CPS CENTER for PRODUCE SAFETY

CPS 2011 RFP – Rapid Response
FINAL PROJECT REPORT

Project Title
Microbial Food Safety On-Farm Risk Assessment

Project Period
August 1, 2011 – December 31, 2011

Principal Investigator
Trevor Suslow
Department of Plant Sciences
University of California, Davis
Email: tvsuslow@ucdavis.edu
Phone: 530-754-8313

Co-Principal Investigator
Gabriela Lopez-Velasco
Postdoctoral Scientist
Department of Plant Sciences
Email: glopezvelasco@ucdavis.edu

Objective
The purpose of this Rapid Response field study was to assess the potential public health hazard, within a unique on-farm data gathering opportunity, of a cantaloupe field adjacent to a small dairy operation. Our objective was to document the likelihood of presumed, localized dispersal of contaminants due to ag-traffic, animal activity, and other direct and indirect transfer of fecal indicators and pathogens from the animal facility before the intended initiation of harvest at that location. In addition, the Rapid Response opportunity permitted the assessment of commercial kits and in-house UC Davis developed molecular methods (up to six different methods) to detect EHEC and Salmonella enterica in melons harboring low levels of stress-adapted pathogen populations against a high background of microbial populations and adhering soil.

FINAL REPORT

Abstract

Microbiological assessment, including the potential risk and confirmed presence of human bacterial pathogens, *Escherichia coli* O157:H7, including other Shiga-toxin positive *E. coli*, and *Salmonella enterica*, was performed in a cantaloupe field located, at one edge, in immediate proximity to a dairy operation facility. Evaluation of total coliforms, fecal indicators and detection of human pathogen molecular markers was initiated, following inquiry and invitation of the responsible handler to the Suslow lab, approximately 2 weeks before the estimated first planned harvest date. The sampling and analysis timeline progressed from pre-harvest to after field discing for crop destruction and included melons, soil, and other environmental samples encompassing irrigation water, run-off water, ditch sediments, manure, dairy corral surface material, compost and aerosol-particulates. Quantification of total coliforms on melons resulted in evidence of greater population densities within areas adjacent to animal corrals as compared to other areas of the production parcel. This higher recovery of indicators corresponded with a larger organic matter fraction and nitrogen content in soil from the corresponding area, potentially reflecting a greater deposition and incorporation of pre-plant dairy lagoon solids and manure scrapings known to have occurred in fall 2010. Additionally, detection of fecal indicators such as *Enterococcus* and *E. coli* was observed in most environmental samples. Presumptive pathogen detection was documented by the presence of molecular markers for *E. coli* O157:H7, presumptive pathogenic *E. coli*, particularly carrying the *stx2* gene (shiga-toxin type 2; EHEC/STEC bacteria), and *Salmonella enterica*. Several isolates of *E. coli* O157:H7 (*stx1*$^{-}$; *stx2*$^{+}$) was confirmed by viable, culture recovery from cantaloupe fruit rind in areas near the dairy operation. Specific qRTPCR detection protocol validation studies determined that post-enrichment composites of up to five melons accurately reflected the status of each of the individual enrichments. Therefore, no ‘false negative’ outcomes were observed in a negative-outcome composite with natural contamination. Samples collected and analyzed 48h prior to the time of the scheduled harvest date decision demonstrated that melon surface microbial counts had not changed in total and fecal coliforms populations relative to numbers two weeks earlier. Additionally, detection of pathogenic *E. coli* and *Salmonella* markers was still positive, thus the handler decision was to abandon the field for harvest and the crop was immediately destroyed by multiple flail-chopper and disc passes to prevent fruit gleaning by the individuals observing an abandoned field. Microbiological assessment of soil after discing provided sound evidence for an increase of 3-log (1000-fold) in total coliforms along the entire

field parcel and detection of pathogenic *E. coli* markers (*eae*A and *stx*2) remained positive. The entire parcel was planted to a cover crop of 'green-manure' maize silage and grown for approximately 60 days before harvesting the primary foliage for animal feed and discing the residues according to standard practice. Fifty soil samples taken across the site revealed that indicator *E. coli* populations had uniformly declined in almost all zones to below the limit of detection. All previously documented virulence markers for EHEC/STEC in soil samples from these same sub-zone sample sites were absent in analysis of soil-extract enrichments post-cover cropping. We feel this indicates one potential mitigation strategy for stabilization or recovery of contaminated soil due to events such as manure application or flooding.

Background

Cantaloupes have been associated with multiples outbreaks in the United States from domestic and imported melons since 2000. Among them, various *Salmonella* has been consistently linked to those outbreaks. Diverse serotypes have been involved in these outbreaks including Chester, Poona, Saphra, Anatum and Litchfield (Castillo et al., 2004; Alvarado-Casillas et al., 2010).

Diverse opportunities for primary contamination and cross-contamination during preharvest phases and postharvest handling are recognized, including fecal contamination by animals or transmission by insects, birds or rodents, use of untreated manure, application of contaminated irrigation or foliar contact water, flood water carrying human waste, and direct human hand contact (Beuchat, 1996; Suslow, 2003; Brandl, 2006). The proximity of animal facilities to the vegetable production area is included in the current Good Agricultural Practices and Draft Guidance for Melons documents (FDA, 2008; FDA 2009) as a potential significant risk factor.

Cattle feces are the main reservoir of Shiga toxin-producing *Escherichia coli* (Fremaux et al., 2008). Fecal pathogens may enter the environment by various means including through direct deposition of feces to land, through runoff of overland fecal materials deposited on soils, especially after heavy rainfall events, and by application of non-composted or inadequately treated manure (Suslow et al. 2003; Thurston-Enriquez et al., 2005).

The purpose of this field study was to assess the potential public health hazard of a cantaloupe field adjacent to a small dairy operation, after awareness of the owner about the likelihood of presumed, localized dispersal of contaminants due to ag-traffic, animal activity, and runoff water from the animal facility was observed two weeks before the intended initiation of harvest at that location. Upon investigation, the melon handler determined that large amounts

raw manure solids from the dairy waste lagoon and corral surface scrapings was applied across the planted parcel approximately 41 days prior to the seeding and 135 days prior to intended harvest.

Briefly, in response to a request from the handler for hazard evaluation and risk assessment, a baseline data-gathering opportunity was defined to assess the transfer of Fecal Indicator Bacteria (FIB) and pathogenic *E. coli* and *Salmonella* from an Animal Feeding Operation (AFO) in close proximity to this commercial melon production field by contamination from land-applied manure, contaminated irrigation water, and particulate aerosols. Our immediate focus was to collect directional information on FIB distribution and molecular evidence for pathogen contamination of fruit by sampling from the most distant area of the ranch block to the areas closest to the AFO. We included samples of residual irrigation water and sediments in the ditch from the most recent irrigation event, which occurred 10 days prior to the first sampling date. In addition, preliminary soil samples associated with zones of fruit collection were been taken and processed to initially define the justification for this project. This research response incident also provided an opportunity to bring a rapid PCR system for *Enterococcus* sp. as an improved FIB source-tracking tool in assessing such risks (relative to generic *E. coli*) and adjacent land/proximity issues. The key areas targeted for which we anticipated developing 'real-world' data included;

1. Evidence for gradients and distances of FIB transport and persistence from an AFO to melon fruit

2. Evidence for gradients and distances of FIB in soil relative to the AFO and proximity to the irrigation ditch

3. Evidence for differential recovery of FIB and pathogens in tailwater sediments relative to spatial position from the irrigation source canal and the AFO

4. Evidence for differential spatial detection of pathogens on fruit relative to the AFO and proximity to the irrigation ditch

5. Evidence for internalization of FIB at the stem scar region

6. Functionality of commercial pathogen detection kits on melons produced under conditions with potentially high microbial background

7. Functionality of a rapid, semi-quantitative PCR method for tracking FIB on melon fruit based on Enterococcus

Research Methods and Results

Methods and Materials:

Field Description

Field Dimension and Adjacent Land. A 24 acre (9.8 hectare) farm parcel study site was located alongside an irrigation water canal directionally flowing and extended from east to west. Adjacent lands included a dairy to the east, a cantaloupe field to the west and solids-separation and manure lagoon and cotton field to the south and south east. The small dairy consisted of three corrals and an area for stockpiling of manure and feedlot surface materials, all located to the east, and a manure lagoon to the south. One of corrals and one manure accumulation area were immediately next to the field, within 10 meters from the terminal section of the irrigation ditch and the first several beds with maturing melons. At the time of project initiation the lagoon had fresh manure liquid+solids waste, an elevated post-separation discharge inlet conduit and was 15 meters from the one section of the irrigation ditch and field boundary (Fig. 1).

Irrigation. The source of irrigation for the entire field was irrigation district canal water that entered the irrigation ditch through a gate located on the west boundary of the field. During irrigation events, flow was directed from the turn-out source towards the east along the southern border and terminated as it turned north immediately across from one corral pen. The siphon-tube irrigation was used to establish furrow application to raised beds, in which the water moved into the field from south to north. The water distribution in this particular field appeared to provide a mechanism for water contamination and recontamination along the eastern border (near corrals and manure piles) and the southeast corner (in front of lagoon) throughout the production season.

Agricultural Practices. A reportedly substantial but non-quantifiable mass of five-year-old manure and animal waste was scraped from lagoon located as described above and applied to the field 41 days prior to seeding and approximately 135 days prior to the anticipated first harvest. Observation of the field made it apparent that the distribution of the soil amendment may not have been uniform due to negative impacts on stand establishment and plant productivity in one area (Fig. 1 – Bald Spot).

*Sampling Zones***.** Following the initial discussion with the handler and site visit by Suslow**,** the field was divided into three zones based on the configuration of the parcel and spatial orientation relative to the adjacent AFO features. The criteria used for sampling the

zones were based on their possible or presumed contamination exposure risk and to attempt to delineate proximity effects from soil amendment and ambient environmental effects. Zone A located to the east, near the northern corral, consisted of 1.13 ac (0.46 ha). Zone B was below and diagonal to zone A with 1.9 ac (0.76 ha) on the southeast corner bordering manure piles to the east and the lagoon to the south. Zone C was the largest contiguous area covering more than half of the field and across from the neighboring cotton field to the south and water canal to the north (Fig. 1).

Sampling procedure

The cooperating land owner allowed access to the potentially affected crop to evaluate the microbiological state of the field, microbiological and physicochemical aspects of its soil and environment, and adjacent areas including the dairy lagoon and corral closest to the melon field. The affected area was divided in 3 different sections, zone A, B and C as described above (Fig. 1). A total of 9 dates of sampling were conducted from July to September (fruit) and into December (fallow soil) of 2011. The sampling procedure over this timeframe was divided in three focal areas of data-gathering as described below:

- **Melon field risk assessment**. Microbiological evaluation of the field and adjacent areas.
    - Melon fruit
    - Soil
    - Irrigation and Tailwater/Algae in the irrigation ditch/Sediments
    - Stacked Manure/Corral Surface Material/Lagoon
    - Aerosol particulates
- **Melon field assessment at pre-harvest**
    - Pathogen detection
    - Determination of fecal indicators
    - Potential pathogen and coliform transference during washing and cutting
    - Evaluation of bacterial endophytes in fruit
    - Taxonomic identification of selected, representative colonies
- **Soil & field assessment after discing.**
    - Soil
    - Residual post-discing irrigation water

Melon processing

For each sampling point, ¼ to full-slip melons were collected and transported to the Mann Lab where they were fully peeled by using a sterile knife. Each melon peel was placed in a sterile bag containing 150 mL of potassium phosphate buffer (3.9 mM KH_2PO_4 and 6.1 mM K_2HPO_4) supplemented with 0.05% Tween 20, and vigorously massaged, shaken, and manipulated by hand for 1 min to remove as many of the attached bacteria from the melon surface. Tenfold serial-dilutions were prepared in 9 mL of sterile 0.1% buffered peptone water (BPW). Total coliforms and presumptive *Escherichia coli* were recovered from the melon samples by plating 100μl onto Chrom-ECC (Chrom Agar, Paris, France) agar incubated at 37^0C for 24 h. All microbial counts are reported as log CFU per fruit.

