

VALIDATION OF AGRICULTURAL WATER AND ANIMAL INTRUSION METRICS FOR
PRODUCE SAFETY IN FLORIDA



By

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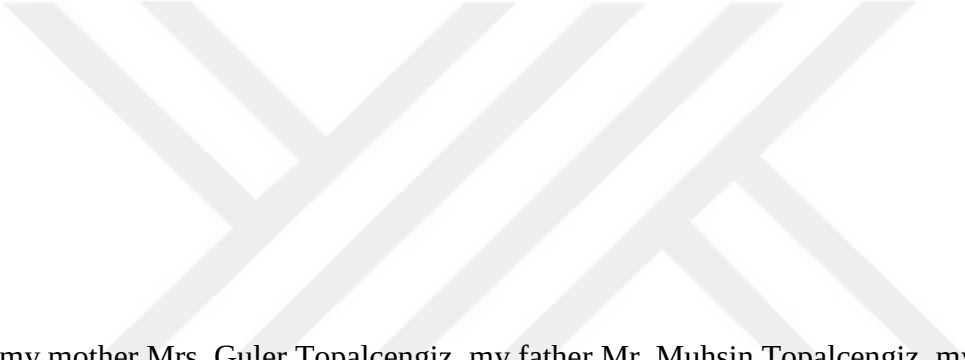
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To my mother Mrs. Guler Topalcengiz, my father Mr. Muhsin Topalcengiz, my brother, Mr. Muharrem Yekta Topalcengiz and my lovely nephew, Mr. Hasan Topalcengiz, for their endless support

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LIST OF ABBREVIATIONS

APHA	American Public Health Association
BSA	Bismuth sulfite agar
BSAR	Bismuth sulfite agar with rifampicin
CDC	Centers for Disease Control
CFU	Colony forming unit
CYC	Cycloheximide
DI	Deionized
<i>E.</i>	<i>Escherichia</i>
EPA	Environmental Protection Agency
FAWN	Florida Automated Weather Network
FDA-BAM	Food and Drug Administration – Bacteriological Analytical Manual
FSMA	Food Safety Modernization Act
GAPs	Good Agricultural Practices
GCREC	Gulf Coast Research and Education Center
GM	Geometric mean
H	Flagellar antigen
HACCP	Hazard Analysis Critical Control Point
HE	Hektoen enteric agar
HER	Hektoen enteric agar with rifampicin
LGMA	Leafy Greens Marketing Agreement
LIA	Lysine iron agar
mPCR	Multiplex polymerase chain reaction
mBPWp	Modified buffered peptone water with pyruvate
MDP	Mulch distant to tomato plant

MPN	Most probable number
MUP	Mulch under tomato plant
NFREC	North Florida Suwannee Research and Education Center
NVSL	National Veterinary Services Laboratory
O	Somatic antigen
ORP	Oxidation reduction potential
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RV	Rappaport-Vassiliadis
S.	<i>Salmonella</i>
spp.	species (pulural)
SMAC	Sorbitol MacConkey agar
STEC	Shiga-toxigenic <i>Escherichia coli</i>
STV	Standard threshold value
T-GAPs	Tomato – Good Agricultural Practices
TIP	Tomato inside of plant
TOP	Tomato outside of plant
TSA	Tryptic soy agar
TSAR	Tryptic soy agar supplemented with rifampicin
TSAN	Tryptic soy agar supplemented with nalidixic acid
TSB	Tryptic soy broth
TSBR	Tryptic soy broth supplemented with rifampicin
TSBN	Tryptic soy broth supplemented with nalidixic acid
TSI	Triple sugar iron agar
TT	Tetrathionate

UF/IFAS	University of Florida/Institute of Food and Agricultural Sciences
USA	United States of America
USDA-ERS	United States Department of Agriculture: Economic Research Service
USDA-NASS	United States Department of Agriculture-National Agricultural Statistical Service
MWQP	Microbiological Water Quality Profile
XLD	Xylose lysine desoxycholate agar
XLDR	Xylose lysine desoxycholate agar with rifampicin
XLT4	Xylose-lysine-Tegertol4

Abstract of Dissertation Presented to the Graduate School
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VALIDATION OF AGRICULTURAL WATER AND ANIMAL INTRUSION METRICS FOR
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Agricultural water quality and animal intrusion are substantial food safety concern in produce fields. Validation of metrics for agricultural water and animal intrusion is essential for produce safety in Florida.

Water samples from six agricultural ponds were collected during two consecutive growing seasons (540 samples). Microbial indicator populations (total coliforms, generic *Escherichia coli*, and Enterococci) were enumerated. A microbial water quality profile (MWQP) was established for all ponds. Water (150 mL) was filtered for pathogen analysis by traditional and multiplex PCR. *Salmonella* isolates were serotyped. All ponds met the current FDA MWQP recommendations 100.0% of the time. The *invA* gene (*Salmonella*) was detected in 26/540 (4.8 %) samples, in all ponds and growing seasons. Fourteen different *Salmonella* serotypes were detected. Six STEC genes were detected: *hly*-83.3%, *fliC*-51.8%, *eaeA*-17.4%, *rfbE*-17.4%, *stx-I*-32.6% *stx-II*-9.4%.

A STEC cocktail and generic *E. coli* were inoculated into 100 mL EPA Worst Case water, sterile, and non-sterile surface water at 15°C and 25°C. At day 168, *E. coli* populations decreased ≤ 0.5 Log CFU/mL in non-sterile surface water and were $2.8 \leq$ and ≤ 6.5 Log CFU/mL in sterile surface water and EPA Worst Case water.

Rifampicin resistant cocktails of STEC and *Salmonella* were inoculated into domestic cattle, pig, waterfowl, deer, and raccoon feces at room temperature. Models to predict STEC, *Salmonella* populations based on generic *E. coli* were developed. Over the 364 day storage, STEC and *Salmonella* populations declined by ≤ 2.4 Log CFU/g in the cattle and deer feces; declined to ≤ 2.6 Log CFU/g in pig, waterfowl, and raccoon feces over the first 56 days. The calculated predictive power (R^2) of the models relating pathogen declines to generic *E. coli* in each fecal type ranged from 0.4362 to 0.9774.

Fate of generic *E. coli* in cattle fecal pats under different tomato field conditions was evaluated, and *Salmonella* and STEC survival was predicted using the developed models. Generic *E. coli* concentration stayed high under field conditions for a week, and different conditions did not impact survival dramatically. The concentration differences between generic *E. coli* and predicted pathogen populations in fecal pats was less than Log CFU/g at each time interval.

CHAPTER 1 INTRODUCTION

Annual costs of illnesses related to the consumption of produce have reached almost \$39 billion in the U.S. (Scharff, 2010). In a report from the Centers for Disease Control and Prevention, number of foodborne disease outbreak-associated illnesses attributed to leafy vegetables is 49 bacterial, 150 viral, 4 parasites and 2 chemical and toxin between 1998 and 2008 (CDC, 2013). Shiga toxin-producing *Escherichia coli* (STEC) was leading bacterial agent with 22 outbreaks and 874 cases followed by 11 *Salmonella* outbreaks with 306 cases (CDC, 2013). The U.S. Food and Drug Administration (FDA) also announced that 131 outbreaks associated with contaminated produce occurred with the result of 14,132 illnesses, 1,360 hospitalization and 27 deaths between 1996 and 2010 (FDA, 2015). In 2006, multistate outbreak of *E. coli* O157:H7 associated with consumption of contaminated spinach resulted in total of 183 cases with 95 hospitalization and three deaths in two weeks (MMWR, 2006).

In 1998, FDA published a document entitled “Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables”, shortly named Good Agricultural Practices (GAPs) guidance (FDA, 1998). GAPs have generally been adopted as safety procedures by the produce industry (FDA, 2010), and particularly by the leafy green and tomato industries to minimize microbial food safety hazards from pre-harvest to post harvest. In Florida, Tomato-GAPs (T-GAPs) are required for all tomato producers, packers and re-packers under Florida Rule 5G-6 (FAC&FAR, 2008). The California Leafy Greens Marketing Agreement (LGMA), a mandatory audit program, was established to ensure the safety of lettuce and leafy greens (Villaneva, 2011). LGMA program certifies member companies for implementing GAPs and getting audited by a third party.

The U.S. Congress authorized FDA to set up minimum safety standards for production and harvesting of fruit and vegetables that may be consumed without any further processing (Harris, et al., 2012). FDA (2015) proposed a produce safety rule under Food Safety Modernization Act (FSMA). Science based standards to minimize the risk of outbreaks related to the consumption of fruit and vegetable have been proposed in this rule. FDA (2015) proposed safety standards regarding the microbial contamination risk routes for produce under the name of five general topics including agricultural water, biological soil amendments, worker training and health and hygiene, domesticated and wild animals, and lastly equipment, tools and buildings. Soil amendments, irrigation water, intrusion by farm and wild animals are major pre-harvest contamination risks in produce safety.

Direct evidence of agricultural water causing foodborne outbreaks is infrequent; surface irrigation water has been implicated as the sources of pathogen contamination with confirmed cases (Pachepsky et al., 2011). The activities of domestic and wild animals are also a substantial food safety concern in produce fields. *Salmonella* Newport isolated from pond water used for irrigation of tomatoes led to a multistate outbreak (Greene et. al., 2008). An outbreak of *Escherichia coli* O157:H7 due to accidental contamination of well water for irrigation with water from lagoon used by dairy was investigated by the California Food Emergency Response Team (CalFERT, 2008). In 2006 multistate outbreak of *E. coli* O157:H7 associated with consumption of contaminated spinach, it was concluded that pathogen in surface water reached wells on the ranch and caused the contamination. The outbreak strain was found in cattle and wild pig feces that could have been possibly introduced the river close to well. Contaminated water from San Benito River recharged the well since water table level in the well dropped during late growing season (CalFERT, 2007).

The safety of agricultural water is important due to high contamination potential at all produce production levels. The produce safety final rule was published with new agricultural water quality criteria under FSMA on November 13, 2015. A rolling system for calculation of a Microbiological Water Quality Profile (MWQP) standards is required for untreated agricultural surface waters. Water quality standards, geometric mean (GM) and a Statistical Threshold Value (STV), have remained the same and ≤ 126 *E. coli* and ≤ 410 *E. coli* in 100 mL water sample, respectively. In the final rule, development of the MWQP must be established with a baseline survey of a minimum of 20 water samples as close as practical to harvest in minimum two maximum four years. After the baseline survey, the MWQP must be verified with minimum of five sampling as close as practical to harvest, annually. Generic *E. coli* populations from five annual samples must be combined with the most recent 15 samples from previous MWQP to calculate a rolling GM and STV. The new calculated GM and STV values should be compared the agricultural water criteria for confirmation. For ground water, sampling must be done four times to develop MWQP, followed by one annual sampling for rolling GM and STV values to compare to standards.

Currently, LGMA (2013) requires agricultural water quality to meet populations of ≤ 126 MPN (or CFU)/100 mL generic *E. coli* (rolling geometric mean $n=5$) and ≤ 235 MPN/100 mL for any single sample for foliar and ≤ 126 MPN /100 mL (rolling geometric mean $n=5$) and ≤ 576 MPN/100 mL for any single sample for non-foliar systems. T-GAPs require that any foliar application of water to tomatoes at time of harvest shall meet the microbial standards for potable water. For non-foliar applications, T-GAPs adopted U.S. EPA recreational water quality metrics contained in 40 CFR Part 131.41(c) that *E. coli* should be ≤ 126 MPN (or CFU)/100 mL (rolling geometric mean $n=5$) and ≤ 235 MPN/100mL for any single sample. LGMA requires the testing

water prior to use; however, T-GAPs requires at least quarterly testing for surface waters. For animal intrusion, T-GAPs requires for exclusion of domestic animals and the minimization of wild animal intrusion. If contamination with animal/bird feces is identified, T-GAPs prohibits the harvesting of contaminated tomatoes and requires an assessment of potential impact of fecal material on field harvestability. LGMA (2013) requires monitoring of wildlife activity and application of remedial action in the case of intrusion. If fecal material is present in the field, a food safety assessment done by qualified personnel should be conducted and a no harvest zone should be established with a minimum 5-foot radius around fecal matter for all type of feces. Real life situations raise questions about the applicability and reliability of published pre-harvest safety standards. The diversity of agricultural water sources, water availability, type of crops and irrigation techniques are some of the important parameters that should be considered. Under FSMA, animal intrusion is considered a main route of contamination. FDA requires the monitor of animal activity and adequate waiting time until harvesting if animal intrusion occurs.

It is important to improve produce safety standards to minimize the risk of illnesses or deaths. Validation and development of standards are essential in many topics for the safe production and harvesting of produce. Important metrics have been proposed and used to minimize the potential risk of contamination to produce. However, the produce industry needs to validate the proposed metrics and evaluate more effective and cost effective approaches to increase microbiological safety, particularly, in regards to agricultural water quality, soil amendments, and intrusion by animals that are major pre-harvest contamination routes. The risk factors related to agricultural water quality and the presences of animal feces in Florida produce production are not well characterized.

The overall objective of this work is to validate agricultural water and animal intrusion safety metrics for produce safety in Florida. There are four main research objectives: (1) To identify and evaluate the influence of events that may result in the increase of episodic contamination of surface irrigation pond water; (2) to determine the fate of STEC and generic *E. coli* in Central Florida surface water under typical environmental conditions; (3) *to determine the* survival of different STEC and *Salmonella* serotypes in different wild animal feces that may potentially contaminate produce; (4) to evaluate the fate of generic *E. coli* and predict *Salmonella* and STEC survival in fecal pats under different field conditions in Florida.

CHAPTER 2

AGRICULTURAL WATER AND ANIMAL INTRUSION ASPECTS OF PRODUCE SAFETY: A LITERATURE REVIEW

Fresh fruits and vegetables have occupied a larger place on consumer plates in the U.S. for decades (Hoelzer et al., 2012). Consumption of fresh produce has increased due to studies regarding the health benefits of fresh products and health conscious consumers (Bassett and McClure, 2008; Hoelzer et al., 2012). Fresh market vegetable production reached a value of 11.4 billion dollars in 2013 according to United States Department of Agriculture, National Agricultural Statistical Service (USDA-NASS, 2014). The three largest produced commodities, onions, head lettuce, and watermelons, accounted for 36% of total production in 2013; however, tomato, head lettuce, and onions constituted the highest total value of 28% (USDA-NASS, 2014). Per capita consumption of fresh fruit and vegetables increased 67% from 1970 to 2008, and reached 180.5 lbs per year (USDA-ERS, 2008). As an expected result of massive production and high demand, the safety of fresh products has become a priority and a challenging task for everybody from production to consumption.

Annual costs of illnesses related to the consumption of produce have reached almost \$39 billion in the U.S. (Scharff, 2010). In a report from the Centers for Disease Control and Prevention, number of foodborne disease outbreak-associated illnesses attributed to leafy vegetables is 49 bacterial, 150 viral, 4 parasites and 2 chemical and toxin between 1998 and 2008 (CDC, 2013). Shiga toxin-producing *Escherichia coli* (STEC) was leading bacterial agent with 22 outbreaks and 874 cases followed by 11 *Salmonella* outbreaks with 306 cases (CDC, 2013). The U.S. Food and Drug Administration (FDA) also announced that 131 outbreaks associated with contaminated produce occurred with the result of 14,132 illnesses, 1,360 hospitalization and 27 deaths between 1996 and 2010 (FDA, 2013). In 2006, a multistate outbreak of *E. coli*

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In 1998, FDA published a document entitled “Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables”, shortly named Good Agricultural Practices (GAPs) guidance (FDA, 1998). GAPs have generally been adopted as safety procedures by the produce industry (FDA, 2010), and particularly by the leafy green and tomato industries to minimize microbial food safety hazards from pre-harvest to post harvest. In Florida, Tomato-GAPs (T-GAPs) are required for all tomato producers, packers and re-packers under Florida Rule 5G-6 (FAC&FAR, 2008). The California Leafy Greens Marketing Agreement (LGMA), a mandatory audit program, was established to ensure the safety of lettuce and leafy greens (Villaneva, 2011). LGMA program certifies member companies for implementing GAPs and getting audited by a third party.

On November 13, 2015, FDA published the final Produce Safety Rule under Food Safety Modernization Act (FSMA). In this rule, science based standards from pre-harvest to post-harvest have been proposed to minimize the risk of pathogen contamination and related outbreaks due to the consumption of fruit and vegetable. FDA (2015) covers safety standards regarding the microbial contamination risk routes for produce under five general topics including agricultural water, biological soil amendments, worker training and health and hygiene, domesticated and wild animals, and lastly equipment, tools and buildings.

Soil amendments, irrigation water, intrusion by domestic and wild animals are pre-harvest contamination routes of contamination in produce fields (FDA, 2015). Leafy greens and tomatoes are high risk products since they are consumed raw most of the time. In this review, field practices during tomato and leafy green production and produce related outbreaks will be

summarized. Agricultural water and animal intrusion will be explained as focus of two main contamination routes of produce in pre-harvest level. This review will give details about outbreaks and the current regulation linked to agricultural water and animal intrusion. Interactions of enteric pathogenic bacteria with leafy greens and tomatoes will be discussed. The behavior, survival, and occurrence of indicator microorganisms and pathogens in agricultural water and animal feces will be explored. Current safety requirements and mitigation strategies will be covered at state and nationwide level.

Field Practices during Leafy Greens and Tomato Production

The California Leafy Green Marketing Agreement (LGMA, 2013) covers fourteen types of leafy greens including arugula, baby leaf lettuce, butter lettuce, cabbage, chard, endive, escarole, green leaf lettuce, iceberg lettuce, kale, red leaf lettuce, romaine lettuce, spinach and spring mix. Three lettuce crops, head, leaf, and romaine, constitute the majority of the leafy green production in the U.S., followed by spinach and cabbage. According to USDA-NASS (2013), the total value of lettuce production was \$1.87B in 2012 in the U.S. California and Arizona are by far the leading states producing leafy greens, particularly head, leaf and romaine lettuce (USDA-NASS, 2014). Florida has a growing leafy green industry. Lettuce, spinach, endive and escarole are some of the leafy greens commercially produced in Florida (Ozores-Hampton et al., 2013). The production of leafy greens is expected to increase significantly to meet high demand.

Leafy greens and tomatoes are widely produced commodities used in home kitchens and large food service establishments all around the world. In the fresh market, they have an important place in terms of production volume and value. Improvements in marketing facilities, longer shelf life, and gradual constant increase in production have met high demand for fresh

produce. Optimal leafy green and tomato production practices have been developed, modified and performed for greater yield and safer products in different locations throughout the world.

Leafy green production practices in the field are modified according to type of leafy green. Since lettuce represents the majority of demanded leafy greens, head, leaf and romaine lettuce production practices have been improved significantly due to achieve higher yields in smaller fields. In California, lettuce is considered as cool season crop with 23°C optimal growing temperature (Turini et al., 2011). High or low temperatures may result in defective products. California and Florida are similar in terms of planting time of lettuce. Planting and harvesting are completed mainly during cool season depending on the region of states and type of lettuce (Turini et al., 2011; Ozoires-Hampton et al., 2013). Growers plants total of 388,000 seed/ha pelleted lettuce seeds with a distance of 1.75 to 3 inch from each other in California (Turini et al., 2011). In Florida, similar practices are applied; however, transplantation is more common. Both seeded and transplanted plants are irrigated with a sprinkler system every 2 to 3 days until seedlings emerge (Stephen, 2004; Turini et al., 2011). After three weeks, plants are thinned when the height of plants is about 3 inches to provide enough space, which is 8 to 12 inches wide for big plants (Stephen, 2004). Leafy greens are primarily hand-harvested; some mechanical harvesting systems have been developed, especially for baby leaf products. Field packing is common for all leafy greens excluding Brussel sprouts and baby leaf; outer leaves of lettuce are stripped from the head during harvest and field packing (Cantwell and Kasmire, 2011). The primary reasons for field packing product is to reduce transportation time, contamination risks during postharvest practices and to have more profitable marketing goods by reducing mechanical damage. If not distributed as whole heads, lettuce heads can be cooled, wrapped, washed, cored, shredded, cut, and packed as fresh cut or ready-to-eat products.

Tomatoes, originate from the West coast of South America, were first domesticated in Mexico (Olson et al., 2010). Thousands of tomato varieties have been developed for better adaptation and yield potential, disease resistance, and consumer preferences. Commercially, tomato varieties are sold under four type based on shape including round, cherry, grape, and Roma. Fresh-market tomato production is concentrated in the southeastern states of the U.S.; California, and Ohio are also main tomato producers (USDA-ERS, 2016). In 2012, Fresh-Market tomato production reached to 2.76 million lbs in the U.S. with a value of \$0.86 billion, in the leading of two states: California and Florida (USDA-ERS, 2013). Seasonality distinguishes these two states from other tomato producing states, and production continues almost all year long.

Tomato production practices in the field may differ depending on weather conditions, technological capabilities, and the availability of water and fertile lands (Sims, 1980). California and Florida are leading tomato producers due to natural and technological advantages (USDA-ERS, 2015). Tomato production may start from direct seeding or transplantation. In California most tomato plants are direct seeded in to raised beds, whereas they are transplanted in Florida (Gould, 1992; Estabrook, 2011). Tomatoes are grown throughout the Florida, but production is concentrated in areas located in Central and South part of state. Tomatoes are a transplanted twice a year, typically in February and August in Central Florida (Santos et al., 2013). Tomato beds are typically 36-40 in wide and 6 to 8 in high in Miami-Dade County, Florida (Li et al., 2009). To reduce the evaporation and weed growth, transplantation beds are covered with black or bright color polyethylene mulch depending on the soil temperature regime of area. Drip irrigation tube establishment and fumigation are done right before mulch coverage of beds (Li et al., 2009). Tomato plants are attached to the stakes to avoid fruits contacting to ground (Hedberg et al., 1999). Tomatoes are harvested at the mature green stage, and time of the day is critically

important for harvesting. The amount of condensation and weather events should be followed, carefully. Fruits should be dry during harvesting to increase shelf-life. Mature green tomatoes are mostly harvested for market mechanically in California, by hand in Florida (Estabrook, 2011). Hand-picked tomatoes are typically transferred to 1,200 lbs holding plastic bins from plastic picking buckets holding 40 to 50 lbs of tomato (Felkey et al., 2006). Finally, tomatoes are transferred to packinghouses for further processes including washing, waxing, sorting, packing, and shipping to retailers.

The fresh produce market has an important impact on the Florida economy. The safety of products from the field to the final consumer should be managed and regulated carefully. Agricultural practices for tomatoes and leafy greens have some similarities and differences. Produces without any killing step during production and packed in the field has to maintain at least the minimum safety standard to avoid outbreaks that threaten people's lives and cause devastating economic impacts. Agricultural practices may change according to region, technology, and grower; however, the maximum yield, high quality, and safe products are aimed from all producers.

Leafy Greens and Tomato Related Outbreaks

A large number of agents, including bacteria, parasites, viruses, have been implicated in produce related outbreaks. For a long time, bacterial agents were considered as the primary cause of foodborne outbreaks (Bean et al., 1990; Mead et al., 1999). However, Scallan et al., (2011) estimated that 58% of foodborne illnesses is caused by Norovirus in the U.S., and followed by *Salmonella* spp. 11%. In the same study, non-typhoidal *Salmonella* spp., is listed as the leading causative agent of hospitalization and the cause of death associated with foodborne illnesses. In a comprehensive review regarding outbreaks associated with fresh produce, some of the primary bacterial agents are noted as *Campylobacter* spp., *Clostridium botulinum*, *Escherichia coli*

O157:H7, enteropathogenic *E. coli*, *Salmonella enterica*., *Shigella* spp., *Listeria monocytogenes* (Harris et al., 2003). *Cryptosporidium* spp., and *Cyclospora* spp. were listed as some of the parasites, and Hepatitis A and Norovirus virus were indicated as the most common viral agents found in fresh produce (Harris et al., 2003). All of these pathogens originate from diverse environmental and enteric sources. Enteric bacterial pathogens, *Salmonella* spp., and STEC causing the bloody diarrhea (hemolytic uremic syndrome), are considered in high importance because of the severity of illnesses, rapid growth, long survival, and internalization abilities on and in fresh produce.

Fresh produce related outbreaks have forced the recognition of the potential safety concerns associated with commodities consumed raw or minimally processed from farm to fork. From 1973 through 1997, the proportion of outbreaks associated with fresh produce amplified almost to 6% at all reported foodborne outbreaks (Sivapalasingam et al., 2004). A survey conducted by the Center for Science in Public Interest (CSPI) reported that produce linked outbreaks accounted for 13% of total outbreaks between 1990 and 2005 (CSPI, 2005). Improvements in surveillance help to researchers to track the source of outbreaks better. However, epidemiological case studies and traceback procedures for a produce related outbreak is challenging task for investigators and officials. Time between harvesting and consumption can be very short depending on the type of produce. The complexity of distribution system, short shelf-life, consumer abuse increase time required for the recognition, investigation, and traceback of fresh produce related outbreaks.

Several factors can contribute the increase the number of produce related outbreaks, including increase in per capita consumption, importation of produce throughout the year due to high demand, additional and changed processing practices for preparation of fresh-cut products,

produce fields planted close to areas where farm and wild animal activities are observed (Lynch et al., 2009). Produce related outbreaks peaks during the hot season, possibly due to increase in consumption of fresh fruit and vegetables and more favorable conditions for growth and survival of foodborne pathogens (Harris et al., 2003; Hedberg et al., 1994). Fresh and fresh-cut produce has been well-recognized vehicle for foodborne pathogens for decades.

Outbreaks associated with the consumption of leafy greens constitute the majority of produce related outbreaks. Painter et al., (2013) estimated that 45.9% of foodborne illnesses are attributed to domestically acquired produce annually, and leafy greens are responsible for almost half of the produce related outbreaks between 1998 and 2008 in the U.S. based on recent reports submitted to CDC through the Foodborne Disease Outbreak Surveillance System. In this report, viral contamination (81%) was the primary microbiological cause of most leafy green related outbreaks, followed by bacterial (12%), and parasitic agents (7%). Although the LGMA covers fourteen types of leafy greens as commercial, the majority of leafy green linked outbreaks are attributed to contaminated lettuce proportional to lettuce consumption rates in all type of leafy greens. An outbreak of *E. coli* O157:H7 infection associated with leaf lettuce consumption affected 40 residents in Montana with laboratory confirmation and 52 bloody diarrhea without laboratory confirmation (Ackers et al., 1998). In 1996, a multistate outbreak of *E. coli* O157:H7, associated with the consumption of mesclun lettuce, occurred in Connecticut and Illinois, and lettuce samples were implicated to a single growing and processing facility with cattle near the fields (Hilborn et al., 1999). Two taco restaurant producing Mexican food were related to multi state *E. coli* O157:H7 outbreaks due to consumption of contaminated lettuce in the north-eastern and mid-western U.S. in 2006 with total of 152 cases (FDA, 2006; FDA 2007). One of these *E. coli* O157:H7 outbreak in a Taco restaurant was summarized in the final report by the California

Food Emergency Response Team, indicating that accidental contamination of well water for irrigation with water from lagoon used by dairy was the source of contamination in iceberg lettuce (CalFERT, 2008). Another multistate *E. coli* O157:H7 outbreak in 2011 was linked to the consumption of romaine lettuce, and caused 58 cases in 10 states with 67% hospitalization and 6.4% hemolytic uremic syndrome development in 2011 (Slayton, et al., 2013). In 2000, lettuce was implicated as the vehicle for a national outbreak of *Salmonella enterica* serotype Typhimurium definitive phage type (DT) 104 with 361 cases in England (Horby et al., 2003). The source of contamination was never identified due to lack of identifying markers on salad and the complexity of food chain. A multi-national *Shigella sonnei* outbreak associated with the consumption of iceberg lettuce occurred in Europe in 1994 (Kapperud et al., 1995). In this outbreak, investigators suggested three potential routes of contamination: irrigation with incompletely treated sewage effluent or polluted drinking water, the use of sewage sludge or manure contaminated with human feces, and accidental contamination due to runoff (Kapperud et al., 1995). Sivapalasingam et al., (2004) identify there were 17 different reported lettuce associated outbreaks, with known pathogens including Norovirus, *Giardia*, *Shigella* spp., Hepatitis A, *Salmonella enterica* spp., *E. coli* O157:H7, *Cyclospora*, in the U.S. between 1973 and 1997.

Spinach is second among the leafy greens in terms of the quantity of consumption. In 2006, a multistate outbreak of *E. coli* O157:H7 was associated with consumption of contaminated spinach, and a total of 205 confirmed illnesses and three deaths were reported from 26 states (FDA, 2007). A subsequent outbreak of *E. coli* O157:H7 was linked to pre-packed organic spinach and spring mix blend in 2012 with a total of 33 infected individuals in five states in the northeastern U.S. The source of contamination was not identified during the FDA

investigation (CDC, 2012). A list of worldwide outbreak investigations associated with leafy greens and herbs from 2000 to 2011 can be found in a project website¹ under the title of “A systems approach to minimize *E. coli* O157:H7 food safety hazards associated with fresh- and fresh-cut leafy greens”.

Tomatoes and complex foods including tomatoes have been associated with primarily outbreaks of *Salmonella enterica* spp. Valadez et al., (2012) report all tomato related outbreaks between 1990 and 2009 have been caused by Hepatitis A (1 outbreak) and *Salmonella enterica* spp. (11 outbreaks) including *S. Javiana*, *S. Montevideo*, *S. Newport*, *S. Braenderup*, *S. Anatum*, *S. Munchen*, *S. Typhimurium*, *S. Thompson*, *S. Berta*, and *S. Saintpaul*. Shiga-toxin producing *E. coli* and *Listeria monocytogenes* have not been linked to directly any tomato related outbreaks (Beuchat and Brackett, 1991; Valadez et al., 2012). Two outbreaks of *Salmonella* (*S. Javiana*-1990 and *S. Montevideo*-1993), associated with the consumption of fresh tomatoes, resulted in total of 276 illnesses in Midwestern states including Minnesota, Illinois, Michigan, and Wisconsin (Hedberg et al., 1999). Investigations demonstrated that both of these *Salmonella* outbreaks came from a single tomato packinghouse located in South Carolina, and a common dump tank for field grown tomatoes was the source of contamination (Hedberg et al., 1999). Another multistate raw tomato associated outbreak traced back to two tomato grower/packer cooperatives caused by *Salmonella* Baildon, led to 86 illnesses with three deaths in eight states from December 1998 to January 1999 (Cummings et al., 2001). In 2006, two tomato fields in Florida were implicated in outbreak of *S. Braenderup*. The environmental investigations reported that potential animal reservoirs of *Salmonella* were observed in and around drainage ditches and these animals were tested positive for *Salmonella*, but different strains from outbreak strain

¹<http://www.ugacfs.org/producesafety/Pages/Basics/WorldIllnesses.html>

(CDC, 2007). Two *Salmonella* outbreaks caused by serotypes Typhimurium and Berta were linked to raw tomato consumption with total of 24 cases (CDC, 2011). Finally, *S. Saintpaul* outbreak was linked to consumption of raw tomatoes with 21 confirmed cases in 2009 (CDC, 2011).

Two outbreaks of *Salmonella* serotype Newport originated from the eastern shore of Virginia in 2002 and 2005. The strain of *S. Newport* that was isolated from an irrigation pond and strains from both outbreaks had the same pulsed-field gel electrophoresis (PFGE) pattern (CDC, 2007; Greene et al., 2008). During the 2005 investigation, it was observed that the irrigation pond water was released in the soil bed and was not in contact with fruit. One year later, in 2006, environmental inspections for the 2005 outbreak around the tomato fields resulted in the isolation of *S. Javiana* and a different strain of *S. Newport* (Greene et al., 2008). High frequency of animal activity, including geese and turtles were observed in the ponds. In 2006, another multistate outbreak of *S. Newport* associated with raw tomatoes occurred mostly in eastern part of the U.S., but the source of implicated tomatoes was never identified (CDC, 2007). In this outbreak, a total of 115 culture-confirmed *S. Newport* isolates with indistinguishable PFGE patterns were obtained from stool specimens and the PFGE pattern matched with the pattern of *S. Newport* from 2005 outbreak (CDC, 2007).

Interactions of Enteric Pathogenic Bacteria with Leafy Greens and Tomatoes

To cause illness, pathogens have to survive all environmental challenges and agricultural practices in the field, sanitation and processing procedures in packing facilities, gastrointestinal tract, and all defense mechanisms of the human body. In the field, enteric pathogens have to deal with extreme physicochemical conditions and diverse microbial community on raw fruit and vegetables. The complexity of microbial ecosystem on plant surfaces depend on the type of produce, agricultural practices, weather conditions, geographical factors in production area,

differences in plant surface morphology, and metabolic functions of plant (Burnett and Beuchat, 2001). Limited nutrient availability and ultraviolet protection reduce the growth or survival chance of pathogens on plant surface (Brandl, 2006). Most aerial plant surfaces are covered with a hydrophobic material called cuticle, which protect them from dehydration and microbial invasion (Yeats and Rose, 2013). Once human pathogens are introduced to a plant surface from fecal material or contaminated agricultural water, they may contact microenvironments on plant surface in which conditions are more favorable for them (Brandl, 2006). After contacting to plant surface, enteric pathogens can attach plant surface, form biofilm, and internalize to survive and proliferate.

Attachment and adhesion of foodborne pathogens to host and plant environments are very important features for survival until they reach primary host. When bacteria run into plant surface, weak, reversible and unspecific initial adhesion finishes in a few seconds depending on physical factors (hydrophobicity, charge, etc.), followed by strong irreversible attachment (Dunne, 2002). Cell components, fimbriae, flagella, and polysaccharides are synthesized for this process (Dunne, 2002). *S. enterica* serovars, and *E. coli* serotypes grow on and adhered to alfalfa sprouts; however, *S. enterica* serovars adhered more strongly than *E. coli* O157:H7 after repeated washing steps (Barak et al., 2002). Different host and bacterial factors influence the attachment to plant surface. Beuchat (1999) reported that irreversible *E. coli* attachment occurred to lettuce leaves from bovine feces after a few hours of application. *S. Agona* and *S. Arizonae* demonstrated less attachment than *S. Typhimurium* and *S. Senftenberg* on basil, spinach, or lettuce surfaces (Berger et al., 2009). Irreversible attachment of *Salmonella* and *E. coli* on green pepper in a short time was concluded in two different studies (Liao and Cooke, 2001; Han et al., 2000). Pathogens and other microorganisms preferred the base of trichomes, stomata, epidermal

cell wall junctions, grooves along veins, damaged plant tissues, and cut surfaces for colonization (Beattie and Lindow, 1999; Seo and Frank, 1999; Boyer et al., 2007). Attachment of enteric pathogens depends on environmental factors, plant type, and bacterial properties. The removal of bacteria with washing and dipping practices is practically difficult after attachment to plant surface (Castillo et al., 2014).

Development of viable and protective structures is important for microorganisms to complete their lifecycles. Attachment and adhesion of pathogens is followed by biofilm formation on plant surfaces. Harsh conditions on plant surface promote the biofilm formation. Lindow and Brandl (2005) estimated that biofilms on plant surface harbor 30 to 80% of the total bacterial population. Microorganisms can form biofilm or become entrapped in a biofilm (Annous et al., 2005). Biofilm producing serotypes of *Salmonella* have greater ability to attach produce and plant surfaces (Patel and Sharma, 2010; Cevallos-Cevallos et al, 2012). The sanitation practices are not effective once microorganisms get into the plant.

