Research Abstract for the
California Lettuce Research Board Research Program
April 2007 to March 2008

Project Title: Survival of generic *E. coli* under different irrigation systems

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Summary

Outbreaks of foodborne pathogens on leafy vegetables have highlighted the need for practical information on the dynamics of such organisms in the field. Applied field-oriented research is needed so that industry and regulators can make informed decisions on growing practices and food safety policies. This project has initiated efforts to develop information on how nonpathogenic (generic) *E. coli* strains persist under Salinas Valley farming conditions; these nonpathogenic strains have been the subject of earlier CLRB funded research (Suslow lab) and are suggested as model substitutes (surrogates), under controlled greenhouse and field studies, for human pathogenic *E. coli* such as the O157:H7 strain. Our objectives were to (1) Examine soil survival of generic *E. coli* under model commercial field situations, and (2) Compare impacts of different irrigation systems and fertilizer levels on generic *E. coli* survival in an open field environment. All experiments used generic *E. coli* strains that were selected for spontaneous, natural resistance to the antibiotic rifampicin (*E. coli*^{rif}). We used rifampicin-amended medium to selectively recover and obtain presumptive identification of the applied bacteria.

In a small plot field experiment, generic *E. coli* strains were sprayed onto beds, irrigated with overhead sprinklers, and the soil periodically tested to examine persistence of the applied strains. With catch-buckets, the irrigation rates in the plots were recorded. All of our *E. coli*^{rif} strains, inoculated to soil at high rates (10^6 or 10^8 CFU/ml), were recoverable from soil for only a short period. By 8 days after inoculation, recovery was at or near the practical detection limit for most plots. By 14 days, *E. coli*^{rif} was no longer detected from soil. Catch-bucket data demonstrated that the downwind side of the plots received significantly more water, and had significantly higher *E. coli* recovery rates, than the upwind plots that had less water and lower *E. coli* recovery rates. In addition, we did not detect *E. coli* in uninoculated plots, indicating that inter-plot contamination did not occur at a detectable level.

For the large plot field experiment, fertilizer (grower standard and supplemented rates) and romaine seed were first placed into the replicated plots. A three isolate mixture of generic *E. coli*^{rif} , originally obtained from central coast environments, was then sprayed onto beds at a high rate (10^7 CFU/ml). Half of the plots were irrigated with overhead sprinklers and half with surface drip tape. Lettuce was germinated and grown to harvestable size. Soil, irrigation runoff (sprinkler plots only), and romaine plants were periodically tested for the bacterium. *E. coli*^{rif} was recovered from soil for only a short period (up to 3 days post inoculation). By 6 days after inoculation, *E. coli*^{rif} was not detected from soil using standard techniques. Bacteria did not live long enough to determine if irrigation (sprinkler vs. drip) or fertilizer (standard vs. supplemental) treatments influenced survival. We did not recover *E. coli*^{rif} from lettuce plants, growing in inoculated beds, at any stage of growth. An enrichment detection method used on lettuce seedlings also did not recover applied strains. *E. coli*^{rif} was not recovered from soil or romaine in uninoculated control beds, indicating that inter-plot contamination did not occur at a detectable level. For runoff water from the sprinkler plots, we recovered *E. coli*^{rif} for up to 12 days post-inoculation. However, late in the crop cycle, detection assays gave presumptive positives for some water runoff and lettuce leaf samples. It was later determined, however, that these bacteria were not *E. coli* but species of *Enterobacter* that grew on selective, differential media.

Conclusions: Our simulation of a one-time, high level contamination event resulted in very short persistence of *E. coli*^{rif}. Water and environmental conditions likely influence bacterial survival, though we could not document significant differences between drip and sprinkler plots. The field environment allowed for the occurrence and survival of abundant background coliform bacteria, arriving from environmental sources, that resulted in positive reactions (fluorescence, blue colonies) in tests used to detect *E. coli*.

Project Report to the
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Introduction

Recent outbreaks of foodborne pathogens on leafy vegetables have highlighted the need for practical information on the dynamics of such organisms in the field. For example, studies are lacking that directly answer how *E. coli* O157:H7 enters agricultural systems, how it spreads in fields, its ability to survive and persist under field conditions, and other related aspects. Applied field-oriented research is needed so that industry and regulators can make informed decisions on growing practices and food safety policies. This project has initiated efforts to develop information on how nonpathogenic (generic) *E. coli* persists under Salinas Valley farming conditions. Environmental data developed with generic *E. coli* can be the basis for gaining grower, handler, agency, and public acceptance of future controlled field trials with reduced-risk strains of *E. coli* O157:H7.

