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Effect of Repeated Irrigation with Water Containing Varying Levels of Total Organic Carbon on the Persistence of *Escherichia coli* O157:H7 on Baby Spinach†

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ABSTRACT

The California lettuce and leafy greens industry has adopted the Leafy Greens Marketing Agreement (LGMA), which allows for 126 most-probable-number (MPN) *Escherichia coli* per 100 ml in irrigation water. Repeat irrigation of baby spinach plants with water containing *E. coli* O157:H7 and different levels of total organic carbon (TOC) was used to determine the epiphytic survival of *E. coli* O157:H7. Three irrigation treatments (0 ppm of TOC, 12 or 15 ppm of TOC, and 120 or 150 ppm of TOC) were prepared with bovine manure containing *E. coli* O157:H7 at either low (0 to 1 log CFU/100 ml) or high (5 to 6 log CFU/100 ml) populations, and sprayed onto baby spinach plants in growth chambers by using a fine-mist airbrush. MPN and direct plating techniques were used to determine the *E. coli* O157:H7 populations on the aerial plant tissue. Plants irrigated with high *E. coli* O157:H7 populations, regardless of TOC levels, showed a 3-log reduction within the first 24 h. Low levels of *E. coli* O157:H7 were observed for up to 16 days on all TOC treatments, ranging from 76.4 MPN per plant (day 1) to 0.40 MPN per plant (day 16). No viable cells were detected on spinach tissue 24 h after irrigation with water containing fewer than 126 CFU/100 ml *E. coli* O157:H7. Under growth chamber conditions in this study, *E. coli* O157:H7 populations in irrigation water that complies with the LGMA standards will not persist for more than 24 h when applied onto foliar surfaces of spinach plants.

Foodborne outbreaks continue to be associated with fresh produce at an alarming frequency, with 713 outbreaks occurring between 1990 and 2005 *(27)*. Almost one-fourth (24.5%) of all foodborne illness outbreaks from 1996 to 2005 were associated with produce *(11)*, including leafy greens *(28, 33)*, melons *(20)*, tomatoes *(10, 22)*, and sprouts *(7)*. In 2007, 14% of all outbreaks that were attributed to a single food commodity were linked to leafy greens, making leafy greens the produce commodity most likely associated with foodborne pathogen contamination *(8)*. As foodborne outbreaks associated with produce continue to occur, all aspects of the farm-to-fork spectrum are under close scrutiny for areas where contamination could occur.

Irrigation water became a recognized source for the preharvest contamination of produce because of its association with outbreaks of *Escherichia coli* O157:H7 on leafy greens. Environmental sampling after the 2006 shredded-lettuce outbreak in the United States traced the source of contamination to irrigation water tainted with dairy effluent from a herd adjacent to the implicated lettuce field *(5)*. Several guidance documents have since been developed to address the microbiological safety of irrigation water, including the Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens *(6)*. The current guidelines set by the LGMA state that irrigation water used for foliar applications may contain ≤126 most probable number (MPN)/100 ml of *E. coli* (rolling geometric mean of five samples), and any single sample may not exceed 235 MPN/100 ml.

Several studies have investigated the persistence of *E. coli* O157:H7 in the phyllosphere of baby spinach plants after foliar irrigation with contaminated water or spot-inoculation with contaminated manure slurry *(9, 15, 18, 21)*. None of these studies, however, involved application of *E. coli* populations in irrigation water containing fewer than 126 MPN/100 ml onto the plant surfaces. Previous studies have not addressed specific parameters regarding irrigation water quality, which could affect the bacterial pathogen populations in the water. Total organic carbon (TOC) content is one indicator of water quality, which measures organic molecules present in water. These molecules (amino acids, sugars, and fatty acids), even when present at very low levels, may be used as nutrient sources by all epiphytic microbiological populations, which might include *E. coli* *(14)*. Environmental survey data has shown that the TOC

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concentration for on-farm irrigation water would likely be between 5 and 10 ppm *(12, 19, 23)*. It is possible that higher TOC levels could occur after a rain event, caused by runoff from wildlife droppings, stockpiles of manure, or incompletely composted manure from neighboring fields *(25, 30, 31)*.

Baby spinach crops are fast growing, cool-weather crops that are generally harvested between 30 and 45 days after sowing. Leaf maturity may have a role in the epiphytic persistence of *E. coli* O157:H7 on leafy green commodities, as greater population sizes of *E. coli* O157:H7 have been found on younger (inner) lettuce leaves than on older (outer) lettuce leaves *(3)*. While baby spinach leaves do not exhibit the same dense, spiral growth pattern as some lettuce cultivars exhibit, previous research has found that the younger baby spinach leaves might be more susceptible to surface contamination because of physiological and microbiological community differences on the plant surfaces *(24)*.

