I. Abstract.

Project Title: Estimation of the area affected by animal feces in vegetable field under overhead sprinkle irrigation system

Project Investigator(s):
Jorge M. Fonseca[1], Sadhana Ravishankar[2], Charles Sanchez[1].
[1]The University of Arizona, Yuma Agricultural Center, Yuma, Arizona.
[2]The University of Arizona, Department of Veterinary Sciences and Microbiology, Tucson, Arizona.

Summary:
A series of experiments were conducted in Yuma, Arizona during the harvest season 2007-2008 to investigate the level of threat posed by possible animal fecal contamination in vegetable fields when exposed to overhead irrigation. Recent food safety guidelines suggested by the Leafy Greens California Marketing Agreement recommend avoiding harvest of crop within a minimum 60-inch radius buffer distance from the spot of contamination. However, little information to conclude the accuracy of this distance in preventing potential fecal contamination of lettuce was available at the time the guidelines were established. In this work we aimed to determine the area affected by feces in a sprinkled-irrigated field and identify factors that affect the area impacted by contamination from feces in the field. Cow feces, which had previously been inoculated with high levels of a non-pathogenic strain of *E. coli*, were placed in romaine lettuce fields, prior to irrigation using overhead sprinklers. Water and lettuce samples were collected from around the feces location and tested for the presence of *E. coli*. Our results showed that currently guidelines underestimate the potential area that can be affected by feces. In this study, *E. coli* from feces was recovered as far as 151 inches. When sprinklers ran with wind speeds of <12 mph the maximum distance travelled by recovered *E. coli* ranged from 24 to 96 inches We found this distance between feces and the farthest location of recovered *E. coli* was influenced by sprinkler run time and maximum wind speed, thus, our data may be conservative as sprinklers in commercial fields run for longer time than the maximum run time in our trials (120 minutes). Wind direction was also found to be a key factor influencing the area of contamination. Moreover, it was found that the survival of *E. coli* O157:H7 and *Salmonella* Newport depend both on the environmental conditions (relative humidity and temperature) and on the type of animal excrement used as substrate. Survival of pathogens in dog feces was less than a week whereas in cow feces the pathogens were still recovered after 225 days. This study has direct implications on current food safety regulations.

II. Main Body of Report.

Project Title: Estimation of the area affected by animal feces in vegetable field under overhead sprinkle irrigation system

Project Investigator(s):
Jorge M. Fonseca. The University of Arizona, Yuma Agricultural Center. Phone: 928-782-3836. Email: jfonseca@ag.arizona.edu
Sadhana Ravishankar. The University of Arizona. Department of Veterinary Sciences and Microbiology. Phone: 520-626-1499. Email: sadhravi@email.arizona.edu
Charles Sanchez. The University of Arizona, Yuma Agricultural Center. Phone: 928-782-3836. Email: sanchez@ag.arizona.edu.

Cooperating Personnel: Shelagh Fallon, Libin Zhu, Ramiro Galvez, Cody Hurlock, Diana Rios. The University of Arizona

Objectives: The overall goal of this project was to estimate the risk level posed by animal feces in vegetable fields irrigated using overhead sprinklers. Specific objectives include: 1) to determine the maximum distance traveled by *E. coli* when irrigation water splashes animal feces previously dropped onto the soil and plants; 2) to elucidate the survival of *Escherichia coli* O157:H7 and *Salmonella* sp. in animal feces exposed to different environmental conditions.