After plating, the bags containing the manually masticated melon samples and potassium phosphate buffer were brought to a final volume with 300 mL double strength (2X) Universal Pre-enrichment Broth (UPB; Difco, Sparks, MD) and incubated at 37 °C for 12-14 h. For *E. coli* O157:H7 recovery, 10 mL of UPB enrichment were transferred to 90 mL of modified EHEC (Enterohemorrhagic *E. coli*) media (mEHEC, Biocontrol; Bellevue, WA) and incubated at 42ºC for 24 h. For Salmonella spp. Recovery, 10 mL of UPB enrichment were transferred to 90 mL of tetrathionate broth (TBB; Difco, Sparks, MD) followed by 6 h of incubation at 42ºC. Then, 20 mL of the enriched samples with TBB were transferred to 180 mL of Bacto M broth (Bacto, Sparks, MD) and incubated at 37 °C for 18 h. These enriched samples were used for pathogen detection which is further described. A schematic process flow diagram is provided in Figure 3.

A separate validation study was conducted to specifically address the issue of compositing melon rind samples to maximize the number surveyed from a lot and conserve cost per test to screen for pathogen presence. Briefly, the entire rinds of 5 melons collected from the study site were tested for the presence of EHEC as post-enrichment composites of boiled DNA. The individual DNA samples and end-point enrichments (retained with the addition of 26% glycerol) were held at -80C to allow both individual confirmation of a positive or negative outcome in qRTPCR and secondary enrichment to recover cultures of positive samples. For this validation study, one strong positive, one weak positive, and one negative composite was selected for assessment of PCR outcome on the individual enrichments in each mixture.

Distribution of indicators on cantaloupe fruit surfaces

To determine whether coliform and indicator *E. coli* populations were differentially distributed on melon rind in contact with the soil bed vs. oriented towards the sky, a separate, simple-design study was conducted to obtain a rough quantitative assessment of indicator populations by removing rind from upper and ground-spot sides of 10 melons harvested from Zone A. Methods for processing and enumeration of FIB were as described above.

Soil processing

Soil samples were collected from multiple locations across each zone and used to evaluate the microbiological state of the soil and the presence of human pathogens. From a composite of approximately 0.5kg, mixed as uniformly as possible after collection, a subsample 100 g of soil was saturated with 0.1M sodium phosphate supplemented with 0.05 % Tween 20 at a 2:1 ratio (v/w) and thoroughly mixed to release bacteria from clay and silt. The suspension was allowed to settle for 30 min and aliquots taken from the upper layer containing the excess extraction buffer. Ten-fold dilution series were prepared in 9 mL of sterile 0.1% of BPW. Total coliforms and presumptive *E. coli* were recovered from the soil samples with Chrom-ECC agar incubated at 37^0C for 24 h. All microbial counts were reported as log CFU per gram.

The sequential enrichment steps procedure previously described for the melon samples was also used for the soil samples, which were further employed for human pathogen detection. Samples of manure, lagoon and algae were processed using the same methodology.

Soil physicochemical properties

Soil samples collected during this investigation were analyzed for mineralized nitrate and ammonium following the modified methods described by Miranda et al. 2001 and Foster, 1995 respectively. Soil pH and electrical conductivity (Ec) was measured following the method described by the Soil Survey Investigations Report No. 42. (USDA, 2004). The sodium adsorption ratio (SAR) was calculated after determining Ca, Mg and Na concentrations in a saturation extract following the method described by USDA Agric. Handb. 60. The total amount of nitrogen and carbon in soil was determined following the method described by AOAC Official Method 972.43. Total organic carbon was determined following the methods of Harris et al. 2001 and AOAC Official Method 972.43.

The cation exchange capacity (CEC) of soil was determined following the method of Rible et al. 1960; where Barium is used to quantitatively displace soil exchangeable cations. The hydrometer method was used to quantitatively determine the proportion of three sizes of primary soil particles as described by Sheldrick and Wang 1993. Finally the soil moisture content under a constant preset pressure potential of 0.33 ATM was determined following the method described by Klute, 1986. This estimation was done to determine the available water capacity of the soil where melons were cultivated.

Water processing

The QuantiTray Colilert System (Idexx Laboratories Inc., Westbrook, ME) was used to determine viable coliform bacteria and presumptive *E. coli* suspended in water. Samples of 100 mL of water from selected areas (above lagoon, in front of cows, next to cows, far west and canal) were processed following the specific technical directions provided with the QuantiTray kit. Total coliforms and *E. coli* are reported as most probable number (MPN) per 100mL of water (MPN/100mL).

Eight liters of water from selected areas were filtered using modified Moore swab (MMS) system to enhance the pathogen detection by capture-filtration of large volumes of water. After filtration, the swab was subjected to the sequential pathogen enrichment procedures as described above.

Aerosol samples processing

Four different locations, two near Zone A (4 subsamples from north and 4 subsamples south corner) and 2 inside Corral in Zone A (3 subsamples near and 3 subsamples far) were selected for aerosol sampling collection. Two different conditions were tested: a) disturbing the soil/manure mimicking animal movement in corrals b) without disturbing the corral soil/manure.

Air samples were taken using the microbial Air Sampler MAS-100 Eco® (MVB; Microbiology and Bioanalytic, Switzerland) by collecting 1000L in 10min onto 20% Bacto Agar (Difco, Sparks, MD). Then, aerosol impaction samples on the soft agar were extracted from the plates and placed in sterile bags containing 30 mL of double strength (2X) BPW and homogenized using a stomacher for 30 s at medium speed. To evaluate the presence of generic *E. coli*, *E. coli* O157:H7 and STEC the homogenates were plated onto Chrom-ECC agar and Chromagar-O157 (DRG International Inc.; Mountainside, NJ). Homogenized samples were

incubated at 37ºC for 24h and further subjected to molecular pathogen detection by PCR and cultural methods described below.

Pathogen detection

Melon, soil, water and air sample enrichments were subjected to qualitative evaluation of presence/absence of *E. coli* O157:H7 and *Salmonella spp*. In order to optimize the sampling size and analysis within practical time and resource constraints, samples were composited in 5 pooled enrichments from each sample and source. An independent assessment of individual vs. composited enrichments on a subset of 15 fruit samples to validate the pooled-analysis methodology.

Evaluation of the presence/absence of Escherichia coli O157:H7

Phenotypic confirmation on selective media. To evaluate the presence of *E. coli* O157:H7, the samples enriched with mEHEC were plated onto Chromagar-O157 (DRG International Inc.; Mountainside, NJ) and Rainbow ® agar O157 (Biolog, Hayward, CA). Plates were incubated for 24 h at 42ºC. Typical mauve and black/grey colonies were considered a positive result for Chromagar-O157 and Rainbow agar plates, respectively.

Rapid detection kits. Assurance GDS and BAX. BAX ® System real-time *E. coli* O157:H7 (real time) (Dupont/Qualicon, Wilmington, DE) and GDS® O157:H7 (BioControl, Bellevue, WA) were used in this study as rapid detection kits following manufacturer instructions, with the exception of the sample enrichment which was performed as previously described. The specific technical directions provided with each kit were followed for all PCR conditons.

Probe base real time PCR. For DNA extraction, an amount of 200 µL of each enriched mEHEC samples was transferred to tubes and placed in a heating block at 95°C for 10 min. Probes and primers in PCR reactions used for each gene are reported in Table 1. Each 20 µL reaction contained 10 µL of a 2× Taqman® Gene expression master mix (Applied Biosystems Inc., Foster City, CA, USA), 0.5 µM of forward and reverse primers, 2.5 pmol of probe targeting genes (Table 1) and 2 µL of enrichment (mEHEC) that was previously boiled for 95 °C for 10 min. Amplification of selected genes was carried in a thermocycler (7300 Real Time PCR

System, Applied Biosystems Inc., Foster City, CA, USA) with a protocol consisting in one cycle of 50 °C for 5 min, one cycle of denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and annealing at 60 °C for 1 min. Amplification greater or equal to the *Ct* value of a standard containing 1 copy of the target gene was classified as positive.

Colony confirmation. Enrichments that were positive for any of the commercial kits or molecular markers tested were plated on ChromO157:H7 and Rainbow agar to isolate presumptive *E. coli* O157:H7. Isolated colonies were purified and one colony was resuspended in 200 µL of Butterfields Phosphate Buffer (3M) and the suspension was boiled at 95˚C for 10 min. Multiplex PCR was utilized to genotype the colonies as previously described (Haack et al., 2009)

Evaluation of the presence/absence of Salmonella spp.

Rapid detection kits. Assurance GDS and BAX. BAX ® Salmonella (Dupont/Qualicon, Wilmington, DE) and GDS® Salmonella (BioControl, Bellevue, WA) were used in this study as rapid detection kits. The specific technical directions provided with each kit, as previously described, were followed carefully. Enriched samples with Bacto M broth were used in this experiment to determine the presence/absence of *Salmonella enterica*.

Probe base real time PCR. For detection of *Salmonella spp*., amplification of the virulence marker *inv*A was done using probe based real-time PCR. Amplicons were generated using forward primer invA-F (5′- TGGGCGACAAGACCATCA- 3′), reverse primer invA-R (5′-TTGTCCTCCGCCCTGTCTAC-3′) and invA probe (6FAMCAATGGTCAGCATGGTATA-MGBNFQ). Each 20 µL reaction contained 10 µL of a 2× Taqman® Gene expression master mix (Applied Biosystems Inc., Foster City, CA, USA), 0.5 µM of forward and reverse primers, 2.5 pmol of probe targeting invA (Applied Biosystems Inc., Foster City, CA, USA) and 2 µL of Bacto M broth enrichment that was previously boiled for 95 °C for 10 min. Amplification was conducted in a thermocycler (7300 Real Time PCR System, Applied Biosystems Inc., Foster City, CA, USA) with a protocol consisting in one cycle of 50 °C for 5 min, one cycle of denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and annealing at 60 °C for 1 min. Amplification greater or equal to the *Ct* value of a standard containing 1 copy of *inv*A was classified as positive.

Phenotypic confirmation on selective media. To evaluate the presence of *Salmonella spp*, the enriched samples with Bacto M broth were plated onto Xylose Lysine Tergitol-4 (XLT-4) agar (Oxoid, Basingstoke, Hampshire, UK) and incubated for 24 h at 37ºC. Typical black colonies were considered a positive result and colonies purified for further determinative confirmation tests.