Objectives

(1) Examine soil survival of generic *E. coli*^{rif} under model commercial field situations.
(2) Compare impacts of different irrigation systems and nutrient levels on generic *E. coli*^{rif} survival in an open field environment.

Procedures and Results

(1) Examine soil survival of generic *E. coli*^{rif} under model commercial field situations.

Procedures

A replicated, small plot (plot dimensions: one 40-inch bed x 20 ft) field trial was established in the Salinas Valley. To simulate a one-time contaminated irrigation water event, three different strains of generic (nonpathogenic) *E. coli* were applied on May 21, 2007 in different concentrations and combinations after plot beds were formed. The equipment used to apply the inoculum was a CO_2 powered, handheld, backpack sprayer, two nozzle spray boom, and Teejet 8005 spray tips (selected to reduce spray drift); the sprayer was set at approx. 25-27 psi. Directed spray applications were made so that only the bedtops (approximately a 22 inch band) received

the inoculum. All strains were selected from central coast sources (irrigation water, lettuce, and soil from a lettuce crop) for spontaneous, natural resistance to the antibiotic rifampicin (*E. coli*^{rif}). Tryptic soy agar (TSA) amended with 80 μg/liter rifampicin and 70 μg/liter MUG (substrate for bacterial fluorescence) was used to facilitate bacteria recovery and identification. Soils at the small plot site contained *Mucor* and *Rhizopus* species that were able to grow on TSA plates incubated at 37 and 44 °C, thereby interfering with the counting of bacterial colonies. To reduce overgrowth by these soil fungi, TSA was therefore amended with 1.0% pentachloronitrobenzene (PCNB) fungicide. Each bacterial strain was shown to lack key genetic determinates of virulence associated with *E. coli* O157:H7. Each strain also has a distinct DNA-fingerprint, facilitating subspecies differentiation following recovery from field trial samples. Earlier research (W. Miller, L. Quon, and T. Suslow, manuscript in progress) demonstrated a high level of *E. coli* diversity in central coast lettuce environments, making it unlikely to encounter any one individual type in multiple locations. Control plots received no bacterial inoculum. The treatments were the following:

Treatment	Source of strain	Concentration(s)*
E. coli^{rif} W353	water	10^6 and 10^8 CFU/ml
E. coli^{rif} P354	lettuce	10^6 and 10^8 CFU/ml
E. coli^{rif} S355	soil	10^6 and 10^8 CFU/ml
W353/P354/S355	“cocktail” mixture	10^6 CFU/ml
non-inoculated control	---	---

* CFU/ml = colony forming units per milliliter

After inoculation, plots were watered with overhead sprinklers to simulate the germination irrigation for direct seeded lettuce. Irrigations were subsequently applied at 2, 4, 6, and 8 days post-planting. Irrigations were made with a single sprinkler line located at the center of the trial so that less water was applied to plots located further from the water source. With catch-buckets, the water volumes were recorded throughout the plots for each irrigation. Soil was collected from each plot immediately after inoculation and at 3, 7, and 15 days post-inoculation. Soil was collected using sanitized collection scoops; 8 soil subsamples were taken from the top 0.5 to 1.0 inch of undisturbed bedtops, bulked into sterile bags, stored on ice, and taken to the lab for analysis.

Bulked soil samples were thoroughly mixed, and a subsample of 100 grams was weighed out into Whirl-Pak plastic bags. 200 ml of 0.01 M sodium phosphate buffer plus 0.05% Tween 20 were added to the soil and the mixture was thoroughly agitated. The mixture was allowed to settle for 1 hour at room temperature. 25 ml of the supernatant were removed and placed in sterile Falcon tubes (50 ml). This liquid was vortexed for 30 seconds, then a 200 μl aliquot was plated and spread onto each of two TSA + rif + MUG + PCNB plates. Plates were incubated at room temperature for 2 hours, then placed in an incubator for 48 hours at 42 °C. After 48 hours, the number of colonies was determined for all plates. 21 random colonies were selected and sent to the Suslow lab for pulsed-field gel electrophoresis (PFGE) analysis to confirm whether the recovered bacteria were the specific *E. coli*^{rif} strains that were introduced to the soil plots.

