Center for Produce Safety 2010 RFP
Final Report
February 28, 2013

Title of Research Proposal: Science-based evaluation of regional risks for Salmonella contamination of irrigation water at mixed produce farms in the Suwannee River watershed

Project Period: October 1, 2010 through January 31, 2013

Principal investigator:
Anita Wright
University of Florida
acw@ufl.edu, 352-392-1991 x 311

Co-principal investigators: Ariena van Bruggen, University of Florida; George Vellidis, University of Georgia BAE*; Michelle Danyluk, University of Florida CREC

Objectives

The original research objectives were modified during revisions of the proposal, and the current proposal includes the following research objectives:

1. **Determine the spatial and temporal incidence of *Salmonella* in irrigation water and the sediments associated with the different irrigation sources.** On-site sampling of 10 farms in the Suwannee River watershed will determine *Salmonella* distribution by using methods that will maximize the recovery.
2. **Evaluate the utility of generic microbial, chemical, and environmental indicators as predictors of *Salmonella* contamination of irrigation water and the sediments associated with the different irrigation sources.** *Salmonella* will be monitored and related to fecal indicator assessments, microbial diversity of irrigation sources and types (pond vs. river or other, open vs. closed systems, reclaimed water, drip, pivot, sprinkler, etc.), environmental parameters (temperature, rainfall, nitrogen, phosphate, dissolved oxygen, carbon, conductivity, and flood events), and the extent of buffering capacity and encroachment by wildlife and/or domestic animals.

Accomplishments – October 1, 2010 through January 31, 2013

FINAL REPORT

Abstract

Outbreaks of human illness associated with produce have resulted in questions about the safety of the water used for irrigating these products. Therefore this project examined factors that may contribute to the regional contamination of irrigation water by *S. enterica* and evaluated methodologies for improved detection, recovery, and characterization of environmental isolates

The proposed research focused on the upper Suwannee River Watershed (SRW), as prior research indicated sustained prevalence of *Salmonella* and unusually high disease incidence associated with the region. *S. enterica* levels were determined monthly in irrigation water and sediments from a diverse set of farms (n=10) within the SRW in 2010-2011 with a more intensive investigations on a limited set of ponds (n=5) in 2012. Various (n=21) physical/biological parameters were assessed, including *E.coli* and fecal indicators. Molecular typing (DiversiLab rep-PCR) was used to determine the relationship of pond isolates (n=96) to clinical vs. environmental strains (n>300). Multiplex PCR was performed to predict serotypes (n=104), and antibiotic resistance of strains (n=193) was also evaluated. To date, all 10 ponds were *Salmonella* positive for both water and sediment samples at some time point during the study. Levels ranged from non-detectable to 4.6 MPN/100ml for water or from non-detectable to >110.0 MPN/100g for 2 sediment samples at ponds VH1 and MD1. The % of positive samples ranged from 11.1 to 50% for each pond with some ponds showing significantly ($p<0.05$) higher levels than others (Figure 3). Highest prevalence was seen in LV (44.7%), MD1 (39.6), CC2 (35.4) with lowest for RT2 (16.7%) and NP (8.5%). Highest mean levels for *Salmonella* from all samples were obtained from ponds CC2 and VH1 when the two sediment samples with >110 MPN/100 g were not considered. Positive samples were more frequent for surface (45.0%) and subsurface (33.3%) water samples, as compared to perimeter (14.2%) and benthic (26.7%) sediment samples. Seasonality was observed for both the % of positive samples and the MPN values (not shown), as the occurrence of *Salmonella* was significantly higher in summer and fall than in winter (ANOVA, $p<0.05$). Although correlations for any single parameter were all $r<0.3$. Based on data collected through March 2012, *Salmonella* levels showed significant positive correlation ($P<0.01$) with fecal coliform ($r=0.26$); however, negative correlation ($r=0.20$) was found for *Salmonella* occurrence. No correlation was seen for generic *E. coli* and *Salmonella* levels but positive correlation ($r=0.16$) was observed for *Salmonella* occurrence. Multiplex PCR patterns corresponded to 10 known serotypes, including Newport, but most (81%) showed no identifiable patterns. Pond isolates clustered into 12 genotypes (based on >85% similarity by rep-PCR), and more strains (50%) were associated with isolates from clinical infections than with strains from the Suwannee River (35%), while some strains (15%) were unique to this study. Antibiotic resistance was observed mostly to streptomycin (100%) and kanamycin (10.4%), with 19.7% resistant to two or more antibiotics. These data suggest that these ponds harbor a diverse population of *Salmonella,* which may pose potential health risks due to their genetic similarity to strains from clinical origin.

Background

Science-based data are needed to establish and validate Good Agricultural Practices (GAPs) metrics to prevent or mitigate contamination of produce by human pathogens at the production level. In order to evaluate, modify, and implement GAPs, studies must establish appropriate assessment protocols for risk evaluation that are specific for the agricultural practices of that region. Irrigation water quality standards are not currently regulated or determined by scientifically based metrics. Coliform bacteria are widely used as indicators of fecal contamination, but their validity as indicators of bacterial

pathogens is questionable. Current methods may not be appropriate for detecting specific pathogens and/or lack the sensitivity needed to detect low pathogen levels. The relationship between the occurrence and distribution of these indicator bacteria with that of a specific pathogen, namely *Salmonella enterica* was examined. Growers in the region agreed to allow periodic collection of water samples from irrigation ponds to provide preliminary data and validate methodologies. Proposed research will systematically examine bacteria in water and sediment from 10 irrigation pond sites for a one-year period, followed by more intensive surveillance of 5 ponds in year 2 of the project. This research attempted to identify management practices, environmental parameters, and locale characteristics associated with increased risk of pathogen contamination by irrigation water and will provide a research-based comparison of indicator organisms and *Salmonella* in a major fruit and vegetable growing area.

Objective 1 Methods and Results:

Methods evaluation protocol. In order to optimize protocols for collection and processing of samples, Dr. Wright's lab evaluated several methods for recovery and enumeration of *Salmonella* from pond water and sediment samples. Samples were collected by Dr. Adams' or Dr. Vellidis' lab personnel from the ponds (n=1 for Oct, 2010; n=4 for Nov and Dec, 2010; n=10 for Jan, 2011 to Feb, 2012) and various physical parameters were recorded at each site. They also conducted assays for fecal indicator bacteria and basic water chemistry. Dr. van Bruggen's lab extracted DNA from water samples for determination of the genetic diversity of bacterial populations. Her lab also provided data on dissolved organic carbon (DOC) and nitrogen (DON). Dr. Danyluk, who is doing a similar survey in central Florida for CPS, is providing consultation.

Evaluation of the *Salmonella* most probable number (MPN) enumeration protocol used primary enrichment growth in either buffered peptone water (BPW) or Lactose Broth (LB), followed by selective enrichment in tetrathionate (TT) broth and isolation on either XLT4 or CHROMagar. Typical colonies were confirmed by PCR of the *invA* gene. PCR for pre-screening and immunomagnetic separation (IMS) of enrichment samples were examined as described in Figure 1.

