroponic systems. The safety issues associated with the use of reclaimed water have been evaluated in tomatoes, and results suggested that urban wastewater in combination with a production system that minimizes contact with the edible part of the crop does not represent a microbial risk (López-Gálvez, Allende, Pedrero-Salcedo, Alarcon & Gil, 2014). The *Codex Alimentarius* (2007) highlights that plants grown in hydroponic systems absorb nutrients and water, which constantly change the composition of the nutrient solution. Because of this, Codex recommends that water used in hydroponic culture should be changed frequently, or if recycled should be treated to minimize microbial contamination (Allende & Monaghan, 2015).

There is some information available on the behaviour of plant pathogens in nutrient solutions used in hydroponic systems (Vallance, Déniel, Le Floch, Guérin-Dubrana, Blancard, 2011), but little about the potential of such solutions to sustain the survival of faecal indicators or human pathogenic microorganisms (Settani, Miceli, Francesca, Cruciata, & Moschetti, 2013). Staley, Rohr, and Harwood (2010) observed that the addition of agrochemicals, including inorganic fertilizers, affected the levels of fecal indicator bacteria in irrigation water. To gain insights regarding the microbial risk associated with water sources in a commercial greenhouse for hydroponically grown peppers, the present study focused on the evaluation of *Escherichia coli* prevalence in two types of irrigation water, reclaimed and surface water, as well as in the peppers and the impact of fertilizer solutions.

2. Materials and methods

2.1. Experimental design and growing conditions

Bell pepper plants (*Capsicum annuum* L. cv Tamarin) were grown in a commercial greenhouse located in Balsicas (Murcia, Spain) from December 2013 until August 2014. Details related to the greenhouse location, climatological monitoring and acquisition data, irrigation water characteristics and crop management were previously described (Lopez-Galvez et al., 2014). Two types of water sources were used for irrigation: reclaimed water (Reclaimed) from the tertiary treatment effluent of a wastewater treatment plant (Roldán-Balsicas, Murcia, Spain), and surface water (Surface) from an Irrigation Community. Irrigation water was supplemented with fertilizer solutions as needed to obtain the fertilized irrigation water (Fertilized Reclaimed and Fertilized Surface Water). Five different fertilizer solutions (FS) were used: **FS1** (Peak® MKP 0-52-34, 75 g/L KH_2PO_4, ICL Fertilizers, Beer Sheva, Israel); **FS2** (Multi-K GG, 75 g/L KNO_3, Haifa Chemicals, Haifa, Israel); **FS3 (**YaraLiva Calcinit, 75 g/L $Ca(NO_3)_2$, Yara Iberian, Madrid, Spain); **FS4** (Biomad micro®, 8 mL/L micronutrients solution, Agroquímicos Los Triviños, Murcia, Spain + Bastion®, 0.5 g/L Fe, Agroindustrial Kimitec, Almería, Spain); and **FS5** (40 mL/L HNO_3 54%, Agroquímicos Los Triviños, Murcia, Spain). Nutrients were supplied to the plants as required through the irrigation water. Five different fertilizing solutions prepared in individual tanks using surface water were used to this end. In all cases, coconut fibre (Pelemix, Alhama de Murcia, Spain) was used as substrate for the hydroponic system. A total of 120 plants were divided into two treatments depending on the irrigation water (Reclaimed and Surface treated plants) with 3 replicates of 20 plants each. During the growing period, minimum and maximum temperatures inside the greenhouse were 12.3 °C and 28.2 °C, respectively, with an average of 21.0 ± 3.9 °C. The relative humidity (RH) in the greenhouse ranged from 55.8% to 99.9% with an average of 79.2 ± 7.6%. The total amount of irrigation water applied was 5022 and 5012 m^3/ha for reclaimed and surface water, respectively.

2.2. Sampling points for water and fertilizer solutions

E. coli prevalence and concentration were monitored in samples (2 L) of irrigation water before (Reclaimed and Surface) and after fertilization (Fertilized Reclaimed and Fertilized Surface). The drainage obtained from the hydroponic substrate lines was another sampling point where water was tested (Drainage Reclaimed and Drainage Surface). Samples were also taken from the different fertilizer solution tanks (FS1-FS5). Atomized water from the nozzles to control RH inside the greenhouse was also evaluated. Sampling was performed 1–2 times per week during 16 weeks from April 28th until August 12th 2014, with a total of 240 water samples. *E. coli* analyses were carried out as previously described (Lopez-Galvez et al., 2014).