Determination of fecal indicators – Enterococcus

Quantification and presence of *Enterococcus spp*. as a specific fecal indicator in melon, soil, manure and water samples was carried out on subset timepoints of the samples described above . Power Soil DNA extraction kit was utilized (MO BIO Laboratories Inc., Carlsbad, CA) for DNA purification of the target environmental samples following manufacturer instructions with some modifications; for melon samples, bacteria was detached from the rind and adhering soil with 100 mL of potassium phosphate buffer (3.9 mM KH_2PO_4 and 6.1 mM K_2HPO_4) supplemented with 0.05% Tween 20, and vigorously homogenized by hand for 1 min. The 100 mL of bacterial suspension from melon wash and 200 mL of water samples were centrifuged at 4500 rpm for 15 min, supernatant was discarded and the pellet was utilized as starting material for the Power Soil DNA extraction kit.

Quantification of *Enterococcus spp*., was followed as described by Haugland et al., 2005 using probe based real-time PCR. Amplicons were generated using forward primer ECST-F (5′- AGAAATTCCAAACGAACTTG - 3′), reverse primer ENC854-R (5′- CAGTGCTCTACCTCCATCATT -3′) and GPL813TQ probe (6FAM - TGG TTC TCT CCG AAA TAG CTT TAG GGC TA - TAMRA). Each 20 µL reaction contained 10 µL of a 2× Taqman® Gene expression master mix (Applied Biosystems Inc., Foster City, CA, USA), 0.5 µM of forward and reverse primers, 2.5 pmol of probe targeting XXX (Applied Biosystems Inc., Foster City, CA, USA) and 5 µL of purified DNA. Amplification was carried in a thermocycler (7300 Real Time PCR System, Applied Biosystems Inc., Foster City, CA, USA) with a protocol consisting in one cycle of 50 °C for 2 min, one cycle of denaturation at 95 °C for 10 min, followed by 45 cycles of 45°C for 15 s and annealing at 60 °C for 1 min.

Identification of coliform bacteria and presumptive E. coli colonies

Bacterial colonies from ChromECC were randomly selected at different sampling point and sources. Pure bacterial colonies were further identified based on partial amplification 16S rRNA gene sequence using a 1.5 Kb fragment with primers 6F/1510R as previously described

by Dulla and Lindow, 2009. Sequence alignment and classification was done using software from the Ribosomal Data Base Project (RDB, release 10) (Cole, et al., 2009, Wang et al., 2007). Aligned sequences were utilized to construct a phylogenetic tree with BioEdit v.7.0.9.0 utilizing unweighted pair group method with arithmetic mean (UPGMA) and Neighbor-joining algorithm (Hall et al. 1999). Additionally, a total of 89 colonies isolated from various sample sources and zone areas were analyzed through ribotyping in generous collaboration with Dupont/Qualicon®. Sample patterns generated were unique to each isolate Ribotyped and results were reported on the basis of similarity to the existing 6,950 bacterial isolates contained within the RiboPrinter® Database. During identification, individual sample patterns were compared to ID reference patterns and were automatically reported if a similarity value was greater than 0.85. Closely matched IDs in the form of a “nearest neighbor” report will be calculated for this purpose.

Effectiveness of melon washing after harvest

Melons from Zone A, B, and C were harvested at full-slip maturity and, due to restrictions on human resources, media supplies, and space constraints at the time, were stored for 8 days before processing started. Due to the circumstances, a modified goal was established with this procedure and melons were held at 10C (50F) to reproduce sub-optimal distribution and retail handling had this field actually been field packed and marketed. Further processing was done simulating best household washing conditions and practices that may be conducted by consumers. A total of 25 melons were used in the evaluations. Each melon was washed using a new vegetable brush and 200 ml of tap water. The entire surface of the melon was brushed in two full vigorous passes. After the melon was brushed, each fruit was rinsed with a clean source of 200 ml of tap water. In between each melon, the brush was disinfected with 95% ethanol for at least 5 min and then rinsed with water before it was used again to brush another melon.

After washing, each melon was cut in half, seeds were removed and each half was placed cut-side down on top of a clean cutting board before peeling. Melons were peeled from the stem scar end to the blossom end. The peel was then placed inside a large sterile bag. The remaining flesh was cubed and placed in a separate bag. A total of 3 bags per melon were analyzed and consisted of; 1-wash water, 2- peel and 3- fruit cubes. Each bag received 500 mL

of buffered peptone water (first enrichment) followed by the procedure for the detection of pathogenic *E. coli* O157:H7 and *Salmonella* as described above. From each bag, 200 □L were plated on RAINBOW® AgarO157 (RA) (BIOLOG, Hayward CA,USA), ChromECC and/or ChromO157. An approximation to the total aerobic bacteria present in the peel of the melon was determined based on the outcome of colony formation on RA plates.

Results:

Microbiological assessment of a commercial melon field located in close proximity to a cattle operation facility was performed in this study. Following the primary discussion with the handler/shipper, an initial assessment was conducted to evaluate the microbiological status of the field and surrounding areas in order to establish the impact of the presence of a dairy waste lagoon as well as the animal operation and influence of animal waste spreading prior to planting. In anticipation of the expected delineation of a buffered, no-harvest zone and an acceptable risk harvest zone, the field was divided in three main areas as previously described; Zones A, B and C which refers to close proximity to animal corral, lagoon and the remaining area of the field respectively (Fig. 1).

Population of coliforms in both melon and environmental samples was assessed (Tables 1 and 2). Population of the fecal indicator *Enterococcus sp.* was not significantly different among melons from the three zones and uniformly present on these fruit but undetectable on fruit harvested in unrelated fields. Coliform population in melons collected from Zone A had significantly greater populations, but not exceeding 1-log (10-fold) higher, than those in Zones B and C. In addition, the maximum and minimum population sizes, determined among all melon samples, were located in Zones A and C respectively. Additionally, on two occasions, a set of melons from a different commercial field under the same handler were used for comparison to the assessed field. The total coliform population in melons from the commercial harvested field was significantly lower than melons coming from the research assessment field of this project and *Enterococcus sp.* was not detected (Table 2).

Environmental samples including, water, algae in ditches, soil, lagoon, manure and compost material as well as air samples were evaluated (Table 3). Water samples were collected from different areas around the melon field. Overall, no significant difference in the population of total coliforms and *E. coli* was determined among the different sources. However, the largest populations for both bacterial groups were determined in Zone A (next to the non-covered animal corral), which corresponded to the only point where *Enterococcus sp.* was detected outside the cropped area. Similarly, algae collected from the irrigation ditch around the

melon field had larger populations of coliforms and *E. coli* in Zones A and B than in Zone C, but those differences were not significant. For soil and compost samples, only total coliforms and generic *E. coli* were quantified. *E. coli* population density was below the limit of detection, in contrast with manure samples that had the largest population of *E. coli* and *Enterococcus sp.* from all environmental samples.

In order to determine the potential contribution of particulate aerosols (fugitive dust) to the transfer of coliforms and other bacteria, air samples were collected onto soft-agar plates. Although direct quantification was not possible, enrichment of the agar-capture plates provided clear evidence of the presence of viable coliforms but not *E. coli* , under the limited sample dates, in more than 50% of the collected samples (Table 3).

Detection of virulence or diagnostic molecular markers of pathogenic *E. coli* including serotype O157:H7 and *Salmonella enterica* was done using Taqman® assays as well as commercial kits (Table 4). Evidence of pathogenic *E. coli* markers (TPEC and *eae*A) was determined in melons from all zones and all environmental samples, with exception of aerosol samples. Additionally there was a consistent detection of *stx2* gene in most of the melon and environmental samples with exception of grid-points for melons from Zone C. The detection of *E. coli* O157:H7 was evidenced in one melon composite from Zone A after the detection of $rfbE_{O157}$ and then supported by positive reactions with both GDS and BAX detection systems. From this composite, colony confirmation was assessed and genotype corresponded to an *E. coli* O157:H7 (*stx*1^{-} *st*$^{-}$*x*2+) isolates. In contrast, confirmed *S. enterica* was only detected in one of the melon composites from Zone B and was not detected on melons from other zones or other environmental samples (Table 4).

The results of the sample processing validation study from one strong positive, one weak positive, and one negative composite, selected for assessment of PCR outcome on the individual enrichments in each mixture, revealed that 1 of 5 enrichments from the strong positive composite was positive for the same virulence markers and 0 of 10 enrichments for the weak and negative groups. We feel this demonstrates and provides some assurance that PCR test kits may be applied to composites of naturally contaminated cantaloupe with a high microbial background and detect positive contamination. No evidence for ‘false negative’ (negative composite shown subsequently to have a positive individual that was diluted out in compositing) reactions was observed for up to 5 melons.

Soil analysis in the three zones indicated that sand was the major component followed by silt and clay, which could be considered a Loam or Sandy Clay Loam (Fig. 2). Sections A

and B particle size distribution was very similar while Zone C deviated from these other sections in the percentage of sand and clay. Loam soils in general retain water and nutrients more effectively. Water retention capacity of Zone A and B was identical and greater than the retention capacity of Zone C. Based on field observations; Zone A and B had greater manure-derived organic content based on difference in soil color, color of the irrigation peak-level band on the sides of beds in these zones, soil texture properties, and the inherent proximity to the dairy liquid/solid waste lagoon. It was apparent that a lagoon discharge conveyance, though not in current use or apparent use in recent years, had been a employed to distribute liquid waste from the lagoon to land and silage crops previously grown on this parcel.

Macronutrient analysis from all three zones indicated that Total N, Mg and K were present at greater concentrations in Zone A and B than in C. Total carbon and organic matter were also present at greater concentrations in Zone A and B, which corresponded to higher CEC and Ec composition. Total mineralized N, nitrate and ammonium was similar in all sections. The pH in all three sections was different; however sections A and B had the lowest pH values. Differences between pH were small and all sections are within the neutral to slightly alkaline profile. No difference in the sodium adsorption ratio SAR was observed between zones and this was mainly attributed to the presence of high and identical concentrations of Ca and Na (Data not shown; available on request and planned for inclusion in journal manuscript submissions) and to small but significantly different Mg concentrations in all sections.

Overall, the soil physicochemical properties appeared to be significantly modified by recent manure waste application and potentially long-term applications prior to conversion of the parcel to a rotation which included a crop intended for human consumption. The properties evaluated in this study diverge greatly from those reported by the California Soil Resource Lab for the two soil types present in this field and is most likely associated with manure inputs. On site observations indicated a light color soil in Zone C associated with lower organic matter, nutrient content and water retention capacity while the opposite traits were observed in Zone A and B. The latter two sections had greater total N and C that improved soil fertility and that are known to directly stimulate microbial activity, growth, and diversity.

After assessment of the field, a second evaluation was performed close to the date established for melon harvest. This assessment was more focused on Zone C, which includes the main cropped area farthest from the animal operation and the lagoon. The objective was to determine whether a data-based setback buffer could be established to allow or salvage part of the field for commercialization. In contrast to the first assessment, no significant difference was found between the coliform populations in Zones A and C. *Enterococcus sp.* was detected on

melons from the three zones, however its population was not significantly different among the three zones evaluated (Table 6A). For melons collected in zone A, a comparison between the rind-side of melon fruit in direct contact with the soil to the upper-rind side of the melon revealed a significantly greater population of coliforms in the upper half of the melon though the difference in practical terms is questionable (Table 6B). Consistent with the first assessment, analysis for pathogen detection showed presence of the *stx2* gene in 2 out of 4 composites from Zone A, but not from Zones B and C. However, in 1 out the 10 composites from Zone C was positive for *Salmonella* using the GDS detection system.