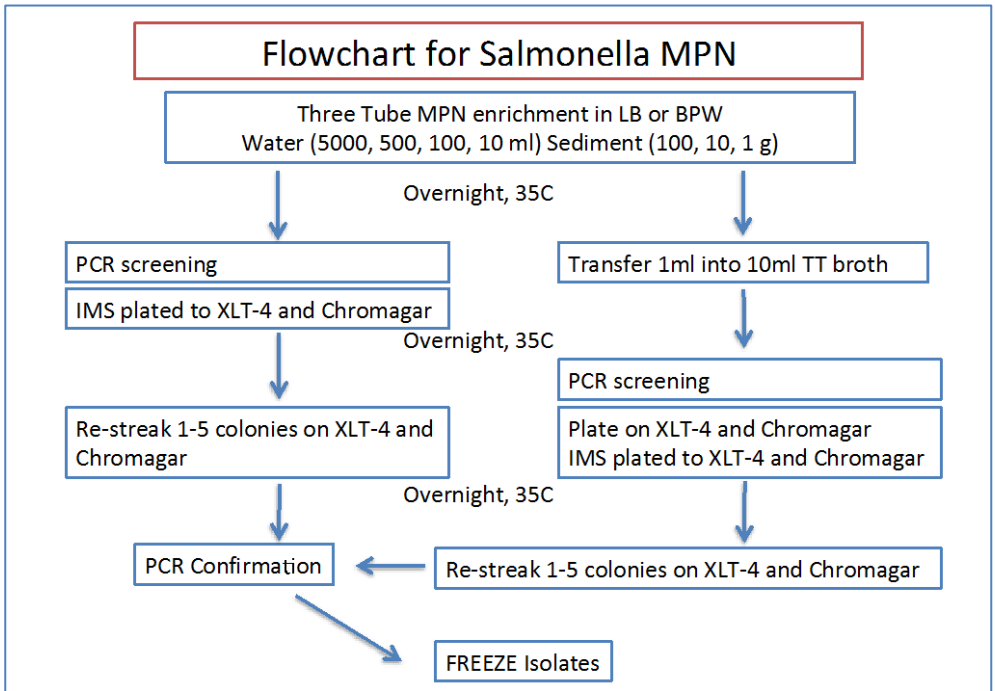

Figure 1. Evaluation of methods for *Salmonella MPN*

In summary, the following variables were examined*:*

1. Media for enrichment and isolation of *Salmonella*: BPW vs. LB and XLT4 vs. CHROMagar
2. Use of PCR screening for *Salmonella* from LB or TT broth enrichment
3. Use of the Modified Morse Swab for concentration of *Salmonella* from pond water (5 L) vs. smaller volumes or the filtrate
4. Use of IMS in LB vs. TT broths and plated on XLT4 vs. CHROMagar agars

All PCR-confirmed *Salmonella* isolates were stored as frozen stocks for future genetic and phenotypic evaluation in her laboratory.

Results of Methods Evaluation. Recovery of *Salmonella* from various MPN samples (n=36) using primary enrichment in either LB or BPW was examined using secondary enrichment in TT broth that was plated onto either XLT4 or CHROMagar. LB showed somewhat (but not significantly) better results for both agars (11% positive on each) compared to recovery from BPW, which was 8 and 2%, respectively. We also found that attempts to concentrate *Salmonella* by IMS did not increase the recovery of *Salmonella,* as recovery with the use of IMS in TT yielded 14% positive on either agar, but was 16 and 14%, respectively, without IMS. As no difference was observed in these 2 assays, subsequent enrichment was limited to LB due to cost constraints and efforts to keep protocols consistent with similar studies being conducted in central Florida by Dr. Danyluk.

We also found that pre-screening of MPN enrichment samples by PCR (n=1706) did not yield results that were consistently predictive of subsequent confirmation by PCR of individual colonies on selective agar. For example, *Salmonella* was never detected in LB by PCR, and the sensitivity of detection in screening TT by PCR was 48% less than subsequent confirmation by PCR of individual colonies on selective agar from TT broth. However, it should be noted that these samples included mostly negative TT tubes, and no false positive were noted by PCR screening.

The use of the modified Morse Swab to filter 5.0 L volumes of pond water samples in triplicate (n=45) also did not increase recovery of *Salmonella*, and enrichment of the filter was often negative in samples where the enrichment of smaller volumes was confirmed positive for *Salmonella*. Furthermore, samples with positive results for enrichment of the filter were also frequently positive for the enrichment of 500ml of filtrate as well, indicating that the filtration was not providing a concentration step. Similar results were obtained for membrane filtration (0.2 milipore) or of tangential flow filtration (data not shown).

Evaluation of 1904 MPN tubes from various sources showed differences in recovery of Salmonella on XLT4 agar vs. CHROMagar. The average recovery based on the % of samples showing positive PCR confirmation of typical colonies isolated on XLT4 agar vs. Salmonella CHROMagar averaged 55 and 44%, respectively, (Table 1). Similarly the % of false positives based on recovery of typical colonies that were not confirmed by PCR differed between agars. However, it should be noted that sequential isolation of typical colonies by restreaking colonies from the primary isolation agar to the alternate agar was highly predictive of confirmation by PCR and showed 100% agreement with PCR confirmation (see cross-plating results below). By applying both agars for primary isolation, we recovered 224 confirmed positive samples, while XLT4 or CHROMagar alone yielded 210 and 177, respectively. These results indicate a loss of sensitivity of 5 and 21%, respectively for each agar.

Table 1. Comparison of recovery of *Salmonella* on XLT4 vs. CHROMagar from results of MPN enrichment (not individual colonies)

Samples:	Both Agars[a] (%)[b]	XLT4 only	CHROM only
PCR confirmed /Positive on agar	223/578 (39%)	210/379 (55%)	177/400 (44%)
False positive rate	355/578 (61%)	169/379 (45%)	223/400 (56%)
Salmonella recovery	High (223)	Medium (210)	Low (177)
Labor intense	High	Low	Low
Cost	High	Low	Medium

a) Number of MPN tubes confirmed positive by PCR/number of tubes showing typical colonies on both agars or on XLT4 only or on CHROMagar only
b) Mean %= average number of tubes confirmed by PCR/number of tubes with typical colonies x 100

Cross-plating results. In order to simplify the *Salmonella* MPN protocol and reduce costs we assessed the validity of a cross-plating methodology. Basically, TT tubes for MPN were initially streaked for primary isolation onto either XLT4 or CHROMagar or onto both agars and then sequentially plated (cross plated) to the alternative agar. Typical colonies were assessed for species identity by PCR.

To date, all isolates that were positive on both agars were also confirmed as *Salmonella* by PCR in this study (n=1611). Furthermore, we examined *Salmonella* isolates from a prior study (Rajabi et al., 2011, n=30). To date we have found only a single isolate from the Suwannee River that was PCR positive, CHROMagar positive, but negative on XLT4 (Figure 2).

Thus, we propose cross-plating as an alternative to PCR for applications where PCR may not be available or practical. The use of less expensive XLT4 ($24/L) for primary isolation significantly reduces costs compared to the more expensive CHROMagar ($55/L). Although some loss of recovery was seen using only XLT4, simplifying the assay would permit evaluation of more samples and could actually improve recovery.

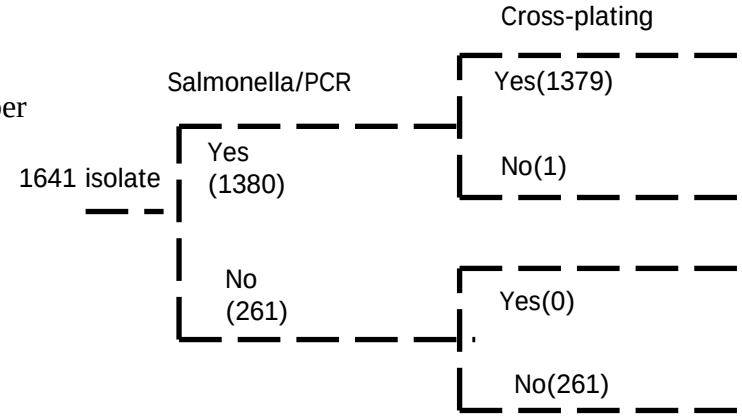

Figure 2. Validation of Cross-plating Methodology. Comparison of the number of PCR-confirmed Salmonella isolates that were positive on both agars for cross plating vs. presumptive but not-PCR confirmed stains.