Results

The water, plant, and soil strains of *E. coli*^{rif}, inoculated to soil at two high rates (10^6 or 10^8 CFU/ml), were recoverable from soil for only a short period (Fig. 1). By 8 days after inoculation, recovery was at or near the detection limit for most plots. By 14 days, *E. coli*^{rif} was no longer detected from soil. The "cocktail" mixture of the three strains experienced a similar decline over time. Survival for the 0 and 4 day samples was highest for the water and cocktail strains of *E. coli*^{rif} and for the log 8 concentrations. The log 6 concentration of the plant strain was not detected after the 0 day sample and the log 6 concentration of the water strain was not found after the 4 day sample. We did not detect *E. coli*^{rif} in uninoculated plots, indicating that inter-plot contamination did not occur at a detectable level. This finding has significance for future studies in that we demonstrated that applications of bacteria could be managed and controlled. The 21 randomly selected strains were analyzed using PFGE, and these strains were confirmed to be the introduced strains used in the experiment. From the relatively small sample of colonies, the frequency of recovery was approximately 9%, 43%, and 48% for water, plant and soil isolates, respectively.

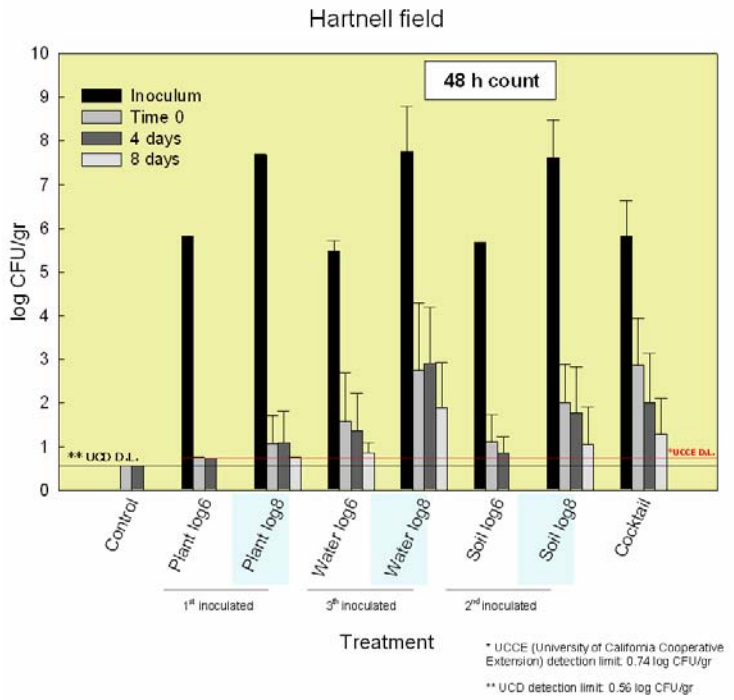

Figure 1. Recovery of generic *E. coli*^{rif} strains that were inoculated to soil in a small plot field study. Inocula were sprayed in the following order: plant strain, soil strain, water strain. D. L. = detection limit.

Catch-bucket data demonstrated that the downwind, south side of the plots (block B) received significantly more water (Fig. 2), and had significantly higher *E. coli*^{rif} recovery rates (Fig. 3). The upwind, north plots (block A) had less water (Fig. 2) and lower *E. coli*^{rif} recovery rates (Fig.

3). Catch-bucket numbers also indicated that the primary difference in total irrigation volumes was due to the very first watering. Because of prevailing breezes on the inoculation day, the south plots received more water than the north plots. This initial watering appears to have significantly influenced the survival results because for irrigations 2 through 5 the south and north sides received comparable water volumes (Fig. 2).