Spinach plants are sometimes irrigated frequently during dry seasons *(4)*. Therefore, we included repeated irrigation events (two times per week) with a water source containing dilute dairy manure to evaluate the persistence of enterohemorrhagic *E. coli* (EHEC) on the foliar surfaces of spinach plants. We hypothesized that the biweekly introduction of moisture and the accumulation of TOC on the plant surfaces via irrigation water would sustain an epiphytic *E. coli* O157:H7 population. Our study examined TOC levels, as adjusted with diluted dairy manure, in irrigation water to determine its effect on the persistence of *E. coli* O157:H7 on spinach leaves.

MATERIALS AND METHODS

Preparation of irrigation solutions. Fresh bovine manure was obtained from a U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS), Beltsville Area Research Center (BARC) Holstein dairy herd that was not exposed to antibiotics. The dairy solids were suspended in a 1:5 ratio of solids:deionized water, and stirred for 6 h at 25°C. Particulate matter was removed via three sequential centrifugation (Allegra 25 R, Beckman Coulter, Fullerton, CA) events at 10,700 × *g* for 20 min, and the supernatant was then sterilized at 121°C for 60 min. The manure extract was analyzed for TOC (Phoenix 8000, Teledyne Tekmar, Mason, OH), ammonia, and nitrate content (QuickChem 8000 FIA Lachat, Fort Collins, CO), and used to prepare three irrigation stock solutions (2 liters each). These solutions were prepared by diluting extracts in sterile deionized water to obtain three experimental TOC concentrations: treatments A, 0 ppm; B, 12 or 15 ppm; and C, 120 or 150 ppm. Treatment A (sterile, deionized water) was used as the control irrigation solution. All sterile irrigation stock solutions were adjusted to pH 7.0 with either 10 N NaOH or 33% HCl and stored at 4°C. Measurements taken throughout refrigerated storage indicated that TOC of solutions were unchanged throughout the duration of the experiment.

Preparation of *E. coli* O157:H7 inoculum. Three human clinical *E. coli* O157:H7 isolates, epidemiologically linked to produce outbreaks, were used to inoculate the irrigation solutions: RM4406 (lettuce outbreak), RM4407 (spinach outbreak), and 5279 (bagged-vegetables outbreak), kindly provided by Robert Mandrell (USDA-ARS, Albany, CA). All strains were adapted to grow in the presence of nalidixic acid (50 μg/ml), as described previously *(26)*. Strains were inoculated from frozen stocks onto MacConkey agar (BD, Franklin Lakes, NJ) supplemented with 50 μg/ml nalidixic acid (MACN; Sigma, St. Louis, MO) and incubated at 37°C for 24 h. All *E. coli* O157:H7 strains were inoculated into 25-ml aliquots of irrigation treatment C (120 or 150 ppm of TOC), and incubated at 37°C for 48 h with agitation (150 rpm). The night prior to each irrigation event where *E. coli* O157:H7 was to be applied to spinach plants, the population of each *E. coli* O157:H7 strain was individually determined by spiral plating (WASP2, Don Whitley Scientific, Frederick, MD) onto MACN plates, which were incubated at 37°C for 24 h. Each liquid manure culture was maintained on ice at 4°C overnight to prevent further growth. Appropriate volumes of each strain (as determined by plate count data) were added to irrigation solutions as prepared above to yield the experimental *E. coli* O157:H7 populations (≤126 CFU/100 ml for the low inocula and 5 to 6 log CFU/100 ml for the high inocula).

Spinach plant cultivation. The spinach (*Spinacia oleracea*) cultivar Avenger, a hybrid semi-Savoy cultivar that is resistant to downy mildew lines 1 through 7 and used in the Central Valley in California, was donated by Seminis, Inc. (Oxnard, CA). Seeds were soaked in 10% commercial sodium hypochlorite solution for 10 min, and then washed three times for 5 min each in sterile deionized water. Seeds were sown in sterile 164-ml cone-shaped plastic vessels (Cone-tainers, model SC10, Stuewe and Sons, Inc., Tangent, OR) filled with 125 g of fine, sandy loam (Keyport-Matawan) soil obtained from the USDA-ARS BARC north farm. Soil was steam pasteurized (85°C for 15 h) to eliminate potential phytopathogens and invertebrates. Cone-tainers with spinach plants were placed into custom trays (RL98, Stuewe and Sons), with one tray for each irrigation treatment to prevent cross-contamination. Plants were maintained in an environmentally controlled growth chamber (CMP 4030, Conviron, Winnipeg, Manitoba, Canada) set to 70% humidity and light intensity of 450 microeinstein units/m^2/s for a 14-h photoperiod at 18°C and 10-h dark period at 13°C. All plants were fertilized and irrigated once per week with diluted (1.32 g/liter) Jack's Classic All Purpose 20-20-20 fertilizer (J. R. Peters, Inc., Allentown, PA), which was applied via pipette to the soil, without contacting spinach leaves.