2.3. Microbiological analysis of peppers

Collection of pepper samples was performed every 2 weeks during the harvest period, from May until August 2014, for a total number of 222 samples over 8 sampling times. At each sampling time, 10–15 samples were taken from each growing condition, which corresponded to peppers irrigated with reclaimed and surface water. Green peppers grade U.S. No. 1 or grade U.S. Fancy, as defined in the United States standards for grades of sweet peppers (USDA., 1989), were randomly picked from the plants at a height of 0.25–1.5 m above the surface of the substrate and transferred aseptically into sterile bags. For the analysis of each sample, pepper flesh was diced in pieces of approximately 3 × 3 cm using a stainless steel knife under aseptic conditions. Samples of 25 g of pepper were taken randomly and diluted 1:5 in buffered peptone water (20 g/L; Scharlab, Barcelona, Spain) and then homogenized using a stomacher for 1 min. Homogenized samples were used for direct plating, filtration, and enrichment. For direct plating, 1 mL of sample was poured in a petri plate and mixed with melted agar before incubation. In order to reduce the detection limit, 25 mL of homogenized sample was filtered through a 0.45 μm membrane filter (Sartorius, Madrid, Spain). The remaining contents of the bags were incubated at 37 °C for 18–24 h for enrichment of viable populations. After enrichment, fluids were applied to the surface of Chromocult coliform agar (Merck, Darmstadt, Germany) with a bacteriological loop and were incubated at 37 °C for 24 h before interpretation of results.

2.4. Survival and growth of E. coli in irrigation water and fertilizer solutions

Plate count experiments were performed in triplicate to determine potential survival or growth of *E. coli* in the irrigation water types (Reclaimed and Surface) as well as in the different fertilizer solutions (FS1 to FS5) using a lab-scale test where reclaimed and surface water were used for the preparation of the solutions. A cocktail of three *E. coli* strains (CECT 471, 515, and 516) was prepared by mixing cells washed twice by centrifugation (4500 *g*, 10 min) in sterile distilled water after overnight incubation (BHI, 37 °C, 20 h). The *E. coli* strains were inoculated in the irrigation water with and without fertilizer solutions to reach a level of 2 log

cfu/100 mL. Initial levels of *E. coli* were determined as described in paragraph 2.3. Inoculated fertilizer solutions were kept in darkness at 25 °C during 7 days and sampled during storage after 1, 2, 3, 6 and 7 days of preparation to monitor the levels of *E. coli*. Physicochemical measurements including pH, oxidation-reduction potential (ORP), turbidity, and conductivity were recorded for all the solutions. A portable multimeter (pH & Redox 26, Crison, Barcelona, Spain) was used to determine pH and ORP. Turbidity was measured by using a turbidimeter (Turbiquant 3000 IR, Merck, Darmstadt, Germany). Conductivity was measured by using a conductivimeter (CM 35, Crison, Barcelona, Spain).

2.5. Statistical analysis

Counts derived from microbiological analysis were log transformed and entered in an Excel spreadsheet (Microsoft Excel, 2010). Results of water samples with values below the detection limit were given a value of 0 log cfu/100 mL for calculation of means. Results were compiled and graphs were made using Sigma Plot 12.0 Systat Software, Inc. (Addilink Software Scientific, S.L. Barcelona). IBM SPSS statistics 20 was used for statistical analysis. The Kolmogorov–Smirnov test and Levene's test were used to assess normality and equality of variance, respectively. When normality could be assumed, ANOVA and Welch tests were used depending on the homogeneity of the variances. When data did not follow a normal distribution, non-parametric tests were applied. The chi-square (χ^2) test was used to compare prevalence of positive samples for *E. coli*.

3. Results

3.1. Presence of E. coli in irrigation water and fertilizer solutions

Limit of detection in water samples was 1 cfu/100 mL. Prevalence of *E. coli* in the different types of irrigation water is shown in Table 1. *E. coli* was detected more often in reclaimed water than in surface water. Furthermore, levels of this indicator bacteria were significantly higher in reclaimed water than in surface water, both before and after fertilization ($p < 0.005$) (Fig. 1). On the other hand, *E. coli* was detected in 20% of the samples obtained from the humidity control nozzles located in the greenhouse. Detection of *E. coli* was less frequent in the fertilizer solutions (Table 2) than in irrigation water with and without fertilizers (Table 1). However, FS4 was an exception as *E. coli* prevalence was similar to Fertilized Surface water (Table 2). Furthermore, *E. coli* level detected in FS4 was the maximum when compared to any other water samples (Table 2). Fertilizer solution FS5 (HNO_3 solution) was always negative for the presence of *E. coli* (see Table 2).

