CENTER for PRODUCE SAFETY

CPS 2014 RFP
FINAL PROJECT REPORT

Project Title
Improved sampling and analytical methods for testing agricultural water for pathogens, surrogates and source tracking indicators

Project Period
January 1, 2015 – December 31, 2016

Principal Investigator
Vincent Hill
Waterborne Disease Prevention Branch, Centers for Disease Control and Prevention
1600 Clifton Road NE
Mailstop D-66, Atlanta, GA 30329
404-718-4151, veh2@cdc.gov

Co-Principal Investigators
George Vellidis
Department of Crop and Soil Sciences, University of Georgia
2360 Rainwater Road, Tifton, GA 31793-5766
229-386-3442, yiorgos@uga.edu

Karen Levy
Department of Environmental Health Epidemiology, Emory University
1518 Clifton Rd, NE
Claudia Nance Rollins Bldg, Room 2019, Atlanta, GA 30322
404-727-4502, karen.levy@emory.edu

Objectives
1. *Develop DEUF protocols and determine recovery efficiencies for pathogens (Salmonella, C. parvum, and C. cayatenensis) and alternative water quality parameters (e.g., Bacteroides) in 50-L surface water samples of varying quality (e.g., different turbidity levels). Establish protocols for minimizing the effect of inhibitors on the molecular methods used for qPCR and isothermal DNA amplification.*
2. *Utilize DEUF to determine if seasonality or weather-related events, such as rainfall, affect the occurrence or concentration of pathogens, surrogates and MST indicators in agricultural water.*
3. *Assess whether utilizing DEUF to collect large volume water samples improves detection and quantitation of target pathogens and pathogen surrogates versus small-volume (1-L) grab samples.*
4. *Determine if the presence and/or concentration of pathogen surrogates correlates with the occurrence and/or concentration of pathogens in agricultural water.*
5. *Demonstrate the utility of DEUF to facilitate detection of MST indicators in agricultural water.*

Funding for this project provided by the Center for Produce Safety through:
CDFA SCBGP grant# SBC14060

FINAL REPORT

Abstract

Currently, fresh produce growers rely on the fecal indicator bacteria, *Escherichia coli*, for characterizing agricultural water quality. In addition to monitoring for a generic indicator, such as *E. coli*, risk-based collection and testing of agricultural source water for pathogens and alternative water quality surrogates can provide farm operators with a better understanding of the quality of water used in crop production. In this project, an emerging water sampling technique—dead-end ultrafiltration (DEUF)—was used to collect large-volume irrigation water samples from three farms in Georgia to investigate the benefits of collecting large-volume samples for pathogens and alternative microbial water quality parameters. UF and grab samples were collected at least every month for one year from the primary irrigation pond at each of the three farms. Precipitation-impacted samples were collected after rain events to enhance the comparison of large- versus small-volume collection procedures and evaluate this risk-based sampling approach. DEUF and grab samples were analyzed for *Salmonella*, *Cryptosporidium*, *E. coli* O157:H7, microbial indicators, and microbial source tracking (MST) markers, which are indicators of fecal contamination that can identify potential sources of fecal contamination contributing to elevated microbial levels. Human-specific MST markers included *Bacteroides* Hf183, *Methanobrevibacter smithii*, and human polyomaviruses. Animal-specific MST markers included mitochondrial markers for wildlife and livestock (bird, cow, chicken, pig). Controlled laboratory experiments were also conducted to determine the efficiency of DEUF for recovering *Salmonella*, *Cryptosporidium, Cyclospora cayetanensis,* and MST markers from agricultural water.

This two-year project was a collaboration between the Centers for Disease Control and Prevention (CDC), University of Georgia (UGA), and Emory University (Emory). UGA staff collected DEUF samples, grab samples, and field water quality data from three farms in the area of Tifton, Georgia, to enable statistical comparison of microbial detection rates using small-volume and large-volume sampling. UGA staff provided the samples to CDC and Emory staff, who processed and analyzed the samples at CDC in Atlanta, Georgia. Samples were analyzed for *E. coli*, enterococci, F+ coliphages, *E. coli* O157:H7, and *Salmonella* using quantitative culture techniques. *Salmonella* isolates were subtyped using traditional antigen-based serotyping and molecular-based pulsed-field gel electrophoresis (PFGE) to identify potential host-specific serovars. *Cryptosporidium* and MST makers included were analyzed by real-time PCR.

This project demonstrated the use of large-volume water sample collection for sensitive detection of pathogens, pathogen surrogates, and fecal source tracking markers in surface- and ground water–fed produce irrigation water. This work also provided an evidence base for evaluating seasonal and precipitation-related sampling as risk-based approaches for agricultural water quality monitoring. Produce industry operators and technical consultants can use these tools to better characterize the microbial quality of irrigation water.

Background

Irrigation water must be of adequate sanitary quality prior to application to food crops in order to limit potential human health risk associated with exposure to waterborne pathogens. Currently, produce growers evaluate irrigation water quality using the fecal indicator bacteria, *Escherichia coli*. *E. coli* is commonly used as an indicator of fecal contamination in drinking water and recreational water because it is considered fecal-specific and can be detected and quantified using simple methods. However, *E. coli* may not be an appropriate indicator for parasites, viruses or even other bacterial pathogens in water. For example, male-specific (F+) coliphages (bacteriophages that infect *E. coli*) are often cited in the literature as more appropriate indicators for viral pathogens given similarities in size,

physical and genetic characteristics and survival in the environment [1, 2]. Additionally, by relying on a single microbe, such as *E. coli*, as a determinant of water quality, produce growers may be underestimating the risk associated with use of some agricultural water sources. Guidelines for periodic *E. coli* monitoring use a geometric mean (GM) cutoff of 126 MPN/100 mL and statistical threshold value (STV) of 410 MPN/100 mL as a measure of microbial water quality. However, evidence is lacking to demonstrate whether these *E. coli* levels are effective for indicating a higher likelihood for the presence of foodborne pathogens in irrigation water. Detection of generic *E. coli* also does not provide information regarding potential sources of contamination, making the development of targeted contamination prevention controls and mitigation strategies difficult. Microbial source tracking (MST) markers, which include various human- and animal-specific microbes and genetic markers, can be used to determine potential sources of contamination in a water supply. Determination of contamination inputs could help guide prevention and mitigation plans.

In addition to water quality monitoring using microbial indicators, producers and food safety investigators need sensitive testing techniques for detecting pathogens associated with disease outbreaks or identifying produce contamination. Pathogens and alternative microbial indicators can be present in low concentrations, necessitating the collection of large-volume water samples. Hollow-fiber ultrafiltration (UF) utilizes dialysis filters to concentrate bacteria, parasites, and viruses simultaneously in water samples. UF represents a cost-effective technique for co-concentrating diverse microbe types in large volume (10–100 liter) water samples. Two approaches to UF have been developed in recent years: tangential-flow and dead-end. Tangential-flow UF recirculates water samples through the ultrafilter while collecting the sample concentrate in a separate 1-L bottle. While tangential-flow UF has been shown to effectively recover diverse microbes from water, it requires significant operator training and can be difficult to set up and perform in a field setting [3-6]. Dead-end UF (DEUF) allows water to flow straight through the ultrafilter while retaining microbes in the filter cartridge. Ultrafilters are later backflushed in a lab to recover the sample concentrate for microbial analysis. DEUF requires little operator training and is easily implemented in the field, making it an attractive option for sampling agricultural waters [7]. The CDC laboratory team has led the development of both of these methods.

The need for such sampling techniques was highlighted in 2013 in response to concurrent outbreaks of cyclosporiasis (reportedly associated with leafy greens and cilantro) causing >600 cases in 25 states. Established methods with known performance characteristics were not available for sampling and analyzing agricultural water samples for *Cyclospora cayetanensis*. DEUF was used to collect irrigation water samples from a farm in Mexico, but the method was not employed until weeks after the outbreaks were identified and the procedures used had not been validated for *C. cayetanensis* recovery. DEUF is routinely used for sample collection by the CDC during waterborne disease outbreak investigations and has resulted in successful detection of numerous microbes, including *Vibrio cholerae, Cryptosporidium*, *Giardia*, norovirus, hepatitis A virus, and hepatitis E virus [8-10].

Numerous analytical techniques have been verified for the detection of diverse microbe types in DEUF concentrates. Fecal indicator bacteria (e.g., *E. coli*, enterococci), pathogens (e.g., *Salmonella*) and viruses (e.g., F+ coliphages) can be directly cultured from UF concentrates. Detection of microbes by real-time polymerase chain reaction (qPCR) requires smaller final sample concentrate volumes, so secondary concentration techniques such as centrifugation and polyethylene glycol (PEG) precipitation are often used to achieve water sample concentrate volumes on the order of 1-5 mL prior to nucleic acid extraction [11]. By utilizing DEUF as the sampling technique for monitoring agricultural water quality, produce growers would have the capacity to obtain water quality data for a wide variety of pathogens, microbial indicators or pathogen surrogates, and MST markers from a single water concentrate.

Method recovery efficiencies for *Salmonella* have been reported for tangential-flow UF applied to tap water [12]. DEUF recovery efficiencies have been reported for *Cryptosporidium parvum* in surface

water [13], but not for *C. cayetanensis*. The effectiveness of DEUF for *Cryptosporidium* oocyst recovery were reassessed in this project with irrigation water and will provide a reference point for new data on the recovery of *C. cayetanensis, Salmonella,* and MST markers in irrigation water.

In four previous Center for Produce Safety (CPS) projects, “Science-based evaluation of regional risks for *Salmonella* contamination of irrigation water at mixed produce farms in the Suwannee River watershed” (University of Florida), “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” (UGA and Emory), "Does *Salmonella* move through the irrigation systems of mixed produce farms of the southeastern United States?" (UGA, Emory, and UC Davis), and "Does splash from overhead sprinkler irrigation systems contaminate produce with Salmonella in the southeastern United States?" (UGA and Emory), members of our team studied irrigation water quality on produce farms in southern Georgia, focusing primarily on sampling strategies and testing for fecal indicators, *Salmonella*, and *E. coli* O157:H7. All study farms were located in the Southeastern Coastal Plain (SECP), an important agricultural region of the US. Farms selected for the studies were considered representative of farms across the SECP based on similarities in agricultural practices, water sources used for crop irrigation, and length and climate of the growing season. Most farms in this region utilize constructed farm ponds, often built by damming a nearby stream, for irrigation purposes. Irrigation ponds are typically recharged by the inflow stream, on-site wells, and precipitation runoff. By allowing ponds to be replenished by direct precipitation runoff, the potential for contamination may be high. However, results from the University of Florida study demonstrated the occurrence of *E. coli* O157:H7 in only 14.6% samples [14]. Preliminary results from the ongoing UGA/Emory study have shown that *Salmonella* is commonly detected in irrigation ponds, and is also detected in irrigation water, but concentrations are often low. Both studies used low-volume sampling techniques to collect pond samples, potentially limiting their ability to detect the pathogens if present in low concentrations.

Implementation of this project is both timely and relevant given recent agriculture water–related disease outbreaks and development of new water sampling and analytical tools. Large-volume sampling using DEUF and new analytical tools (such as rapid molecular testing, alternative indicators) provide new opportunities to equip produce growers and other food safety investigators with sensitive, field-portable, and user friendly tools for testing the microbial quality of agricultural water supplies.

Research Methods and Results

Objective 1. Development and validation of DEUF protocols

Laboratory studies were conducted to determine the effectiveness of DEUF for recovering *C. parvum, C. cayetanensis, Salmonella* spp., generic *E. coli* and two MST markers of human fecal contamination, *Bacteroides* Hf183 and human polyomaviruses (HPyVs), from irrigation water. Real-time quantitative PCR (qPCR) assays have been reported for these human-specific fecal markers, but not in conjunction with DEUF [15, 16]. Irrigation water for the recovery experiments was obtained from one surface-fed and one ground water–fed irrigation pond in the Suwannee River watershed in southern Georgia (ponds SC and LV). Five replicate experiments each were conducted using 50-L volumes of low turbidity (LV, mean 0.707 NTU) and high turbidity (SC, mean 23.2 NTU) irrigation water. Turbidity and conductivity of the irrigation water samples were measured before each experiment using a Hach 2100P turbidimeter and Oakton CON 100 conductivity meter, respectively. For each microbe or MST marker, DEUF method recovery was determined by dividing the quantity of analyte in the final DEUF concentrate by the quantity of each analyte added to the 50-L samples.

Mean percent recoveries and standard deviations were calculated for each analyte. For each analyte,

statistical differences in percent recoveries between high and low turbidity waters was evaluated using a two-sample t-test assuming unequal variances. *P*-values ≤ 0.05 were considered statistically significant.

Parasite Recovery

Quantified *C. parvum* and *C. cayetanensis* stocks were obtained by flow cytometry sorting and were seeded into 50-L irrigation water samples at average input levels of 3,000 *C. parvum* oocysts and 8,000 *C. cayetanensis* oocysts as determined by microscopy. The bulk water was concentrated by the DEUF method as previously described, with additional concentration of the DEUF concentrate by centrifugation [7, 13]. A portion of the final concentrate was subjected to immunomagnetic separation (IMS) and fluorescent antibody (FA) microscopy to detect and quantify *C. parvum* recovery [17]. Nucleic acid extraction was performed on an additional portion of the final concentrate [18]. Recovery of *C. cayetanensis* was determined by real-time PCR (qPCR), as there are no available IMS or FA microscopy methods for *C. cayetanensis* and the sample matrix precluded direct visualization by light microscopy [19].