On-site monthly sampling. Sediment and water samples were obtained from ten irrigation ponds in the southern Georgia region of the upper Suwannee River Watershed from October, 2010 through February, 2012 in order to complete an entire year of data collection for all samples. A total of 530 water and sediment samples were evaluated from 10 diverse ponds that varied in size, depth, amount of associated vegetation or buffer, and the type of irrigated crops in the immediate area. To date, all 10 ponds were *Salmonella* positive for both water and sediment samples at some time point during the study. Levels ranged from non-detectable to 4.6 MPN/100ml for water or from non-detectable to >110.0 MPN/100g for 2 sediment samples at ponds VH1 and MD1. The % of positive samples ranged from 11.1 to 50% for each pond with some ponds showing significantly ($p<0.05$) higher levels than others (Figure 3). Highest prevalence was seen in LV (44.7%), MD1 (39.6), CC2 (35.4) with lowest for RT2 (16.7%) and NP (8.5%). Highest mean levels for *Salmonella* from all samples were obtained from ponds CC2 and VH1 when the two sediment samples with >110 MPN/100 g were not considered. Positive samples were more frequent for surface (45.0%) and subsurface (33.3%) water samples, as compared to perimeter (14.2%) and benthic (26.7%) sediment samples. Seasonality was observed for both the % of positive samples and the MPN values (not shown), as the occurrence of *Salmonella* was significantly higher in summer and fall than in winter (ANOVA, $p<0.05$).

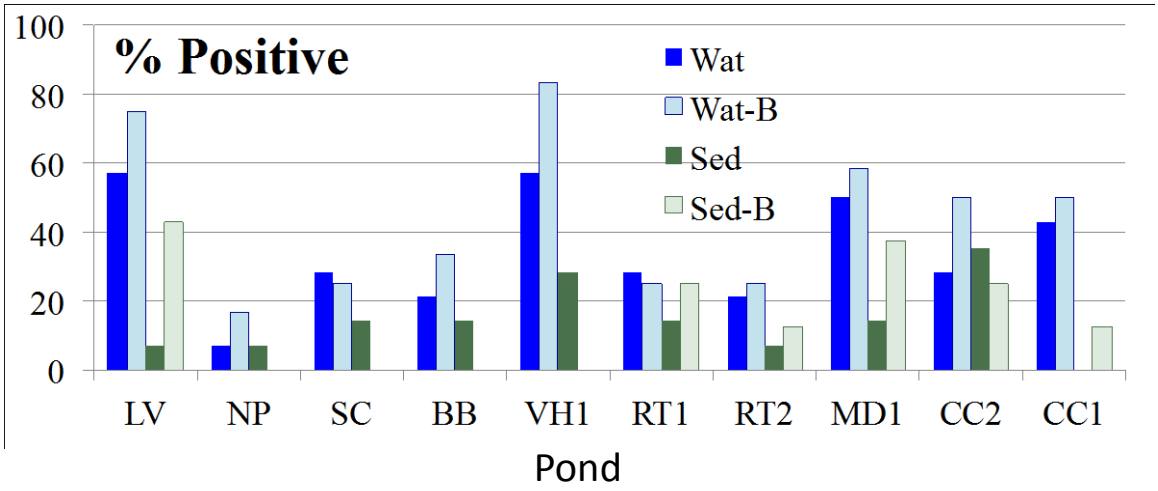

Figure 3. *Salmonella* analysis by pond. Data for each pond show the % positives for all samples collected Jan 2011 to Feb 2012 for surface (Wat) and subsurface (Wat-B) water and perimeter (Sed) and benthic (Sed-B) sediments samples. (Sed-B samples were collected from July, 2011 to Feb, 2012 for all ponds.)

Objective 2 Methods and Results:

Methods for Statistical analyses of Salmonella occurrence and levels with spatial, temporal, and environmental factors. Various environmental factors were evaluated as potential indicators for the risk of *Salmonella* contamination and included the following parameters: generic *E. coli*, fecal coliforms, total suspended solids, soluble reactive portion of total phosphorus, total organic carbon, total nitrogen, copiotrophic bacteria, oligotrophic bacteria, average temperature of the pond region between sampling, total rainfall of the pond region between sampling, temperature of the pond water when sampling, specific conductance, dissolved oxygen percentage, dissolved oxygen concentration, dissolved oxygen charge, oxidation reduction potential, and the richness of bacterial community based on Shannon-Wiener diversity index and Simpson diversity index. Pearson's correlation coefficients

were calculated to evaluate the correlations between fecal indicators and the population of *Salmonella* spp. isolated from irrigation ponds, and biserial correlation coefficients for the occurrence of *Salmonella* spp. (1).

Results for statistical analyses. Statistical Based on data collected through March 2012, *Salmonella* levels showed significant positive correlation ($P<0.01$) with fecal coliform ($r=0.26$); however, negative correlation ($r=0.20$) was found for *Salmonella* occurrence. No correlation was seen for generic *E. coli* and *Salmonella* levels but positive correlation ($r=0.16$) was observed for *Salmonella* occurrence. More detailed statistical analyses on these parameters and other variables are still in progress.
All microbial counts were log transformed to obtain a normal distribution for statistical analysis. Chi square test/pared t test were conducted to compare the occurrence/logMPN of Salmonella in surface and subsurface water samples. ANOVA was conducted to compare the Salmonella difference between ponds and months. Biserial correlation coefficients/Pearson's correlation were calculated to evaluate the correlations between the 27 environmental factors and the occurrence/logMPN of Salmonella from irrigation ponds. Discriminant analyses were performed on (1) the selected environmental factors and (2) DGGE data to group the 240 water samples according to the predicted presence or absence of Salmonella. Chi square and t test were done in Excel, biserial correlation was calculated using online tools. Other statistical analyses were performed using SAS 9.3.

Water samples ($n=24$) were analyzed for samples collected from each pond between Mar 2011 to Feb. 2012 (240 samples in total). Salmonella were detected in 41.7% of the samples (100/240) and detection ranged from 12.5% at pond NP to 70.8% positive at pond VH (Figure 4). The highest geometric mean of the logMPN was at pond VH and the lowest at pond NP. Significant difference ($p<0.0001$) was observed between ponds. The % positive samples and log MPN level were consistence by pond. For evaluation of the temporal variability of water samples ($n=20$) for 10 ponds, the % positive samples and MPN were evaluated by month (Figure 5). Salmonella were detected in all months. The highest detection was found in September. Significant difference ($p=0.0043$) was observed between months. The % positive and log MPN level were consistence by month.

Overall, 27 environmental factors were evaluated as potential indicators for the risk of Salmonella contamination. Based on pearson correlation and biserial correlation, strong correlations were not observed for any factor as r values never exceeded 0.3d. However, the significant factors are high lighted in Table 2 and included *E. coli*, Fecal coliforms, oligotrophic bacteria (oligo), average temperature of the pond region in the last week before sampling (L avg Tm) or 2 weeks before sampling (L avg Tm-2), copiotrophic bacteria (Copio), soluble reactive portion of total phosphorus (ortho-P) and average temperature (ave Tm), which were significant in both correlations.

