CPS CENTER for PRODUCE SAFETY

CPS 2011 RFP
FINAL PROJECT REPORT

Project Title

Evaluation of sampling protocol to provide science-based metrics for use in identification of Salmonella in irrigation water testing programs in mixed produce farms in the Suwannee River watershed

Project Period

January 1, 2012 – December 31, 2013

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Objectives

1. *Objective 1. Compare the utility of a composite sample collected from multiple locations in surface water to a single grab sample for maximizing probability of detection of Salmonella and indicator bacteria.*
2. *Explore the role of precipitation on Salmonella and indicator bacteria concentrations by a) Comparing 5-day geometric means of samples collected near vegetated buffer/pond interfaces to field/pond interfaces and b) Comparing 5-day geometric means to background levels established in Objective 1.*

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FINAL REPORT

Abstract

The overall goal of this proposal is to develop knowledge which will allow vegetable producers who rely on untreated surface sources of irrigation water to effectively address recently proposed FDA rules. In January 2013, the FDA proposed that all *agricultural water* must be *safe* for its intended use. Although data may be available with which to respond to the FDA proposals on *safe agricultural water* for some parts of the United States, there are still significant knowledge gaps in the southeastern United States that prevent us from doing so. Before a science-based response can be formulated, this knowledge gap must be closed. In the Southeast, a variety of irrigation sources are used by vegetable producers with the most common source a constructed farm pond. A companion CPS-funded study led by Dr. Anita Wright at the University of Florida consistently found measurable concentrations of *Salmonella*, shiga toxin-producing *E. coli* (STEC) and *Campylobacter jejuni* in water samples collected from near the irrigation system intake of 10 ponds in the Southeast. The intake is usually 10 to 20 ft from the bank and at a depth of 3 to 6 ft. Collecting samples at the intake typically requires a boat, specialized sampling equipment, and time, all of which make it difficult for vegetable producers to collect samples during the growing season – especially if it is to be done weekly as FDA is proposing.

Our study developed and evaluated two different producer-friendly sampling strategies designed to reflect *Salmonella* concentrations at the irrigation system intake. Strategy 1 consisted of collecting 3 grab samples from the bank near the intake of the irrigation system, approximately 10ft apart. Strategy 2 consisted of collecting 3 grab samples distributed along the perimeter of the pond. For each strategy, a composite sample was created from the 3 grab samples. We collected samples from 5 ponds used to irrigate produce and other crops for 19 months beginning March 2012 and ending September 2013. *Salmonella* concentrations in the ponds were low, averaging below 1 MPN/100mL for all ponds. Of the 507 samples analyzed, 217 samples (42.8%) were confirmed positive for *Salmonella* and 290 (57.2%) were negative. There were statistically significant differences in both concentrations and percent positives between ponds and between calendar months. Statistically, both sampling strategies represented the intake well. However, we also evaluated how frequently the analytical results from the intake matched the analytical results for each sampling strategy. Overall, there was a 70% match rate between the intake and strategies' composite samples and this match was statistically significant. In other words, 70% of the time the analytical results for *Salmonella* from the intake matched the analytical results of the composite sample (positive intake = positive composite and negative intake = negative composite). However, this also means that the samples did not match about 30% of the time. For individual ponds, the results were more variable – the lowest match rate was 50% while the highest was 89%. These results indicate that sampling from the bank does not reliably represent water near the irrigation system intake.

We also evaluated the effect of storm-driven surface runoff events on two of the study ponds. For 12 storms (6 per pond) occurring between January and August 2013, 33% of pond water samples collected shortly before rainfall events were positive for *Salmonella* while 58% were positive immediately after rainfall events. Surface runoff samples from agricultural fields were positive 38% of the time, and samples from forested areas were positive 40% of the time. Small streams feeding the ponds were positive 100% of the time. This indicates that hydrologic features which accumulate water and flow into ponds during storm events are more likely sources of contamination than direct surface runoff. Finally, we found that the 235 CFU per 100 mL generic *E. coli* threshold proposed by FDA is not a good indicator of the presence of *Salmonella* in irrigation ponds and surface runoff in the Southeast.

Background

Irrigation is an essential component of fruit and vegetable production. Yet irrigation water has been shown to be a vector for the contamination of fresh produce by pathogenic bacteria and has been implicated in outbreaks of foodborne human disease and deaths (CDC, 2008; Greene et al., 2008). The FDA recognizes this and in its recently released "Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption", it proposes to establish the requirement that all *agricultural water* must be *safe* and of adequate sanitary quality for its intended use (proposed § 112.41.) FDA's definition of *agricultural water* is water that comes into *direct contact* with produce and includes irrigation water that is applied using direct water application methods such as overhead sprinkler irrigation. It does not include indirect water application methods such as drip or furrow irrigation. However, the proposed rules do acknowledge that use of contaminated water in drip or furrow irrigation may still serve as a vehicle for bringing contaminants into the growing environment, which may potentially be transferred to produce by rain splash, workers, or equipment. Proposed § 112.42 and § 112.43 would require producers to ensure that their water sources (including ponds) are safe, that the sources' watersheds are protected, and that their water distribution systems (such as irrigation systems) are free of pathogens. Proposed §112.45(b)(1) requires weekly sampling during the growing season of any untreated surface water (for example, a river or pond) which receives precipitation-driven surface runoff and is used as agricultural water. FDA also proposes that agricultural water be considered *safe* if test results do not exceed 235 CFU per 100 mL generic *E. coli.* FDA seeks comment on these proposed rules.

Although data may be available with which to respond to the FDA proposals on *safe agricultural water* for some parts of the United States, there are still significant knowledge gaps in the southeastern United States that prevent us from doing so. Before a science-based response can be formulated, this knowledge gap must be closed. Our project addresses this knowledge gap on *safe agricultural water* and specifically addresses irrigation water used by vegetable producers in the southeastern Coastal Plain (SECP) of the United States. The information from this region will also be relevant to other agricultural regions of the United States that rely on untreated surface water and on-farm reservoirs as sources of irrigation water. The SECP is an ecoregion which spans portions of Louisiana, Mississippi, Tennessee, Alabama, Florida, Georgia, South Carolina, North Carolina, and Virginia. It is an important vegetable production area of the United States with a long growing season that allows for at least two vegetable crops per year in most areas. Southern Georgia is in the heart of SECP and has been identified by federal agencies and researchers as being representative of the agricultural practices, climate, and water resources of the SECP (Jang et al., 2013; Cho et al., 2010; Sheridan et al. 1992).

In the SECP, a variety of irrigation sources are used by vegetable producers with the most common source a constructed farm pond. These ponds are typically created by damming a 2nd or 3rd order stream (Figure 1). During the growing season, the ponds serve as source waters for on-farm irrigation systems (Figure 2). They are replenished by the stream, direct surface runoff during precipitation events, and sometimes by ground water from nearby wells. Even when a ground water well is available, vegetable producers frequently irrigate directly from the pond because they can withdraw water from the pond at a much higher capacity than from the well. If the water is withdrawn for use in drip irrigation, it passes through a series of sand filters before entering the piping of the irrigation system. If the water is withdrawn for use in overhead sprinkler irrigation systems (center pivot or solid set), then it enters the piping of the irrigation system directly without any filtration.

A companion CPS-funded study led by Dr. Anita Wright at the University of Florida consistently found measurable concentrations of *Salmonella*, shiga toxin-producing *E. coli* (STEC) (Gu et al., 2013a), and *Campylobacter jejuni* (Gu et al., 2013b) in water samples collected from 10 ponds in the SECP used for irrigation (Figure 3). The study focused on quantifying the presence of pathogens in water samples

collected at the irrigation system intake of the pond. The intake is usually 10 to 20 ft from the bank and at a depth of 3 to 6 ft (Figure 4). Collecting samples at the intake typically requires a boat, specialized sampling equipment, and time, all of which make it difficult for vegetable producers to collect samples during the growing season – especially if it is to be done weekly as FDA is proposing. Our study developed and evaluated two different producer-friendly sampling protocols designed to reflect *Salmonella* concentrations at the irrigation system intake.

Research Methods and Results

We selected 5 of 10 ponds used by the Wright project for our study (Figure 3). These were the ponds with the 3 highest percent positives for *Salmonella*, a pond with median percent positives, and the pond with the lowest percent positives. The first four ponds were used to irrigate vegetables with either drip or overhead sprinkler systems during the life of the study. The fifth pond was used to irrigate bioenergy feedstocks (Miscanthus). However, the team thought it prudent to include this pond in the study because it provides a benchmark for our analytical methods. Figure 2 is a composite of images from some of the study sites.

Objective 1 – Sampling Strategies

From March 2012 to September 2013 (19 months), water samples were collected from the five irrigation ponds using two different sampling strategies (Figure 5). Strategy 1 consisted of collecting 3 grab samples (1.5L each) from the bank near the intake of the irrigation system, approximately 10ft apart. Strategy 2 consisted of collecting 3 grab samples along the perimeter of the pond. One was located at the bank near the intake, one on the pond dam, and the third some distance away from either of the other two sampling locations. The sampling points were selected to represent the landscape around the perimeter. For both strategies, a composite sample was also created by combining 0.33 L aliquots from each of the three grabs. In addition samples were collected at the irrigation system intake. For the first six months of the project, one surface sample was collected. For the final year of the project, two samples were collected: the surface sample and a deep sample (0.5m). We assumed that the samples collected at the intake are representative of the water entering the intake during irrigation. During statistical analyses of the results, the bank samples collected with Strategy 1 and Strategy 2 were compared to the intake samples.

Strategies were sampled on alternating months; during Month 1, samples for Strategy 1 were collected and during Month 2, samples for Strategy 2 were collected (Figure 6). Because of the large travel distances between ponds and to distribute the sampling load in the analytical laboratories, two ponds were sampled on week one of each month and three ponds were sampled on week three of each month.

In addition to the water samples, temperature, pH, dissolved oxygen concentration, turbidity, and specific conductivity were measured *in situ* at each pond with a multiparameter water quality sonde. Finally, precipitation events at each pond were recorded with a tipping bucket rain gage connected to a data logger. The rain gages were installed on the bank nearest the intake. Data were downloaded monthly during the regular sampling visits to the ponds.

Water Sample Analyses

All water samples were placed in coolers with ice and transported to the Water Chemistry Laboratory at the University of Georgia's Tifton Campus. For the first 6 months of the project, 5 water samples were analyzed per pond per month (4 original samples + composite sample). For the final 13 months of the project, 6 water samples were analyzed per pond per month (5 original samples + composite sample). The theoretical maximum number of water samples was 540. Because of low water levels in some of the ponds during 2012, we collected and analyzed a total of 507 water samples during the project period.

All samples were analyzed for *Salmonella*, *E. coli*, Total Coliform, NO_3-N, NH_4-N, TN, PO_4-P, TP, Cl^-, and total suspended solids under the supervision of Ms. Debbie Coker – the Water Chemistry Laboratory's manager. A novel cross-streaking method developed by Dr. Anita Wright (Luo et al., 2014) was used to isolate, confirm, and enumerate *Salmonella*. The method uses a MPN protocol with three dilutions cultured in enrichment broth, selective broth, and plated on two types of selective media. With the dilutions we were using, our lower detection limit was 0.0548 MPN/100mL and our upper detection limit was 11 MPN/100mL. All presumptive positive *Salmonella* colonies were confirmed by PCR targeting the InvA gene in Dr. Sree Rajeev's laboratory at the University of Georgia's Veterinary Diagnostic Laboratory in Tifton. The IDEXX Colilert reagent and the Quanti-tray system were used to analyze water samples for *E. coli* (IDEXX Laboratories, 2013). Samples were analyzed for NO_3-N, NH_4-N, TN, PO_4-P, TP, Cl^- with colorimetric autoanalyzers using standard methods (Clesceri et al., 1998).

General *Salmonella* Sampling Results

Salmonella concentrations in the ponds were low, averaging below 1 MPN/100mL for all ponds. Of the 507 samples analyzed, 217 samples (42.8%) were confirmed positive for *Salmonella* and 290 (57.2%) were negative. We evaluated the sampling results in several different ways to better understand the dynamics that drive *Salmonella* in these ponds. For data analyses, samples below our lower detection limit (No Detect or ND) were assigned a value of 0.001 MPN/100mL. Samples above our upper detection limit were assigned a value of 11 MPN/100mL. All samples were then multiplied by 1000 and log-transformed (natural log) to approximate a normal distribution prior to any statistical analyses.

We compared the 5 ponds spatially and temporally. Figure 7 shows mean *Salmonella* MPN/100mL concentrations in the ponds. Ponds 4 and 5 had the highest mean concentrations. While most samples collected from ponds 4 and 5 were similar in concentration to those collected from ponds 1-3, some samples were at or above our upper detection limit which increased the ponds' mean concentrations. We analyzed the log-transformed data using a variance GLM procedure followed by a means separation LSD test. There were statistically significant differences between the ponds as shown in Figure 8 and Table 1. Pond size and watershed size did not affect the results however there may be a link between pond age and *Salmonella* concentrations. Pond 3 which had the lowest concentrations was constructed less than 10 years ago while Pond 4 was constructed more than 60 years ago. We are currently attempting to locate construction records for the other ponds. Figures 7 and 8 also show the percent positive samples from each pond while Figure 9 shows a strong correlation between percent positive samples and *Salmonella* log MPN/100L. This result indicates that and these two parameters are good predictors of each other.