Procedures:

Field Work
Romaine Lettuce cv. Fresh Heart (Dic – March, 2008) and cv. Sun Belt (January- April, 2008) were grown in 2-acre fields at the Yuma Agricultural Center following common commercial agricultural practices. Irrigation was provided through sprinklers. Sprinklers pipes were match with rain bird 14VH-5/64" nozzles. Distance between nozzles was 30 ft, and distance between parallel pipes was 38.5 ft. Animal feces (cow or dog) were placed in the field (middle of four nozzles) during various phases of the plant's growth cycle. The first attempts to estimate the approximate area travelled by feces particles was conducted using chemical markers (red dye, starch). Following these preliminary trials animal feces were inoculated with a specific non-pathogenic *E. coli.*

Bacterial Strains and Growth Conditions
A streptomycin resistant *E. coli* K-12 strain (ATCC 25253), was used throughout the field work. This strain was stored at –80^{o}C in tryptic soy broth (TSB) containing 25 % glycerol. For culture preparation the stock was streaked for individual colonies on tryptic soy agar and grown at 37^{o}C overnight. A single colony was used to inoculate TSB and grown overnight at 30^{o}C with agitation at 140 rpm. This was used to inoculate TSB and grown at 37^{o}C with agitation at 140 rpm until there were approximately 10^{8} - 10^{9} cells/ml to give a final concentration of 10^{7} – 10^{8}

cells/g in the feces. The culture was immediately mixed with feces and used in the irrigation study.

Inoculation of feces and field irrigation

Approximately 1300 g or 500 g of feces (cow or dog respectively) was mixed with sufficient *E. coli* culture (500 ml for cow feces, 50 ml for dog feces) in a Ziploc bag. The feces was then transported to the field and placed on the top of the field bed, and the surrounding 4 lettuce (romaine) were removed where appropriate. Catch cans were places 1-1.5 ft apart in the furrows prior to irrigation. The field was then irrigated by overhead sprinklers for 75-120 min. Lettuce samples were taken along with the catch cans and analyzed for the presence of *E. coli*. Dog feces were only analyzed once as it was determined that the pattern of spread from the feces was the same as the cow feces. Scheme 1 illustrates the distribution of each catch cans and plants in the field and subsequent results.

Determination of *E. coli* presence

The feces were tested for the *E. coli* population by serial dilution prior to the addition of the cultured *E. coli* and also after to determine the approximate level/g in the final feces sample. The water samples from the catch cans in the different field locations were plated (1 ml) onto Petrifilm EC plates (3M). When appropriate lettuce samples were also taken and tested for the presence of *E. coli*. The outside leaves were removed from each lettuce sample and the used to determine the *E. coli* population by blending with 0.1 % peptone water for 1 min in a blender at 230 rpm. A 1 ml sample from this was than plated onto Petrifilm EC plates (3M). These were incubated at 35^{o}C for 48 h, checking for blue colony formation after 24 and 48 h. The results were recorded based on the formation of blue colonies with or without entrapped gas (as we observed that the *E. coli* strain ATCC 25253 does not produce the entrapped gas on petrifilms, which allowed us to differentiate between native *E. coli* present in the feces and the added culture). On two of the trials the *E. coli* negative samples were mixed with TSB and incubated overnight at 37 ^{o}C to determine if there was *E. coli* present but in a level too low to detect by immediate plating, however it was determined that the results were similar, so the procedure was not repeated for each of the trials.

Test Bacteria and Animal Feces

The test bacteria used in this study included *Escherichia coli* O157:H7 and *Salmonella enterica*. Three strains of *E. coli* O157:H7 (F4546, 960218 and SEA 13B88) were used and made into a cocktail. *S. enterica* serotype Newport was used. The test animal feces included cow, dog and bird feces, which were obtained from Yuma, AZ.

Bacterial Culture Preparation

Each bacterial culture was prepared by inoculating cryo-preserved cells in tryptic soy broth (TSB) and incubating overnight (18-20 h) at 37°C with shaking at 150 rpm. Two transfers were done before a working culture was prepared. Cells were harvested by centrifugation (2,000 x g for 10 min) and washed twice in sterile buffered peptone water (BPW). Cells were finally suspended in BPW to a concentration of about 10^{9} CFU/ml and formed the initial inoculum needed for experiments. To prepare the cocktail for *E. coli* O157:H7, equal volumes (3 ml) of the inoculum suspended in BPW, of each strain, were dispensed in a test tube and mixed well.