Irrigation of baby spinach plants. The irrigation treatments began when all plants had developed two true leaves (approximately 14 days after sowing). For the three irrigation solutions (treatments A, B, and C), 200 ml of each irrigation solution was prepared by adding appropriate volumes of each *E. coli* O157:H7 culture (as prepared above) to achieve a low or high population of *E. coli* O157:H7 inocula. During each irrigation event, individual spinach plants were transferred to a biological safety hood and sprayed using a hand-held airbrush (model 200, Badger Air Brush Company, Franklin Park, IL) connected to an air compressor (model 180-10, Badger Air Brush Co.). This technique deposited a fine mist of each irrigation solution onto the entire foliar surface of each plant, including the stem, adaxial, and abaxial surfaces of each leaf (Fig. 1). Each plant was sprayed until total coverage was achieved, without runoff, which varied between 15 and 25 s, depending on the amount of tissue, resulting in the application of 1.5 to 3 ml of the irrigation treatment onto the foliar surface of each plant. Twice a week, each irrigation solution (treatments A, B, and C) was sprayed on all plants within each treatment block. Depending on the day of harvest, the plants were exposed to at least 1 irrigation event (day 0 harvest) and up to 5 irrigation events for plants exposed to the low *E. coli* O157:H7 inoculum, and 10 irrigation events for plants exposed to the high *E. coli* O157:H7

FIGURE 1. *Leaf tissue of a 14-day-old baby spinach plant after an irrigation event with hand-held airbrush.*

inoculum. Plants were harvested daily for microbiological analyses. If 2 consecutive days of foliar microbial analyses of baby spinach plants were negative for *E. coli* O157:H7, then plants would receive an additional inoculated irrigation treatment at the next scheduled event. If these analyses revealed the presence of *E. coli* O157:H7, then the next scheduled irrigation event would contain the appropriate irrigation treatment, but without the addition of the *E. coli* O157:H7 inoculum.

Microbiological analyses. At each harvest, randomly selected plants were excised with sterile scissors, 5 mm above the soil surface. The entire aerial tissue from each plant was placed into a sterile 80-ml stomacher bag (Secure T, Fisher Scientific) containing 40 ml of mEHEC broth (BioControl, Bellevue, WA) supplemented with 50 μg/ml of nalidixic acid. Samples were then homogenized in a Stomacher 80 (Seward, Ltd., Basingstoke, UK) for 2 min at 230 rpm, and then sonicated for 30 s (Astrason ultrasonic cleaner, Plainview, NY). A three-tube MPN was performed on each sample homogenate with 10, 1, and 0.1 ml of the homogenate added to each of three tubes, containing 0, 9, and 9.9 ml, respectively, of sterile mEHEC broth supplemented with 50 μg/ml nalidixic acid. The tubes were incubated for 24 h at 37°C, before 10 μl of each tube was isolated on MACN for identification of positive tubes containing *E. coli* O157:H7 populations. The freeware MPN calculator (VB6 version, www.i2workout.com/mcuriale/mpn/index.html) was used to calculate the final MPN per gram for each sample. Each MPN was performed in triplicate, and the results were expressed as the mean MPN per plant.

The MPN assays for the plants exposed to the low *E. coli* O157:H7 populations in irrigation treatments were based on a single plant through the first three inoculation events. Beginning with irrigation event 4, three plants were harvested per MPN analysis, which increased the detection sensitivity from 1.2 MPN per plant to 0.4 MPN per plant. The plants receiving high *E. coli* O157:H7 population inocula were analyzed, with one plant per MPN assay. Additionally, remaining homogenate was spiral plated (100 μl, in triplicate) onto MACN plates.

Liquid from irrigation treatments containing low and high populations of *E. coli* O157:H7 was collected after each spray event and analyzed with filtration (low inocula) or spiral plating (high inocula). For the low inoculum, 25 ml of liquid was vacuum filtered through each of four sterile, hydrophobic grid membrane filter monitors (56 mm/100 ml, 0.45 μm; BioPath, Inc., West Palm Beach, FL). Membranes were removed aseptically from containers, placed onto MACN plates, and incubated for 24 h at 37°C, after which colonies were visualized and counted on the surface of the membrane.