Mean recovery for *C. parvum* by the DEUF method was 70% (standard deviation [SD] 82%) and 149% (SD 72%) for high and low turbidity irrigation water, respectively (Table 1). *C. parvum* percent recovery was significantly higher in the low turbidity water compared to the high turbidity (t-value = 2.53, *P* = 0.04). Mean recovery percentages for *C. cayetanensis* by the DEUF method could not be calculated due to the different quantification methods used for the DEUF input and output samples (oocyst count vs. qPCR, respectively). However, at the seeding levels used for this study, *C. cayetanensis* was successfully recovered by the DEUF method for all high and low turbidity replicate experiments. These results suggest that the DEUF method is effective for recovering *C. cayetanensis* from irrigation waters.

Salmonella, E coli, and MST Marker Recovery

Quantified stocks of *Salmonella enterica* serovar Typhimurium were seeded into 50-L irrigation water samples at average input levels of 7×10^4 CFU. Primary settled sewage was used to seed naturally occurring *E. coli*, human *Bacteroides*, and HPyV. The bulk water was concentrated by DEUF as for the parasite recovery experiments. *Salmonella* was enumerated before and after DEUF by a three volume, three-tube most probable number (MPN) culture method, with qPCR confirmation of positive replicates [3, 20]. *E. coli* was enumerated by the Colilert-18 Quanti-Tray 2000® method. The remaining DEUF concentrate was further concentrated using polyethylene glycol (PEG) precipitation to simultaneously pellet bacteria and viruses. Sodium chloride and PEG 8000 were added to the sample concentrate sequentially to achieve final concentrations of 0.3 M sodium chloride and 8% PEG. Samples were incubated at room temperature for 2 hours and centrifuged at 10,000 x *g* for 30 minutes. Nucleic acid extraction was performed on a portion of the PEG precipitate and gene copies of the *Bacteroides* Hf183 target and genome equivalents of the HPyV target were enumerated using qPCR standard curves [15, 16]. Briefly, ten-fold serial dilutions were made of the synthetic plasmid DNA and genomic viral DNA standards for quantification of *Bacteroides* Hf183 and HPyV, respectively, which ranged from 10^0 to 10^5 copies per 5 µL of standard. Standard dilution series were run in triplicate with each 96-well plate run of samples, and the standard curve was used to determine the target copy number in each unknown sample, as well as the efficiency of the assay. The average qPCR assay efficiencies were 101.9% and 90.6% for *Bacteroides* Hf183 and HPyV, respectively.

Mean recovery for *E. coli* by the DEUF method was 77% (SD 26%) and 54% (SD 14%) for high and low turbidity irrigation water, respectively (Table 2). This is similar to previous recovery estimates from surface waters, indicating that the DEUF method performs as well for irrigation water as other surface water types [21]. Calculated mean recovery percentages for *Salmonella* by the DEUF method were 537% (SD 719%) and 510% (SD 735%) for high and low turbidity irrigation water, respectively. The

higher than 100% recovery estimates for *Salmonella* are likely due to the imprecision of the MPN quantification method. MPN methods are generally thought to be semi-quantitative, as the statistical calculation of MPN generates a wide confidence interval around the estimated quantity. This imprecision was compounded by using two MPN estimated quantities (pre- and post-DEUF) to calculate recovery efficiency. Future work will include running qPCR on input and output samples to refine the recovery efficiency calculations. Mean recovery for the *Bacteroides* Hf183 gene target by the DEUF method was 3.8% (SD 2%) and 9.9% (SD 5%) for high and low turbidity irrigation water, respectively. Mean recovery for HPyVs by the DEUF method was 7.8% (SD 5%) and 7.2% (SD 4%) for high and low turbidity irrigation water, respectively. Hf183 percent recovery was significantly higher in the low turbidity water compared to the high turbidity (t-value = 2.36, $P = 0.05$). *E. coli, Salmonella*, and HPyV recoveries were not significantly different between high and low turbidity irrigation water.

Objective 2. Effect of weather-related events on microorganism detection and concentration

Water samples were collected from three irrigation ponds in the Suwannee River watershed in southeastern Georgia. Two of the ponds are surface water–fed (SC and NP) and one is ground water–fed (LV). Water samples were collected at least monthly during scheduled sampling events from May 2015 to May 2016. To investigate the impact of weather-related events on pathogens, indicator microorganisms, and MST markers in produce irrigation ponds, water samples were collected within 24–48 hours of ≥0.5 inch of rainfall. Scheduled sampling events that occurred within 24–48 hours after rainfall of ≥0.5 inch were considered rain event samples. In total, irrigation water samples were collected for 23 scheduled and rain events. To characterize irrigation water quality associated with harvest periods at the farms, water samples were collected at least twice per month during June–July (corresponding to harvests for crops planted in March) and during October–November (corresponding to harvests for crops planted in July/August). Irrigation water samples also were collected twice per month in August–September, a period corresponding to the highest incidence of reported cases of salmonellosis and cryptosporidiosis in the US [22, 23].

Water Sample Collection, Processing, and Analysis

Water samples were collected from two locations per pond, designated as sides A and B. Fifty liters of water were filtered by DEUF and two 1-L grab samples were collected at each sample location. *In-situ* temperature, turbidity, dissolved oxygen, conductivity, oxidation reduction potential (ORP), and pH readings were taken at the time of sampling using a YSI multiparameter meter. Ultrafilters and grab samples were transported on ice to CDC in Atlanta on the day of collection and held at 4°C before analysis the following morning.

Ultrafilters were backflushed and the DEUF concentrate was directly analyzed for generic *E. coli*, enterococci, male-specific (F+) coliphages*, E. coli* O157:H7, and *Salmonella*. *E. coli* and enterococci were respectively enumerated by the Colilert-18 and Enterolert Quanti-Tray 2000® methods (IDEXX), respectively. F+ coliphages were analyzed by the USEPA Single Agar Layer (SAL) method [24]. *Salmonella* was enumerated by a three volume, three-tube most probable number (MPN) culture method, with qPCR confirmation of presumptive positive isolates from each MPN replicate [3, 20]. *Salmonella*-positive isolates were serotyped and subjected to pulsed-field gel electrophoresis (PFGE). The presence of *E. coli* O157:H7 was determined by enrichment in modified tryptone soya broth with novobiocin, followed by IMS and plating on CT-SMAC agar and confirmation of presumptive positive samples by qPCR [5]. Grab samples were tested for generic *E. coli*, enterococci, F+ coliphages*, Salmonella*, and *E. coli* O157:H7 using the same methods described for the DEUF samples, with modifications of sample volume.

The remaining DEUF concentrate volume and 500-mL of the grab sample were further concentrated by PEG precipitation, and nucleic acid was extracted from a portion of the PEG precipitates. Nucleic

acid extracts were assayed for the presence of *Cryptosporidium* [25] and MST markers by end-point real-time PCR. The MST marker gene targets representing human sources of contamination included *Bacteroides* Hf183, human polyomaviruses (HpYVs), and *Methanobrevibacter smithii* [15, 16, 26]. Animal-specific qPCR assays targeted mitochondrial DNA of cows, chickens, pigs, and birds [27]. All real-time PCR assays were performed with an internal control (ABI TaqMan Exogenous Internal Positive Control) to monitor method performance and potential PCR inhibition. Molecular assays were considered positive if duplicate reactions resulted in crossing threshold (CT) values <45.

The remaining volume from the grab sample was used to determine total suspended solids (TSS) according to Standard Method 2540 D [28].

Statistical analyses were performed to determine if rain events affected the occurrence or concentration of pathogens, indicators, or MST markers in irrigation ponds. Logistic and linear mixed-effects models were used to evaluate the associations between analyte detection and concentration, respectively, while controlling for pond variables using restricted maximum likelihood. Weather events were modeled as a binary (rain event Yes/No) predictor and analyte detection (binary Yes/No) or concentration (culture-based *Salmonella, E. coli,* enterococci, and phage) as the response variable. Pond-level variables controlled for included: sample type (grab or DEUF) and sample location (A or B) as a fixed effects, pond as a random effect intercept (or water source type, ground water or surface, as a fixed effect instead). *P*-values ≤ 0.05 were considered statistically significant.

Results

In total, we collected 19, 15, and 17 baseline samples (<0.50 inch of rain 24–48 hours before sampling event) from ponds SC, LV, and NP, respectively, and 3, 8, and 5 rain events from the three ponds, respectively. The differences in number of baseline and rain events for each pond were related to spatial differences in rainfall amounts, with some sampling events qualifying as rain events for some ponds but baseline for others. Physical water quality parameters showed that rain events were associated with higher solids loading (e.g., TSS and turbidity) and lower pH in the irrigation ponds, especially for surface water–fed ponds SC and NP (Table 3). It has been shown in other microbial studies that enteric pathogens in surface water are more likely to be associated with solid particles than be free-floating [29].

Of the molecular analytes (Table 4), including *Cryptosporidium* and the MST markers, only the human-specific *Bacteroides* (Hf183) detection was significantly associated with sampling after a rain event (β = 1.17, P = 0.003), corresponding to a 3.2-fold increased odds of detection after a rain event. Sampling method (P < 0.0001) had a significant effect on the model, while pond, water source, and sample location did not (P > 0.05). F+ coliphage (Figure 3) detection was also significantly associated with sampling after a rain event (β = 1.01, P = 0.02), corresponding to a 2.8-fold increased odds of detection after a rain event. Sampling method (P = 0.0003) and water source (P = 0.02) had a significant effect on the model, while pond and sample location did not (P > 0.05). Neither *E. coli* (Figure 1)**,** enterococci (Figure 2), nor *Salmonella* (Figure 4) were detected significantly more frequently after rain events compared to non-rain events. There were too few detections of *E. coli* O157:H7 for any statistical analyses to be performed.

F+ coliphage, *E. coli*, and enterococci concentrations were positively associated with a water sample being collected after a rain event (vs. a baseline event) (β = 0.17, P = 0.02; β = 0.86, P < 0.001; and β = 0.56, P < 0.001, respectively), meaning that these analyte concentrations were 1.48 to 3.63 plaque-forming units [PFU] or MPN/100 mL higher after rain events compared to baseline sampling events. *Salmonella* concentrations were not significantly associated with rain events (P > 0.05).

E. coli concentration exceeding the geometric mean threshold (126 MPN/100 mL) was significantly associated with rain events (β = 1.59, P = 0.002), equivalent to an approximate 5-fold increased odds of a water sample being above the geometric mean after a rain event. Pond and water source had a

significant effect on the model, while sample location and sampling method did not ($P > 0.05$). *E. coli* concentration exceeding the statistical threshold value (410 MPN/100mL), was not significantly associated with rain events when controlling for pond variables ($P > 0.05$).

Due to the high prevalence of the fecal indicator bacteria, *E. coli* and enterococci, in the irrigation ponds, it is unsurprising that increased detection of these microbes was not observed after rain events. However, it is notable that the concentration of both organisms, but not *Salmonella*, were positively correlated with sampling after a rain event, meaning that *E. coli* monitoring occurring after rainfall resulted in significantly higher concentration measurements, but this increase was not associated with increased *Salmonella* detection or concentration. The increased detection of Hf183 and increased detection and concentration of F+ coliphage following rain events suggest a human contamination source related to run-off to these ponds. This could potentially suggest an increased risk of viral contamination of these surface water sources.

Objective 3. Assessment of large-volume sample collection

Qualitative detection results (positive vs. negative) were used to determine if large-volume DEUF sampling allowed for increased detection rates of F+ coliphages or *Salmonella* compared to small-volume grab sampling. Statistical comparisons for *E. coli* and enterococci could not be performed because there were too few non-detects of these indicators during the study. Logistic mixed-effects models were used to evaluate the difference in detection between grab samples and DEUF while controlling for pond variables using restricted maximum likelihood: sample method (grab vs. DEUF) as the binary predictor, sample location (A vs. B) as a fixed effect, sample event number nested within pond as a random effect intercept (or water source type, ground water or surface, as a fixed effect instead), and analyte detection (binary Yes/No) as the response variable. DEUF samples were associated with an increased probability of detecting *Salmonella* compared to grab samples ($\beta = 1.04$, $P = 0.00006$), equivalent to an approximate 3-fold increased odds of detecting *Salmonella* in a sample if the DEUF was used. DEUF samples were associated with an increased probability of detecting F+ coliphages compared to grab samples ($\beta = 2.46$, $P < 0.0001$), equivalent to an approximate a 12-fold increased odds of detecting F+ coliphage when DEUF is used. Sample location did not have a significant effect on either model ($P > 0.05$), but pond and water source did have a significant effect.