Bacterial community analysis was examined in relationship to the occurrence of *Salmonella* in 240 water samples using DGGE. In total, 65 Operational taxonomic units (OTUs) were generated through DGGE analysis of the bacterial communities based on 16s PCR. Based on biserial correlation, the intensities of 7 bands were significantly correlated to Salmonella occurrence ($p<0.05$, for 3 positive and 4 negative correlations). Another 6 bands showed significance at $0.05< P <0.1$. None of these bands were highly correlated ($r>0.7$) to each other. Discriminate analysis for DGGE was based on 100 water samples that were positive for Salmonella and the same number of randomly selected Salmonella negative water samples. The separation of samples with and without Salmonella is significant which is shown in a plot of canonical variable one versus band 24 (Figure 6). Step-wise discriminate analysis for the presence or absence of Salmonella in each water sample and 24 DGGE OTUs (data not shown) that contributed to the classification. A different set of random negative samples won't change the OTUs.

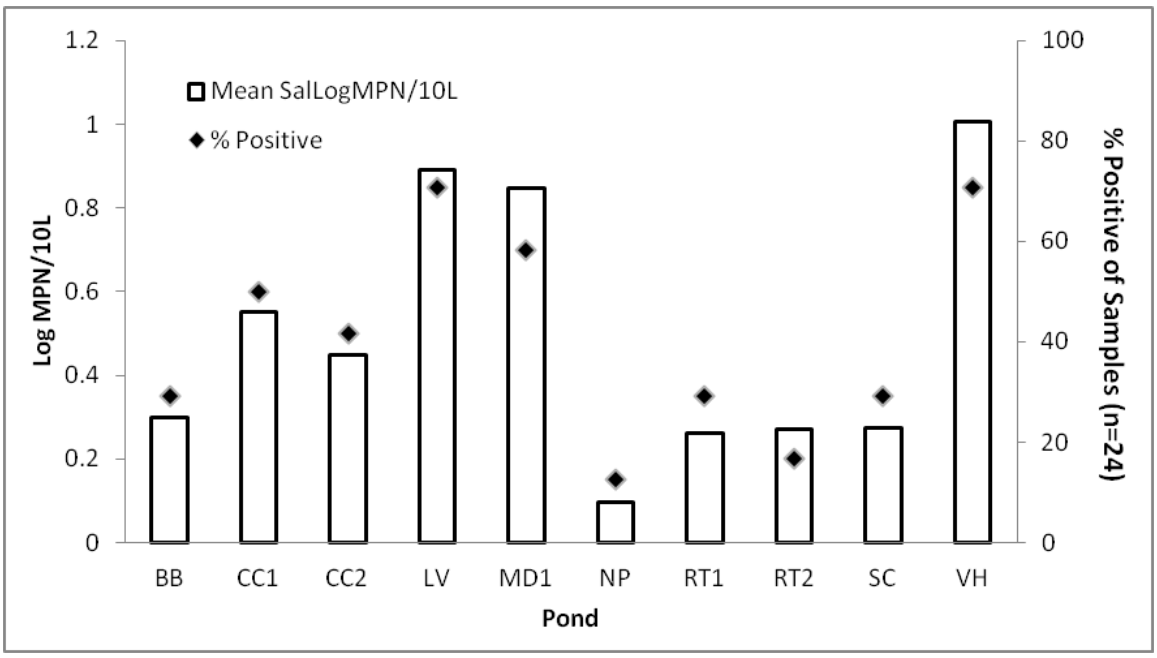

Figure 4. Spatial distribution of *Salmonella* by pond. Occurrence (% positive) and MPN levels (Mean Sal LogMPN/10L) of *Salmonella* from pond water samples collected October 2010 – February 2012.

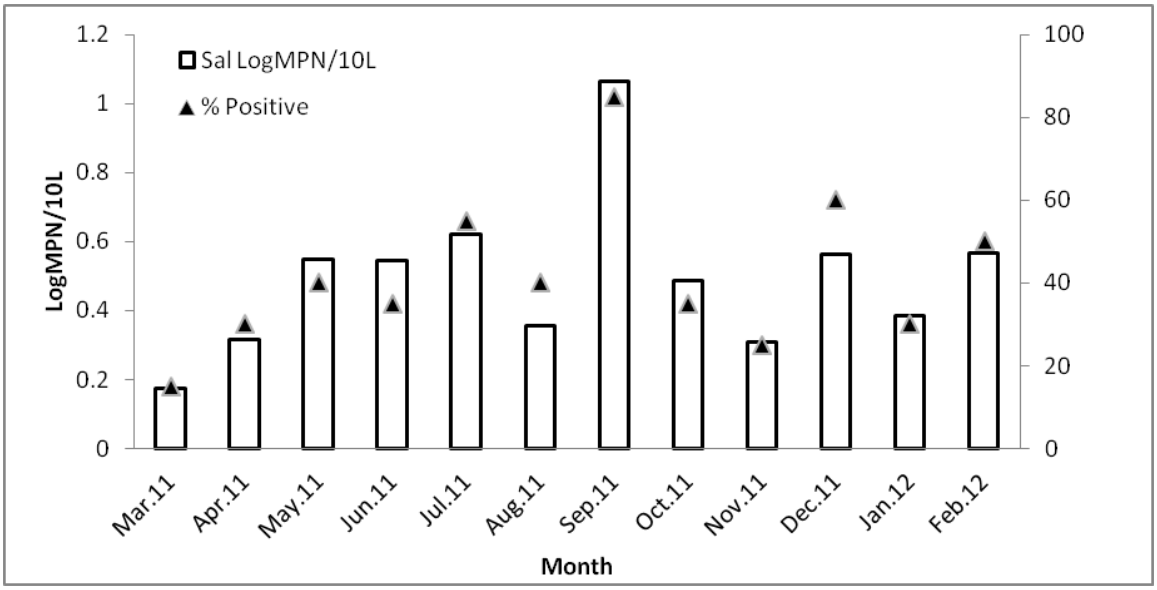

Figure 5. Temporal variability of *Salmonella* by month. Occurrence (% positive) and MPN levels (Mean Sal LogMPN/10L) of *Salmonella* from pond water samples collected October 2010 – February 2012.

Table 2. Relationships among environmental factors and the logMPN (Pearson) and occurrence (Biserial) of Salmonella in pond water

	Pearson	P		Biserial	P
E. Coli	0.270511	0	**E. Coli**	0.2855	0
Fecal Coliforms	0.284204	0	**Fecal Coliforms**	0.2908	0
Copio	0.158676	0	**Oligo**	0.1948	0.0024
Oligo	0.210147	0.0002	average Rainfall	0.1593	0.0135
pH	-0.18497	0.004	**L Avg Tm**	0.1486	0.0213
Ortho-P	0.176415	0.0061	**Copio**	0.1477	0.0221
L Avg Tm	0.167527	0.0093	**Ortho-P**	0.146	0.0237
DO Conc	-0.16306	0.0114	ORP	-0.1457	0.024
average Tm	0.157676	0.0145	**average Tm**	0.1443	0.0254
Nitrate	-0.15681	0.015	Temp	0.1256	0.0519
Temp	0.143229	0.0265	Nitrate	-0.1252	0.0528
Species Richnes	-0.12123	0.0608	DON	-0.1155	0.0742
Shannon-Wiene	-0.12005	0.0634	pH	-0.1132	0.08
DON	-0.11868	0.0666	DO Conc	-0.1112	0.0857
L2 avg Tm	0.11415	0.0776	L tot rain	0.1061	0.1009
L tot rain	0.110429	0.0878	Shannon-Wiene	-0.0985	0.1281
average Rainfal	0.107052	0.098	L2 avg Tm	0.0977	0.1313
ORP	-0.10245	0.1136	Species Richnes	-0.0971	0.1338
Simpson Divers	0.078393	0.2263	Simpson Diversi	0.0665	0.3051
DO Charge	0.073613	0.256	DO Charge	0.0638	0.3249
DO%	-0.07332	0.258	Evenness	-0.0442	0.496
L2 tot rain	-0.04879	0.4527	DO%	-0.0381	0.5571
Turbidity	0.020717	0.7496	DOC	-0.0251	0.6985
SpCond	-0.01988	0.7602	TSS	0.0182	0.779
Evenness	0.013092	0.8401	Turbidity	0.0148	0.8199
TSS	-0.0093	0.8873	L2 tot rain	0.0032	0.9602
DOC	0.002036	0.975	SpCond	-0.0012	0.9849

