# Annual Leafy Greens Research Report

## For the Period April 1, 2014 to March 31, 2015

1. **SUMMARY:** Please submit as a separate file. This paragraph, which should be written in layman's terms, will be used in a flyer mailed to the leafy greens industry. The flyer is intended to give industry members a quick overview of what has been accomplished over the past year and to direct them to the CLGRP web site for more in depth information.

**REPORT**

**Project Title: Development of an alternative irrigation water quality indicator and risk management tool for pathotoxigenic *E. coli*.**


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**Abstract:** Growers and handlers of lettuce and leafy greens have years of experience in routine monitoring and assessment of the microbiological quality of agricultural water used for irrigation and crop protection applications using the current standards prescribed by the California Leafy Green Products Handler Marketing Agreement (LGMA) based on quantitative levels of generic (nonpathogenic indicator) *E. coli*. *E. coli* levels are intended to indicate or provide presumptive evidence for chronic or acute fecal contamination of the water source or the water conveyance and distribution system. Prior to and upon the release for public comment of the proposed regulations implementing the provision in the FDA Food Safety Modernization Act (FSMA) related to produce safety (Subpart E § 112.41 *Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; Proposed Rule* (Produce Safety Rule)), there was a significant level of concern surrounding the specifics associated with these existing industry standards and anticipated federal agricultural water regulatory limits. In response to thousands of public comments on agricultural water standards alone, the 2014 Proposed Supplemental Rule substantially modified the anticipated requirements and provided for a "die-off" provision between irrigation and harvest dates but retained generic *E. coli* as the fecal indicator bacteria (FIB) for compliance. An alternative and well-established FIB to monitor contamination of water sources is the Gram-negative obligate anaerobic bacterium *Bacteroides*. *Bacteroides*, in general, make up a substantial proportion of bacterial populations in fecal matter, as much as 40% of the bacterial populations and 10% of the fecal mass, and are not known to have growth potential in the environment. Recent developments in rapid, quantitative, real-time PCR methods and first generation commercial Total Bacteroides qRTPCR kits make application in service labs for routine agricultural water testing a plausible

reality. With these improved techniques, substantial gains in knowledge regarding the association of this indicator to fecal sources of water contamination and correlation to presence of bacterial, viral, and parasite pathogens have been achieved. Perhaps more importantly, the costs of current rapid test kits have put this FIB in reach and within typical operator skill-level for high volume testing by service labs.

This project was initiated to develop preliminary data to support a *proof of concept* to replace quantitative irrigation water standards based on generic *E. coli* with a qualitative (semi-quantitative) presence-absence standard based on Bacteroides as an improved indicator of fecal contamination.

**Objectives**:

1. To develop an initial baseline of comparative data for indicator *E. coli*, Total Bacteroides, enterohemorrhagic *E. coli* (EHEC) and Salmonella in surface water sources used for leafy greens irrigation management in CA production districts.
2. Assess a novel pre-commercial PCR test that specifically differentiates clinically-relevant shigatoxin-producing *E. coli* (STEC) from those lacking this critical virulence marker but have been known to potentially trigger a crop destruct decision in current STEC/EHEC screening of leaf greens.