During our temporal analysis, we first compared the samples collected during 2012 (249 samples) to the samples collected during 2013 (258 samples). Means of the log-transformed data were 0.967 MPN/100L for 2012 and 0.908 MPN/100L for 2013. There were no statistically significant differences between the two means (Table 2). Because the project spanned 19 months of sampling, May through September were sampled twice – once in 2012 and again 2013. Data from both years were pooled together for each of these months since there were no statistical differences between years. Figure 10 shows the temporal distribution of *Salmonella* in the ponds. There is an upward trend in both concentrations and % positives during the summer with the peak in October. There is a decreasing trend during winter with the lowest values measured in March. November 2012 is somewhat of an outlier to these trends. It was unusually cold during November 2012 following a very dry year so we investigated the relationship between *Salmonella* concentrations and temperature. Although in Figure 10, temperature generally follows the same trend as concentrations, the correlation between the two was weak ($R^2 = 0.40$ – see Figure 11) indicating that more factors were at play. There are statistically significant differences between the lowest and highest months (Table 3).

Salmonella Sampling Strategy Results

As indicated earlier, we compared two sampling strategies. Strategy 1 consisted of collecting 3 grab samples from the bank near the intake of the irrigation system, approximately 10ft apart. Strategy 2 consisted of collecting 3 grab samples along the perimeter of the pond. The three locations were selected to characterize the landscape around the pond (cultivated, marshy, wooded, etc.). We compared the log-transformed data from the intakes, the grab samples, and the composite samples as shown in Table 4. Within strategies, there were no statistical differences between the intake samples and the composite sample created from the three grab samples. For Strategy 1, there were no statistical differences between the intake samples, the composite sample, and the three adjacent bank samples (Edge 1, Edge 2, Edge 3). However for Strategy 2, Edge 6 was significantly different from Edge 8. Edge 6 samples were collected from the same location as Edge 1 samples but on alternating months (Figure 5). Although there were no significant differences between the intake samples associated with each of the strategies (Intake1 = Strategy 1; Intake 2 = Strategy 2), there were significant differences between the two composites (Composite1 = Strategy 1; Composite2 = Strategy 2) likely driven by the grab sample collected from Edge 8. The mean of Edge 8 was affected by several samples above our detection limit from Pond 5.

The statistical analyses described in the previous paragraph indicate that both sampling strategies represent the intake well. However, we also evaluated how frequently the analytical results from the intake matched the analytical results for each strategy. Overall, there was a 70.5 % match rate between the intake and composite samples and this match was statistically significant (Table 5). Likewise using data from all ponds, Strategy 1 and Strategy 2 had about a 70% match rate which was statistically significant (Table 5). In other words, 70% of the time the analytical results for *Salmonella* from the intake matched the analytical results of the composite sample (positive intake = positive composite and negative intake = negative composite). However, this also means that the samples did not match about 30% of the time. For individual ponds, the results were more variable and the match rate was not always statistically significant (Table 5). The lowest match rate was at Pond 1 (50% for Strategy 1 – not significant) while the highest was at Pond 5 (89% for Strategy 2 – significant).

Salmonella and Other Water Quality Parameters

The analysis of the other water quality parameters measured during sampling (*E. coli*, Total Coliform, NO_3-N, NH_4-N, TN, PO_4-P, TP, Cl^-, pH, DO, conductivity) and their association with the presence of *Salmonella* is being conducted by Ms. Camilla Borgato, one of our project's graduate students. Ms. Borgato is pursuing a M.S. in Environmental Science and is currently analyzing her data. Her thesis defense is scheduled for May 26th, 2014. *We will send CPS a supplement to this report when Ms. Borgato has completed her analyses*.

Objective 2 – Surface Runoff and Pond Watersheds

This component of the project was conducted primarily by Ms. Casey Harris, our project's second graduate student who is pursuing a M.S. in Ecology. This component of the project forms her thesis research project and will be published in her thesis and journal articles resulting from her thesis. Her thesis defense is scheduled for March 25th, 2014.

Surface runoff and pond water samples were collected from Ponds 1 and 3 during six different rainfall events at each pond between January and August 2013. Pond water samples were collected before and after each rainfall event to assess any change in pathogen concentrations in the pond as a result of surface runoff. These pond water samples were collected and composited from the same locations as the composites in Strategy 1 and Strategy 2 of our regular monthly sampling. Surface runoff was collected by opening and pinning sterile 2-liter Whirl-Pak® bags to positions in the landscape where surface flow concentrated in the immediate pond watershed (Figure 12). Surface runoff bags were

approximately evenly spaced around the perimeter of the pond. The bags were installed in anticipation of a rainfall event and collected within two hours after the storm or at sunrise following an overnight storm (Figure 13). Each surface runoff sample was a composite of these Whirl-Pak bags totaling 4-6 liters depending on the intensity of the storm. Bags were composited according to sampling location and recorded under six sample categories (field edge, forest edge, etc.) (Table 6). Only composite samples were analyzed. Because of logistics, only one pond was sampled per rain event.

An automated ISCO sampler was installed at a stream feeding each pond. At Pond 1, the stream flowed regularly during wet conditions. At Pond 3, the stream was ephemeral and flowed only during and shortly after rainfall events. The intake of each ISCO sampler was set slightly above base flow to capture storm flow entering the ponds from upstream (Figure 14). The samplers collected 60 mL samples in sterile 10 L glass jars at regular intervals to create a composite of storm flow (Figure 15). Other ephemeral streams feeding each pond were sampled using Whirl-Pak bags like any other surface runoff sample. One of the streams at Pond 3 only drained agricultural fields, and thus was included in the field runoff sample category. All other ephemeral streams and the larger stream at Pond 1 drained large areas with multiple land use types and were included in the stream sample category (Table 6).

Approximately 192 pond water sample bags, 255 surface runoff sample bags, and 10 ISCO samples were collected from which 127 composites were created and analyzed. All samples were analyzed as described under Objective 1.

Surface Runoff Results

Between January and August 2013, 25% of the composite samples collected from Pond 1 and Pond 3 during our regular monthly sampling (Objective 1) were positive for Salmonella. For 12 storms occurring during that time period, 33% of composite pond water samples collected shortly before rainfall events were positive while 58% were positive immediately after rainfall events. Surface runoff samples from agricultural fields were positive 38% of the time, and samples from forested areas were positive 40% of the time. Small inflow streams feeding the ponds were positive 100% of the time. Surface runoff and pond water samples from Pond 1 tended to have higher Salmonella levels than Pond 3 (Figure 16), though not significantly. Samples collected in January through March tended to have lower Salmonella levels overall, with the highest Salmonella samples collected in June through August.

Fifty-three percent of samples collected overall did not contain detectable *Salmonella* by our laboratory method. Each of these “non-detects” was assigned a value of 0.001 MPN per 100 mL. We evaluated a linear mixed-effects model and determined profile confidence intervals for *Salmonella* in each sample type using the *lmer* program from the *lme4* package (version 1.0-5; Bates et al., 2013) for the R Language and Environment for Statistical Computing (version 3.0.2; R Core Team, 2013) (Table 7). Mixed-effects models are capable of accounting for multiple levels of non-independence between data points. In this case, the sources of non-independence between data points were the ponds, locations, and dates the samples were collected. The outcome variable of the model, *Salmonella*, was defined on a scale from 0.0548 to 11 MPN per 100 ml sample (Jarvis, 2010). The *Salmonella* data was converted to a 100 L scale and log-transformed for model analysis.

It is important to note that our method for measuring *Salmonella* in any sample of water could not detect less than 0.0548 MPN per 100mL; this was our detection limit. Field runoff, forest runoff, and pond water prior to rainfall events had model-estimated levels of *Salmonella* significantly below the detection limit. Levels of *Salmonella* in ponds following rainfall events were not significantly different from the detection limit. Field runoff, forest runoff, and pond water did not differ significantly from one another, but small streams flowing into the ponds had significantly higher *Salmonella* levels than most other locations. Statistically significant differences between sample types are indicated by non-overlapping 95% confidence intervals (Figure 17). All of these relationships between *Salmonella*

estimates and the detection limit were influenced by the substitution of 0.001 MPN/100mL for non-detects, and would differ if another substitution were used.

Samples from one particular location, a forest doubling as a popular fishing spot at Pond 1, frequently had higher *Salmonella* levels than other forested areas. If this particular location had been eliminated from the model, the estimated overall forest *Salmonella* level would appear slightly lower than the level for fields.

The same model was applied to our *E. coli* data. The IDEXX method for measuring *E. coli* in any sample of water has a detection limit of 1 CFU per 100 ml. *E. coli* levels in ponds before and after rainfall events did not differ significantly from one another. *E. coli* levels in field runoff, forest runoff, and small streams flowing into the ponds also did not differ significantly from one another. However, *E. coli* levels in field runoff, forest runoff, and small streams were all significantly higher than *E. coli* levels in ponds before and after rainfall events (Figure 18).

E. coli in storm runoff from fields, forests, and small streams appears high, significantly above the 235 *E. coli* CFU limit for safe water usage under the proposed Food Safety Management Act, but these inputs seem to be diluted by the ponds. In contrast, *Salmonella* concentrations in ponds after rainfall events appear higher (though not significantly) than *Salmonella* concentrations in storm runoff from nearby fields or forests.

Samples of pond water exceeded 235 MPN/100mL for *E. coli* in four out of 60 samples collected in this study, but none of those four contained detectable *Salmonella* (Table 8). Of the 60 samples, 26 did contain detectable *Salmonella* but had *E. coli* levels below the 235 MPN/100mL threshold. *E. coli* was a slightly better predictor of *Salmonella* in samples of runoff. Overall, *E. coli* correctly predicted the presence or absence of *Salmonella* only about 55% of the time (sum of correctly positive and correctly negative in Table 9).

Landscape Analysis

The project's five pond watersheds as well as the five additional pond watersheds used in the recently completed Anita Wright project were identified using the USGS National Elevation Dataset (Gesch 2007, Gesch et al. 2002) and hydrology tools available in ArcGIS Desktop 10.1 (ESRI Corp. 2011) (Figure 19). The land uses within each watershed were classified into five main categories using the 2011 USDA NASS Cropland Data Layer. Land use within a 250 meter radius of each pond edge was classified manually based on aerial imagery from the 2010 National Agricultural Imagery Program for greater accuracy. (Table 10, Figures 20 and 21)

Wildlife

Ms. Harris installed automated wildlife cameras in strategic positions around Ponds 1 and 3 and is using the photographs to qualitatively assess the presence of wildlife around the ponds (Figure 22). A large variety of the same small and large mammals and birds visit each pond.

Serotyping

The species *Salmonella enterica* includes over 2500 serotypes potentially capable of causing human infections. In investigations of outbreaks of foodborne illness, identifying the particular serotype involved is necessary to link individual cases of illness together and trace them back to the original source of contamination. Although serotyping was not included in our proposal, our team decided that it was important for us to understand if the *Salmonella* we were finding in our samples was associated with human illness. We serotyped a portion of the isolates collected during the sampling strategy and surface runoff components of the project. Salmonella serotyping is an expensive and laborious process and is performed only in few reference laboratories in the United States. Our samples were serotyped at the National Veterinary Services Laboratory Ames, Iowa. So far we have received results from only a portion of the surface runoff sample isolates we have sent to the NVSL and a summary of those results is

presented further below. *We will send CPS a supplement to this report when we have received all the serotyping results*.

Sampling Strategy Samples

We serotyped one isolate from every positive sample collected between October 2012 and September 2013, not including composite samples. This totals 115 isolates, and should provide an efficient and relatively balanced view of the most common serotypes found at these five ponds each month. We would like to identify whether the same serotypes are present at geographically distant ponds, and whether particular serotypes appear to have any seasonal/temporal trends.

Surface Runoff Samples

To obtain an idea of the diversity of *Salmonella* present across these samples, we used seven vials of different *Salmonella* O antisera representing the most common serogroups and tested every isolate collected from storm sampling. Diversity was surprisingly high – some of our samples, especially those collected from streams, contained up to four different *Salmonella* serogroups. Since one serogroup may encompass hundreds of serotypes, we decided to serotype up to three isolates from every serogroup in every positive sample. This totaled 150 isolates, of which 112 have been serotyped so far. With the results, we hope to determine whether the serotypes collected from fields, forests, or streams during storms reflect the serotypes collected from pond water before or after the same storms. In general, *Salmonella* levels do appear to be slightly elevated in these ponds after storms, and we would like to see whether the same serotypes persist in pond water even in periods without rain. It is possible that other serotypes, such as those more closely associated with aquatic animals and not known to commonly cause human illness, could be the main serotypes present in pond water in periods without rain.