Concentration data from the culturable microbes were incorporated into linear mixed-effects models to evaluate the impact of sampling volume on measured concentration while controlling for pond variables using restricted maximum likelihood: sample method (grab vs. DEUF) as a binary predictor, sample location (A vs. B) as a fixed effect, pond as a random effect intercept (or water source type, ground water or surface, as a fixed effect instead), and the $\log_{10}$ concentration of analyte as the response variable. *Salmonella* concentration was negatively associated with a sample processed by DEUF filtration ($\beta = -0.7268$, $P < 0.0001$), equivalent to an average 6.1 MPN/100 mL lower *Salmonella* concentration in DEUF samples versus grab samples. *E. coli* concentration was negatively associated with a sample processed by DEUF filtration ($\beta = -0.1210$, $P = 0.0039$), equivalent to an average 1.3 CFU/100 mL lower *E. coli* concentration in DEUF samples versus grab samples. F+ coliphage concentration was also negatively associated with a sample processed by DEUF filtration ($\beta = -0.2626$, $P = 0.0014$), equivalent to an average 1.8 PFU/100 mL lower F+ coliphage concentration in DEUF samples versus grab. These results are not surprising because there is a likelihood of some irreversible microbial attachment to DEUF filter media, but this “recovery efficiency” factor differs between microbes. For example, enterococci concentrations were not associated with a sample processed by DEUF filtration, meaning there was no significant difference in the concentration in DEUF samples versus grab. For *Salmonella*, F+ coliphage, and *E. coli* models, pond and water source had a significant effect on the model while sample location did not.

For detection of lower-level indicators or pathogens (coliphage and *Salmonella*), DEUF increased the detection rates and gave a better estimation of water quality than grab sampling for these analytes. Even with loss during DEUF processing, the concentration estimates between DEUF and grab sampling were only different by a small amount (<10 MPN or PFU/100 mL), so the concentrations were not meaningfully underestimated when DEUF was used. DEUF would not be necessary for quantification of *E. coli* or enterococci in irrigation water because both indicators are present at sufficiently high concentrations to be effectively detected by grab sampling. However, DEUF can improve detection rates for low concentration pathogens or indicators during more advanced monitoring.

Objective 4. Evaluation of indicators and *E. coli* microbial water quality thresholds

Indicator and pathogen data were evaluated to determine whether *E. coli* geometric mean and STV thresholds were associated with pathogen presence in irrigation water. Detection of *Salmonella* was compared to *E. coli* concentration data to specifically examine how well the *E. coli* thresholds indicate a higher likelihood for the presence of this common foodborne pathogen. Logistic regression (controlling for pond variables) was used to evaluate whether *Salmonella* presence (detected in DEUF samples) was associated with *E. coli* concentrations exceeding the geometric mean level (126 MPN/100 mL) or the statistical threshold value (410 MPN/100 mL). *Salmonella* presence was not significantly associated with *E. coli* concentrations above the geometric mean (β = 0.17, P = 0.07) or the statistical threshold value (β = -1.21, P = 0.31) (Figure 5). This suggests that the microbial water quality thresholds for *E. coli* were not predictive of *Salmonella* presence in these irrigation ponds during this study.

The utility of *E. coli*, enterococci, F+ coliphages, and the MST markers were evaluated as tools for predicting the presence of pathogens in irrigation water. Logistic and linear mixed-effects models were used to evaluate associations between indicator and MST marker presence or concentration (when applicable) and *Salmonella* presence and concentration, respectively, while controlling for pond variables. *Salmonella* detection or $\log_{10}$ concentration (in DEUF samples) was modeled with the indicator/MST marker binary or continuous predictor (when applicable), sample location as a fixed effect, and sample event nested within pond as a random effect intercept.

E. coli concentrations in DEUF and grab samples were positively associated with the probability of detecting *Salmonella* in the samples (β = 0.900, P = 0.0008 and β = 0.852, P = 0.0016, respectively), equivalent to a 1-$\log_{10}$ increase in *E. coli* concentration, increasing the odds of detecting *Salmonella* by 2.5-fold. *E. coli* concentrations were not associated with *Salmonella* presence in grab samples. Enterococci concentrations in DEUF and grab samples were also positively associated with the probability of detecting *Salmonella* in DEUF samples (β = 1.17, P = 0.0015 and β = 1.10, P = 0.0013, respectively), equivalent to a 1-$\log_{10}$ increase in enterococci concentration, increasing the odds of detecting *Salmonella* by 3.2-fold. Enterococci concentrations were not associated with *Salmonella* presence in grab samples. Neither F+ coliphage detection nor concentration in DEUF or grab samples was associated with *Salmonella* DEUF presence (P > 0.05). The association between *Salmonella* detection and the detection of *E. coli* or enterococci could not be evaluated due to the high prevalence of both microbes in all three irrigation ponds. Neither F+ coliphage detection nor concentration was associated with *Salmonella* presence in DEUF or grab samples.

Cryptosporidium, *Bacteroides* Hf183, HPyVs, and *M. smithii* were not associated with *Salmonella* presence in DEUF samples when controlling for pond variables (P > 0.05). None of the animal MST markers (bird, chicken, cow, pig) were significantly associated with *Salmonella* presence in DEUF samples (P > 0.05), likely due to the low detection rate of the MST markers.

E. coli concentrations in DEUF and grab samples were positively associated with *Salmonella* concentration in DEUF samples (β = 0.18, P < 0.0001 and β = 0.18, P = 0.0003, respectively), equivalent to a 1-$\log_{10}$ increase in *E. coli* concentration being associated with a 0.18-$\log_{10}$ increase in *Salmonella* concentration in DEUF samples. *E. coli* concentrations were not associated with *Salmonella* concentrations in grab samples. Enterococci concentrations in DEUF and grab samples were also positively associated with *Salmonella* concentration in DEUF samples (β = 0.15, P = 0.013 and β = 0.19, P = 0.0008, respectively), equivalent to a 1-$\log_{10}$ increase in enterococci concentration being associated with a 0.15- or 0.19-$\log_{10}$ increase in *Salmonella* concentration, depending on which sample method was used. Grab sample enterococci concentrations were not associated with *Salmonella* concentrations in grab samples. F+ coliphage concentration was positively associated with *Salmonella* concentration in DEUF samples (β = 0.16, P = 0.036), equivalent to a 1-$\log_{10}$ increase in coliphage concentration being associated with a 0.16-$\log_{10}$ increase in *Salmonella* concentration. Grab sample F+ coliphage concentrations were not associated with *Salmonella* concentrations in grab or DEUF samples.

E. coli and enterococci concentrations, measured by either grab or DEUF methods, correlated with *Salmonella* detection and concentration in DEUF samples but not in grab samples. This suggests that monitoring for fecal indicator bacteria, such as *E. coli* and enterococci, by grab sampling is appropriate, but that when indicator levels trigger further monitoring, large-volume sampling would be more effective for determining the extent of low-concentration pathogen contamination. Geometric mean and STV *E. coli* thresholds were not found to be associated with *Salmonella* presence in South Georgia produce-farm irrigation ponds. Therefore future research is needed to determine risk-based thresholds for indictor monitoring in irrigation water. While enterococci were not found to have a stronger association with *Salmonella* in this study, future work should consider other microbial alternative indicators and standards for each.

Objective 5. Utility of DEUF to facilitate detection of *Cryptosporidium* and MST markers

DEUF versus Grab

The prevalence of *Cryptosporidium* and the MST markers in each pond using the two sampling methods is described in Table 4. Logistic mixed-effects models were used to evaluate the associations between sampling method (DEUF vs. grab) and *Cryptosporidium* or MST detection by real-time PCR while controlling for pond variables. Sample method (grab vs. DEUF) was modeled as a binary predictor, and analyte detection (Yes/No) as the binary response variable. The pond level variables controlled for included: sample location (A vs. B) as a fixed effects, sample event (to only compare within samples) nested within pond as a random effect intercept (or water source as a fixed effect [ground- vs. surface-fed] instead).

Cryptosporidium (β = 2.05, P < 0.0001) and *Bacteroides* Hf183 (β = 2.78, P < 0.0001) detections were significantly associated with a sample being collected with DEUF compared to grab sampling, meaning testing was significantly more likely to detect one of these analytes if the DEUF method was used instead of grab sampling. Sample location did not significantly affect either analyte's model (P > 0.05), but sample event and pond significantly affected both models. Water source significantly affected the *Cryptosporidium* detection model (the ground water–fed pond was less likely to have detects than the surface water–fed ponds) but not the Hf183 model. No other MST markers were significantly associated with the sampling method after controlling for pond variables (P > 0.05).

Cryptosporidium subtyping was not possible for this study because the sensitivity of the genotyping PCR assay was not high enough to detect the low concentrations of *Cryptosporidium* found in the irrigation pond samples. The *Cryptosporidium* and Hf183 results provide evidence for the benefits of large-volume sample for the detection of low-concentration MST markers and pathogens. This work

also demonstrates the utility and feasibility of performing MST analyses on irrigation water samples using DEUF despite low recovery efficiencies measured for these analytes.

Salmonella Antigen-based Serotyping

Salmonella was detected in at least one of the three irrigation ponds for 20 out of the 23 sampling events that occurred over the course of one year (SC = 19 positive events, LV = 7 positive events, and NP = 14 positive events). For three sampling events, *Salmonella* was not detected in any of the three ponds. Only one isolate from each positive MPN dilution was selected for typing, meaning up to 9 isolates per sample could be typed. A total of 131 isolates were serotyped, representing 71 unique water samples (*N* = 48 positive DEUF samples and 23 positive grab samples out of the 276 total grab and DEUF samples collected during the study period).

Eighteen different *Salmonella* serotypes were detected among the 131 typed isolates. Overall, serotypes Muenchen and Rubislaw were most frequently detected in the three ponds over the 23 sampling events, being detected in 23% and 20% of samples, respectively (Table 5). Up to five different serotypes were detected in a pond at a single sampling event (either in the grab or DEUF sample from either collection site), and up to two serotypes were detected within a single water sample (a sample collected with one sampling method from one sampling site on one pond). In total, 11 different *Salmonella* serotypes were detected in SC, 11 in LV, and 12 in NP. Figure 6 shows the proportion of *Salmonella* serotypes detected in positive samples for each of the three irrigation ponds.

All of the *Salmonella* serotypes detected are considered potentially pathogenic to humans, and none of the serotypes detected were those commonly associated with animals (e.g., serotypes Kentucky [chickens], certain strains of Typhimurium [birds], Gallinarum [chickens], Dublin [cows], Choleraesuis [pigs]). Three of the serotypes detected in the ponds, Javiana, Muenchen, and Saintpaul, were among the top 10 serotypes causing human *Salmonella* cases in the United States in 2015 [30].

Salmonella PFGE and PulseNet Query

Salmonella isolate subtyping was conducted using pulsed-field gel electrophoresis (PFGE). PFGE using the XbaI enzyme was performed on all *Salmonella* isolates (*N* = 131), and the resulting patterns were queried against the CDC PulseNet database of clinical *Salmonella* isolates submitted in 2015 and 2016 [31]. Pattern analysis and UPGMA dendrogram generation were performed using BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium) with the Dice coefficient and tolerance of 1.5% (Figure 7). A matching PulseNet pattern was identified for 65 out of the 131 isolates from the irrigation pond samples (Table 6). In total, 21 different PulseNet patterns were matched to the 65 isolates, some with multiple patterns matching to different isolates of the same serotype (e.g., PulsoTypes JN6X01.0044 and JN6X01.0092 are both antigen-based serotype Saintpaul).

Because all *Salmonella* isolates detected in the study samples were identified as potentially pathogenic serotypes to humans, and no association was found between the presence of *Salmonella spp.* and human MST markers, it was not possible to evaluate any association between pathogenic *Salmonella* presence and the human MST markers. The association between animal MST markers and *Salmonella* serotypes could not be determined because no animal-associated serotypes were detected.

Outcomes and Accomplishments

This project resulted in a number of positive outcomes and accomplishments. The partnership between CDC, Emory University, and the University of Georgia resulted in a successful laboratory and field-based study that will provide timely and useful scientific data to the fresh produce industry. We were able to utilize and validate CDC's DEUF large-volume water collection method, used extensively for drinking and recreational water, for agricultural water types such as irrigation water. The DEUF method proved effective for enabling reliable detection of low-level pathogen and indicator concentrations, making it a valuable tool for advanced irrigation water monitoring. During a yearlong field study, we collected a diverse set of irrigation water samples that allowed us to answer a host of questions related to small- vs. large-volume sampling, correlations between indicators and pathogens, and the usefulness of innovative water quality analytes for irrigation water monitoring. We successfully completed the goals of this study, the findings of which will help inform decision making by regulators, the produce industry, and fresh fruit and vegetable producers.

Summary of Findings and Recommendations

- DEUF procedures were established for agricultural irrigation water applications, and recovery efficiencies for the DEUF method were determined for pathogenic bacteria, human parasites, and human-specific fecal bacteria and viruses from irrigation water.
- The DEUF method had higher detection rates of the low-level microbes, *Salmonella* and F+ coliphage, compared to grab samples, but minimal impact on concentration estimates. The DEUF method did not adversely affect detection of *E. coli* and enterococci compared to grab sampling. The DEUF method also increased detection rates of the molecular analytes *Cryptosporidium* and the *Bacteroides* Hf183 human-associated microbial source tracking marker.
- DEUF is recommended for advanced monitoring of irrigation water when target analytes are suspected to be present at low concentrations, as with pathogens or alternative indicators such as fecal source tracking markers.
- The *Bacteroides* Hf183 human-associated marker and F+ coliphage were detected more frequently after rain events, suggesting a runoff-related human contamination source.
- *E. coli* concentration increased after a rain event and was more likely to exceed the geometric mean level, but this increase was not associated with an increase in *Salmonella* detection or concentration.
- Overall, *Salmonella* detections were not associated with *E. coli* concentrations above the geometric mean or statistical threshold value levels, indicating that these microbial water quality thresholds for *E. coli* were not predictive of *Salmonella* presence in the irrigation ponds studied during this project. However, increased *E. coli* and enterococci concentrations (by either grab or DEUF sampling) were associated with increased *Salmonella* detection and concentration (by DEUF only).
- Neither *Cryptosporidium* nor any of the MST markers were associated with *Salmonella* detection.
- Of the 131 subtyped *Salmonella* isolates collected from the irrigation ponds, approximately half matched PFGE patterns of clinical *Salmonella* isolates in the PulseNet database, and all isolates cultured from the ponds were serotypes potentially pathogenic to humans.