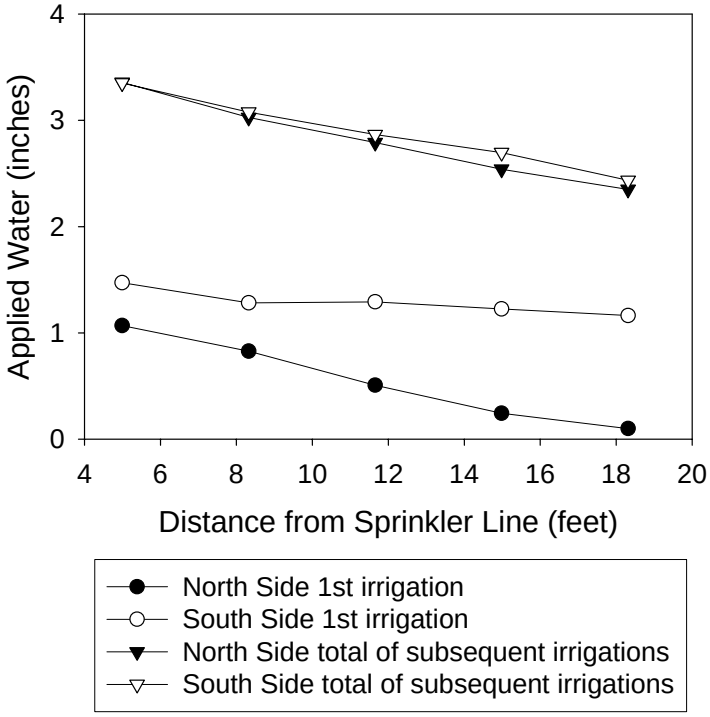

Figure 2. Applied water (inches) for north and south sides of the inoculated small plot experiment.

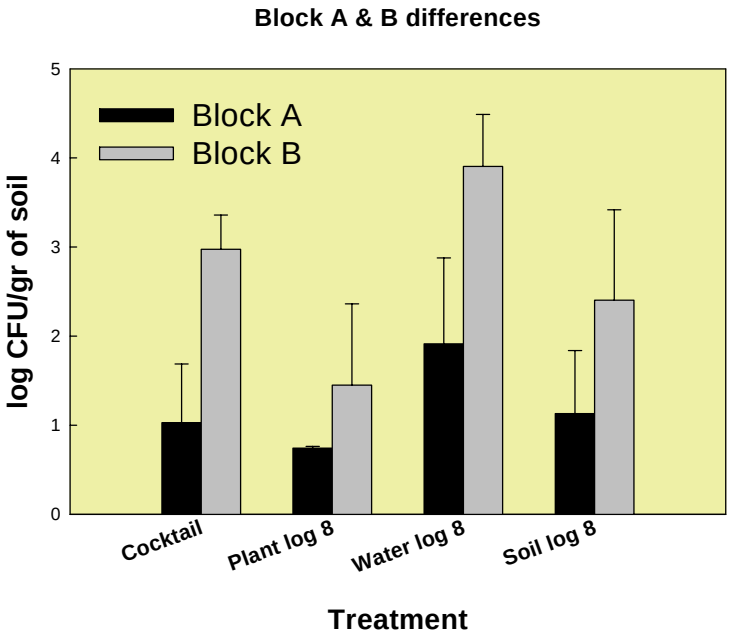

Figure 3. Soil survival of *E. coli*^{rif} in the wetter south (block B) vs. drier north (block A) sides of the small plot study.

(2) Compare impacts of irrigation systems and nutrient levels on generic *E. coli*^{rif} survival in an open field environment.

Procedures

A replicated (3 blocks), large plot, open field trial (SVR 51) was established in the Salinas Valley and used a randomized complete block design. Irrigation treatments (sprinkler or drip) were applied to the main plots of nine beds (40-inch) wide x 145 feet long, and fertilizer treatments (grower standard or supplemental N and P) were incorporated prior to bed shaping into sub-plots of three beds (40-inch) wide x 145 feet long. Beds were seeded with romaine lettuce. Inocula from three strains (water, plant, and soil) of generic *E. coli*^{rif} were combined and then sprayed onto beds at a high rate (10^7 CFU/ml) on July 20, 2007. This start date was selected so that the romaine would reach harvestable stage by mid-September. The strains were applied using the same equipment and methods as used for the small plot study. Directed spray applications were made so that only the bedtops (approximately a 22 inch band) received the inoculum. Control plots received no bacterial inoculum. The treatments were the following:

E. coli^{rif}	Irrigation method	Fertilizer level*
inoculated	sprinkler	standard rate
inoculated	drip	standard rate
inoculated	sprinkler	supplemented rate
inoculated	drip	supplemented rate
non-inoculated	sprinkler	standard rate

non-inoculated	drip	standard rate
non-inoculated	sprinkler	supplemented rate
non-inoculated	drip	supplemented rate

* Standard fertilizer rate = 300 lb per acre of 7-7-7; supplemented fertilizer rate = 300 lb per acre of 7-7-7 plus 350 lb N and 250 lb P per acre.