Growth of *E. coli* O157:H7 in irrigation solutions. Each irrigation solution (150 ml), as described above to contain TOC concentrations of 0, 12, or 120 ppm, was transferred into each of three separate 250-ml Pyrex screw-cap bottles (Fisher Scientific). Each bottle was inoculated with the three-strain *E. coli* O157:H7 inoculum at populations of either 0 or 4 log CFU/ml. Solutions were incubated without agitation, with the caps loosened, and maintained under the same growth chamber temperatures as were baby spinach plants (14 h at 18°C, 10 h at 13°C) in a low-temperature, illuminated programmable incubator (model 818, Thermo Electron Corp., Waltham MA). *E. coli* O157:H7 populations in irrigation solutions were quantified daily for up to 44 days by making appropriate serial dilutions and spiral plating, in duplicate, onto MACN plates, which were incubated at 37°C for 18 h.

RESULTS

Microbiological and nutrient content of irrigation treatments. The *E. coli* O157:H7 populations that were applied to spinach plants in inoculated irrigation events are shown in Tables 1 and 2. For irrigation events involving the low inocula, populations of *E. coli* O157:H7 ranged from 5 to 71 CFU/100 ml. For irrigation events involving high inocula, populations of *E. coli* O157:H7 ranged from 5.13 to 6.48 log CFU/100 ml. Treatment A contained 0 ppm of TOC, 0 ppm of nitrate, and 0 ppm of ammonia; treatment B contained 12 or 15 ppm of TOC, 0.58 ppm of ammonia, and 0.02 ppm of nitrate; and treatment C contained 120 or 150 ppm of TOC, 5.42 ppm of ammonia, and 0.11 ppm of nitrate.

Irrigation with low *E. coli* populations (≤126 CFU/100 ml). Spinach plants reached the two-leaf stage at approximately 17 days, when the biweekly foliar irrigation events commenced (Table 1). Epiphytic *E. coli* O157:H7 populations were all below the MPN detection limit (1.2 MPN per plant) for the plants harvested immediately after irrigation events 1, 2, and 3, on days of analysis 0, 2 and 7, respectively. *E. coli* O157:H7 populations on spinach plants were also below detection limits (<1.2 MPN per plant) for subsequent days of analysis for each of these respective irrigation events. Very low populations of *E. coli* O157:H7 were recovered from plant tissues harvested from the first

TABLE 1. *Low populations of* Escherichia coli *O157:H7 in irrigation solutions containing varying TOC concentrations and on spinach plants 0, 1, and 2 days after repeated irrigation events*

			Treatment:					
			A (0 ppm of TOC)		B (15 ppm of TOC)		C (150 ppm of TOC)	
Irrigation event[a]	Day of analysis[b]	Plant age (days)[c]	CFU/100 ml	MPN/plant	CFU/100 ml	MPN/plant	CFU/100 ml	MPN/plant
1	0	17	5	<1.20	56	<1.20	15	<1.20
	1	18		<1.20		<1.20		<1.20
	2	19		<1.20		<1.20		<1.20
2	2	19	31	<1.20	6	<1.20	55	<1.20
	3	20		<1.20		<1.20		<1.20
	4	21		<1.20		<1.20		<1.20
3	7	24	37	<1.20	36	<1.20	31	<1.20
	8	25		<1.20		<1.20		<1.20
	9	26		<1.20		<1.20		<1.20
4	9	26	22	3.74	60	4.13	50	49.2
	10	27		<0.40		<0.40		<0.40
	11	28		<0.40		<0.40		<0.40
5	16	33	11	0.32	31	0.16	71	0.41
	17	34		<0.40		<0.40		<0.40
	18	35		<0.40		<0.40		<0.40

[a] Irrigation events containing low *E. coli* O157:H7 (≤126 CFU/100 ml) inocula were applied in irrigation events 1 through 5.
[b] Spinach plants were irrigated with low populations of *E. coli* O157:H7 on days 0, 2, 7, 9, and 16.
[c] Plant age is indicated from the time of germination of spinach seedlings.

day of irrigation events 4 and 5 (sampling days 9 and 16, respectively). *E. coli* O157:H7 detection on spinach plants was facilitated by increasing the MPN sensitivity to <0.4 MPN per plant by using a composite sample of three plants for each MPN assay, which were performed in triplicate. No viable *E. coli* O157:H7 cells, however, were recovered 1 or 2 days after irrigation events 4 and 5 (Table 1).

On all days of analysis where *E. coli* O157:H7 populations were below detection limits for MPN analyses, the remaining liquid from each plant sample homogenate (approximately 7 ml) was enriched for 48 h in mEHEC broth and plated onto MACN. All enrichments were negative for the presence of *E. coli* O157:H7.