Survival of *E. coli* O157:H7 and *S. enterica* in Cow and Dog Feces

Cow and dog feces samples (10 g each) were dispensed into stomacher bags and pressed into a thin layer. These samples were then quickly dipped in hot water to reduce background microflora, and cooled to room temperature. Each bag of sample was inoculated with 0.1 ml overnight culture of *S.* Newport or *E. coli* O157:H7 cocktail, and stomached for 1 min at normal speed to mix well. Samples were then stored at a combination of either 26°C and 40% relative humidity or 15°C and 80% relative humidity conditions. Samples were taken at appropriate time intervals to enumerate the surviving bacteria. At each sampling, 90 ml of BPW were added to the samples in stomacher bags, and serial dilutions were done as needed in BPW. Samples were plated on Sorbitol MacConkey (SMAC) agar for *E. coli* O157:H7 and Xylose Lysine Dexosycholate (XLD) agar for *Salmonella* Newport. Plates were incubated at 37^{o}C for 24-48 hrs and counted. The pH of the feces was measured.

Survival of *E. coli* O157:H7 and *S. enterica* in Bird Feces

Bird feces samples (100g) were placed on a sheet of aluminum foil and pressed into a thin layer. Overnight culture of *Salmonella* Newport or *E.coli* O157:H7 cocktail (1 ml) was inoculated on the entire layer evenly. Samples were then dispensed into stomacher bags and mixed well by manually shaking with massaging for 1 min. Samples were then stored at combinations of either 26°C and 40% relative humidity or 15°C and 80% relative humidity conditions. Samples were taken at appropriate time intervals to enumerate the surviving bacteria. Feces samples (3 g) were transferred into centrifuge tubes and 27 ml of BPW were added. The mixtures were vortexed well for 5 minutes and serial dilutions were done as needed in BPW. Samples were plated on XLD with chloramphenicol and tetracycline for enumeration of *Salmonella* Newport, and on Chromagar O157 for *E. coli* O157:H7. Plates were incubated at 37^{o}C for 24-48 hrs and counted. The pH of the feces was measured.

Results and Discussion:

The results with multiple runs of sprinklers in three different fields showed diverse results (table 1) which may be due to the variable factors encountered in each trial. E. coli inoculated in feces was observed as far as 151 inches from the initial spot of the feces. Factors such as humidity, temperature, wind speed (average and maximum), wind direction, plant size/maturity, sprinkler run time and type of feces were hypothesized to affect the final area affected by the presence of feces in a sprinkled-irrigated. In preliminary trials we did not observe differences with different temperature and relative humidity levels or between dog feces and cow feces (data not shown). Thus, we focused on using cow feces and on the rest of the evaluation factors. Although some initial results suggested some effect of the plant maturity it was clear to us that factors such a run time and wind speed and wind direction override any other factor. A 0.70 correlation factor was found between sprinkler run time and maximum distance travelled by feces particles (based on our recover of the specific *E. coli*). Wind speed (average and maximum) and maximum distance produced a 0.40-0.45 correlation factor. When the wind speed was <12 mph the distance ranged from 24 to 96 inches, but then again sprinkler run time was the main factor influencing this range of values. It is possible that the high correlation of sprinkler run time and distance was due to increased volume sample at the farthest distance that allowed us to recover it. Moreover, it may

also be possible that the increased run time and subsequent higher relative humidity allows for increased "continuous splashing" of water drops. This suggests that future studies in this area should include work with sprinklers running for longer time.

We also need to take into consideration that in all the trials conducted in this study the animal feces were placed right in the center of four sprinkle heads. However, the distance travelled by feces particles (and *E. coli*) may also be function of the location of the feces from the sprinkler heads. In separate experimentation (data not shown) running sprinklers without feces in the field and collecting water with catch cans positioned in different locations of the field we observed more water accumulating in containers where the sprinklers heads were (again, opposite side of the wind direction). Thus, it is possible that placing the feces in the middle of two sprinkler head will yield different areas than positioning the feces in the middle of sprinkler heads.