In order to determine the suitability of coliforms as indicators, and in consequence pathogenic bacteria to be present inside the fruit, endophytic coliforms were determined after surface sterilization of melons from the three areas. Coliforms were detected after enrichment inside the fruit in almost 100% of the melons evaluated from the three zones (Table 6A). Additionally, melons collected from the field were stored for 8 days under intentionally poor, but not hypothetical, distribution temperature conditions and then vigorously washed to mimic best consumer handling practices. After washing, approximately a 1-log reduction was observed, but large counts of total bacteria were present in both the melon washwater and remaining on the rind after washing as compared to melons obtained from a different commercially harvested source (Table 7). Populations in the fruit flesh once the melons were washed and peeled was approximately log 5 [100,000] CFU/melon (Table 7).

Analysis of partial 16s rRNA sequences from coliforms and *E. coli* established the presence of *Enterobacter sp.* as a predominant coliform colony isolated from on melon and soil. In contrast, a deeper analysis of selected pure colny isolates by ribotyping revealed the presence of other bacteria species including *Enterobacter cloacae, Enterobacter cancerogenous, Enterobacter aerogenes, Klebsiella oxytoca, Pantoea agglomerans,* and *Serratia marcescens* which were isolated from melon samples around the field. Other isolates were identified as *Providencia rettgeri* and *Providencia spp.* were exclusively isolated from stacked manure and dairy waste lagoon related samples. In some cases, clusters were formed likely providing evidence that the same microorganism was isolated from two different samples or from two different zones which indicate that there was not a particular distribution of single group of microorganisms within a particular zone (Fig.4). Similarly, different *E. coli* clusters where formed for isolates from different sources and zones (Fig. 4B). Ribotyping of *E. coli* isolates, revealed the formation of 12 unique clusters, which likely belong to 12 different *E. coli* strains among the subset of total retained isolates submitted to Dupont/Qualicon technical staff for RiboPrinter® analysis. Among the 12 clusters of *E. coli* identified, clusters 1, 2 and 3

corresponded to *E. coli* isolated from animal corral surface material as well as from melon and soil samples across the field area (zones A, B and C). This demonstration of Bacterial Source Tracking output results suggests that the fecal indicator bacterial composition on fruit and crop production soil could be impacted by direct and indirect transport or point-source and non-point source transference of 'contamination'. The bacterial composition of the major crop-animal clusters was in proximity zones most related to corrals and dairy-waste handling (Fig 5). The remaining nine clusters were only assigned to a single location of the field, but likely this is due to the limited number of isolates analyzed in this preliminary technique demonstration assessment .

After evidence of potentially pathogenic *E. coli* (STEC), confirmation of *E. coli* O157:H7 , isolated but detectable presence of *Salmonella*, and the consistent detection of *stx2* in the sample enrichments, it was recommended not to harvest the melons for commercialization. The cooperating handler made this decision and the crop was destroyed by complete, multi-pass flail chopping and discing. After soil discing, a final microbiological assessment of the soil was performed. Compared to the first assessment, the population of total coliforms and *E. coli* increased around 3-log, likely due to the incorporation of organic matter and a release of sugars and nutrients from the melon. In this case, no significant difference was found for both bacterial groups among the three zones. Further molecular evidence of residual populations of potentially pathogenic *E. coli* (*eaeA* and *stx2*) was detected around the entire disced area in the three zones. The entire parcel was planted to a cover crop of 'green-manure' maize silage and grown for approximately 60 days before harvesting the primary foliage for animal feed and discing the residues according to standard practice. Fifty soil samples taken across the site revealed that indicator *E. coli* populations had uniformly declined in almost all zones to below the limit of detection, with the exception of four replicates (1 in Zone C and 3 in Zone A) . Closest to the corrals in Zone A, two sampling sectors had residual populations of log 3.62-3.78 CFU/g. Irrigation ditch water samples taken during the establishment of the cover crop revealed that, as with the initial samples taken at the beginning of the Rapid Response, high populations of *E. coli* were detected in the water only at the ditch area closest to the animal corral. This water was introduced to the field in Zone A and may explain the elevated levels after cover crop incorporation as compared to the rest of the field. Regardless, all previously documented virulence markers for EHEC/STEC in soil samples from these same sub-Zone sample sites were absent in analysis of soil-extract enrichments post-cover cropping. We feel this indicates one potential mitigation strategy for stabilization or recovery of contaminated soil due to events such as manure application or flooding.

Outcomes and Accomplishments

Since the 2006 *E. coli* O157:H7 outbreak associated with spinach consumption in addition to multiple outbreaks and produce related food-borne illness, including various *Salmonella* on imported cantaloupe, and more recently *Listeria monocytogenes* on domestic cantaloupe, there is a clear awareness and concern regarding the potential for contamination of fresh produce at any point along the supply-chain. Many potential sources of contamination are recognized including water and animal waste, whether applied as a soil amendment or due to proximity to and Animal Feeding Operation (AFO). In this study, production of cantaloupe intended for commercialization and broad distribution marketing was grown in close proximity (less than 10 m at one field boundary) to a dairy facility (AFO) which harbored approximately 200 animals and the usual associated potential animal-vectors of contamination dispersal. Although lagoon solids and non-composted manure was applied and incorporated in alignment with some schemes for current Best Practice recommendations of Good Agricultural Practices (GAP's) – application more than 120 days before harvesting-, this interval appears not to have been adequate or sufficient timeframe for the amount (unspecified but reported to be large) and soil-type/conditions at this site. Other Best Practice guidance, such as the Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens specify a one year interval, ***prior to planting***, following non-composted manure applications. At this location, independent of manure waste applications, domestic animal presence (waste and activity) and potential vector activity (birds, rodents, insects) as well as the presence of an active dairy waste lagoon, including aerosols generated, may have combined to significantly compromise the safety of this crop for human consumption.

- Coliform populations on melon were greater in fruit located closer to the animal corrals. Levels of total coliform exceeded, by as much as 1000-fold, that of cantaloupe taken from fields more distant from the dairy during the same harvest timeframe.
- Fecal indicators, *E. coli* and *Enterococcus,* were homogenously found on melon surfaces from this location but absent in melons harvested in other regional fields
- The specific quantitative, non-homogeneous distribution of indicators and detectable presence/absence of pathogens on fruit and in soil provide insights to both the importance of a comprehensive sampling regime to establish lot acceptance criteria and to verify any mitigations or corrective actions applied to minimize the contamination or survival of bacterial pathogens in a field.

- Pathogenic *E. coli*, *E. coli* O157:H7 molecular markers were consistently detected on melon surfaces and soil samples from areas closer to animal operations, corrals and lagoon, but pathogenic *E. coli* markers (*eae*A and *stx* genes) were also consistently found in other field zones across the cropped parcel that were farther from the animal operations, and many of the environmental samples collected including water, algae in irrigation source ditches, stacked manure, lagoon sediments, and animal corral surface material. Similar markers were not detected in cantaloupes taken from other regional fields in the same timeframe.
- Following secondary evaluations of initial PCR reactions, confirmed *Salmonella* molecular markers were found on one sampling date at a location distant from the animal operation but closer to the area associated with tailwater drainage and collection.
- Soil analysis provided evidence of the effect of substantial non-composted manure application on physicochemical parameters and nutrient composition that can favor microbial growth.
- Within this Rapid Response study, validation of a compositing protocol for cantaloupe surveys was demonstrated with post-enrichment pooling of five melons with no 'false-negative' outcomes due to dilution of individual samples during compositing.
- An enhancement of aerosol sampling technique using a soft-agar approach was validated at an off-site location (UCD feedlot) and we feel this data has practical applications for researchers using the agar-based capture method for particulate transport studies. Applied to the study site, limited transference from the AFO under the conditions evaluated was observed.
- Recovery of soil, as measured by decline of indicator *E. coli* populations to below the limit of detection in 46 of 50 soil samples and absence of residual EHEC/STEC virulence markers in all samples, following green-manure cover cropping provides some insights to mitigation strategies applicable to all fresh produce production.

Outbreaks and surveillance-associated recalls have been predominantly associated with *S. enterica* rather than with *E. coli* O157:H7 or other non-O157 EHEC/STEC. However, this study provides solid evidence of *E. coli* O157:H7 and other pathogenic *E. coli* to contaminate and persist on melon surfaces due to separate or combined contributions of site-specific soil amendment practices, potentially long-term and cumulative, and an immediately adjacent AFO. Limited but diagnostic evidence that viable *Salmonella* could be present on melon and in soil associated with this specific field supports that conclusion that the subject crop represented a

public health risk. Concerns for elevated risk were identified had harvest operations commenced and the field, based on observations during the study timeframe, would likely have attracted various potential vectors to damaged and over-ripe fruit from the AFO-side across multiple harvests.

All GAPs industry and federal guidance and industry standards strongly advise that Best Practice includes maximizing the distance between horticultural food operations (fresh produce production) and animal activity (including both domestic animal production, feeding operations, and concentrated resident or transient wildlife populations). However it is clear that there is a paucity of science-based evidence related to setting rational and functional setback distances and to minimize risks from adjacent land use and agricultural production. In this study we have provided evidence of the presumptive effect of both application of large amounts of manure over an extended period of time, potential inadequacy of current preharvest intervals following application of non-composted manure to soil, and the importance of hazard analysis of adjacent land use for potential risk exposure during production of cantaloupe and, likely, other fresh-consumed produce.

One additional accomplishment of this project that we feel has significant value was the rapid organization and collaborative leadership and facilitation achieved by the key parties intimately involved. The combined efforts and actions of the following groups, including the collective funding resources and in-kind donations, made this project possible in a rapid timeline and may serve as a template for organizing and guiding the execution of similar on-farm data-gathering opportunities;

- Individual handler/shipper responsible for cantaloupe marketing
- Suslow USDA SCRI grant related to melon food safety
- CPS – supplemental funding and Rapid Response Industry Steering Committee specific to this event (combined five conference calls and in-person review of study design and progress of research effort)
- CA Melon Research Board and CA Cantaloupe Advisory Board
- Western Growers Association
- Prof. S. Wuertz and Dr. A. Schriewer– UCD Environmental Engineering for rapid biomaterials transfer of *Enterococcus* probes
- Dupont/Qualicon – dontaiton of time, materials, and technical expertise for RiboPrinter® analysis

Summary of Findings and Recommendations

In this study, production of melons intended for commercial marketing were grown in close proximity to a dairy cattle facility. Proximity issues due to animal presence and activity, abundant vector potential between the dairy and the crop, as well as crop and dairy-related human activity appear to have significantly compromised the safety of this crop. However, the pre-plant application of manure and lagoon waste, including prior applications over a long period of time, appear to have made the establishment of clear spatial buffer and gradient between the AFO and cantaloupes targeted for harvest impossible. While we feel that a case for in-season transfer of STEC from the AFO, within 200 feet of the eastern boundary, might be made scientifically the risks associated with this site necessitated and supported the full crop-destruct decision.

Within this opportunity to evaluate natural persistence and contamination events, our findings clearly and strongly support existing guidance to maximize the distance between agricultural operations (fresh produce production) and animal activity, however it remains inconclusive to establish a science and data-based spatial limit. We feel broad dissemination of the results from this study, especially the documented spatial distribution of fecal indicators and pathogenic bacteria in both crop and soil across a large parcel, will be of significant value to the research community and public health agencies in refining ag-environmental sampling plans for food safety assessments or outbreak investigations. Equally, the key outcomes from this Rapid Response study site may be used by industry to reassess or validate current and evolving standards.