Internalization may occur during pre-harvest or postharvest. Some are the routes for internalization are the immersion of fruit into an inoculated solution, and entrance of bacteria through naturally open, damaged, or cut tissue (Yaron, 2014). Pre-harvest internalization has been studied with numerous aspect of plant growing since pre-harvest variables from plantation to harvesting are diverse and easy to manipulate. Internalization can start from seedling to mature plant. Several factors can affect the route of foodborne pathogen into produce including, type of pathogen and produce, and stage of plant or fruit development (Yaron, 2014). Aerial portion of plants, stoma, scar tissue, and wounds, are susceptible for contamination. For example, pathogens can colonize in stoma due to contamination from irrigation or rain drop splashes (Pachepsky et al., 2011). In another study, *E. coli* O157:H7 colonization was observed

around and inside of stoma (Jeter and Matthysee, 2005). Both *E. coli* O157:H7 and *Salmonella* cells were detected at after nine days in hydroponically grown lettuce and radish; however, *E. coli* O157:H7 and *Salmonella* did not internalize in cress and spinach (Jablasone et al., 2005). Solomon et al. (2002) observed that *E. coli* O157:H7 internalized in tissues of lettuce via roots after five days of high population pathogen exposure in contaminated irrigation water and manure slurry.

Attachment and biofilm formation are well studied abilities that help enteric pathogens to survive in harsh conditions different from human and animal intestine. The role of internalization is still controversial for pathogen survival. These survival capabilities of microorganisms indicate the importance of tomato and leafy green safety starting from field. The mechanisms and more products need to be explained clearly for the improvement of produce safety from farm to fork.

Agricultural Waters

The produce industry requires abundant, unpolluted, and sustainable water sources to provide safe products to the consumer. The primary water consumption occurs in the field during plant production in produce industry. Agricultural water refers to water that is used for all agricultural purposes including irrigation, frost protection, fertilizer and pesticide application, crop cooling, equipment cleaning, and removal of salt from soil (FDA, 1998). The source of agricultural water may vary for farmers depending on the availability of natural sources and the presence of water distribution canals. Water can be obtained from single source or multiple sources to supply fields. The use of surface water sources is seen for agricultural purpose depending on the area in Florida. Rivers, lakes, irrigation ponds fed with ground water, canals, and creeks are some of these surface water sources that are open to environmental impacts.

The safety of produce is directly related to agricultural water quality since it is considered as one of main vehicle for pathogen transmission at the pre-harvest level (FDA, 2015).

Contamination of agricultural water might occur due to natural and agricultural resources as wild animals and livestock farms. Direct evidence of agricultural water causing foodborne outbreaks is infrequent; surface irrigation water has been implicated as the sources of pathogen contamination with confirmed cases (Pachepsky et al., 2011). *Salmonella* Newport isolated from pond water used for irrigation of tomatoes lead to a multistate outbreak (Greene et. al., 2008). CalFERT (2008) investigated that well water used for irrigation was contaminated from a dairy lagoon accidentally and caused an outbreak of *E. coli* O157:H7. A multistate outbreak of *E. coli* O157:H7 resulting 205 confirmed cases and three deaths in 26 states occurred due to consumption of contaminated spinach (FDA, 2007). “No definitive determination could be made regarding how the *E. coli* O157:H7 pathogens contaminated spinach in this outbreak” was announced, however the same team concluded that causative agent could have reached the wells used for irrigation and caused contamination (CalFERT, 2007). The outbreak strain was found in cattle and wild pig feces that could have been possibly introduced the river close to well. Contaminated water from the San Benito River recharged the well since the water table level in well dropped during late growing season (CalFERT, 2007).

The safety of agricultural water is important due to high contamination potential at all production levels of produce. The Final Rule was published with new agricultural water quality criteria under FSMA on November 13, 2015 under Produce Safety Rule. Water quality requirements for agricultural waters under Produce Safety Rule required the establishment of a “microbial water quality profile (MWQP)” by sampling twenty times as close as practical to harvest for minimum two maximum four years and calculating a geometric mean and a

“statistical threshold value (STV)”. To meet the water quality standard, the geometric mean and STV should have been ≤ 126 *E. coli* and ≤ 410 *E. coli* in 100 mL water sample, respectively. This has been changed from the previous proposed rule where a grower would look a simple sample maximum and rolling geometric mean of 5 samples. In final rule, development of MWQP must be established with a baseline survey of a minimum of 20 water samples as close as practical to harvest in minimum of two and a maximum of four years. After the baseline survey, MWQP must be verified with minimum of five sampling as close as practical to harvest, annually. Generic *E. coli* populations from five annual samples must be combined with the most recent 15 samples from previous MWQP to calculate a rolling GM and STV. The new calculated GM and STV values should be compared the agricultural water criteria for confirmation. For ground water, sampling must be done four times to develop MWQP, followed by one annual sampling for rolling GM and STV values to compare to standards.

In the final rule, growers can still continue use their water sources even if water quality requirements are not met similar to supplemental notice published in 2014. There are three options for the water exceeding these numbers in proposed rule:

1. Pre-harvest interval: Application of time interval as days between last irrigation and harvest to reduce count into standards by assuming a die off rate of 0.5 log per day. Final rule allow maximum interval of four days pre-harvest (2 logs).
2. Harvest and storage interval: Application of time interval as days between harvest and of storage or microbial removal rates during treatments to reduce the counts into standards with a supported data as peer reviewed paper.
3. Discontinuation of water source.
4. Treatment of water.

Different standards are required by other organizations. These requirements may be more strict or easier to monitor. Currently, LGMA (2013) requires agricultural water quality to meet populations of ≤ 126 MPN (or CFU)/100 mL generic *E. coli* (rolling geometric mean $n=5$) and ≤ 235 MPN/100 mL for any single sample for foliar and ≤ 126 MPN /100 mL (rolling geometric

mean $n=5$) and ≤ 576 MPN/100 mL for any single sample for non-foliar systems. T-GAPs require that any foliar application of water to tomatoes at time of harvest shall meet the microbial standards for potable water. For non-foliar applications, T-GAPs adopted U.S. EPA recreational water quality metrics contained in 40 CFR Part 131.41(c) that *E. coli* should be ≤ 126 MPN (or CFU)/100 mL (rolling geometric mean $n=5$) and ≤ 235 MPN/100mL for any single sample. LGMA requires the testing water prior to use; however, T-GAPs ask for test at least quarterly for surface waters.

Indicator Microorganisms in Agricultural Waters

The monitoring of fecal indicator microorganisms is a commonly accepted method in produce safety; however, there is no ultimate indicator to assess potential pathogen risks in agricultural waters. Regardless, the microbial quality of water that comes into the edible portion of produce is commonly believed to directly relate to the safety of produce, and metrics describing indicator organisms are commonly used in the produce industry to ensure safety of a product. Indicator microorganisms indicate general fecal contamination and should not be used interchangeably with Index microorganism. Indicator microorganism exceeding specific limits does not represent the presence of pathogen; index microorganism shows the possible occurrence of ecologically similar pathogens (Cordier and ICMSF, 2013; Mossel et al., 1995). The intention for the use of index microorganism is to predict the presence of a specific pathogen, whereas the intention of the use of indicator microorganism is indirect prediction of pathogen presence due to fecal contamination (Cordier and ICMSF, 2013).

In general, bacterial indicators from fecal matter have been used and recommended to monitor fecal contamination in water sources. Difficulties, cost and time, in the detection of waterborne pathogens encourage the use of indicator microorganisms. There is not sufficient scientific evidence reporting that present standards are reflecting the presence and population of

enteric viruses and protozoan parasites in agricultural waters (Gerba and Rock, 2014). The World Health Organization (WHO) recommends the use of indicator microorganisms in guidelines for safe and recreational water environments that meet criteria including the presence and absence of indicator in correlation with pathogen of concern, not multiply in the environment, in response to environmental conditions as pathogen of concern but not a pathogen, easy to isolate, identify, enumerate and inexpensive (WHO, 2006). American Society for Microbiology (ASM) recommended to monitor generic *E. coli* as a primary microbial indicator (ASM, 2001). Ashbolt et al. (2001) listed the key fecal indicator microorganisms in WHO water quality guideline as coliforms, thermotolerant coliform, *E. coli*, fecal streptococci, enterococci, sulphite-reducing clostridia, *Clostridium perfringens*, bifidobacteria, bacteriophages, coliphages, *Bacterioides fragilis* bacteriophage. *E. coli*, fecal streptococci, enterococci, and total coliform are typically recommended as indicator microorganisms for fecal contamination and microbial water quality in surface waters (Steele et al., 2004).

Agricultural surface water quality is monitored less frequently than drinking and recreational waters in practice (Pachepsky et al., 2011). FDA (2015) requires the measurement of generic *E. coli* by looking at population in 100 mL (MPN/100 mL) water samples as indicator microorganisms in agricultural waters. Agricultural water quality shall meet the recommendations that were adopted from United States Environmental Protection Agency (EPA) recreational water standards (EPA, 2012).

Pathogens in Agricultural Waters

Common agricultural water sources are ground water, surface water, and reclaimed water. Unless it is contaminated with runoff or wastewater, ground water typically maintains good quality. More than 50% of the waterborne illnesses in the U.S. are caused by the consumption of contaminated groundwater via septic tanks, landfills and artificial recharge of

ground water, and treated sewage effluent from irrigated crops (Yates et al., 1985). Virus contamination to ground water occur more often since their small size and survival capabilities help viral agents to pass through soil (Gerba and Rock, 2014). In a surveillance study, a total of 141 out of 448 sites were positive for at least one of following viruses: enterovirus, rotavirus, Hepatitis A or Norwalk virus in the U.S. ground water sources (Abbaszadegan et al., 2003). Bacterial prevalence was reported as 9.9% total coliform, 8.1% enterococci, and 1.8% *Clostridium* in the same study. Pathogens can be introduced directly to the ground water via well opening. For example, the use of contaminated ground water used for irrigation caused a multistate outbreak of *E. coli* O157:H7 (CalFERT, 2007). The Florida administrative code (FAC, 2011) sets effluent limits from reclaimed water as maximum 25 fecal coliforms/100 mL. Reclaimed water has the poorest water quality and only allowed for direct irrigation of the crops that must be peeled, cooked, skinned, or thermally processed in Florida. For other crops consumed without any killing or elimination step of microorganisms, no direct contact with reclaimed water is allowed and subsurface and drip, ridge and furrow irrigation techniques are required (FAC, 2011).

Surface water sources are more vulnerable to contamination due to direct interaction with human based contamination sources, animal activities, weather events, and other environmental factors. A large number of pathogens can survive long enough in agricultural water to contaminate the produce and to reach humans during consumption. The presence of pathogens in agricultural water sources has been reported for different regions and type of pathogens. In a survey conducted in Canada, Johnson et al., (2003) reported that the prevalence of *E. coli* O157:H7 and *Salmonella* spp. was 0.9% and 6.25% in 90 mL water samples in more than 1,400 samples, respectively. In another study done in South Alberta, Canada, *E. coli* O157:H7 were

detected 1.3% and 2% of surface waters in the Oldman River Basin for each year over 2 year sampling (Gannon et al., 2004). In the same study, *Salmonella* was isolated 5.5% in the first year and 14.9% in the second year of sampling in 90 mL of samples (Gannon et al., 2004). Fecal indicators and *E. coli* O157:H7 were found in 11% in 100 mL and 2.7% in 100 and 1000 mL of private water supply samples filtered through 0.45 µm pore size cellulose nitrate membrane filters used as drinking waters in Netherlands, respectively (Schets et al., 2005). In Argentina, surface water samples in 48 cattle feedlots yielded 12.7% STEC positive in runoff from corrals exposed surface waters and 10.0% STEC positive in non-exposed surface waters sampled as two liter with Moore swabs (Tanaro et al., 2014). Micallef et al., (2012) found *Salmonella* spp. positive samples in up to 250 mL irrigation pond water (3/39) and in 10 g irrigation pond sediments (2/111) in the Mid-Atlantic region of the U.S. In a survey in Texas, Duffy et al., (2005) found *Salmonella* in 16 out of 170 (9.4%) surface irrigation surface water samples. In a comprehensive study, the prevalence of *L. monocytogenes*, *Salmonella* spp. and STEC was 48, 16, and 4 out of 174 in 250 mL water samples from surface water close to produce fields in the North East U.S. (Strawn et al., 2012). *Salmonella* and *E. coli* O157:H7 were isolated from irrigation waters and sediments in irrigation and non-irrigation water sources in the farms in Central California (Benjamin et al., 2013). Parasites are also commonly found in environmental water samples that are used for agricultural application. In a study conducted for irrigation water samples in Costa Rica, Mexico, Panama and the U.S., a total of 28% of the irrigation water samples were tested positive for microsporidia, 60% tested positive for *Giardia* cysts, and 36% tested positive for *Cryptosporidium* oocysts (Thurston-Enriquez et al., 2002). A comprehensive review by Pachepsky et al. (2011) about irrigation water as a source of contamination covers more details for further reading.

The presence of pathogens in Southeastern U.S. surface waters has been reported in several studies (Haley et al., 2009; Luo et al., 2015; McEgan et al., 2013; Patchanee et al., 2010; Rajabi et al., 2011). *Salmonella* was detected in 79.2% of water samples (2 liters) from six sampling stations in Suwannee Basin in Georgia during monthly sampling for one year (Haley et al., 2009). In North Carolina, different *Salmonella* serotypes in 25 mL water samples were isolated from four type of watersheds including residential/industrial area (58.8%), forestry (57.1%), crop agriculture (50%), and swine production (41.7%) (Patchanee et al., 2010). A number of studies were completed in the upper Suwannee River basin in southern Georgia and North Florida to understand distribution and occurrence of pathogens. Rajabi et al. (2011) found that 96% of water samples up to 500 mL collected from four sites in Suwannee River were positive for *Salmonella*. 500 mL water samples from irrigation ponds were positive 28.2% of the time for *Salmonella* in an agricultural region in southern Georgian and North Florida (Luo et al., 2015). In the same area, Gu et al., (2013) isolated *E. coli* O157:H7 from all 10 vegetable irrigation ponds sampled as three 500 mL aliquots of each water sample in monthly sampling. In Central Florida, all 202 water samples from water sources close to agricultural areas and wetlands, including lake, pond, creek, and stream were positive for *Salmonella* spp. after tangential flow filtration of 10 L of water (McEgan et al., 2013). The occurrence of pathogens is common in agricultural areas in the Southeast U.S.

Pathogen and Indicator Microorganism Relationships in Agricultural Waters

Both pathogens and indicator microorganisms can be introduced to agricultural waters; however, interpretation of indicator microorganism data for pathogen concentrations is problematic (Pachepsky et al., 2011). The correlations between pathogens and indicator microorganisms have been studied for a long time to evaluate the success of recommended water quality standards. It has been reported that the correlations are present, weak, or temporal

between microbial indicators and the presence of pathogens. High correlations between total and fecal coliforms and the presence of *Salmonella* was reported in canal and rivers in South Africa (Ijabadeniyi et al., 2011). On the central coast of California, the presence of *Salmonella* and *E. coli* O157:H7 was not accompanying significantly with generic *E. coli* concentration in irrigation water and sediments from streams and ponds over two and half year sampling period (Benjamin et al., 2013). Smith et al. (2009) did not find a correlation between the abundance of generic *E. coli*, enterococci, and *stx* (shiga toxin gene) encoded by STEC in recreational waters in Pennsylvania. Fecal indicator bacteria concentrations (enterococci and generic *E. coli*) correlated significantly with *Salmonella*, but not with *E. coli* O157:H7 and occurrence of *stx* in fourteen waterbodies in central California (Walters et al., 2011). In another study, the authors proposed that STEC levels in freshwater microcosms do not correlate with generic *E. coli* and other fecal indicator bacteria (Mauro et al, 2013). In surface waters, the occurrence of pathogens can be predicted based on microbial indicators without specific counts although many of pathogens do not behave similar to fecal microbial indicators (Payment and Locas, 2011). Correlations between fecal indicator microorganisms and pathogens is sought, but not proven for all conditions and type of pathogen of interest.

Fate and Temporal Variability of Microorganisms in Agricultural Waters

Surface irrigation waters are constantly subject to various environmental factors. Pathogens and indicator microorganism concentrations may change with internal and external variables in agricultural water sources. Some of internal factors includes type of water source, the presence of predators, bacteriophage lysis, physicochemical attributes of water, the presence of algae, nutrient availability, and assemblage with other organisms (Chandran and Hatha, 2005). Natural microbiota reduced culturable *E. coli* and *Salmonella* cells in estuarine environment particularly under combined action with sunlight (McCambridge and McMeekin, 1979;

McCambridge and McMeekin, 1981; Rhodes and Kator, 1990). The significance of starvation on microbial population was tested in sewage and lake water, and concentrations was associated with the availability of organic nutrient (Sinclair and Alexander, 1984). The disappearance of fecal coliforms was connected with the presence of bacteriophages in Boston Harbor and Great Lakes (Ricca and Cooney, 1998; Vijayavel et al., 2014). These internal factors may be affected by external factors.

Agricultural activities around water sources, presence of wild animals, time of day (solar radiation), weather events, temperature, runoff, and precipitation can be included in external factor list that may affect the population of pathogens and indicator microorganisms in addition to internal factors. The presence of wild animal and agricultural activities around agricultural water sources are discussed under in animal intrusion section. Generic *E. coli* concentration decreased from morning to midday in three Canadian streams (Meays, et al., 2006), and the concentration differences reached 0.5 log on sunny days and 0.2 log on cloudy days in Lake Michigan (Whitman et al., 2004). The inactivation rate of *E. coli* was reported greater under sunlight exposure than in the darkness in eustary and irrigation pond water (Chandran and Hatha, 2005; Maiga et al., 2009). All other external factors related to weather events are connected to each other and will be discussed around two leading variables: temperature and rainfall (precipitation) that affect other factors strongly.

Survival studies of pathogens and indicator microorganisms in water have been done in various agricultural, recreational, and other fresh water sources in different parts of the world. In general, studies have focused on survival of generic *E. coli*. Survival studies in natural water sources reveal a decline in microbial concentrations over time. In general, three phases of biphasic microorganism inactivation were observed in survival studies in water: a lag period

followed by fast log-linear inactivation, and slow log linear inactivation (Pachepsky et al., 2011; Blaustein et al., 2013). In a recent study, Blaustein et al., (2013) assembled 450 *E. coli* survival datasets in different water sources to show temperature dependence of inactivation rates. Fish and Pettibone (1995) observed that *E. coli* concentrations decreased gradually in two weeks in fresh water. *E. coli* survived up to 260 days in filtered and autoclaved river water at temperatures from 4 to 25°C, but only 15 days in non-sterile river water at 4°C with inverse proportionality to temperature (4°C > 15°C > 25°C > 37°C) in any sterile and non-sterile river water in U.K. (Flint, 1987). In Australia, survival of *E. coli* had an inverse relationship to temperature in recreational waters with trials at 10°C differences (Craig et al., 2004). The same relationship between *E. coli* and temperature was found in estuarine water in the Rhode River estuary in Maryland (Faust et al., 1975). Contradictory and different findings have been reported about temperature and *E. coli* survival in waters. Temperature difference affected the survival of *E. coli* hardly at 4°C and 25°C in water samples from eutrophic lake in Finland (Korhonen and Martikainen, 1991). Both generic *E. coli* and *E. coli* O157:H7 had lower die-off rate in colder water from Five-Mile Creek in Jefferson County, Alabama (Easton et al., 2005). *E. coli* survived better than *Salmonella* in tropical estuary under different conditions (Chandran and Hatha, 2005). The effect of temperature is important on the survival of both pathogens and especially on indicator microorganisms.

Rainfall events and irrigation may cause the distortion of sediments, and result in increase of contamination of surface irrigation water (Pachepsky et al., 2011). Population of generic *E. coli* and incidence of *E. coli* O157:H7 increased as a consequence of flow rate increases in rivers and streams after precipitation in California (Cooley et al., 2007). *Salmonella* Agona isolates significantly correlated with rainfall in marine environment conducted in shellfish production

areas of Galicia in Spain (Martines-Urtaza et al., 2004). *Salmonella* levels in surface waters from Upper Suwannee Basin in Georgia correlated average daily watershed rainfall (Haley et al., 2009). In the same area, Gu et al. (2013) found a positive correlation between rainfall and *E. coli* O157:H7 concentration in irrigation pond waters. In Ohio, higher total coliform and generic *E. coli* concentrations were detected after precipitation >20 mm, but no association was noted (Won et al., 2013). The dilution effect of rain on populations of thermotolerant coliforms was reported following 10.2 mm rain in 24 h prior to sampling with no correlation in Puerto Rico (Santago-Rodriguez et al., 2012). In the same study, concentration of indicator microorganisms in watershed increased after >25.4 mm of rain 48 h prior to sampling, and enterococci also correlated positively with rainfall (Santago-Rodriguez et al., 2012). Conflicting results were obtained in South Eastern part of the U.S. McEgan et al. (2013) reported that the average rainfall from previous day, week, and month prior to sampling did not correlate with *Salmonella*, coliforms, *E. coli* and aerobic plate counts levels significantly in Central Florida surface waters. However, *Salmonella* levels in irrigation pond waters from southern Georgia and North Florida correlated with rainfall (Luo et al., 2015). These studies suggest that different amount of precipitation and different locations may influence the concentration of indicator microorganisms and pathogens.

Mitigating Agricultural Water Quality

Agricultural waters are commonly accepted as potential conveyor for microbial contamination to fresh and minimally processed fruit and vegetables. The risk of produce contamination with pathogen can be reduced in agricultural waters with several mitigation strategies. These strategies are the use of different and safer irrigation methods, treatment of water to bring microbial load in a safe level before irrigation, and reduction of pathogen from input sources and reservoirs (Pachepsky et al., 2011). Irrigation method may affect the risk of

produce contamination sourced from agricultural water. Cantaloupe, lettuce and, bell pepper irrigated with bacteria and virus inoculated water were contaminated greater in furrow irrigation than subsurface drip irrigation (Song et al., 2006). Water quality requirement in the Produce Safety Rule do not apply to agricultural waters that does not contact directly harvestable portion of the produce as drip irrigation of tree crops (FDA, 2015). Treatment of water is another alternative strategy to reduce pathogen population. Filtration of water through sand and soil, waste stabilization ponds, oxidation reduction, chlorination, ozonation, and heat treatment are some of potential techniques (Pachepsky et al., 2011). The most effective approach to minimize produce contamination in the field is the prevention of agricultural water sources from potential contamination sources. For example, cattle are considered reservoirs for *E. coli* O157:H7 and other STEC serotypes (Ferens and Hovde, 2011). Surface water samples exposed to liquid discharges in cattle feedlots tended to have more STEC positive in runoff from corrals non-exposed surface waters (Tanaro et al., 2014). Agricultural water sources should be conserved minimize contaminations from potential source of contaminations with preventive methods as fencing, sediment basin, hedgerows, runoff protection, grass waterways, filter strips (Robins, 2012). There are other mitigation strategies for agricultural water including biofilm control, concurrent use of irrigation, and break time between final irrigation and harvesting (Pachepsky et al., 2011). Control of irrigation water contamination is essential for the production of safer fresh products.

Animal Intrusion

FDA (2015) considers animal intrusion is considered a primary route of contamination in fruit and vegetable fields. The activities of domestic and wild animals are also a substantial food safety concern in produce fields. Taking proactive precautions can reduce the presence of animals, but all wildlife cannot be avoided. The location of the field, type of crop, the presence

of water and food sources around fields, the occurrence of animal husbandry, and neighborhood activities may affect the prevalence and type of animals in produce fields. Domestic and wild animals and their excreta can contaminate crops directly and water sources used for agricultural purposes. Fecal deposits may harbor various pathogen species for prolonged time even in field conditions.

The prevalence and presence of microorganisms in domesticated and wild animal excreta poses a high likelihood of contamination of produce. The source of contamination for outbreak of *E. coli* O157:H7 associated with leaf lettuce consumption in Montana could not be identified; however, improperly composted cattle manure or contaminated irrigation water from uphill stream or flood or direct animal presence in irrigation water source was proposed as the origin of contamination (Ackers et al., 1998). A multistate outbreak of *E. coli* O157:H7 occurred in 1996 was investigated that the consumption of contaminated mesclun lettuce from a single grower and processor caused outbreak in Connecticut and Illinois. Cattle was seen near the field; however, only generic *E. coli* was isolated from wash water and finished product (Hilborn et al., 1999). Unpasteurized apple juice was implicated as the source of *E. coli* O157:H7 outbreak in 1996, and the presence of deer in orchard was concluded as possible source of pathogen even though outbreak strain was not isolated from deer pellets (Cody et al., 1999). Another multistate outbreak of *E. coli* O157:H7 was associated with the consumption of contaminated spinach in 2006. Outbreak strain was isolated from cattle and wild pig feces around fields and was possibly introduced the well water, which was concluded as the source of contamination (CalFERT, 2007). In 2008, an outbreak of *Campylobacter jejuni* occurred due to the consumption of raw peas collected from fields on which Sandhill crane stopped in while breeding in Alaska; isolates cultured from the Sandhill crane feces were the same as strains obtained from human stools and

peas (Gardner et al., 2011). Laidler et al. (2013) concluded that the consumption of locally grown contaminated strawberries with deer pellets caused outbreak of *E. coli* O157:H7 in Oregon in 2011. The outbreak strain was identified in 10 out of 11 initial environmental samples, and all positive samples had deer feces. The presence of animals and their excreta near the field represent important part of outbreak investigations.

Safety requirements for animal intrusion vary in the field based on local and nationwide organization. In Florida, Tomato-Good Agricultural Practices (T-GAPs) requires the exclusion of domestic animals and minimization of wild animal intrusion for animal intrusion. If contamination with animal/bird feces is identified, T-GAPs prohibit for harvesting contaminated tomatoes and require an assessment of potential impact of fecal material on the field harvestability (FAC&FAR, 2008). The California Leafy Greens Marketing Agreement (LGMA) requires monitoring of wildlife activity and application of remedial action in the case of intrusion. In the presence of fecal material in the field, a food safety assessment done by qualified personnel should be conducted and a no harvest zone should be established with a minimum 5-foot radius around fecal matter for all type of feces (LGMA, 2013). Under the FSMA Produce Safety Rule, animal intrusion is considered a main route of contamination. FDA requires monitoring of animal activity and allowing an adequate waiting time until harvesting if animal intrusion occurs (FDA, 2015). Real life situations, agricultural practices, and environmental factors bring questions about the applicability and reliability of proposed standards.

Pathogens from Animals

Domestic and wild animals are natural reservoirs for human enteric pathogens (Jay-Russell, 2013). The feces of animal can introduce these pathogens to produce fields directly or with contaminated agricultural water sources. There are several produce related outbreak caused

by animals as discussed above. *E. coli* O157:H7 represents the most prevalent animal originated pathogen in produce related outbreaks. It has been isolated from waterfowl, wild birds, opossum, raccoon, black-tailed deer, white-tailed deer, and feral pigs in the U.S. (Langholz and Jay-Russel, 2013). Cattle, sheep, goats, deer, and swine are generally accepted as the most important potential source of *E. coli* O157:H7 that could be introduced to leafy greens (Jay-Russell, 2013). Particularly, cattle are considered primary reservoirs for *E. coli* O157:H7 and other STEC serotypes (Ferens and Hovde, 2011). Animal associated outbreak strains of STEC have been isolated cattle pats, deer pellets, and the pig feces (Ackers et al., 1998; CalFERT, 2007; Laidler et al., 2013). *Salmonella* infections are perceived as associated with the consumption of contaminated animal sourced foods; produce has been rising source of salmonellosis outbreaks for decades (Fatica and Schneider, 2011). *Salmonella* have been isolated from both warm and cold-blooded animals including gulls, wild boars, rats, raccoons, and white-tailed deer samples in different countries mostly in low percentages of samples (Compton et al., 2008; Meerburg et al., 2006; Renter et al., 2006; Wacheck et al., 2010; Wahlström et al., 2003). In California, *Salmonella* was isolated from domestic cattle, birds, coyotes, deer, elk, wild pig, skunk, amphibians, and reptiles (Gorski et al., 2011; Gorski et al., 2013). Parish et al. (1998) isolated *Salmonella* spp. from toads and frogs after an unpasteurized orange juice outbreak in Florida. *C. jejuni* have been isolated in samples from wild birds, wild boars, moose in Sweden (Wahlström et al., 2003). *Yersinia* spp. have been detected in animal samples from wild boar, wild birds, beaver, and raccoon feces (Hacking and Slieo, 1974; Wacheck et al., 2010). Similarly, *L. monocytogenes* were isolated from beef and dairy cattle, deer, moose, otter, and raccoon feces (Fenlon, 1985; Lyautey et al., 2007; Wacheck et al., 2010). In addition to bacterial pathogens, protozoal and nematodal parasites, avian influenza, norovirus, and other viruses were detected in

animals (Jay-Russell, 2013). Wild and domestic animals are potential contamination sources in produce field with various bacterial species.

Fate of Microorganisms in Animal Feces

Domestic and wild animal feces have a high potential for pathogen transfer to crops in produce fields. Particularly, fecal materials from warm-blooded animals are considered natural reservoirs for various pathogens (Geldreich, 1996). There are some practical challenges that impact the relevance and quality of wildlife-pathogen studies about fecal deposits including unknown age, origin, contamination after deposition, the use of laboratory conditions and failure to mimic field conditions, waiting time before collection that may change bacterial population in feces (Langholz and Jay-Russell, 2013). Current published data about the survival of pathogens are mostly on cattle feces and slurries. High inocula (10^5 Log CFU/g) *E. coli* O157:H7 strains survived in dairy cattle pats for 49 days at 37°C, 56 days in 22°C, and 70 days at 5°C (Wang et al., 1996). In the same study, a human isolate was the only surviving strain at 37°C and 22°C based on chromosomal DNA fingerprinting isolates. To test the effect of environmental conditions on the survival of *E. coli* O157:H7, Kudva et al., (1998) inoculated sheep manure and observed that *E. coli* O157:H7 survived for 21 months. In a second set of experiment, aerated ovine manure was positive for *E. coli* O157:H7 for 4 months (Kudva et al., 1998). Fukushima et al. (1999) inoculated cattle feces with STEC strains O26, O111, and O157 at 10^1 , 10^3 and 10^5 CFU/g concentrations. All three strains survived for 2 to 14 weeks at 5°C, 1 to 18 weeks at 15°C, and 3 to 12 weeks at 25°C on samples with middle and high inoculations. The manure to soil ratio affected the survival of *E. coli* O157:H7 proportionally along with times in both manure amended autoclaved and un-autoclaved soils (Jiang et al., 2002). *E. coli* O157:H7 and *S. Typhimurium* survived better in cow manure at 20°C compare to 4 or 37°C, and *E. coli* O157:H7 and *S. Typhimurium* concentrations decreased six log CFU/g in 38 and 48 days with a decimal

reduction time of six days to three weeks, respectively. *S. Choleraesuis* was recovered from dry and wet stored swine feces from infected 8 week old pigs over 13 and 3 months of storage at 26°C (Gray and Fedroka-Cray, 2001). Another serotype, *Salmonella* Newport, the concentration increased during first three days of storage followed by steady decrease until day 107 and day 158 in manure amended non-sterile and sterile soils, respectively (You et al., 2006). In the same study, *S. Newport* was recovered with a most probable number method until day 332 in the manure amended non-sterilized soil and 405 days in manure amended sterilized soils. Sinton et al. (2007) reported that 90% of inactivation rate of bacteria in bovine feces on pasture were: *C. jejuni* - 6.2 days, fecal streptococci - 35 days, *S. enterica* - 38 days, *E. coli* - 48 days, and enterococci - 56 days. In another study, generic *E. coli* survived more than 45 days cattle manure during summer in Canada (Meays et al., 2005). All these studies show that pathogens can survive in animal feces for a long time, particularly in laboratory conditions, but shorter in field conditions.

Survival Models for Manure-borne Microorganisms

Different type of models have been developed for pathogens and indicator microorganisms in animal feces. Developed models focus on release of manure-born microorganisms from the source, portioning and attachment of microorganisms after release, survival and inactivation, and transport manure-born microorganisms (Pachepsky et al 2006). In here, survival and inactivation models will be covered. First order exponential decay models are predominantly used in the estimation of manure-borne microorganism die-off and temperature is considered as the only environmental parameter that can affect the die-off rate of microorganisms (Pachepsky et al 2006; Wang et al., 2004). More environmental parameters are present that may affect the survival of microorganisms in feces. Microbial die-off in manure does not follow first order exponential die-off all the time. Rather than a direct decline, tested

microorganisms initially regrow followed by a decay in survival studies in domestic and wild animal feces (Kudva et al., 1998; Wang et al., 1996; Wang et al., 2004; You et al., 2006). In addition, a two stage of decline was observed, including regrowth of lag phase in different studies and field studies (Kudva et al., 1998; Meays et al. 2005; Wang et al., 2004). Die-off rate is essential parameter used in different type of models including Arrhenius and Chick model (Pachepsky et al., 2006). Differences among die-off rates of tested microorganisms in manure from literature represent an important challenge in models. Die-off rates might be affected by numerous factors including type of microorganism, temperature, diet applied to animal, moisture content, the presence and amount of soil in manure, manure management practices, field conditions as rain solar radiation, and several others. Development of die-off models for pathogen and indicator microorganism require more complex models with the inclusion of more parameters and further data sets.

Managing Wildlife and Domestic Animal Intrusion

Increased demand for fruits and vegetables have amplified the amount of production and initiated the use of faster practices with improved technologies for massive capacities. Produce may be contaminated at any stage from farm to fork. Animal intrusion is considered as one of the main source of contamination in pre-harvest level (FDA, 2015). Domestic and wild animal excreta have a high potential to contaminate produce in the field and during further level of production. A sanitizing wash step is applied to produce to reduce potential pathogen population (Gill et al., 2009). Elimination of produce contamination that occurs in pre-harvest level may be difficult with post-harvest sanitizing applications (Materon, 2003). Wash water can be the source of cross-contamination particularly in reused and recirculated systems (Luo et al., 2011). Management of animal intrusion is essential starting in pre-harvest level in produce industry.

In the field, animal intrusion management strategies will vary based on field and environmental conditions. In a survey, responding growers revealed that at least one control measure was adopted by 89% of all growers to discourage and eliminate wild animals from the fields (Bianchi and Lowell, 2016). These control measures included use of poison baits (42%), removal of non-crop vegetation (37%), removal or abandonment of water source conservation practices (21%). Some of these practices mentioned above may affect the water quality adversely (Bianchi and Lowell, 2016). Standard operation procedures (SOPs) to minimize wild animal intrusion have been published by institutions working on produce safety. For example, the National Good Agricultural Practices Program (The National GAPs Program) leaded by Cornell University published educational materials, decision tree documents, SOPs for produce fields online for growers (2016). To minimize food safety risks associated with animal intrusion. The National GAPs Program (2016) recommends identification and risk assessment due to wild and domestic animals, use of methods to prevent animal entry to fields, reduction and elimination of animal attractions, monitor and documentation of animal activity, and training all workers about procedures and company policies. Co-management that covers the management domestic and wild animal intrusion is accepted by LGMA in California. LGMA (2013) defines co-management as “An approach to conserving soil, water, air, wildlife, and other natural resources while simultaneously minimizing microbiological hazards associated with food production.” Examples of co-management of water quality and food safety include consultation with professionals and other expertise, planning, communication among growers, handlers, buyers, and third-party auditors, and updating (Robins, 2012). There are more animal intrusion management practices applied by growers in practice and groups providing consultation and detailed information to growers.