As mentioned earlier, our results so far are incomplete. Some *Salmonella* serotypes associated with human illness (such as var. Muenchen and Saintpaul) do appear in these two vegetable farm environments. However, some of the most common *Salmonella* serotypes associated with human illness (including var. Javiana, Enteritidis, Typhimurium, Montevideo, Heidelberg) were not detected at all. Some of the more persistent *Salmonella* serotypes found in repeated months at these two ponds appeared in every sample type (field, forest, stream, ponds pre- and post- storms). A list of the *Salmonella* serotypes found in each pond and sample type is shown in Table 11 along with the total number of months the serotype appeared.

Outcomes and Accomplishments

This project provided many challenges ranging from logistical to scientific but resulted in a series of positive outcomes and accomplishments. With support from the Center for Produce Safety, we developed a strong and dynamic multi-state, multi-institutional team dedicated to developing knowledge which will allow vegetable producers who rely on untreated surface sources of irrigation water to effectively address recently proposed FDA rules. The partner institutions include the University of Georgia, Emory University, the University of Florida, the University of California at Davis, and the Western Center for Food Safety. The team consisted of microbiologists, water quality experts, hydrologists and several vegetable producers in southern Georgia. The partnerships and trust we developed with the vegetable producers will be long-lasting and will allow us to conduct important on-farm projects in the future. In addition, we trained several young scientists during the project's two years. Two graduate students conducted their M.S. thesis on components of the project. A third graduate student assisted with the project. We also employed two post-doctoral researchers, two undergraduate student workers, a field technician and a lab analyst.

We developed innovative analytical techniques for the laboratory and innovative sampling techniques for the field which will make future projects easier, more cost-effective, and more productive. We implemented a novel cross-streaking method developed by Dr. Anita Wright (Luo et al., 2014) to isolate, confirm, and enumerate *Salmonella* in our laboratories at the University of Georgia and Emory University. Dr. Wright trained our lab manager and post-doc in her laboratory and then sent her graduate student to our laboratory to help us establish the method in our laboratories. For one year, both Dr. Wright's laboratory and Dr. Vellidis' laboratory analyzed samples from the same ponds using the same methods. The results are being used to quantitatively assess the robustness of the method.

We were excellent stewards of the Center for Produce Safety funds provided to us for this project. The existing infrastructure and technical expertise at Dr. Sree Rajeev's laboratory at the University of Georgia's Veterinary Diagnostic Laboratory in Tifton allowed us to expeditiously confirm all presumptive positive *Salmonella* colonies by PCR targeting the InvA gene at a relatively low cost despite the fact that we analyzed a much larger number of samples by PCR than originally expected. We were also able to leverage the project's personnel costs with related ongoing projects and thus realized significant savings. As a result, we were able process and send samples confirmed positive by PCR for serotyping to the National Veterinary Services Laboratory (NVSL) in Ames, Iowa. This will allow us to understand if the *Salmonella* we found in our samples is associated with human illness – something which will make our results more powerful and more meaningful to the produce industry.

We successfully completed the goals of our proposal although the experimental approach used for our second objective (surface runoff) was modified from that originally proposed. This allowed us to better understand the contribution of the landscape to the ponds' *Salmonella* load. A summary of our findings and recommendations is provided in the following section.

Summary of Findings and Recommendations

- The bank sampling strategies we evaluated do not consistently represent presence of *Salmonella* in the water near the irrigation system intakes so they are probably not the best approach for weekly sampling used for GAP and other similar protocols.
- Bank sampling can be used to assess longer-term trends in the ponds and to assess the potential risk of using the water for irrigating produce.
- The most representative sample of water entering the irrigation system can be collected by installing a sampling valve in the supply line of the irrigation system. This will allow producers to easily collect samples while the irrigation system is operating. However, this approach does not prevent contaminated irrigation water from being distributed by the irrigation system. In a recently initiated CPS-sponsored study, we will be installing sampling valves in the supply lines of several irrigation systems and we will compare samples collected from near the intake, the sampling valve, and the irrigation system during irrigation events.
- Precipitation driven surface-runoff does increase the concentration of *Salmonella* in the ponds. It is not clear however if this is an effect of inflowing water disturbing pond sediments or an effect of *Salmonella* being added to the ponds directly by runoff and storm-driven stream flow. The concentrations of *Salmonella* in surface runoff and the percentage of runoff samples found positive for *Salmonella* were similar to those found in the ponds during monthly sampling.
- The 235 CFU per 100 mL generic *E. coli* threshold proposed by FDA is not a good indicator of the presence of *Salmonella* in irrigation ponds and surface runoff in the Southeast.

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APPENDICES

Publications and Presentations

Presentations

To date, we have made two presentations on this project:

- Poster presentation at the 2012 Center for Produce Safety Symposium
- Oral presentation at the 2013 Center for Produce Safety Symposium.

Planned presentations include:

- June 2014 Center for Produce Safety Symposium
- January 2015 Southeast Regional Fruit and Vegetable Conference. This conference is the largest educational conference and trade show in the southeastern United States that unites growers, vendors and suppliers.

Publications

The results from this work have not yet been published, but several publications are in progress and most will either be published or submitted to scientific journals by 30 June 2014. Below is a list of publications *already in progress*.

- Borgato, Camilla. 2014. Correlation of *Salmonella* with physical, chemical, and biological water parameters in irrigation ponds of the Southeastern USA. M.S. thesis, University of Padova, Italy. (We anticipate two journal articles resulting from this thesis)
- Harris, Casey. 2014. Storm runoff and land use related to Salmonella irrigation ponds of the Southeastern USA. M.S. thesis, University of Georgia, Athens, USA. (We anticipate two journal articles resulting from this thesis – one describing the results of the surface runoff study and the second analyzing the relationship between the landscape surrounding the ponds and *Salmonella* measured in the ponds.)
- Journal articles in progress
    - Chemical and physical water quality parameters associated with the presence of *Salmonella* in irrigation ponds
    - Is *E. coli* a good predictor of *Salmonella* in irrigation ponds
    - Effect of precipitation and landscape position on *Salmonella* and *E. coli* in surface runoff around irrigation ponds
    - The relationship between the landscape surrounding irrigation ponds and *Salmonella* measured in the ponds
    - Producer-friendly sampling protocols designed to reflect *Salmonella* concentrations at the irrigation system intake

Budget Summary

The funds provided by the Center for Produce Safety were appropriate for the effort required to accomplish the project's objectives. Funds were expended as indicated below. During Year 2 of the project, we requested a line item shift in the budget of the project. We moved funds from Personnel to Supplies and Materials to cover higher than expected analytical costs because we were conducting significantly more PCR analyses than originally estimated. The request was approved and the spending table below reflects that shift of funds.

Expenditures 01 Jan 2012 – 31 Dec 2013		
Total Salaries	$	115,831.68
Total Benefits	$	22,873.50
Travel	$	3,225.88
Operating*	$	104,791.94
Indirect Costs	$	6,935.00
Total Expenditures	$	253,658.00

*Operating includes Supplies and Materials, Subaward to Emory University, and Other Costs.

Account Balance	$	1,230.00

Tables and Figures

Table 1. Statistical comparison of *Salmonella* Mean log MPN/100L concentrations for the project's five ponds. Means with the same t Grouping letter are not significantly different.

t Grouping		Mean log MPN/100L	N	Pond
	A	1.2166	99	Pond 5
	A	1.1238	102	Pond 4
B	A	0.9959	102	Pond 1
B	C	0.8105	102	Pond 2
	C	0.5474	102	Pond 3

Table 2. Statistical comparison of *Salmonella* Mean log MPN/100L concentrations for 2012 and 2013. Means with the same t Grouping letter are not significantly different.

t Grouping	Mean log MPN/100L	N	Year
A	0.9673	249	2012
A	0.9081	258	2013

Table 3. Statistical comparison of *Salmonella* Mean log MPN/100L monthly concentrations. Means with the same t Grouping letter are not significantly different.

t Grouping			Mean log MPN/100L	N	Month
	A		1.5829	55	Sep
	A		1.4935	25	Oct
B	A		1.2785	54	Aug
B	A		1.1970	25	Dec
B	C		0.9972	54	Jun
B	C		0.9803	55	Jul
B	C	D	0.9655	55	Apr
B	C	D	0.8799	54	May
E	C	D	0.7044	25	Jan
E	F	D	0.4986	25	Feb
E	F		0.2327	25	Nov
	F		0.1370	55	Mar

Table 4. Statistical comparison of *Salmonella* Mean log MPN/100L concentrations for the two sampling strategies evaluated. Means with the same t Grouping letter are not significantly different.

t Grouping			Mean log MPN/100L	N	Month	Strategy
	A		1.2723	44	Edge8	2
B	A		1.1964	45	Composite2	2
B	A	C	1.0841	45	Edge7	2
B	A	C	1.0723	14	Intake2 (subsurface)	2
B	A	C	1.0542	45	Intake2 (surface)	2
B	A	C	0.9889	50	Edge3	1
B	A	C	0.9874	50	Intake1 (surface)	1
B	A	C	0.8631	19	Intake1 (subsurface)	1
B		C	0.7704	45	Edge6	2
B		C	0.7442	50	Edge2	1
		C	0.6915	50	Composite1	1
		C	0.6488	50	Edge1	1

Table 5. Frequency with which the analytical results for *Salmonella* from the intake matched the analytical results for *Salmonella* each for sampling strategy (positive intake = positive composite and negative intake = negative composite).

Metrics	Number of Matching/Not Matching Pairs of Observations (Percent Matching/Not Matching Paired Observations)					
	Pond 1			Pond 2		
	Strategy 1	Strategy 2	Overall	Strategy 1	Strategy 2	Overall
Matched	8 (80%)	6 (67%)	14 (74%0	7 (70%)	6 (67%)	13 (68%)
Not Matched	2 (20%)	3 (33%)	5 (26%)	3 (30%)	3 (33%)	6 (32%)
Total	10 (100%)	9 (100%)	19 (100%)	10 (100%)	9 (100%)	19 (100%)
χ^2	3.6	1	4.26	1.6	1	2.57
P-value	0.05	0.31	0.03	0.2	0.32	0.108

Metrics	Pond 3			Pond 4		
	Strategy 1	Strategy 2	Overall	Strategy 1	Strategy 2	Overall
Matched	5 (50%)	7 (78%)	12 (63%)	8 (80%)	5 (56%)	13 (68%)
Not Matched	5 (50%)	2 (22%)	7 (37%)	2 (20%)	4 (44%)	6 (32%)
Total	10 (100%)	9 (100%)	19 (100%)	10 (100%)	9 (100%)	19 (100%)
χ^2	0	2.77	1.31	3.6	0.11	2.57
P-value	1	0.09	0.25	0.057	0.73	0.1

Metrics	Pond 5			Overall By Strategy		Overall
	Strategy 1	Strategy 2	Overall	Strategy 1	Strategy 2	
Matched	7 (70%)	8 (89%)	15 (79%)	35 (70%)	32 (71%)	67 (70.5%)
Not Matched	3 (30%)	1 (11%)	4 (21%)	15 (30%)	13 (29%)	28 (29.5%)
Total	10 (100%)	9 (100%)	19 (100%)	50 (100%)	45 (100%)	95 (100%)
χ^2	1.6	5.44	6.36	8	8.02	16.01
P-value	0.2	0.01	0.01	0.0047	0.0046	<0.0001

Table 6. Descriptions of sample type classifications and locations. Occasionally more than one sample was collected per location per month.

Sample Type		Pond 1		Pond 3	
		Location	Months sampled	Location	Months sampled
Pond before precipitation	Pond water collected during dry periods, a few hours before expected storms	Near intake	6	Near intake	6
		Pond edges	6	Pond edges	6
Pond after precipitation	Pond water collected immediately following storms	Near intake	6	Near intake	6
		Pond edges	6	Pond edges	6
Pond monthly	Pond water collected at regular monthly intervals, regardless of rainfall	Near intake (alternate months)	4	Near intake (alternate months)	4
		Pond edges (alternate months)	4	Pond edges (alternate months)	4
Inflow streams	Water collected from streams or major ditches flowing into ponds during storms	Intermittent stream	6	Intermittent stream	3
		Large ditch next to paved road	3	-	-
Fields	Runoff collected at the interface between agricultural fields and ponds during storms	Peanut field	6	Miscanthus (biofuel feedstock) field	6
		Tomato field	6	Peanut/Corn fields	4
Forests	Runoff collected at the interface between large patches of non-agricultural land and ponds	House with pines, grass	5	Mixed species forest A	6
		Shrubs, partially wet	6	Mixed species forest B	6
		Mixed species forest	5	-	-

Table 7. Linear mixed-effect model specification for the *lme4* package. The model was fit by a restricted maximum likelihood method. *lmer* Model: Salmonella = Type + (1|Pond) + (1|Month) + (1|Location)

Variable	Variable type	Levels	Transform-ation	Description of variable
Type	Fixed factor	6	-	Identifies the sample type (Fields, Forests, etc.)
Pond	Random factor*	2	-	Identifies sample from Pond 1 or Pond 3
Month	Random factor*	6	-	Date range (out of 6 full sampling cycles) of sample collection
Location	Random factor*	24	-	Identifies specific locations of repeated sampling
Salmonella	Outcome		natural log	Salmonella present in each sample

*Random factors were defined with random intercepts [(1|…) in *lmer* notation], but not random slopes.