3.2. Monitoring the levels of E. coli in peppers

The detection limit for *E. coli* on peppers was 0.5 cfu/g. All the samples of pepper irrigated with reclaimed water were negative for the presence of *E. coli* (n = 111). However, *E. coli* was detected in 5 samples (4.5%) of peppers irrigated with surface water (n = 111) corresponding to 3 different sampling dates (3 in May 5th, 1 in June 4th, and 1 in August 11th). It was possible to quantify the counts of *E. coli* in 2 out of 5 positive samples, with levels of 130 and 3 cfu/g in June 4th and August 11th, respectively. The other 3 samples were positive only after enrichment.

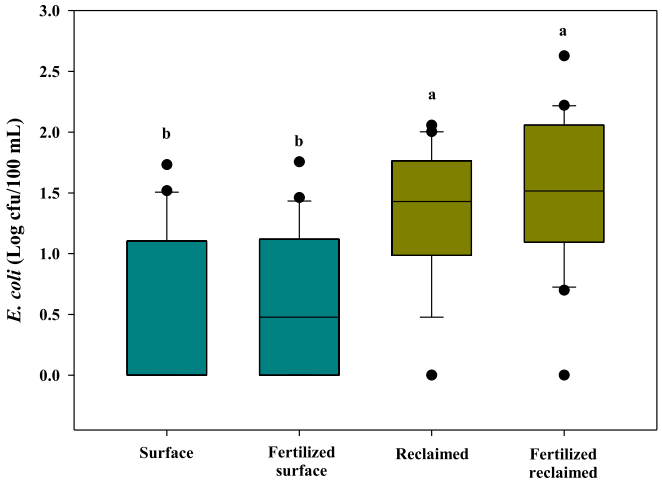

Fig. 1. Boxplot representing *Escherichia coli* levels in different water sources including reclaimed water (Reclaimed), surface water (Surface), fertilized reclaimed water (Fertilized Reclaimed) and fertilized surface water (Fertilized Surface). Bottom and top of the boxes represent the 25th and 75th percentiles. The box plot whiskers represent the minimum and maximum values. Dots represent outlier values. Different letters represent significant difference at $P < 0.005$.

3.3. E. coli survival and growth in irrigation water and in fertilizer solutions

In the preliminary test of the lab-scale studies, FS5 showed a rapid inactivation of *E. coli* in the nitric acid solution and therefore FS5 was not included in other trials. When the physicochemical characteristics of irrigation water with and without fertilizer solutions were measured, some differences were observed particularly for pH, ORP, turbidity and conductivity (Table 3). Changes of *E. coli* levels in reclaimed and surface irrigation water are shown in Fig. 2. A native level of *E. coli* of about 0.9 log cfu/100 mL was detected in both types of water before inoculation. After inoculation the level increased to approximately 2 log cfu/100 mL. In both types of water (reclaimed and surface) the same tendency was observed over time, with a decrease in the *E. coli* concentration during the first 2–4 days and a constant level during the rest of storage (Fig. 2). *E. coli* levels reached a concentration of about 1 log cfu/100 mL at the end of the experiments in both types of irrigation water samples. Changes of *E. coli* levels in fertilizer solutions

Table 1
Prevalence of *E. coli* in reclaimed and surface irrigation water at different sampling points.

Water type	Sampling point	Number of positive samples	Prevalence (%)
Reclaimed	Receiving tank	19/20	95^{a*}
Fertilized Reclaimed	After fertilization	19/20	95^{a}
Drainage Reclaimed	Drainage	14/20	70^{ab}
Surface	Receiving tank	8/20	40^{bcd}
Fertilized Surface	After fertilization	12/20	60^{bc}
Drainage Surface	Drainage	6/20	30^{cd}
Pulverized Surface	Humidity nozzles	4/20	20^{d}

*Different letters in the same column indicate significant differences ($P < 0.05$).

Table 2
Prevalence of *E. coli*, maximum levels and interquartile range (IQR) in the different fertilizer solutions analysed.