Table 3. Operational taxonomic units (OTUs) of DGGE data with significant correlations to the occurrence of Salmonella ($p<0.1$)

Relative Band position(%)	Band No.	Biserial Correlation	P-value
47.9	**b37**	**0.1933**	**0.0026**
3.6	**b4**	**0.1838**	**0.0043**
64.7	**b48**	**-0.1654**	**0.0103**
28.9	**b23**	**0.1528**	**0.0179**
25.2	**b20**	**-0.1266**	**0.0231**
63.5	**b47**	**-0.1372**	**0.0336**
97.4	**b64**	**-0.1312**	**0.0422**
90.4	b61	-0.1254	0.0523
69.1	b50	-0.1208	0.0616
46.2	b36	-0.1183	0.0673
43	b34	0.1085	0.0936
71.3	b51	-0.1085	0.0936
99.1	b65	-0.1067	0.0992

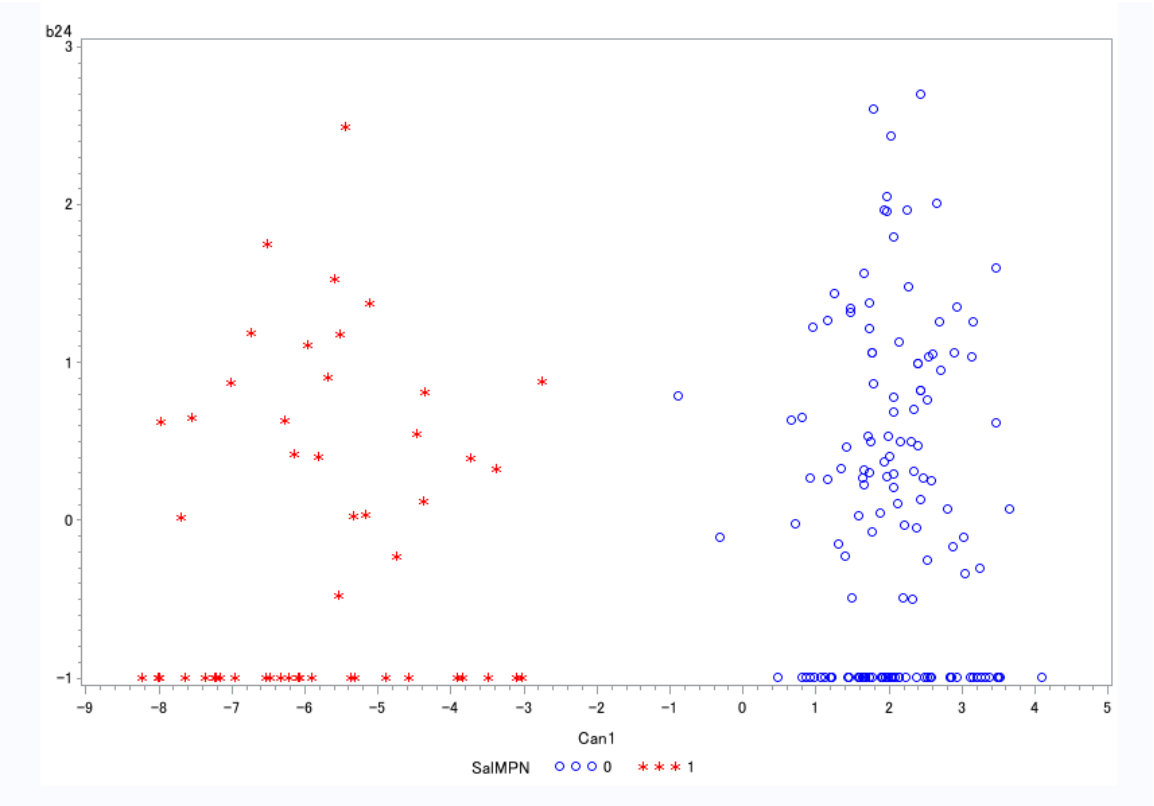

Figure 6. Discriminant Analyses for DGGE. The separation of samples with and without Salmonella is significant which is shown in a plot of canonical variable one versus band 24.

Characterization of Salmonella isolates. Over 2000 confirmed *Salmonella* isolates were recovered to date, and a multiplex PCR was used to screen strains (n=96) for identification via previously described patterns for known serotypes (Kim et al. 2006). Ten Serotypes were identified by multiplex patterns, including Branderup (1,2 and 2; n=1); Derby (1,2,3,4 and 5; n=2); Munchen (1,2,5 and 0; or n=1 or 1,2,5, and 5; n=3); Newport (1,2,3,5 and 0 n=1). Most strains (81%) presented patterns that were not associated with previously reported patterns that are commonly associated with clinical isolates. The multiplex assay showed no association among multiplex types with pond or seasonal distribution. Antibiotic resistance was observed mostly to streptomycin (100%) and kanamycin (10.4%), with 19.7% resistant to two or more antibiotics.

Representative strains were also examined for genetic diversity using DiversiLab rep-PCR, which revealed that pond isolates were distributed among at least 12 genotypes (based on <85% similarity). Strains from irrigation ponds clustered with strains from other environmental aquatic sources (35%), but similarity of some pond strains (50%) to strains from clinical infections was also observed (Figure 7). Several isolates from different ponds occasionally appeared to be clonal (>95% similarity). These data highlight the genetic diversity of *Salmonella* in irrigation sources in this region.