Table 8. Using *E. coli* samples above 235 MPN/100mL to predict *Salmonella* presence.

***E. coli* Threshold** (235 MPN/100mL)	***Salmonella***		
	Present	**Absent**	**Total**
Runoff Samples (includes fields, forests, and inflow streams)			
Above	26	22	48
Below	5	8	13
Pond Samples (includes before/after precip. and monthly)			
Above	0	4	4
Below	26	34	60

Table 9. Using *E. coli* samples above 235 MPN/100 ML to predict *Salmonella* presence – percentages.

***E. coli* Prediction of *Salmonella* Presence**	**Sample Type**		**All** (%)
	Pond (%)	**Runoff** (%)	
Correctly positive	0	43	21
Correctly negative	53	13	34
Incorrectly positive	6	36	21
Incorrectly negative	41	8	25

Table 10. Pond size and watershed size for each pond in this study as well as percent cover by various land use types within a 250 m radius of each pond edge.

Pond	**Pond Area** (m^2)	**Pond Area** (ac)	**Watershed Area** (m^2)	**Watershed Area** (ac)	**Cropland** (%)	**Forest / Wetland** (%)	**Other** (%)	**Water** (%)	**Paved** (%)
Pond 1	79,935	20.0	2,745,691	686.4	42.9	40.4	15.9	0.0	0.8
Pond 2	5,799	1.4	6,822	1.7	81.0	15.2	3.8	0.0	0.0
Pond 3	46,722	11.7	658,244	164.6	36.8	53.8	9.4	0.0	0.0
Pond 4	10,955	2.7	204,309	51.1	28.8	31.3	39.9	0.0	0.0
Pond 5	21,637	5.4	877,759	219.4	57.3	33.7	2.8	5.7	0.6

Table 11. Partial serotyping results from surface runoff samples.

Number of months Each Serotype Was Found in Each Habitat					
More Frequent			**Less Frequent**		
Serotype Name			**Serotype Name**		
Sample Type	**Pond 3**	**Pond 1**	**Sample Type**	**Pond 3**	**Pond 1**
Muenchen			**Gaminara**		
Fields	1	1	Forests	1	
Forests		1	Inflow streams	1	
Inflow streams	1	3	Pond after precip.	1	
Pond after precip.	2	1	Pond before precip.	1	
Pond before precip.	1		**Braenderup**		
Saintpaul			Forests	1	
Fields		2	Inflow streams		1
Forests		2			
Inflow streams	1	3	**Inverness**		
Pond after precip.		2	Forests		2
Pond before precip.		1			
			Anatum		
Bareilly			Inflow streams		1
Fields	1	1			
Forests		2	**Newport**		
Inflow streams		2	Fields		1
Pond after precip.	1				
Pond before precip.		1	**Meleagridis**		
			Fields	1	
Rubislaw					
Forests		1	**Give_var._15+**	1	
Inflow streams	1	4	Inflow streams		
Pond after precip.		1			
Pond before precip.		1	**I_6,7:-:e,n,z15**		1
			Inflow streams		
III_60:r:e,n,x,z15					
Inflow streams		4	**III_16:z10:e,n,x,z15**		1
Pond after precip.	1	1	Inflow streams		
Pond before precip.	1				
			III_50:nonmotile	1	
I_38:k:-			Pond after precip.		
Fields		1			
Forests		1	**III_50:r:-**	1	
Inflow streams		2	Inflow streams		
Pond after precip.		1			
Pond before precip.	1	1	**III_60:r:-**		1
			Pond before precip.		

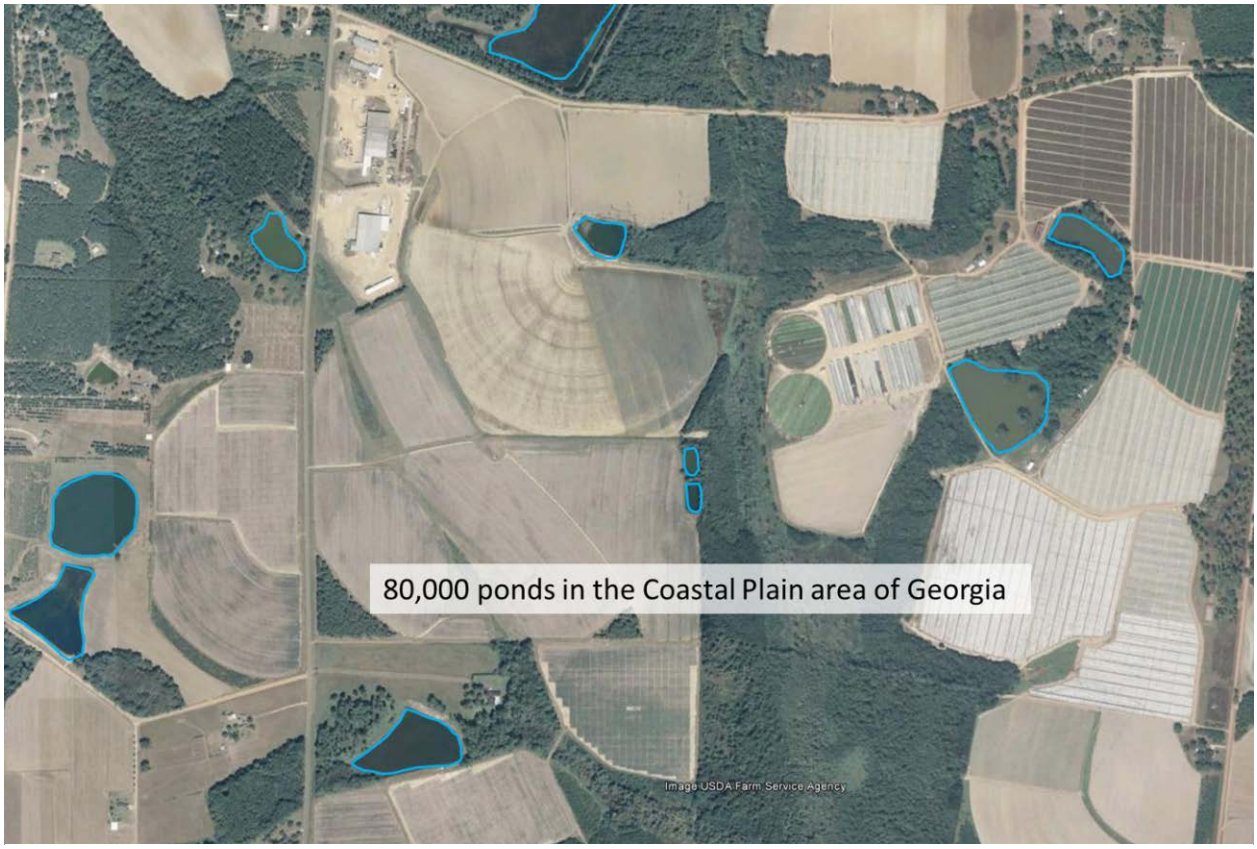

Figure 1. Typical landscape of the Southeastern Coastal Plain (SECP) near Tifton, Georgia. There are 80,000 ponds in the SECP half of which are man-made and used for irrigation.

Figure 2. Three types of irrigation systems typically used to irrigate produce in the SECP. Clockwise from top left – center pivot, drip, and solid set sprinkler systems. All three of the systems shown here use water from irrigation ponds.

Figure 3. The 10 ponds used by the Wright study were located in the Upper Suwannee, Little, Upper Ochlockonee and Lower Ochlockonee HUC8 watersheds. This study used a subset of 5 ponds located in the Little, Upper Ochlockonee and Lower Ochlockonee HUC8 watersheds.

Figure 4. The intake of irrigation pump stations is usually 10 to 20 ft from the bank and at a depth of 3 to 6 ft. Collecting samples at the intake typically requires a boat, specialized sampling equipment, and time, all of which make it difficult for vegetable producers to collect samples during the growing season.

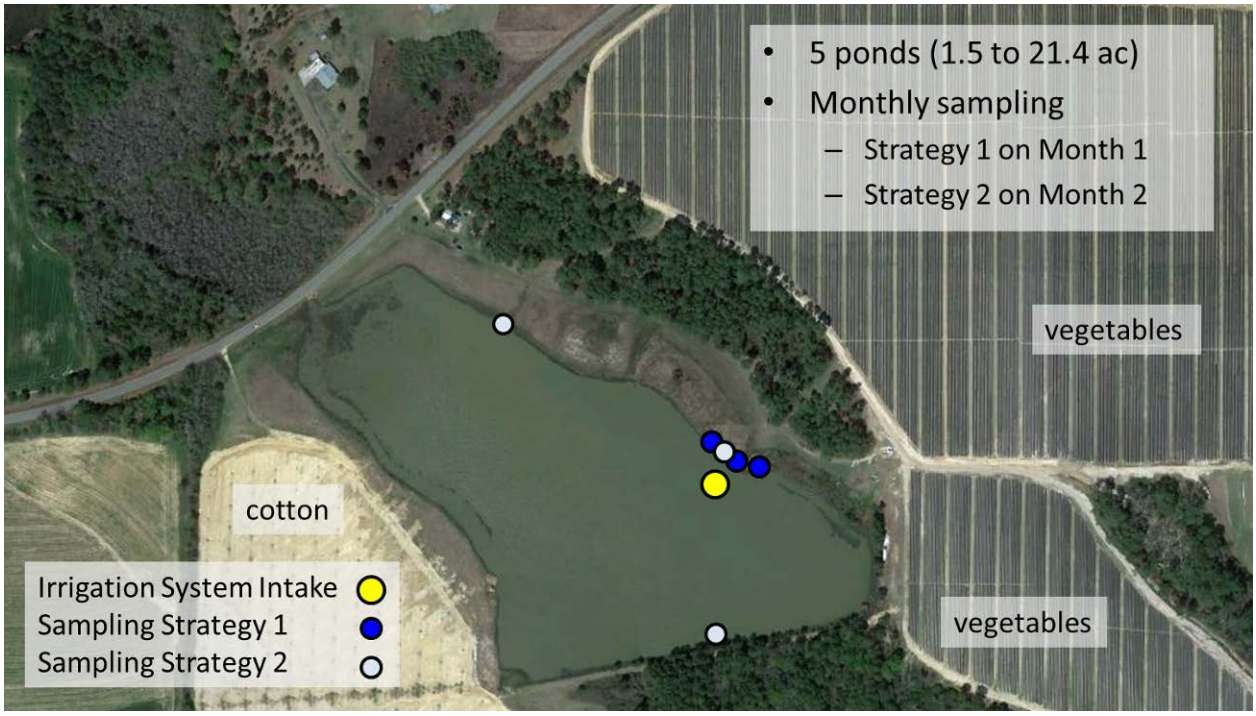

Figure 5. The two strategies used in the study as implemented at Pond 1. Strategy 1 consisted of collecting 3 grab samples (1.5L each) from the bank near the intake of the irrigation system, approximately 10ft apart. Strategy 2 consisted of collecting 3 grab samples along the perimeter of the pond. The three locations were selected to characterize the landscape around the pond (cultivated, marshy, wooded, etc.).

Figure 6. Ms. Camilla Borgato, one of the project's graduate students, collects a grab sample from one of the Strategy 2 bank sampling positions at Pond 4.

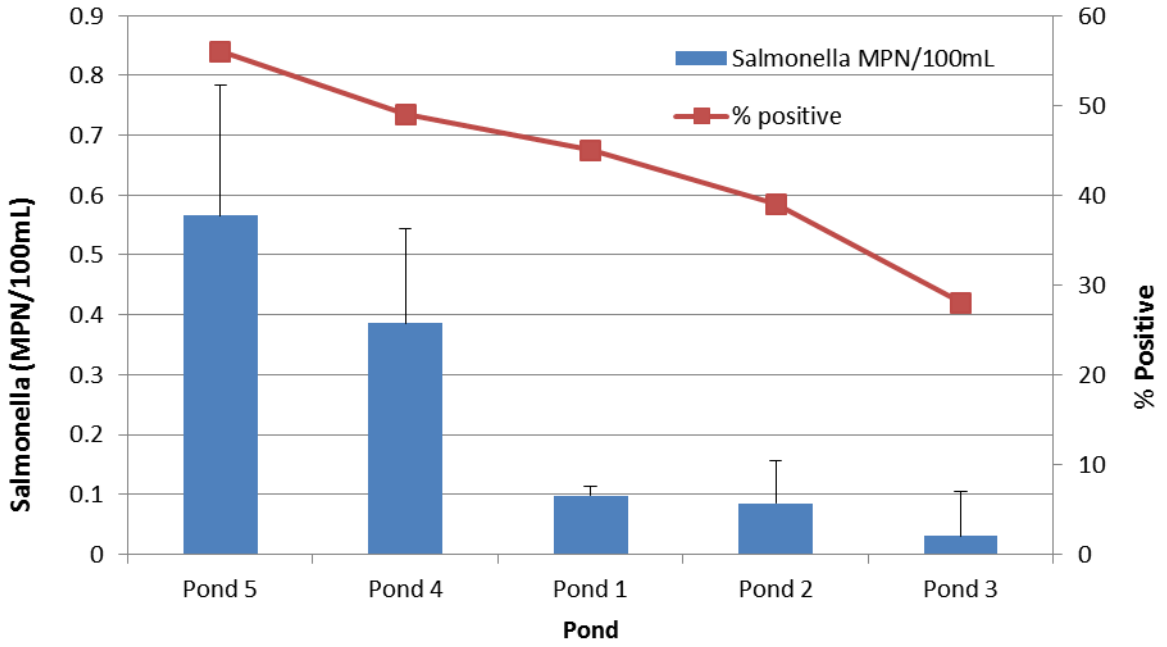

Figure 7. Mean *Salmonella* MPN/100mL concentrations (bars) and percent positives (line) in the project's five ponds.