Figures 1 and 2 show the results of *E. coli* O157:H7 and *S. enterica* survival in cow feces. Two batches of feces were tested. Both the organisms survived the longest in cow feces among the 3 types of feces tested. For both organisms, survival of the cells was longer at 26^{o}C and 40% relative humidity compared to those at 15^{o}C and 80% relative humidity. The 2 graphs show batch to batch variability which could be due to the differences in feces composition, however, the trend in survival was similar in both batches.

Figure 3 shows the survival of *E. coli* O157:H7 and *S. enterica* in dog feces. Cells exposed to 15^{o}C and 80% relative humidity survived for 7 days, while cells exposed to 26^{o}C and 40% relative humidity survived only for 3 days for both organisms. This is contrary to the survival of both organisms in cow feces and need further investigation. In general, dog feces did not support the survival of both *E. coli* O157:H7 and *S. enterica* compared to the other 2 feces tested. This could be attributable to the lower pH of dog feces (pH 5.0), compared to the other 2 feces (cow feces- pH 8.7; bird feces- pH 6.2).

The results of the survival of *E. coli* O157:H7 and *S. enterica* in bird feces are shown in figure 4. As seen from the figure, survival of *S. enterica* was better than that of *E. coli* O157:H7. This could explain why *S. enterica* is more prevalent in birds compared to *E. coli* O157:H7. The bird feces were dry, while the other 2 feces were wet. *S. enterica* has better desiccation resistance than *E. coli* O157:H7, and this resistance could have played a role in better survival. The survival of *E. coli* O157:H7 was similar under both storage conditions, while in case of *S. enterica*, the survival was better at 15^{o}C and 80% relative humidity compared to 26^{o}C and 40% relative humidity conditions.

In summary, the results produced in this project indicated the potential contamination in the field can be at least 151 inches from the location of the feces. The area may be reduced to <96 inches if maximum wind speed during the irrigation is <12 mph. The area impacted increased in this study when sprinklers ran for longer time. This research has direct implications with current food safety guidelines in the field.

Table 1: Distance travelled by *E. coli* from inoculated feces with regard to wind direction and speed. Farthest positive sample indicate direction from feces were the sample was located with respect to the feces. Trials 6 and 18 had the majority of wind direction show by the top value however some changes in direction as shown by the bottom value. The weather information was an average of values compiled from AZMET between the hours of 11 am – 3 pm which is the timeframe when each trial was conducted.

Trial	Sprinkler Time	Plant Stage	Wind Direction	Wind Speed (mph) aver	Wind Speed (mph) max	Farthest Positive Sample - Direction from Feces	Max Dist. Inches	Indicator
1	120	No plants					63	Red Dye
2	120	No plants					24	Red Dye
3	90	No plants					N/A	Starch
4	75	No plants					N/A	Starch
5	80	No plants	NE-S	8.72	15.38	NE	□ 75	Microbial
6	80	mature	SE- SW	2.28	5.16	NE	61	Microbial
7	80	mature	N	5.4	13.54	SE	42	Microbial
8	90	mature	W-NW	9.16	14.78	E	□ 64	Microbial
9	90	very mature	N-NE	5.64	11.64	Center	24	Microbial
10	90	20 leaves	S-W	5.04	9.98	NE-SE	□ 79	Microbial
11	90	20 leaves	S-W	5.04	9.98	NE-SE	□ 79	Microbial
12	90	20-30 leaves	NW - W	10.34	17.24	SE	□ 64	Microbial
13	90	20-30 leaves	NW-N	8.28	16.78	S	□ 73	Microbial
14	90	30-40 leaves	N-NE	9.84	17.4	S	□ 83	Microbial
15	90	30-40 leaves	NW-NE	9.52	17.26	S	□ 83	Microbial
16	90	40 leaves	SW-W	8.66	16.02	E-NE	123	Microbial
17	120	mature	SE-SW	11.24	17.22	N	125	Microbial
18	120	mature	S-SE, NW	4.08	9.48	SW	96	Microbial
19	120	very mature	W-NW	9.84	18.32	E-NE	□ 151	Microbial