Irrigation with high *E. coli* O157:H7 populations (5 to 6 log CFU/100 ml). Spinach plants reached the two-leaf stage at approximately 13 days, when the biweekly foliar irrigation events commenced (Table 2). The initial irrigation event (event 1) contained 5.49 log CFU/100 ml for treatment A (0 ppm of TOC), 5.88 log CFU/100 ml for treatment B (12 ppm of TOC), and 6.48 log CFU/100 ml for treatment C (120 ppm of TOC), as shown in Table 2. Recovered populations of *E. coli* O157:H7 on the plant tissues harvested immediately after irrigation on day 0 for irrigation event 1 were all above the maximum limit (too numerous to count, >440 MPN per plant) for the MPN assay. Calculations based on the irrigation population density and amount of volume that was applied to the plants suggested that populations were ≥4 log MPN per plant. *E. coli* O157:H7 populations declined within 24 h to 34 MPN per plant for treatment A, 76 MPN per plant for treatment B, and 76 MPN per plant for treatment C. Low populations of *E. coli* O157:H7 were recovered sporadically in both treatments A and C, until the 15th day of analysis (28-day-old plants), where means of 0.5 and 67 MPN per plant were recovered, respectively. Populations of *E. coli* O157:H7 were detected on plants in treatment B until the 16th day of analysis (29-day-old plants), where 0.48 MPN per plant were recovered. Plants were harvested for the next 3 consecutive days; the results were consistently below MPN detection limits (<1.20 MPN per plant). All remaining liquid from the plant homogenates were enriched but remained negative for the presence of *E. coli* O157:H7.

The second application of *E. coli* O157:H7 population occurred during irrigation event 7, on 34-day-old spinach plants, where the irrigation water contained 5.13 log CFU/100 ml (treatment A), 5.47 log CFU/100 ml (treatment B), and 5.54 log CFU/100 ml (treatment C) (Table 2). Recovered populations immediately after irrigation were 1,467, 2,467, and 3,133 MPN per plant for respective treatments A, B, and C. *E. coli* O157:H7 populations declined by over 3 log MPN per plant after 1 day, with 0.48, 1.23, and <1.20 MPN per plant for treatments A, B, and C, respectively. *E. coli* O157:H7 persisted for 2 days after irrigation event 7 (harvest day 23) for treatments A and C, when 1.23 and 2 MPN per plant were recovered, respectively.

The third and final application of irrigation water containing *E. coli* O157:H7 occurred during event 9, on 41-day-old spinach plants, where 5.54 log CFU/100 ml (treatment A), 6.01 log CFU/100 ml (treatment B), and 6.13 CFU/100 ml (treatment C) were applied onto the foliar surfaces of each plant. Recovered populations of *E. coli* O157:H7 immediately after irrigation on day of analysis 28 were 2,467, 5,267, and 6,000 MPN per plant for treatments A, B and C, respectively. *E. coli* O157:H7 populations declined within 24 h (day of analysis 29) after application to

TABLE 2. *High populations of* Escherichia coli *O157:H7 in irrigation solutions containing varying TOC concentrations and on spinach plants 0, 1, and 2 days after repeated irrigation events*

			Treatment:					
			A (0 ppm of TOC)		B (12 ppm of TOC)		C (120 ppm of TOC)	
Irrigation event[a]	Day of analysis[b]	Plant age[c]	log CFU/ 100 ml	MPN/plant	log CFU/ 100 ml	MPN/plant	log CFU/ 100 ml	MPN/plant
1	0	13	5.49	TNTC[d]	5.88	TNTC	6.48	TNTC
	1	14		34.21		76.4		76.4
	2	15		3.07		1.71		15.87
2	2	15		6.13		0.96		1.71
	3	16		3.55		2		10
	4	17		0.48		3.07		61.33
	7	20		1.71		<1.20		<1.20
3	7	20		403		3.23		25.47
	8	21		1.23		<1.20		2
	9	22		3.07		<1.20		0.48
4	9	22		<1.20		<1.20		0.48
	10	23		<1.20		<1.20		<1.20
	11	24		<1.20		32		<1.20
	14	27		3.07		<1.20		2
5	14	27		10		<1.20		<1.20
	15	28		0.48		<1.20		66.67
	16	29		<1.20		3.07		<1.20
6	16	29		<1.20		0.48		<1.20
	17	30		<1.20		<1.20		<1.20
	18	31		<1.20		<1.20		<1.20
	21	34		<1.20		<1.20		<1.20
7	21	34	5.13	1,467	5.47	2,467	5.54	3,133
	22	35		0.48		1.23		<1.20
8	23	36		1.23		<1.20		2
	24	37		<1.20		<1.20		<1.20
	25	38		<1.20		<1.20		<1.20
9	28	41	5.54	2,467	6.01	5,267	6.13	6,000
	29	42		2.69		0.48		0.48
	30	43		0.48		0.48		<1.20
10	30	43		<1.20		12.4		<1.20
	31	44		<1.20		<1.20		0.48
	32	45		<1.20		<1.20		<1.20
	35	48		<1.20		<1.20		<1.20

[a] Irrigation events containing high populations of *E. coli* O157:H7 (5 to 6 log CFU/100 ml) inocula were applied in irrigation events 1, 7, and 9.
[b] Spinach plants were irrigated with high populations of *E. coli* O157:H7 on day 0, 21, and 28.
[c] Plant age is indicated from the time of germination of spinach seedlings.
[d] TNTC, too numerous to count.