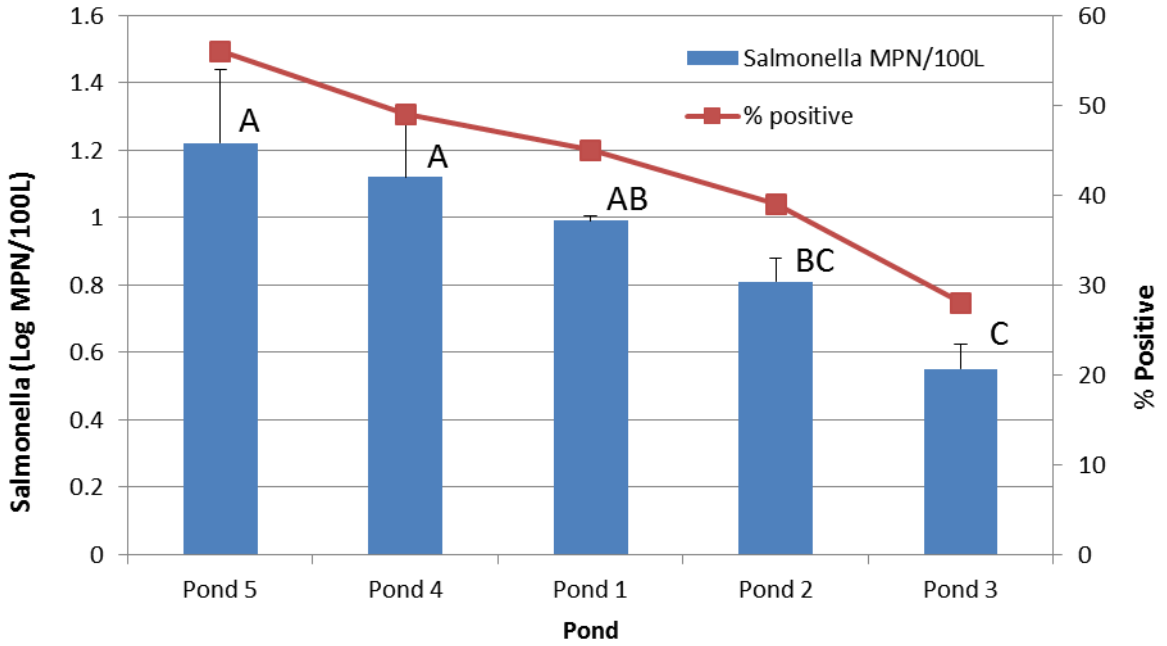

Figure 8. Statistical comparison of *Salmonella* Mean log MPN/100L concentrations (bars) and percent positives (line) in the project's five ponds. Bars with the same t Grouping letter are not significantly different.

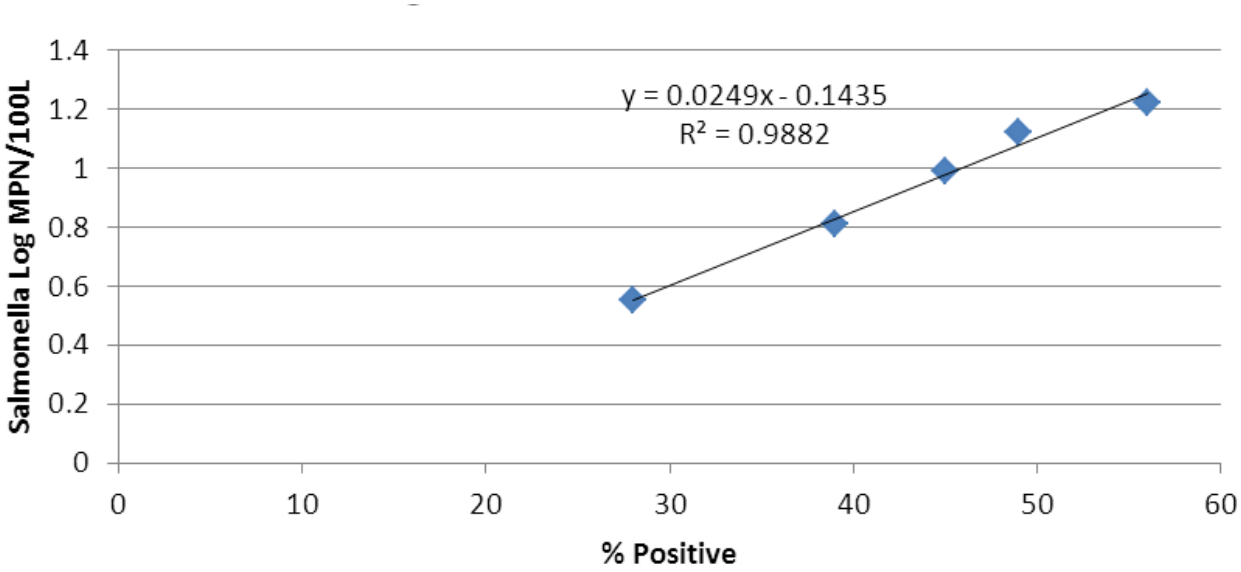

Figure 9. Correlation between % positives and *Salmonella* log MPN/100L for the project's five ponds.

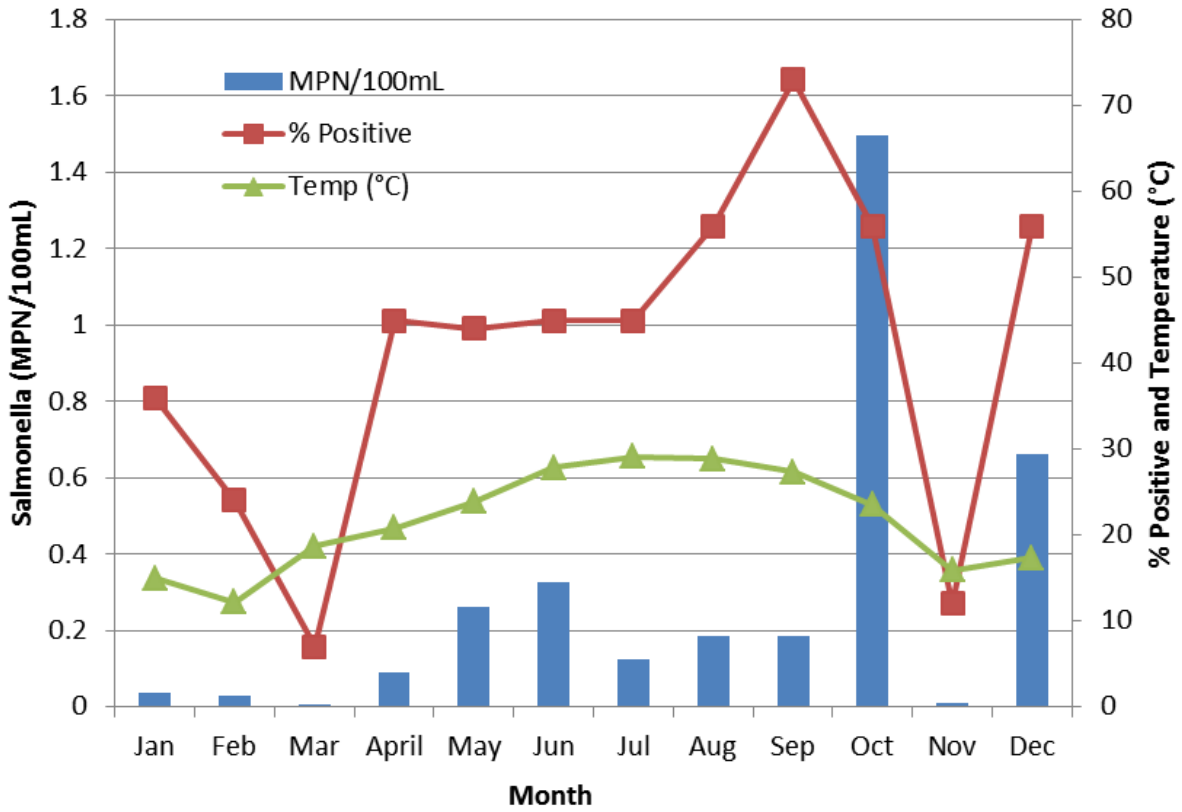

Figure 10. Mean *Salmonella* MPN/100mL concentrations (bars) and percent positives (red line) in the by month. The numbers above the bars indicate months during which concentrations exceeded the upper detection limit. The green line indicates mean water temperature.

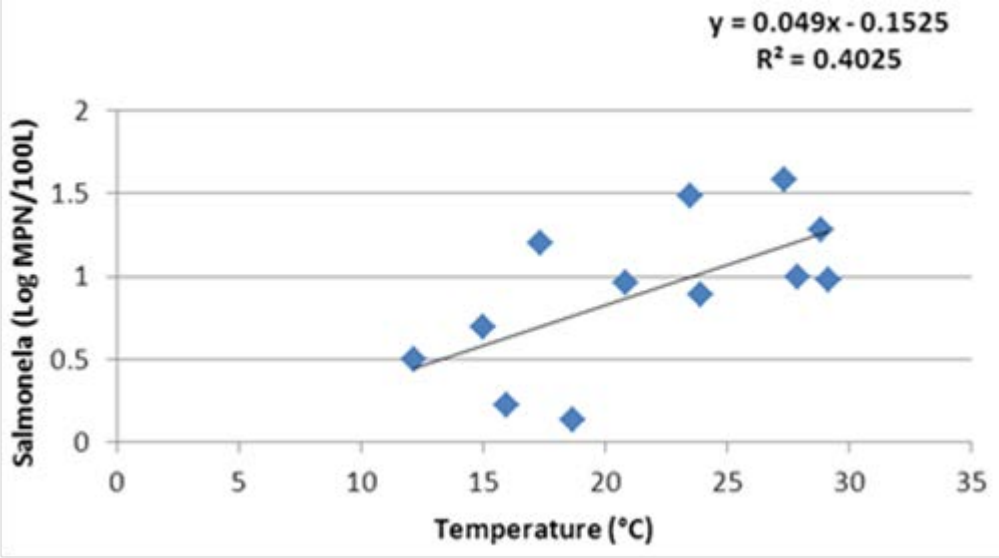

Figure 11. Correlation between monthly temperature and *Salmonella* log MPN/100L.

Figure 12. Pinned sterile 2 L Whirl-Pak® sample bags at Pond 1 prior to and after a runoff event.

Figure 13. Whirl-Pak® bags with runoff samples collected from a forest edge (top) and field edge (bottom) at Pond 3.

Figure 14. In the top photograph, Mr. Rodney Hill and Ms. Casey Harris install sterile Tygon tubing for the ISCO sampler at Pond 1 prior to a precipitation event. The bottom photograph shows the sampler intake installed just above base flow. The vertical position of the intake is easily adjusted.

Figure 15. Approximately 5 L of sample collected with the ISCO sampler at the ephemeral stream at Pond 3 during a precipitation event.