Fertilizer solutions	Number of positive samples	Prevalence	Maximum levels (log cfu/100 mL)	IQR
FS1	4/20	20[b*]	0.6	0.0
FS2	4/20	20[b]	0.9	0.0
FS3	1/20	5[b]	1.7	0.0
FS4	12/20	60[a]	2.8	1.5
FS5	0/20	0[b]	0.0	0.0

*Different letters in the same column indicate significant differences ($P < 0.05$).

Table 3
Physicochemical characteristics of reclaimed and surface irrigation waters with and without the fertilizer solutions (FS) used in lab-scale experiments: FS1 (KH_2PO_4), FS2 (KNO_3), FS3 ($Ca(NO_3)_2$) and FS4 (micronutrients solution + Fe).

Water type	Solution	pH	ORP[a]	Turbidity (NTU)	Conductivity (ms/cm)
Reclaimed	**Water**	7.8 ± 0.1	393.0 ± 0.0	3.9 ± 0.4	1.6 ± 0.0
	FS1	4.3 ± 0.0	468.0 ± 1.4	3.7 ± 0.3	43.9 ± 0.1
	FS2	8.4 ± 0.0	381.0 ± 4.2	4.8 ± 0.1	81.9 ± 0.2
	FS3	7.1 ± 0.0	430.5 ± 2.1	3.5 ± 0.2	63.5 ± 0.2
	FS4	8.2 ± 0.0	138.0 ± 2.8	NM	4.3 ± 0.0
Surface	**Water**	7.2 ± 0.1	454.5 ± 4.9	40.6 ± 1.4	0.9 ± 0.0
	FS1	4.0 ± 0.0	590.5 ± 26.2	13.3 ± 1.4	38.1 ± 1.2
	FS2	7.2 ± 0.1	445.5 ± 36.1	9.8 ± 0.8	61.8 ± 11.9
	FS3	6.3 ± 0.2	491.0 ± 28.3	12.7 ± 2.1	48.9 ± 9.5
	FS4	8.5 ± 0.1	395.0 ± 40.7	NM	2.8 ± 0.1

[a] ORP: oxidation-reduction potential. NM: Not measurable.

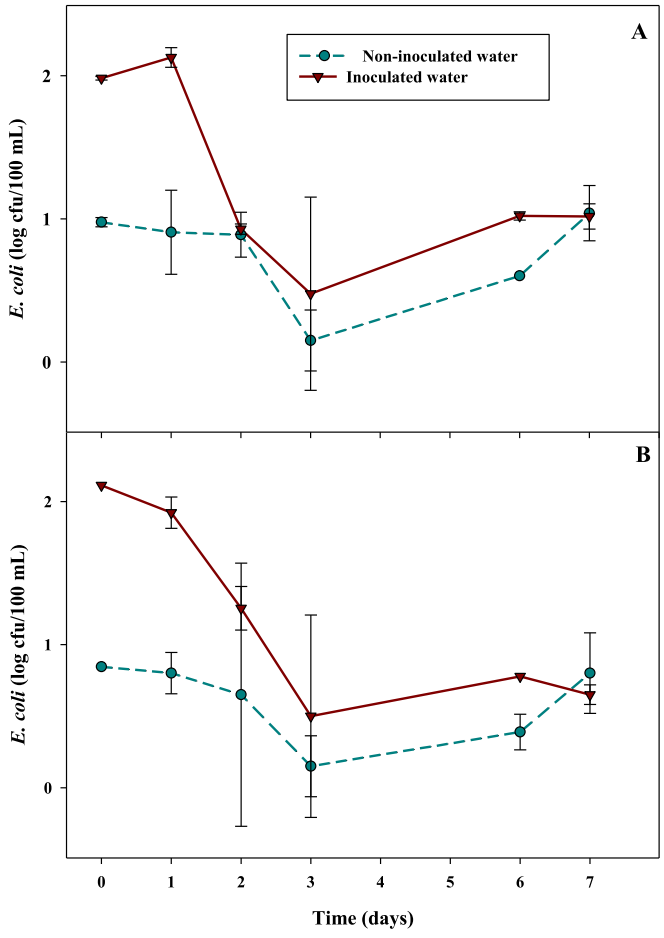

Fig. 2. Changes in *E. coli* levels (log cfu/100 mL) during 7 days at 25 °C in: A) reclaimed water, and B) surface water.