2.69, 0.48, and 0.48 MPN per plant for treatments A, B and C, respectively. *E. coli* O157:H7 populations in treatments A and B declined to 0.48 MPN per plant, while those in treatment C declined to below the detection limit. After irrigation event 10 (day of analysis 30), which did not contain *E. coli* O157:H7 inocula, spinach plants irrigated with treatment B had 12.4 MPN *E. coli* O157:H7 per plant, whereas treatments A and C were below detection limits. The plant harvest continued until day 48, after all remaining plants were negative for *E. coli* O157:H7 for at least 2 consecutive days.

***E. coli* O157:H7 growth in irrigation treatments.** Low (0 log CFU/ml) and high (4 log CFU/ml) populations of *E. coli* O157:H7 were inoculated into irrigation solutions containing 0, 12, or 120 ppm of TOC and incubated under the same conditions used to grow the spinach plants. In all trials with low (Fig. 2) or high (Fig. 3) initial starting populations of *E. coli* O157:H7, the populations rapidly increased over several days before reaching approximate maximum population densities of 7 and 8 log CFU/ml, respectively. These high population densities were sustained by the nutrients contained in both TOC irrigation solutions of 12 and 120 ppm for longer than 44 days. Conversely, in all irrigation solutions containing 0 ppm of TOC, the *E. coli* O157:H7 were not recovered after 6 days when starting with a low initial population (Fig. 2) and 13 days for the high initial population (Fig. 3).

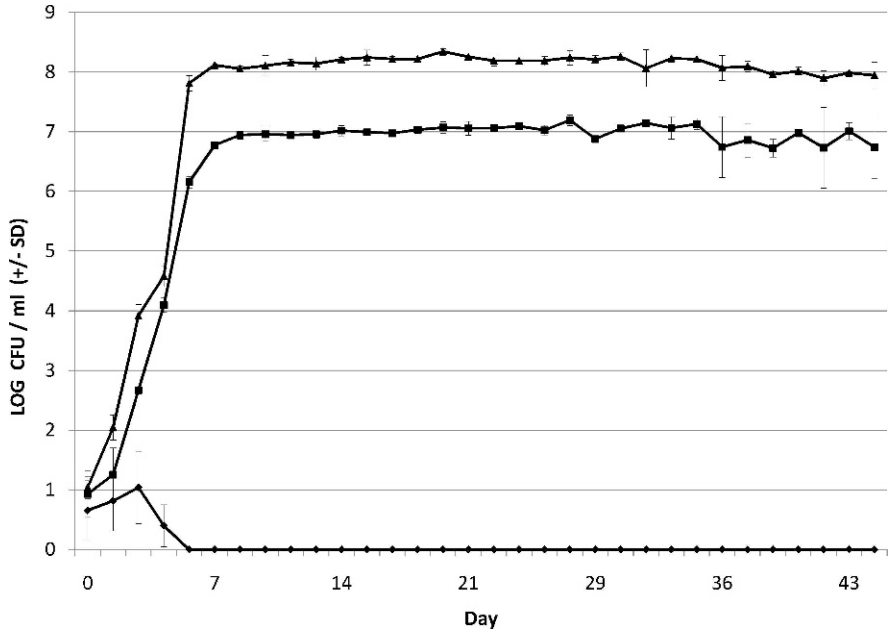

FIGURE 2. *Growth of low initial populations (0 log CFU/ml) of* Escherichia coli *O157:H7 in irrigation treatments containing varying amounts of total organic carbon (TOC): treatment A (0 ppm), treatment B (12 ppm), and treatment C (120 ppm).* ◆, *0 ppm TOC;* ■, *12 ppm TOC;* ▲, *120 ppm TOC.*

DISCUSSION

Irrigation water quality has recently become a contentious issue, as current irrigation water microbiological standards for leafy greens (LGMA) are based on the recreational water standards determined by the U.S. Environmental Protection Agency. These standards do not take into account the fate of human pathogens on foliar surfaces of leafy greens, where they appear to decline rapidly in population size. For example, the microbiological standards for irrigation water intended for leafy greens in the LGMA do not take into account the chemistry regarding irrigation water quality, prevalence, or persistence of foodborne pathogens on foliar surfaces as applied via overhead irrigation. This study replicated on-farm irrigation water by adding organic carbon content, using a dairy manure source, to prepare water representative of various irrigation water sources found in the United States.