Conclusion

Fresh produce contamination may occur in every stage of production. In field, agricultural water, domestic and wild animals, and soil amendments are main contaminations routes. Numerous type of pathogens including pathogenic bacteria, viruses, or parasites can survive long enough in agricultural water and animal feces in the field conditions. Survival studies in pre-harvest routes have been conducted for generic *E. coli*, STEC, and *Salmonella* in general. In agricultural waters, results show differences depending on location and water source. Also, microbial population changes in agricultural water have been observed due to environmental factors. Limited data have been published regarding the survival capabilities of different STEC strains and *Salmonella* spp. in wild animal feces to justify if any harvest stipulations in produce safety guidelines are applicable for wild animal feces. Florida is one of the massive producer of fresh fruit and vegetables, and the safety of produce remains as a big concern that needs more improvement. Particularly, contamination risks during pre-harvest need more research in Florida due to special wild nature, weather, and agricultural practices.

CHAPTER 3

EVALUATION OF MICROBIAL QUALITY OF AGRICULTURAL SURFACE WATER IN CENTRAL FLORIDA

Introduction

The U.S. Congress authorized FDA to set up minimum safety standards for production and harvesting of fruit and vegetables that may be consumed without any further processing (Harris, et al., 2012). Science based standards to minimize the risk of outbreaks related to the consumption of fruit and vegetable have been covered in the Produce Safety Rule. Agricultural water is considered as one of the main microbial contamination risk routes for produce (FDA, 2013). On November 13, 2015, water quality criteria for untreated water that contacts harvested crops was released in final Produce Safety Rule under Food Safety Modernization Act (FSMA) by FDA. This rule requires the development of microbiological “microbial water quality profile (MWQP)” by calculating a geometric mean and a “statistical threshold value (STV)”. To meet the water quality standard, the geometric mean and STV should be ≤ 126 *E. coli* and ≤ 410 *E. coli* in 100 mL water sample, respectively. The safety of agricultural water is important due to high contamination potential at all produce production levels. Direct evidence of agricultural water causing foodborne outbreaks is infrequent; surface irrigation water has been implicated as the sources of pathogen contamination with confirmed cases (Pachepsky et al., 2011).

The microbial monitoring of agricultural surface waters is less frequent than for drinking and recreational waters. Generic *E. coli*, fecal streptococci, enterococci, and total coliforms are common indicator microorganisms for fecal contamination and microbial water quality (Steele et al., 2005). The presence of pathogens, particularly *Salmonella* spp. and STEC, in agricultural water sources has been reported for different regions (Gu et al., 2013; Haley et al., 2009; Johnson et al., 2003; Luo et al., 2015; McEgan et al., 2013; Micallef et al., 2012; Patchanee et al., 2010; Rajabi et al., 2011; Strawn et al., 2013). Rainfall events and agricultural practices that cause the

distortion of sediments and run-off water often result in resuspension of microorganisms in agricultural surface water (Goyal et al., 1977; Pachepsky et. al, 2011). The population of indicator microorganisms and pathogens, *E. coli* O157:H7, *Salmonella* spp., are affected differently depending on rainfall events in different regions and time of the year (Cooley et al., 2007; Martines-Urtaza et al., 2004; Santiago-Rodriguez et al., 2012; Steele et al., 2005; Strawn et al., 2013; Won et al., 2013). No strong or clear correlation between precipitation and microbial water quality were also reported in some studies (Goyal et al., 1977; McEgan et al., 2013). These studies raise questions about the effect of different amount of precipitation, sampling times, and sites that may influence the concentration of indicator microorganisms and pathogens. More data are needed regarding the populations of indicator microorganism and pathogen in agricultural surface waters throughout growing season and the effects of weather events on them.

The objectives of this study were to a) monitor indicator microbial populations in surface water weekly over the course of two growing seasons; b) increase monitoring frequency during high water usage or weather events to determine their impact on microbial populations; c) evaluate FSMAs proposed agricultural water requirements for Central Florida surface water; d) determine correlation between microbial indicators and physicochemical water attributes; e) determine correlation of indicator organisms and the presence of *Salmonella* and STEC genes in the tested water samples.

Material and Methods

Water Sampling

Surface water samples (500 mL) were collected from ponds in West Central Florida weekly, and every other day after rain events of 2.0 cm or more within 24 h, and after intensive surface water usage due to initial planting stage or freeze protection. The six ponds sampled were in use for agricultural purposes. Physical conditions of the ponds are shown in Table 3-1. Surface

waters samples were collected during dry season in Florida when irrigation of fields was essential; crops in all fields were irrigated by drip irrigation under plastic mulch. Sampling was done prior to solar noon to minimize the influence of sunlight. Samples were collected during three consecutive growing seasons from 2012 to 2014: 46 times in first year and 44 times in second year. Total of 540 samples were collected during the 2012-2013 (276) and 2013-2014 (264) growing seasons from six ponds. During the third year (2014/2015 growing season), water samples were collected 5 times from each pond for a total of 30 sample, to simulate the 5 samples, required under the Produce Safety Rule to be collected annually. Geometric mean and STV values, to establish MWQPs for all ponds, were calculated using excel tool developed by Western Center for Food Safety, University of California, Davis (<http://wcfs.ucdavis.edu/>). Water (150 mL) from each sampling time and pond was filtered and the filters were stored at -20°C for identification of *Salmonella* and STEC profile genes with PCR analysis for first two growing seasons.

Microbiological Analysis

Surface water samples were returned to laboratory on ice immediately after sampling and microbial analysis of samples began within three hours. The populations of target microorganism were enumerated via most probable number (MPN/100 mL) in required dilutions with an IDEXX Quanti-Tray® 2000 (IDEXX Laboratories, Westbrook, ME). Three different fecal contamination indicators were monitored. Quanti-Tray® 2000/Colilert® was used to examine of total coliform and generic *E. coli* populations, and Quanti-Tray® 2000/Enterolert® for enterococci populations, respectively. Total coliform and *E. coli* populations were examined through the activity of β -galactosidase and β -glucuronidase, respectively. The activity of β -glucosidase was used for the enterococci population identification.

Water Attributes

Physicochemical attributes of water were tested on 100 mL samples for each pond with relevant instruments. Temperature of water, turbidity, conductivity, pH and oxidation-reduction potential (ORP) of samples were measured in triplicate. Turbidity was measured in Formazin Attenuation Units (FAU) with a portable colorimeter (DR/850, Hach Company, Loveland, CO, USA). Water temperatures ($^{\circ}\text{C}$) were measured with a portable temperature probe (SH66A, Cooper Instrument Corporation, Middlefield, CT, USA). ORP (mV) were measured with a portable ORP meter (pH6 Acorn series, Oakton, 126 Vernon Hills, IL USA). pH was measured with lab scale pH meter (Accumet[®] AB15 Basic, Fischer Scientific, Pittsburg, PA USA). Conductivity ($\mu\text{S}/\text{cm}$), was measured with a portable conductivity tester (HI98304 DIST[®] 4 EC, HANNA Instruments, Woonsocket, RI).

Salmonella and STEC Genes

For STEC, 25 mL of modified peptone water with pyruvate (mBPWp; Neogen, Lansing, MI) was added to frozen filters, and incubated at $35 \pm 1^{\circ}\text{C}$ for 24 h. After preenrichment, DNA extraction was done using MoBio UltraClean DNA isolation kit (MoBio, Carlsbad, CA). For *Salmonella*, an extra enrichment was performed in Rappaport-Vassilidas (RV) (Difco, Bectin, Dickinson) medium at $42 \pm 1^{\circ}\text{C}$ for 48 h and DNA extraction was applied starting from RV.

The presence of *invA* gene was used to detect *Salmonella* DNA. The *invA* primer set protocol was modified from Rahn et al. (1992). Primer sequences were Forward: GAATCCTCAGTTTTTCAACGTTTC and Reverse: TAGCCGTAACAACCAATACAAATG. All reagents were obtained from Fisher *exACTGene*[™] PCR kit and Core Reagent Sets (Fisher Scientific). The PCR reagent concentrations for 50 μL reaction was as follows: 31 μL water, 10 μL 5x Go Taq Flexi buffer, 3 μL 25 mM MgCl_2 , 1 μL 10 mM dNTPs, 0.25 μL Go Taq DNA polymerase, and 2 μL 10 μM of each primer, and 1 μL DNA template. In practice, the PCR

reaction mix was prepared with 20 µL water, 25 µL AmpliTaq Gold 360 (A&B Applied Biosystems, Foster City, CA) that contains all reaction ingredients as listed above except for primers, 2 µL 10 µM of each primer and 1 µL DNA template. The ideal concentration of each primer pair were 200 nmol/L for *invAF/invR*. PCR conditions were: 10 min 94°C for melting, followed by 20 cycle of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and with a final elongation of 72°C for 5 min. Gel electrophoresis was performed on 2.0% agarose gel with 1X TBE buffer (BIOAID, Hercules, CA) at 120 V for 60 min. Then, gel was placed in 400 mL of ethidium bromide (40 mg/mL) for 20 min for staining. Before UV light (MultiDoc-It Digital Imaging System; UVP, Upland, CA, USA) visualization, gels were de-stained in 400 mL deionized water twice for 20 min.

Extracted DNA via MoBio UltraClean® DNA isolation kit (MoBio, Carlsbad, CA) was run in multiplex PCR to look at 6 STEC gene with a protocol modified from Hu et al. (1999). The PCR assay was designed to amplify 6 genes including, *hly*, *fliC*, *eaeA*, *rfbE*, *stx-I*, and *stx-II*. Six pairs of primers and volume of primers in primer mix were listed in Table 3-2. For 50 µL reaction, 15.64 µL water, 25 µL AmpliTaq Gold 360 (A&B Applied Biosystems, Foster City, CA) 8.36 µL primer mix and 1 µL DNA template were used. The ideal concentration of each primer pair were 60 nmol/L for *fliCF/fliCR*, 75 nmol/L for *eaeAF/eaeAR*, 100 nmol/L for *rfbEF/rfbER*, and 200 nmol/L for *hlyF/hlyR*, *stx-IF/stx-IR*, and *stx-IIF/stx-IIR*. PCR conditions were: 10 min 95°C for melting, followed by 20 cycle of 94°C for 30 s, 59°C for 1 min with 0.5°C decreasing temperature per cycle, 72°C for 1 min, and a second 20 cycle of 94°C for 30 s, 49°C for 1 min, 72°C for 1 min and with a final elongation of 72°C for 7 min. Electrophoresis and staining was the same as the protocol for conventional PCR for *Salmonella* except that 130 V was used for 60 min during gel electrophoresis.

***Salmonella* Isolation and Serotyping**

Following PCR analysis, *Salmonella* positive samples were enriched by FDA Bacteriological Analytical Manual (BAM) to obtain a culture (Andrews et al., 2011). After RV enrichment, a loopful of *Salmonella* positive samples were streaked onto bismuth sulfite agar (BSA), xylose-lysine-desoxycholate (XLD), and hektoen enteric agar (BSA, XLD, HE; Difco, Becton, Dickinson, Sparks, MD). XLD and HE was incubated at $35 \pm 2^{\circ}\text{C}$ for 24 h, and BSAR was incubated at $35 \pm 2^{\circ}\text{C}$ for 48 h. Following incubation, if typical colonies were present, they were transferred into triple sugar iron (TSI; Difco, Becton, Dickinson, Sparks, MD) and lysine iron agar (LIA; Difco, Becton, Dickinson, Sparks, MD) slants. Positive isolates were frozen in 30% glycerol and 70% tryptic soy broth mixture (TSA; Difco, Becton, Dickinson, Sparks, MD) for further confirmation tests. Isolations were streaked on xylose-lysine-tergitol-4 (XLT4; Difco, Bectin, Dickinson, Sparks, MD) and confirmed using a latex agglutination test (Oxoid, Hampshire, UK) as described on manual for double confirmation before sending isolates to the National Veterinary Services Laboratories (USDA, NVSL) for serotyping. XLT4 media was incubated for $24 \pm 2^{\circ}\text{C}$ at $35 \pm 2^{\circ}\text{C}$ and latex agglutination test was performed at room temperature.

Rainfall Data Collection and Animal Activity Observation

Rainfall data was collected from the Florida Automated Weather Network (FAWN) (<http://fawn.ifas.ufl.edu/>). The closest weather recording FAWN tower (Dover station) to all agricultural ponds was chosen and precipitation rates were followed daily during growing seasons. Animal activity in and around ponds was observed and noted during sampling.

Statistical Analysis

Statistical analysis was achieved by analyzing data with JMP 11 software (SAS® Institute Inc., Cary, NC, USA 2013). All statistical analyses were performed on data collected

only during 2012/2014 and 2013/2014 growing seasons. Pearson product moment correlation coefficients (“r”) among the physicochemical characteristics of waters and indicator microorganism were analyzed ($P < 0.05$). Linear regression of precipitation and microbial indicators was calculated in R^2 .

Results

Water from six agricultural irrigation ponds were sampled for three consecutive year during growing seasons in Central Florida. MWQP for the first year, a two year baseline, and third year rolling consisting of geometric means (GM) and statistical threshold values (STV) of generic *E. coli* were calculated for each pond, and are listed in Table 3-3. All agricultural ponds tested in Central Florida met the current recommendations ($GM \leq 126$ *E. coli* and $STV \leq 410$ *E. coli* in 100 mL) for MWQP 100.0% of the time after establishment of baseline survey for two years. The GM values for all pond were under recommended limits all the time. GM values ranged from 1 to 32 for all ponds except for Pond 2 in first year growing season, which was 118, and close to water quality criteria. STV values were lower than recommendation for all ponds during two year baseline and third year rolling MWQPs. With the exception of Pond 2, all STV values were ≤ 303 and under produce safety agricultural water criteria in the first season. The highest STV, from Pond 2 in the first year only, was 5571 and higher than criteria (≤ 410). The correlation coefficients among microbial indicators Log MPN/100 mL and measured physicochemical characteristics are discussed below. Microbial indicators, their correlations with characteristics, and results of the presence of *Salmonella* and STEC genes is explained, separately. Observed animal activity during sampling included turtles, fishes, frogs, alligators, and birds including ibis, heron, Sandhill cranes, wood storks, wild and farm ducks, and vultures.

Microbial Indicators

All microbial indicators, total coliform, enterococci, and generic *E. coli* followed similar trends (Figure 3-1 and Figure 3-2). The population of indicators fluctuated during sampling time for 204 days in season 1 and 260 days in season 2. At all ponds, total coliform population was the highest at almost all sampling times ranging from log 0 to 8 MPN/100 mL. Population of enterococci was the second highest and ranged from log 0 to 5.2 MPN/100 mL. Population of Generic *E. coli* stayed below the population of other microbial indicators at most of the time, and ranged from log 0 to 4.2 MPN/100 mL.

The amount of rain exceeded 2.0 cm within 24 h 6 and 10 times in growing season 1 and 2, respectively. Maximum precipitation was measured as 7.5 cm within 24 h. Following rain events, total coliform populations increased up to 4.2 Log MPN/100 mL from the previous sampling. Microbial populations within ponds varied up to 4 Log MPN/100 mL between sample points. The highest correlations (R^2) between rainfall and total coliform were observed in Pond 1 and Pond 6 that were 0.15 and 0.40, respectively. Between rainfall and enterococci, the highest correlations (R^2) were lower than other indicators and observed in Pond 2 and Pond 6 that were 0.11 and 0.22, respectively. Rainfall of 24 h (mm/day) did not correlated with generic *E. coli* population for Pond 1,3,4,5 ($R^2 < 0.1$). Generic *E. coli* correlated the most strongly with rainfall in Pond 2 and Pond 6 where correlations (R^2) were 0.16 and 0.42, respectively. Rest of the correlations (R^2) between indicators and precipitation are below 0.1 and shown in Table 3-4.

Results for *Salmonella* and STEC Gene Presence

The percentage of each gene detected, in each pond, can be seen in Figure 3-3 for the first two growing seasons. All STEC genes were detected in 2.6% of the samples. Individual STEC genes were detected in every sample (540) as following percentages: *stx-I*-32.6%, *stx-II*-9.4%, *eaeA*-17.4%, *hly*-83.3%, *rfbE*-17.4%, *fliC*-51.8%. The individual percentage of each STEC gene

in each pond was shown in Table 3-5 and ranged as follows: *stx-I*: 28.9-35.6%, *stx-II*: 7.8-13.3%, *eaeA*: 12.2-24.4%, *hly*: 80-86.7%, *rfbE*: 13.3-22.2%, *fliC*: 50-54.4%. The *invA* gene was detected in 26/540 (4.8 %) samples, in all ponds and both growing seasons. However, 57.7 % (15/26) of the *invA* positive samples were from Ponds 2 and 4. The *invA* gene was detected in all ponds and the percentages were 4.4% in Pond 1, 7.8% in Pond 2, 2.2% in Pond 3, 8.9% in Pond 4, 2.2% in Pond 5, and 3.3% in Pond 6. At least one *Salmonella* or STEC gene was detected in 91.3% of samples.

***Salmonella* Serotyping**

Only 21/26 samples isolated successfully and sent to NVSL for serotyping after *Salmonella* confirmation tests. NVSL reported 14 different serotypes were detected out of 21 *Salmonella* isolates as shown in Table 3-6. *S. Saintpaul* and *S. 4, 12,i:-* was isolated twice and three times from separate ponds, respectively. *S. 4, 5, 12:i:-* was isolated four times in three different ponds. Other serotypes isolates from ponds included *Anatum_var._15+*, *Hartford*, *Baildon*, *Javiana*, *Anatum*, *Newport*, *Muenchen*, *Rough O:i:-*, *Florida*, *IV_50:z4,z23:-* and *Rubislav*.

Water Attributes and Microbial Indicators

Physicochemical characteristic for all six agricultural irrigation ponds were plotted for the first two growing seasons. Water and air temperature plots are displayed in Figure 3-4. pH – ORP and turbidity and conductivity are plotted together in Figure 3-5 and Figure 3-6, respectively. Multiple linear regression analysis with population of total coliform, enterococcus, and generic *E. coli*, air temperature, water temperature, conductivity, pH, oxidation reduction potential, and turbidity indicated that all microbial indicators correlated with conductivity, pH, and turbidity were performed ($P < 0.05$). Pearson product moment correlation coefficients (r) and P values for all attributes are listed on Table 3-7. Correlation coefficients demonstrate

stronger positive and negative linear relationship when they are closer to 1 and -1. The highest correlation, 0.75, was between air and water temperatures. Microbial indicators correlated with conductivity and pH from environmental variables ($P < 0.05$). The most prominent correlation was calculated for pH with all microbial indicators ($r \geq 0.2$). The correlations between microbial indicators and water attributes were below $r < 0.2$ except for pH. Correlation coefficients among microbial indicators were above 0.58 ($P < 0.0001$). The highest correlation coefficient among the microbial indicators, 0.63, was calculated between generic *E. coli* and enterococcus.

Discussion

The microbial quality of water that comes into contact with the harvested portion of produce is commonly believed to directly relate to the safety of produce, and metrics describing indicator organisms are commonly used in the produce industry to ensure safety of a product (Harris et al., 2012). The new Produce Safety Rule requires growers to develop a microbial water quality profile (MWQP) for their agricultural water source based on the population of generic *E. coli*. Developed MWQPs for all ponds tested in Central Florida indicate that all ponds met the FDA recommendations for microbial water quality, however this does not guarantee the safety of these agricultural waters. Several times population of generic *E. coli* passed the previously proposed water quality recommendations by FDA that the highest allowed generic *E. coli* population level for produce contact water is 235 MPN/100 mL in a single sample or with a geometric mean ($n=5$) not to surpass 126 MPN/100 mL (FDA, 2013). LGMA (2013) still uses these recommendations, and T-GAPs only recommends the use of potable water for fruit contact (FAC&FAR, 2008). The final Produce Safety Rule allows growers to use water exceeding these standards under some conditions. How the microbiological quality of surface water relates to, and should be evaluated, in regard to produce safety requires further evaluation. Understanding

the relationships between indicator microorganisms, the presence of pathogen, and physicochemical attributes of water allows a greater understanding of agricultural water risks.

Although only generic *E. coli* is considered a fecal contamination indicator and microbial water quality in the Produce Safety Rule, fecal streptococci, enterococci, and total coliforms are commonly used (Steele et al., 2005). Pachepsky et al. (2015) summarized experimental data about the relationships between thermotolerant coliform, pathogens, and generic *E. coli* that proposed relationships were significant 35% in surface waters suitable for irrigation. In this study, three type of microbial indicators including total coliform, enterococci, and generic *E. coli* were monitored to have better understanding correlations and how population of microbial indicators behaved. Microbial indicators tested in this study yielded moderate Pearson product moment correlation coefficients correlations (r) ranging 0.58 to 0.63. This situation suggests the use of generic *E. coli* or other indicator microorganisms tested as fecal contamination will yield acceptable results for surface waters in Central Florida.

All microbial indicators correlated with each other moderately ($r \geq 0.58$), but poorly with physicochemical water attributes. When all the water attribute data from the six ponds were pooled for analysis, a total of 540 for input for each attribute, pH and ORP negatively correlated, and conductivity and turbidity positively correlated, with all microbial indicators; none of these attributes led to correlations in acceptable range to predict the microbial populations (Table 3-7). Gu et al. (2013) also reported that the occurrence of *E. coli* O157:H7 negatively correlated with ORP. The highest correlation with all microbial indicators was calculated for pH ($r > 0.2$). Generic *E. coli* and enterococcus have a statistically significant correlation ($P < 0.05$) with all physicochemical water attributes; R^2 were below 0.08 indicating that they are not enough to predict microbial populations. The positive conductivity correlation with microbial indicators in

this study conflicted with previously reported inverse correlation results in estuary waters in North Carolina and surface waters in Central Florida (Gonzalez et al., 2012; Gonzales and Noble, 2014; McEgan et al., 2013). All studies suggested that inverse correlation of conductivity with *E. coli* and enterococcus concentration is due to storm and run-off water changing salinity and other dissolved solids into water and having a dilution effect on *E. coli* concentrations. The confliction might be explained that pond waters in this study are close-system surface water sources feed with well water, and that the length of the sampling period was longer, more frequent, and concentrated on only six ponds. McEgan et al. (2013) and Gonzales et al. (2012) sampled total of 202 and 151 times, respectively compare to 540 data points in this study. Temperature and fecal coliform concentration were positively correlated with *E. coli* O157:H7 (Gu et al., 2013). In a study conducted in South Africa, total and fecal coliforms did not correlate with chemical oxygen demand; enterococcus correlated with pH, turbidity, and rainfall (Ijabadeniyi et al., 2011).

Precipitation and agricultural activities can cause contamination of agricultural surface water and increase in concentration of indicator microorganisms (Pachepsky et al., 2011). None of our indicator population showed consistent relationship with precipitation rates. Rainfall occasionally influenced the indicator population; a limited numbers of increases in indicator concentration from specific ponds was observed after high precipitation rates. This might be explained by the physical conditions of each pond and their run-off exposure probability. The highest indicator populations after rain were monitored in the over 40 year old pond coded Pond 2 with open soil edges and high run-off possibility. This pond is not elevated and connected to a creek. The highest precipitation and microbial indicator correlations were obtained in Pond 6 for total coliform, enterococcus and generic *E. coli*. No particular reason was observed for the

exceptional results of Pond 6; however, strong effect of sampling site difference was witnessed on microbial indicator population.

Similar and conflicting results have been reported previously in studies evaluating correlations between precipitation and microbial populations including pathogens. In California, generic *E. coli* and *E. coli* O157:H7 population increase correlated with flow rate increase in rivers after rainfall (Cooley et al., 2007). *Salmonella* Agona isolates correlated significantly with rainfall in marine environment conducted in shellfish production areas of Galicia in Spain (Martines-Urtaza et al., 2004). Santiago-Rodriguez et al. (2012) established that 10.2 mm of rain 24 h prior to sample collection decreased the concentration of thermotolerant coliforms owing to possible dilution effect; whereas the number of these indicator microorganisms increased after >25.4 mm of rain 48 h prior to sample collection due to possible sediment distribution. In the same study, they also suggested that no correlation was calculated between the concentrations of thermotolerant coliforms and rainfall reported for one week prior to sample collection. In a recent study, the average rainfall from previous day, week, and month prior to sampling did not significantly correlate with bacterial levels in Central Florida Surface waters (McEgan et al., 2013). In Georgia, *E. coli* O157:H7 and *Salmonella* positively correlated with precipitation levels (Gu et al., 2013; Haley et al., 2009). Won et al. (2013) reported that total coliform and generic *E. coli* counts in canals were higher after heavy precipitation (>20 mm) compare to lighter rainfall amounts whereas no association were found between microbial indicators and precipitation in reservoirs in Ohio. All microbial indicators tested here showed similar fluctuations in population after weather events in consistent with calculated statistical correlations among them ($0.63 \geq r \geq 0.58$). Different results suggest that there might be some correlation on microbial population and precipitation in surface waters, whereas degree and type

of effect might change based on other characteristics of water source. In this study, correlation variability in different six agricultural pond in a 4 mile radius area indicates unreliability of the use of only rainfall data in the prediction of precipitation effect on microbial water quality. Instead, present microbial water quality, physical conditions of water source, and run-off exposure assessment should be considered for prediction and risk assessment.

The presence of pathogens in surface water is expected due to possible exposure to animals, humans, weather and other environmental factors (Pachepsky et al., 2011). *Salmonella* can be found in any surface water, particularly in the South Eastern U.S. where they have been reported in different types of surface water sources at the overall frequencies between 37 to 100% in water samples sizes ranging from 1 mL direct MPN enrichment to 10 L tangential flow filtration followed by enrichment in North Carolina, Georgia, Central and North Florida (Haley et al., 2009; Luo et al., 2015; McEgan et al., 2013; Patchanee et al., 2010; Rajabi et al., 2011). McEgan et al. (2013) reported that *Salmonella* can be found in Central Florida surface water throughout the year similar to other water survey studies conducted in the South Eastern U.S. (Haley et al., 2009; Luo et al., 2015; Patchanee et al., 2010; Rajabi et al., 2011). The *invA* gene was detected in all ponds and both growing seasons. These results show that the presence of *Salmonella* in agricultural pond waters in Central Florida is similar to other type of surface water as reported in the South East U.S., previously. However, the distribution of detected *invA* positive samples was not equal for each pond, and more than half of the positive samples were obtained from ponds 2 and 4 with the MWQPs under required standards.

The number of studies evaluating prevalence of STEC in irrigation water in the South East region of the U.S. is limited compare to *Salmonella*. Gu et al., (2013) reported that all 10 irrigation ponds from Suwannee River Watershed in Georgia were positive for *E. coli* O157:H7

some of the time during sampling period. Several water survey studies in other regions have showed the presence of STEC in agricultural water. The prevalence of *E. coli* O157:H7 was 0.9% and 2% in two independent surveys about surface water in Southern Alberta, Canada (Johnson et al., 2003; Gannon et al., 2004). In Netherlands, *E. coli* O157:H7 was found in 3% of private wells (Schets et al., 2005). In Argentina, surface water samples in 48 cattle feedlots yielded 70.5% *rfb* positive and 12.7% STEC in runoff from corrals exposed surface waters and 60.0% *rfb* positive and 10.0% STEC in nonexposed surface waters (Tanaro et al., 2014). In this study, all six genes were detected in different percentages. The percentage of all these STEC genes were similar in all ponds and in the order of Hemolysin (*hly*), H7 (*Flic*), and *stx-I* are the most abundant *stx-II* least found.

The correlations between microbial indicators and the presence of pathogens were concluded as possible in previous studies with similar findings, whereas this might be temporal, unsystematic or place and time specific. Ijabadeniyi et al. (2011) reported high correlations between total and fecal coliforms and the presence of *Salmonella* in sampling sites including canal and two rivers in South Africa. However, generic *E. coli* population did not significantly associate with the presence of either *E. coli* O157 or *Salmonella* in water or sediment samples in a study done along the Central Coast of California (Benjamin et al., 2013). Generic *E. coli* and enterococci did not correlate with *stx* gene encoded by STEC in recreational waters in Pennsylvania and water bodies in Central California (Smith et al., 2009; Walters et al., 2011). Fecal indicators correlated with *Salmonella*, significantly in the same study (Walters et al., 2011). Higher correlations were observed at sites where indicator microorganisms and pathogens are detected in high populations for extended period of time (Payment and Locas, 2011). Surface waters tested in Central Florida meet the FDA recommendations for microbial water quality,

however at least one *Salmonella* or STEC gene was detected in 91.3% of samples. This suggests the potential presence of the pathogens in Central Florida surface agricultural water that meets the required microbial water quality water safety standards. During a risk assessment of microbial acceptability of agricultural water, the MWQP based on indicator organisms might not be enough to represent the safety of surface waters.

The source of pathogens in these ponds are potentially wild and domesticated animals, naturally found pathogens in water and sediment, and carried pathogens via runoff water from around ponds (Pachepsky et al., 2011). These ponds, except for Pond 2, are small bodies of water with no connection to other natural water sources. Turtles, fishes, frogs, alligators, and various types of bird including ibis, heron, Sandhill cranes, wood storks, wild and farm ducks, and vultures were observed in and around the ponds. It has been reported that reptiles, wild and domesticated animals, household animals, and birds are some of the biotic sources of *Salmonella* (Gaertner et al., 2008; Johnston et al., 2010; Moore et al., 2003). Turtles were almost always observed in the ponds with high *invA* detection. Gaertner et al. (2008) isolated *Salmonella* from wild turtles, but negative water samples. This suggests that the presence of *Salmonella* in turtle is not enough for prediction of *Salmonella*, however, *Salmonella* is harbored by normal turtle intestine, and defecation can increase the potential risk of contamination (DuPonte et al., 1978). Ponds can be cleared from turtles easily by the instalment of short barriers around ponds.

Table 3-1. Physical conditions of the ponds sampled over the 2012-2013 growing season.

Pond Code	Approximate Pond Age (years)	The conditions around pond	Sunlight exposure
Pond 1	3	Grassed, elevated, low run off	High
Pond 2	45-49*	Open soil, not elevated, high run off, connected to a creek	Low
Pond 3	3	Grassed, not elevated, low run off, tailwater recovery	High
Pond 4	24	Grassed, not elevated, low run off	High
Pond 5	4-5	Grassed, not elevated, low run off	High
Pond 6	7	Lightly grassed, not elevated, medium run off	Medium

* Expanded 6 years ago.

Table 3-2. Primers used in conventional *invA* and multiplex PCR assays for *Salmonella* and STEC.

Target	Gene carrier	Size (bp)	Primer	Sequence 5' –3'
Invasion	<i>Salmonella</i>	284	<i>invAF</i>	GAATCCTCAGTTTTTCAACGTTTC
			<i>invAR</i>	TAGCCGTAACAACCAATACAAATG
Hemolysin	O157 & Non-O157	774	<i>hlyF</i>	CCCTGGCAGACCTTTGATG
			<i>hlyR</i>	CCGTGTCTTTTCTGATACTCA
H7	O157	625	<i>fliCF</i>	GCGCTGTCGAGTTCTATCGAGC
			<i>fliCR</i>	CAACGGTGACTTATCGCCATTCC
STX-II	O157 & Non-O157	484	<i>stx-IIIF</i>	GACTGTGATGCATCAGGCAAAG
			<i>stx-IIIR</i>	TTGGAGTATTAACATTAACCCCAGG
Intimin	O157	368	<i>eaeAF</i>	GTGTCCATTTATACGGACATCCATG
			<i>eaeAR</i>	CCTATAACGTCATGCCAATATTGCC
O157	O157	292	<i>rfbEF</i>	TGTAACCTGGAAAGGTGGAGTATAC
			<i>rfbER</i>	GCTATTCTGAGTCAACGAAAAATAAC
STX-I	O157 & Non-O157	210	<i>stx-IF</i>	GTTTTTCTTCGGTATCCTATTCCG
			<i>stx-IR</i>	GATGCATCTCTGGTCATTGTATTAC

Table 3-3. Calculated MWQP values for all ponds for three consecutive growing seasons.

Pond Code	First year		Two year baseline MWQP		Third year rolling MWQP	
	GM	STV	GM	STV	GM	STV
Pond 1	7	94	1	2	1	4
Pond 2	118	5571*	8	103	11	137
Pond 3	3	19	2	6	2	4
Pond 4	14	166	9	140	6	51
Pond 5	6	54	5	30	8	95
Pond 6	32	303	7	70	8	76

* Value that does not meet the produce safety rule criteria.

Table 3-4. Precipitation and microbial indicator correlations for all ponds.

Pond Code	Precipitation Correlation (R^2)		
	Total Coliform	Enterococci	Generic <i>E. coli</i>
Pond 1	0.1468	0.0631	0.0335
Pond 2	0.0026	0.1090	0.1586
Pond 3	0.0094	0.0406	0.0113
Pond 4	0.0392	0.0264	0.0195
Pond 5	0.0000	0.0150	0.0021
Pond 6	0.3956	0.2261	0.4242



Table 3-5. The positive percentages of STEC genes for all ponds.

Pond Code	Positive STEC Genes (%)						All
	<i>stx-I</i>	<i>stx-II</i>	<i>eaeA</i>	<i>hly</i>	<i>rfbE</i>	<i>fliC</i>	
Pond 1	32.2	7.8	20	83.3	22.2	52.2	0.2
Pond 2	35.6	7.8	16.7	82.2	13.3	50	0.4
Pond 3	35.6	10	24.4	80	22.2	52.2	0.6
Pond 4	32.2	8.9	12.2	84.4	15.6	52.2	0.0
Pond 5	28.9	8.9	17.8	86.7	16.7	54.4	0.9
Pond 6	31.1	13.3	13.3	83.3	14.4	54.4	0.6

Table 3-6. List of *Salmonella* isolates and summary of tests applied to each *invA* positive sample.

Sample information			From RV	Isolation and Confirmation tests			NVSL, USDA*
Isolate code	Pond code	Sample date	PCR (<i>invA</i>)	BAM	XLT4	Latex	Serotype
SA4	Pond 4	12/23/2012	+				
SA32	Pond 2	04/22/2013	+				
SA40	Pond 4	05/02/2013	+				
SA55	Pond 1	12/12/2012	+	+	+	+	Saintpaul
SA70	Pond 4	02/15/2013	+	+	+	+	4, 5, 12:i:-
SA80	Pond 2	04/20/2013	+	+	+	+	Saintpaul
SA86	Pond 2	04/24/2013	+	+	+	+	Anatum_var._15+
SA92	Pond 2	05/24/2013	+	+	+	+	Hartford
SA103	Pond 1	12/26/2012	+	+	+	+	4, 12:i:-
SA106	Pond 4	12/26/2012	+	+	+	+	4, 12:i:-
SA133	Pond 1	04/30/2013	+	+	+	+	Baildon
SA139	Pond 1	05/21/2013	+	+	+	-	Javiana
SB38	Pond 2	05/07/2014	+				
SB63	Pond 3	12/30/2013	+	+	+	+	4, 5, 12:i:-
SB76	Pond 4	03/09/2014	+	+	+	+	4, 5, 12:i:-
SB77	Pond 5	03/09/2014	+	+	+	+	4, 12:i:-
SB78	Pond 6	03/09/2014	+	+	+	+	Anatum
SB90	Pond 6	05/15/2014	+	+	+	+	Newport
SB92	Pond 2	06/04/2014	+	+	+	+	Muenchen
SB111	Pond 3	01/15/2014	+	+	+	+	Rough_O:i:-
SB112	Pond 4	01/15/2014	+	-			
SB138	Pond 6	05/14/2014	+	+	+	-	Florida
SB218	Pond 2	03/12/2014	+	+	+	-	IV_50:z4,z23:-
SB220	Pond 4	03/12/2014	+	+	+	+	4, 5, 12:i:-
SB244	Pond 4	02/26/2014	+	+	+	+	Rubislaw
SB245	Pond 5	02/26/2014	+	+	+	+	4, 5, 12:i:-

* NVSL, USDA: National Veterinary Services Laboratories, US Department of Agriculture.

Table 3-7. Pearson product moment correlation coefficients (r) with *P*-values determined between each of the physical, chemical, and biological water attributes for all ponds combined.