North

	A			B			C		D		E			F			G		
1	P	P	X	P	**P**	X	**P**		**P**	X	**P**	P	X	P	P		P	P	
2	P	P	X	P	P	X	**P**		**P**	X	P	P	X	P	P		P	P	
3	P	P	X	P	**P**	X	**P**		**P**	X	**P**	P	X	**P**	P		P	P	
4	P	P	X	P	**P**	X	**P**		**P**	X	**P**	P	X	P	P		P	P	
5	P	P	X	P	**P**	X	X	**S**	X	X	**P**	P	X	**P**	P	X	P	P	
6	P	P	X	P	**P**	X	**P**		**P**	X	**P**	P	X	P	P	X	P	P	
7	P	P	X	P	**P**	X	**P**		**P**	X	**P**	P	X	**P**	P	X	P	P	
8	P	P	X	P	P	X	**P**		**P**	X	P	P	X	P	P	X	P	P	
9	P	P	X	P	**P**	X	**P**		**P**	X	**P**	P	X	**P**	P	X	P	P	
10	P	P		P	P	X	**P**		**P**	X	P	P	X	P	P	X	P	P	
11	P	P		P	**P**		**P**		**P**	X	**P**	P		**P**	P		P	P	

West **East**

South

Scheme 1: Example of the distribution of plants, catch cans, feces and *E. coli* recovered in the field after sprinkle irrigation. P: Plant sample; X: Water in catch can sample; S: Sample of cow feces. Gray cells indicates areas with coliforms and dark cells indicate location where inoculated *E. coli* was recovered.

Figure 1. Survival of *Escherichia coli* O157:H7 and *Salmonella* Newport in cow feces (Batch 1)

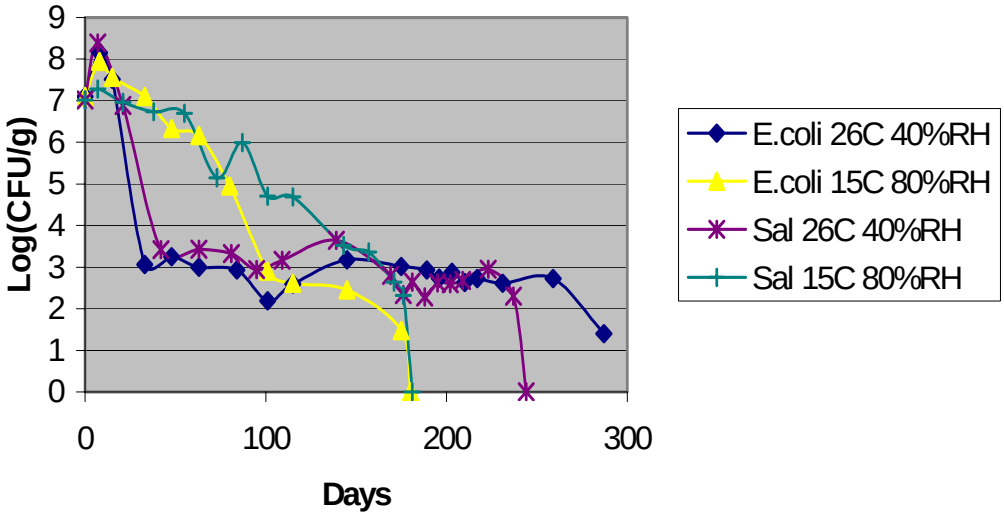

Figure 2. Survival of *Escherichia coli* O157:H7 and *Salmonella* Newport in cow feces (Batch 2)