BACKGROUND REFERENCES

1. A. Castillo, I. Mercado, L.M. Lucía, Y. Martínez-Ruiz, J. Ponce de León, E.A., Murano and G.R. Acuff, Salmonella contamination during production of cantaloupes: a binational study. Journal of Food Protection, 67 (2004), pp. 713–720
2. AOAC Official Method 972.43, Microchemical Determination of Carbon, Hydrogen, and Nitrogen, Automated Method, in Official Methods of Analysis of AOAC International, 16th Edition (1997), Chapter 12, pp. 5-6, AOAC International, Arlington, VA.
3. B. Fremaux, C. Prigent-Combaret, M.L. Delignette-Muller, M. Dothal, B. Mallen, A. Gleizal and C. Vernozy-Rozand, Persistence of Shiga-toxin producing Escherichia coli O26 in various manure-amended soil types. J. Appl. Microbiol., 104 (2008), pp. 296–304.

4. Bertrand, R. and B. Roig (2007). "Evaluation of enrichment-free PCR-based detection on the rfbE gene of Escherichia coli O157--Application to municipal wastewater." Water Research 41(6): 1280-1286.
5. Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37 (Database issue): D141-D145; doi: 10.1093/nar/gkn879.]
6. Dulla, G. F. J., & Lindow, S. E. (2009). Acyl-homoserine lactone-mediated cross talk among epiphytic bacteria modulates behavior of Pseudomonas syringae on leaves. ISME J, 3(7), 825-834.
7. Food and Drug Administration (FDA), Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables, Center for Food Safety and Applied Nutrition, Washington, DC (2008) http://www.gpo.gov/fdsys/pkg/PLAW-111publ353/pdf/PLAW-111publ353.pdf.
8. Food and Drug Administration (FDA), Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Melons; Draft Guidance. 2009.
9. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ProduceandPlanProducts/ucm174171.htm
10. Haack, S. K., Duris, J. W., Fogarty, L. R., Kolpin, D. W., Focazio, M. J., Furlong, E. T., Meyer, M. T. (2009) Comparing wastewater chemicals, indicator bacteria concentrations, and bacterial pathogen genes as fecal pollution indicators. J. Environ. Qual. 38:248-258.
11. Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.
12. Harris, D., Horwáth, W. R. and Van Kessel, C. 2001. Acid fumigation of soils to remove carbonates prior to total organic carbon or CARBON-13 isotopic analysis. Soil Science Society of America Journal 65:1853-1856.
13. Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., and Dufour, A. P. (2005). Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. 36:559-568.
14. J.A. Thurston-Enriquez, J.E. Gilley and B. Eghball. (2005). Microbial quality of runoff following land application of cattle manure and swine slurry. J. Water Health, 3:157–171.
15. Klute, A. (1986). Water retention: laboratory methods. p. 635-662. In: A. Klute (ed.) Methods of soil analysis: Part 1. Physical and mineralogical methods. ASA Monograph Number 9.

16. L.R. Beuchat. (1996). Pathogenic microorganisms associated with fresh produce. Journal of Food Protection, 59: 204–216.
17. M.T. Brandl. (2006) Fitness of human enteric pathogens on plants and implications for food safety. Annual Review of Phytopathology, 44 :367–392.
18. Rible, J. M. and Quick, J. (1960). Method S-19.0. Cation Exchange Capacity. In: Water soil plant tissue. Tentative methods of analysis for diagnostic purposes. Davis, University of California Agricultural Experiment Service. Mimeographed Report.
19. S. Alvarado-Casillas, L.S. Ibarra-Sánchez, N.E., Martínez-González and A. Castillo. (2010). Validation of a washing a sanitizing procedure for cantaloupes at a Mexican packing facility. Journal of Food Protection, 73:362–365.
20. Sheldrick, B. H. and Wang, C. (1993). Particle-size Distribution. pp. 499-511. In: Carter, M. R. (ed), Soil Sampling and Methods of Analysis, Canadian Society of Soil Science, Lewis Publishers, Ann Arbor, MI.
21. T.V. Suslow, M.P. Oria, L.R. Beuchat, E.H. Garret, M.E. Parish, L.J. Harris, J.N. Farber and F.F. Busta. (2003). Production practices as risk factors in microbial food safety of fresh and fresh-cut produce. Comprehensive Reviews in Food Science and Food Safety, 2 :38–77.
22. U.S. Salinity Laboratory Staff. (1954). Choice of determinations and interpretation of data. p. 26. In: L. A. Richards (ed.) Diagnosis and improvement of saline and alkali soils. USDA Agric. Handb. 60. U.S. Government Printing Office, Washington, D.C.
23. United States Department of Agriculture, Natural Resources Conservation Service. (2004). Soil survey laboratory methods manual. Version No. 4.0. Soil Survey Investigations Report No. 42. http://soils.usda.gov/technical/lmm/
24. Wang, Q, G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 73(16):5261-7.
25. Yoshitomi, K. J., K. C. Jinneman, et al. (2006). "Detection of Shiga toxin genes stx1, stx2, and the +93 uidA mutation of E. coli O157:H7/H-using SYBR® Green I in a real-time multiplex PCR." Molecular and Cellular Probes 20(1): 31-41.

APPENDICES

Publications and Presentations (required)

No publications for this project have been submitted at the time of filing this Final Report but this activity will be the next step to enter the methodologies addressed and data developed into the public domain within a peer-reviewed journal.

Overviews of the objectives and outcomes of this project have been presented to the CA Melon Research Board (Annual Research Meeting January 12, 2012; San Diego, CA) and the United Fresh Produce Association Winter Leadership Meeting (Food Safety and Technology Council; January 24, 2012; San Diego, CA).

Budget Summary (required)

The accomplishment of this project involved the combined funding of three sources. From the budgeted amounts of $9550 for supplies, and $450 for travel, all funds allocated by CPS were used for microbiological media, disposable labware, PCR reagents, and partial support for travel to the study site for sampling on nine dates. The disposition of the final accounting is in progress.

Suggestions to CPS (optional)

Given the nature of the sensitivity of the natural contamination event and the speed at which definition of a Rapid Response project and supporting incremental funding had to be organized we feel that all involved at CPS did a remarkable job. There was a narrow window of time for a crop very near harvest maturity to maximize the data collection for a range of areas of interest. The integrated effort of the CPS Executive Director, members of the Advisory Committee, Chair of the Technical Committee, and selected members as project reviewers, to facilitate the capture the unplanned opportunity was highly effective.

Going forward, we are open to and suggest that CPS coordinate with the PI the definition and dissemination of key outcomes and recommendations from this specific project and the value of positioning regional researchers and industry to evaluate and execute Rapid Response efforts on an annual basis.

Tables and Figures (optional)

Table 1. Taqman ® probes and primers utilized in this study for detection of *E. coli* O157:H7

Probe/primer	Sequence (5'- 3')	Reference
TPEC (Total pathogenic E. coli)		
TPEC (probe)	6FAM-TGCTTCTGTGTATCAGGG-MGBNFQ	
TPEC(forward)	TGATCACTGGCGGCGATA	
TPEC(reverse)	TATGATGTCCTCATCTTCAGAGAGAAC	
eaeA (probe)	FAM-AAAACGCTGACCCGCAC-MGBNFQ	
eaeA (forward)	CCAACATGTTTGCAGGAAGGA	Modified from Yoshitomi, et al. 2006
eaeA (reverse)	CCCGCTTTACGGCAAATTTA	
$rfbE_{O157}$ (probe)	NED-CAAAAGCACCCTATAGCT-MGBNFQ	
$rfbE_{O157}$ (forward)	GATGCCAATGTACTCGGAAAAAT	Bertrand and Roig 2007
$rfbE_{O157}$ (reverse)	CCACGCCAACCAAGATCCT	
stx1 (probe)	FAM-TGATGAGTTTCCTTCTATGTGTC- MGBNFQ	
stx1 (forward)	GTGGCATTAATACTGAATTGTCATCA	Modified from Yoshitomi, et al. 2006
stx1 (reverse)	GAAGAGTCCGTGGGATTACGC	
stx2 (probe)	FAM-CCGCCATTGCATTAACAGA- MGBNFQ	Modified from Yoshitomi, et al. 2006
stx2 (forward)	TGGAAAACTCAATTTTACCTTTAGCA	
stx2 (reverse)	GCAAATAAAACCGCCATAAACATC	

Table 2. Assessment of total coliforms population on melons collected at the field.

	Population of coliforms (log CFU/melon)			*Enterococcus sp.* (log cells/melon)[*]
Field zone	**Miminum population size**	**Mean population[§]**	**Maximum population size**	
A	6.10	6.88 ± 0.40^{a}	7.62	3.02 ± 0.11^{a}
B	6.14	6.52 ± 0.22^{b}	6.90	2.24 ± 0.65^{a}
C	4.75	6.30 ± 0.54^{b}	6.95	2.57 ± 0.77^{a}
Outsource sample[¥]	3.13	5.19 ± 1.20^{c}	6.50	Not detected

(*) Values represent mean ± standard deviation (n=2, 5 and 10 for zones A, B and C respectively). Different low case letters within the same column indicate significant difference among the samples ($p>0.20$).
(§) Values represent mean ± standard deviation (n=20). Different low case letters within the same column indicate significant difference among the samples ($p<0.0001$).
(¥) Samples were collected from a different commercial field.

Table 3. Assessment of total coliforms, *E. coli* and *Enterococcus sp.* populations in environmental samples collected around the melon field area.

Sample type and location	Total coliforms	*E. coli*	*Enterococcus*
Water samples[*]	(log MPN/100 mL[§])		(log cells/100 mL)
Above lagoon	3.79 ± 0.09	1.89 ± 0.04	Not detected
In front of cow corral (zone A)	3.85 ± 0.00	1.64 ± 0.00	Not detected
Next to corral (zone A)	3.89 ± 1.99	2.70 ± 1.53	2.16 ± 0.00
Zone C	3.53 ± 0.41	0.30 ± 0.00	Not detected
Near canal	3.29 ± 0.30	1.09 ± 0.41	Not detected
	(log CFU/g of sample[§])		(log cells/100 g)
Algae[*]			
Zone A	4.51 ± 1.34	2.17 ± 0.77	ND
Zone B	3.63 ± 0.06	2.48 ± 0.56	ND
Zone C	3.45 ± 0.14	1.43 ± 0.00	ND
Soil[*]			
Zone A	3.95 ± 0.37	ND	Not detected
Zone B	3.07 ± 0.74	ND	Not detected
Zone C	3.24 ± 1.25	ND	Not detected
Lagoon	1.93 ± 0.83	1.69 ± 0.36	ND
Manure	1.43 ± 0.00	3.25 ± 0.34	4.45 ± 0.73
Compost	3.86 ± 0.38	1.43 ± 0.00	Not detected
Air sampling[¥]			
South	7/10	0/10	ND
North	6/10	0/10	ND

(§) Values represent mean ± standard deviation (n=2 samples). Detection limit log -0.05 MPN/100 mL and log 1.43 CFU/g of sample.
(*) Not significant difference ($p>0.2$) was determined among different samples for both assessed populations.
(¥) Populations were below the limit of detection and thus samples were enriched. Results represent positive samples/total samples collected after enrichment. Enrichments were considered positive when presence of purple colonies was evidenced on Chrom ECC.
(ND) Not determined

Table 4. Pathogen detection in melon and environmental samples during microbiological assessment of field.