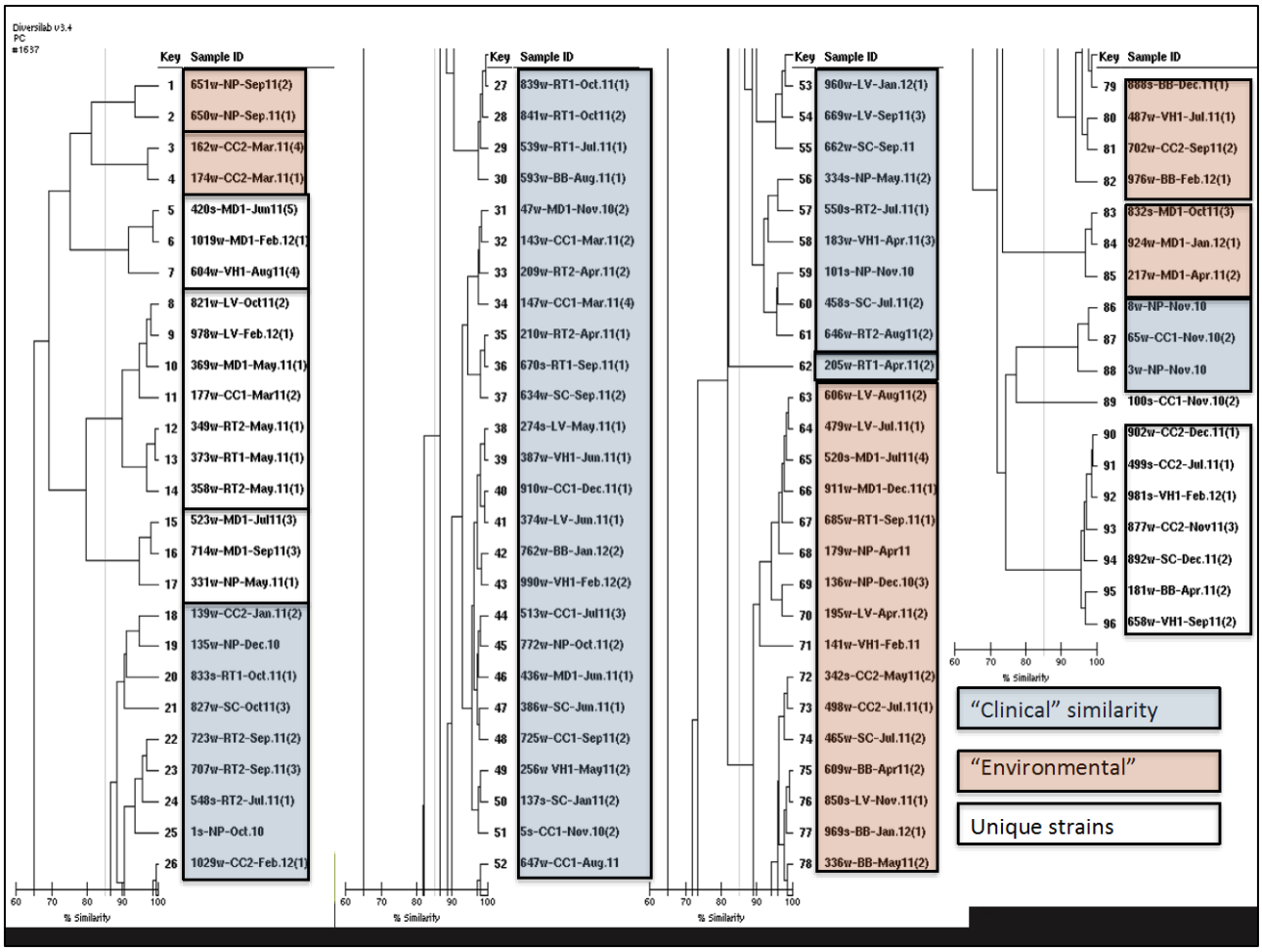

Figure 7 DiversiLab rep PCR for *Salmonella* isolates. Distribution of genetic similarity among these *Salmonella* isolates from pond samples. Strains are grouped by those with closest similarity to primarily clinical vs. environmental isolates vs. strains unique to this study.

Methods and results from analysis of selected ponds in year 2 of project. Beginning March 2012, sampling efforts focused on 5 ponds based on the levels of *Salmonella* observed for 2010-2012. These efforts coordinated with another CPS project headed by Dr. Vellidis that evaluated intensive sampling from the same ponds. Ponds were selected that showed consistently higher (LV, MD1) vs. lower (NP, CC2, SC) levels of *Salmonella* occurrence relative to the other ponds. A total of 20 water samples were collected from each pond between Mar 2012 to Dec. 2012 (100 samples in total). *Salmonella* was detected in 32% of the samples (32/100). The highest geometric mean of log was in pond CC2 and the lowest in pond NP (Figure 8). There were no significant differences among ponds (p=0.1795), and the % positive samples vs. log MPN level were consistent by pond.

A total of 20 sediment samples were also collected from each pond between Mar 2012 to Dec. 2012 (100 samples in total). Salmonella were detected in 17% of the samples (17/100) and detection ranged from 5% at pond LV and SC to 50% positive at pond CC2 (Figure 9). The highest geometric mean of log was at pond CC2 and the lowest at pond LV. Significant difference was observed between ponds (p=0.0003), and the % positive of positive samples vs. log MPN level were consistent by pond.

A total of 10 water and 10 sediment samples were collected each month from the 5 ponds. Salmonella were detected in all months in water samples but was absent from sediments in May, August and

October. The highest prevalence for water samples was found in August-October (p=0.0731), while April, June and September showed the highest levels in sediments (p=0.0974). The % of positive samples and log MPN levels were consistence by month for both water and sediment.

A total of 19 environmental factors were evaluated as potential indicators for the risk of *Salmonella* contamination in water and sediment. Based on Pearson and biserial correlations, the significant factors ($p<0.05$) are highlighted in Tables 4 and 5, and included *E. coli* MPN and the average temperature (Ave Temp) at the time of sampling or 1 (L Ave Temp) or 2 (L2 Ave Temp) weeks before sampling.

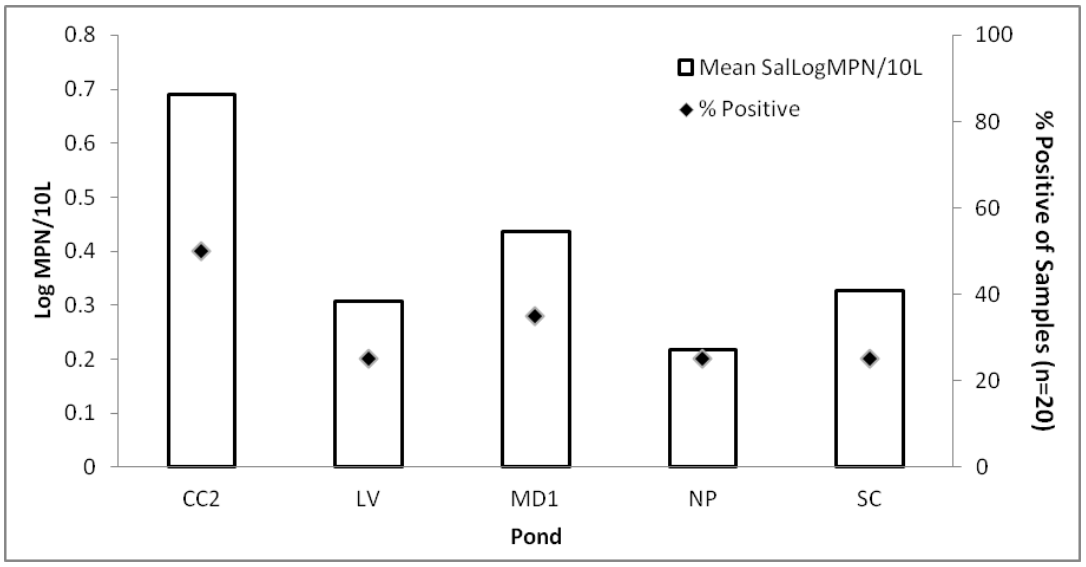

Figure 8. Salmonella in pond water. Occurrence (% positive) and MPN levels (Mean Sal LogMPN/10L) of *Salmonella* from pond water samples collected March – December 2012.