Our recovery methods detected low populations of the surface-applied *E. coli* O157:H7. Our methodologies might have underestimated these populations, as some cells may have been internalized through the stomata, the recovery of which has not been evaluated with our methodology. *Salmonella* has recently been shown to exhibit a chemotactic motility response toward the photosynthetic nutrients produced by lettuce plants, resulting in colonization surrounding, and subsequent internalization through, stomata *(13),* but it is unclear whether *E. coli* O157:H7 cells possess this chemotactic ability.

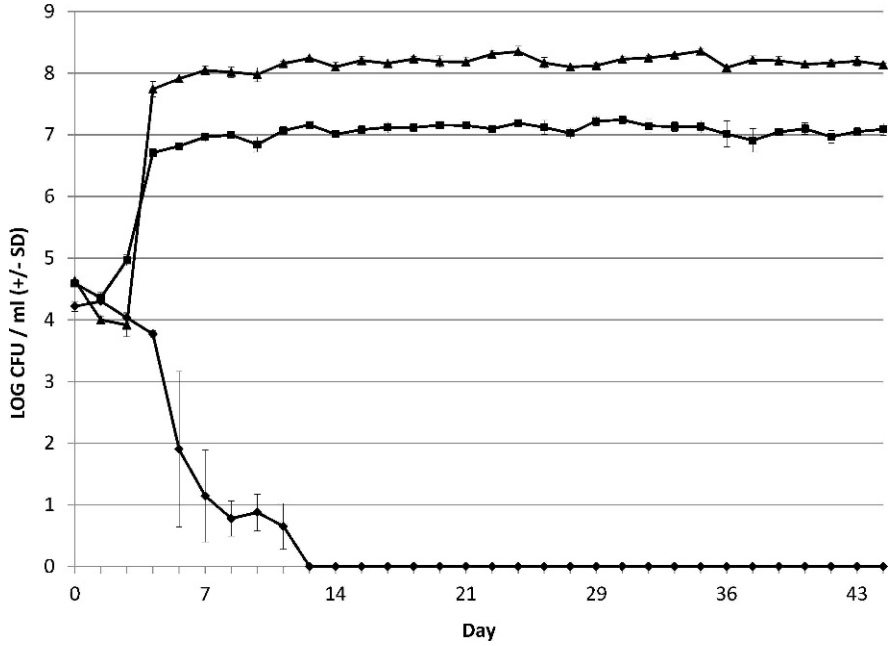

FIGURE 3. *Growth of high initial populations (4 log CFU/ml) of* Escherichia coli *O157:H7 in irrigation treatments containing varying amounts of total organic carbon (TOC): treatment A (0 ppm), treatment B (12 ppm), and treatment C (120 ppm).* ◆, *0 ppm TOC;* ■, *12 ppm TOC;* ▲, *120 ppm TOC.*

As the spinach plants grew, more tissue was present during each subsequent irrigation event and, therefore, older plants should have collected greater *E. coli* O157:H7 populations than the younger plants. For example, older plants containing four or more true leaves had more surface area, which would be more likely to collect larger *E. coli* O157:H7 populations as deposited from the irrigation water than would younger plants containing fewer leaves. However, our results did not support this hypothesis. When young plants (13 days old) were inoculated with high populations of *E. coli* O157:H7, these populations persisted at low levels for 16 days postinoculation. In addition, we observed a temporary (but substantial) population increase in treatments A and C when the plants were 20 days old after irrigation event 3, which did not contain additional *E. coli* O157:H7. In contrast to the inoculated 13-day-old plants, *E. coli* O157:H7 populations that were inoculated onto 34- and 41-day-old plants did not survive for more than 2 and 4 days, respectively. These findings suggest that the spinach plant tissues could support *E. coli* O157:H7 survival at various capacities, depending on the developmental stage (age) of the plant. Pu et al. *(24)* reported significant plant-age effects on the ability of populations of *E. coli* O157:H7 in soil to transfer to the surfaces of baby spinach. They concluded that the epiphytic surfaces of baby spinach tissues were most susceptible to contamination with *E. coli* O157:H7 between 3 and 5 weeks in development. Our findings are in agreement with those of others *(3),* who suggest that baby spinach tissue is more likely to support *E. coli* O157:H7 growth and survival at specific ages.