After inoculation, all plots were watered once with overhead sprinklers to initiate the germination of the lettuce. Subsequently, plots were irrigated with either surface drip or overhead sprinklers. To germinate the lettuce, irrigations were applied 2, 4, 7, 11, and 14 days after the first watering. Soil moisture tension was monitored in the irrigation plots at 6-inch and 12-inch depths using watermark blocks from the first irrigation until harvest except for a 7-day period when the crop was thinned and cultivated. In one of the sprinkler irrigated plots, relative humidity and air temperature were monitored hourly at a height of 8 inches above the bed top using an HMP35C relative humidity/air temperature sensor and a Campbell Scientific CR1000 datalogger. The lettuce was irrigated, grown, thinned, and managed according to standard commercial practices. The experiment was terminated when the lettuce reached harvest size and a final plant sample taken at that time. Total water applied to the entire crop was 19 inches for the sprinkler areas and 11 inches for the drip areas (Fig 4).

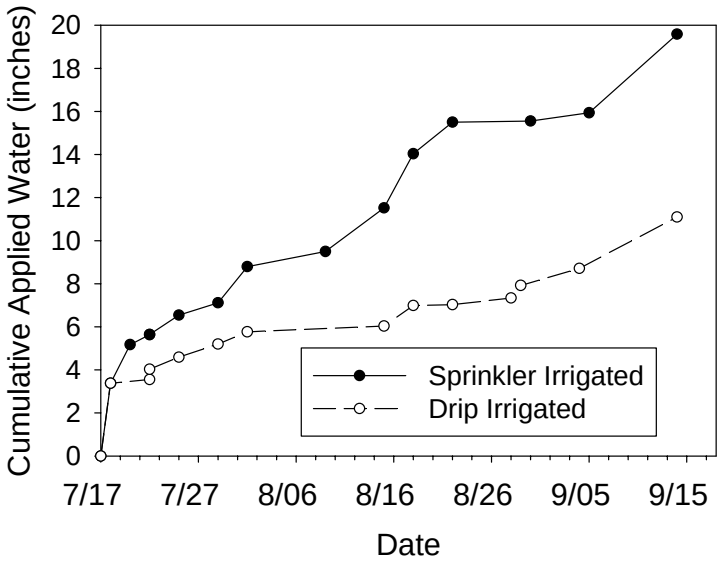

Figure 4. Cumulative applied water in drip and sprinkler irrigated plots at the large plot open field (SVR 51) trial.

All soil and plant samples were collected from undisturbed areas on the center bed of the three-bed plots. Soil was collected from each plot immediately after inoculation and at 3, 6, and 13 days post-inoculation. Soil was collected, stored, and tested for *E. coli*^{rif} according to the same procedures as described for the small plot study.

At 13 days post-planting, a bulked romaine seedling sample was collected from each plot and tested for *E. coli*^{rif}. For each plot, 50 random plants were unearthed and the roots and adhering soil placed in one collection bag. The foliage (crowns and leaves) was placed in a second bag. Roots and rhizosphere soil were tested by adding 100 ml 0.01 M sodium phosphate buffer + 0.05% Tween 20, smashing the plant material to expose vascular tissue (xylem), shaking and agitating the mixture vigorously for 1 minute, allowing the mixture to settle for 10 minutes, then plating 250 µl aliquots onto each of 2 TSA + rif + MUG plates. Plates were incubated and evaluated according to the same procedures as described for the small plot study. Lettuce foliage was tested in a similar manner except that 100 ml of buffered peptone water (Difco Laboratories) was added to this material. To further test lettuce seedlings for internalized *E. coli*^{rif}, an additional set of individual subsamples (5 plants per sample per plot) was collected and bagged in groups of five in separate bags. Each set was tested for *E. coli*^{rif} in a similar way.

As the romaine crop developed, later plant collections consisted only of the above ground portion of the lettuce. Ten plants per plot were cut at the soil level (leaving behind the oldest, lower 4 to 5 wrapper leaves), placed in one large plastic bag, stored on ice, and transported to the lab. Plants were analyzed by chopping all leaves and crowns into approximately 1 inch square pieces, mixing all chopped pieces in a tray, then removing 3 replications of 25 grams each and placing them in Whirl-Pak bags. 150 ml of buffered peptone water were added to the bag, bag contents were thoroughly mixed and agitated for 1 minute, and 50 ml of this mixture were filtered. A 250 µl aliquot was removed and plated onto each of two TSA + rif + MUG plates. Plates were incubated and evaluated as previously described.