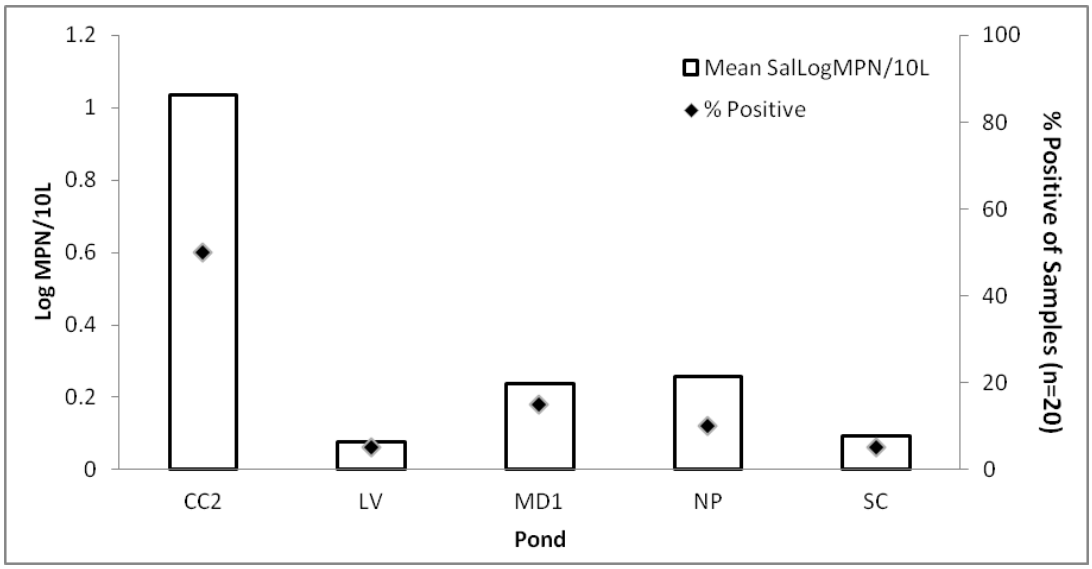

Figure 9. *Salmonella* in sediments. Occurrence (% positive) and MPN levels (Mean Sal LogMPN/10L) of Salmonella from pond sediment samples collected March – December 2012.

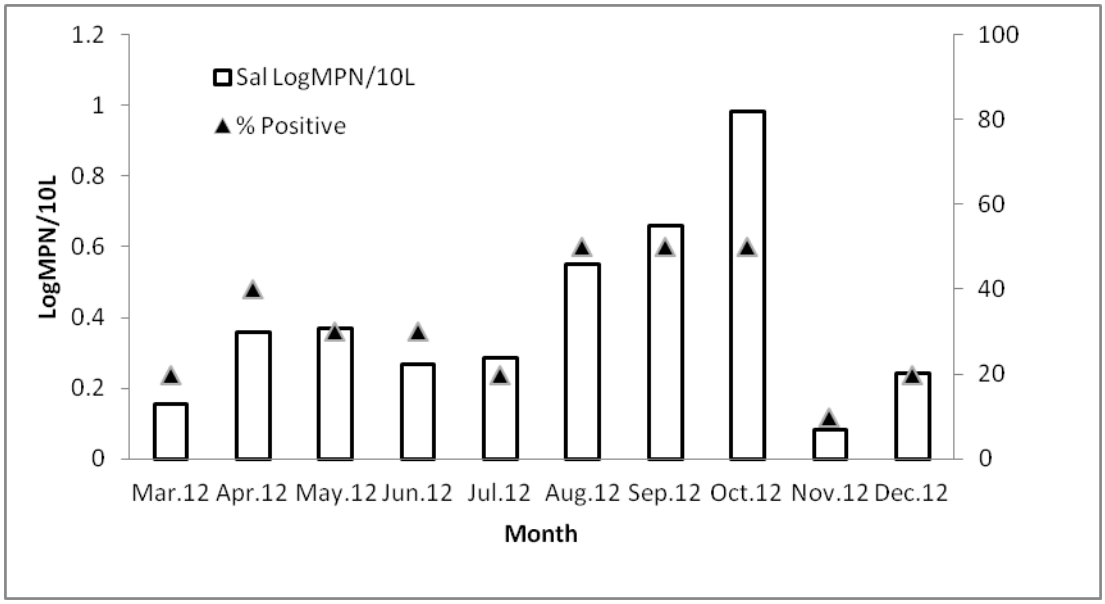

Figure 10. Temporal variability of *Salmonella* in pond water by month. Occurrence (% positive) and MPN levels (Mean Sal LogMPN/10L) of *Salmonella* from pond water samples collected March– December 2012.

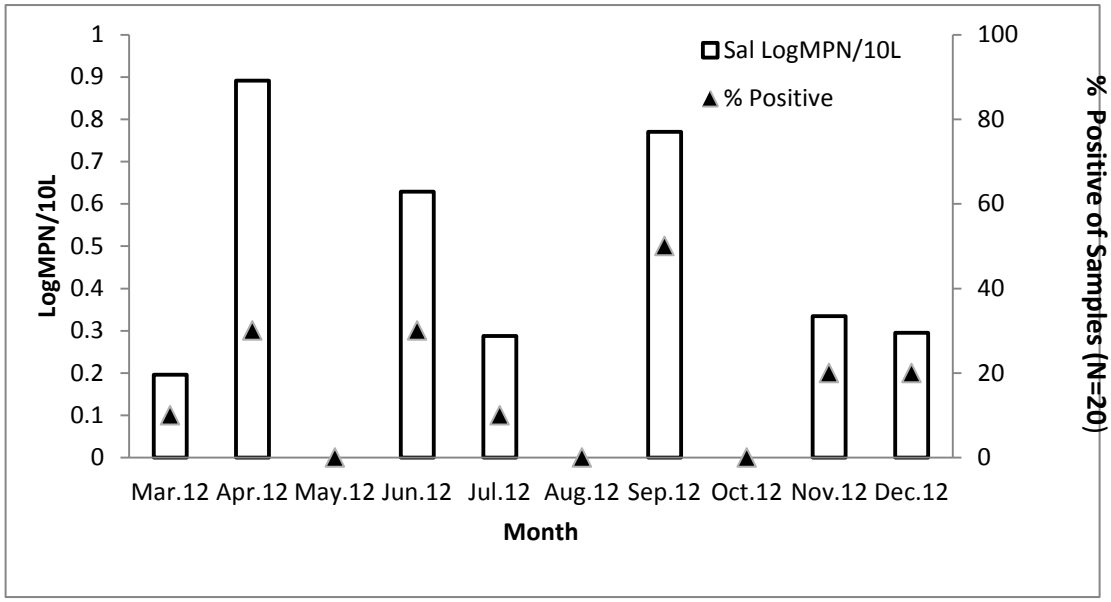

Figure 11. Temporal variability of *Salmonella* in sediments by month. Occurrence (% positive) and MPN levels (Mean Sal LogMPN/10L) of *Salmonella* from pond water samples collected March– December 2012.

Table 4. Relationships among environmental factors and the occurrence and LogMPN of Salmonella in water samples.

	Biserial-r	P value		Person-r	P value
E. Coli	0.2469	0.0133	E. Coli	0.284029	0.004187
L2 avg Tm	0.2461	0.0136	L Avg Tm	0.246907	0.013271
Ave Temp	0.2152	0.0315	Ave Temp	0.23638	0.017897
L Avg Tm	0.2093	0.0366	L2 avg Tm	0.234057	0.019092
DO Conc	-0.1742	0.0831	DO Conc	-0.19072	0.057367
Rainy Days	0.1655	0.0998	pH	-0.16472	0.101527
Ortho-P	-0.1538	0.1266	Turbidity	0.155588	0.122185
ORP	-0.133	0.1873	Temp	0.135309	0.179541
L tot rain	0.1213	0.2293	Rainy Day	0.103122	0.307273
Temp	0.116	0.2506	Ortho-P	-0.09907	0.327104
Nitrate	-0.104	0.3031	SpCond	0.090246	0.371922
SpCond	0.096	0.3422	Nitrate	-0.0878	0.385044
DO%	-0.0914	0.4207	L tot rain	0.085789	0.396115
pH	-0.0768	0.4473	DO%	-0.08445	0.403785
L2 tot rain	-0.0511	0.6138	ORP	-0.0779	0.441664
Total Rainfall	-0.0325	0.7483	Total Rain	-0.05875	0.561833
Turbidity	0.0312	0.7582	L2 tot rain	-0.0308	0.761729
TSS	-0.0185	0.8548	TSS	0.028841	0.775772
DO Charge	0.0094	0.926	DO Charge	0.025072	0.804454

Table 5. Relationships among environmental factors and the occurrence and LogMPN of Salmonella in sediment samples.