Water Attributes	<i>E. coli</i>	Enterococcus	Air temperature (°C)	Water temperature (°C)	Conductivity (µS/cm)	pH	ORP (mV)	Turbidity (FAU)
Coliforms	0.5868* (<0.0001)	0.5787* (<0.0001)	0.0905* (0.0365)	0.1563* (0.0003)	0.1191* (0.0058)	-0.2094* (<0.0001)	-0.0672 (0.1209)	0.0851* (0.0495)
<i>E. coli</i>		0.6328* (<0.0001)	-0.0492 (0.2561)	-0.0004 (0.9919)	0.1805* (<0.0001)	-0.1983* (<0.0001)	-0.1170* (0.0068)	0.2187* (<0.0001)
Enterococcus			-0.0248 (0.5675)	0.1011* (0.0195)	0.1049* (0.0153)	-0.2104* (<0.0001)	-0.1027* (0.0176)	0.2880* (<0.0001)
Air temperature (°C)				0.7449* (<0.0001)	-0.1537* (0.0004)	0.3286* (<0.0001)	-0.0158 (0.7154)	0.0344 (0.4284)
Water temperature (°C)					-0.1568* (0.0003)	0.2484* (<0.0001)	-0.0597 (0.1682)	0.0028 (0.9494)
Conductivity (µS/cm)						0.0564 (0.1929)	-0.1949* (<0.0001)	0.1050 (0.0153)
pH							-0.1727* (<0.0001)	0.1063* (0.0141)
ORP (mV)								-0.0424 (0.3284)

* Correlation coefficients in bold are statistically significant ($P < 0.05$).

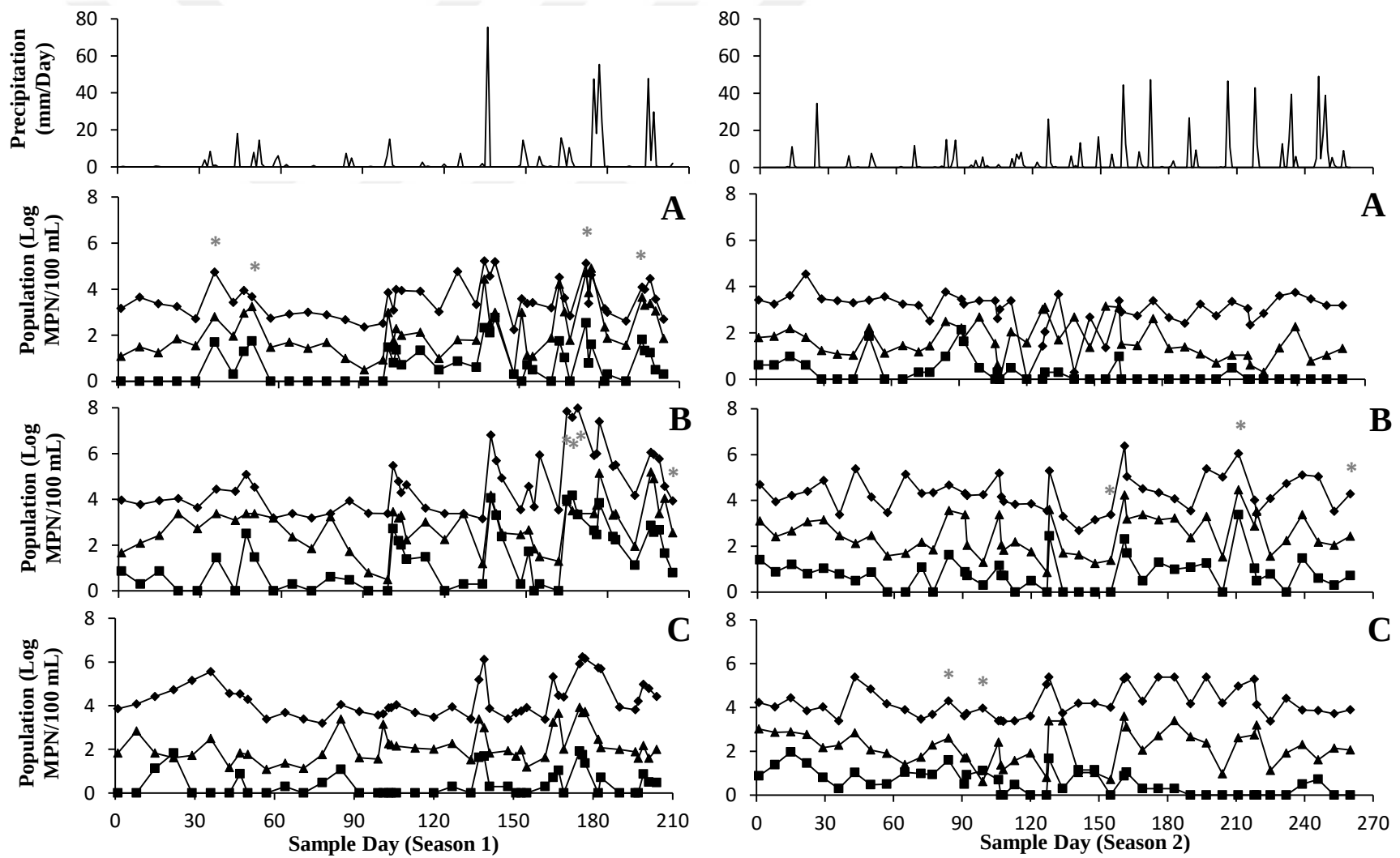


Figure 3-1. Most Probable Number of Total coliform (♦), Generic *E. coli* (■), and enterococci (▲) (Log MPN/100 mL) from six agricultural ponds (Pond 1: A, Pond 2: B, Pond 3: C, Pond 4: D, Pond 5: E, Pond 6: F) in Central Florida. *invA* positive sampling days were shown with “*”.

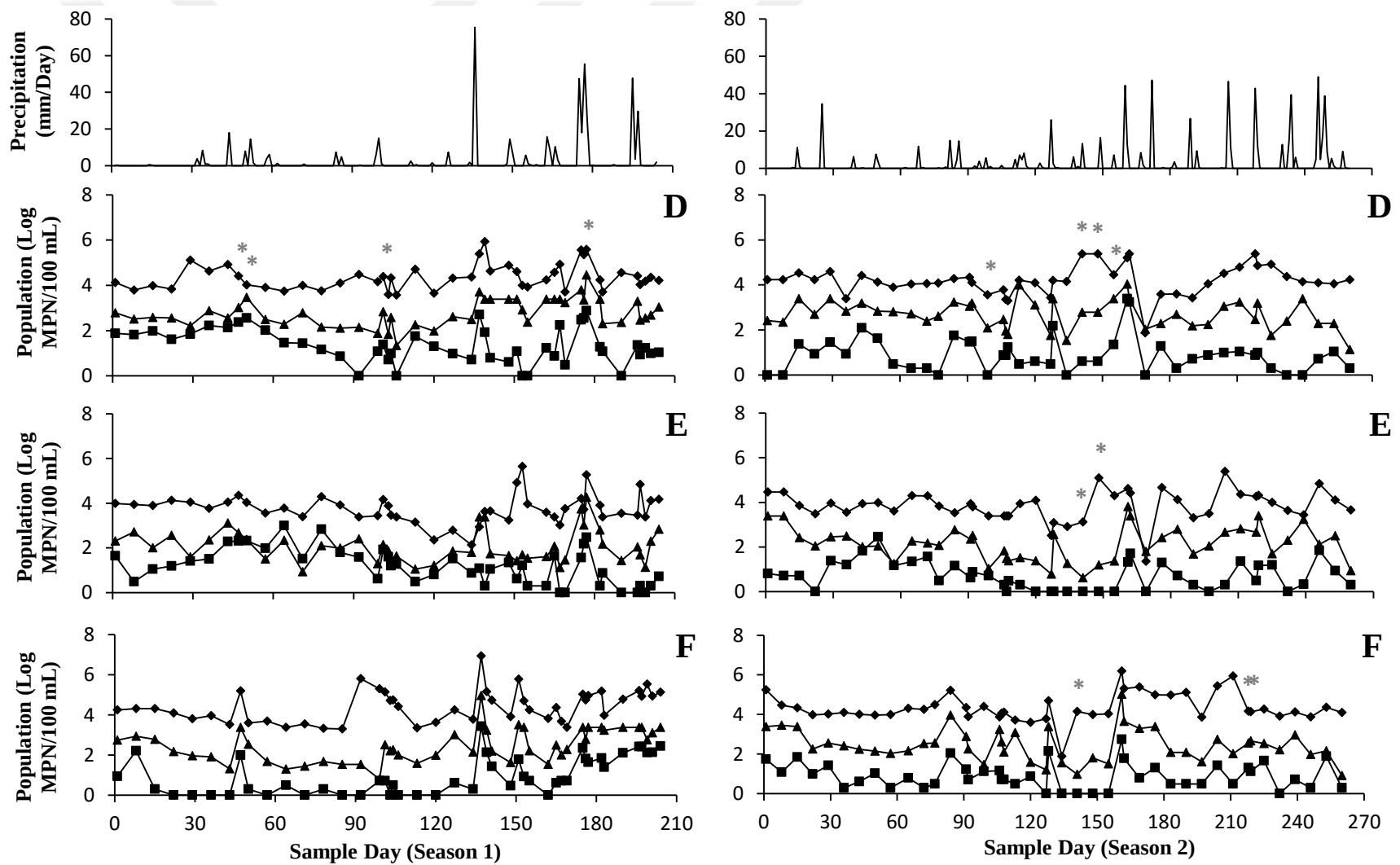


Figure 3-2. Most Probable Number of Total coliform (♦), Generic *E. coli* (■), and enterococci (▲) (Log MPN/100 mL) from six agricultural ponds (Pond 1: A, Pond 2: B, Pond 3: C, Pond 4: D, Pond 5: E, Pond 6: F) in Central Florida. *invA* positive sampling days were shown with “*”.

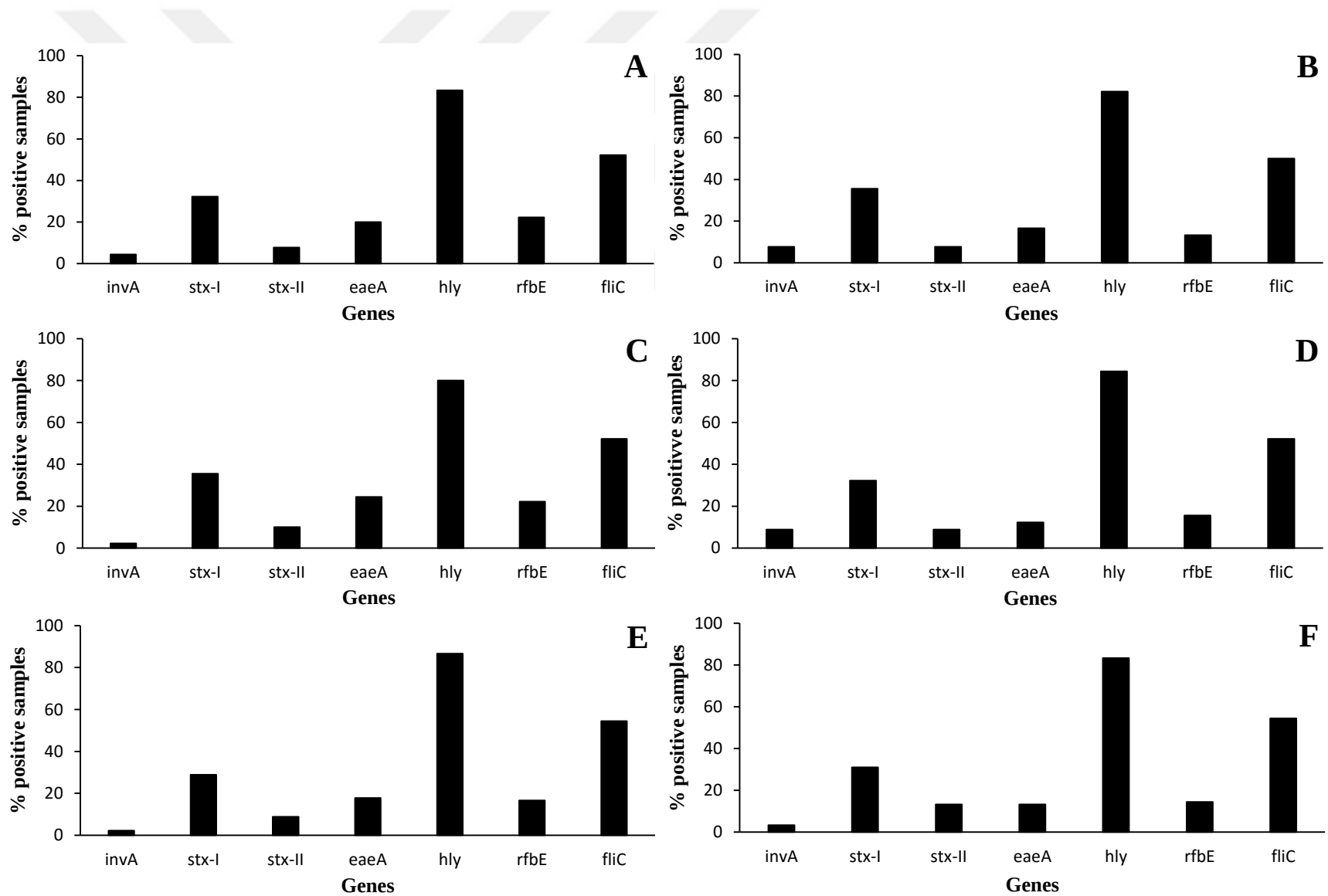


Figure 3-3. The presence of pathogen genes in six agricultural ponds (Pond 1: A, Pond 2: B, Pond 3: C, Pond 4: D, Pond 5: E, Pond 6: F) in central Florida (n=90 samples/pond).

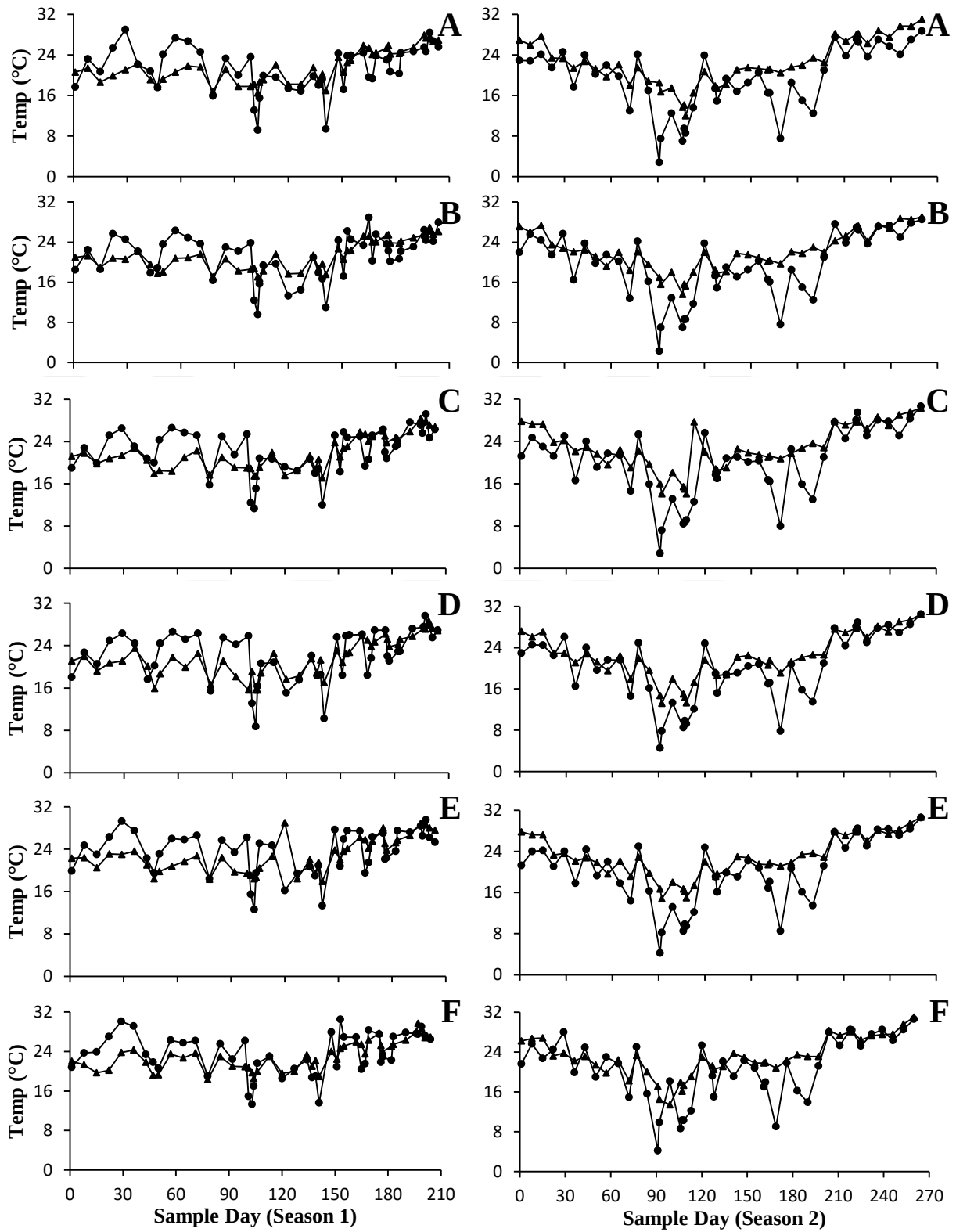


Figure 3-4. Air temperature (●) and Water temperature (▲) from six agricultural ponds (Pond 1: A, Pond 2: B, Pond 3: C, Pond 4: D, Pond 5: E, Pond 6: F) during 2012-2013 (46 data points) and 2013-2014 (44 data points) growing season in Central Florida.

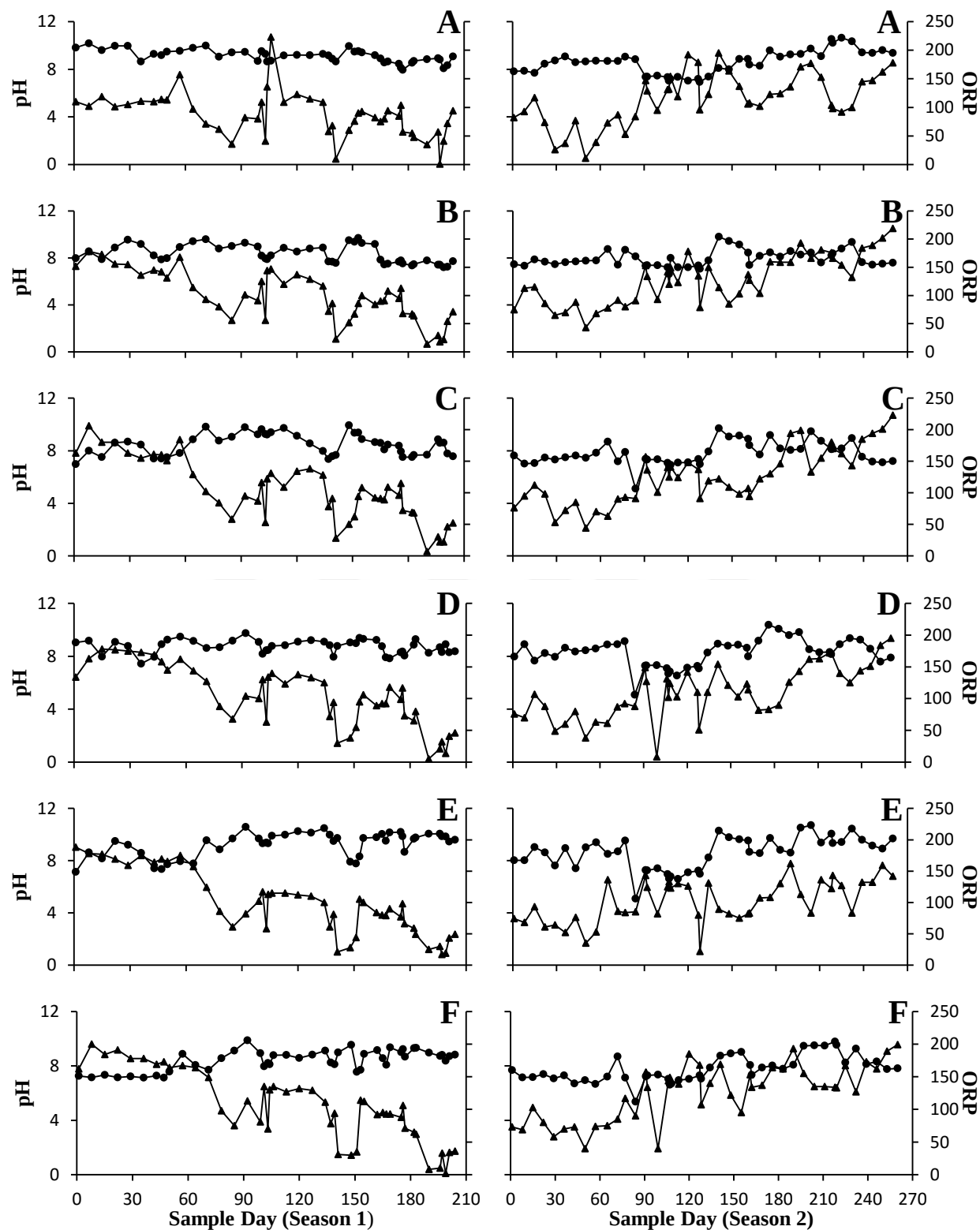


Figure 3-5. pH (●) and ORP (mV) (▲) from six agricultural ponds (Pond 1: A, Pond 2: B, Pond 3: C, Pond 4: D, Pond 5: E, Pond 6: F) during 2012-2013 (46 data points) and 2013-2014 (44 data points) growing season in Central Florida.

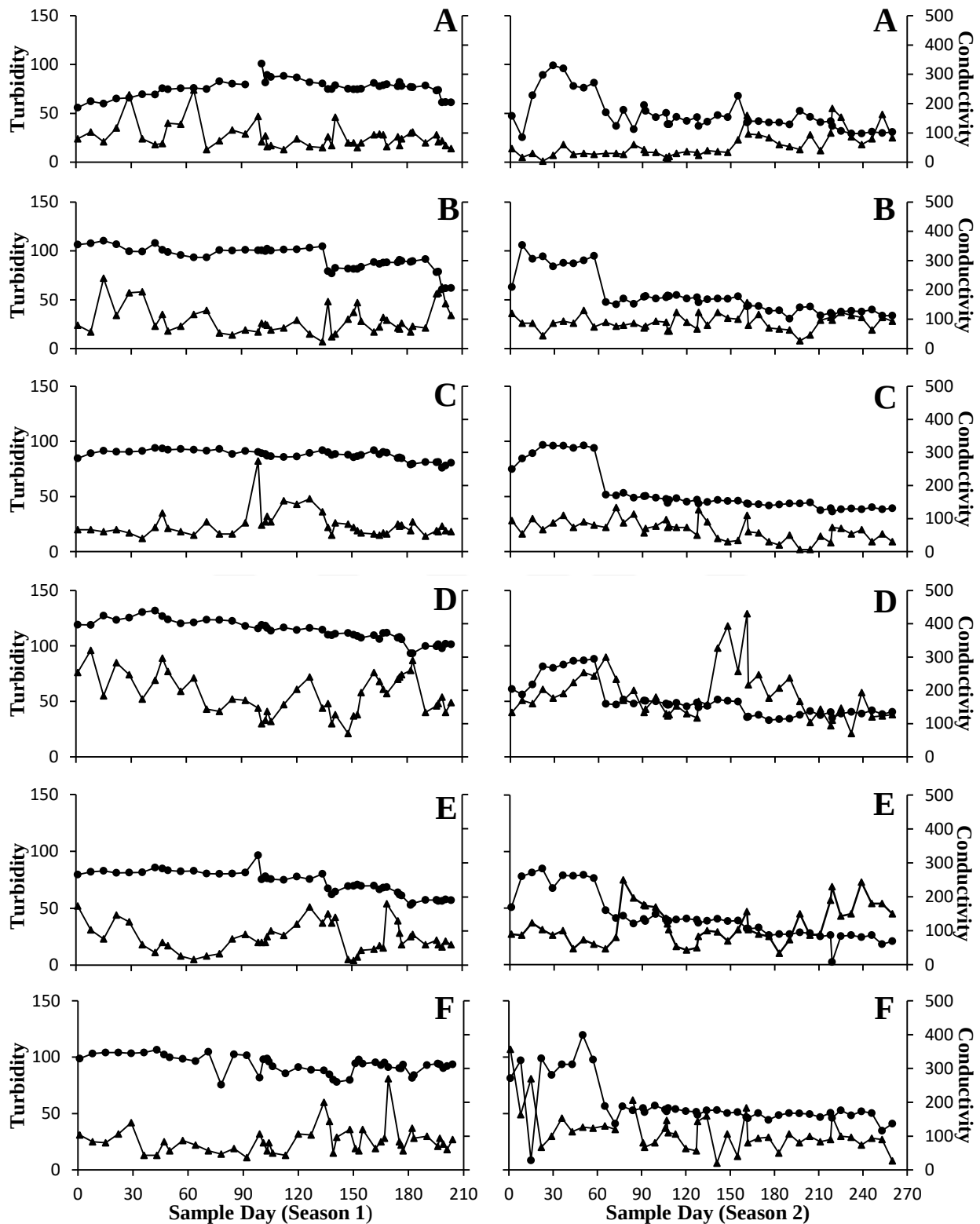


Figure 3-6. Conductivity ($\mu\text{S}/\text{cm}$) (●) and Turbidity (FAU) (▲) from six agricultural ponds (Pond 1: A, Pond 2: B, Pond 3: C, Pond 4: D, Pond 5: E, Pond 6: F) during 2012-2013 (46 data points) and 2013-2014 (44 data points) growing season in Central Florida.

CHAPTER 4

FATE OF STEC AND GENERIC *E. COLI* IN CENTRAL FLORIDA SURFACE WATERS AT DIFFERENT TEMPERATURES

Introduction

Agricultural water quality can be contaminated by direct human impact, animal activities, agricultural inputs, and environmental sources (Pachepsky et al., 2013). Pathogens can survive long enough in agricultural water to contaminate the produce and to reach primary host (Easton et al., 2005; Korhonen and Martikainen, 1991). Besides generic *E.coli* used as microbial indicator, shiga toxin-producing *Escherichia coli* (STEC) have been detected and isolated from surface waters that are used as agricultural surface water sources (Gu et al., 2013; Johnson et al., 2003; Strawn et al., 2013). Outbreaks due to the use of contaminated irrigation water is not commonly concluded with direct evidences, however, the potential risk cannot be ignored. Jacobsen and Bech (2012) suggests that one of the main sources of fresh produce contamination is irrigation water. An *E. coli* O157:H7 outbreak due to accidental contamination of well water for irrigation with water from lagoon used by dairy was investigated by the California Food Emergency Response Team (CalFERT, 2008). In 2006 multistate outbreak of *E. coli* O157:H7 associated with consumption of contaminated spinach, it was concluded that pathogen in surface water reached wells on the ranch and caused contamination (CalFERT, 2007).

The population of indicator microorganisms and pathogens in agricultural surface waters may vary depending on location and type of water source, agricultural activities around water source, presence of wild animals, weather events, runoff, precipitation, and natural microbial reservoirs in contact with water as bottom sediment, periphyton (a complex assemblage of algae, microinvertebrates, cyanobacteria, and detritus) (Anderson et al., 2005; Pachepsky et. al, 2011). The population of water quality indicator microorganisms and pathogens in irrigation waters tend to decrease along with time, and inversely proportional decay to temperature is noted. The effect

of temperature on *E. coli* in surface waters has been studied on samples from different region of the world under distinct conditions (Craig et al., 2004; Flint, 1987; Korhonen and Martikainen, 1991). Hellweger et al. (2009) established that microbial decay is biphasic and impacted by types of indicator microorganism or pathogen, temperature, solar radiation, various chemical parameters in water, and salinity. Competition with natural flora was implicated as the primary factor of decay, and the rate of decay varied in water samples from different sources (Flint, 1987). The diversity of physicochemical and biological of water sources in these studies make comparison of results difficult for researchers. The use of a standard water is important for comparison in microcosms studies. United States Environmental Protection Agency (EPA, 1987) developed a standard and easy to prepare water called as EPA Worst Case water for testing points-of-use microbiological water purifiers.

The objective of this study is to determine the fate of STEC and generic *E. coli* in Central Florida surface water at different temperatures. The study will provide better understanding of STEC survival in agricultural surface waters and more detailed risk assessment materials for growers and researchers in Florida.

Material and Methods

Temperature Determination

Temperature values (90 per pond) from six agricultural ponds were collected over two consecutive growing seasons in Central Florida as described in Chapter 3. The water temperature values at all sampling sites fluctuated between 15°C and 30°C. Mini experiments with a single *E. coli* O157:H7 strain were designed to see survival trends for 14 days in Pond 1, Pond 2, and Pond 3 water samples at 15 ± 1 , $22 \pm 1^\circ\text{C}$, and $30 \pm 1^\circ\text{C}$. For subsequent experiments, temperatures of 15 ± 1 and $25 \pm 1^\circ\text{C}$ and Pond 2 and Pond 3 water samples were used based on

water temperature data and mini survival experiment results. High and low water quality (Chapter 3) was also considered for the selection of ponds.

Pond Water Selection and EPA Worst Case Water

Water was collected in 10 L autoclavable carboy (Nalgene, Rochester, NY, USA) from the edge of ponds close to the extraction hose without causing sediment removal. The two ponds were sampled twice, with a one-week gap between each sampling. Enough water was collected at each sampling to complete a replicate was collected from each pond at a depth of 10 to 20 cm below the surface. Samples were transported to the laboratory (1 h) and stored at 4°C until the start of the experiment. Chemical and physical characteristic of water samples was measured within two hours of sampling as described in Chapter 3.

Sterile surface waters were prepared by autoclaving pond waters in 1 L glass bottles at 121°C, 15 psi, for 15 min. Psychochemical attributes were measured for sterile waters. EPA Worst Case water was prepared by mixing deionized water, 1500 mg/L dissolved sea salts (Sigma-Aldrich, St Louis, MO) and 10 mg/L humic acid (Sigma-Aldrich). Fine test dust (Powder Technology Inc., Burnsville, MN) were used to adjust final turbidity to 30 FAU. The pH of the EPA Worst Case water was 5.3. The same autoclave procedure as sterile surface water was applied to EPA Worst Case water.

Bacterial Cultures and Sample Preparation

Rifampicin (Rif; Fisher Bioreagents, Fair Lawn, NJ) resistant STEC (*E. coli* O145: Clinical isolate, Romaine Outbreak, O104: Clinical isolate, sprout outbreak, O111: Clinical isolate, apple juice outbreak, NY, O103: Venison outbreak, and O157: Clinical isolate, spinach outbreak) isolates and naladixic acid (Nal; Sigma-Aldrich, St Louis, MO) resistant generic *E.coli* (K-12) were used in the experiment. Rif resistant STEC strains were tested for Nal resistance to ensure that only desired population were counted on media supplemented with rifampicin.

Similarly, Nal resistant generic *E. coli* was tested for rifampicin resistance to ensure that only desired population were counted on media supplemented with Nal.

Rif resistant STEC strains and Nal resistant generic *E. coli* stored at $-80\text{ }^{\circ}\text{C}$, were streaked onto tryptic soy agar with Rif (TSAR $80\text{ }\mu\text{g /mL}$; Difco, Becton, Dickinson, Sparks, MD) and Nal (TSAN $50\text{ }\mu\text{g /mL}$), respectively. Agar plates were incubated at $35 \pm 2\text{ }^{\circ}\text{C}$ for $24 \pm 2\text{ h}$. One isolated colony of Rif resistant STEC and Nal resistant generic *E.coli* was transferred into tryptic soy broth with rifampicin (TSBR; Difco, Becton, Dickinson, Sparks, MD) and tryptic soy broth with Nal (TSBN), respectively. After incubation at $35 \pm 2\text{ }^{\circ}\text{C}$ for 18 h, one loopful of overnight growth was transferred into an additional tube of TSBR and TSBN for another incubation at $35 \pm 2\text{ }^{\circ}\text{C}$ for 18 h. Following incubation, cells were collected by centrifugation at $3000 \times g$ for 10 min. The supernatant was removed and 10 mL of 0.1% peptone water was vortexed with the pellet to wash cells. Rif resistant STEC and Nal resistant generic *E.coli* cells were washed for three times, and resuspended in 5 mL peptone to obtain concentration of cells ($10^9\text{--}10^{10}\text{ CFU/mL}$). Then cells in cocktail were diluted to get desired concentration (10^7 CFU/mL) before inoculation of the water samples.

During the 14 day mini experiment, survival of only the *E. coli* O157:H7 strain was evaluated at three temperatures, 15 ± 1 , 22 ± 1 , and $30 \pm 1^{\circ}\text{C}$, in agricultural waters from Pond 1, Pond 2, and Pond 3. Microcosms (100 mL) were prepared by filling in 120 mL sterile polypropylene cups, aseptically (Oxford, Mansfield, MA, USA). Inocula (1 mL) was added to each 100 mL sample, to achieve a final concentration of 10^5 CFU/mL . Lids were screwed tightly onto cups. Samples (1 mL) were taken and diluted if necessary for spread plating on days 0, 1, 3, 5, 7, and 14. Six repetitions were performed ($n=6$).

Water from two ponds (Pond 2 and Pond 3) and two incubation temperature (15 ± 1 and $25 \pm 1^\circ\text{C}$) were used for the rest of the study. Five STEC strains and generic *E. coli* were mixed to prepare one cocktail of STEC and generic *E. coli*. The same inoculation procedure was applied for non-sterile surface water, sterile surface water and EPA Worst Case water. Samples (1 mL) were taken and diluted if necessary for spread plating on days 0, 1, 3, 5, 7, 14, 28, 56, 84, 112, 140, and 168. Duplicate experiments with triplicate samples were performed at all temperatures (n=6).

The effect of protozoans was tested in a 14 day experiment in non-sterile agricultural surface waters at 15 ± 1 and $25 \pm 1^\circ\text{C}$. Cycloheximide (LeJeune et al., 2001: 20 mg/1 mL; MP Biomedicals Inc, Solon, OH, USA) was added to non-sterile surface water samples to eliminate protozoans 24 h before inoculation with STEC and Generic *E. coli* cocktail. Three replicates of the cycloheximide experiment were completed (n=3).

Microbiological Analysis

Both STEC and generic *E. coli* populations were determined by spread plating one mL inoculated samples from direct microcosm cups and following serial dilutions (0.1 mL) in 0.1% peptone water onto TSAR for STEC and TSAN for generic *E. coli* at each sampling time. Each sample was spread plated in duplicate. Plates were incubated at $35 \pm 2^\circ\text{C}$ for 24 h to achieve full recovery of survived and injured cells prior to counting. To increase the sensitivity and lower the limit of detection to (1 CFU/mL), 1 mL of the initial dilution was plated over 4 plates (0.25 mL/plate). When no colonies were observed, a MPN method was used to enumerate the population of STEC and generic *E. coli* in samples. A three-by-four tube MPN was performed to determine the cell concentration. Dilutions were as follows: 10 mL water in 10 mL double strength modified peptone water with pyruvate (mBPWp), 1 mL water in 9 mL single strength (mBPWp), and 0.1 mL water in 9 mL single strength (mBPWp). Triple strength mBPWp was

added to the remaining water sample (around 60 mL) and enriched in the original sample cup. MPN tubes were incubated for 24 ± 2 h at $35 \pm 2^\circ\text{C}$. After enrichment, a loopful of enrichment (10 μL) was streaked onto sorbitol MacConkey agar with rifampicin (SMACR; 80 $\mu\text{g/mL}$) for STEC and incubated for 24 h at $35 \pm 2^\circ\text{C}$. For generic *E. coli*, CHROMagar ECC (DRG, Mountainside, NJ, USA) with Nal was used. When typical colonies were observed, the result was noted as positive and MPN values were calculated using the U.S. Food and Drug Administration-Bacteriological Analytical Manual table (FDA, 1998).