A previous study determined that *E. coli* O157:H7 persistence on lettuce was not statistically different when using water or cow manure extract as an inoculum carrier *(34).* This study, however, did not adapt *E. coli* O157:H7 strains to growth in the carrier (sterile cow manure), as performed in our study. We also confirmed that *E. coli* O157:H7 cells were able to grow and sustain high population densities in irrigation solutions containing TOC (treatments B and C), under the same environmental conditions in which spinach plants were grown. However, no differences in the survival of *E. coli* O157:H7 on spinach leaves were observed, based on the TOC content in the irrigation solutions. These results indicate that TOC concentrations in the irrigation water at or below 150 ppm, even after repeated irrigation events on the same plants, do not appear to have any effect on the persistence of *E. coli* O157:H7. Repeated irrigation events containing high or low levels of TOC did not confer any enhanced survival to *E. coli* O157:H7 on foliar surfaces. The rapid die off of *E. coli* O157:H7 observed on all surfaces of the spinach leaves indicates that the TOC treatments did not aid in the formation of *E. coli* O157:H7 aggregates on baby spinach tissues, which have been shown to aid microbial survival on foliar surfaces *(17, 32).* In addition, the TOC treatments did not enhance the survival of *E. coli* O157:H7 that were reintroduced through repeated irrigation at later stages of spinach development. The repeated, low-level introduction of *E. coli* O157:H7 populations onto spinach plants did not appear to help the pathogen in overcoming the obstacles necessary for survival, including limited nutrient sources, desiccation, and microbial competition found on foliar surfaces *(2).*

Our overhead-irrigation strategy involved the airbrush application of the treatments to all aerial tissues until saturation was achieved and terminating application just before runoff. However, it is possible that if more irrigation volume were introduced to the foliar surfaces (e.g., with the incorporation of chemical ''spreader-stickers,'' commonly used in commercial horticulture to enhance the ability of liquid to cover and stick to leaf surfaces), the survival of *E. coli* O157:H7 might have been enhanced. In cases where high populations of *E. coli* O157:H7 were applied, and two plant harvests occurred on the same day within the same treatment, the recovered *E. coli* O157:H7 populations were higher in plants harvested immediately after the uninoculated irrigation treatment was applied (Table 2). For example, two plant harvests occurred on the seventh day of analysis where one harvest occurred prior to, and one occurred after, irrigation event 3. Greater populations of *E. coli* O157:H7 were recovered from all treatments immediately after the irrigation event. This effect was most likely the result of the residual *E. coli* O157:H7 population's ability to utilize the brief application (and availability) of moisture. This additional moisture also could have facilitated detection by enhancing the separation of *E. coli* O157:H7 cells from the surface matrix of spinach leaves. Because this effect was seen among all irrigation treatments (including A, 0 ppm of TOC), the surface-applied soluble carbon did not appear to provide any protective or selective advantage to the residual *E. coli* O157:H7 populations in the spinach phyllosphere. In fact, even after the application of 10 irrigation events containing high concentrations of TOC (treatment C, 150 ppm of TOC), the harvested spinach tissues did not exhibit any accumulated carbon or nitrogen effects (data not shown). It is possible that the small volumes of the irrigation solutions introduced onto foliar surfaces were insufficient to influence the overall concentrations of the available carbon sources on foliar surfaces.

The literature is replete with a wide range of reports describing various survival and persistence data of *E. coli* O157:H7 on leafy green commodities. These varying results could be due to the deposition of cells in some places on the leaf surfaces that are more favorable for bacterial survival than are others, i.e. stomata, grooves along the leaf veins, and the base of the trichomes and cell wall junctions *(1, 16).* Textured microenvironments of certain spinach cultivars (e.g., Savoy) have been determined to be more suitable for *E. coli* O157:H7 survival than spinach varieties with smooth surfaces *(15).* Solomon et al. *(29)* determined low levels of *E. coli* O157:H7 persistence on lettuce for 30 days after irrigation with 6 log CFU/100 ml, whereas Erickson et al. *(9)* did not recover residual surface or internalized *E. coli* O157:H7 cells in spinach tissue 6 days after irrigation with 6 log CFU/100 ml. Mootian et al. *(18)* found persistence of *E. coli* O157:H7 on lettuce leaves for at least 30 days postexposure with water containing 3 log CFU/100 ml.

This study was designed to determine the efficacy of the current LGMA irrigation water microbiological stan-

dards. We determined that baby spinach plants do not support the survival of *E. coli* O157:H7 for more than 1 day after overhead irrigation with water that complies with the LGMA standards for *E. coli* content. It should be noted that our results were obtained in a controlled growth chamber environment by using *E. coli* populations that were <126 CFU/100 ml. Further study involving field experiments are encouraged to corroborate these findings. Our results also suggest that the repeated introduction of *E. coli* O157:H7 at low populations in irrigation water does not enhance the survival or persistence of the pathogen on foliar surfaces. Furthermore, the TOC content of irrigation water ≤150 ppm, even with repeated irrigation, does not appear to affect the persistence of *E. coli* O157:H7 on spinach tissues. Further work establishing microbiological standards for irrigation water should consider the fate of specific microbial populations on foliar surfaces.

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