		Taqman® assays						Commercial detection kits			
Sample source		TPEC	*eaeA*	*rfbE*$_{O157}$	*stx1*	*stx2*	*invA*	GDS O157	BAX O157	GDS Salmonella	BAX Salmonella
Melon	Zone A*	4/8	4/8	1/8*	0/8	1/8*	0/8	2/8	1/8	0/8	0/8
	Zone B	1/10	1/10	0/10	1/10	1/10	1/10	0/10	0/10	0/10	1/10
	Zone C	1/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Water		2/2	2/2	0/2	1/2	2/2	0/2	0/2	0/2	0/2	0/2
Algae		2/2	2/2	0/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2
Lagoon		1/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1
Manure		1/1	1/1	0/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1
Air		0/4	0/4	ND	ND	ND	ND	ND	ND	ND	ND
Soil	Zone A	1/1	1/1	0/1	ND	ND	ND	0/1	0/1	0/1	0/1
	Zone B	1/1	1/1	1/1	ND	ND	ND	1/1	0/1	0/1	0/1
	Zone C	1/1	1/1	0/1	ND	ND	ND	0/1	0/1	0/1	0/1

Results represent total positives/total number of composites. Each composite consisted in 5 polled enrichments from each sample and source. In the case of melons, each sample consisted of 3 melons.
(*) Colony confirmation was obtained from one of the composites. The colonies were obtained from one of the samples. A typical colony was further screened for genotyping which evidenced presence of *rfbE*$_{O157}$, *fliC*$_{H7}$, *eaeA* and *stx*2 genes.
(ND) not determined

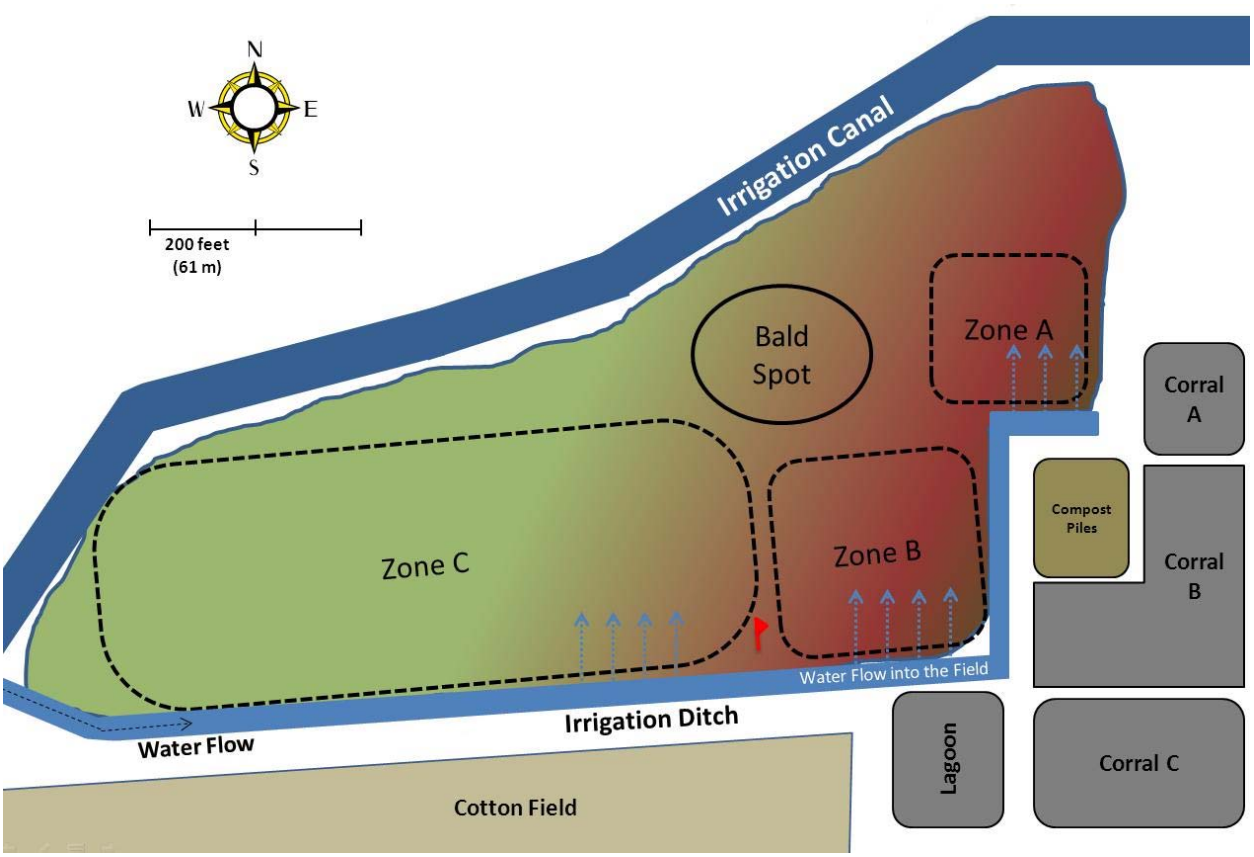

Figure 1. Schematic of melon field and surrounding areas.

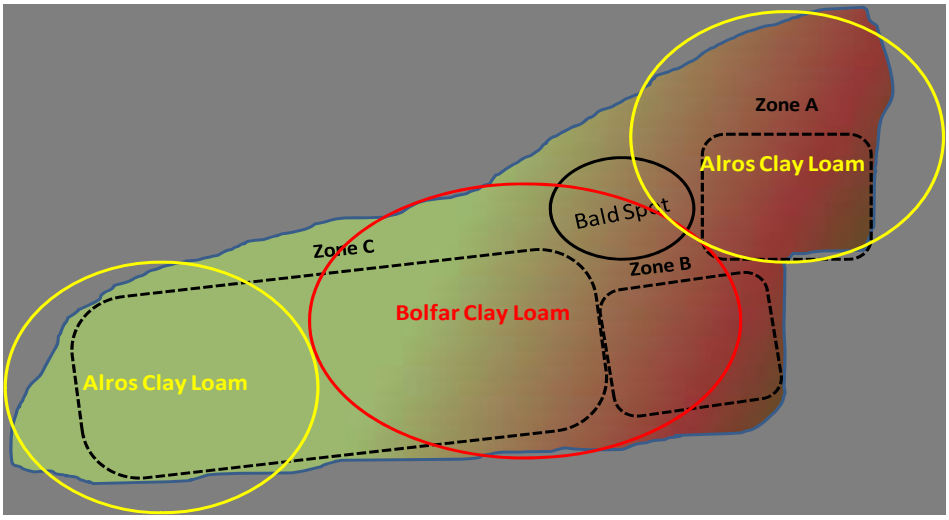

Figure 2. Soil type distribution at the evaluation site based on the California Soil Resource Lab survey.

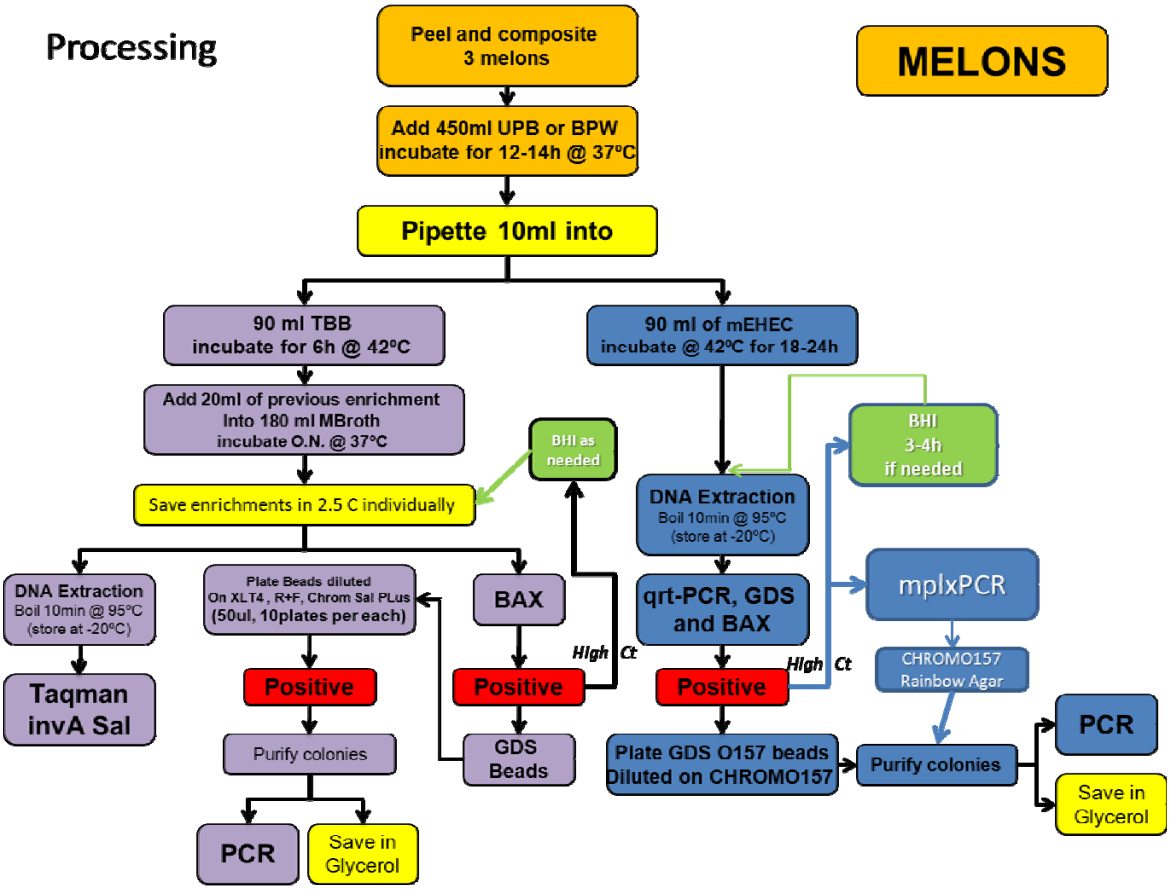

Figure 3. Process Flow diagram for sampling, processing, detection, recovery, and verification of *E. coli* O157:H7, non-O157 Shigatoxin positive *E.coli*, and *Salmonella* on cantaloupe. For details contact tvsuslow@ucdavis.edu