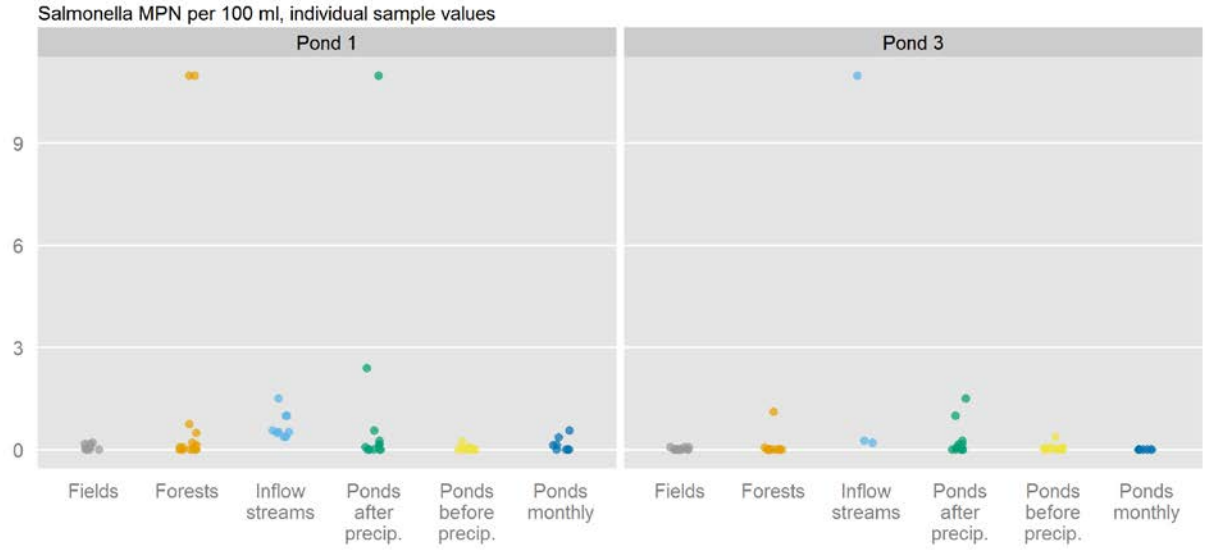

Figure 16. Individual samples collected during this storm runoff portion of the project. Four samples had concentrations above our upper detection limit and were assigned concentrations of 11 MPN/100mL

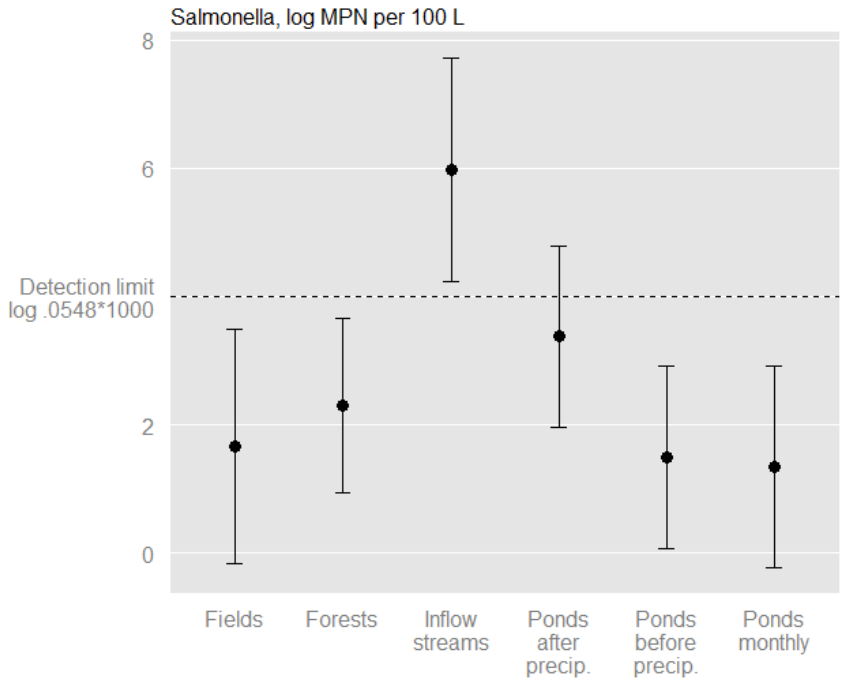

Figure 17. Model-estimated *Salmonella* levels by sample type, shown with 95% confidence intervals.

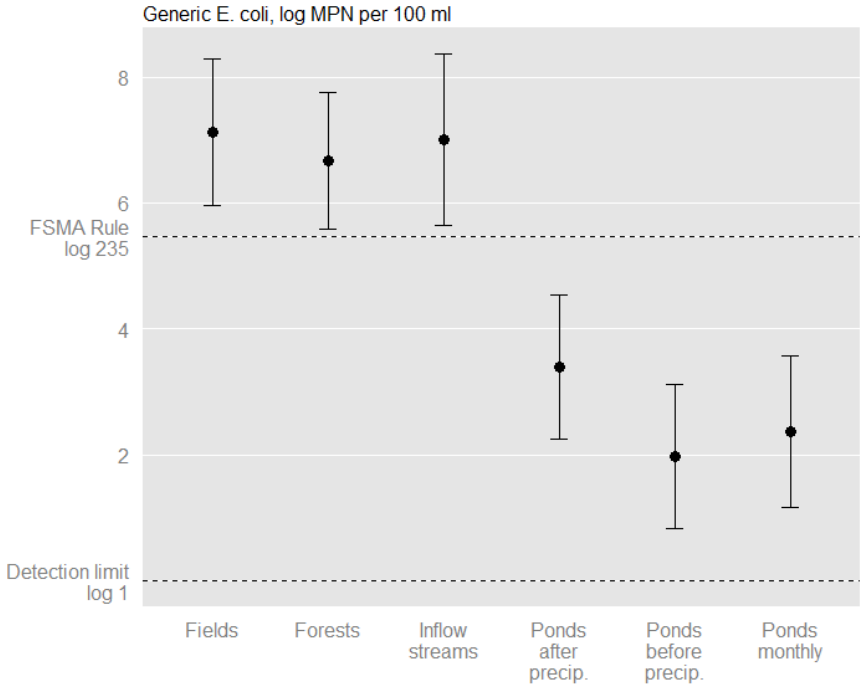

Figure 18. Model-estimated *E. coli* levels by sample type, shown with 95% confidence intervals.

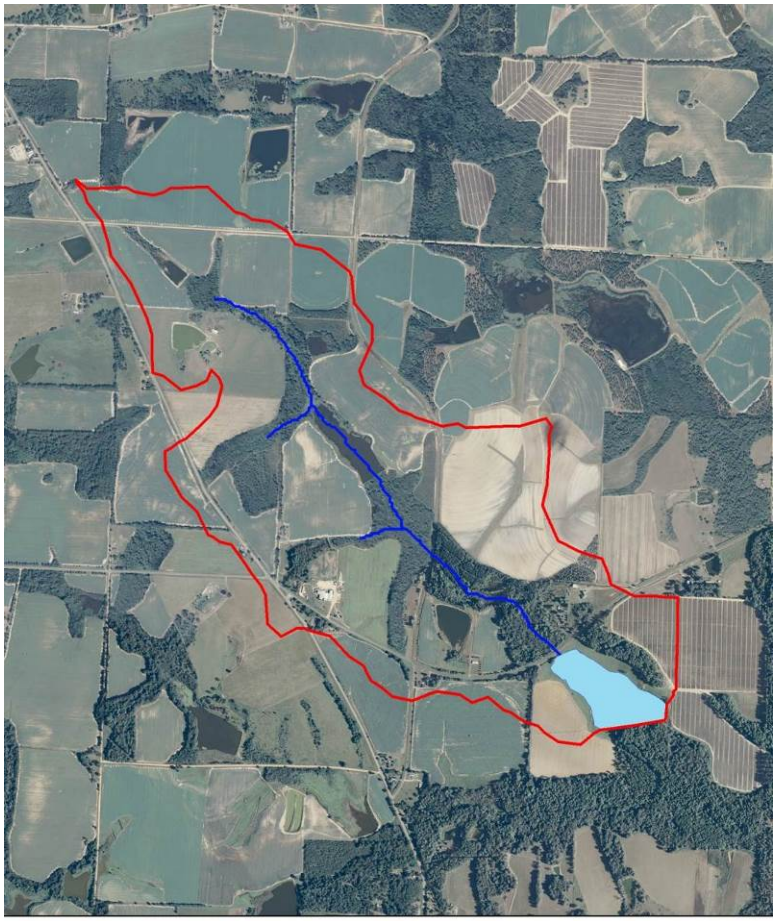

Figure 19. Map showing the watershed boundary of Pond 1. This is the largest pond and the largest watershed included in the study. The watershed's perimeter is indicated by the red line. The pond's area is 21.4 ac and the watershed's area 676 ac.

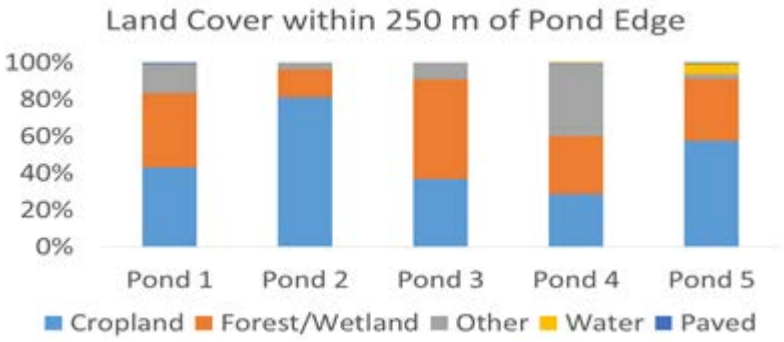

Figure 20. Graphical representation of land cover classification within a 250 m radius of each pond.

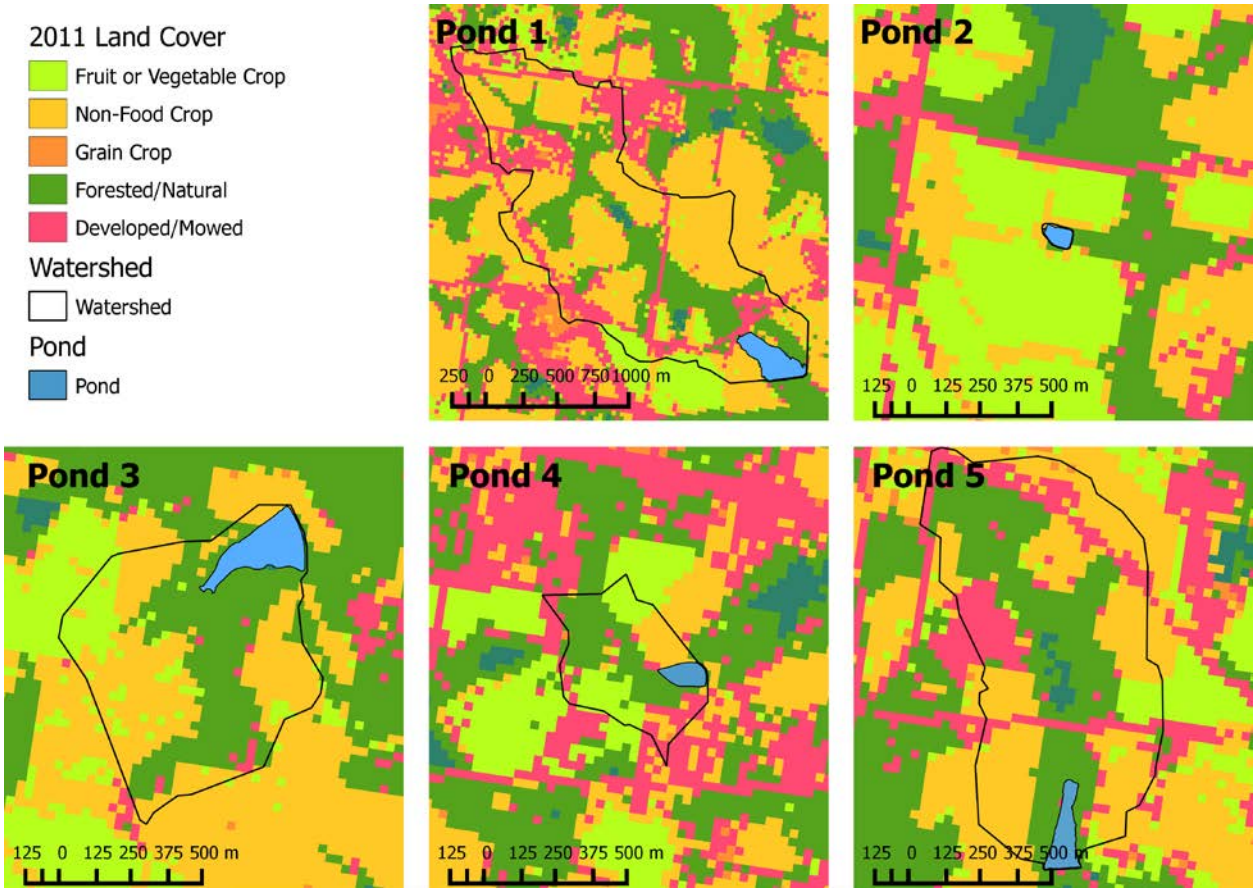

Figure 21. Pond and watershed boundaries and land cover classification. Land cover was classified into five main categories using the 2011 USDA NASS Cropland Data Layer which has a ground resolution of 30 meters.