APPENDICES

Publications and Presentations

Abstracts:

Mattioli, M., A. Kahler, C. Miller, M. Tertuliano, G. Vellidis, V. Hill, and K. Levy. 2017. Evaluating the Effects of Rainfall on Foodborne Pathogen and Fecal Indicator Presence in Surface Irrigation Waters on Southeastern USA Produce Farms. 2017 University of North Carolina Water Microbiology Conference, Chapel Hill, 15-17 May (scheduled).

Kahler, A., C. Miller, M. Mattioli, M. Tertuliano, K. Levy, G. Vellidis, and V. Hill. 2017. Evaluation of FSMA *E. coli* Guidelines as Predictors of Foodborne Pathogens in Irrigation Water. 2017 International Association for Food Protection Annual Meeting, Tampa, 09-12 July (scheduled).

Professional Presentations:

Kahler, A. 2016. Improved sampling and analytical methods for testing agricultural water for pathogens, surrogates and source tracking indicators. 2016 Center for Produce Safety Research Symposium, Seattle, Washington, 28-29 June.

Kahler, A. 2016. The Water We Eat. American Chemistry Council Water Quality & Health Council and ACC Disinfection Work Group Meeting, Atlanta, Georgia, 29 February.

Kahler, A. 2015. Improved sampling and analytical methods for testing agricultural water for pathogens, surrogates and source tracking indicators. 2015 Center for Produce Safety Research Symposium, Atlanta, Georgia, 23-24 June.

Vellidis, G., and K. Levy. 2016. Is Your Irrigation Water Safe? Southeast Regional Fruit and Vegetable Conference, Savannah, Georgia, 07-09 January.

Budget Summary

All project funds ($196,569) were expended.

Tables and Figures

Table 1. Measured water quality parameters and *Cryptosporidium parvum* recovery efficiency by DEUF

Experiment ID	Turbidity (NTU)	Conductivity (µS/cm)	*C. parvum* Percent Recovery
High Turbidity Water			
SC-1	19.4	200	51
SC-2	23.3	155	53
SC-3	33.6	153	59
SC-4	12.6	145	60
SC-5	27.3	110	127
Mean	**23.2**	**153**	**70**
Low Turbidity Water			
LV-1	0.867	126	88
LV-2	0.8	219	208
LV-3	0.867	126	172
LV-4	0.5	250	239
LV-5	0.5	250	36
Mean	**0.707**	**194**	**149**

Table 2. Measured water quality parameters and recovery efficiencies of *E. coli*, *Salmonella*, and MST markers by DEUF

Experiment ID	Turbidity (NTU)	Conductivity (µS/cm)	*E. coli* Percent Recovery	*Salmonella* Percent Recovery (95% CI)	*Bacteroides Hf183* Percent Recovery	HPyV Percent Recovery
High Turbidity						
SC-1	10.4	220	37	1800 (123, 26202)	7.1	5.1
SC-2	7.68	216	80	113 (9, 1448)	4.5	11.1
SC-3	7.75	216	77	61 (4, 998)	1.6	4.7
SC-4	12.8	218	83	361% (25,5227)	1.5	2.7
SC-5	7.13	217	108	350 (21, 5710)	4.3	15.1
Mean	**9.15**	**217**	**77**	**537**	**3.8**	**7.8**
Low Turbidity						
LV-1	1.82	209	50	299 (20, 4218)	1.9	0.6
LV-2	0.67	166	48	53 (3, 4036)	10.6	10.9
LV-3	2.24	164	46	354 (23, 23369)	9.9	6.5
LV-4	2.89	164	80	1802 (145, 23619)	16.9	10.7
LV-5	2.62	164	47	44 (3, 644)	10.3	7.3
Mean	**2.11**	**165**	**54**	**510**	**9.9**	**7.2**

Table 3. Average physical and chemical water quality results for irrigation ponds SC, LV, and NP; May 2015 – May 2016

Sampling Event	Farm Pond	pH	Water Temp (°C)	Turbidity (NTU)	TSS (mg/L)	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)	ORP
Baseline	SC	7.75	23.2	12.1	7.85	197	10.2	170
	LV	8.10	21.8	1.55	1.19	234	10.3	135
	NP	7.50	23.6	19.3	9.55	71.9	10.2	159
Rain Event	SC	7.23	18.1	16.7	10.5	180	9.75	173
	LV	7.81	23.1	1.16	1.33	226	10.2	142
	NP	6.94	18.9	22.3	12.0	73.7	9.07	186

Table 4. The number of molecular-based pathogen and microbial source tracking (MST) marker detects by real-time PCR over the total number of samples tested (%). Results are grouped by pond (SC, LV, NP) and sample collection type (grab, DEUF).

Analyte	No. of Positive PCR Results (% Positive)					
	SC-Grab	LV-Grab	NP-Grab	SC-DEUF	LV-DEUF	NP-DEUF
Cryptosporidium	30 (68)	21 (46)	23 (52)	42 (95)	30 (65)	37 (84)
***Bacteroides* Hf183**	4 (9)	0	1 (2)	13 (30)	10 (22)	14 (32)
Human Polyomavirus	1 (2)	0	0	0	3 (7)	1 (2)
M. smithii	0	0	0	1 (2)	0	0
Bird (Goose)	0	0	0	0	2 (4)	0
Chicken	1 (2)	2 (4)	0	1 (2)	1 (2)	2 (5)
Cow	0	0	0	2 (5)	1 (2)	0
Pig	1 (2)	2 (4)	1 (2)	2 (5)	2 (4)	3 (7)

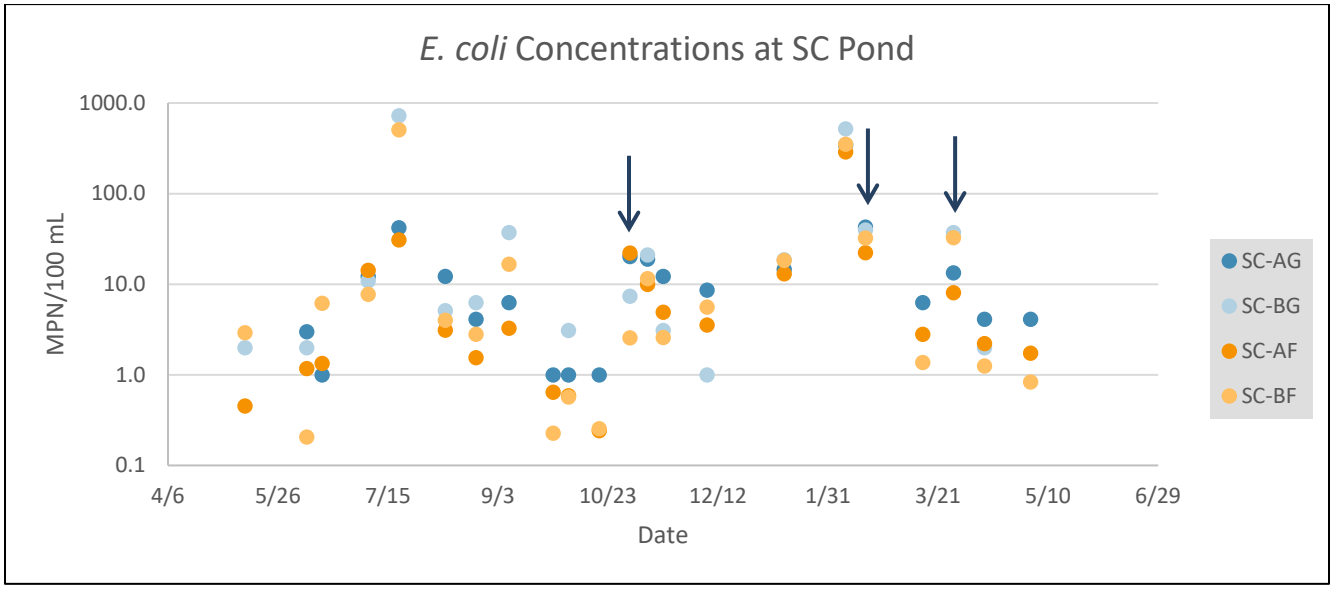
N = 44 for SC and NP ponds
N = 46 for LV pond

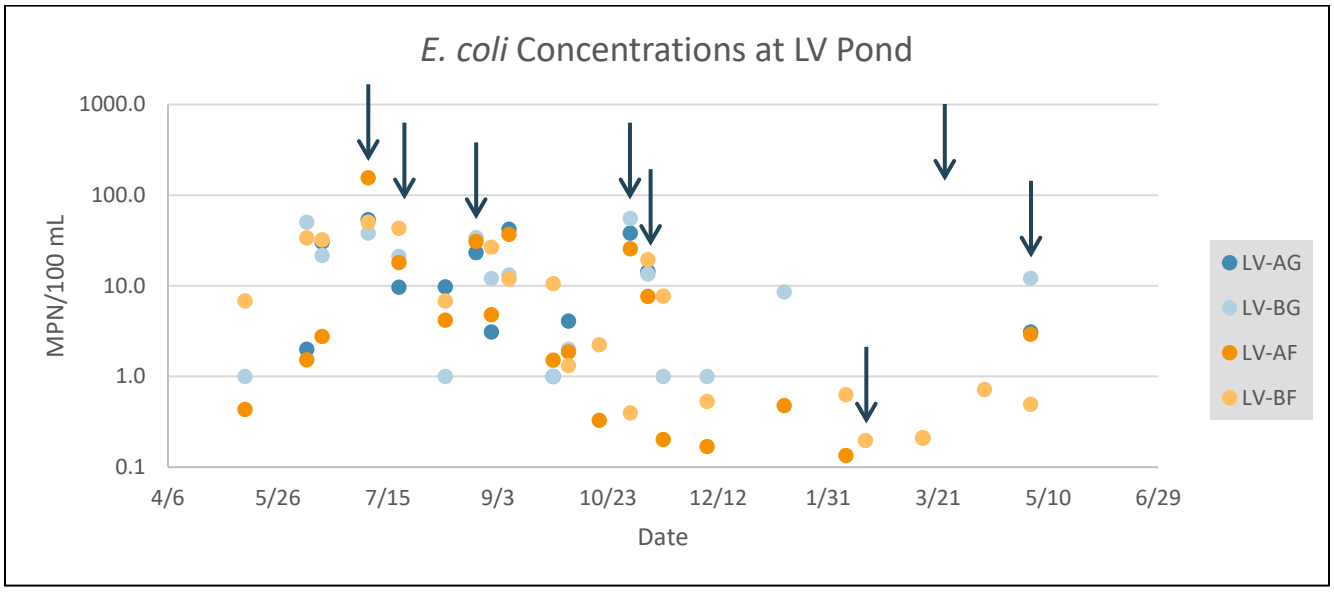
Table 5. The number of water samples in which each *Salmonella* serotype was detected by pond (SC, LV, or NP), sample location (site A or B), and sample collection method (DEUF [F] or grab [G]). The total represents the total number (%) of water samples in which each serotype was detected out of the 71 *Salmonella* positive water samples. More than one serotype was isolated in several water samples.

	SC				LV				NP				
	A		B		A		B		A		B		
Serotype	**F**	**G**	**F**	**G**	**F**	**G**	**F**	**G**	**F**	**G**	**F**	**G**	**Total**
Alabama	0	0	0	0	1	0	0	0	0	0	0	0	1 (1%)
areilly	0	0	1	0	0	0	0	0	0	0	1	1	3 (4%)
Braenderup	0	0	0	0	0	0	0	0	0	0	0	1	1 (1%)
Gaminara	0	0	0	0	0	0	0	0	1	1	2	2	6 (8%)
Give	0	0	2	0	0	0	0	0	1	1	1	1	6 (8%)
Hartford	0	0	1	1	0	0	0	0	0	0	0	0	2 (3%)
I 38:k:-	1	0	1	0	0	0	0	0	0	0	0	0	2 (3%)
I 4,5,12:b:-	0	0	0	0	0	0	0	0	6	1	4	0	11 (15%)
IIIb 16:z10:e,n,x,z15	1	0	1	0	0	0	0	0	1	0	1	0	4 (6%)
IIIb 38:(k):z35	0	0	0	0	0	0	0	0	1	0	0	0	1 (1%)
IIIb 38:(k):z35:z56	0	0	0	0	0	0	0	0	0	0	1	0	1 (1%)
IIIb 60:r:e,n,x,z15	1	0	1	0	3	1	0	0	1	2	0	1	10 (14%)
Inverness	1	1	0	0	0	0	0	0	0	0	0	0	2 (3%)
Javiana	1	0	0	0	0	0	0	0	0	0	0	0	1 (1%)
Montevideo	0	0	0	0	0	0	0	0	1	0	0	0	1 (1%)
Muenchen	3	3	1	2	0	0	0	1	2	0	3	1	16 (23%)
Rubislaw	3	1	3	0	1	0	2	0	1	0	3	0	14 (20%)
Saintpaul	1	3	0	0	2	0	1	0	0	0	0	0	7 (10%)

Table 6. Number of *Salmonella* isolates from pond samples collected in 2015 and 2016 for each matching PulseNet PFGE PulsoType, as well as the number of clinical PulseNet entries and states reporting cases with matching PulsoType in 2015 and 2016. (The total number of clinical cases in the PulseNet database and associated antigen-based serotype for each PulseNet PFGE PulsoType is also listed.)