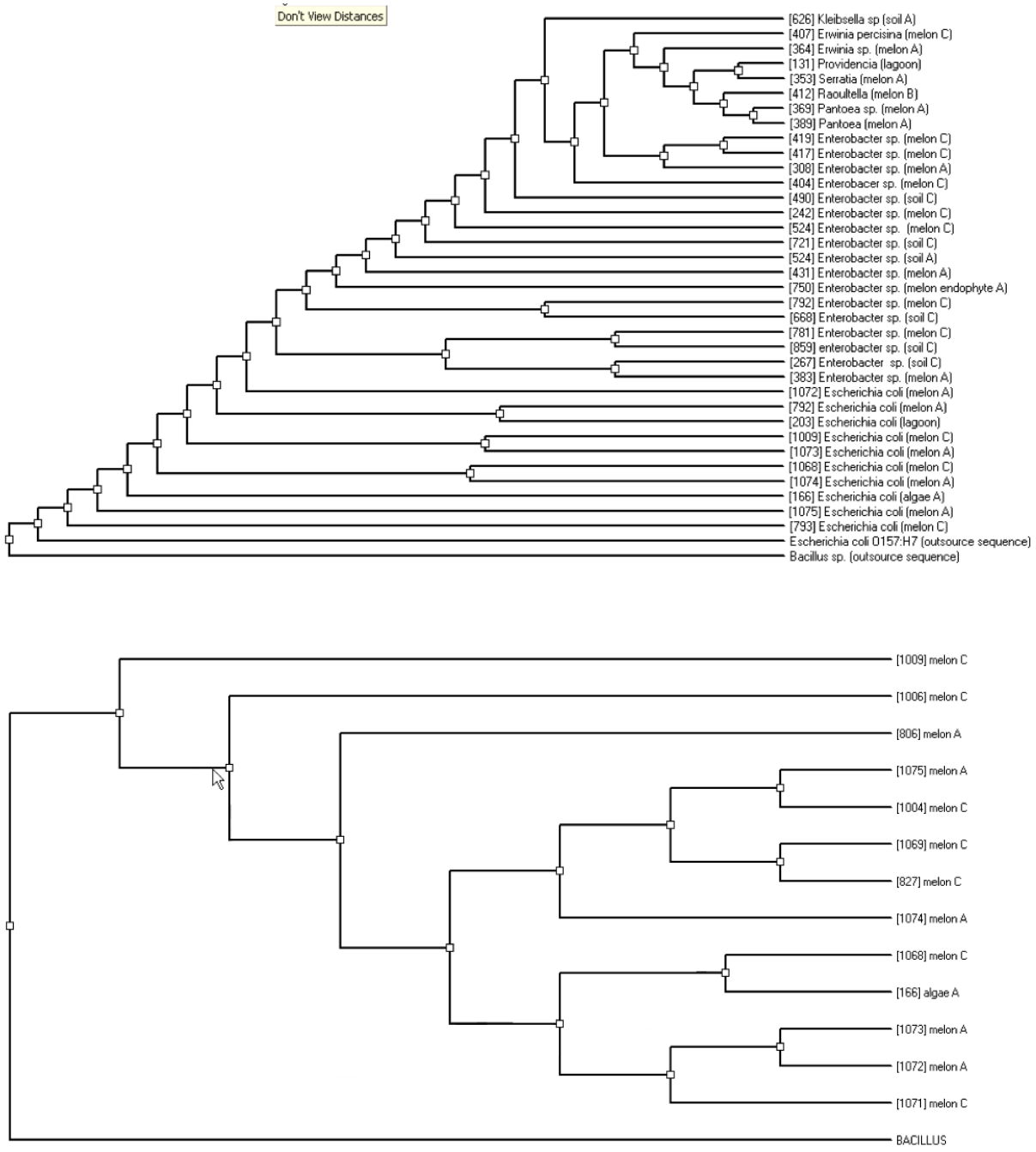

Figure 4 A (upper) and B (lower). Phyllogenetic tree of 16S rRNA sequences from colonies isolated from melon and environmental samples. [colony identification number] Bacterial classification (sample type and zone source). Outsource sequences were obtained from the National Center for Biotechnology Information (NCBI).

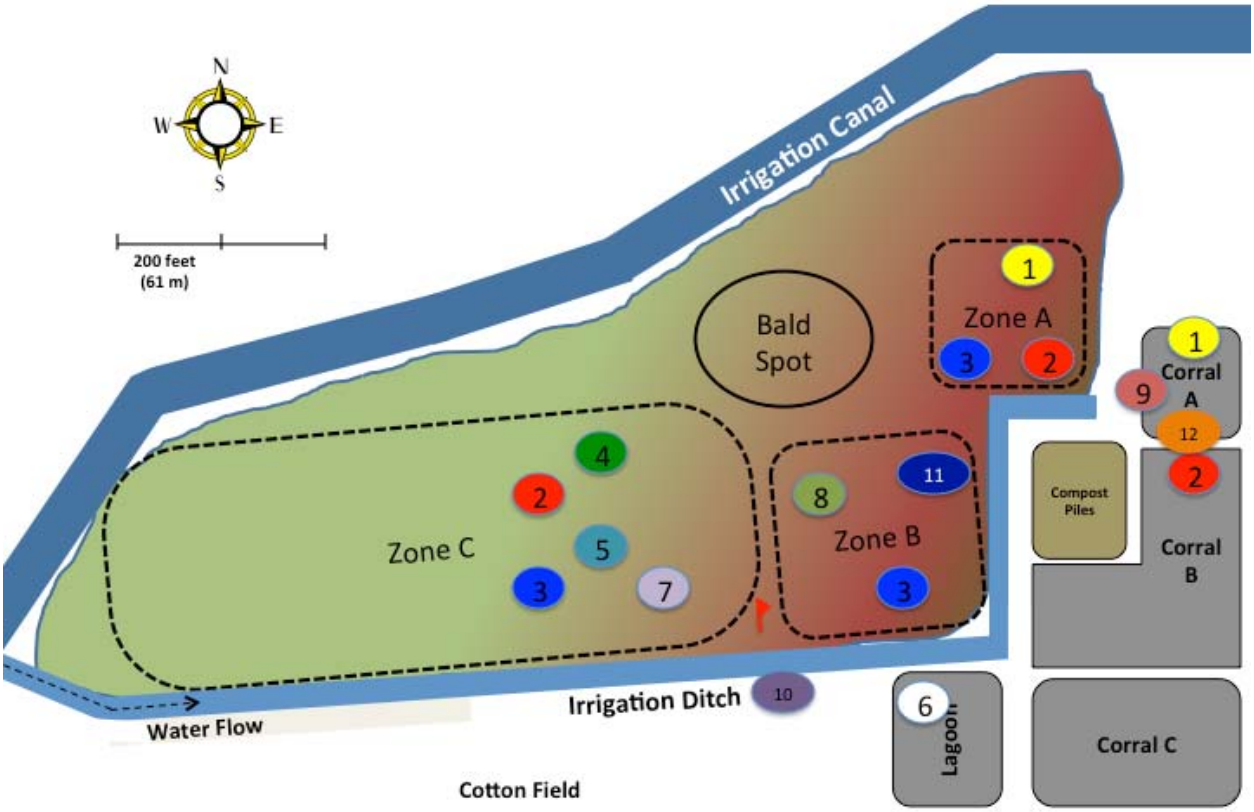

Figure 5. Different *E. coli* clusters (represented in colored/numbered circles) isolated from different sections in the field. Circles with same color/number indicate that isolates obtained from each source and spatial location shared at least 85% similarity after classification through ribotyping and thus was considered the same genetic sub-type of indicator *E. coli.*

Center for Produce Safety

2011 Final Report – All Technical Contributors to Research Accomplishments and Outcomes

Project title: Rapid Response Melon Food Safety

Project Director :

Trevor Suslow
One Shields Ave.
University of California, Davis
Department of Plant Sciences
Mann Lab Mail Stop 3
Davis, CA 95616-8780
530-754-8313
530-752-4554 fax
tvsuslow@ucdavis.edu

Key personnel:

Adrian Sbodio; Staff Research Associate
Gabriela Lopez-Velasco; Postdoctoral researcher
Alejandro Tomas-Callejas; Postdoctoral researcher
Eduardo Gutierrez; Doctoral candidate
Polly Wei; Junior Specialist II
Xuan Pham; Junior Specialist I
Dawit Diribsa; Junior Specialist I

Other personnel:

Alex Camacho; Undergraduate lab assistant
Lee Ann Richmond; Mann Lab manager
Ravi Nakrani; Junior Specialist I
Jocelyn Lam ; Undergraduate lab assistant
Laura Huang; Undergraduate lab assistant
Heather Abbot; Undergraduate lab assistant

Dupont/Qualicon

Julie Kraynak
Stacey Stolenberg
Amy Smith
Casey Simmons

Location of Work:

UC Davis Mann Lab and Department of Plant Sciences Research Farm Facility. Cooperating Grower fields.

Table 5. Soil analysis during field assessment

Zone	N (Total) %	C (Total) %	Nitrate (Kg/Ha)	Ammonium (Kg/Ha)	Total mineralized N (Kg/ha)	OM (LOI) %	C-Org-LOI %	Ec (mS)	CEC (meq/100g)
A	0.27 ± 0.03[a]	2.38 ± 0.31[a]	124 ± 33.9[a]	2.81 ± 0.08 [a]	127 ± 34.0 [a]	4.01 ± 0.29[b]	2.33 ± 0.16[b]	0.41 ± 0.06[a]	26.6 ± 0.35[a]
B	0.30 ± 0.00[a]	2.73 ± 0.01[a]	149 ± 21.2 [a]	2.77 ± 0.09 [a]	151 ± 21.2 [a]	5.07 ± 0.13[a]	2.94 ± 0.08 [a]	0.30 ± 0.40[b]	28.4 ± 1.20[a]
C	0.19 ± 0.01[b]	1.72 ± 0.08[b]	128 ± 28.5 [a]	2.82 ± 0.09 [a]	131 ± 28.5 [a]	3.03 ± 0.15[c]	1.76 ± 0.08 [c]	0.29 ± 0.04[b]	22.0 ± 1.37[b]

Zone	pH	SAR	Ca (meq/100g)	Mg (meq/100g)	K (meq/100g)	Sand %	Silt %	Clay %	Water Retention (0.33 ATM) %
A	7.17 ± 0.08[b]	2	15.2 ± 0.32 [a]	6.65 ± 0.16 [a]	3.08 ± 0.90 [a]	42.0 ± 1.00[b]	32.0± 1.00[a]	26.0 ± 0.00[a]	27.3 ± 1.33[a]
B	6.52 ± 0.29[c]	2	16.1 ± 1.98 [a]	6.06 ± 0.28 [b]	1.48 ± 0.00 [b]	43.0 ± 4.24[a,b]	31.0 ± 2.38[a]	26.0 ± 1.41 [a]	24.8 ± 1.13[a]
C	7.50 ± 0.17[a]	2	17.8 ± 1.67 [a]	5.63 ± 0.03 [c]	1.07 ± 0.10 [b]	47.6 ± 2.57[a]	29.0 ± 1.41[a]	23.4 ± 1.27[b]	22.4 ± 0.84[b]

Results represent mean ± standard deviation (n=2, 2 and 6 samples from zones A, B and C respectively. Each sample is a representation of a composite of 5 subsamples)

Different low case letter within the same column represent significant difference ($p<0.05$) among the three zones per each soil parameter analyzed.

Table 6. General Characteristics for each soil within a depth range of 15in from soil surface (California Soil Resource Lab)

Soil Type	Organic Matter %	Clay %	Sand %	Silt %	CEC Meq/100g	pH	Ec (ds/m)	SAR
Alros Clay Loam	0.75	31	35.4	33.6	15	8.2	3	10
Bolfar Clay Loam	2	31	35.4	33.6	20	7.9	0	0

From the investigated locations; zone A is composed mainly of Alros Clay Loam, zone B is composed mainly of Bolfar Clay Loam while zone C is a combination of both soil types. Samples 1 through 45 are located within the Alros Clay Loam portion of the field while samples 46 through 90 are located within the Bolfar Clay Loam portion.