Background generic *E. coli* population in pond waters used for inoculation were decided by using Quanti-Tray[®] 2000/Colilert[®] as described in Chapter 3 after water collection.

Statistical Analysis

STEC and generic *E. coli* concentration data from each of the replicates on each sampling time from microcosms at tested conditions were average and plotted against time after log-transformation. JMP Pro 11.0 (SAS, Cary, NC, USA) was used in determination of statistical significances using ANOVA and Tukey's HSD test. The critical *P*-value was set at 0.05.

Results

Microbiological and Physicochemical Properties of Waters

Water data was not collected at the time of water sampling for the mini experiment. Non-sterile surface water samples had generic *E. coli* population between <1 and 1 MPN/100 mL. In pond waters used for first replicate, the pH, conductivity ($\mu\text{S/cm}$), ORP (mV), and turbidity (FAU) values of non-sterile surface were 6.94, 378 $\mu\text{S/cm}$, 97 mV and 13 FAU in Pond 2 and 9.14, 192 $\mu\text{S/cm}$, 42 mV, and 31 FAU in Pond 3. The same physicochemical values for sterile water were in the same order as follows: 7.64, 291 $\mu\text{S/cm}$, 84 mV, and 7 FAU in Pond 2, and 9.73, 130 $\mu\text{S/cm}$, 88 mV, and 18 FAU in Pond 3.

In water samples used for second replicate, the pH, conductivity ($\mu\text{S}/\text{cm}$), ORP (mV), and turbidity (FAU) values of non-sterile surface were 7.40, 153 $\mu\text{S}/\text{cm}$, 96 mV, and 1 FAU in Pond 2 and 7.63, 132 $\mu\text{S}/\text{cm}$, 91 mV, and 13 FAU in Pond 3. The same psychochemical values for sterile water were as follows in the same order: 7.87, 156 $\mu\text{S}/\text{cm}$, 81 mV, and 1 FAU in Pond 2, and 7.64, 131 $\mu\text{S}/\text{cm}$, 96 mV, 12 FAU in Pond 3.

***E. coli* O157:H7 Non-sterile Surface Water**

Three temperatures, 15, 22, and 30°C, were tested to see survival capabilities of *E. coli* O157:H7 in the non-sterile surface water samples from Ponds 1, 2, and 3 for 14 days (Figure 4-1). Inoculated *E. coli* O157:H7 populations were between 5.2 and 5.3 Log CFU/mL with no statistical significance in non-sterile surface water microcosms at all tested temperatures and ponds at Day 0 ($P > 0.05$). All means with standard deviations for *E. coli* O157:H7 in non-sterile surface water are presented in Table 4-1. *E. coli* O157:H7 populations at all temperatures and pond waters increased or decreased maximum 0.3 Log CFU/mL at Day 1. At Day 3, the decline in *E. coli* O157:H7 population in Pond 1 was 0.3 Log CFU/mL at 15°C, 1.3 Log CFU/mL at 22°C, and 2.9 Log CFU/mL at 30°C, and concentrations were statistically significant from those in Pond 2 at 15°C and 22°C and in Pond 3 at 30°C ($P < 0.05$). In Pond 2, *E. coli* O157:H7 concentration decreased in the range from 2.4 to 3.1 Log CFU/mL at all tested temperatures at Day 3 ($P > 0.05$). The decrease in *E. coli* O157:H7 population was greater at warmer temperatures, similar to the other ponds, and was between 0.4 and 1.4 Log CFU/mL in Pond 3 (Figure 4-1). *E. coli* O157:H7 populations in at 15, 22, and 30°C were between 2.3 and 4.9 Log CFU/mL in Pond 1, 2.3 and 2.8 Log CFU/mL in Pond 2, and 3.8 and 4.9 Log CFU/mL in Pond 3 at Day 3. After seven days, *E. coli* O157:H7 concentration dropped below 1.0 Log CFU/mL in Pond 1 and Pond 2 at 22 and 30°C; and was 2.2 and 2.5 Log CFU/mL in Pond 3 at the same temperatures. *E. coli* O157:H7 populations stayed higher at 15°C in Day 7, and were 1.8 Log

CFU/mL in Pond 1, 1.7 Log CFU/mL in Pond 2, and 3.6 Log CFU/mL in Pond 3. On the last day of trial (Day 14), no statistically significant difference among *E. coli* O157:H7 concentrations were seen in all ponds and at all temperatures (≤ 1.0 Log CFU/mL) with the exception of Pond 3 at 15°C ($P > 0.05$), where populations remained higher. Based on these findings, only ponds 2 and 3, and storage at 15 ± 1 and 25 ± 1 °C were used for subsequent experiments.

Non-Sterile Surface Water

Inoculated populations of STEC and generic *E. coli* were between 5.4 and 5.8 Log CFU/mL in all non-sterile surface water microcosms from Pond 2 and Pond 3 at 15°C and 25°C at Day 0. All means with standard deviations for STEC and generic *E. coli* in non-sterile surface water are presented in Table 4-2. Declines started in both pathogenic and non-pathogenic *E. coli* concentrations at all tested conditions except for generic *E. coli* at 15°C, started at Day 1. Generic *E. coli* behaved similarly in both ponds until Day 28 at 15°C and until Day 14 at 25°C. The same pattern that was seen for generic *E. coli* was observed for STEC over the same time period; STEC population stayed higher than generic *E. coli* concentration by at least 0.4 Log CFU/mL at 15°C and 0.9 Log CFU/mL starting from Day 5 (Figure 4-2). At Day 28, STEC and generic *E. coli* populations ranged from 2.5 to 3.0 Log CFU/mL in non-sterile surface water from both ponds at 15 and 25°C. After Day 28, all tested samples demonstrated similar survival behaviors until the last sampling day (Figure 4-2). No statistically significant difference was calculated among both pathogen and non-pathogen *E. coli* concentrations for all conditions in non-sterile surface water samples at Day 56 at both temperatures ($P > 0.05$). STEC cocktail and generic *E. coli* concentration was above 1.3 and below 2.0 Log CFU/mL at 15 and 25°C in both ponds at Day 84 with the exception of generic *E. coli* population in Pond 3 samples incubated at 15°C. Similarly, both Pond 2 and Pond 3 microcosms had STEC and generic *E. coli* populations

between 1.0 to 1.9 Log CFU/mL at both temperatures on Day 112 except for generic *E. coli* population in Pond 3 samples incubated at 15°C. Generic *E. coli* populations in Pond 3 were constantly and significantly in lower concentrations than others and below 1 Log CFU/mL at 15°C on days 84 and 112 ($P < 0.05$). Starting at Day 140, no statistically significant differences in STEC and generic *E. coli* populations were seen amongst all tested samples at all conditions ($P > 0.05$). On the last sampling day (Day 168), the concentration of both STEC and generic *E. coli* remained in plate count ranges. At Day 168, STEC and generic *E. coli* decreased below 0.5 Log CFU/mL at all conditions. Enrichment was performed for only 1 to 5 replications out of 6 when it was necessary. Percentages for positive enrichment replications were noted on Table 4-2.

Calculated Log MPN/mL enrichment results for each STEC replicate are as follows: Pond 2-Day168-25°C 1.97 (1 sample); Pond 3-Day140-15°C 1.36 (1 Replicate); Pond 3-Day168-15°C 1.97, 1.97, 1.10 (3 samples). Calculated Log MPN/mL enrichment results for each generic *E. coli* replicate are as follows: Pond 2-Day168-15°C 1.97, 0.56, 1.46, 3.04, and 0.56 (5 samples); Pond 2-Day168-25°C 1.97 (1 sample); Pond 2-Day140-25°C 1.73 (1 sample); Pond 2-Day168-15°C <0.47, 0.56, 0.24, 0.24 (4 samples).

Sterile Surface Waters and EPA Worst Case Water

Inoculated population of STEC and generic *E. coli* were between 5.4 and 5.8 Log CFU/mL in all sterile surface water from Pond 2 and Pond 3 and EPA worst case water microcosms at 15°C and 25°C at Day 0. In general, very similar survival trends for pathogenic and non-pathogenic *E. coli* were seen at all tested conditions (Figure 4-3). Both generic *E. coli* and STEC stayed high in concentrations over 3 Log CFU/mL in sterile pond waters over the 168 day storage.

With the exception of inoculations in Pond 3 water, concentrations of both generic and STEC remained stable in all sterile surface water and EPA worst case water at 15°C until the end

of sampling period, and ranged between 4.8 and 6.4 Log CFU/mL. Constant and slow STEC population decline occurred in Pond 3 at 15°C in total of 2 Log CFU/mL until Day 168. Generic *E. coli* concentration in Pond 3 at 15°C dropped 1.3 Log CFU/mL at Day 3, however, remained over 6.1 Log CFU/mL for rest of the experiment starting from Day 28. Generic *E. coli* populations in Pond 2, Pond 3, and EPA Worst Case water were more than STEC populations up to 2.8 Log CFU/mL all the time at 15°C. At day 168, sterile surface water samples, and EPA Worst Case water had pathogenic and non-pathogenic *E. coli* populations ranging from 3.7 to 6.5 Log CFU/mL at 15°C.

STEC and generic *E. coli* populations behaved slightly different in sterile pond water and EPA Worst Case water at higher temperatures. At Day 14, STEC and generic *E. coli* populations in Pond 2, Pond 3, and EPA Worst Case water increased up to 0.8 Log CFU/mL, and reached over 6.0 Log CFU/mL at 25°C. STEC and generic *E. coli* populations in all sterile water samples at 25°C ranged between 4.4 and 6.6 Log CFU/mL from Day 0 to Day 84. STEC concentrations stayed higher than generic *E. coli* in Pond 2 and Pond 3 water starting at Day 28 until the last sampling day at 25°C. At day 168, sterile surface water samples had pathogenic and non-pathogenic *E. coli* populations ranging from 4.4 to 5.4 Log CFU/mL at 25°C. A similar survival trend to sterile pond waters was observed in EPA Worst Case water after day 112. STEC and generic *E. coli* concentrations in Pond 2 and Pond 3 ranged from 4.4 to 4.9 Log CFU/mL at Day 168. Generic *E. coli* population in EPA Worst Case water at 25°C decreased 3.1 Log CFU/mL in total, and was 2.8 Log CFU/mL that was 1 Log CFU/mL less than STEC at the last day of sampling at 25°C as seen in Figure 4-3.

Effect of Temperature on *E. coli* Survival in EPA Worst Case Water, Sterile and Non-sterile Surface Water

During the course of the 168-day sampling period, both STEC and generic *E. coli* populations remained in high concentrations in sterile surface water and EPA Worst Case water at all temperatures. Starting at day 7, STEC concentrations at 15°C entered a slow decline trend in Pond 3 water, and dropped to 3.7 Log CFU/mL at last sampling day. Both STEC and generic *E. coli* concentrations remained between 4.1 and 6.5 Log CFU/mL in Pond 2 and EPA Worst Case water at 15°C at Day 168 over the 168 day storage. Both pathogenic and non-pathogenic *E. coli* populations in sterile surface water were not significantly different from those in EPA Worst Case water for 168 days of experiment (Figure 4-3, Figure 4-4). No significant decrease in STEC and generic *E. coli* populations occurred in EPA Worst Case water except for samples incubated at 25°C over the 168 day storage. At higher temperatures, STEC and generic *E. coli* populations were lower in EPA Worst Case water than sterile pond waters after Day 84. The population of STEC and generic *E. coli* was 3.8 and 2.8 Log CFU/mL in EPA Worst Case water at 25°C at Day 168, respectively. Sterile water samples from both ponds at 25°C had STEC and generic *E. coli* concentrations between 4.4 and 5.4 Log CFU/mL at day 168.

STEC and generic *E. coli* populations in non-sterile water samples from Pond 2 and Pond 3 were very close to those in sterile water and EPA Worst Case water one day after inoculation. Starting at Day 3, the non-sterile surface water had significantly lower both pathogenic and non-pathogenic *E. coli* concentrations compared to the sterile surface water and EPA Worst Case water at all tested temperatures. At Day 28, both STEC and generic *E. coli* populations decreased below 3.0 Log CFU/mL in non-sterile pond water samples; sterile pond water and EPA Worst Case water had the *E. coli* populations between 4.3 and 6.3 Log CFU/mL. Over the 168 day storage, declines in both STEC and generic *E. coli* populations were completely different in

sterile pond waters from non-sterile samples. STEC and generic *E. coli* populations dropped below 1 Log CFU/mL in non-sterile surface water at all temperatures at Day 168 compare to those in sterile surface water and EPA Worst Case water that were over 2.8 Log CFU/mL.

At Day 0 and 1, *E. coli* O157:H7 concentration was not significantly different than STEC and generic *E. coli* concentration in Pond 2 and Pond 3 at 15°C. *E. coli* O157:H7 population in non-sterile surface water were significantly different from STEC and generic *E. coli* populations in the same water at Day 14 (Figure 4-2, Figure 4-1). At Day 14, STEC and generic *E. coli* concentrations were around three times higher than *E. coli* concentrations in non-sterile pond water samples (Table 4-2, Table 4-1).

Effect of Protozoans in Non-sterile Surface Water

STEC and generic *E. coli* were tested in non-sterile surface water supplemented with cycloheximide in triplicate (n=3). Differences mentioned below were not statistically compared. The survival of STEC and generic *E. coli* at all conditions (Pond 2 and Pond 3; 15°C and 25°C; with cycloheximide and without cycloheximide) are shown in Figure 4-5. At Day 0, STEC and generic *E. coli* concentrations in Pond 2 and Pond 3 waters with and without cycloheximide were not significantly different and in the range from 5.4 to 5.8 Log CFU/mL. No important changes occurred in pathogenic and non-pathogenic *E. coli* populations at Day 1 at all conditions except for the samples with cycloheximide at 15°C that had increases up to 0.8 Log CFU/mL (Figure 4-5 A1). At Day 3, both STEC and generic *E. coli* populations in both pond waters with cycloheximide were up to 0.7 Log CFU/mL at 15°C and 1.0 Log CFU/mL 25°C higher than those in the same water without cycloheximide. Both STEC and generic *E. coli* populations in Pond 2 and Pond 3 waters with cycloheximide had higher concentration than water samples without cycloheximide at 15°C at Day 5; *E. coli* populations in non-sterile surface water samples without cycloheximide at 25°C were lower or close to those in samples with cycloheximide. At

Day 7, both STEC and generic *E. coli* concentrations in non-sterile surface water with cycloheximide was higher than ones in samples without cycloheximide. Population differences between cells in pond waters with and without cycloheximide was 1.3 Log CFU/mL higher for STEC and 1.5 Log CFU/mL greater for generic *E. coli* at 15°C. Opposite situation happened at 25°C, and STEC and generic *E. coli* populations in the samples without cycloheximide stayed greater up to 0.9 Log CFU/mL at Day 7 with the exception of generic *E. coli* concentration in Pond 3. Similarly, STEC and generic *E. coli* populations stayed higher in water samples with cycloheximide at 15°C and lower 25°C at Day 14.

Discussion

Two representative temperatures were chosen as incubation temperatures based on the water temperature ranges measured for two consecutive growing seasons (2012/2013, 2013/2014) in Central Florida as mentioned in the surface water survey study in Chapter 3. The temperatures measurements in previous work were performed in the field (90 times) during sampling that means actual water temperatures in the field conditions were used in the determination of experiment parameters. The measured temperatures fluctuated between 15°C and 30°C. The measured water temperatures did not go below 15°C even during freeze events in winter months. In general, the growing season in Central Florida starts at the beginning of fall and slows down at the end of spring (Brown et al., 2015). Crops are not planted in the summer due to excessively hot weather for crops.

During the initial trial with *E. coli* O157:H7, three temperatures (15°C, 22°C, and 30°C) were used to develop preliminary results prior to determining final temperatures for the long term experiment. For subsequent experiments, temperatures of 15 ± 1 and 25 ± 1 °C and Pond 2 and Pond 3 water samples were used based on water temperature data and *E. coli* O157:H7 survival experiment results. The selected temperatures of 15°C and 25°C were considered

representative daily temperatures during growing seasons. Only effect of temperature was tested as external factor on survival of pathogenic and non-pathogenic *E. coli* in surface waters from Central Florida. It is important to note that the effect of sunlight was ignored in here. The inactivation rate of *E. coli* in estuary and irrigation pond water was greater under sunlight than in the darkness (Chadran and Hatha, 2005; Maïga et al., 2009).

In previous work (Chapter 3), a total of six ponds were monitored to establish microbial water quality profile for each pond. Two of them, the one with the lowest water quality (Pond 2) and the one with the greatest water quality (Pond 3) were selected for use in the survival study. It should be noted that both agricultural ponds used in this study met the current recommendations by FDA (2015) ($GM \leq 126$ *E. coli* and $STV \leq 410$ *E. coli* in 100 mL) for establishing a microbial water quality profile 100.0% of the time. No statistical difference was calculated between non-sterile Pond 2 and Pond 3 for STEC concentrations at any time point at 15 and 25°C until the last sampling day (Day 168) ($P > 0.05$). Similar results were obtained for generic *E. coli* in general, and generic *E. coli* populations were statistically the same in Pond 2 and Pond 3 at any sampling time except for Day 84 and 112 at 15°C and Day 1 at 25°C ($P > 0.05$). The limited effect of different non-sterile surface water sources was observed on STEC and generic *E. coli* survival in Central Florida surface waters.

The survival capabilities of pathogenic and non-pathogenic *E. coli* in non-sterile surface waters was different over the first 14 days, however not significant long term. Fish and Pettibone (1995) observed that *E. coli* densities in fresh water decreased gradually over two weeks. There is no statistical difference between STEC and generic *E. coli* populations in Pond 2 at 15 and 25°C after Day 7 ($P > 0.05$). In Pond 3, no statistical significance was calculated between STEC and generic *E. coli* populations after Day 28 at 15°C and after Day 7 at 25°C ($P > 0.05$). These

findings are supported by a study that compare the survival capabilities of STEC and non-STEC in fresh water ecosystem over 4.5 days (Mauro et al., 2013). In this study, the authors suggest that the presence of *stx* gene in *E. coli* provide the bacteria an advantage for survival compare to those without *stx* genes by escaping from grazing and killing protists with *stx* production (Mauro et al., 2013). Here, both pathogenic and non-pathogenic *E. coli* survived for 168 days in non-sterile surface water at all tested conditions. Almost all enrichment process was applied at last sampling day of experiment when population is below the detection limit of 1 CFU/mL. Similar survival trends and close generic *E. coli* and STEC populations observed for long term might be explained that non-pathogenic *E. coli* obtained *stx* gene from pathogenic cells or environment. Both STEC and generic *E. coli* were inoculated microcosms together, and the possibility of cell interaction cannot be ignored. Although no test for the presence of *stx* gene for survived generic *E. coli* cells was applied here, the effect of protozoa on survival of *E. coli* was tested. Cycloheximide added for inhibition of eukaryotes reduced differences in STEC and generic *E. coli* concentrations that was seen in non-sterile pond water without cycloheximide during first two weeks (Figure 4-5 A2, B2). This supports acquisition and use of *stx* gene against predators by generic *E. coli* in microcosms.

Higher temperatures did not have important impact on speed of decay for STEC and generic *E. coli* in non-sterile surface waters in this study. *E. coli* K12 survived up to 15 days at 4°C in untreated river water, and die-off were proportional to increase of temperature, 4 > 15 > 25 > 37°C (Flint, 1987). In Finland, contradictory results were reported that *E. coli* inactivation rates at 4°C and 25°C in water sample from eutrophic lake were significantly different in 44 day survival study (Korhonen and Martikainen, 1991). Craig et al. (2004) found that *E. coli* survival had an inverse relationship with temperature in recreational coastal water. In another study,

generic *E. coli* and *E. coli* O157:H7 had lower die-off rate in colder water (Easton et al., 2005). This suggest that contamination risk is high throughout growing season in Central Florida due to colder surface water during the growing season.

E. coli O157:H7 populations in non-sterile surface water decreased more rapidly than the STEC. Three temperatures in three ponds were tested for survival of *E. coli* O157:H7 to be able to compare the characteristics of individual strain besides the determination of experiment parameters based on preliminary results. At Day 14, *E. coli* O157:H7 concentration went below 1 Log CFU/mL almost at all conditions except for those in Pond 3 at 15 and 22°C. However, STEC populations remained above 2.7 Log CFU/mL. This suggests the importance of using multiple serotypes in survival microcosm survival studies.

The use of generic *E. coli* as microbial water quality indicator may not be the best, but demonstrated similar survival to STEC concentration in Central Florida surface waters. The decay of pathogenic and non-pathogenic *E. coli* is statistically different in the first week of survival period at most of the tested conditions as mentioned before ($P < 0.05$). Though it is a difference around 0.1 Log CFU/mL, STEC survived better over the first week than generic *E. coli*. However, highly similar survival trends between pathogenic and non-pathogenic *E. coli* help us to estimate potential risk of contamination by looking at indicator microorganism concentration for the course of experiment. Easton et al. (2005) reported that *E. coli* O157:H7 had twice faster die-off than generic *E. coli*. In this study, sewage contaminated waters were evaluated, so the effect of nutrient availability and the presence of place to hide from predators would be high. Higher nutrient content increases the survival of both non-pathogenic *E. coli* and STEC in water (Hutchinson et al., 2005; McFeters and Stuart, 1972). Also, Easton et al. (2005) quantified generic *E. coli* by using a MPN based method (Colilert®-18, IDEXX) and enumerated

E. coli O157:H7 with direct plate count technique. The sensitivity differences between methods should be considered.

FDA (2015) requires the establishment of a microbial water quality profile by sampling agricultural water at least twenty times in two year period. Surface waters are open to direct human impact and animal exposure that are considered as contamination sources to open water bodies. The survival difference between generic *E. coli* and STEC lessen along with time in non-sterile surface water. There are studies that supports and do not support the correlation between pathogen and the presence of generic *E. coli* as microbial indicator. In a study conducted in Central Coast of California, Benjamin et al. (2013) observed that generic *E. coli* population did not significantly associate with the presence of either *E. coli* O157 or *Salmonella* in water or sediment. The abundance and distribution of *stx* gene did not correlate with generic *E. coli* in recreational waters and coastal waterbodies (Smith et al., 2009; Walters et al., 2011). Mauro et al. (2013) also found that STEC levels do not correlate with the overall generic *E. coli* levels in freshwater ecosystem. However, higher correlations were observed at sites where indicator microorganisms and pathogens are detected in high populations for extended period of time (Payment and Locas, 2011).

Survival studies of pathogen and indicator microorganisms in water samples have been done in various type of waters including, agricultural, recreational, estuary, and other sources at different part of world. Indicator microorganisms and pathogens populations may change with internal factors including type of water source, the presence of predators, bacteriophage lysis, physicochemical attributes of water, the presence of algae, nutrient availability, and its assemblage with other organisms (Chadran and Hatha, 2005). These internal factors may vary drastically depending on environmental factors. Environmental factors can be eliminated in

water survival studies, but internal factors that decide fate of cells will remain in water samples. Comparison of mi without any tools as inactivation rate and type between water survival studies is challenging, and not convincing most of the time. EPA Worst Case water can be used as standard medium to compare water survival studies based on its performance in this study as mentioned below.

Generic *E. coli* and STEC survived in sterile agricultural surface waters and EPA worst case water until the course of experiment with fluctuations in concentration. *E. coli* survived more than 260 days in filtered and autoclaved river water at 25°C (Flint, 1987). Increase in *E. coli* population was seen until Day 28, especially at 25°C. Similarly, Fish and Pettibone (1995) reported the growth of *E. coli* in sterile creek water for ten days after inoculation. Fluctuation in both pathogenic and non-pathogenic *E. coli* population were around 5.0 ± 1.1 and 5.0 ± 1.5 Log CFU/mL at 15°C in all type of tested sterile waters, respectively. STEC and generic *E. coli* concentrations in EPA Worst Case water stayed 95.8% and 58.3% of time higher than those in sterile surface waters at 15°C. Particularly, generic *E. coli* population in sterile surface water were greater all the time after Day 56 at 15°C. EPA Worst Case water can be considered as a convenient standard medium at 15°C for STEC survival studies in surface water from Central Florida. At 25°C, pathogenic and non-pathogenic *E. coli* concentrations fluctuated between 5.4 and 6.6 Log CFU/mL until Day 84 in sterile agricultural surface waters and EPA worst case water. However, generic *E. coli* and STEC concentrations in EPA Worst Case water were up to 1.1 and 2.0 Log CFU/mL less than those in sterile Pond waters starting from Day 112. STEC and generic *E. coli* concentrations in EPA Worst Case water stayed 33.3% and 58.3 of time higher than those in sterile surface waters at 25°C. STEC and generic *E. coli* population in sterile surface water was greater than those in EPA Worst Case water all the time starting from Day 28

and Day 56 at 25°C, respectively. These results suggest that EPA Worst Case water does not remain more cells than sterile surface water at 25°C for both Generic *E. coli* and STEC in long term *E. coli* survival studies. However, it might be used as standard medium for comparison due to small differences.



Table 4-1. Comparison of the concentration of *E. coli* O157:H7 at different temperatures.

Days	Mean $\pm$ SD <i>E. coli</i> O157:H7 population (Log CFU/mL)*								
	15°C	Pond 1 22°C	30°C	15°C	Pond 2 22°C	30°C	15°C	Pond 3 22°C	30°C
0	5.2 $\pm$ 0.0A	5.2 $\pm$ 0.1A	5.2 $\pm$ 0.0A	5.2 $\pm$ 0.0A	5.2 $\pm$ 0.1A	5.3 $\pm$ 0.0A	5.2 $\pm$ 0.1A	5.2 $\pm$ 0.1A	5.2 $\pm$ 0.1A
1	5.1 $\pm$ 0.1AB	5.0 $\pm$ 0.0AB	4.9 $\pm$ 0.1B	5.2 $\pm$ 0.1AB	5.2 $\pm$ 0.0AB	5.3 $\pm$ 0.2A	5.1 $\pm$ 0.2AB	5.3 $\pm$ 0.4AB	5.3 $\pm$ 0.5A
3	4.9 $\pm$ 0.1A	3.9 $\pm$ 0.2B	2.3 $\pm$ 0.2C	2.8 $\pm$ 0.2C	2.5 $\pm$ 0.3C	2.2 $\pm$ 0.4C	4.8 $\pm$ 0.3A	4.9 $\pm$ 0.3A	3.8 $\pm$ 0.9B
5	3.8 $\pm$ 0.6AB	1.4 $\pm$ 0.4D	1.2 $\pm$ 0.2D	2.1 $\pm$ 0.3CD	1.7 $\pm$ 0.2D	1.3 $\pm$ 0.1D	4.6 $\pm$ 0.1A	3.7 $\pm$ 0.4AB	2.9 $\pm$ 1.1BC
7	1.8 $\pm$ 0.4BC	0.9 $\pm$ 0.6CD	0.5 $\pm$ 0.4D	1.7 $\pm$ 0.3BC	0.9 $\pm$ 0.3CD	0.3 $\pm$ 0.3D	3.6 $\pm$ 0.4A	2.2 $\pm$ 0.5B	2.5 $\pm$ 1.1B
14	0.3 $\pm$ 0.7B	0.0 $\pm$ 0.0B	0.0 $\pm$ 0.0B	0.8 $\pm$ 0.7AB	0.5 $\pm$ 0.6AB	0.0 $\pm$ 0.0B	1.6 $\pm$ 0.4A	1.0 $\pm$ 0.9AB	0.8 $\pm$ 0.9AB

* Mean $\pm$ standard deviation of duplicate samples from each of six replications. Within rows, means with different upper case letters indicate significant difference among the compared values at a particular time interval ($P \leq 0.05$). No enrichment applied.

Table 4-2. Comparison of the concentration of STEC and generic *E. coli* in non-sterile pond water at different temperatures.

Mean $\pm$ SD <i>E. coli</i> population (Log CFU/mL)*								
Days	Pond 2				Pond 3			
	STEC		Generic <i>E. coli</i>		STEC		Generic <i>E. coli</i>	
	15°C	25°C	15°C	25°C	15°C	25°C	15°C	25°C
0	5.8 $\pm$ 0.1AB	5.8 $\pm$ 0.1A	5.6 $\pm$ 0.1BC	5.7 $\pm$ 0.2AB	5.7 $\pm$ 0.1AB	5.7 $\pm$ 0.1AB	5.4 $\pm$ 0.1C	5.6 $\pm$ 0.2BC
1	5.5 $\pm$ 0.2AB	5.4 $\pm$ 0.1AB	5.6 $\pm$ 0.1A	5.5 $\pm$ 0.2AB	5.6 $\pm$ 0.1A	5.3 $\pm$ 0.2BC	5.5 $\pm$ 0.2AB	5.2 $\pm$ 0.1C
3	4.9 $\pm$ 0.4AB	4.6 $\pm$ 0.2B	4.8 $\pm$ 0.1AB	3.4 $\pm$ 0.2C	5.0 $\pm$ 0.1A	4.8 $\pm$ 0.1AB	4.7 $\pm$ 0.1AB	3.5 $\pm$ 0.2C
5	4.4 $\pm$ 0.5A	4.2 $\pm$ 0.1AB	3.8 $\pm$ 0.1BC	3.2 $\pm$ 0.1D	4.3 $\pm$ 0.2A	4.4 $\pm$ 0.1A	3.5 $\pm$ 0.3CD	3.3 $\pm$ 0.2D
7	3.8 $\pm$ 0.2A	4.0 $\pm$ 0.2A	3.3 $\pm$ 0.2B	3.1 $\pm$ 0.2B	3.9 $\pm$ 0.4A	4.2 $\pm$ 0.1A	3.2 $\pm$ 0.2B	2.8 $\pm$ 0.5B
14	3.3 $\pm$ 0.1ABC	3.2 $\pm$ 0.1ABC	2.9 $\pm$ 0.3CD	3.1 $\pm$ 0.5BCD	3.6 $\pm$ 0.2A	3.4 $\pm$ 0.1AB	2.8 $\pm$ 0.2D	3.1 $\pm$ 0.1BCD
28	2.8 $\pm$ 0.1ABC	2.7 $\pm$ 0.2ABC	2.8 $\pm$ 0.2ABC	2.6 $\pm$ 0.3BC	3.0 $\pm$ 0.2A	3.0 $\pm$ 0.2AB	2.5 $\pm$ 0.2C	2.9 $\pm$ 0.2AB
56	2.1 $\pm$ 0.2A	2.3 $\pm$ 0.6A	2.2 $\pm$ 0.5A	2.2 $\pm$ 0.5A	2.1 $\pm$ 0.4A	2.2 $\pm$ 0.4A	1.6 $\pm$ 0.5A	2.5 $\pm$ 0.6A
84	1.5 $\pm$ 0.3A	1.9 $\pm$ 0.5A	1.7 $\pm$ 0.3A	1.6 $\pm$ 0.4A	1.3 $\pm$ 0.4AB	1.7 $\pm$ 0.5A	0.6 $\pm$ 0.7B	1.8 $\pm$ 0.6A
112	1.4 $\pm$ 0.4AB	1.6 $\pm$ 0.5AB	1.4 $\pm$ 0.3AB	1.7 $\pm$ 0.5AB	1.0 $\pm$ 0.7BC	1.8 $\pm$ 0.4A	0.5 $\pm$ 0.5C	1.9 $\pm$ 0.6A
140	1.1 $\pm$ 0.4A	1.1 $\pm$ 0.7A	0.6 $\pm$ 0.6A	1.0 $\pm$ 0.8A	0.7 $\pm$ 0.6 ^a A	1.6 $\pm$ 0.6A	0.5 $\pm$ 0.6 ^a A	1.5 $\pm$ 0.6A
168	0.3 $\pm$ 0.3A	0.3 $\pm$ 0.2 ^a A	0.1 $\pm$ 0.2 ^a A	0.1 $\pm$ 0.2 ^a A	0.3 $\pm$ 0.3 ^a A	0.3 $\pm$ 0.4A	0.0 $\pm$ 0.0 ^b A	0.5 $\pm$ 0.4A

* Mean $\pm$ standard deviation of duplicate samples from each of six replications. Within rows, means with different upper case letters indicate significant difference among the compared values at a particular time interval ($P \leq 0.05$).

a Enrichments were applied; 100% of enriched samples are positive with STEC.

b Enrichments were applied; 75% of enriched samples are positive with STEC.

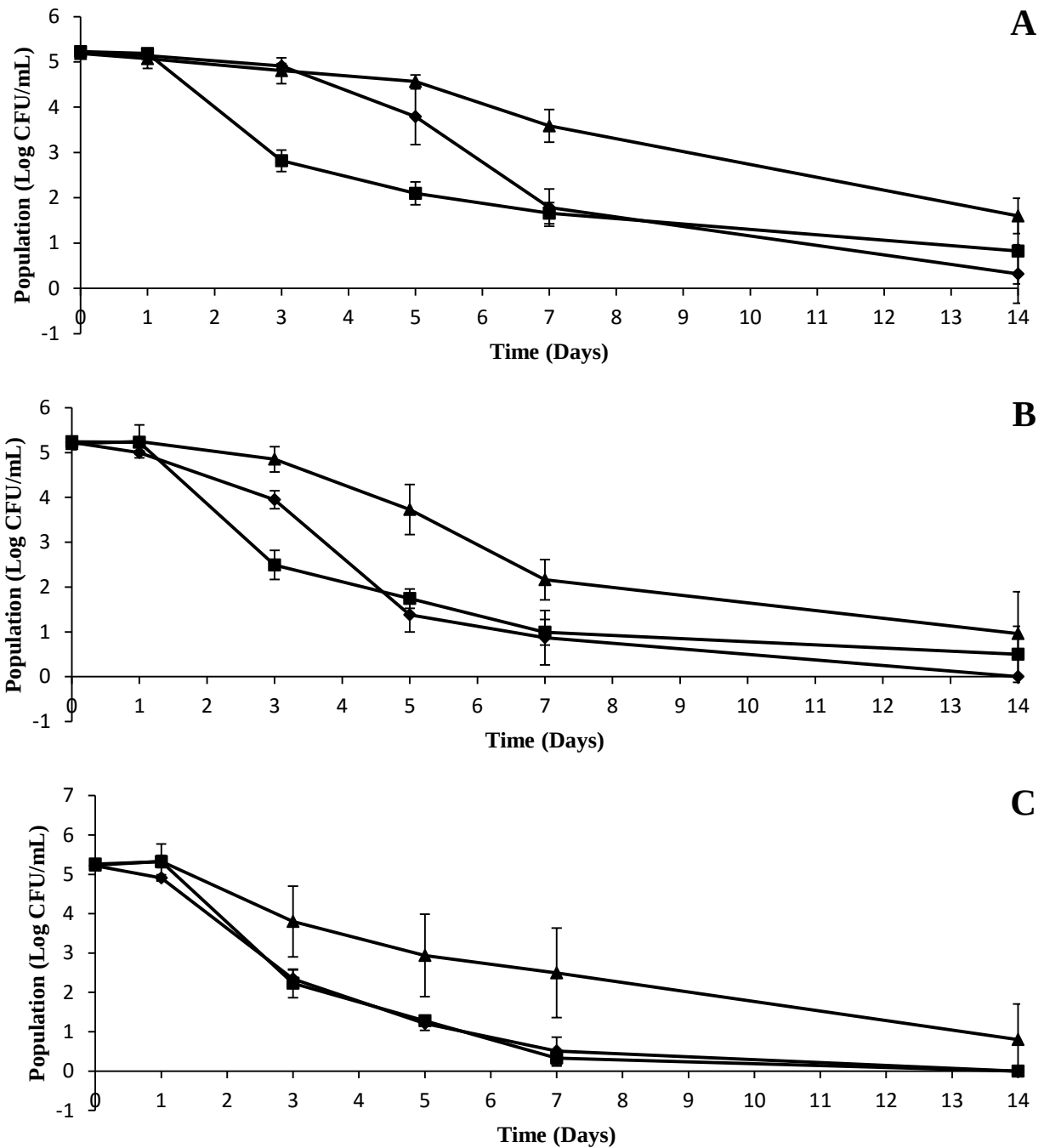


Figure 4-1. Survival of *E. coli* O157:H7 in non-sterile surface waters from three different ponds: (♦) Pond 1 (■) Pond 2, (▲) Pond 3 at (A) 15°C ± 2, (B) 22°C ± 2 (C) 30°C ± 2 (No enrichment applied; Mean ± standard deviation, n=6).