PulsoType	No. Pond Isolates 2015	No. Clinical Entries 2015	No. States Reporting Cases 2015	No. Pond Isolates 2016	No. Clinical Entries 2016	No. States Reporting Cases 2016	Total No. Clinical Cases in Database	Associated Serotype
Unknown	38	-	-	28	-	-	-	-
JN6X01.0044	2	46	17				471	Saintpaul
JKXX01.0059	8	22	13	5	29	12	300	I 4,5,12:b:-
JN6X01.0092	2	14	9				167	Saintpaul
JBPX01.0466	1	6	4				87	Braenderup
JIXX01.0076				1	10	3	69	Montevideo
JRLX01.0032				2	5	3	42	Inverness
JGGX01.0108				1	1	1	41	Javiana
JJ6X01.0239	3	2	2	2	3	3	16	Muenchen
JLPX01.0125	4	0	0				13	Rubislaw
JHAX01.0034	3	0	0				13	Hartford
JAPX01.0226				2	0	0	11	Bareilly
JLPX01.0139				3	1	1	9	Rubislaw
JAPX01.0157				1	0	0	8	Bareilly
JRLX01.0050	2	0	0				5	I 38:k:-
JLPX01.0060				2	0	0	5	Rubislaw
JJ6X01.3847	6	5	4				5	Muenchen
RN5X01.0012				2	0	0	2	IIIb 60:r:e,n,x,z15
JN6X01.1182	6	2	2				2	Saintpaul
JLPX01.0130	1	0	0				2	Rubislaw
JLPX01.0131	5	0	0				1	Rubislaw
JJ6X01.4409				1	0	0	1	Muenchen


E. coli Concentrations at SC Pond
MPN/100 mL
1000.0
100.0
10.0
1.0
0.1
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
SC-AG
SC-BG
SC-AF
SC-BF


E. coli Concentrations at LV Pond
MPN/100 mL
1000.0
100.0
10.0
1.0
0.1
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
LV-AG
LV-BG
LV-AF
LV-BF

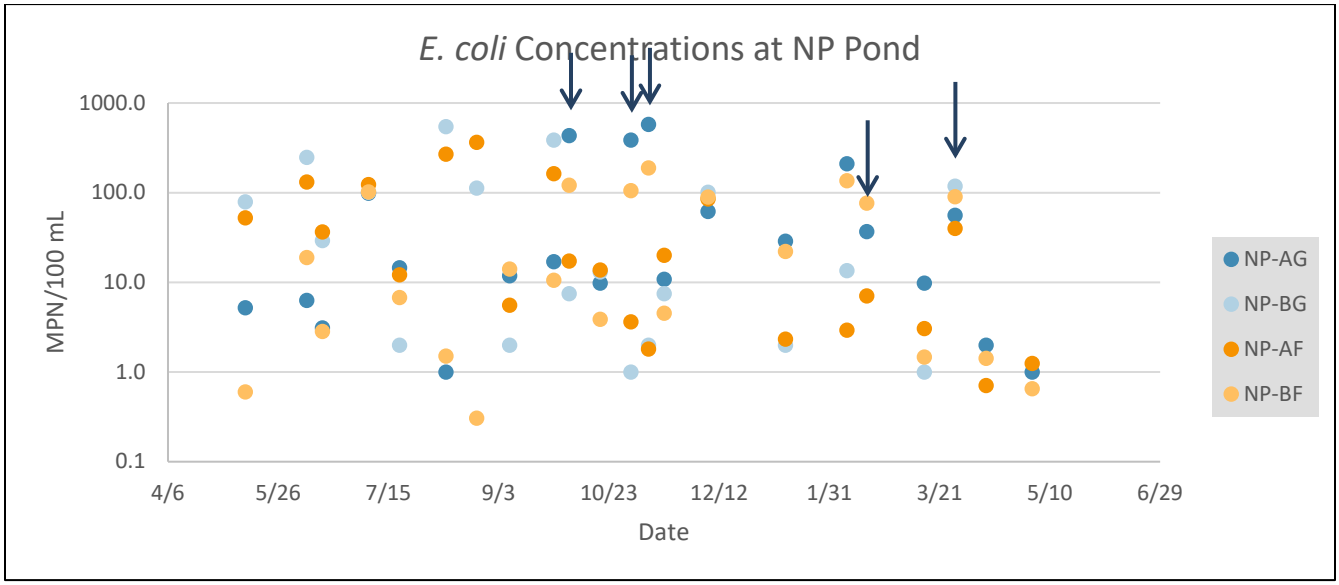
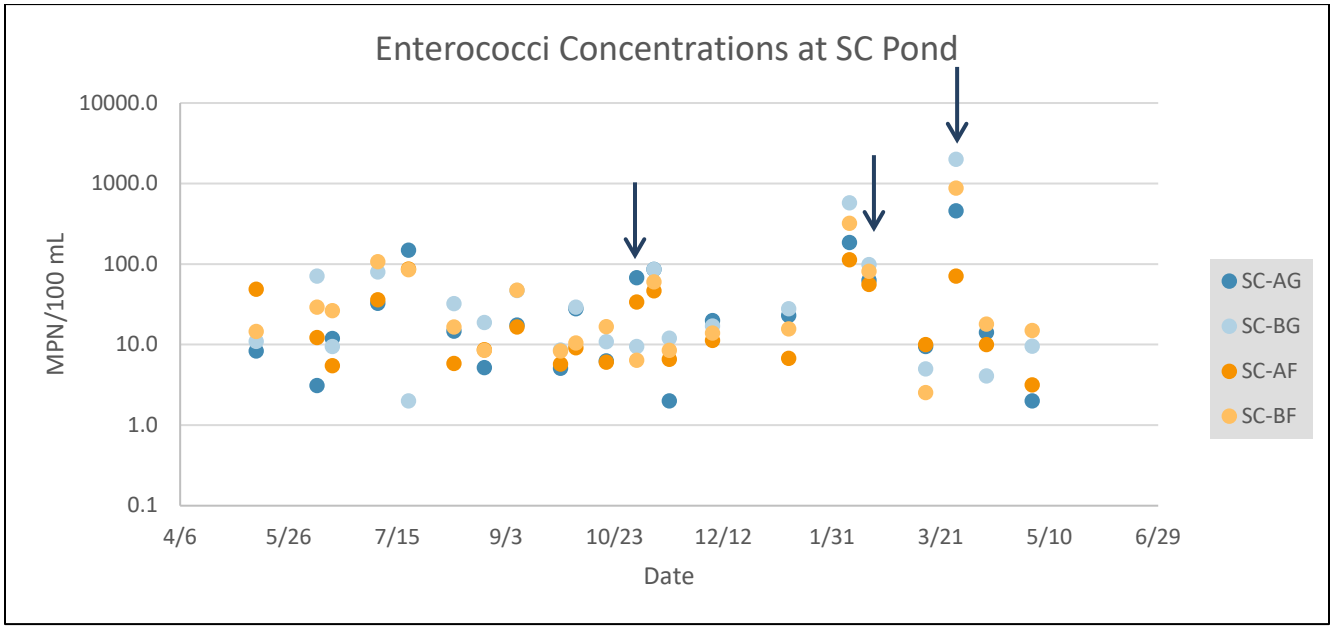
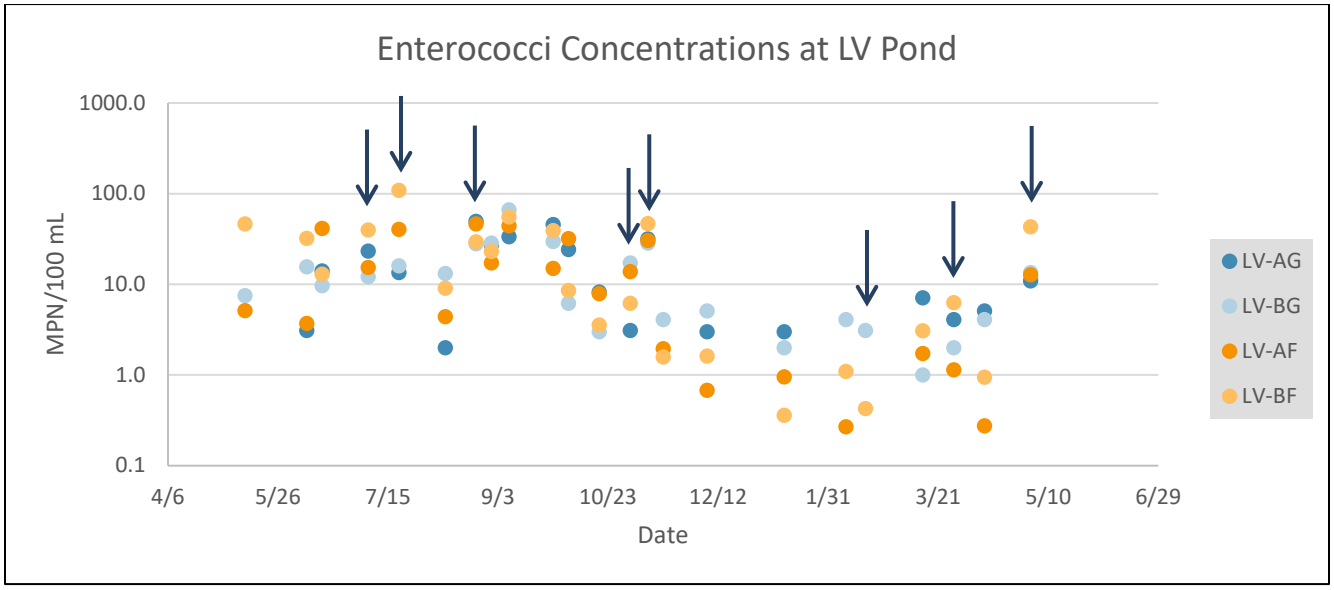


Figure 1. *E. coli* concentrations at ponds SC, LV, and NP (data only shown for detections). Blue circles are for small-volume grab samples collected from two locations (A and B) in the pond. Orange circles are for large-volume DEUF samples collected from these same locations. Blue arrows denote rain events.


Enterococci Concentrations at SC Pond
MPN/100 mL
10000.0
1000.0
100.0
10.0
1.0
0.1
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
SC-AG
SC-BG
SC-AF
SC-BF


Enterococci Concentrations at LV Pond
MPN/100 mL
1000.0
100.0
10.0
1.0
0.1
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
LV-AG
LV-BG
LV-AF
LV-BF