**Procedures:**

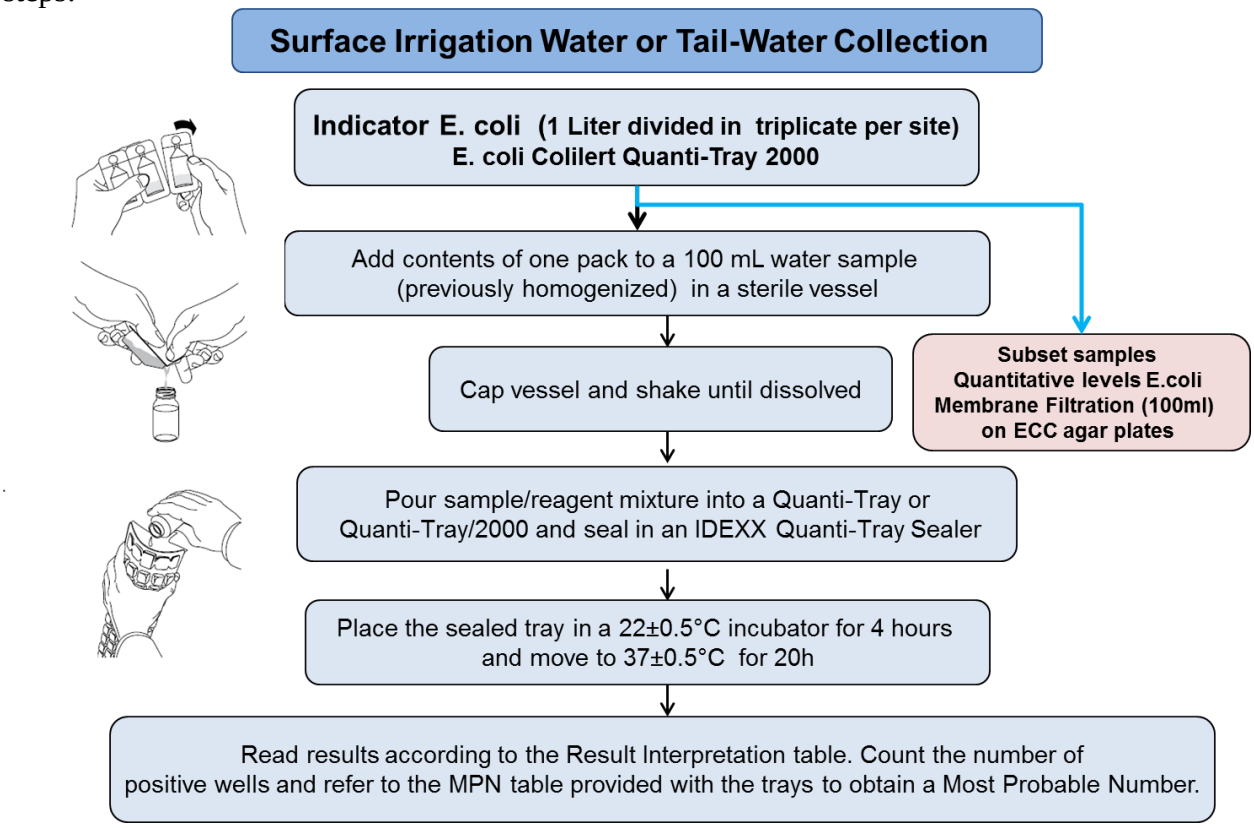
OBJECTIVE 1- The initiation of this project focused on the optimization of real-time PCR detection of Total Bacteroides (Total Bacteroidales) in water of decreasing quality and validation of quantitative detection limits. This activity was completed and a Standard Operating Procedure for sampling, filtration, cell lysis, and PCR standard curves for predicting "cell number" (copy-number equivalents) has been applied to seasonally collected water samples. In conjunction with an expended version of this project, co-funded by Center for Produce Safety which began at the mid-point of this CLGRB project, standard protocols for post-enrichment detection of pathogens in water samples was also completed and a pre-survey verification of performance with ROKA Atlas Transfer Tubes as the primary pathogen screen was completed. The methods and data developed to establish the SOP validation are not included in this report to the industry but are available upon request. The methods and results, including graphs of Standard Curves for Total Bacteroides, and performance of commercial kits and custom PCR systems will be included in planned peer-reviewed journal manuscripts.