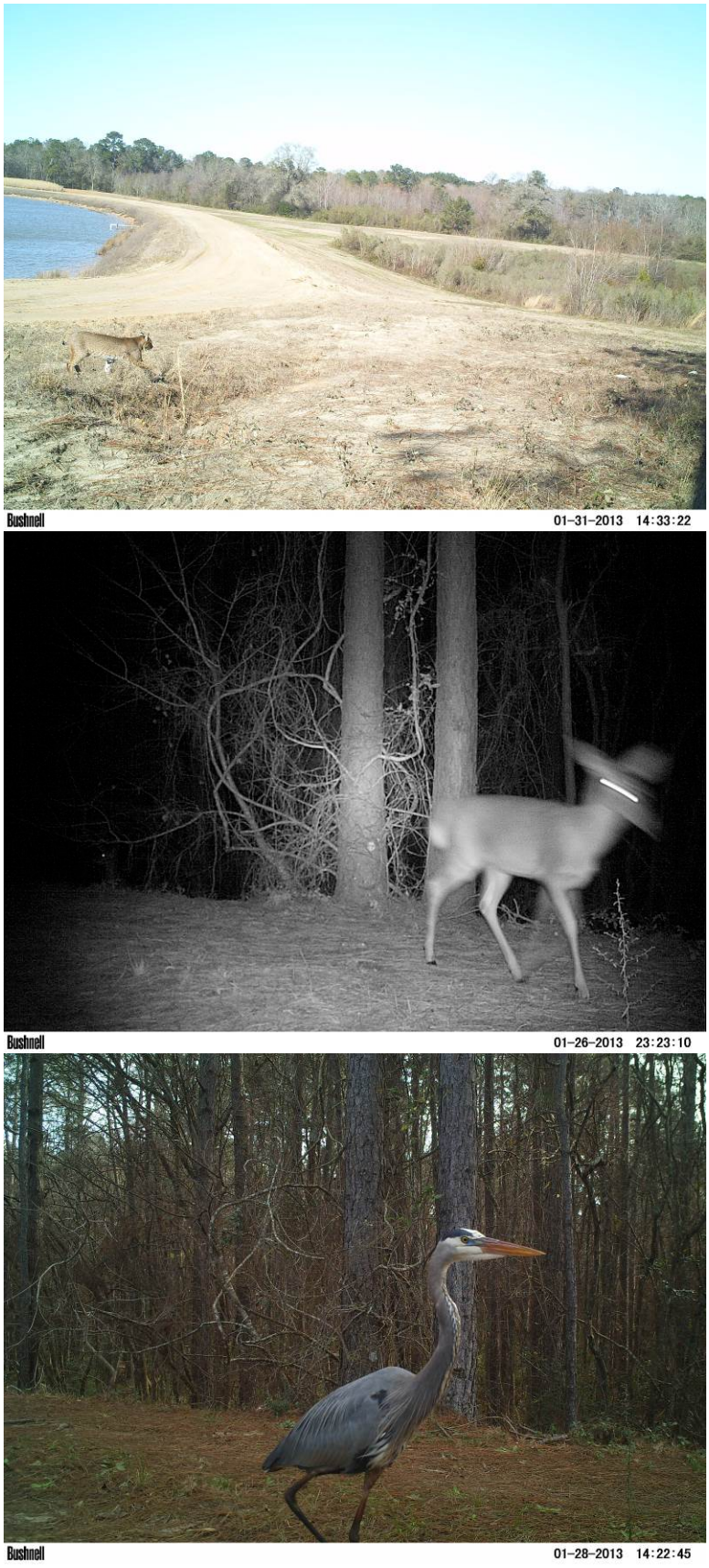

Figure 22. A bobcat near the dam of Pond 3 in late January, 2013 (top) and a deer, a great blue heron, and a coyote on the dam of Pond 1 in late January and early February, 2013 (center and bottom). The images were recorded by a wildlife camera.

Suggestions to CPS (optional)

CPS staff members were supportive and helpful whenever needed. Thank you for your support.

SUPPLEMENT TO FINAL PROJECT REPORT

Project Title
Evaluation of sampling protocol to provide science-based metrics for use in identification of Salmonella in irrigation water testing programs in mixed produce farms in the Suwannee River watershed

Project Period
January 1, 2012 – December 31, 2013

Principal Investigator
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Objectives

1. *Objective 1. Compare the utility of a composite sample collected from multiple locations in surface water to a single grab sample for maximizing probability of detection of Salmonella and indicator bacteria.*
2. *Explore the role of precipitation on Salmonella and indicator bacteria concentrations by a) Comparing 5-day geometric means of samples collected near vegetated buffer/pond interfaces to field/pond interfaces and b) Comparing 5-day geometric means to background levels established in Objective 1.*

Funding for this project provided by the Center for Produce Safety through:
CDFA SCBGP grant #SCB11063

SUPPLEMENT TO FINAL PROJECT REPORT

Serotypes of *Salmonella* from Irrigation Ponds and Storm-Driven Surface Runoff in Southern Georgia Fresh Produce Farm Landscapes

Background

The species *Salmonella enterica* includes over 2500 serotypes potentially capable of causing human infections. In investigations of outbreaks of foodborne illness, identifying the particular serotype involved is helpful for linking individual cases of illness together and tracing them back to the original source of contamination. Although serotyping was not included in our proposal, our team decided that it was important for us to understand whether the *Salmonella* found in our samples was associated with human illness. We serotyped a portion of the isolates collected during the sampling strategy and surface runoff components of the project. *Salmonella* serotyping is an expensive and laborious process and is performed only in a few reference laboratories in the United States. Our samples were serotyped at the National Veterinary Services Laboratory (NVSL) in Ames, Iowa.

Research Methods and Results

Objective 1 – Sampling Strategies

One isolate was chosen at random from each of the samples from the last 12 months of the sampling strategies portion of the project. The sampling strategies portion of the project included monthly sampling of five irrigation ponds, two of which were also used in the surface runoff portion of the project. These isolates were revived in tetrathionate broth (BD Difco, Sparks, MD), streaked onto CHROMagar *Salmonella* Plus agar (CHROMagar, Paris, France), then streaked onto tryptic soy broth agar slants (BD Difco, Sparks, MD) and sent to the NVSL. A total of 109 pond water isolates were serotyped. Composite samples were not included.

Twenty *Salmonella* serotypes were identified among the 109 tested isolates from 117 positive samples of our 273 total samples (excluding composite samples) collected from October 2012-September 2013. Isolates from eight of the samples were no longer viable and could not be serotyped. Eighty-three percent of the isolates belonged to serotypes found in cases of human salmonellosis in our south Georgia study region in 2012-2013. *Salmonella* Muenchen was identified at all five ponds, and Saintpaul and Rubislaw were identified at four of the five ponds (Table 1). Muenchen, Saintpaul, and Rubislaw were responsible for approximately 6%, 6%, and 1% (respectively) of cases of human salmonellosis with known serotypes in our study region in 2012-2013 (GA Dept. of Health, email communication). Javiana and Newport, the two serotypes responsible for approximately 28% and 23% (respectively) of cases of human salmonellosis with known serotypes in our study region in 2012-2013, were found in three ponds, Ponds 2, 4, and 5 – but not in Ponds 1 or 3, which were the two ponds sampled during the surface runoff portion of the project. In September 2013, Javiana was found in all three of those ponds.

Objective 2 – Surface Runoff

All of the frozen stored *Salmonella* isolates from the surface runoff portion of the project were revived in tetrathionate broth and streaked onto CHROMagar *Salmonella* Plus agar. The surface runoff portion of the project included two irrigation ponds and their surrounding watersheds, sampled during a total of 12 rain events. To get an idea of the serotype diversity present, the isolates were tested with *Salmonella* O poly antisera (each containing multiple O antigens) in the following order, until a positive agglutination reaction was observed: Poly B, Poly A, Poly D, Poly G, Poly C, Poly E, and Poly F (BD Difco, Sparks, MD). This process revealed that each of our original water samples sometimes contained isolates representing up to four different O poly groups. Since each O poly group consists of many different

serotypes, we decided to determine the serotypes of up to three isolates representing each O poly group found in each sample. These isolates were picked at random from the CHROMagar plates, streaked onto tryptic soy broth agar slants, and sent to the NVSL. A total of 163 isolates were serotyped for this portion of the project, including isolates obtained from surface runoff from fields and forests, pond water before and after storms, and stream water during storm flow.

Nineteen *Salmonella* serotypes were identified among the 163 tested isolates from 55 positive samples of our 109 total samples collected from January-August 2013. Sixty-four percent of the isolates belonged to serotypes found in cases of human salmonellosis in the Little watershed in 2013.

Several serotypes were found in every type of sample from pond water, surface runoff, and stream water: Muenchen, Saintpaul, I 38:k:-, and Bareilly (Table 2). Rubislaw and Gaminara were found in every type of sample except surface runoff from fields. Muenchen, Saintpaul, I 38:k:-, and Rubislaw were also found during at least six of the eight months of the project (Table 3). Muenchen and Saintpaul were responsible for 3% and 8% (respectively) of cases of human salmonellosis in the Little watershed in 2013 (GA Dept. of Health, email communication). I 38:k:-, Rubislaw, and Gaminara were each responsible for only one case of human salmonellosis, and Bareilly was responsible for none.

Streams harbored the widest variety of serotypes: 13 of the 19 serotypes identified overall were found in streams, and five of these were found only in streams. Saintpaul and Rubislaw were especially prevalent in streams. Saintpaul is commonly associated with cases of human salmonellosis in the greater U.S. (3). Many of the samples from streams contained more than one serotype, and some contained more than four serotypes.

Forests and fields harbored the smallest variety of serotypes on a per-sample basis. Several serotypes associated with human salmonellosis in the study region were recovered from fields and forests, including Saintpaul and Muenchen. Newport, also frequently associated with human illness, was only recovered from fields and only during one rain event (in August).

Eight serotypes were found in pond water before rain events, and ten serotypes were found in pond water after rain events. Of the eight serotypes found before rain events, only one was found in more than one sample – no particular serotypes were regularly present in pond water before rain events throughout the study. The most common serotypes found in pond water after rain events reflected the most common serotypes present in stream water and surface runoff.

Comparison with Cases of Human Salmonellosis

To compare the *Salmonella* serotypes found in fresh produce farm landscapes in our study and the serotypes commonly involved in cases of human salmonellosis, we used 2012 disease incidence reports and data from the CDC's FoodNet Program (1) and Foodborne Disease Outbreak Surveillance system (2), and 2012-2013 data from the Georgia Department of Health (GA Dept. of Health, email communication). FoodNet operates a *Salmonella* surveillance program across 10 states including Georgia, compiling information on all cases of human salmonellosis submitted by each state. The Foodborne Disease Outbreak Surveillance system compiles information on all documented foodborne outbreaks in the U.S., defined as "the occurrence of two or more cases of a similar illness resulting from ingestion of a common food", rather than all individual cases of salmonellosis. The Georgia Department of Health maintains records of salmonellosis for each county in the state, including the date of illness and the serotype involved, if known. To summarize the incidence of salmonellosis in our study region, we pooled records from the Georgia counties intersecting the HUC-8 watersheds involved in our study (Figure 1). The two ponds for the surface runoff portion of the project were located in the Little watershed, and the three additional ponds for the sampling strategies portion of the project were located across three watersheds, including the Little, Upper Ochlockonee, and Lower Ochlockonee.

In our 11-county study region, the incidence of human salmonellosis per 100,000 people was 83 in 2012 (319 cases) and 74 in 2013 (281 cases), based on Georgia Department of Health records and

population estimates of 384,649 for 2012 and 382,035 for 2013 (4). This incidence is higher than the 10-state incidence reported by FoodNet, which was 16 per 100,000 people in 2012 (7,800 cases) and 15 per 100,000 in 2013 (7,277 cases) (5, 6). The CDC estimates that for every reported case of human salmonellosis, 29 more remain unreported because many of those infected do not seek medical treatment and/or do not obtain a laboratory-confirmed diagnosis of *Salmonella* infection (7).

Likewise, many foodborne outbreaks likely go unreported or unrecognized as connected cases rather than individual cases of illness. However, past records of *Salmonella* outbreaks do provide an idea of the serotypes commonly associated with various types of food. The most common serotype in our study overall, Saintpaul, was responsible for only one reported foodborne outbreak in 2012 affecting only two people (both in Minnesota), and it was traced back to smoked turkey (8). The second most common serotype in our study, Muenchen, was responsible for two foodborne outbreaks in 2012 affecting a total of 26 people (in Idaho and South Carolina); one outbreak was traced back to prepared macaroni and cheese, and the other outbreak could not be traced back to a particular product (8).

Rubislaw, despite being very commonly found in our water samples, was not a particularly common cause of illness in south Georgia in 2012-2013 (only five cases) and was not implicated in any reported foodborne outbreaks in the U.S. 2012 (8). Outbreaks of Javiana, the 4th most common serotype recovered from our pond water samples, were traced back to baked chicken, cucumber, iceberg lettuce, and mixed fruit in 2012 (8). Outbreaks of Newport, the 5th most common serotype recovered from our pond water samples, were traced back to cantaloupe, chicken, lettuce, tomatoes, and prepared meat and beans (8). Some of the outbreaks of Javiana and Newport affected multiple states.