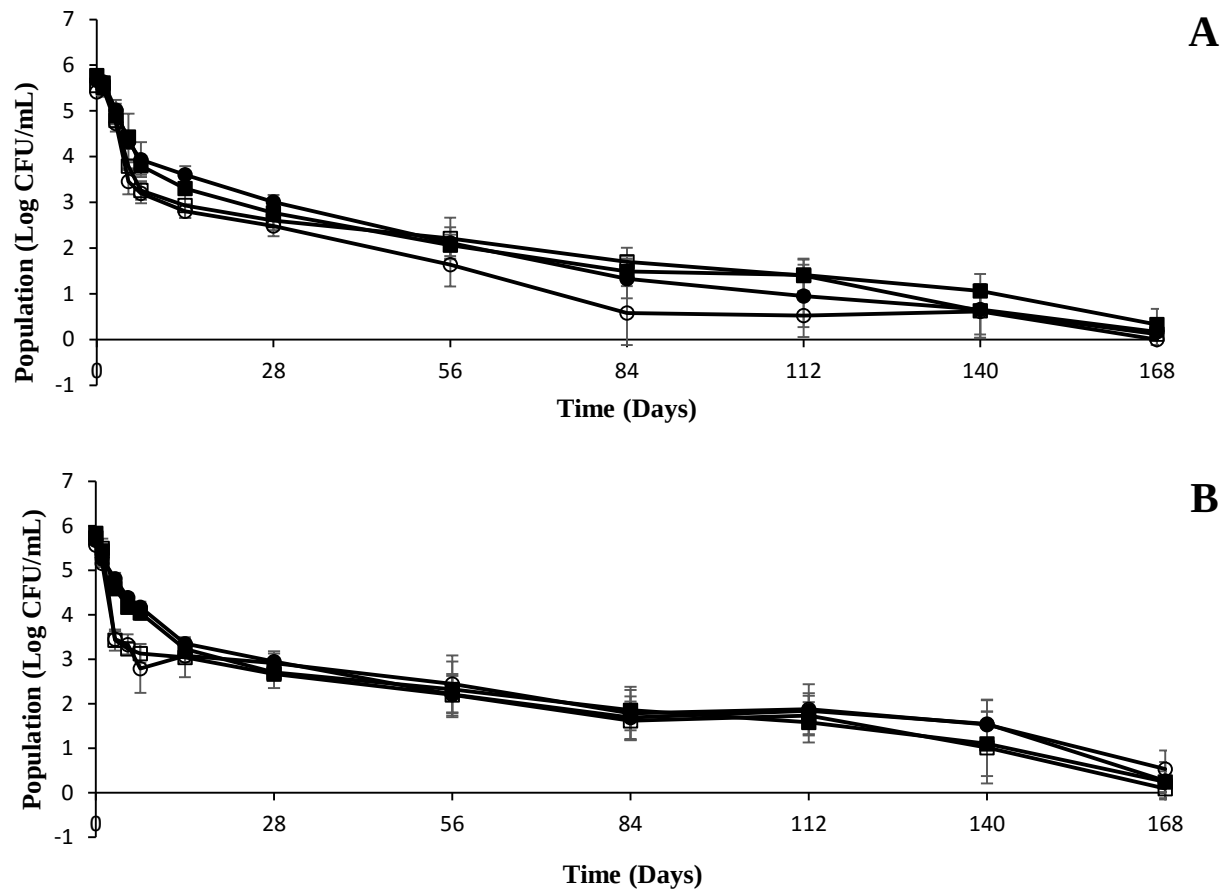


Figure 4-2. Survival of STEC and generic *E. coli* in non-sterile surface waters from two different ponds: (■) STEC in Pond 2, (□) Generic *E. coli* in Pond 2, (●) STEC in Pond 3, (○) Generic *E. coli* in Pond 3 at (A) 15°C ± 2, (B) 25°C ± 2 (Mean ± standard deviation, n=6).

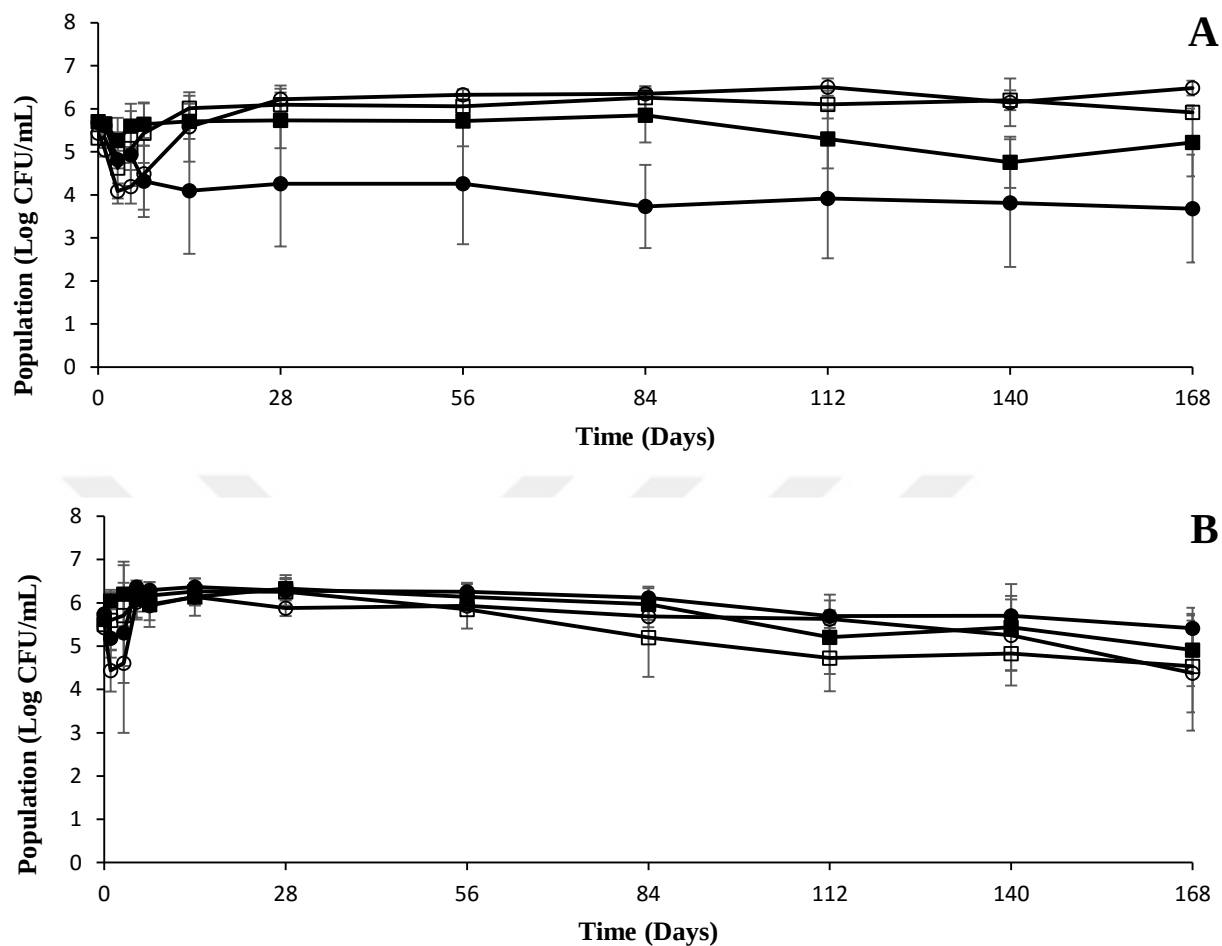


Figure 4-3. Survival of STEC and generic *E. coli* in sterile surface waters from two different ponds: (■) STEC in Pond 2, (□) Generic *E. coli* in Pond 2, (●) STEC in Pond 3, (○) Generic *E. coli* in Pond 3 at (A) 15°C ± 2, (B) 25°C ± 2 (Mean ± standard deviation, n=6).

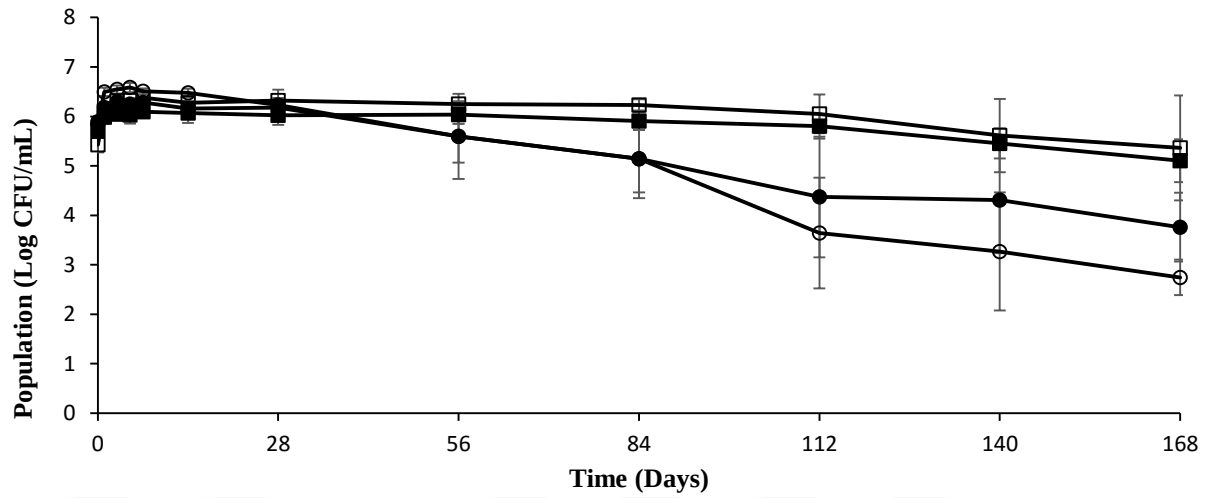


Figure 4-4. Survival of STEC and generic *E. coli* in EPA Worst Case Water (WCW): (■) STEC at 15°C ± 2, (□) Generic *E. coli* 15°C ± 2, (●) STEC 25°C ± 2, (○) Generic *E. coli* 25°C ± 2 (Mean ± standard deviation, n=6).

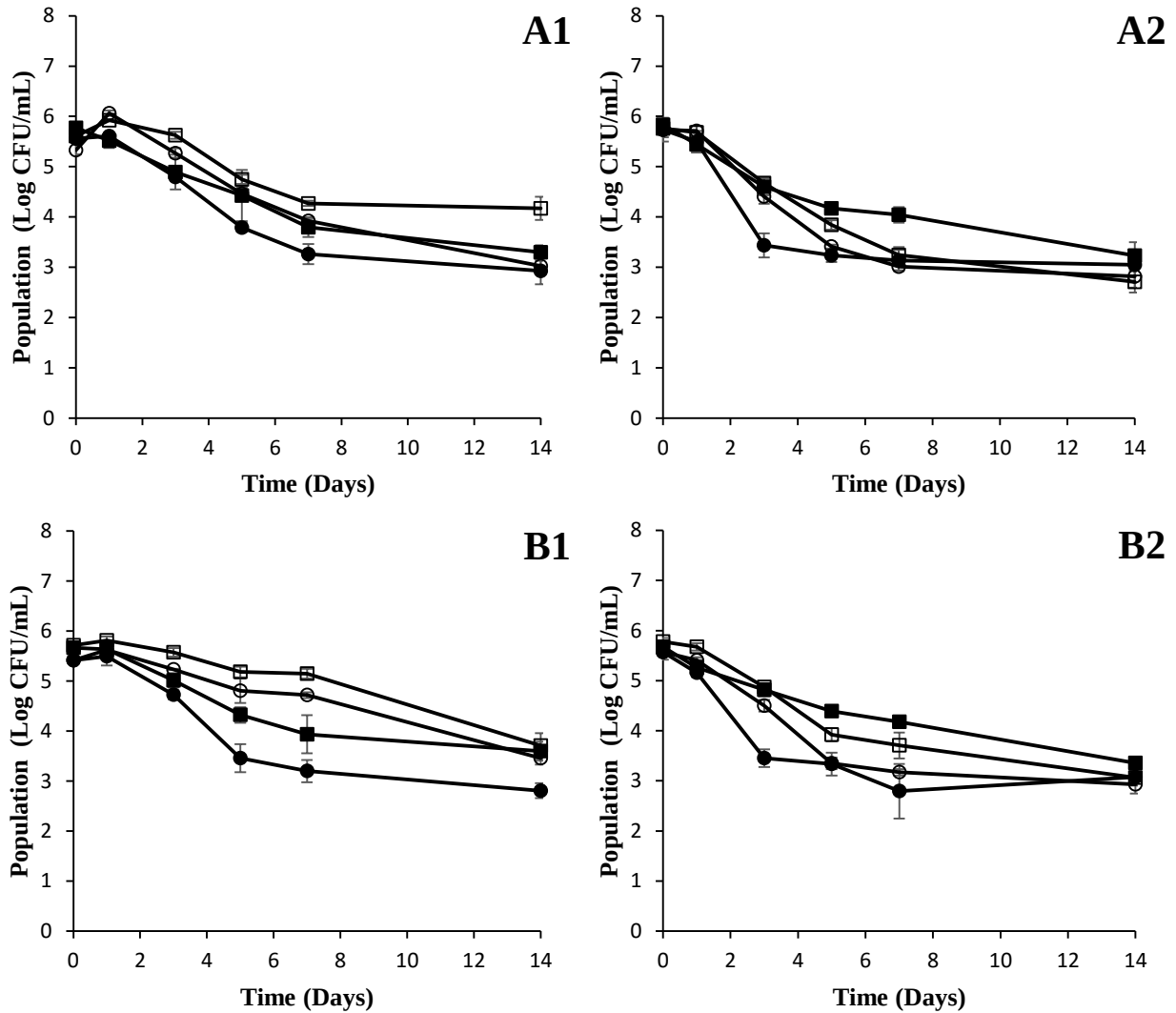


Figure 4-5. Survival of STEC and generic *E. coli* in non-sterile surface waters from two different ponds supplemented with and without cycloheximide: (■) STEC, (●) Generic *E. coli*, (□) STEC in cycloheximide treated water, (○) Generic *E. coli* in cycloheximide treated water at (A1) 15°C ± 2 in Pond 2, (A2) 25°C ± 2 in Pond 2, (B1) 15°C ± 2 in Pond 3, (B2) 25°C ± 2 in Pond 3 (Mean ± standard deviation, n=3).

CHAPTER 5

THE SURVIVAL OF STEC AND SALMONELLA SEROTYPES IN DIFFERENT WILD ANIMAL FECES THAT MAY POTENTIALLY CONTAMINATE PRODUCE

Introduction

Domestic and wild animal intrusion represents a significant route of pathogen transfer during produce production (FDA, 2015). Warm blooded animal feces are implicated as natural reservoirs for numerous pathogens that thread public health (Geldreich, 1996). Investigations regarding multistate outbreak of *E. coli* O157:H7 associated with the consumption of mesclun lettuce occurred in Connecticut and Illinois in 1996 found that fecal contamination indicator, generic *E. coli*, was cultured from wash water and final product that were from the cattle near the field (Hilborn et al., 1999). In 2006 a multistate outbreak of *E. coli* O157:H7 was associated with the consumption of contaminated spinach; the outbreak strain was found in cattle and wild pig feces that could have been introduced to well water used for irrigation (CalFERT, 2007). In 2011, outbreak of *E. coli* O157:H7 associated with the consumption of locally grown strawberries occurred and contaminated strawberries with the deer feces was concluded as the cause of outbreak. Large numbers of deer pellets with and without the outbreak strain were identified in the area, suggesting that multiple animals were present in the field (Laidler et al., 2013). The activities of domestic and wild animals are a substantial food safety concern in produce fields.

Safety requirements for corrective actions following animal intrusion in the field vary based on local and nationwide organization. In Florida, Tomato-Good Agricultural Practices (T-GAPs) require the exclusion of domestic animals and the minimization of wild animal intrusion for animal intrusion. If contamination with animal/bird feces is identified, T-GAPs prohibit for harvesting contaminated tomatoes and require an assessment of potential impact of fecal material on field harvestability (FAC&FAR, 2008). The California Leafy Greens Marketing Agreement

(LGMA) requires monitoring of wildlife activity and application of remedial action in the case of intrusion. In the presence of fecal material in the field, a food safety assessment done by qualified personnel should be conducted and a no harvest zone should be established with a minimum 5-foot radius around fecal matter for all type of feces (LGMA, 2013). Under Food Safety Modernization Act (FSMA), animal intrusion is considered a main route of contamination. The U.S. Food and Drug Administration (FDA) requires the monitor of animal activity and adequate waiting time until harvesting if animal intrusion occurs (FDA, 2015). Real life situations bring questions about the applicability and reliability of proposed standards.

Current published data about the survival of pathogens are mostly on the cattle feces and fecal slurries. Cattle may shed *Salmonella* in the range from 10^2 to 10^7 CFU/g (Himathongkham et al., 1999). A cow typically sheds from 10 to 100 CFU/g of *E. coli* O157:H7, unless they are classified as a super-shedders, where they can shed $>10^4$ CFU/g (Gyles, 2007). Human pathogens, *Salmonella*, *Listeria monocytogenes*, *E. coli* O157:H7, have been isolated from wildlife species including deer, wild pig, cattle, elk, coyote, and various types of birds (Gardner et al., 2011; Fenlon, 1985; Gorski et al., 2011; Jay et al., 2007; Wahlström et al., 2003). Pathogens can survive for a prolonged time in animal feces and manure. *E. coli* O157:H7 survived almost 2 months in dairy cattle feces and more than 6 months in manure-amended non-sterile soils at room temperature (Jiang et al., 2002; Wang et al., 1996). Non-O157 STEC survived for 18 weeks in cattle feces and are a potential risk (Fukushima et al., 1999). *Salmonella* Typhimurium populations decreased by six logs at 37°C over 48 days in cattle manure and a manure slurry (Himathongkham et al., 1999). Based on present survival data, models have been developed to predict pathogen population in animal feces. Recently, Guber et al. (2015) developed models that estimate survival and release of *E. coli* due to irrigation in

white-tail deer pellets. Another deactivation model was developed for *Salmonella* in manure and manure-amended soil (You et al., 2006). The behavior of manure-born pathogens and indicator microorganism behavior in animal feces have been modelled for release from the source, partitioning and attachment, survival and inactivation, and transport (Pachepsky et al., 2006).

Limited data have been published regarding the survival capabilities of different STEC strains and *Salmonella* species in wild animal feces to justify if any harvest stipulations in produce safety guidelines are applicable for wild animal feces. The purpose of this study is to compare the survival of STEC, *Salmonella* serotypes, total aerobic count, and generic *E. coli* in cattle and different wild animal feces found in Florida that may potentially contaminate produce and to develop a model to estimate population of *Salmonella* and STEC based on the population of naturally present generic *E. coli*. This study will help us to understand how pathogens behave in wild animal feces and provide risk assessment tools to produce safer fruits and vegetables.

Materials and Methods

Serotypes Used

Six serotypes of *Salmonella* and five serotypes of STEC were used. All serotypes are available in Dr. Danyluk's culture collection. Rifampicin resistant isolates of *Salmonella* serotypes included Typhimurium (Strain LT2), Montevideo (Human isolate, tomato outbreak), Anatum (Clinical isolate, tomato outbreak), Javiana (ATCC BAA-1593 tomato outbreak), Branderup (04E61556 2-99 Roma tomato outbreak), and Newport (Environmental isolate, tomato outbreak).

Rifampicin resistant STEC serotypes include O145 (Clinical isolate, Romaine outbreak), O104 (Clinical isolate, sprout outbreak), O111 (Clinical isolate, apple juice outbreak, NY), O103 (Venison outbreak), and O157 (Clinical isolate, spinach outbreak).

Inoculum Preparation

Rifampicin (Fisher Bioreagents, Fair Lawn, NJ) resistant STEC and *Salmonella*, stored at -80°C , were streaked onto tryptic soy agar with rifampicin (TSAR; $80\text{ }\mu\text{g}/\text{mL}$; Difco, Becton, Dickinson, Sparks, MD). Agar plates were incubated at $35 \pm 2^{\circ}\text{C}$ for $24 \pm 2\text{ h}$. One isolated colony of Rifampicin resistant strains were transferred into tryptic soy broth with rifampicin (TSBR; Difco, Becton, Dickinson, Sparks, MD) and incubated at $35 \pm 2^{\circ}\text{C}$ for 18 h. One loopful of overnight growth was transferred into an additional tube of TSBR and incubated at $35 \pm 2^{\circ}\text{C}$ for 18 h. Following incubation, cells were collected by centrifugation at $3000 \times g$ for 10 min. The supernatant was removed and 10 mL of 0.1% peptone water was vortexed with the pellet to wash cells. Rifampicin resistant cells was washed for three times, and resuspended in 5 mL peptone to obtain concentration of cells (10^9 – 10^{10} CFU/mL). Then cells were diluted to get desired concentration before inoculation (10^6 – 10^7 CFU/mL). An appropriate volume of serotypes was mixed to have an approximately equal number of cells from each serotype to achieve the total desired inoculating dose of 10^5 to 10^6 CFU/g feces.

Cattle and Wild Animal Feces Preparation

Cattle and wild animal feces (sufficient for the entire experiment) was obtained from different sources in Florida. The types of wild animal feces used in this study included feral pig, water fowl (mostly goose), deer, and raccoon. Beef cattle feces was collected from a local farm (Clermont, FL). Wild pig feces were collected from feral pigs located in University of Florida citrus groves (Lake Placid, FL). A mixture of waterfowl feces (primarily goose) was collected around a small lake (Lakeland, FL) that hosts different types of migratory waterfowl throughout the year. Deer feces was collected from local hunters (Winter Haven, FL) and a wildlife refuge (Orlando, FL). Raccoon fecal samples were collected from the same wildlife refuge (Orlando,

FL). All samples were transported to laboratory in cooler after collection and kept in refrigerator ($4 \pm 2^\circ\text{C}$) until inoculation. Dry weight and pH of fecal samples were measured.

Cocktail Inoculation and Bacterial Enumeration

Fecal samples (25 g) were placed in WhirlPak™ sample bags (Whirl-Pak bag, Nasco), and 2.5 mL of the cocktail inoculum was added to bags. The inoculated sample was mixed with a stomacher for 4 min. Fecal samples (1g) were put into 15 mL conical centrifuge tubes for each time interval and stored at room temperature. Populations were enumerated on Day 0, 1, 3, 5, 7, 14, 28 and every 28 days for up to 1 year. Fecal samples without inoculation were plated on TSAR to observe changes in background biota.

At each sampling time, 9 mL 0.1% peptone (Difco, Becton, Dickinson, Sparks, MD) was added to the centrifuge tube containing 1 g of the feces. Samples were vortexed for 2 min and dilutions were made in 0.1% peptone prior to spread plating onto TSAR. If a low cell concentration was expected, the 1 mL was plated as 0.25 mL onto 4 plates to increase the detection limit to 10^{-1} CFU/g initial concentration. Plates were incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Raccoon samples were additionally plated on bismuth sulfite agar with rifampicin (BSAR; 80 $\mu\text{g/mL}$; Difco, Becton Dickinson, Sparks, MD) and xylose lysine desoxycholate agar supplemented with rifampicin (XLDR; 80 $\mu\text{g/mL}$; Difco, Becton Dickinson, Sparks, MD) for *Salmonella* and sorbitol MacConkey agar with rifampicin (SMACR; 80 $\mu\text{g/mL}$; Difco, Becton Dickinson, Sparks, MD) for STEC to avoid miscounting on TSAR due to high background biota resistant to rifampicin.

An uninoculated set of samples (n=3) was prepared for each animal as mentioned above for inoculated samples. Samples were also spread onto TSA and CHROMagar ECC (DRG, Mountainside, NJ, USA) to monitor the total aerobic count and generic *E. coli* concentration,

respectively, for each sampling time for all animal feces. If no colonies were detected, enrichment process was applied to determine the presence/absence of cells.

Enrichments of STEC and *Salmonella*

If no colonies were detected, enrichment process was applied to determine the presence/absence of pathogens. For STEC enrichment, equal volume of (10 mL) of double strength modified buffered peptone water with pyruvate was added to the sample and incubated at $35 \pm 2^\circ\text{C}$ for 5 h. Following 5 h incubation, stock solution of rifampicin was added to standard concentration (32 μL of rifampicin to 20 mL; 80 $\mu\text{g/mL}$; Difco, Becton Dickinson, Sparks, MD) and incubated again at $42 \pm 2^\circ\text{C}$ for 18-24 h. After overnight incubation, the enrichment was streaked onto SMACR and incubated for 24 h at $35 \pm 2^\circ\text{C}$. After 24 h, if typical colonies are present, enrichment was recorded as positive, and populations were recorded as 0.3 Log CFU/g (limit of detection).

For *Salmonella* enrichment, equal volume (10 mL) of double strength lactose broth was added to the sample and incubated at $35 \pm 2^\circ\text{C}$ for 24 h. After an overnight incubation, 1 mL of enrichment was added to 9 mL of tetrathionate (TT; Difco, Becton Dickinson, Sparks, MD) broth and 0.1 mL of enrichment was added to 9.9 mL of Rappaport-Vassiliadis (RV; Difco, Becton Dickinson, Sparks, MD). TT broth and RV broth tubes were incubated at $35 \pm 2^\circ\text{C}$ for 24 h and $42 \pm 2^\circ\text{C}$ for 48 h, respectively. Following incubation, a 10 μL loopful, from each broth, was streaked onto BSAR, XLDR, and hektoen enteric agar supplemented with rifampicin (HER; 80 $\mu\text{g/mL}$; Difco, Becton, Dickinson, Sparks, MD). XLDR and HER was incubated at $35 \pm 2^\circ\text{C}$ for 24 h, and BSAR was incubated at $35 \pm 2^\circ\text{C}$ for 48 h. Following incubation, typical colonies were present, selected colonies were transferred into triple sugar iron (TSI; Difco, Becton, Dickinson, Sparks, MD) and lysine iron agar (LIA; Difco, Becton, Dickinson, Sparks, MD)

slants. Slants were incubated at $35 \pm 2^{\circ}\text{C}$ for 24 h. When enrichments were positive, populations were recorded as 0.3 Log CFU/g (limit of detection).

Statistical Analysis and Model Development

The means of the data for *Salmonella* and STEC strains was calculated for the fecal samples. The statistical difference between the populations of the bacterial cocktails in the different manure type and for each time interval was evaluated using ANOVA. Multifactor analysis of variance tests for animal and pathogen interactions within each animal and each time interval was calculated. All data was processed using Microsoft Excel, 2013 and JMP 11 software (SAS® Institute Inc., Cary, NC, USA 2013). The alpha value was set at 0.05.

Reduction rates of *Salmonella*, STEC, and generic *E. coli* were calculated for all type of animal feces. To calculate rate of reductions (days required to achieve a 1 Log CFU/g reduction), linear trendlines in survival plots were drawn from Day 0 to first day that cell population dropped to below 1.0 Log CFU/g. ANCOVA was applied for comparison of rate of reduction.

To assess the relationship between generic *E. coli* and *Salmonella* and STEC with estimated reduction rates in the cattle feces, survival data for all animals were used. To determine the population of *Salmonella* and STEC in cattle and wild animal feces by using the population of generic *E. coli*, linear and second order polynomial regression line equations for all type of tested microorganisms were calculated. *Salmonella* or STEC equations were combined with generic *E. coli* equations individually for the same animal to obtain mathematical expression of each pathogen in correlation with the population of generic *E. coli*. New combined equations were plotted to develop model that predicts pathogens population in different animal feces in the relationship with generic *E. coli* population. All used equations and R^2 are listed in Table 5-6. Models were developed with in the form of following combinations of trendline equations:

1. Two second order polynomial

a. Generic *E. coli* : $F_1(x) = ax^2 + bx + c$

$$: 0 = ax^2 + bx + (c - F_1(x))$$

$$: x = [(-b \pm (\Delta)^{1/2})/2a]$$

b. Pathogen : $F_2(x) = dx^2 + ex + f$

c. Model equation: $F_2(x) = d[-b/4a^2 \pm (\Delta/4a^2)^{1/2}]^2 + e[-b/2a \pm (\Delta/4a^2)^{1/2}] + f$

$$*(\Delta) = [(-b \pm (b^2 - (4a(c - F_1(x))))^{1/2})/2a]$$

2. One linear and one polynomial

a. Generic *E. coli* : $F_1(x) = ax + b$

b. Pathogen : $F_2(x) = cx^2 + dx + e$

c. Model equation: $F_2(x) = c [(F_1(x) - b)/a]^2 + d (F_1(x) - b)/a + e$

3. Two linear

a. Generic *E. coli* : $F_1(x) = ax + b$

b. Pathogen : $F_2(x) = cx + d$

c. Model equation: $F_2(x) = c(F_1(x) - b)/a + d$

$F_1(x)$: Population of generic *E. coli*

$F_2(x)$: Population of pathogen

Results

Sample Properties

A total of five type of animal feces were used for this study. Measured pH values and dry matter percentages for each sample as follows: Cattle, 7.39 and 15.44%; pig, 8.44 and 43.51%; waterfowl, 7.56 and 24.5%; deer, 7.07 and 55.26%; raccoon, 5.31 and 30.89%.

Survival

Survival behavior of *Salmonella* varied between different animal species. At Day 0, initial *Salmonella* population were ranged between 5.2 and 5.7 Log CFU/g in all samples, and increased in all type of feces until Day 5 with the exception of the raccoon (Figure 5-1, Figure 5-4, Table 5-1), where populations immediately began to decline. *Salmonella* could not be detected at or beyond Day 28 upon enrichment in raccoon feces. Population increase at Day 5 reached around 1.1 Log CFU/g in the cattle feces, 2.6 Log CFU/g for the pig feces, 0.7 Log CFU/g in the waterfowl feces, and 1.7 Log CFU/g in the deer feces. *Salmonella* populations could only be enumerated from the cattle and the deer feces at the end of the study on Day 364. *Salmonella* population decline was 0.8 Log CFU/g and 1.6 Log CFU/g lower than initial concentration in the cattle and the deer feces at Day 364, respectively. *Salmonella* was detected in the waterfowl and the pig feces until Day 308 and 364, respectively; however, the population ranged from 0 to 2.6 Log CFU/g after Day 28. *Salmonella* behaved statistically the same as in pig, waterfowl, and raccoon samples starting at Day 112 ($P<0.05$). Similar trends were observed in cattle and deer samples until the last sampling day.

Similar to *Salmonella*, STEC behaved differently in feces from different animal species. At Day 0, initial STEC population were ranged between 5.0 and 5.7 in all samples, and increased in all type of feces until Day 5 with the exception of raccoon and waterfowl (Figure 5-2, Figure 5-4, Table 5-2). Direct decline started in raccoon feces after inoculation and STEC could not be detected at Day 28 upon enrichment. Population increase at Day 5 reached around 0.9 Log CFU/g in the cattle feces, 2.4 Log CFU/g for the pig feces, and 1.7 Log CFU/g in the deer feces. STEC population decline was 2.3 Log CFU/g and 2.4 Log CFU/g lower than initial concentration in the cattle and the deer feces at Day 364, respectively. STEC was detected in waterfowl and pig feces until Day 224 and 196; however, the population ranged from 0 to 2.0

Log CFU/g after Day 14. STEC populations could only be enumerated from the cattle and the deer feces at the end of the study on Day 364. STEC behaved statistically the same as in pig, waterfowl, and raccoon samples starting at Day 84 ($P>0.05$). In the pig samples, the population of STEC and *Salmonella* decreased up to five logs, and enumeration of STEC strains was not possible by plate counting on Day 168. Similar trends were observed in cattle and deer samples until the last sampling day.

Naturally present total aerobic microbial population ranged from 5.8 to 8.6 Log CFU/g at Day 0 among the different animal feces. Generic *E. coli* populations at Day 0 were between 2.8 and 6.7 Log CFU/g in all type of wild animal feces. At Day 3, the difference among the population of total aerobic count and generic *E. coli* among tested samples from five type of animal dropped to less than 2.2 and 1.2 Log CFU/g, respectively. Total aerobic microbial count stayed as the highest throughout the experiment for all type of animal feces. The total aerobic count stayed between logs 3 to 10 CFU/g in all type of animal feces over the course of the experiment, and the highest population fluctuation was observed in the pig feces (Figure 5-3, Figure 5-4). Generic *E. coli* survived better in the deer and the cattle feces similar to STEC and *Salmonella*. Similar trends to *Salmonella* and STEC survival were observed for generic *E. coli* in all type of feces (Figure 5-3, Figure 5-4).

Multifactor analysis of variance tests for animal and pathogen interactions among animals revealed similar results to ANOVA and resulted in no significant difference in pig, waterfowl, and raccoon feces starting at Day 112 ($P > 0.05$) as shown on Table 5-3. Statistically significant differences between pooled *Salmonella* and STEC populations among different type of animal feces was calculated at Day 1, 3, 5, 28, 308, 336, 364 ($P < 0.05$) showing that both *Salmonella* and STEC behave the same more than three quarters of the year ($P > 0.05$) (Table 5-

4). Multifactor analysis of variance tests for animal and pathogen interactions indicated that there is no significant animal pathogen interaction after Day 28 ($P > 0.05$) (Table 5-4). In the cattle and deer samples, both *Salmonella* and STEC behaved differently compare to other types of animal feces ($P < 0.05$).

Rates of Reduction

Reductions rates for *Salmonella*, STEC, and generic *E. coli* were calculated for in each animal feces for initial linear decline. R^2 were between 0.49 and 0.96 for all type of feces and microorganisms. All reduction rates and number of days used for initial linear decline data are shown with equations and R^2 in Table 5-5. In cattle and deer feces, high rates of reduction were calculated for all microorganisms, Reduction rates for *Salmonella*, STEC, and generic *E. coli* were 188.7, 109.9, and 112.4 days in cattle feces and 161.3, 107.5, and 172.4 days in deer feces. Generic *E. coli* rates were significantly different from *Salmonella* in cattle feces and STEC in deer feces ($P < 0.05$). All reduction rates remained below 25 days in the pig and waterfowl feces. *Salmonella*, STEC, and generic *E. coli* had rate of reduction as 11.6, 7.3, and 25.2 days in the pig feces, and 23.6, 5.7, and 17.5 in the waterfowl feces. *Salmonella* and STEC rates were statistically the same ($P > 0.05$), but different from generic *E. coli* in the pig feces ($P < 0.05$). All tested microorganisms had different rate of reduction in the waterfowl feces ($P < 0.05$). In the raccoon feces, reduction rates were statistically the same ($P < 0.05$), and number of days to reach a 1 log reduction were 9.8 days for *Salmonella*, 10.7 days for STEC, and 9.0 days for generic *E. coli*.