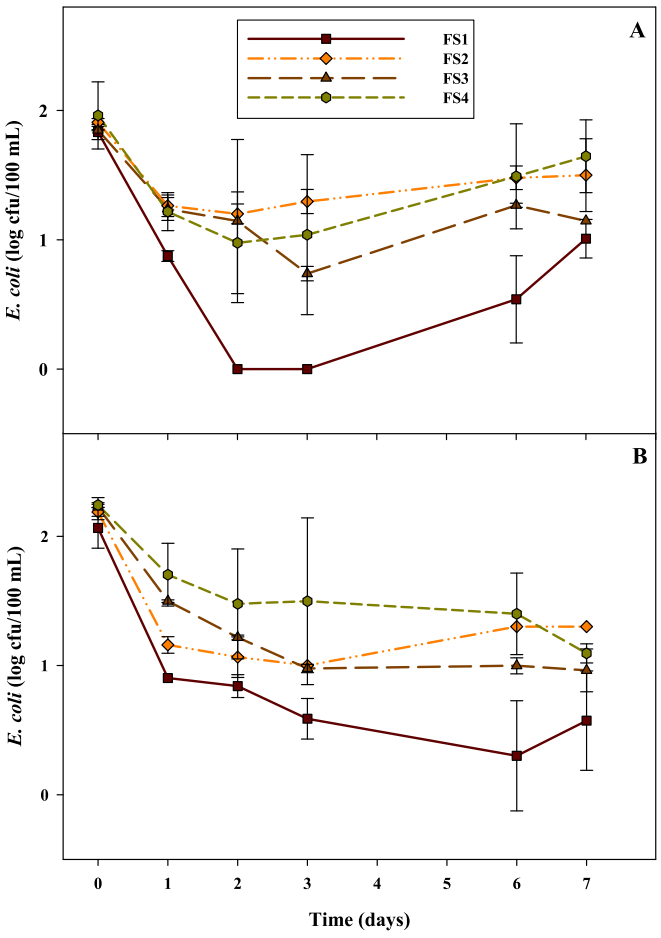

Fig. 3. Changes in *E. coli* levels (log cfu/100 mL) during 7 days at 25 °C in: A) reclaimed water with fertilizer solutions; B) surface water with fertilizer. FS1 (KH_2PO_4), FS2 (KNO_3), FS3 ($Ca(NO_3)_2$), FS4 (micronutrients + Fe).

prepared with reclaimed and surface water are shown in Fig. 3. During storage at 25 °C, *E. coli* levels decreased in all the FS but the rate was different depending on the fertilizer solution. Levels of *E. coli* in FS1 (KH_2PO_4), prepared with both reclaimed and surface water decreased faster after 1–2 days of storage than the rest of FS. However, when FS1 was prepared using reclaimed water, a slight recovery in *E. coli* numbers was observed after 6–7days. For fertilizer solutions of FS2, FS3 and FS4, no significant differences in *E. coli* loads were observed among them and the final levels were about 1 log cfu/100 mL.

4. Discussion and conclusions

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 10^2-10^3 cfu/100 mL. In the present study, where a hydroponic system was applied, there was no direct contact between irrigation water and fruit. However, levels of *E. coli* were always below 10^3 cfu/100 mL, reaching only sporadically values above 10^2 cfu/100 mL mostly in the reclaimed water, the drainage of surface water and in the fertilizer solution containing micronutrients (FS4). Levels of *E. coli* in irrigation water were similar before and after the fertilizers were added in agreement with our previous study where the same experimental setting was used for tomatoes (Lopez-Galvez et al., 2014). Bernstein, Guetsky, Friedman, Bar-Tal, and Rot (2008) in their assessment of the presence of *E. coli* in a hydroponic greenhouse for ornamental flowers irrigated with reclaimed water found similar *E. coli* levels in drainage waters to those detected in our study (0.6–0.7 cfu/mL). However, these authors did not detect *E. coli* in irrigation water (chlorinated secondary effluent), nutrient solution, or substrate. Results from our lab-scale experiments confirmed the inhibition of *E. coli* growth in reclaimed and surface water, but the survival after an initial decrease. Accordingly, literature data on survival of *E. coli* in water suitable for irrigation generally show an initial decrease followed by a second phase of survival in small numbers (Pachepsky et al., 2011).