For sprinkler plots only, irrigation surface runoff water was collected after each irrigation and tested for *E. coli*^{rif}. Using the QuantiTray 2000 Colilert system (Idexx Corporation), 100 ml of collected water were mixed with Colilert reagents, dispensed into the assay trays, sealed, and incubated at 37 °C according to manufacturer recommendations. A set of 10^{-1} dilutions was also prepared and tested with the Colilert system.

Results

E. coli^{rif} was recovered from soil for only a short period of up to 3 days post inoculation (Fig. 5). By 6 days after inoculation, *E. coli*^{rif} was no longer detected from soil. There were no significant differences in populations in the different irrigation (sprinkler vs. drip) or fertilizer (standard vs. supplemental) treatments (Fig. 5); however, bacteria did not live long enough to determine if such factors could influence survival. *E. coli*^{rif} was not recovered from soil in uninoculated control beds, indicating that inter-plot contamination did not occur at a detectable level.

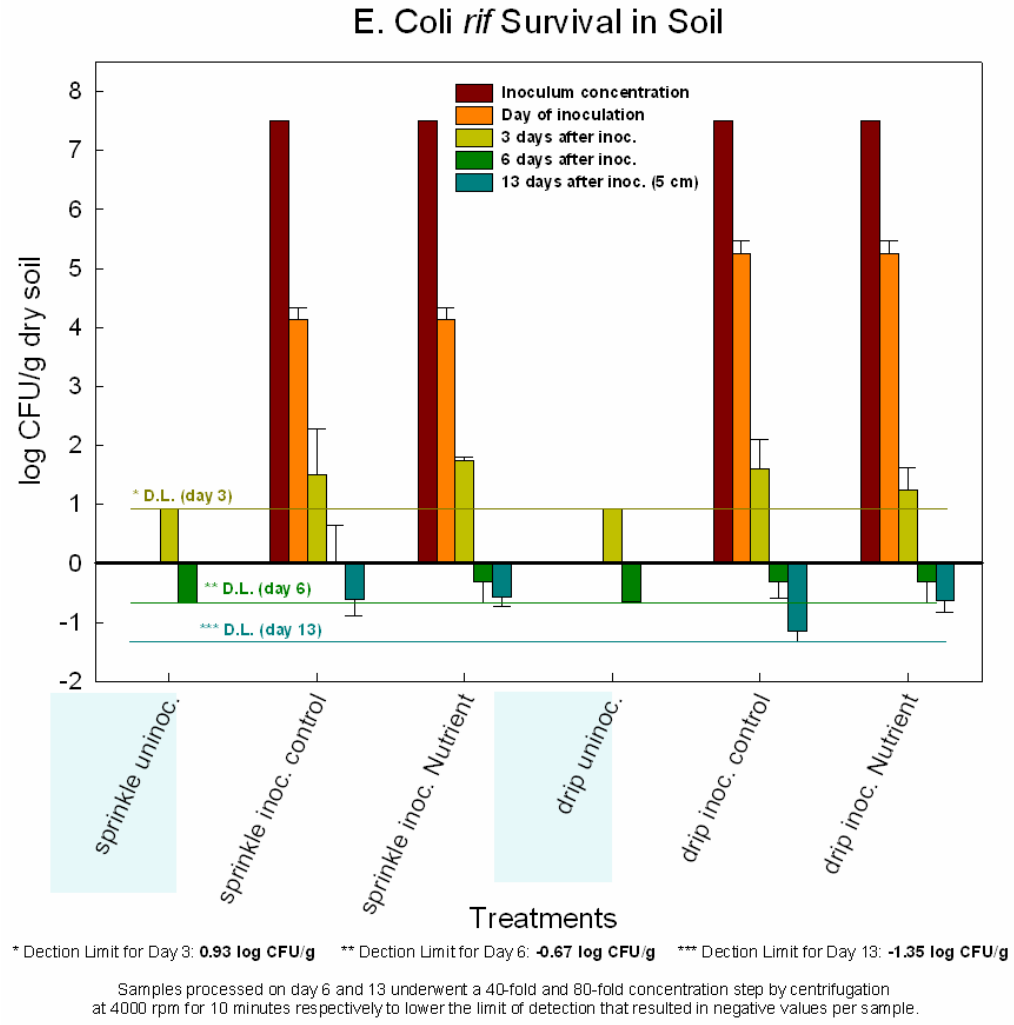

Figure 5. Recovery of generic *E. coli*^{rif} strains that were inoculated to soil in a large plot (SVR 51) field study. D. L. = detection limit.