Table 7A. Pre-harvest microbiological assessment of melon

	Total coliforms[(*)]		***E. coli*** [(*)]		***Enterococcus sp.***[(*)]		**Detection of melon endophytic coliforms**[(§)]		
Melon Source	(log CFU/melon)	n		n	(log cells/melon)	n	F	FSS	SS
Zone A	6.82 ± 0.66[a]	10	4.07 ± 0.65[a]	7	4.12 ± 0.94[a]	5	7/7	7/7	7/7
Zone B	ND		4.06 ± 0.32[a]	3	4.16 ± 0.92[a]	9	4/4	4/4	4/4
Zone C	6.89 ± 0.64[a]	75	3.86 ± 0.43[a]	15	3.91 ± 1.41[a]	17	14/14	13/14	12/14
Outsource[(¥)]	2.49 ± 0.79[b]	18	Not detected	18	Not detected	6	14/42	16/42	18/42

[(*)] Values represent the mean ± standard deviation and n=number of samples (for coliforms and *Enterococcus sp.* each sample was composed of three and one melon respectively). Different low case letters within the same column, indicate significant difference ($p<0.05$) among the samples.

[(§)] Results represent number of positive samples/total number of samples. Positive presence of coliforms was determined after enrichment of the surface sterilized fruit flesh (F), fruit next to the stem scar (FS) and in the melon stem scar (SS).

[(¥)] Samples were collected from a different commercial field.

(ND) Not determined

Table 7B. Comparison of coliform population between upper-rind and soil-contact melon rind hemispheres, collected in Zone A.

	Mean population	Maximum	Minimum	Δ(top-bottom)[*]	p-value
	log CFU/melon				
Top	6.82 ± 0.66	7.41	5.69	0.78	0.027
Bottom	6.04 ± 0.95	7.7	4.81		

Results represent mean ± standard deviation (n=10 melons). Significant difference was established by t-test if p-value <0.05

(*) D represent the log difference between top and bottom coliform populations from melon hemispheres. Soil-contact (bottom) refers to that melon rind half in direct contact with soil, compared to top melon half which is the corresponding rind facing away from the soil.

Table 8. Effect of washing after postharvest storage and handling in total bacterial population

Zone	Melon wash	Melon peel (after washing)	Melon flesh (after washing)
	log CFU/melon		
A	9.03 ± 0.39[a]	8.17 ± 0.45[a]	5.78 ± 0.69[a]
B	9.15 ± 0.07[a]	8.40 ± 0.00[a]	5.70 ± 0.72[a]
C	8.53 ± 0.55[a]	7.82 ± 0.62[a]	5.45 ± 0.68[a]
Outsource melon(¥)	3.53 ± 1.16[b]	ND	ND

(*) Values represent the mean ± standard deviation. Different low case letters within the same column, indicate significant difference (p<0.05) among the samples.

(¥) Samples were collected from a different commercial field.

(ND) Not determined

Table 9. Soil microbiological assessment after field discing.

	Total coliforms	*E. coli*	Pathogen detection(§)	
Zone	**(log CFU/ g of soil)***		***eaeA***	***stx2***
A	6.47 ± 0.23[a]	4.39 ± 0.66 [a]	2/2	1/2
B	6.35 ± 0.26 [a]	4.11 ± 0.17 [a]	2/2	1/2
C	6.46 ± 0.19 [a]	4.35 ± 0.84 [a]	4/6	2/6

(*) Values represent mean ± standard deviation (n=10, 10 and 24 for zones A, B and C respectively). Different low case letter indicate significant difference ($p<0.05$).

(§) Results represent positive composites/total number of composite analyzed. Each composite consisted in 5 polled enrichments from each soil sample. Detection of other molecular markers (*rfbE, stx1, invA*) as well as detection with GDS and BAX systems was negative.

Table 10. Effect of alternating Wet-Dry cycles on indicator bacteria populations in retained study-site soil

Days	***E. coli***			**Total Coliforms**		
	(log CFU/g of dry Soil)*			**(log CFU/g of dry Soil)***		
	Dry	**Wet-Dry**	**difference**	**Dry**	**Wet-Dry**	**difference**
0	2.94 ± 0.24	2.94 ± 0.24	0.00	4.77 ± 0.22	4.77 ± 0.23	0.00
3	2.08 ± 0.27	1.65 ± 0.12	0.43	4.86 ± 0.25	4.02 ± 0.14	0.84
5	1.75 ± 0.31	1.53 ± 0.06	0.22	4.79 ± 0.35	4.23 ± 0.16	0.56

7	1.43 ± 0.00	1.57 ± 0.13	-0.14	4.15 ± 0.44	3.69 ± 0.28	0.46
10	2.14 ± 0.30	1.66 ± 0.22	0.48	4.48 ± 0.13	3.89 ± 0.15	0.59
18	1.43 ± 0.00	1.45 ± 0.01	-0.02	3.55 ± 0.23	2.95 ± 0.14	0.59

(*) Values represent mean ± standard error (n=5). Limit of Detection: log 1.43 CFU/g of dry soil

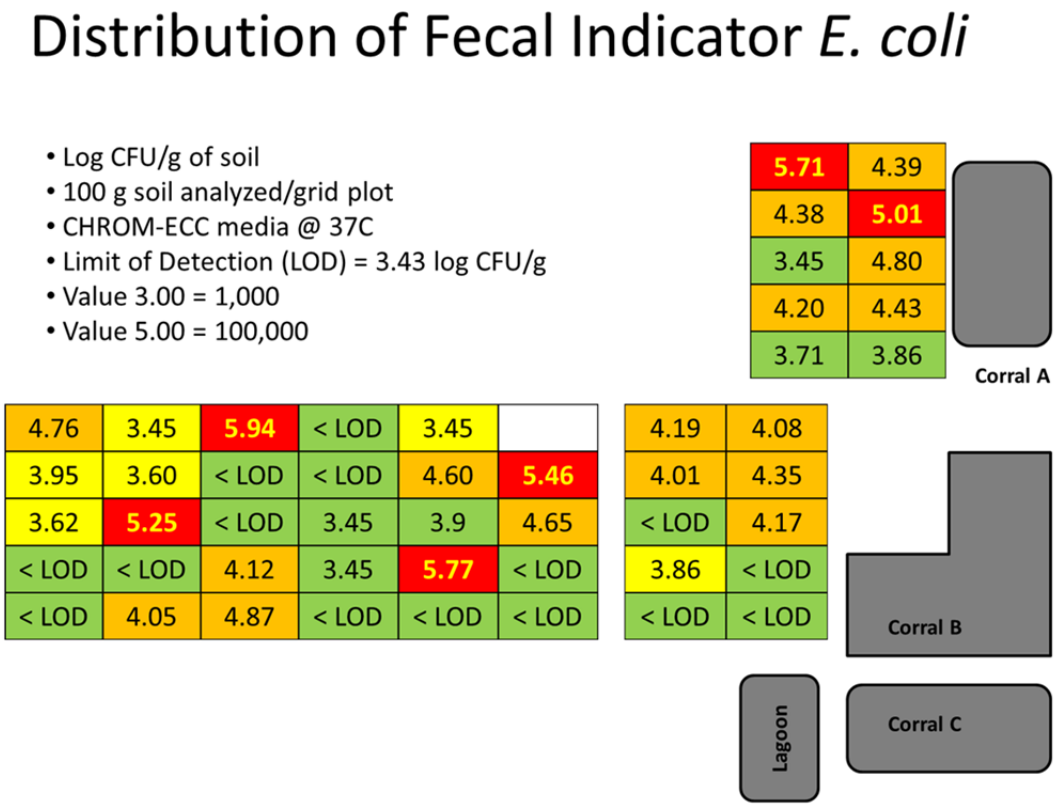

Figure 6. Representation of grid-analysis of fecal indicator E. coli in study-site soil as a function of proximity to the dairy operation.

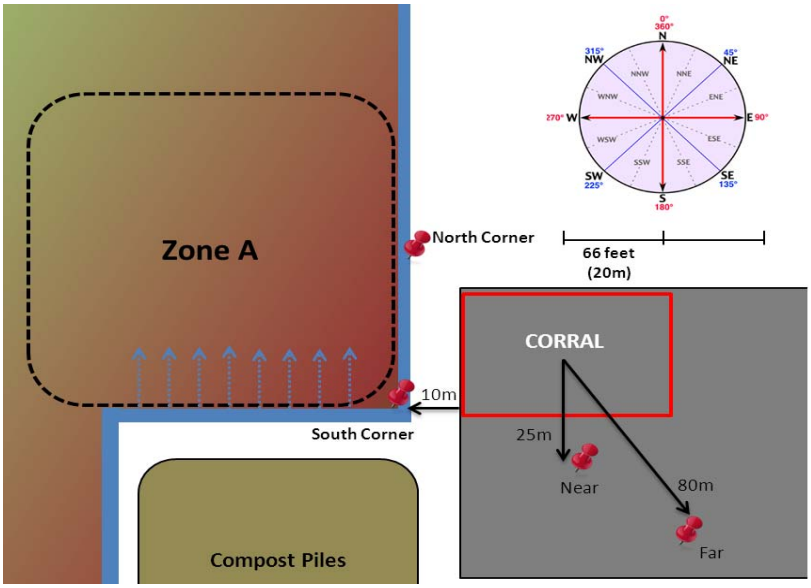

Figure 7. Detailed schematic of ditch water, aerosol capture, and AFO surface material sampling locations at study-site.

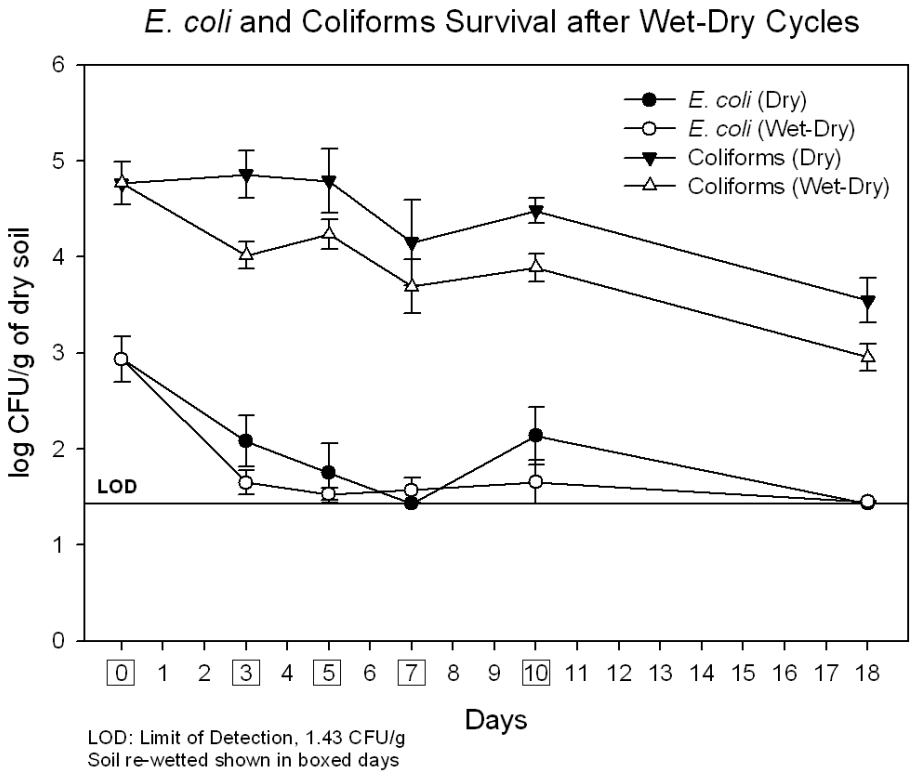

Figure 8. Persistence of total coliforms and generic *E. coli* in composited RR 17 soil collected on sampling date 4 prior to discing of the production field