Typhimurium, Enteritidis, and I 13:23:b- were not found in any of our water samples, yet were responsible for 10%, 7%, and 4% of cases of salmonellosis (respectively) in our south Georgia study region in 2012-2013. Typhimurium and Enteritidis were the most common causes of salmonellosis in the U.S. in 2012 according to FoodNet, and were also the most common causes of *Salmonella* outbreak events in the greater U.S. in 2012 (1, 8). Outbreaks of Typhimurium were traced back to turkey, beef, cantaloupe and prepared/processed foods (8). Outbreaks of Enteritidis were traced back mainly to eggs, chicken, beef, and other prepared or processed foods (8).

Four of the 10 most common serotypes found in our irrigation pond water samples were among the 10 most common serotypes causing human illness in our south Georgia study region in 2012-2013 or in the 10-state FoodNet region in 2012 (Table 5) (1). Only two of these (Javiana and Newport) were among the 10 most common serotypes implicated in foodborne outbreaks in 2012 (8).

Seventy-two percent of cases of human salmonellosis in our south Georgia study region (not including cases with unknown serotypes) were caused by serotypes also recovered from samples at the five irrigation pond sites during the surface runoff or sampling strategies projects (Table 4). This indicates that serotypes present in irrigation water sources and associated environments have the potential to cause illness in humans, and further studies are underway to determine whether produce is actually contaminated by *Salmonella* from these sources.

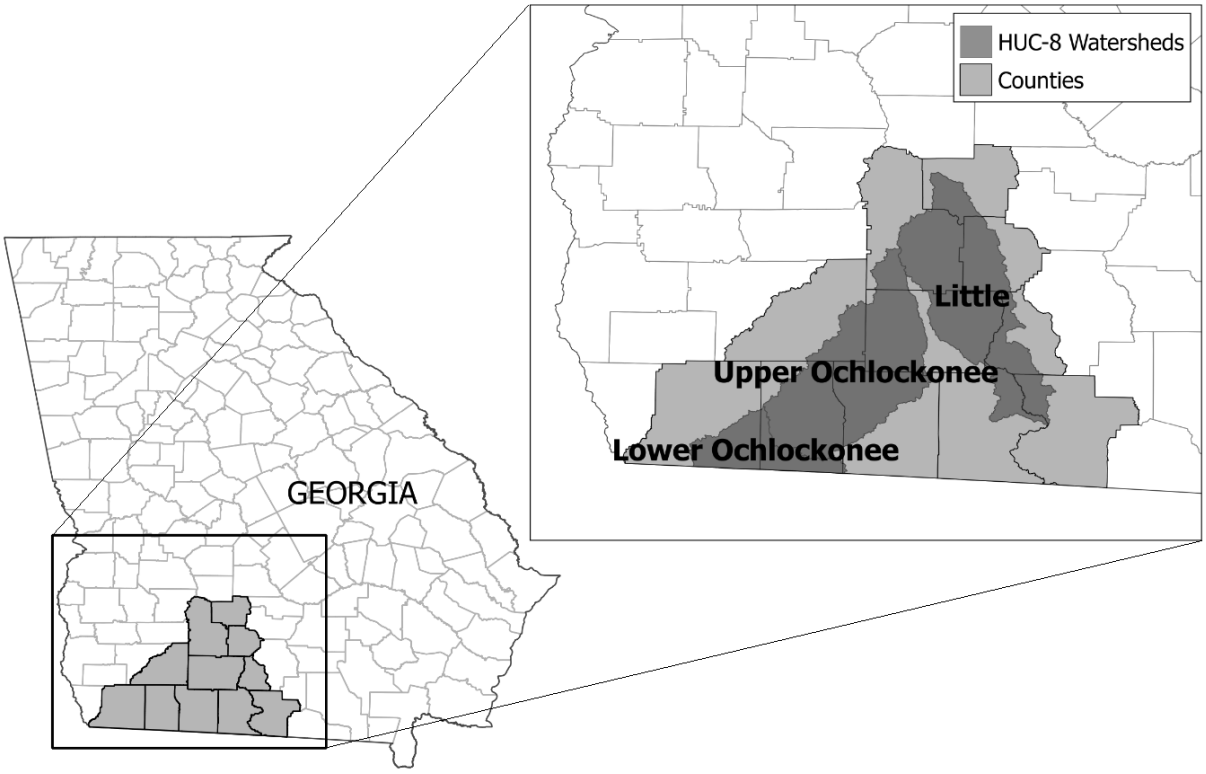
Summary of Findings and Recommendations

- A majority of the *Salmonella* isolates found in our water samples represented serotypes of *Salmonella* known to cause human illness.
- A few of the isolates found in our water samples represented serotypes of *Salmonella* found in outbreaks of foodborne illness from fresh fruit/vegetable products. These include *Salmonella* serotypes Javiana and Newport.
- Based on our main study, very low levels of *Salmonella* (of any serotype) were present in our water samples. Further research is needed to assess the actual likelihood of crop contamination from *Salmonella* present in irrigation water sources at varying levels.

Figures

Figure 1

Map showing our study region. Our study sites were located within the three HUC-8 watersheds shown. Data on salmonellosis from the Georgia Department of Health was pooled for the counties highlighted.



Tables

Table 1

Serotypes found in the sampling strategies portion of the project, with the number of samples in which each serotype was found per pond. Shown with the number of cases of human salmonellosis in the counties intersecting the Little, Upper Ochlockonee, and Lower Ochlockonee watersheds in 2012-2013 (GA Dept. of Health, email communication).

	Number of samples in which each serotype was found						
Pond:	1	2	3	4	5	Total	Human cases
Braenderup		2		1		3	2
Gaminara			4		1	5	2
Give var. 15+			1			1	-
Inverness	1				3	4	3
monophasic var. I 38:k:-	5					5	2
Javiana		2		6	2	10	138
Mbandaka	3					3	-
Montevideo		1				1	14
Muenchen	2	2	6	2	3	15	31
monophasic var. I 6,8:d:-			1			1	-
Newport		3		2	1	6	111
Rubislaw	1	1	1	10		13	5
Saintpaul	10	5		2	10	27	29
I 4,5,12:i:-					1	1	7
I 6,7:k:-	1					1	-
III 16:z10:e,n,x,z15			1	1		2	-
monophasic var. III 16:z10:-					1	1	-
III 35:l,v:z35		1	1		3	5	-
III 59:k:z35	1		1			2	-
III 60:r:e,n,x,z15	1	1	1			3	-
Unknown serotype	2	1	2	3	1	9	112
Other serotype							144
Total positive* / total samples	27/55	19/55	18/55	27/55	26/53	117/273	600
Total serotypes / total samples	9/55	9/55	9/55	8/55	9/53	20/273	

Table 2

Salmonella serotypes found in the surface runoff portion of the project, from two sites in Little watershed. Shown with the number of cases of human salmonellosis attributed to each serotype in counties in the Little watershed in 2013 (GA Dept. of Health, email communication).

	Number of samples yielding each serotype					
	Pond water		Surface runoff			
Serotype	Pre-storm	Post-storm	Fields	Forests	Streams	Human cases
Anatum					1	1
Bareilly	1	2	2	2	2	none
Braenderup				1	1	none
monophasic var. I 6,7:-:e,n,z15					1	none
Gaminara	1	2		1	1	1
monophasic var. I 16:d:-		1				none
Give var. 15+					1	none
Inverness		1		2		none
monophasic var. I 38:k:-	2	3	1	1	2	1
Meleagridis			1			none
Muenchen	1	3	2	3	5	6
Newport			1			51
Rubislaw	1	3		1	7	1
Saintpaul	1	5	2	3	8	15
III 16:z10:e,n,x,z15					2	none
III 50:r:-					1	none
monophasic var. III 50:-:-		1				none
III 60:r:e,n,x,z15	1	4			4	none
monophasic var. III 60:r:-	1					none
Positive samples / total samples	8/24	14/24	8/19	11/28	14/14	76/217**
Total serotypes / total samples*	8/24	10/24	6/19	8/28	13/14	

*Many samples contained more than one serotype.
**The remaining cases of human salmonellosis in Little watershed counties in 2013 were attributed to Javiana (52), Typhimurium (21), Enteritidis (16), Montevideo (7), Miami (6), I 13,23:b:- (6), and Carrau, Heidelberg, I 4,[5],12:i:-, Infantis, Kintambo, Mississippi, and Senftenberg (1 each), and 26 with unidentified serotypes.

Table 3

Salmonella serotypes found in the surface runoff portion of the project, from two sites in Little watershed. Shown with the months each serotype was present in any type of water sample, and the months each serotype was reported as the cause of cases of human salmonellosis in Little watershed in 2013 (GA Dept. of Health, email communication).

Serotype	Months each serotype was found								Human cases
Anatum			Mar						Feb
Bareilly		Feb		Apr		Jun	Jul	Aug	-
Braenderup						Jun	Jul		-
monophasic var. I 6,7:-:e,n,z15								Aug	-
Gaminara					May	Jun	Jul		Sep
monophasic var. I 16:d:-					May				-
Give var. 15+					May				-
Inverness							Jul	Aug	-
monophasic var. I 38:k:-	Jan	Feb	Mar	Apr		Jun		Aug	Jun
Meleagridis					May				-
Muenchen	Jan	Feb		Apr	May	Jun	Jul	Aug	May, Jul, Sep-Nov
Newport								Aug	Jan-Apr, Jun-Dec
Rubislaw		Feb	Mar	Apr		Jun	Jul	Aug	Aug
Saintpaul			Mar	Apr	May	Jun	Jul	Aug	Jan, Jun-Oct, Dec
III 16:z10:e,n,x,z15					May				-
III 50:r:-					May				-
monophasic var. III 50:-:-							Jul		-
III 60:r:e,n,x,z15		Feb		Apr			Jul	Aug	-
monophasic var. III 60:r:-				Apr					-
Total serotypes / total samples	2/8	5/9	4/17	6/17	8/10	7/17	9/20	9/11	

Table 4

Serotypes causing cases of human salmonellosis in counties in the Little, Upper Ochlockonee, and Lower Ochlockonee watersheds in 2012-2013 (GA Dept. of Health, email communication). **Bolded** serotypes are those that were also found among our water sample.

Cases of human salmonellosis in our study region in 2012 and 2013

		2012	2013	Total	% of Total
Top 10 serotypes found in humans in our region	**Javiana**	72	66	138	23.0
	Newport	52	59	111	18.5
	Typhimurium	22	25	47	7.8
	Enteritidis	11	22	33	5.5
	Muenchen	23	8	31	5.2
	Saintpaul	12	17	29	4.8
	I 13,23:b:-	11	7	18	3.0
	Montevideo	7	7	14	2.3
	I 4,[5],12:i:-	3	4	7	1.2
	Miami	-	6	6	1.0
Additional serotypes found in pond samples and/or runoff samples	**Rubislaw**	1	4	5	0.8
	Bareilly	3	1	4	0.7
	Inverness	2	1	3	0.5
	Anatum	1	1	2	0.3
	Braenderup	-	2	2	0.3
	Gaminara	1	1	2	0.3
	I 38:k:-	-	2	2	0.3
	Mbandaka	-	-	-	-
	Give var. 15+	-	-	-	-
	Meleagridis	-	-	-	-
	I 6,7:-:e,n,z15	-	-	-	-
	I 6,7:k:-	-	-	-	-
	I 6,8:d:-	-	-	-	-
	I 16:d:-	-	-	-	-
	III 16:z10:e,n,x,z15	-	-	-	-
	III 16:z10:-	-	-	-	-
	III 35:l,v:z35	-	-	-	-
	III 50:r:-	-	-	-	-
	III 50:-:-	-	-	-	-
	III 59:k:z35	-	-	-	-
	III 60:r:e,n,x,z15	-	-	-	-
	III 60:r:-	-	-	-	-
	Other serotypes	20	14	34	5.7
	Unknown	78	34	112	18.7
	Total cases	319	281	600	100%

Table 5

The most common *Salmonella* serotypes in pond water in our 5-pond sampling strategies study in 2012-2013, compared with the most common serotypes implicated in cases of human salmonellosis in our south Georgia study region in 2012-2013, and in the greater United States in 2012 only (GA Dept. of Health, email communication; 1, 8).

Ranked by:	Present study # of samples	South Georgia # of human cases	US FoodNet reports # of human cases	US Outbreak reports # of outbreak events
Most	Saintpaul	Javiana	Enteritidis	Enteritidis
	Muenchen	Newport	Typhimurium	Typhimurium
	Rubislaw	Typhimurium	Newport	Newport
	Javiana	Enteritidis	Javiana	Javiana
	Newport	Muenchen	I 4,[5],12:i:-	Heidelberg
	III 35:l,v:z35	Saintpaul	Muenchen	Braenderup
	Gaminara	I 13,23:b:-	Bareilly	I 4,[5],12:i:-
	I 38:k:-	Montevideo	Montevideo	Thompson
	Inverness	I 4,[5],12:i:-	Heidelberg	Montevideo
Least	III 60:r:e,n,x,z15	Miami	Saintpaul	Bareilly

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