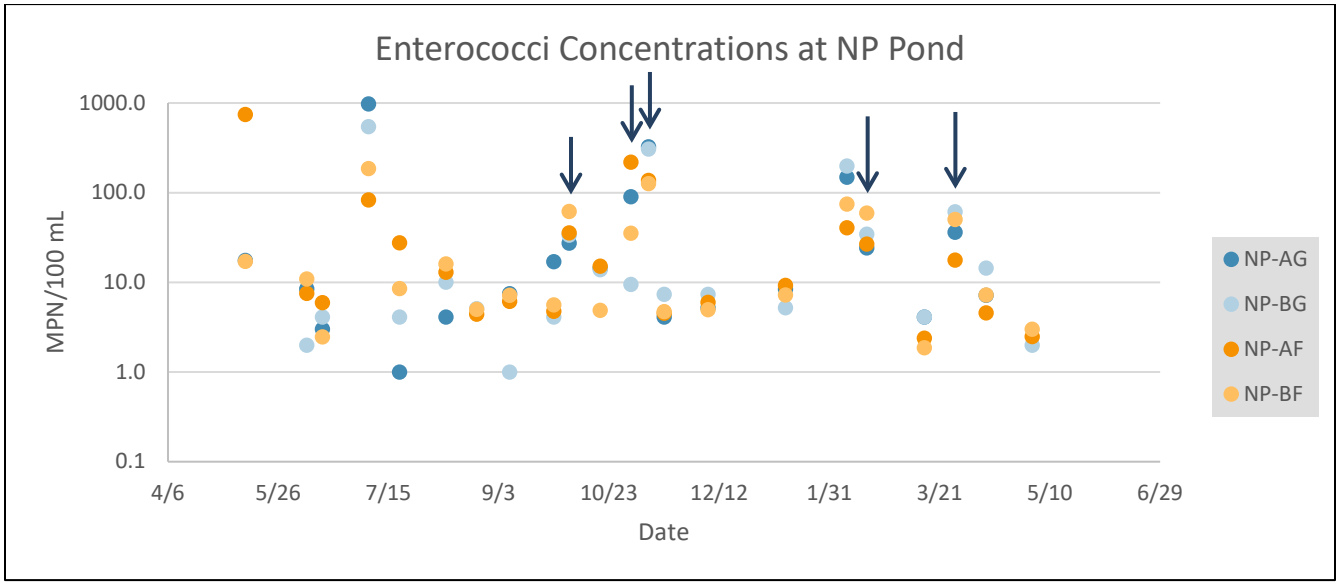
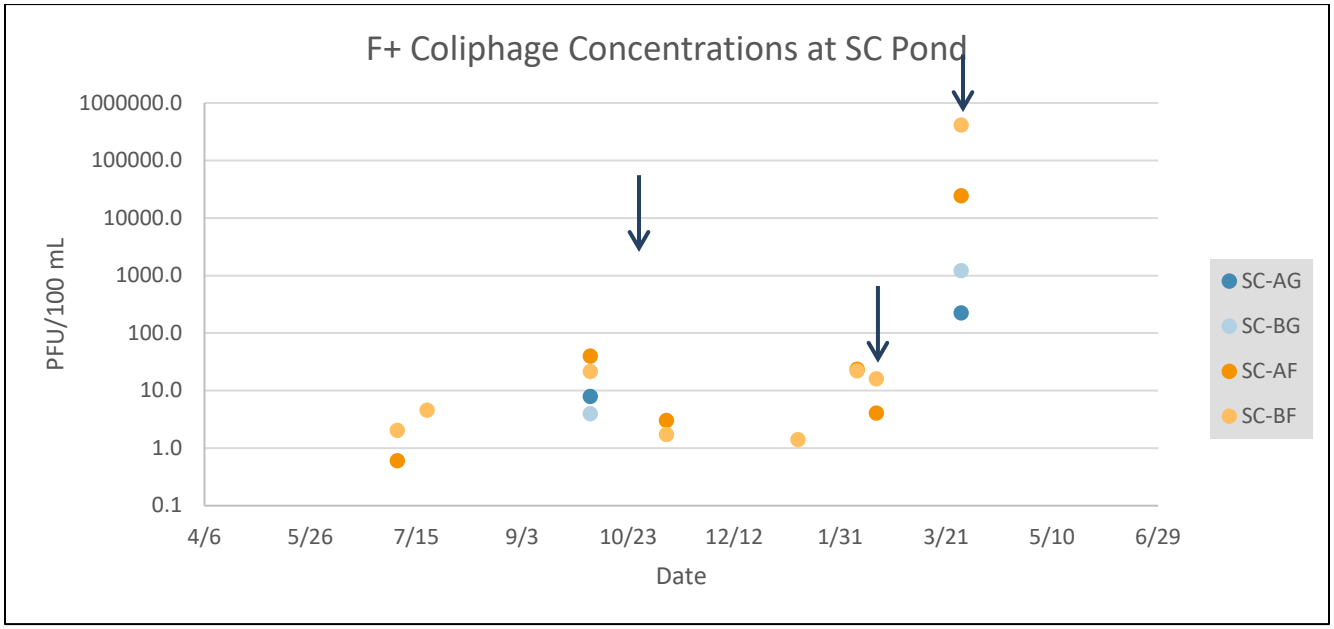
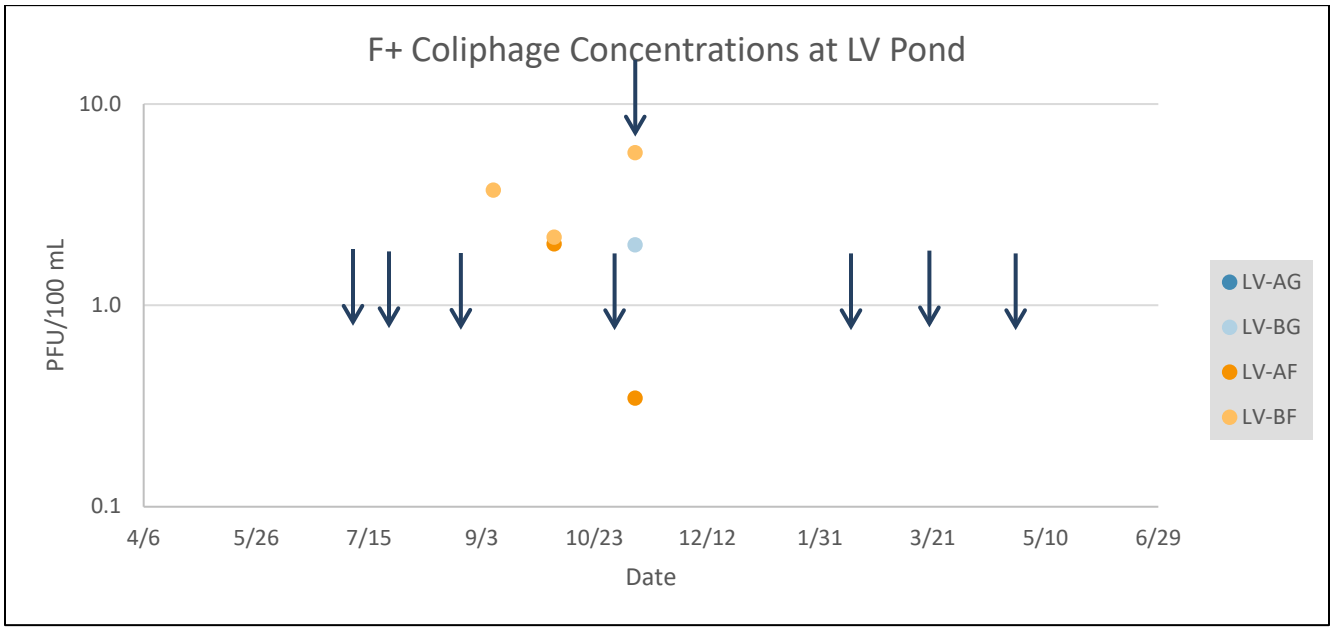


Figure 2. Enterococci concentrations at ponds SC, LV, and NP (data only shown for detections). Blue circles are for small-volume grab samples collected from two locations (A and B) in the pond. Orange circles are for large-volume DEUF samples collected from these same locations. Blue arrows denote rain events.


F+ Coliphage Concentrations at SC Pond
1000000.0
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
PFU/100 mL
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
SC-AG
SC-BG
SC-AF
SC-BF


F+ Coliphage Concentrations at LV Pond
10.0
1.0
0.1
PFU/100 mL
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
LV-AG
LV-BG
LV-AF
LV-BF

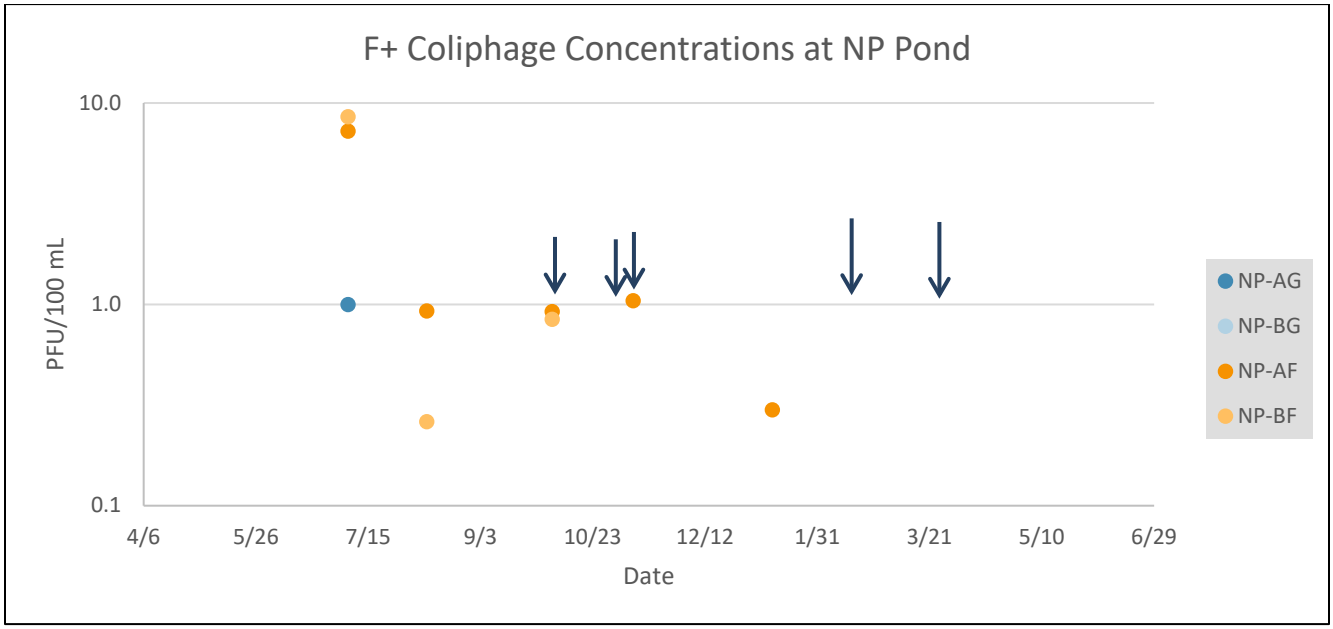
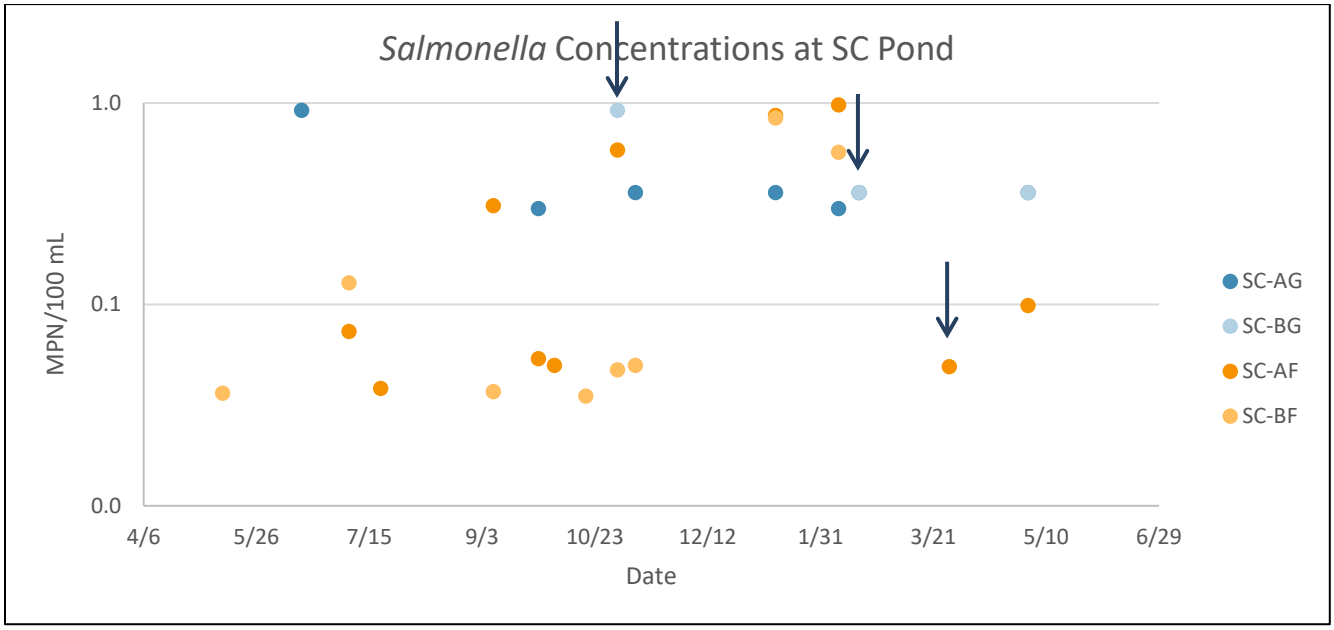
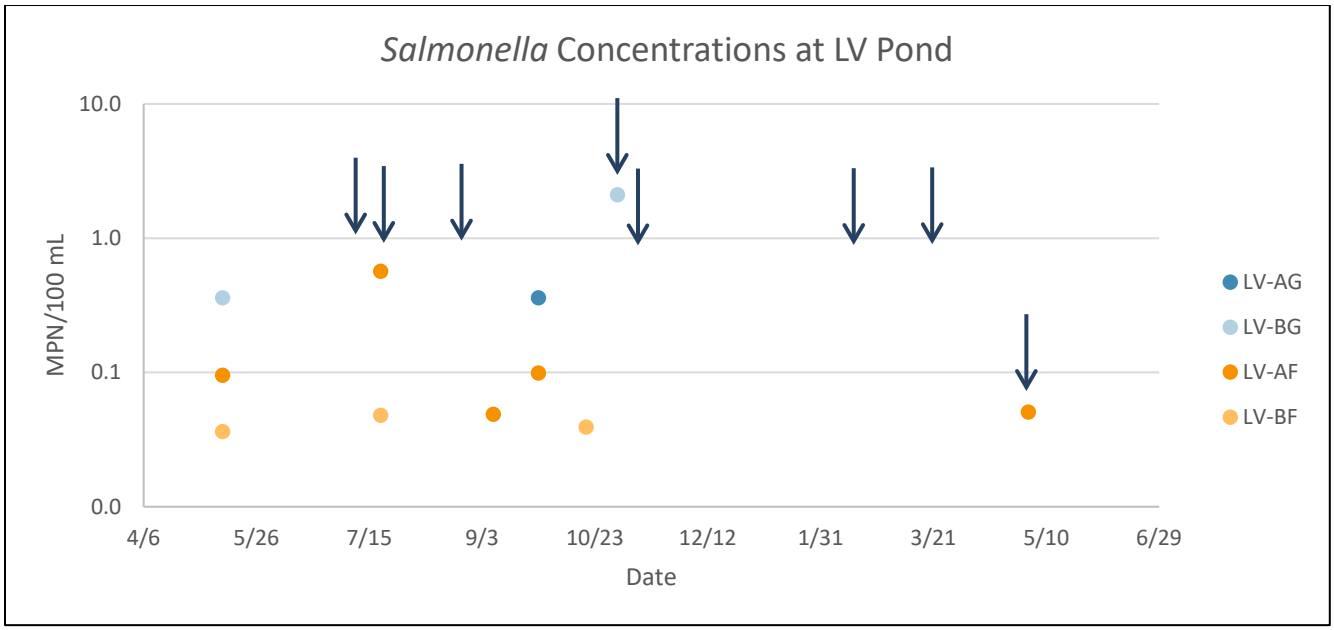


Figure 3. Male-specific (F+) coliphage concentrations at ponds SC, LV, and NP (data only shown for detections). Blue circles are for small-volume grab samples collected from two locations (A and B) in the pond. Orange circles are for large-volume DEUF samples collected from these same locations. Blue arrows denote rain events.