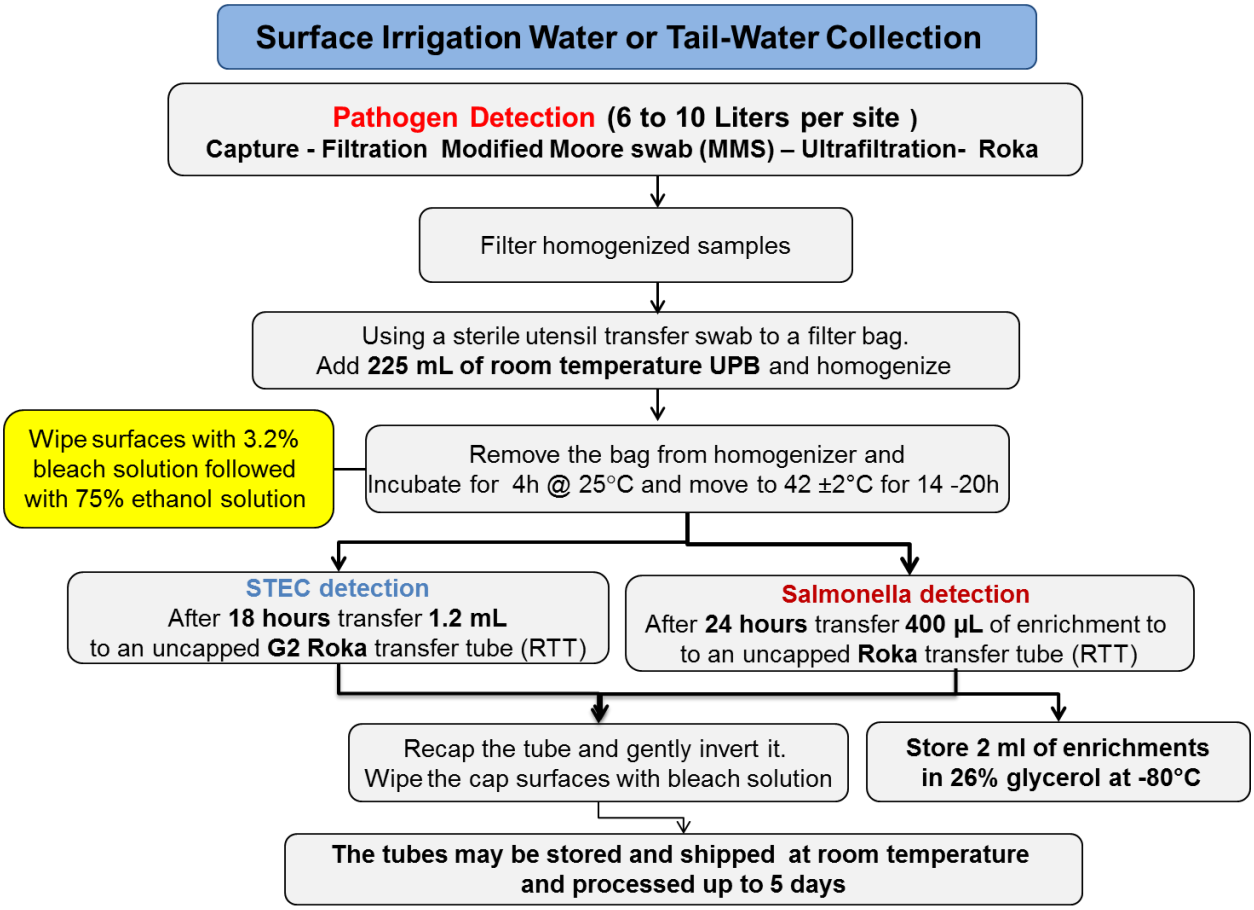
Water samples were collected from surface water sources, primarily within the Central Coast region from north to south Salinas Valley during seasonal production. Within the broader context of the CPS-CLGRB project, samples have been collected from other leafy green production areas in the San Joaquin Valley and later in 2015 this will be further expanded to include the South Coast region and the Imperial Valley. The basic sampling plan includes collection of 6-10L of water, transport to UCD on ice, holding at 2.5C overnight, and processing within 24h. From this homogenized larger volume, replicate 100ml sub-samples are extracted for the standard LGMA *E. coli* tests and additional sub-samples are extracted for Bacteroides and pathogen testing.

During the progress of this project, in response to interactions with growers and handlers, we

initiated the collection of tail-water from cooperating ranches to assist in answering questions about the re-use of irrigation water for crop management. Tail-water was collected in the same manner as other surface water sources but followed the progressive movement of this run-off water through the series of sedimentation ponds and ditches to the final holding impoundment. This preliminary survey is continuing within the CPS-CLGRB project which extends through 2016.

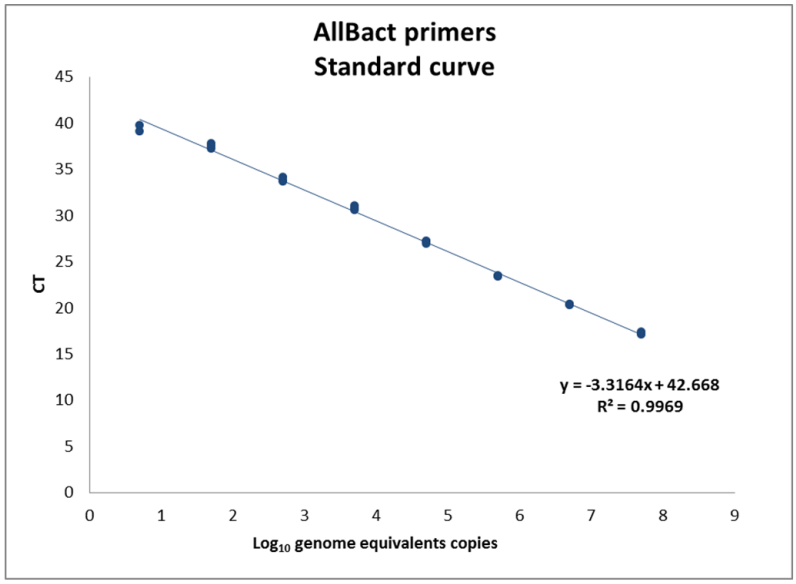
Rather than a lengthy detailed description of methods and procedures, this report includes the flow diagrams used for this project to describe the standard methods and sample processing steps:






An example of the Standard Curve we developed for estimating Total Bacteroides in a water sample is shown below in Figure 1. As this indicator is not cultured, as with *E.coli*, this is the standard used for direct comparison during each run with a water sample, following filtration and lysis, to establish a semi-quantitative value. Upon completion of the full CPS-CLGRB project, a multi-component comparison of both FIB to pathogen presence in the water sources will be conducted.

Figure 1. Development of a standard curve for estimating Total Bacteroides in Surface Water




**Ensure that the efficiency of amplification of the control template is 90–110% (a slope of -3.58 to -3.10) and that the R2 >0.9**

OBJECTIVE 2 – During this project period our analysis of several commercial cultures and isolates associated with leafy greens fields, primarily spinach, which were 'flagged' as contaminated with shigatoxin (*stx*) producing *E. coli* (STEC) and the presence of the additional key virulence factor, *eae*-attachment, in conventional PCR tests were further screened with the ROKA Atlas EG2 system for clinically-relevant STEC and additional virulence factors using techniques standard in our lab. Details are available on request. In addition, a few positive samples tested in a commercial lab using this ROKA system were made available to within this project to conduct a similar analysis of presumptive virulence factors to rule in or out the presence of non-O157 EHEC in the sample.

Briefly, the ROKA system relies on a surrogate marker for clinically-relevant STEC as a diagnostic genetic element to differentiate presumptive EHEC from environmental STEC or mixed cultures of *E. coli* and non-*E. coli* bacteria with *stx* or *eae*, but not both in a single cell. We followed the technical protocol provided by ROKA when using this system in our lab.

**Results and Discussion:**

During the course of this project period, after SOP validation was completed, a number of surface water sources have been tested for constituent quality (temperature on-site, turbidity, conductivity, pH, and TSS), the target FIB, and pathogen presence. An example of the comparative efficiency of the two primary detection systems for Total Bacteroides is provided below in Figure 2 and 3. These results may not be of particular interest or importance to the industry but are essential components of a final test method that would be used by a commercial service lab and are provided merely as examples of our planned deliverables for this project.

Figure 2. Comparison of PCR-based quantification of Total Bacteroides in inoculated surface water samples