Prediction Models for Pathogens in Different Type of Animal Feces

All survival data for each individual type of target microorganism in each animal feces were used to calculate trendline results including R^2 and linear and second order polynomial equations as in Table 5-6. Linear equations were used when developed model in polynomial

format did not provide the prediction range from 0 to 9 Log CFU/g due to their parabolic manner. All R^2 for equations ranged from 0.4919 to 0.9911. For the cattle, linear equations of *Salmonella* (R^2 : 0.4919) and STEC (R^2 : 0.8867) and polynomial equations of generic *E. coli* (R^2 : 0.8656) were used. Only second order polynomial equations were used for the pig, the waterfowl, and the raccoon with following R^2 : Pig-*Salmonella* 0.8845, pig-*STEC* 0.8085, pig generic *E. coli* 0.788; water fowl-*Salmonella* 0.9048, water fowl-*STEC* 0.8380, water fowl generic *E. coli* 0.9581; raccoon-*Salmonella* 0.9872, raccoon-*STEC* 0.9911, raccoon generic *E. coli* 0.9862. For the deer, linear equations of *Salmonella* (R^2 : 0.6769), *STEC* (R^2 : 0.8344), and generic *E. coli* (R^2 : 0.7639) were used. *Salmonella* or *STEC* equations combined with generic *E. coli* equations to obtain mathematical expression of each pathogen in correlation with the population of generic *E. coli* provided new combined equations with R^2 for ranging from 0.4362 to 0.9774.

Predicted and actual *Salmonella* and *STEC* populations in each animal species are plotted in Figure 5-7. Predicted values are always higher than the actual population of pathogens calculated with trendline equations. These differences are very low in the cattle and the deer feces due to use of linear equations. Individual combined models used to predict the population of *Salmonella* and *STEC* in each animals feces are shown in Figure 5-5 and 5-6, respectively.

Salmonella model in Figure 5-5 predicts that pig feces can harbor at least 1 Log CFU/g more *Salmonella* cells compare to cattle, waterfowl, and raccoon feces with the same generic *E. coli* concentration. The predicted *Salmonella* populations in the deer feces are very close to the given generic *E. coli* population. For example, if the population of generic *E. coli* in the deer feces is 5 Log CFU/g, the model predicts the population of *Salmonella* around 5 Log CFU/g. According to the *Salmonella* prediction model, pig feces has the highest risk in case of

contamination, and can have around 4 Log CFU/g *Salmonella* in the absence of generic *E. coli*. Raccoon feces is in the lowest risk group. The model predicts that in the presence of 4 Log CFU/g generic *E. coli* population in the waterfowl, cattle and deer feces *Salmonella* concentration will be between 3 and 4 Log CFU/g. Deer feces becomes as hazardous as pig feces in the presence of generic *E. coli* over 6 Log CFU/g.

The STEC model in Figure 5-6 predicts higher pathogen populations in the order of pig, deer, cattle, raccoon, and waterfowl feces at high generic *E. coli* concentration over 6 Log CFU/g. According to STEC prediction model, pig feces has the highest risk in case of contamination, and can have around 7 Log CFU/g STEC when generic *E. coli* concentration is at 6 Log CFU/g. Until the population of generic *E. coli* reaches around 2.5 Log CFU/g, deer feces does not have STEC in countable concentrations. Model predicts that in the presence of 2.5 Log CFU/g generic *E. coli* population in raccoon, pig, waterfowl, cattle and deer feces, STEC concentration will be between 2 and 3 Log CFU/g. According to STEC prediction model, pig feces has the highest contamination risk, and waterfowl feces is in the lowest risk group.

Discussion

The presence of domesticated, farm, or wild animals must be controlled in produce fields to ensure the safety of products being grown. However, the total exclusion of these animals from fields is not easy to achieve in practice. Various local and nationwide organizations require safety standards, including the observation and exclusion of animals, no harvest zone establishment, adequate waiting time, and a risk assessment as mentioned above (FAC&FAR, 2008; LGMA, 2013; FDA, 2015). Safety metrics are used to establish safety standards and requirements to minimize pathogen contamination risks sourced from animal and animal feces. Currently, studies regarding the survival of pathogens focused predominantly on cattle feces as manure and slurries (Jiang et al., 2002; Himathongkham et al., 1999; Wang et al., 1996).

However, the presence of human pathogens and potential contamination risks from wild animal and wild animal feces studied in different regions (Fenlon, 1985; Gardner et al., 2011; Gorski et al., 2011, Jay et al., 2007; Wahlström et al., 2003). There is no published data about the survival capabilities of *Salmonella* spp., and STEC in cattle and wild animal (pig, waterfowl, deer, and raccoon) feces found in the South Eastern U.S. All chosen animals in this study are commonly found and observed in or around fields, agricultural water sources in Florida. They may act as potential source of produce contamination in pre-harvest level.

Salmonella spp. and STEC survived for extended period of a time in all types of feces in this study. Both genera survived better in the cattle and deer feces than other animal feces. Initial concentrations of pathogens ranged from 5.0 to 5.3 Log CFU/g for deer and cattle samples. No reduction below initial concentration was observed for both STEC and *Salmonella* in the deer samples until Day 252, and maximum 2.3 Log CFU/g reduction was observed in the cattle samples. At the end of the sampling period (Day 364), the highest reduction in pathogen population was less than 2.5 Log CFU/g in the cattle and deer samples. Jiang et al. (2002) reported that *E. coli* O157:H7 survived in manure-amended unautoclaved soils for up to 56 days at 5°C, 152 days at 15°C, and 193 days at 21°C. In another study, *E. coli* O157:H7 and *Salmonella* Typhimurium decreased by six logs at 37°C until Day 38 and 48, respectively in cattle manure and manure slurry (Himathongkham et al., 1999). Wang et al. (1996) inoculated the dairy cattle feces with *E. coli* O157:H7 at 10^3 and 10^5 CFU/g concentrations to determine the fate of this pathogen at different temperatures. *E. coli* O157:H7 survived at 37°C for 42 and 49 days with low and high concentration, respectively, and at 22°C for 49 and 56 days with low and high concentration, respectively. In a similar study, Fukushima et al. (1999) inoculated cattle feces with STEC O26, O111, and O157 at 10^1 , 10^3 and 10^5 CFU/g concentrations. All three

serotypes were no longer counted after 2 to 14 weeks at 5°C, 1 to 18 weeks at 15°C, and 3 to 12 weeks at 25°C on samples with middle and high inoculations. Multi-drug resistance *Salmonella* Newport survived in manure and manure for amended-soil up to 184 and 332 days, respectively (You et al., 2006). The survival time in our study is longer than previously published data. This might be explained with longer sampling period in here, fecal matters with different back biota and physicochemical conditions, type of food resources for animals at different regions, strain variability used in studies.

Both cattle and deer are ruminant animals, and the populations of *Salmonella* and STEC in the feces from ruminants were still in direct plating count and between 2.6 and 4.5 CFU/g at over a year. *E. coli* O157:H7 has been isolated from feral and farm pigs and considered as significant risk; ruminants including cattle, sheep, goat, and deer are recognized as the major reservoirs for this pathogen (CalFERT, 2007; Gyles, 2007; La Ragione et al., 2009; LGMA, 2013). The primary site of *E. coli* O157:H7 colonization in cattle is in the rectal-anal junction mucosa and the carriage of *E. coli* O157:H7 at this site is in correlation with high level of fecal shedding (Sheng et al., 2011). In small ruminants as goat and sheep, the primary site for *E. coli* O157:H7 colonization is defined as the distal intestine including caecum, colon and rectum (La Ragione et al., 2009). It should be noted that fecal samples from these animals are high in cellulosic materials that is part of the major components used by enteric pathogens for attachment and biofilm formation. Teplitski et al. (2009) state that *E. coli* and *Salmonella* use bacterial polymers in addition to aggregative fimbriae to attach plant seedlings including cellulose, poly- β -1,6-*N*-acetyl-D-glucosamine, colanic acid and O-antigen capsule.

In the pig, waterfowl, and raccoon samples, the concentration of these pathogens stayed in very low or fell below the limit of detection. STEC could no longer be identified by

enrichment in the pig feces at Day 168. *Salmonella* population in the pig feces, however, was still at concentration of log 1 to 1.5 CFU/g at Day 308, 336, and 364. *Salmonella* and STEC could not be recovered in waterfowl feces starting at Day 336 and 252, respectively. Immediate reduction of *Salmonella* and STEC population was observed in the raccoon sample. At Day 56, no pathogens were detected upon enrichment in raccoon fecal sample. The survival of microorganisms in the fecal matter may depend on several factors including microbial community and the presence of predators, animal's diet, moisture content, antibiotic residuals, chemical composition, and pH. Raccoon samples used in this study had a lower pH value (5.31) than other animal feces that were close to 7.00 or higher.

Salmonella serotypes and STEC strains followed similar survival trends in all type of feces; however, *Salmonella* stayed in higher concentrations in deer, pig, and waterfowl feces. *Salmonella* strains survived the best in the cattle samples up to 364 day and the worst in the raccoon feces for 28 days. STEC strains survived the best in deer samples up to 364 days and the worst in the raccoon feces for 28 days. *Salmonella* survived better than STEC in almost in all type of feces tested here. The time for *Salmonella* populations to decline by 1 Log CFU/g was longer than STEC in all type of feces except for the raccoon feces. *Salmonella* reduction rates were greater than rate of STEC reduction around 80 days in the cattle feces, 4 days in the pig feces, 17 days in the waterfowl feces, and 50 days in the deer feces. *Salmonella* and STEC reduction rate difference was less than 1 day in the raccoon feces. Previous studies reported that *Salmonella* survived for longer time than STEC similar to this study (Jacobsen and Bech, 2012). The survival capabilities of *Salmonella* reported here are greater in cattle and wild animal feces based on reduction rates.

Total aerobic count and generic *E. coli* populations were monitored along with *Salmonella* and STEC populations to have larger perspective of microbial survival in feral pig, waterfowl, deer, and raccoon feces besides the cattle feces. Total aerobic count stayed as the highest concentration as expected in all type of feces used in here. Generic *E. coli* followed similar survival trends and population decline to tested pathogens for all type of feces (Figure 5-4). Predictive models were developed to calculate *Salmonella* and STEC concentrations in wild animal feces based on the concentration of generic *E. coli*. Although the power of both models (R^2) seem moderate to high, the predictive model developed to estimate the population of pathogens based on generic *E. coli* yielded consistent results with occurred outbreaks due to fecal contamination. According to predictive *Salmonella* model, pig feces has the highest risk of *Salmonella* since the presence of generic *E. coli* in amount of only few cells or no cells indicates the concentration of *Salmonella* around 10^4 Log CFU/g (Figure 5-5). STEC model shows that the pig feces is the highest risk if the population of Generic *E. coli* is more than 10^3 Log CFU/g (Figure 5-6). Also, deer feces becomes the second potential reservoir when generic *E. coli* population reaches Log 10^5 CFU/g which is highly possible based on the survival data. The outbreaks of STEC originated from animals confirm the results of STEC models (CalFERT, 2007; Laidler et al., 2013). This model shows that the exclusion of wild animals, especially feral pigs and deer, from fields is essential to minimize the contamination risk.

FDA has currently adopted the National Organic Program (NOP standards) of at least 90 and 120 day interval between the application of raw manure as biological soil amendment for crops not in contact and in contact with soil under the FSMA final rule (FDA, 2015). Prior to producing leafy greens, one year waiting time is a requirement in case of the application of un-composted, incompletely composted or non-thermally treated (e.g., heated) animal manure to

fields by LGMA (2013). T-GAPs does not allow growers to use raw manure, and requires the detailed information about the treatment of manure (FAC&FAR, 2008). Based on results in this study under laboratory conditions, pathogen populations may still be present under both FDA and LGMA requirements if field contamination occurs with cattle and deer feces. However, environmental factors should be monitored and considered during risk assessment. The survival capabilities of manure-borne *E. coli* is affected by temperature, moisture, pH, solar radiation, diet of animal, strain, and type of soil (Van Kessel et al., 2007). These environmental factors may change continuously and can reach devastating points for microorganisms in the field. We would recommend the use of standards from T-GAPs to ensure the safety of produce in the field. In case of animal intrusion and defecation in the field, remedial actions as no harvest zone should be applied followed by risk assessment by experts.

Table 5-1. Comparison of *Salmonella* cocktail population means with standard deviation in different animal feces.

Days	Mean $\pm$ SD <i>Salmonella</i> spp. population (Log CFU/g)*				
	Cattle	Pig	Waterfowl	Deer	Raccoon
0	5.3 $\pm$ 0.2B	5.3 $\pm$ 0.2AB	5.7 $\pm$ 0.3A	5.3 $\pm$ 0.2AB	5.5 $\pm$ 0.2AB
1	5.7 $\pm$ 0.3B	7.0 $\pm$ 0.7A	6.5 $\pm$ 0.2A	6.9 $\pm$ 0.4A	5.1 $\pm$ 0.2C
3	6.0 $\pm$ 0.2D	7.8 $\pm$ 0.1A	6.6 $\pm$ 0.1CB	7.0 $\pm$ 0.13	4.9 $\pm$ 0.3
5	6.3 $\pm$ 0.3C	7.9 $\pm$ 0.3A	6.3 $\pm$ 0.1C	7.0 $\pm$ 0.2B	4.8 $\pm$ 0.3D
7	5.6 $\pm$ 0.6C	7.5 $\pm$ 0.4A	6.4 $\pm$ 0.2B	7.0 $\pm$ 0.3AB	4.5 $\pm$ 0.5D
14	5.4 $\pm$ 0.3B	6.2 $\pm$ 0.8AB	5.9 $\pm$ 0.5AB	6.8 $\pm$ 0.3A	2.9 $\pm$ 0.7C
28	5.4 $\pm$ 0.3AB	4.9 $\pm$ 2.3AB	4.3 $\pm$ 1.8B	7.0 $\pm$ 0.5A	1.0 $\pm$ 1.5C
56	4.8 $\pm$ 0.3AB	1.2 $\pm$ 2.0C	2.6 $\pm$ 2.8BC	7.0 $\pm$ 0.6A	0.0 $\pm$ 0.0C
84	4.8 $\pm$ 1.0AB	0.8 $\pm$ 1.2CD	2.5 $\pm$ 2.4BC	6.9 $\pm$ 1.0A	0.0 $\pm$ 0.0D
112	4.5 $\pm$ 0.9A	1.2 $\pm$ 2.2B	1.7 $\pm$ 2.0B	6.3 $\pm$ 0.9A	0.0 $\pm$ 0.0B
140	3.7 $\pm$ 0.4B	0.0 $\pm$ 0.0C	0.8 $\pm$ 1.8C	6.5 $\pm$ 1.2A	0.0 $\pm$ 0.0C
168	4.0 $\pm$ 0.8B	0.8 $\pm$ 1.9C	0.9 $\pm$ 2.0C	6.5 $\pm$ 0.8A	0.0 $\pm$ 0.0C
196	3.5 $\pm$ 1.0B	0.0 $\pm$ 0.1C	1.5 $\pm$ 2.2C	6.0 $\pm$ 0.8A	0.0 $\pm$ 0.0C
224	3.0 $\pm$ 0.6B	0.0 $\pm$ 0.1C	2.1 $\pm$ 2.3BC	6.1 $\pm$ 2.0A	0.0 $\pm$ 0.0C
252	3.0 $\pm$ 0.5B	0.3 $\pm$ 0.4C	0.2 $\pm$ 0.6C	5.7 $\pm$ 1.4A	0.0 $\pm$ 0.0C
280	3.8 $\pm$ 1.4A	0.4 $\pm$ 0.8B	0.8 $\pm$ 1.5B	4.9 $\pm$ 2.1A	0.0 $\pm$ 0.0B
308	4.5 $\pm$ 0.9A	1.3 $\pm$ 1.0B	1.2 $\pm$ 1.0B	5.1 $\pm$ 2.2A	0.0 $\pm$ 0.0B
336	4.7 $\pm$ 0.9A	1.5 $\pm$ 1.0B	0.0 $\pm$ 0.0B	5.0 $\pm$ 2.1A	0.0 $\pm$ 0.0B
364	4.5 $\pm$ 0.4A	1.2 $\pm$ 1.2B	0.0 $\pm$ 0.0B	3.7 $\pm$ 2.1A	0.0 $\pm$ 0.0B

*Means with different letter within each row are significantly different ($P < 0.05$).

Table 5-2. Comparison of STEC cocktail population means with standard deviation in different animal feces.

Days	Mean $\pm$ SD STEC population (Log CFU/g)*				
	Cattle	Pig	Waterfowl	Deer	Raccoon
0	5.0 $\pm$ 0.6A	5.4 $\pm$ 0.6A	5.6 $\pm$ 0.2A	5.3 $\pm$ 0.2A	5.3 $\pm$ 0.2A
1	6.1 $\pm$ 0.4BC	6.6 $\pm$ 0.2AB	5.7 $\pm$ 0.1C	6.8 $\pm$ 0.6A	5.0 $\pm$ 0.2D
3	5.7 $\pm$ 0.6C	7.8 $\pm$ 0.1A	5.7 $\pm$ 0.1C	7.1 $\pm$ 0.4B	4.7 $\pm$ 0.2D
5	5.9 $\pm$ 0.3C	7.9 $\pm$ 0.2A	5.5 $\pm$ 0.2C	7.0 $\pm$ 0.3B	4.2 $\pm$ 0.5D
7	5.8 $\pm$ 0.3B	7.4 $\pm$ 0.2A	5.8 $\pm$ 0.2B	7.2 $\pm$ 0.5A	4.0 $\pm$ 0.3C
14	5.5 $\pm$ 0.1AB	5.3 $\pm$ 1.4B	4.1 $\pm$ 1.2BC	7.0 $\pm$ 0.5A	3.6 $\pm$ 0.5C
28	6.3 $\pm$ 0.5A	1.7 $\pm$ 1.0B	0.9 $\pm$ 2.1B	6.6 $\pm$ 0.1A	1.5 $\pm$ 0.7B
56	5.2 $\pm$ 0.5A	0.1 $\pm$ 0.1C	1.9 $\pm$ 2.1B	6.5 $\pm$ 0.5A	0.0 $\pm$ 0.0C
84	5.1 $\pm$ 0.6A	0.8 $\pm$ 1.8B	2.0 $\pm$ 1.9B	6.3 $\pm$ 1.3S	0.0 $\pm$ 0.0B
112	4.1 $\pm$ 0.3B	0.8 $\pm$ 1.8C	0.0 $\pm$ 0.0C	6.4 $\pm$ 0.8A	0.0 $\pm$ 0.0C
140	4.1 $\pm$ 0.5B	0.0 $\pm$ 0.1C	0.6 $\pm$ 1.6C	6.3 $\pm$ 1.1A	0.0 $\pm$ 0.0C
168	3.9 $\pm$ 0.5B	0.0 $\pm$ 0.0C	0.8 $\pm$ 0.9C	5.8 $\pm$ 1.5A	0.0 $\pm$ 0.0C
196	3.4 $\pm$ 0.6B	0.0 $\pm$ 0.0C	0.0 $\pm$ 0.0C	5.1 $\pm$ 1.6A	0.0 $\pm$ 0.0C
224	3.3 $\pm$ 0.5B	0.0 $\pm$ 0.0C	0.8 $\pm$ 1.1C	5.3 $\pm$ 2.0A	0.0 $\pm$ 0.0C
252	3.4 $\pm$ 1.2B	0.0 $\pm$ 0.0C	0.0 $\pm$ 0.0C	5.3 $\pm$ 1.8A	0.0 $\pm$ 0.0C
280	3.9 $\pm$ 1.2A	0.0 $\pm$ 0.0B	0.0 $\pm$ 0.0B	4.3 $\pm$ 1.8A	0.0 $\pm$ 0.0B
308	2.9 $\pm$ 0.7A	0.0 $\pm$ 0.0B	0.0 $\pm$ 0.0B	3.5 $\pm$ 2.4A	0.0 $\pm$ 0.0B
336	2.8 $\pm$ 0.2A	0.0 $\pm$ 0.0B	0.0 $\pm$ 0.0B	3.5 $\pm$ 3.0A	0.0 $\pm$ 0.0B
364	2.6 $\pm$ 0.4A	0.0 $\pm$ 0.0B	0.0 $\pm$ 0.0B	3.2 $\pm$ 1.6A	0.0 $\pm$ 0.0B

*Means with different letter within each row are significantly different ($P < 0.05$).

Table 5-3. Multifactor analysis of variance tests for animal and pathogen interactions within each time interval. *Salmonella* and STEC concentrations were averaged together for each animal.

Days	Mean population (Log CFU/g)*				
	Cattle	Pig	Waterfowl	Deer	Raccoon
0	5.1B	5.4AB	5.6A	5.3AB	5.4AB
1	5.9B	6.8A	6.1B	6.9A	5.0C
3	5.8C	7.8B	6.1C	7.0B	4.8D
5	6.1C	7.9A	5.9C	7.0B	4.5D
7	5.7B	7.5A	6.1B	7.1A	4.2C
14	5.5B	5.8B	5.0B	6.9A	3.2C
28	5.9A	3.3B	2.6BC	6.8A	1.3C
56	5.0B	0.7D	2.3C	6.8A	0.0D
84	4.9B	0.8CD	2.3C	6.6A	0.0D
112	4.3B	1.0C	0.8C	6.4A	0.0C
140	3.9B	0.0C	0.7C	6.4A	0.0C
168	4.0B	0.4C	0.8C	6.2A	0.0C
196	3.5B	0.0C	0.7C	5.6A	0.0C
224	3.2B	0.0D	1.4C	5.7A	0.0D
252	3.2B	0.2C	0.1C	5.5A	0.0C
280	3.8A	0.2B	0.4B	4.6A	0.0B
308	3.7A	0.7B	0.6B	4.3A	0.0B
336	3.7A	0.8B	0.0B	4.3A	0.0B
364	3.5A	0.6B	0.0B	3.4A	0.0B

*Means with different letter within each row are significantly different ($P < 0.05$).

Table 5-4. Multifactor analysis of variance tests for animal and pathogen interactions within each time interval. For animals, *Salmonella* and STEC concentrations were averaged together for each animal. For pathogens, total *Salmonella* and total STEC concentrations from all animals were averaged, respectively. Animal*Pathogen: animal pathogen interactions.

Days	Animal	P-values*	
		Pathogen	Animal*Pathogen
0	0.0061	0.2767	0.5023
1	<0.0001	0.0223	0.0046
3	<0.0001	0.0004	0.0002
5	<0.0001	<0.0001	0.0014
7	<0.0001	0.0535	0.0232
14	<0.0001	0.0809	0.0005
28	<0.0001	0.0017	<0.0001
56	<0.0001	0.2791	0.6723
84	<0.0001	0.7023	0.8951
112	<0.0001	0.1300	0.4122
140	<0.0001	0.9267	0.9706
168	<0.0001	0.2416	0.8548
196	<0.0001	0.0531	0.2577
224	<0.0001	0.2534	0.5089
252	<0.0001	0.6540	0.8030
280	<0.0001	0.2788	0.8738
308	<0.0001	0.0005	0.4459
336	<0.0001	0.0025	0.1554
364	<0.0001	0.0051	0.0860

*Means with different letter within each row are significantly different ($P < 0.05$).

Table 5-5. Rate of reduction for *Salmonella*, STEC, and generic *E. coli* for each animal feces for initial linear decline.

Animal	Microorganism	Days used in equation	Equation	R ²	Reduction rates for 1 log (Day)*
Cattle	<i>Salmonella</i>	364	$y = -0.0053x + 5.3825$	0.4919	188.7A
	STEC	364	$y = -0.0091x + 5.7021$	0.8867	109.9B
	Generic <i>E. coli</i>	364	$y = -0.0089x + 6.2056$	0.8655	112.4B
Pig	<i>Salmonella</i>	84	$y = -0.086x + 7.2986$	0.8595	11.6A
	STEC	56	$y = -0.137x + 7.2104$	0.8195	7.3A
	Generic <i>E. coli</i>	140	$y = -0.0397x + 5.4227$	0.5978	25.2B
Waterfowl	<i>Salmonella</i>	140	$y = -0.0423x + 6.2047$	0.9278	23.6A
	STEC	28	$y = -0.1755x + 6.1942$	0.9283	5.7B
	Generic <i>E. coli</i>	112	$y = -0.0571x + 7.2668$	0.9362	17.5C
Deer	<i>Salmonella</i>	364	$y = -0.0062x + 6.9756$	0.6769	161.3A
	STEC	364	$y = -0.0093x + 6.9774$	0.8344	107.5B
	Generic <i>E. coli</i>	364	$y = -0.0058x + 6.9308$	0.7639	172.4A
Raccoon	<i>Salmonella</i>	56	$y = -0.1023x + 5.0327$	0.9097	9.8A
	STEC	56	$y = -0.0936x + 4.8617$	0.9576	10.7A
	Generic <i>E. coli</i>	56	$y = -0.111x + 6.113$	0.912	9.0A

* Capital letters within columns indicate significant difference among microorganisms for each animal feces ($P < 0.05$).

Table 5-6. Calculated trendline equations from survival data.

Animal	Bacteria	R ²	Linear	R ²	Polynomial (2 nd order)	Final R ²
			Equation		Equation	
Cattle	<i>Salmonella</i>	0.4919	$y = -0.0053x + 5.3825^*$	0.8607	$y = 5E-05x^2 - 0.0213x + 5.8803$	0.4362
	STEC	0.8867	$y = -0.0091x + 5.7021^*$	0.9025	$y = 1E-05x^2 - 0.0133x + 5.8329$	0.7675
	Generic <i>E. coli</i>	0.8655	$y = -0.0089x + 6.2056$	0.8656	$y = 1E-06x^2 - 0.0093x + 6.2179^*$	
Pig	<i>Salmonella</i>	0.5533	$y = -0.0179x + 5.3398$	0.8845	$y = 0.0001x^2 - 0.0657x + 6.8306^*$	0.6971
	STEC	0.575	$y = -0.0189x + 4.8574$	0.8085	$y = 0.0001x^2 - 0.0605x + 6.1547^*$	0.6372
	Generic <i>E. coli</i>	0.6333	$y = -0.0168x + 4.5139$	0.7881	$y = 9E-05x^2 - 0.0455x + 5.4093^*$	
Waterfowl	<i>Salmonella</i>	0.7722	$y = -0.0173x + 5.2892$	0.9048	$y = 8E-05x^2 - 0.0421x + 6.0623^*$	0.8669
	STEC	0.6584	$y = -0.0153x + 4.1522$	0.838	$y = 9E-05x^2 - 0.0431x + 5.0175^*$	0.8029
	Generic <i>E. coli</i>	0.765	$y = -0.0224x + 5.9373$	0.9581	$y = 0.0001x^2 - 0.0615x + 7.1543^*$	
Deer	<i>Salmonella</i>	0.6769	$y = -0.0062x + 6.9756^*$	0.8103	$y = -3E-05x^2 + 0.0033x + 6.68$	0.5171
	STEC	0.8344	$y = -0.0093x + 6.9774^*$	0.8927	$y = -3E-05x^2 - 0.0008x + 6.7113$	0.6373
	Generic <i>E. coli</i>	0.7639	$y = -0.0058x + 6.9308^*$	0.7669	$y = 4E-06x^2 - 0.007x + 6.9697$	
Raccoon	<i>Salmonella</i>	0.8319	$y = -0.0703x + 4.7242$	0.9872	$y = 0.0015x^2 - 0.1853x + 5.4363^*$	0.9736
	STEC	0.8847	$y = -0.0666x + 4.6017$	0.9911	$y = 0.0011x^2 - 0.1541x + 5.1432^*$	0.9774
	Generic <i>E. coli</i>	0.8777	$y = -0.0822x + 5.8348$	0.9862	$y = 0.0014x^2 - 0.1915x + 6.5119^*$	

* The equations used in models.

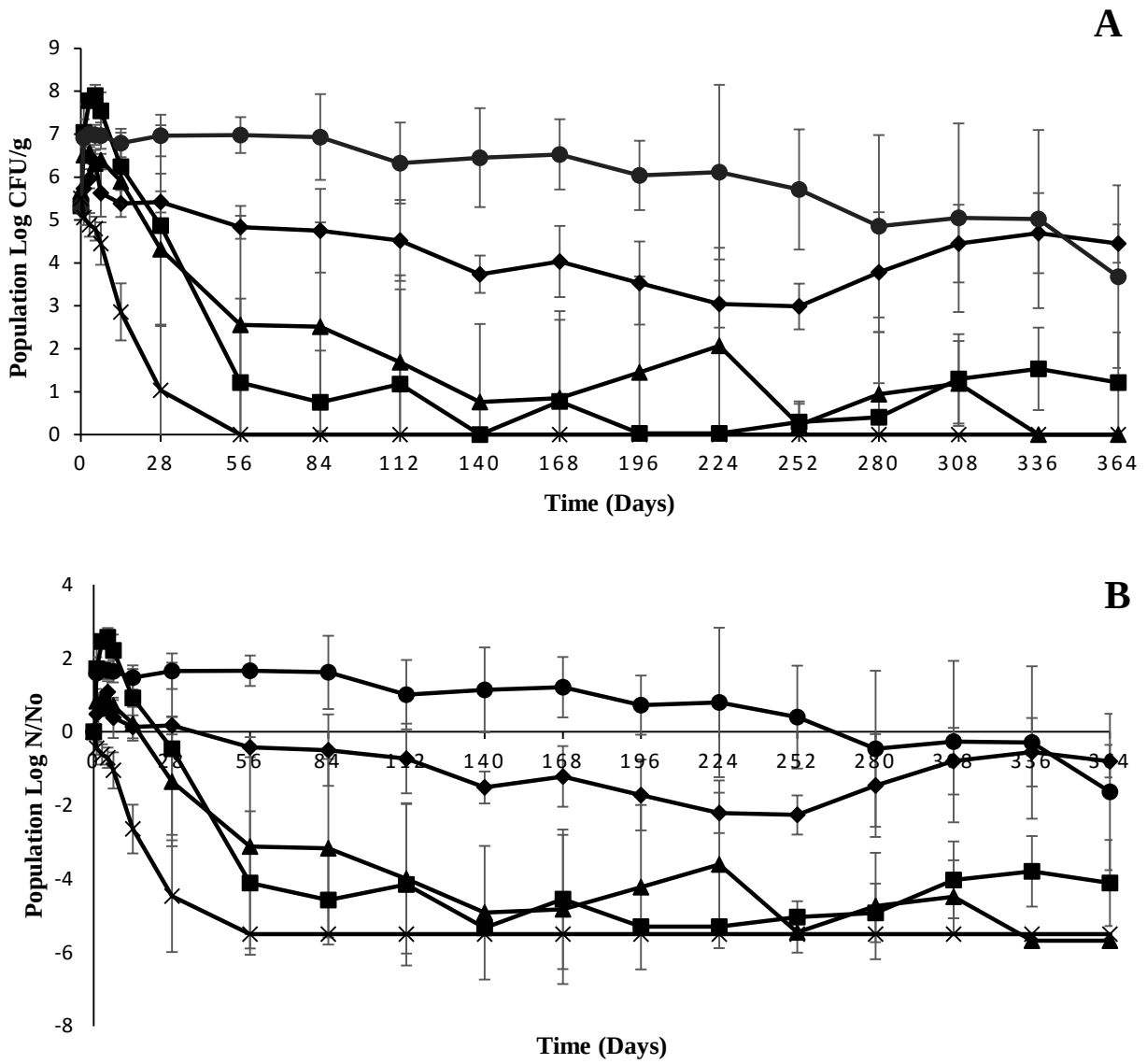


Figure 5-1. Actual (A) and normalized (B) *Salmonella* cocktail population in cattle (◆), pig (■), waterfowl (▲), deer (●), and raccoon (×) feces at room temperature (n=6).

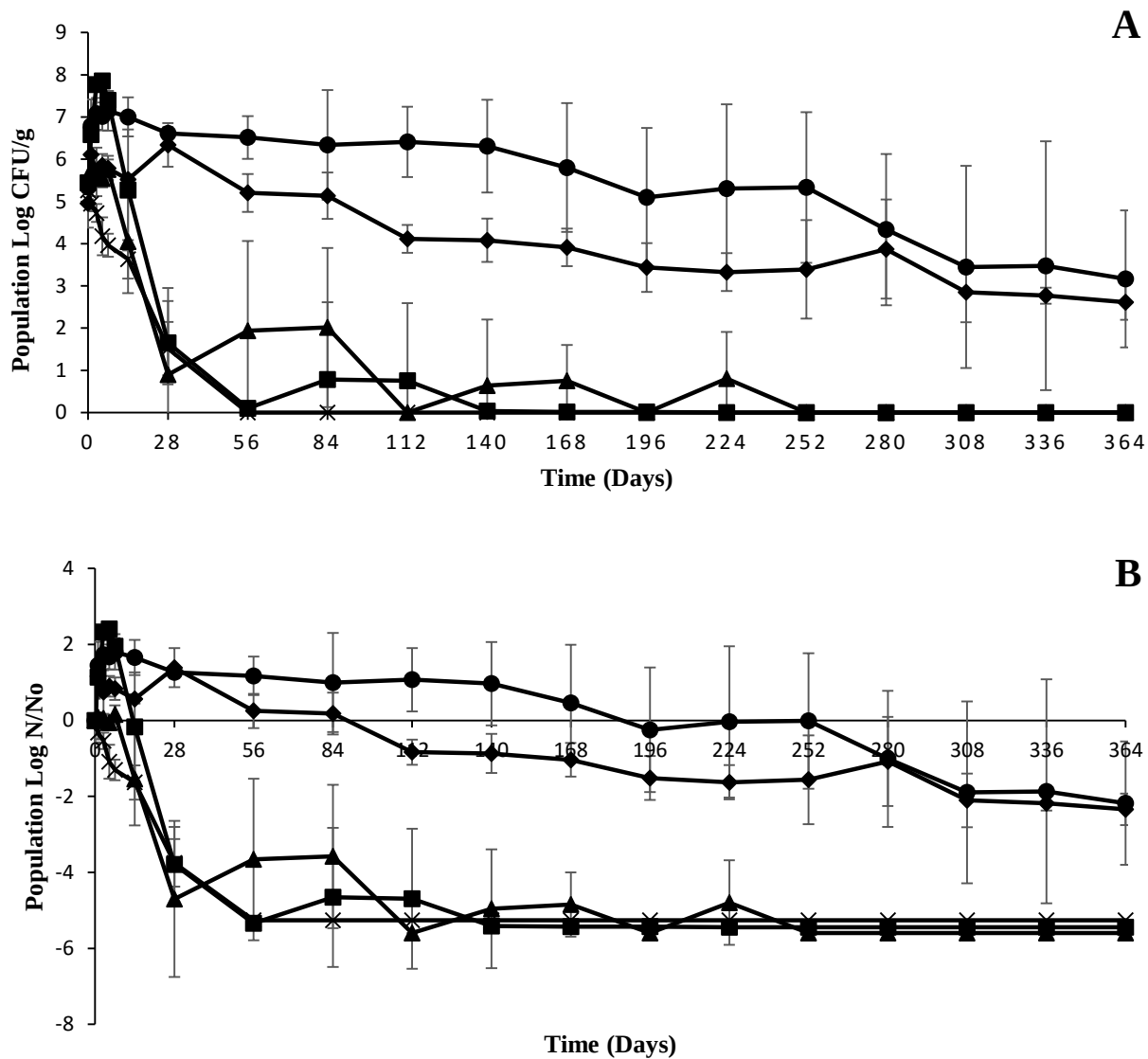


Figure 5-2. Actual (A) and normalized (B) STEC cocktail population in cattle (♦), pig (■), waterfowl (▲), deer (●), and raccoon (×) feces at room temperature (n=6).