Salmonella Concentrations at SC Pond
MPN/100 mL
1.0
0.1
0.0
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
SC-AG
SC-BG
SC-AF
SC-BF


Salmonella Concentrations at LV Pond
MPN/100 mL
10.0
1.0
0.1
0.0
4/6
5/26
7/15
9/3
10/23
12/12
1/31
3/21
5/10
6/29
Date
LV-AG
LV-BG
LV-AF
LV-BF

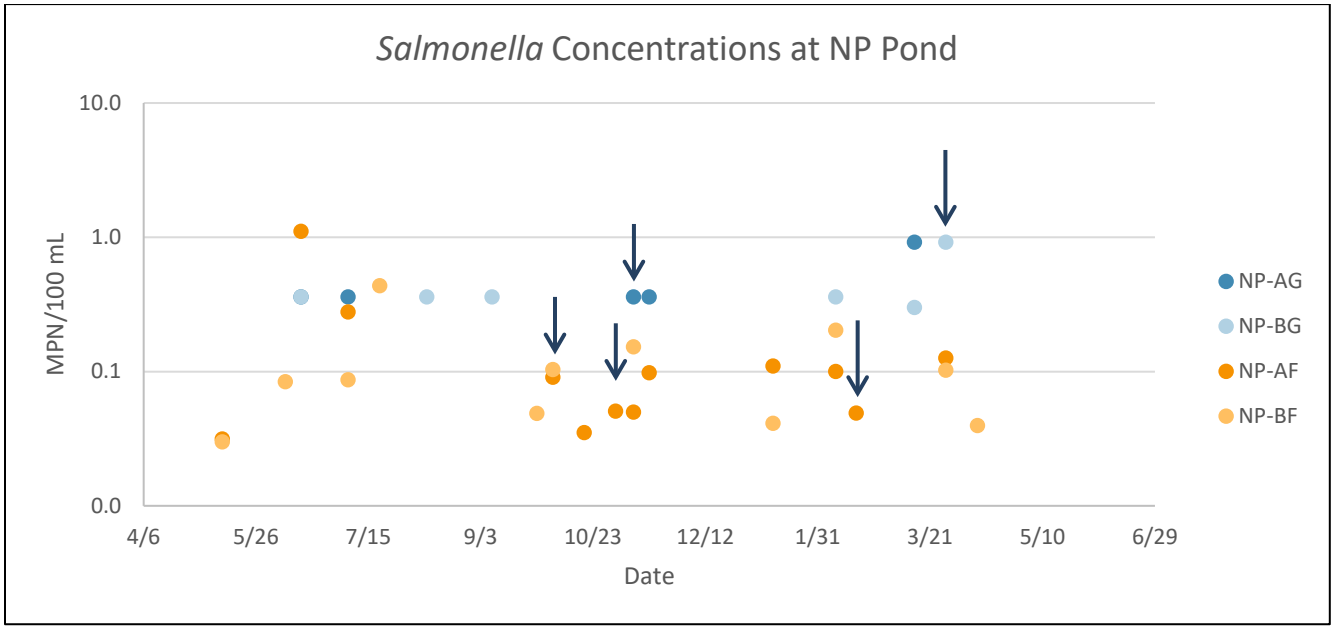
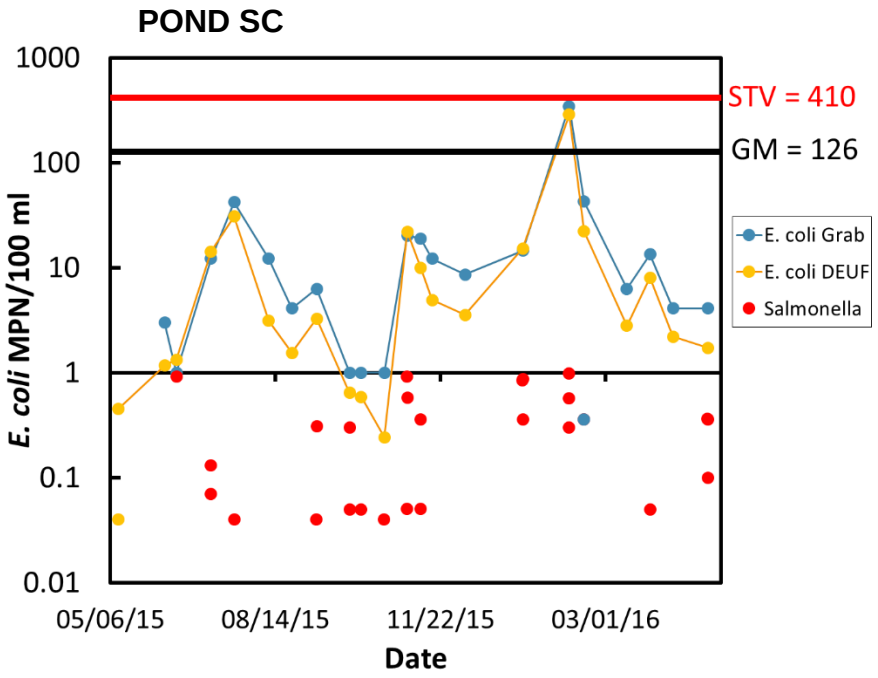
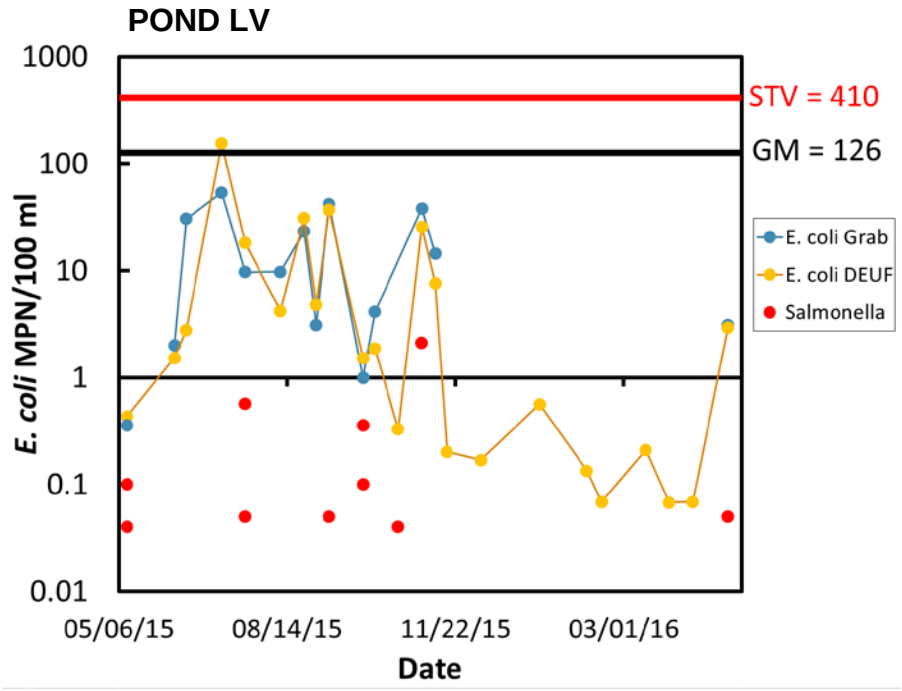


Figure 4. *Salmonella* concentrations at ponds SC, LV, and NP (data only shown for detections). Blue circles are for small-volume grab samples collected from two locations (A and B) in the pond. Orange circles are for large-volume DEUF samples collected from these same locations. Blue arrows denote rain events.


POND SC
1000
100
10
1
0.1
0.01
E. coli MPN/100 ml
STV = 410
GM = 126
E. coli Grab
E. coli DEUF
Salmonella
05/06/15
08/14/15
11/22/15
03/01/16
Date


POND LV
1000
100
10
1
0.1
0.01
E. coli MPN/100 ml
STV = 410
GM = 126
E. coli Grab
E. coli DEUF
Salmonella
05/06/15
08/14/15
11/22/15
03/01/16
Date

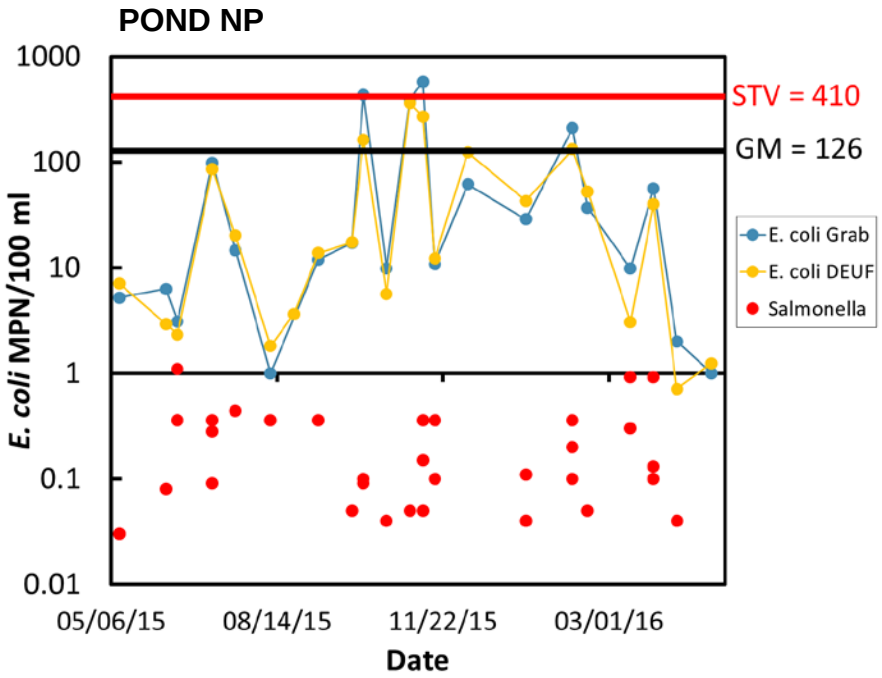


Figure 5. *E. coli* and *Salmonella* concentrations at ponds SC, LV, and NP (data only shown for detections). Blue circles are for small-volume grab samples, and orange circles are for large-volume DEUF samples collected from these same locations. Red circles show *Salmonella* detections (grab or DEUF). The new FSMA standard statistical threshold value (STV) and geometric mean (GM) limit are shown as red and black lines, respectively.