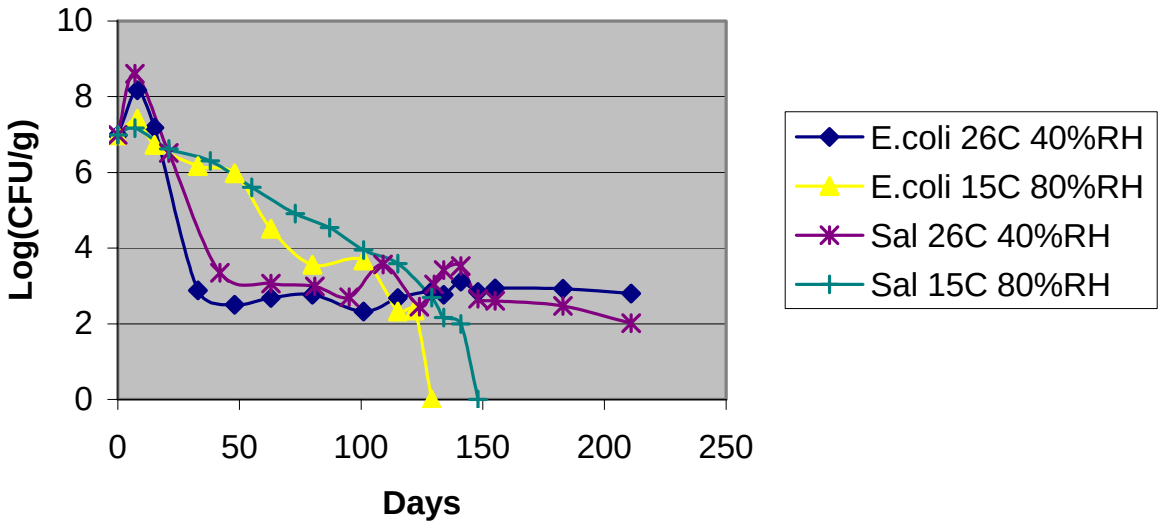

Figure 3. Survival of *Escherichia coli* O157:H7 and *Salmonella* Newport in dog feces

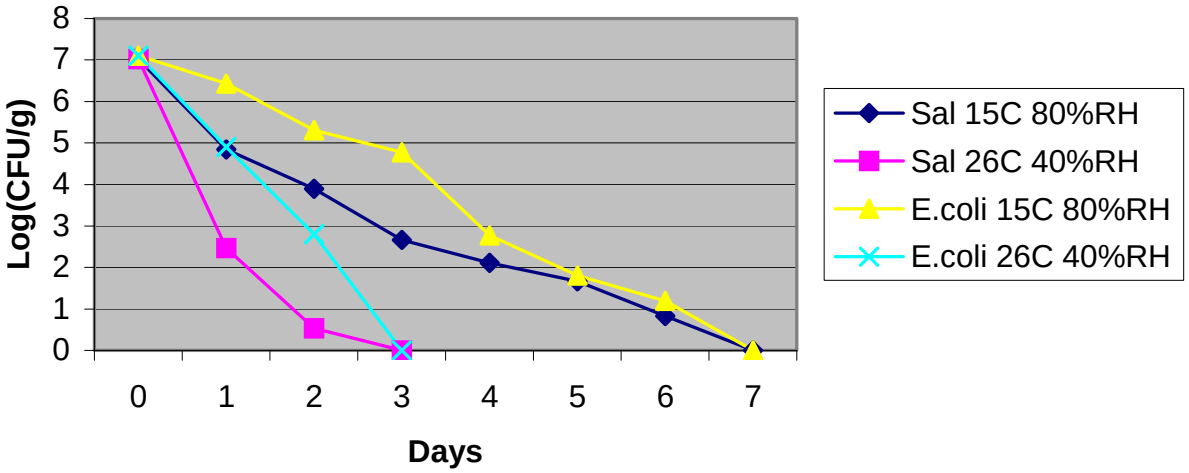

Figure 4. Survival of *Escherichia coli* O157:H7 and *Salmonella* Newport in bird feces