Soil moisture tension ranged between 0 and 15 kPa at the 6 inch depth during the first 2 weeks of the crop, indicating that the soil was moist up to the date that *E. coli*^{rif} was no longer detected (Fig. 6). Soil moisture levels were highest in the sprinkler-irrigated plots (lowest soil moisture tensions) (Fig. 6). During the first 3 days after inoculation, relative humidity of the air above the beds was below 50% during the day and air temperature was above 25 C (Fig. 7).

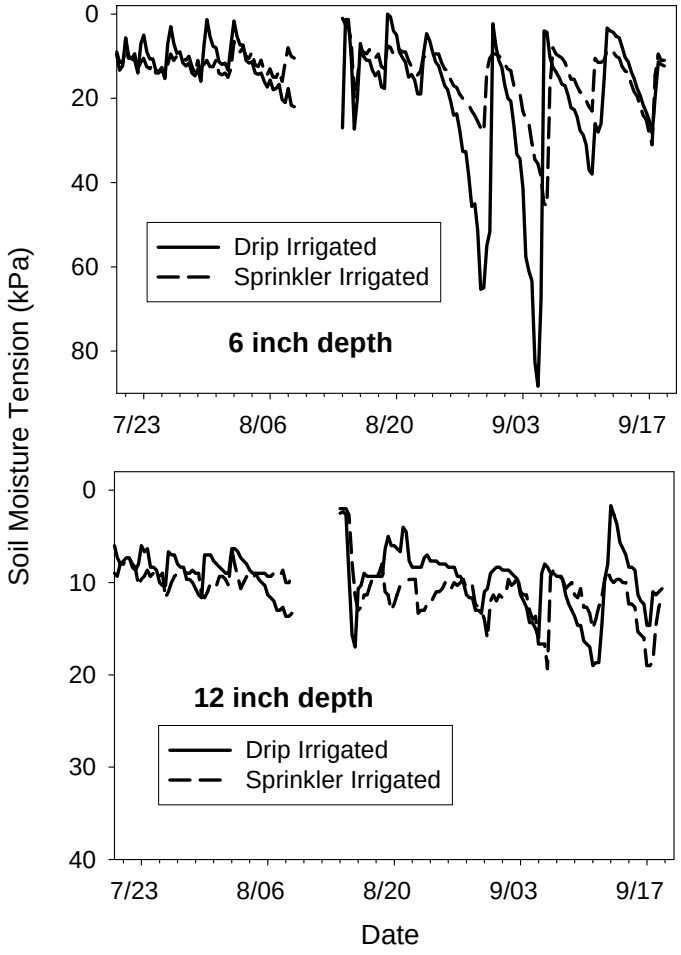

Figure 6. Soil moisture tension at the 6 inch and 12 inch depths in the drip and sprinkler areas of the large plot (SVR 51) field trial. Data are missing from a seven day period when the crop was thinned and cultivated.