Prevalence and levels of *E. coli* in fertilizer solutions used in the greenhouse were low, except in the case of FS4. In the tests performed at lab-scale to assess the behaviour of *E. coli* in fertilizer solutions in controlled conditions, similar levels of *E. coli* were detected after 1 week in FS4 compared with the rest of fertilizer solutions. However, when looking to shorter storage times, levels of *E. coli* 1–2 days after inoculation were lower in all the FS compared with FS4. Physicochemical characteristics of the fertilizer solutions were quite different, with FS4 (micronutrients solution) showing lower ORP and conductivity. Fertilizer solution of FS1 (KH_2PO_4) showed a significant lower pH, causing probably the faster initial decrease in *E. coli* numbers and lower levels at the end of the storage period when compared to the rest of the FS. Settani et al. (2013) also observed bacterial decrease, using a very high initial *E. coli* inoculum of $\approx 10^7$ log cfu/mL, in a mineral nutrient solution used for hydroponic cultivation of radish. Conversely, Staley et al. (2010) observed an increase in *E. coli* levels caused by inorganic fertilizers, although in a former study from the same group (Staley, Rohr, & Harwood, 2011) no effect of fertilizers on faecal indicator bacteria in water was reported. They hypothesized that growth of indicator bacteria could be promoted by the addition of inorganic fertilizers when nutrients are a limiting factor for the growth of these microorganisms (Staley et al., 2011).

E. coli levels of about 1.4 log cfu/100 mL were detected in the pulverized water used for humidity control of the greenhouse. This is not surprising because surface water in which *E. coli* was also detected was used for this purpose. This observation suggests that the microbiological quality of this water is very important because it gets into direct contact with the surface of the fruit.

Despite the presence of *E. coli* in irrigation water, mainly in reclaimed water, and in the pulverized water used to increase the RH, *E. coli* was only detected in five samples of peppers. In our previous work where similar *E. coli* levels were found in irrigation water, *E. coli* was not detected in tomato samples (n = 72) (Lopez-Galvez et al., 2014). Avila-Vega, Álvarez-Mayorga, Arvizu-Medrano, Pacheco-Aguilar, Martínez-Peniche, (2014) detected *E. coli* in 5.1% of greenhouse hydroponic pepper samples (n = 528), a similar percentage to that found in the present study for peppers irrigated with surface water (4.5%, n = 111). The same authors reported that the concentration of *E. coli* in the nutrient solution was 0.6 log MPN/100 mL, the same level as that detected in our study (0.6 log cfu/100 mL). Though lower *E. coli* prevalence was observed in surface water than in reclaimed water, a small number of positive samples were detected in peppers irrigated with fertilized surface water. When Dagianta, Goumas, Manios, and Tzortzakis (2014) analysed a low number of samples (n = 23), *E. coli* were only detected in peppers from plants irrigated with fertilized water, but not in fruits from plants irrigated without fertilizers. Forslund, Battilani, Ensink, Marcussen, Gola, (2013) did not detect *E. coli* in tomatoes although it was present in reclaimed water and soil. A link between levels of indicator bacteria in irrigation solutions and in pepper fruit was not evident as it was previous observed in tomatoes (Lopez-Galvez et al., 2014). Accordingly, Manios, Papagrigoriou, Daskalakis, Sabathianakis, Terzakis, (2006) could not relate presence of enterococci in treated wastewater used for irrigation with its presence in greenhouse grown tomato and cucumber. These results suggest that when using hydroponic cultivation systems for crops with edible parts well separated from the soil, factors other than the microbiological quality of the irrigation water would be more important for the microbiological quality of the fruit (Orozco et al., 2008b). Unrealistically high levels of the pathogenic bacteria are necessary in the nutrient solution to observe such internalization phenomenon (Koseki, Mizuno, & Yamamoto, 2011).

Irrigation with untreated or improperly treated wastewater is a clear food safety risk (Steele & Odumeru, 2004). However, adequate use of properly treated wastewater could help to control microbial risk while increase availability of water for agriculture, (O'Connor, Elliott, & Bastian, 2008). Pachepsky et al. (2011) reported that until 2011 there have not been cases of foodborne illness linked to the use of reclaimed wastewater for irrigation in the USA. In our study, a relationship between *E. coli* levels in irrigation water and in pepper fruit was not observed. Furthermore, lab-scale experiments showed *E. coli* survival but not growth in most fertilizer solutions and in irrigation water. Our results also highlight the importance of monitoring the quality of irrigation water and also of other aqueous solutions used for crop management, especially if they are going to be in direct contact with the edible parts of the crop.

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