	Bi-r	P value		Pearson	P value
SpCond	0.315	0.0014	SpCond	0.333115	0.000708
ORP	0.238	0.0171	E. Coli	0.244294	0.014309
E. Coli	0.2126	0.0337	ORP	-0.23885	0.016725
Nitrate	0.145	0.15	Nitrate	-0.14609	0.147219
DO Conc	0.112	0.2674	Total Rain	-0.09982	0.323191
Total Rain	0.0973	0.3356	DO Conc	-0.09542	0.345087
L2 avg Tm	0.0566	0.5757	L2 tot rain	-0.08641	0.392697
L2 tot rain	0.052	0.6073	L2 avg Tm	0.059591	0.555907
pH	0.0517	0.6094	TSS	0.034959	0.729935
Turbidity	0.0468	0.644	Temp	-0.03289	0.745966
Temp	0.0458	0.6511	DO%	-0.02996	0.767759
DO%	0.0398	0.6942	Turbidity	-0.02826	0.780621
Ortho-P	0.0397	0.6949	Ave Temp	0.021869	0.829083
Ave Temp	0.0209	0.8361	Ortho-P	-0.01529	0.880688
Rainy Day	0.018	0.8588	DO Charge	0.015007	0.882246
L Avg Tm	0.0152	0.8809	pH	-0.00866	0.932325
TSS	0.0129	0.8986	L tot rain	0.00309	0.975659
L tot rain	0.0082	0.9355	Rainy Day	-0.0031	0.976368
DO Charge	0.0026	0.9798	L Avg Tm	-0.00256	0.980306

Outcomes and accomplishments

We have established collaboration with FDA as part of the Next Generation Sequencing Project. This collaboration includes a consortium of laboratories and state health departments throughout the country and will greatly expand our database to provide nationwide whole genome sequence comparisons. Strains collected from these studies will be inputted into this data base in order to determine regional difference in distribution of *Salmonella.* DNA extracted from water samples for DGGE analysis will be used to examine microbial populations using 16S sequence comparisons.

These data were presented at the Annual CPS meetings in 2011 and 2012 by Dr. Wright. Two manuscripts based partially on these data have been published or are in press: Gu et al., 2012 "Factors affecting the occurrence of Escherichia coli O157:H7 contamination in irrigation ponds on produce farms in the Suwannee River Watershed" and Gu et al., 2013"Factors affecting the occurrence of *Camplylobacter* contamination in irrigation ponds on produce farms in the Suwannee River Watershed". Another paper is in preparation: Luo et al., "Distribution and diversity of *Salmonella enterica* from irrigation ponds within the Suwannee River Watershed". We anticipate submission of two additional papers on methodology.

The completion of the last 100 strains for DiversiLab rep-PCR analysis was delayed by equipment failure, but we now have equipment on loan and are completing these studies. We will continue to evaluate data and have plans to coordinate sampling results with the other CPS-funded project titled "Evaluation of sampling protocol to provide science-based metrics for use identification of *Salmonella* in irrigation water testing programs in mixed produce farms in the Suwannee River watershed" led by Dr. Vellidis at the University of Georgia. The Vellidis' project will be using the same 5 ponds but with even larger number of samples. We have received funding from CPS to link with a UC Davis project (Jay-Russell) to examine *Salmonella* isolated from wildlife in the region.

Summary of Findings and Recommendations

The following describes the summary of our findings:

1. *Salmonella* was distributed throughout all irrigation ponds in the Upper Suwannee River watershed. Although there were some ponds with consistently higher levels of pathogen detection, there were no clear pond characteristics or agricultural practices that distinguished these ponds from those with lower levels.
2. Several environmental factors showed significant associations with pathogen occurrence and levels, but the correlations were weak ($r<0.3$)
3. Although we were able to identify significant associations with some environmental parameters and the prevalence of Salmonella, we are still struggling with one of the inherent problems to the investigation of pathogens in the pre-harvest environment, namely the fact that relatively low density of these organisms persist. We have detected *Salmonella* at all sites, but frequently the MPN values are right at the limit of detection, which begs the question of the accuracy of these values. Attempts to increase sensitivity by milipore filtration, modified Morse filter and IMS and the latest efforts with tangential flow filtration were not successful.
4. The practice of "cross-plating" (isolation from enrichment onto XLT4 followed by confirmation of CHROMagar) showed 100% agreement with more standard methods using PCR confirmation. This method could be a cost effective alternative to standard methods that would increase the capacity and sensitivity of *Salmonella* evaluation.

5. Operational taxonomic units (OTUs) from analysis 16S community bacterial DNA by DGGE also did show strong association with *Salmonella* occurrence and levels and could be exploited as molecular markers that could serve as alternatives to fecal indicators.
6. Molecular typing (DiversiLab rep-PCR) indicated an increased incidence (50%) of strains with similarity to clinical isolates as compared to a prior study on the Suwannee River where only 12% showed similarity to clinical strains. Planned genomic studies in collaboration with FDA should help determine the significance of the findings and better define the associated public health risks.

Peer-reviewed publications (PDFs attached)

1. Gu, G., L. Zhiyao, J. Cevallos-Cevallos, P. Adams, G. Vellidis, A. Wright, and A. Van Bruggen. 2012. Factors affecting the occurrence of *Escherichia coli* O157 contamination in irrigation ponds on produce farms in the Suwannee River Watershed. *Can. J. Microbiol.* Published on the web 3 December 2012, 10.1139/cjm-2012-0599
2. Gu, G., L. Zhiyao, J. Cevallos-Cevallos, P. Adams, G. Vellidis, A. Wright, and A. Van Bruggen. 2012. Occurrence and population density of *Campylobacter jejuni* in irrigation ponds on produce farms in the Suwannee River Watershed. *Can. J. Microbiol.* In Press.

Abstracts funded in part by this grant

1. Luo, Z., G. Gu, P. Adams, G. Velidis, A. Van Bruggen, and A.C. Wright. 2011. *Salmonella* Contamination of Irrigation Water from Mixed Produce Farming. Southeastern Branch of the American Society for Microbiology. Gainesville, FL.

2. Luo, Z., G. Gu, P. Adams, G. Velidis, A. Van Bruggen, and A.C. Wright. 2012. Evaluation of Regional Risks for *Salmonella* Contamination of Irrigation Water from Mixed Produce Farming. Emerging Pathogens Research Day. Gainesville, FL.

3. Luo, Z., G. Gu, P. Adams, G. Velidis, A. Van Bruggen, and A.C. Wright. 2012. Evaluation of Regional Risks for *Salmonella* Contamination of Irrigation Water from Mixed Produce Farming. Proceedings of the American Society for Microbiology, San Francisco, CA.

4. Luo, Z., G.Gu, P. Adams, G. Velidis, A. VanBruggen, M. Danyluk, A.C. Wright. 2012 Distribution and genetic diversity of *Salmonella enterica* isolated from Irrigation Water in the Suwannee River Watershed. Southeastern Branch of the American Society for Microbiology. Athens, GA.

Oral Presentations funded in part by this grant

1. Wright, A. C. 2012. Science-Based Evaluation of Regional Risks for *Salmonella* Contamination of Irrigation Water from Mixed Produce Farming. Center for Produce Safety Annual meeting. Davis, C
2. Wright, A. C. 2012. "Environmental Pathogens" Invited seminar for Food Science Departmental Seminar, University of Florida.
3. Wright, A. C. 2012. Distribution and genetic diversity of *Salmonella enterica* isolated from Irrigation Water in the Suwannee River Watershed. Athens, GA.