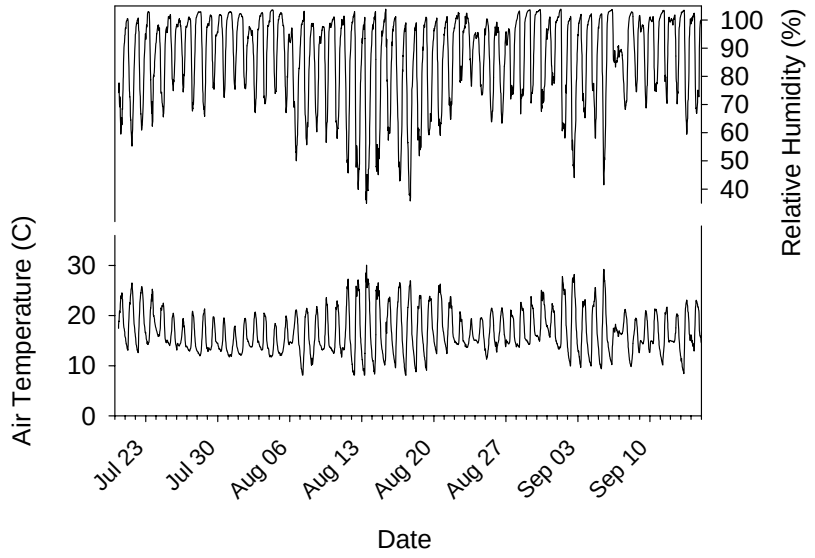

Figure 7. Hourly relative humidity and air temperature at the large plot (SVR 51) field trial.

We did not recover *E. coli*^{rif} from lettuce plants, growing in inoculated beds, at any stage of growth. Bulked and 5-plant lettuce seedling samples failed to test positive for the applied isolates and therefore gave no evidence for internalization of *E. coli*^{rif} (detection limit = 0.26 log CFU/root or stem). An enrichment detection method used on lettuce seedlings also resulted in no recovery (detection limit = -0.70 log CFU/stem). Plants sampled at rosette stage and just prior to harvest were also negative for the inoculation isolates. *E. coli*^{rif} was not recovered from romaine in uninoculated control beds.

For runoff water from the sprinkler plots, we recovered *E. coli*^{rif} strains for a short period after introduction to the soil. At 12 days post-inoculation, *E. coli*^{rif} was recovered from sprinkler runoff from inoculated/standard fertilizer and inoculated/high nutrient beds. By the 23rd day post-inoculation sample, *E. coli*^{rif} was no longer detected (Fig. 8). Late in the crop cycle, however, water runoff assays resulted in presumptive positives for some water runoff (fluorescence in QuantiTray tests) and lettuce leaf (growth and fluorescence on TSA + rif + mug) samples; water runoff from untreated control plots also showed presumptive positive readings (Fig. 8). The Suslow lab later determined that bacteria obtained from these presumptive positive plates were not *E. coli* but species of *Enterobacter* that grew on selective, differential media, indicating these tests were false positives for *E. coli*.

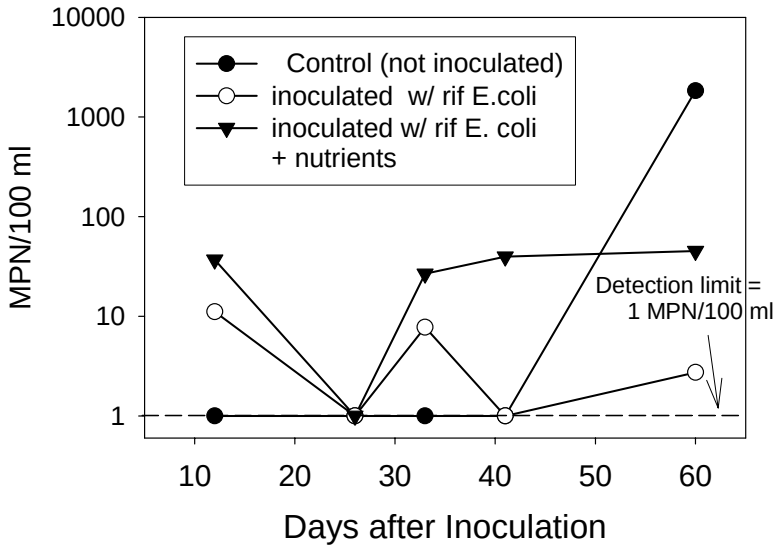

Figure 8. Presumptive *E. coli*[rif] recovered from surface water runoff from sprinkler irrigated plots in the large (SVR 51) field trial.

Summary

Our simulation of a one-time, high level contamination event resulted in very short persistence of *E. coli*[rif]. Increased *E. coli*[rif] survival was associated with higher rates of sprinkler applied water. Though water and environmental conditions likely influence bacterial survival, we could not document significant differences between drip and sprinkler plots due to the very short survival time. *E. coli*[rif] also persisted for a short period in surface water runoff. Romaine grown in the experimental plots did not test positive for *E. coli*[rif] at anytime. The field environment allowed for the occurrence and survival of abundant background coliform bacteria, evidently arriving from environmental sources, that resulted in positive reactions (fluorescence, blue colonies) in tests used to detect *E. coli*.

Acknowledgments

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