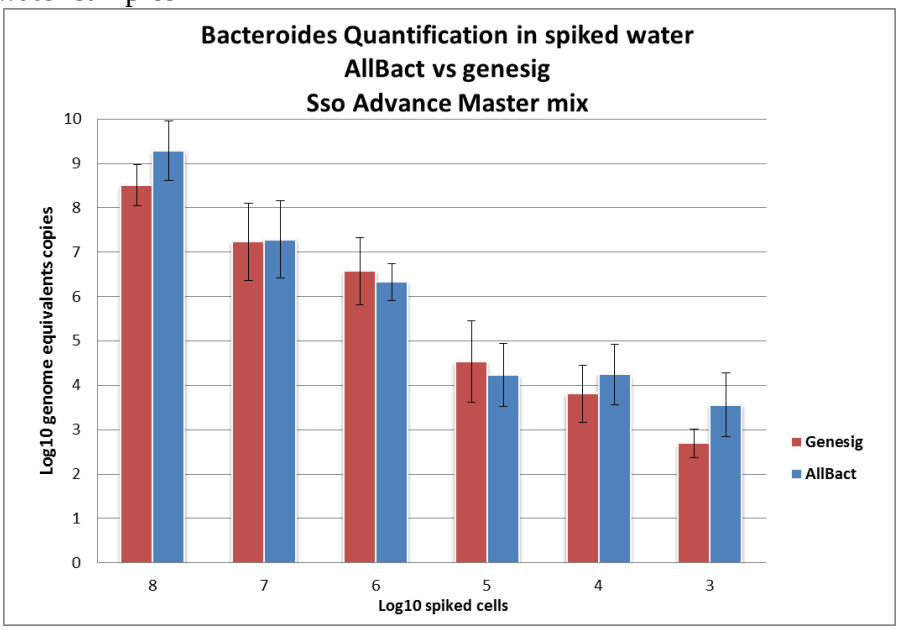
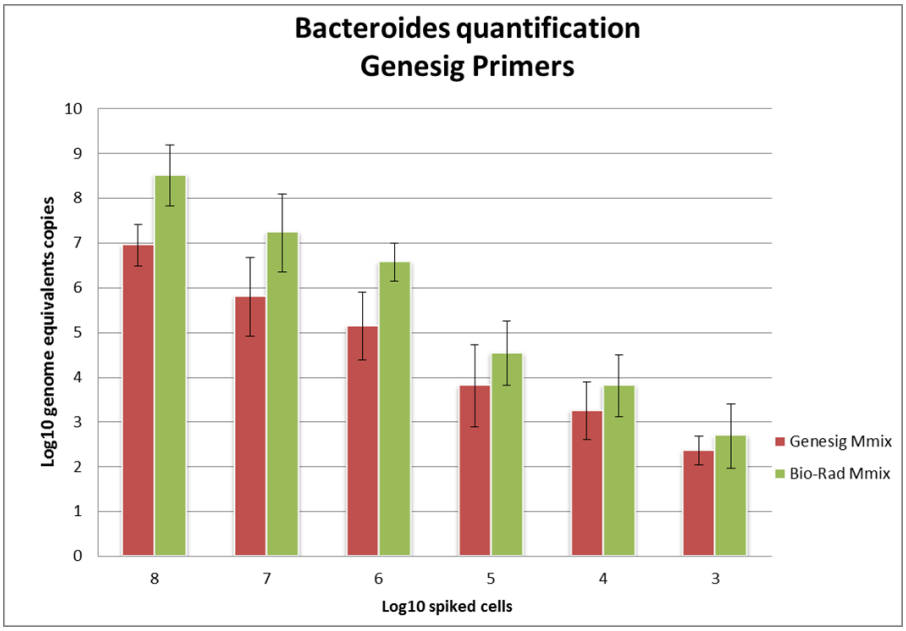



Figure 3. Comparison of different critical PCR components (Master Mix) on quantification of Total Bacteroides



As this is an on-going study, we reserve the reporting of results and analysis until a more extensive and complete data set is completed. We feel it important to withhold reporting of full pathogen testing outcomes until the completion of the full CPS-CLGRB project to merge all sites into one code-protected spreadsheet. An example of FIB results from 2014 is shown below in Figure 4 and Table 1.

Figure 4. Comparison of generic *E. coli* and Total Bacteroides in diverse surface water sources in the Central Coast region