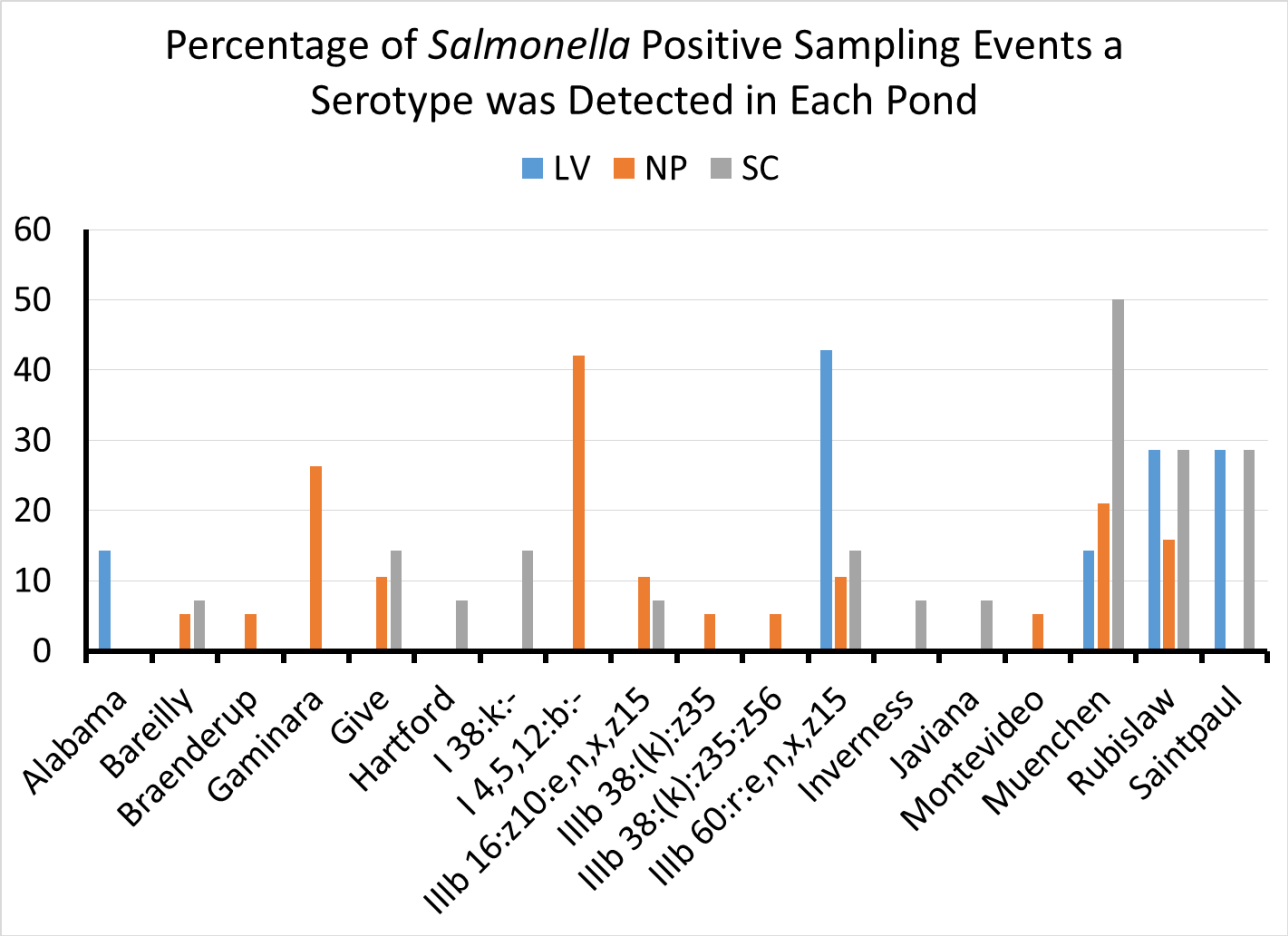


Figure 6. The percentage of *Salmonella*-positive sampling events in each pond in which a serotype was detected. There were 14 positive sampling events in pond SC, 7 positive sampling events in pond LV, and 19 positive sampling events in pond NP. A sampling event was positive for *Salmonella* if any of the samples collected at each sampling event had detectable *Salmonella*: grab or DEUF collected from either site A or B.

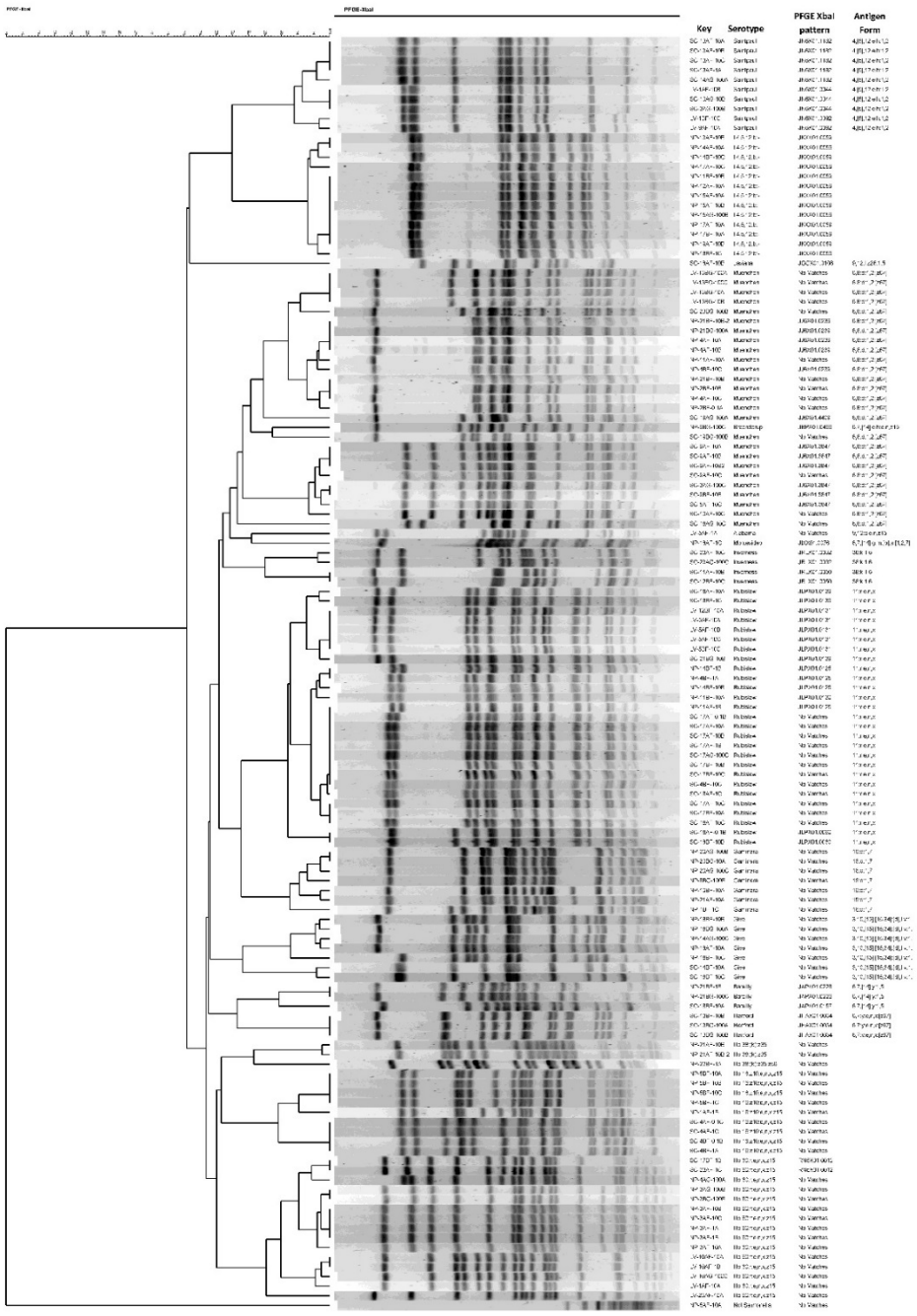


Figure 7. PFGE patterns and the corresponding dendrogram for the 131 *Salmonella enterica* isolates collected from irrigation ponds on three produce farms in southern Georgia. Isolate name (pond-sample event, sample location, sample method-MPN dilution, MPN replicate) and associated antigen-based serotype are listed, as well as the antigen form of the serotype.

References cited:

1. Allwood, P.B., et al., *Survival of F-specific RNA coliphage, feline calicivirus, and Escherichia coli in water: a comparative study.* Applied and Environmental Microbiology, 2003. **69**(9): p. 5707-5710.
2. *Phage Ecology*. Wiley Series in Ecological and Applied Micobiology, ed. R. Mitchell. 1987, New York.
3. Hill, V.R., et al., *Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples.* Applied and Environmental Microbiology, 2007. **73**(13): p. 4218-4225.
4. Hill, V.R., et al., *Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants.* Applied and Environmental Microbiology, 2005. **71**(11): p. 6878-6884.
5. Mull, B. and V.R. Hill, *Recovery and Detection of Escherichia coli O157:H7 in Surface Water, Using Ultrafiltration and Real-Time PCR.* Applied and Environmental Microbiology, 2009. **75**(11): p. 3593-3597.
6. Gibson, K.E. and K.J. Schwab, *Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water.* Appl. Environ. Microbiol., 2011. **77**(1): p. 385-391.
7. Smith, C.M. and V.R. Hill, *Dead-End Hollow-Fiber Ultrafiltration for Recovery of Diverse Microbes from Water.* Applied and Environmental Microbiology, 2009. **75**(16): p. 5284-5289.
8. Wilczynski, J.A., et al., *Outbreak of cryptosporidiosis associated with a firefighting response—Indiana and Michigan.* MMWR, 2012. **61**(9): p. 153-156.
9. Hill, V.R., et al., *Toxigenic Vibrio cholerae O1 in Water and Seafood, Haiti.* Emerging Infectious Diseases, 2011. **17**(11): p. 2147-2150.
10. O'Reilly, C.E., et al., *A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004.* Clinical Infectious Diseases, 2007. **44**(4): p. 506-512.
11. Hill, V.R., et al., *Detection of GI and GII Noroviruses in Ground Water Using Ultrafiltration and TaqMan Real-time RT-PCR.* Food and Environmental Virology, 2010. **2**(4): p. 218-224.
12. Polaczyk, A.L., et al., *Ultrafiltration-based techniques for rapid and simultaneous concentration of multiple microbe classes from 100-L tap water samples.* Journal of Microbiological Methods, 2008. **73**(2): p. 92-99.
13. Mull, B. and V.R. Hill, *Recovery of diverse microbes in high turbidity surface water samples using dead-end ultrafiltration.* Journal of Microbiological Methods, 2012. **91**(3): p. 429-433.
14. Gu, G., et al., *Factors affecting the occurrence of Escherichia coli O157 contamination in irrigation ponds on produce farms in the Suwannee River Watershed.* Canadian Journal of Microbiology, 2012: p. 1-8.
15. McQuaig, S.M., et al., *Quantification of Human Polyomaviruses JC Virus and BK Virus by TaqMan Quantitative PCR and Comparison to Other Water Quality Indicators in Water and Fecal Samples.* Applied and Environmental Microbiology, 2009. **75**(11): p. 3379-3388.
16. Haugland, R.A., et al., *Evaluation of genetic markers from the 16S rRNA gene V2 region for use in quantitative detection of selected Bacteroidales species and human fecal waste by qPCR.* Systematic and Applied Microbiology, 2010. **33**(6): p. 348-357.
17. USEPA, *Method 1623.1: Cryptosporidium and Giardia in Water by Filtration/IMS/FA*. 2012, Office of Water: Washington, D.C. p. EPA 816-R-12-001.
18. Hill, V., et al., *Development of a nucleic acid extraction procedure for simultaneous recovery of DNA and RNA from diverse microbes in water.* Pathogens, 2015. **4**: p. 335-354.

19. Qvarnstrom, Y., et al., *Improved Molecular Detection of Cyclospora cayetanensis in Human Stool Specimens using UNEX DNA extraction and real-time PCR.* J Clin Microbiol, 2017. **Submitted**.
20. Hill, V.R. and M.D. Sobsey, *Removal of Salmonella and microbial indicators in constructed wetlands treating swine wastewater.* Water Science and Technology, 2001. **44**(11-12): p. 215-222.
21. Kahler, A.M., et al., *Evaluation of an ultrafiltration-based procedure for simultaneous recovery of diverse microbes in source waters.* Water, 2015. **7**: p. 1202-1216.
22. Haley, B.J., D.J. Cole, and E.K. Lipp, *Distribution, Diversity, and Seasonality of Waterborne Salmonellae in a Rural Watershed.* Applied and Environmental Microbiology, 2009. **75**(5): p. 1248-1255.
23. Yoder, J.S., et al., *Cryptosporidiosis surveillance--United States, 2009-2010.* MMWR Surveill Summ, 2012. **61**(5): p. 1-12.
24. USEPA, *Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure*. 2001, Office of Water: Washington, D.C. p. EPA 821-R-01-029.
25. Jothikumar, N., et al., *Detection and differentiation of Cryptosporidium hominis and Cryptosporidium parvum by dual TaqMan assays.* Journal of Medical Microbiology, 2008. **57**(9): p. 1099-1105.
26. Johnston, C., et al., *A real-time qPCR assay for the detection of the nifH gene of Methanobrevibacter smithii, a potential indicator of sewage pollution.* Journal of Applied Microbiology, 2010. **109**(6): p. 1946-1956.
27. Schill, W.B. and M.V. Mathes, *Real-time PCR detection and quantification of nine potential sources of fecal contamination by analysis of mitochondrial cytochrome b targets.* Environmental Science & Technology, 2008. **42**(14): p. 5229-5234.
28. (APHA), A.P.H.A., *Standard Methods for the Examination of Water & Wastewater*. 21 ed, ed. A.W.W.A. American Public Health Association, Water Environment Federation. 2005, Washington, DC: American Publich Health Association.
29. Templeton, M.R., R.C. Andrews, and R. Hofmann, *Particle-Associated Viruses in Water: Impacts of Disinfection Processes.* Critical Reviews in Environmental Science and Technology, 2008. **38**: p. 137-164.
30. Centers for Disase Control and Prevention. *Foodborne Outbreak Online Database*. 2016 January 25, 2017]; Available from: https://www.cdc.gov/foodnet/reports/data/incidence-trends.html.
31. Ribot, E.M., et al., *Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet.* Foodborne Pathogen and Disease, 2006. **3**(1): p. 59-67.