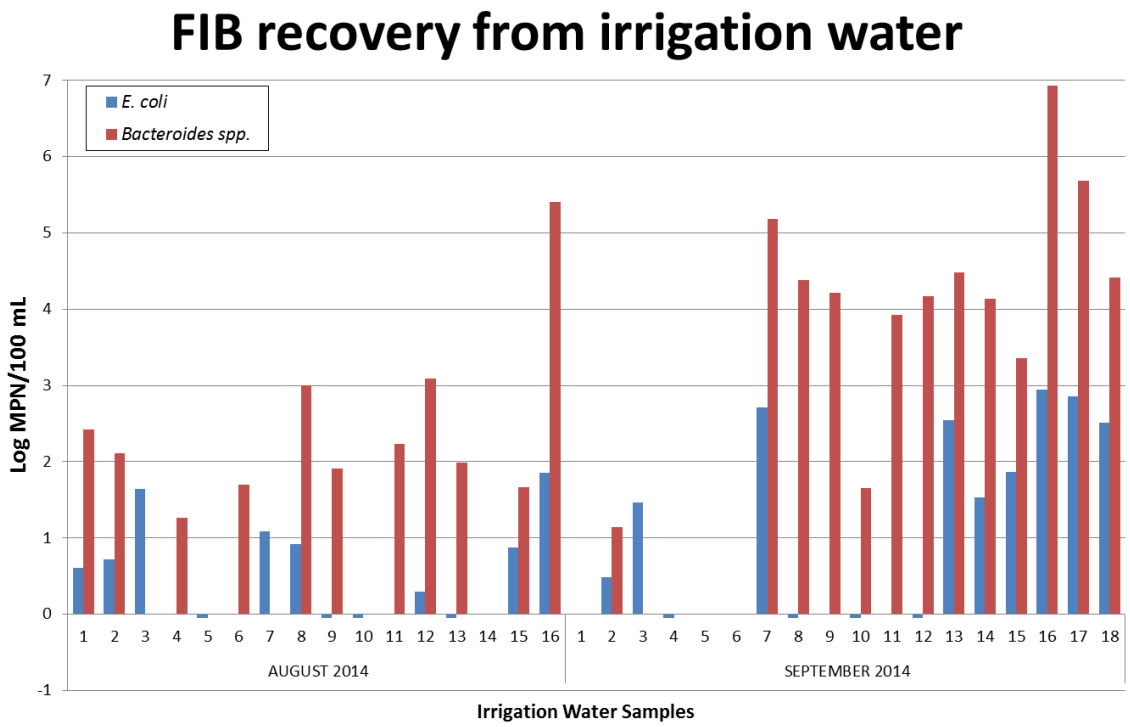



Table 1. Example of relationship among fecal indicator bacteria and human pathogen potential in surface water sources from 2014 Central Coast survey

| | *E. coli* | *Bacteroides* spp | ROKA | |
|---|---|---|---|---|
| | Colilert (Quanti-tray 2000) | qrt-PCR | Salmonella | STEC/EHEC |
| | log MPN/100 mL ± stdev | Log10 genome equivalents copies | | |
| **A** | -0.02 ± 0.03 | 6.63 | + | - |
| **B** | 1.13 ± 0.02 | 7.53 | + | - |
| **C** | 0.30 ± 0.32 | 6.92 | - | + |
| **D** | 2.58 ± 0.51 | 3.51 | - | - |

In Table 1, the log MPN value of Source D, 2.58/100 ml, would be equivalent to 380 MPN/100 ml which would exceed the single value test outcome for LGMA standards for overhead irrigation and would exceed the proposed geometric mean value for the proposed FSMA ag-water Water Quality Profile. However, this water source had a lower genome-equivalent copy number, the basis for quantifying Total Bacteroides prevalence, and no Salmonella or STEC/EHEC detected. In contrast, on the same sample date, Source A, B, and C all had low *E. coli* counts and higher Total Bacteroides values with detection of either Salmonella or STEC/EHEC. A more expansive spreadsheet from the diverse sites and dates is being compiled but as the correlation between FIB and pathogens in water, and more importantly survival on the irrigated crop is not well established the complete details will be presented in the final CPS-CLGRB report.

Very shortly after initiating research under Objective 2, we demonstrated that some commercial test outcomes found to be positive for STEC/EHEC, which resulted in destruction of adjacent field lots of leafy greens, could not be confirmed culturally. Multiple isolates from the primary enrichment were tested and none were found to contain the essential virulence elements (eae, stx 1 and or 2) in a pure cell-line culture. Isolates which were found to contain either *stx* 1 or 2 but not *eae* were shown to lack other virulence markers, including alternative attachment factors to *eae*, which would be indicative of a potential human pathogen. These isolates gave a negative reaction within the ROKA EG2 system which would not have 'flagged' the field as contaminated. Within this timeframe, the two cooperating commercial labs providing testing services to growers and handlers began using the Roka Atlas system. As a consequence, the number of samples available to us diminished completely.

During the remaining period, we analyzed a small number of grower samples which tested positive within the EG2 protocol by commercial labs and submitted to us for further confirmation analysis as well as our own testing of the irrigation sources discussed above. We quickly showed that in several cases, when *E. coli* O157:H7 was not the cause of a positive test result, the purified isolates obtained from the primary enrichment cultures were positive by the ROKA test for the surrogate marker but lacked other virulence factors. Our results as well as parallel testing by Roka Biosciences demonstrated that these isolates were sufficiently common in Central Coast production fields to constituent a significant problem with this test method. These were classified as false positive results which triggered an intensive but short-lived investigation among our lab, commercial test labs, Roka Biosciences, and multiple meetings and discussion with handlers of lettuce and leafy greens.

It is not necessary for this report to recount the steps taken in the months following this evidence but a new procedure was rapidly adopted, based on the evidence, which included a secondary screen for *stx* for any culture positive for the surrogate marker. This is the current procedure and, based on discussions with those conducting preharvest testing the number of positives during the 2015 season were greatly reduced. As a consequence, we stopped receiving cultures to evaluate which limited our accomplishments against this objective. However, we are continuing to collect field samples in a related project funded by CPS with participation of the CLGRB.