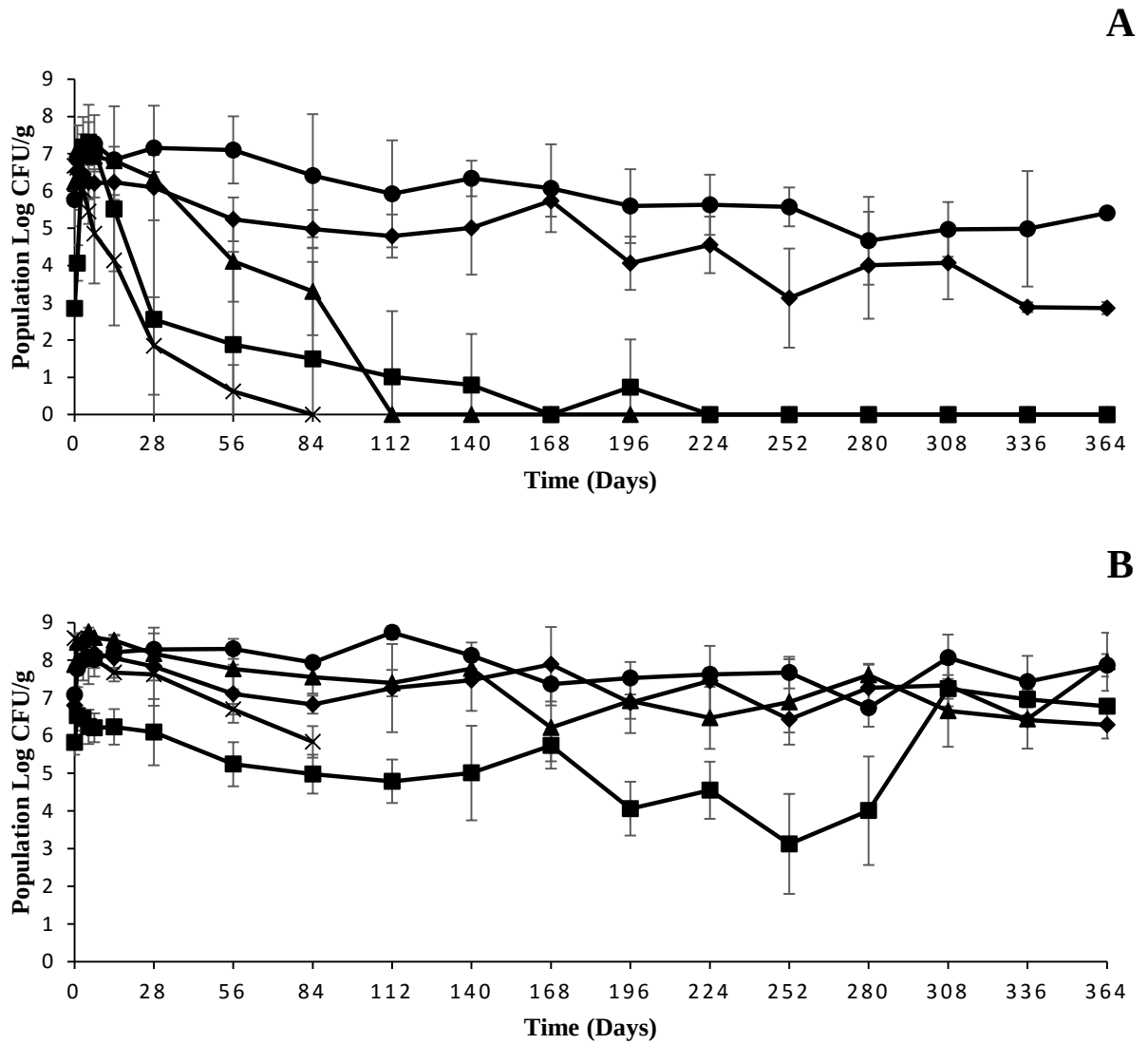


Figure 5-3. Generic *E. coli* (A) and total aerobic count (B) population in cattle (♦), pig (■), waterfowl (▲), deer (●), and raccoon (×) feces at room temperature (n=3).

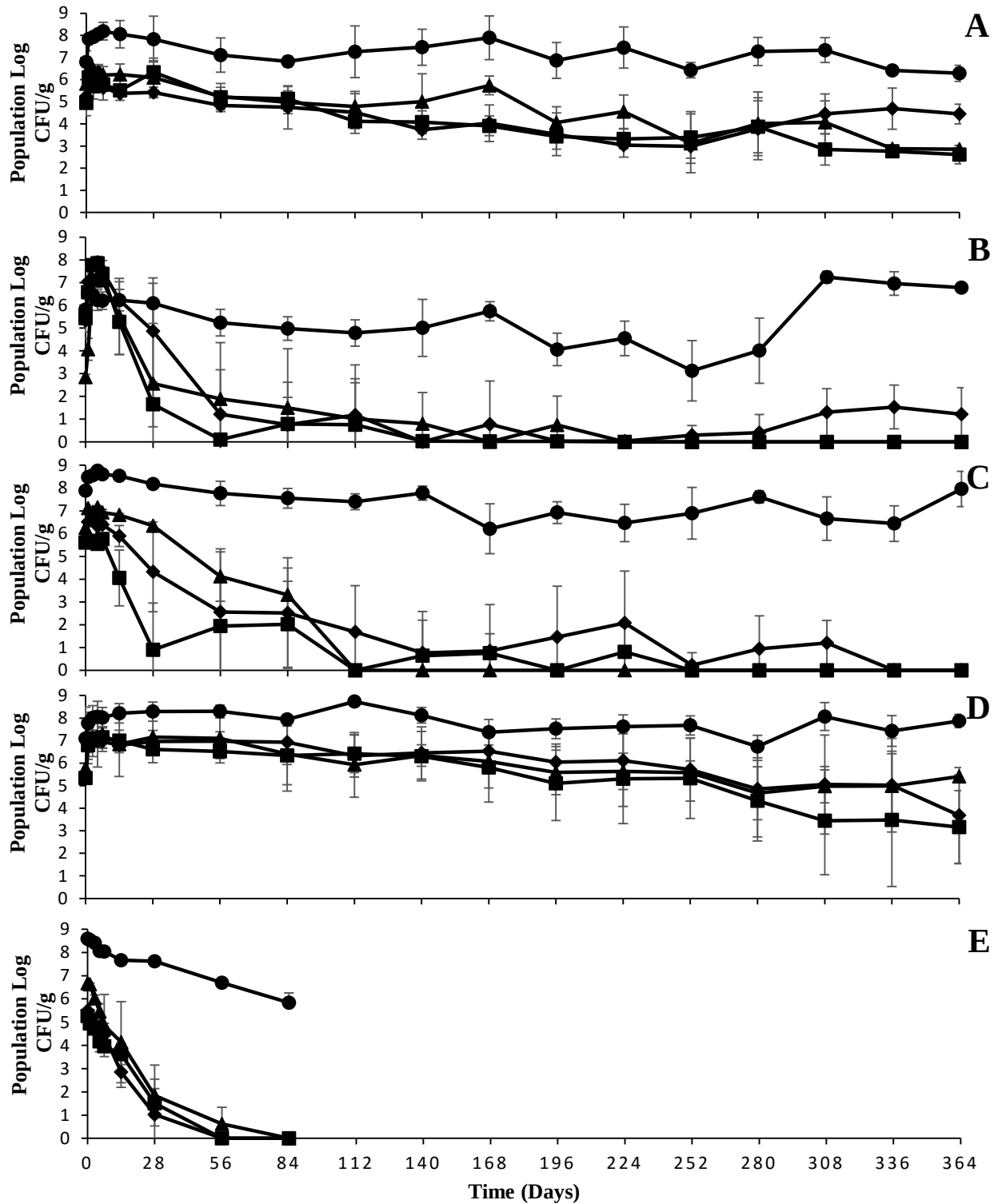


Figure 5-4. Population of *Salmonella* (♦), STEC (■), generic *E. coli* (▲), and total aerobic count (●) in cattle (A), pig (B), waterfowl (C), deer (D), and raccoon (E) feces at room temperature.

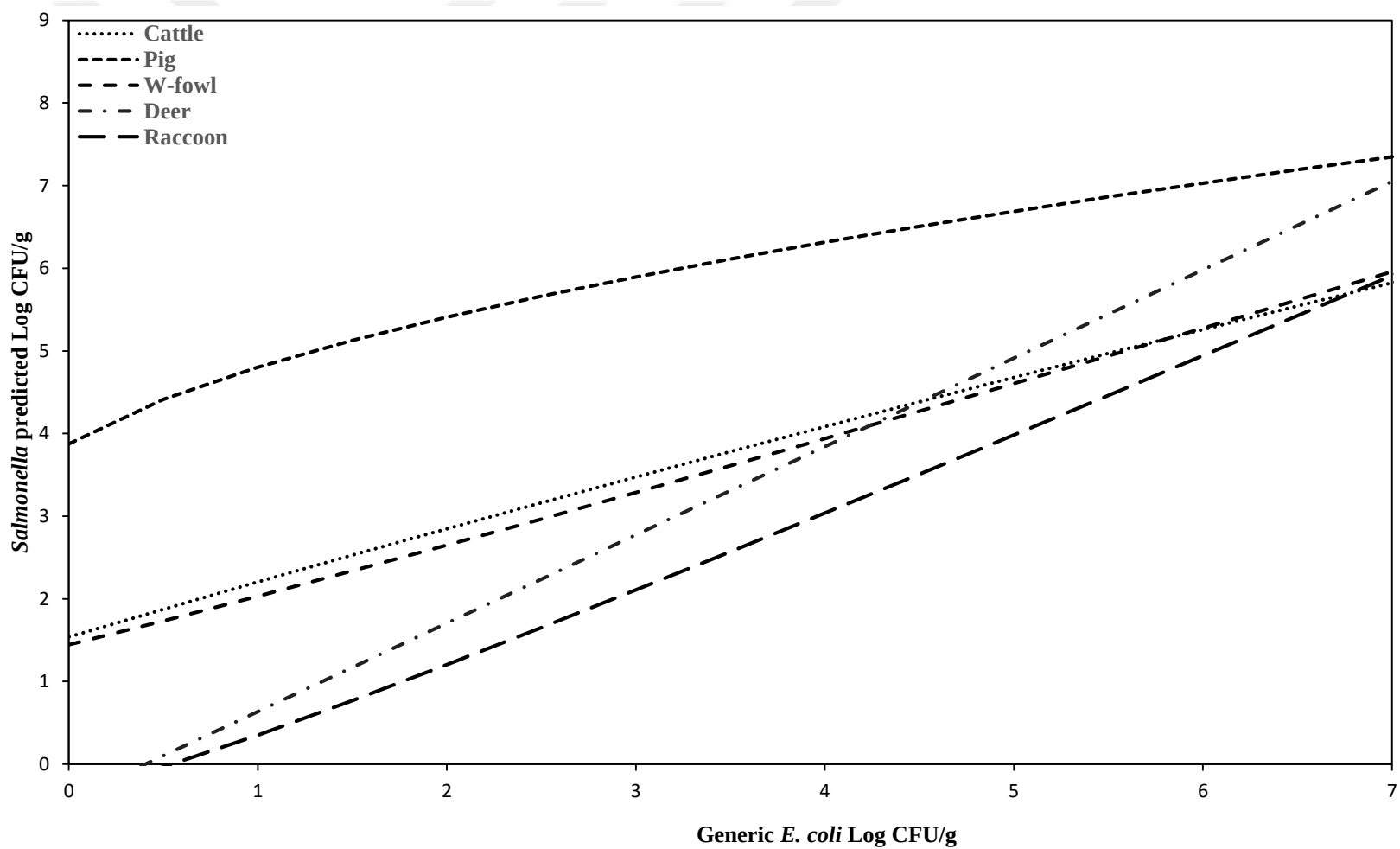


Figure 5-5: *Salmonella* population prediction model based on generic *E. coli* population in the farm cattle and wild animal feces.

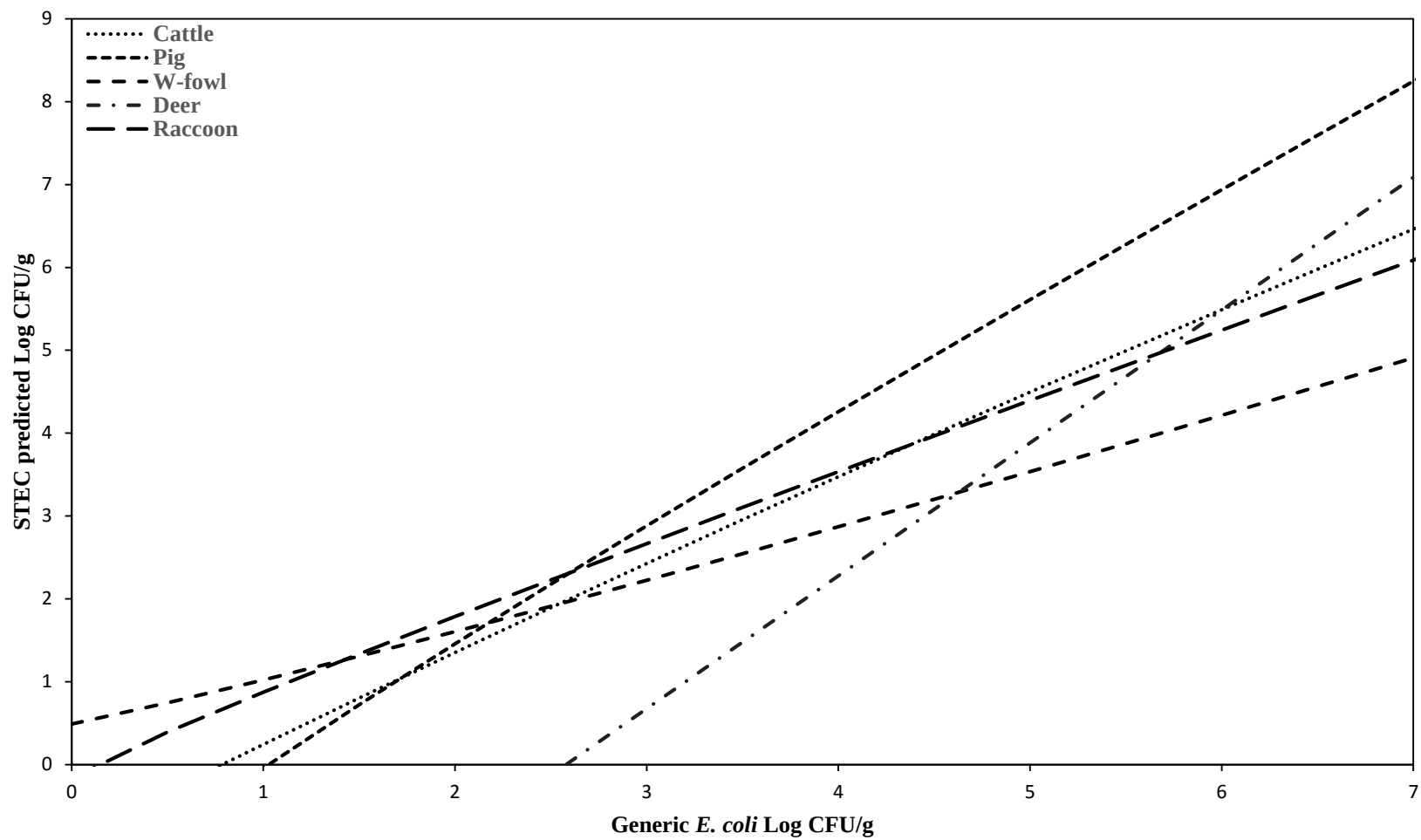


Figure 5-6: STEC population prediction model based on generic *E. coli* population in the cattle and wild animal feces.

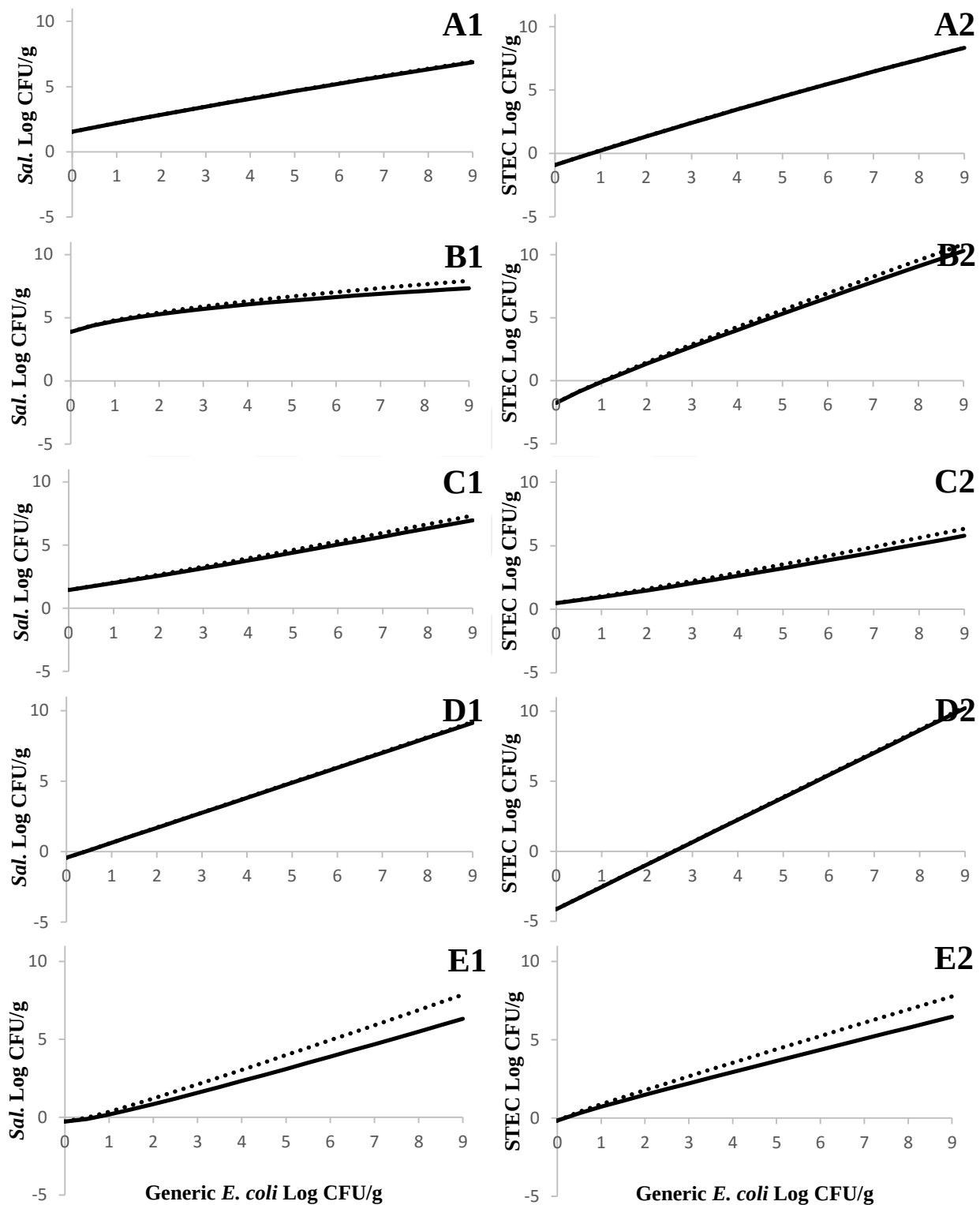


Figure 5-7. Predicted (dotted line) and actual (solid line) population of *Salmonella* (1) and STEC (2) cocktail population in cattle (A), pig (B), waterfowl (C), deer (D), and raccoon (E) feces at room temperature.

CHAPTER 6

FATE OF GENERIC *E. COLI* AND PREDICTION OF *SALMONELLA* AND STEC SURVIVAL IN FECAL PATS UNDER FIELD CONDITIONS IN FLORIDA

Introduction

Tomatoes are grown throughout Florida, and environmental conditions differ significantly from South to North. The prevalence of animal intrusion is highly varied in the field, based on proximity to domestic animals and riparian habitats, but is not uncommon. Efforts to exclude animals from fields to minimize fecal contaminations are not always successful. The evidence of animal intrusion is seen in tomato fields as fecal deposits, footprints, consumed crops, broken and dispersed plants, and dead animals. Animal intrusion is considered one of the main route of contamination in produce fields by FDA (2015).

Pathogen transfer from animals to produce depends on a variety of production practices and environmental variables. Fecal deposits may be direct or indirect cause of outbreak or a contributing factor. Human pathogens, including *Salmonella*, *Listeria monocytogenes*, *E. coli* O157:H7, have been isolated from wildlife species including deer, wild pig, cattle, elk, coyote, and various types of birds (Gardner et al., 2011; Gorski et al., 2011, Fenlon 1985; Jay et al., 2007; Wahlström et al., 2003). Animal feces were implicated as a source of contamination in produce related outbreaks (CalFERT, 2007; Laidler et al., 2013). Several factors may contribute the contamination of produce. Pathogens in animal feces may directly deposited on produce, or spread as a consequence of harvesting practices, the use of contaminated irrigation water, rain splatter, postharvest water, or from excessive water in the flooded fields. *Salmonella* Montevideo isolates from tomatoes, opossum, and mice were identical after wild animal intrusion to several green houses with hydroponic system in Mexico (Orozco et al., 2008). Risk mitigation of animal intrusion and remediation of fecal deposits in the field is essential for safe production.

Generic *E. coli* and human enteric pathogens as *Salmonella* spp., and shiga-toxin producing *E. coli* (STEC) can survive for a long period of time in animal feces and manure depending on temperature differences (Fukushima et al., 1999; Himathongkham et al., 1999; Jiang et al., 2002; Kudva et al., 1998; Wang et al., 1996). Several factors affect contamination potential of animal feces around the site of fecal contamination including amount of feces, type of crop, height of the plant, location of contamination, wind speed and direction, humidity, insect/amphibian vectors, and irrigation method/rain water (Wild Farm Alliance, 2016). Generic *E. coli* was able to live more than 45 days in summer in fresh cattle manure in south-central interior of BC in Canada, and no effect of shade and fecal pat size was observed for the first week, but seen for longer time (Meays, et al., 2005). Shaded fecal pats had slower *E. coli* decline than non-shaded ones (Van Kessel et al., 2007). In a study, generic *E. coli* die-off had the lowest reductions at 27°C and 83% moisture content in dairy cow manure (Wang et al., 2004). The type of environmental conditions may vary dramatically in different areas.

The purpose of this objective was to evaluate fate of *E. coli* in fecal pats under different conditions in the tomato field and to predict *Salmonella* and STEC survival based on the model developed in the previous chapter. Limited data exist on relative risk associated with the presence and fate of pathogens and microbial indicators in animal feces on crops and mulch beyond leafy greens. The data generated here will improve the effectiveness of risk assessment and good agricultural practices performed for the protection of produce from animal originated microbial contamination in Florida.

Material and Methods

Preparation of Samples

The survival of naturally present generic *E. coli* in the cattle feces were tested under four different conditions including: (i) on tomatoes located on the inside of plants (TIP); (ii) on

tomatoes located on the outside of plants (TOP); (iii) on plastic mulch under tomato plants (MUP); and (iv) on mulch distant from tomato plants (MDP). The experiment was designed to test the behavior of generic *E. coli* in fecal pats under actual field conditions. Fields at the Gulf Coast Research and Education Center (GCREC) located in Wimauma, FL, Central Florida and North Florida Suwannee Research and Education Center – Suwannee Valley (NFREC) in Live Oak, FL under the University of Florida’s Institute of Food and Agricultural Sciences (UF/IFAS) were trans-planted with round tomatoes (Cultivars “HM 1823” in GCREC and “BHN 602” in NFREC) for experiment during the spring and fall. Tomatoes were grown under typical commercial conditions as described in Chapter 2. Two trials for each location and season were performed in total of eight trials for each condition.

Fresh cattle feces were obtained for each trial from a local farm in Clermont, FL and kept at 4°C up to three days. Dry matter of feces was measured by drying 5 g of sample at 106°C for three days. Fecal pats were prepared the day before fieldwork. For trials on tomatoes, feces was weighed into 1 g samples and packed. For each trial, two times more weighed fecal samples (≥ 96) than needed were prepared in case of losses due to environmental conditions. For trials on mulch, feces was weighed into 10 g samples and placed onto black polyethylene mulch that was cut into a circle and put into Petri dishes. For each trial, 24 extra fecal samples were prepared and packed in case of losses due to environmental conditions in addition to the 48 required samples. Samples were held at 4°C until use and transferred the field in the cooler.

Field Setup and Sample Collection

On the initial field day, the 1 g previously prepared fecal samples were placed on mature green tomatoes located inside and outside of plant with clear latex gloves. Fecal pats were stuck between fruit and stalk insertion to reduce the losses due to environmental conditions. Petri dishes with 10 g fecal pats on plastic mulch were placed under tomato plants on the beds and at

previously identified open areas distant from tomato plants on the bed in group of six plates. Petri dishes without fecal pats were placed on appropriate areas as control groups. Examples of fecal pats on tomatoes and plastic mulch in the field are shown in Figure 6-1.

Samples were collected at Day 0 immediately after setting and at Day 1, 3, and 7. On each sampling date, six tomatoes with feces on them from inside and outside of plants each were harvested with ethanol wiped scissors from two to three centimeter above stalk. During harvesting, tomatoes were dropped in sterile 24 oz. (710 ml) Whirl-Pak® filter bags (Nasco, Jackson, WI) to minimize contamination. Three tomatoes without fecal samples for each condition were collected randomly and placed in sampling bags as control groups. Six samples on plastic mulch at each conditions were carefully placed in sterile Whirl-Pak® bags with everything that had accumulated on them including leaves, sand, rain water, bugs, and other plant pieces. Three petri dishes without fecal pats for each condition were placed in sampling bags as control groups. Sterile bags were placed into cooler with ice for transfer to laboratory and immediate microbiological analysis.

Environmental conditions including temperature, relative humidity, and leaf wetness were measured with portable weather stations which were already set throughout the field (WatchDog 1000 Series Micro Stations, by Spectrum Technologies, Inc., Aurora, IL). The data were collected with 3 micro stations set as diagonally to the field during all the course of experiment for each season and location. Solar radiation data were obtained from the Florida Automated Weather Network station that was closest the tested fields (<http://fawn.ifas.ufl.edu/>).

Microbiological Analysis

Tomatoes were processed by addition of 9 mL 0.1% peptone water (Difco, Becton, Dickinson, Sparks, MD) into bag. Tomatoes in the bag with peptone water were washed as much as possible before disassembling fecal pats. Then, tomatoes, and stalks were removed from bag

and the peptone feces remaining in the bag were squeezed by hand followed by mixing with a stomacher (Smasher, AES Lab, Cranbury, NJ) for one minute. Similarly, mulch with fecal pats were filled with 90 mL 0.1% peptone water and stomached for one minute prior to plating.

The same procedure was applied to enumerate generic *E. coli* population in the cattle feces on mulch and tomatoes; 1 mL of sample was serially diluted in peptone water. Dilutions (100 μ L) were spread onto CHROMagar™ ECC (DRG, Mountainside, NJ, USA) in duplicates. To increase the sensitivity, 1 mL of the initial dilution was plated over 4 plates (0.25 mL/plate). Plates were incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Blue colonies were counted on plates and number of colonies were recorded. Enrichment was planned, but not applied since generic *E. coli* population never fell below countable levels during all trials.

Statistical Analysis and Prediction of Pathogen Survival

Generic *E. coli* concentration data from each replicates on each sampling time were averaged and plotted against time after log-transformation for each condition. JMP Pro 11.0 (SAS, Cary, NC, USA) was used in determination of statistical significances using ANOVA and Tukey's HSD test. The critical *P*-value was set at 0.05.

For prediction of pathogen survival in fecal pats in the field conditions, average generic *E. coli* concentration for each location were calculated for each sampling day by combining all data for tomatoes and plastic mulch in each season ($n = 24$). Developed predictive models in Chapter 5 were used to calculate STEC and *Salmonella* survival predictions based on generic *E. coli* concentration in the cattle feces.

Results

Four conditions were tested to understand the survival behavior of generic *E. coli* in fecal pats in tomato fields in Central (GCREC) and North Florida (NFREC). Generic *E. coli* concentrations in fecal pats remained above 2.4 Log CFU/g all the time, and reached up to 6.5

Log CFU/g in the cattle feces placed on tomatoes located inside of plants (TIP), on tomatoes located outside of plants (TOP), on mulch under tomato plants (MUP), and on mulch distant from tomato plants (MDP). The most stable and unstable generic *E. coli* population were in the fecal pats on MUP where population levels ranged from 4.5 to 6.5 Log CFU/g and on TIP where populations ranged from 2.4 to 6.5 Log CFU/g in all trials during the two sampling season. In general, *E. coli* populations stayed higher in the fecal pats on mulch; however, there were no constant trends among the tested conditions at all time intervals. No statistically significant difference was calculated conditions between MDP and MUP in Fall 1 and Fall 2 trials performed in both GCREC and NFREC ($P > 0.05$). At Day 7, 5 out of 8 times, generic *E. coli* concentration was statistically the same at all conditions ($P > 0.05$); significant differences were seen almost all the times at Day 1 and Day 3 except for one time for each sampling time done in fall trials ($P < 0.05$). Fluctuations in generic *E. coli* populations were observed more during spring trials under all conditions.

No generic *E. coli* was detected from control groups at all tested conditions and trials. More detailed results for each season and location are included below.

Spring Trials - NFREC

Generic *E. coli* concentrations in the cattle feces stayed close to each other with small fluctuations at all tested conditions (TOP, TIP, MUP, and MDP) during Spring 1 and Spring 2 trials at NFREC as shown in Figure 6-2 A and Figure 6-2 C. All means with standard deviations for generic *E. coli* concentration in the fecal pats on tomatoes and plastic mulch are presented for the spring season at Table 6-2. Initial concentrations at Day 0 ranged from 4.5 to 6.1 Log CFU/g under all conditions. At Day 1, increases in generic *E. coli* populations (Log CFU/g) in fecal pats were 1.3 in TIP, 0.4 in TOP, and 0.9 in MDP, and populations were between 5.8 and 6.0 Log CFU/g. Oppositely, *E. coli* concentrations declined from 5.4 to 5.1 Log CFU/g over the first day

on MUP in the Spring 1 trial. A 1.4 Log CFU/g increase in *E. coli* concentration in the cattle feces was seen on MDP in Spring 2 trial, and decreases (Log CFU/g) were 1.7 on TOP, 1.3 on TIP, and 0.3 on MUP with a range from 4.4 to 6.1 Log CFU/g at Day 1. Generic *E. coli* concentrations in fecal pats on MUP in trial 1 and MDP in trial 2 were statistically different from other conditions at Day 1 ($P < 0.05$). No particular survival trend was observed at Day 3 among tested conditions. Generic *E. coli* population in fecal pats were 5.1 and 5.5 Log CFU/g on TIP and 4.6 and 4.5 Log CFU/g on TOP in Spring 1 and Spring 2 trials, respectively. The cattle feces on MUP had generic *E. coli* concentrations at 5.2 Log CFU/g in both spring trials at Day 3. *E. coli* populations were 4.2 and 5.9 Log CFU/g in the cattle feces on MDP in Spring 1 and 2 trials at Day 3, respectively. At Day 7, no statistically significant difference was calculated among all tested conditions in Spring 1 trial with a generic *E. coli* concentration ranges from 4.6 to 5.2 Log CFU/g ($P > 0.05$). In Spring 2 trial, population of generic *E. coli* (Log CFU/g) in fecal pats was 4.8 on TOP, 4.5 on TIP, 5.7 on MDP and, 5.1 on MUP at Day 7. With the exception of generic *E. coli* concentrations on MUP, statistically significant differences among all sampling days was calculated for all conditions for both trials ($P < 0.05$).

Spring Trials - GCREC

Populations of generic *E. coli* did not behave similarly at all tested conditions (TIP, TOP, MUP, and MDP) in the cattle feces during Spring 1 and Spring 2 trials at GCREC as shown in Figure 6-2 B and Figure 6-2 D. All means with standard deviations for generic *E. coli* concentration in the fecal pats on tomatoes and plastic mulch are presented for spring season at Table 6-2. Initial concentrations at Day 0 were between 5.1 and 5.9 Log CFU/g at all conditions in both Spring 1 and Spring 2. Population of generic *E. coli* in fecal pats on MUP increased 0.5 and 0.9 Log CFU/g at Day 1 in Spring 1 and Spring 2 trials, respectively. Decline was observed at other conditions at Day 1, and statistical difference was calculated between MUP and other

conditions in both trials ($P < 0.05$). Generic *E. coli* concentration dropped to 4.3 Log CFU/g from 5.7 on TIP, to 3.5 Log CFU/g from 5.9 on TOP, and to 4.1 Log CFU/g from 5.6 on MDP in trial 1. Similar to Spring 1 trial at Day 1, concentration declined to 3.9 Log CFU/g from 5.6 on TIP, to 3.4 Log CFU/g from 5.1 on TOP, and to 4.0 Log CFU/g from 5.2 on MDP. At Day 3, increases in generic *E. coli* population (Log CFU/g) in fecal pats were 2.2 on TOP, 0.7 on TIP, 1.6 on MDP, and 0.6 on MUP compare to previous sampling day in Spring trial 1. Statistically significant differences were seen between conditions on tomato and mulch in Spring 2 trial ($P < 0.05$). Generic *E. coli* concentration dropped to around 2.5 Log CFU/g in fecal pats on TOP and TIP; however, populations were 5.0 Log CFU/g on MDP and 4.6 Log CFU/g on MUP in Spring 2 trial at Day 3. No significantly different concentrations were calculated among all conditions in Spring 1 trial at Day 7 ($P > 0.05$), and generic *E. coli* populations (Log CFU/g) in fecal pats were 4.8 on TIP, 4.7 on TOP, 4.5 on MDP, and 5.2 on MUP. In Spring 2 trial, samples on TOP had the lowest population of generic *E. coli* (2.9 Log CFU/g), and concentrations in fecal pats at other conditions were 3.9 on TIP, 4.6 on MDP, and 5.1 Log CFU/g on MUP. No generic *E. coli* concentrations was statistically the same among all sampling days for both trials as seen in Table 6-2 ($P < 0.05$).

Fall Trials - NFREC

Generic *E. coli* behaved similarly and population never decreased below 5.0 Log CFU/g at all tested conditions (TIP, TOP, MUP, and MDP) in the cattle feces during Fall 1 and Fall 2 trials at NFREC as shown in Figure 6-3 A and Figure 6-3 C. All means with standard deviations for generic *E. coli* concentration in the fecal pats on tomatoes and plastic mulch are shown for fall season at Table 6-3. Initial concentrations at Day 0 were close to each other and between 5.0 and 5.3 Log CFU/g at all conditions in Fall 1 trial, and ranged from 5.6 to 6.1 Log CFU/g in Fall 2 trial. With the exception of TOP (remained the same as in Day 0) in Fall 2 trial, generic *E. coli*

concentration increased up to 6.5 Log CFU/g at Day 1 at other conditions. The increases (Log CFU/g) in generic *E. coli* population in fecal pats at Day 1 were 1.1 and 0.0 on TOP, 1 and 0.4 in TIP, 0.5 and 0.6 on MDP, and 0.4 and 0.9 on MUP in Fall 1 and Fall 2 trial. At Day 3, population of generic *E. coli* (Log CFU/g) in the cattle feces were 6.4 and 5.7 on TOP, 6.2 and 5.5 on TIP, 5.8 and 6.0 on MDP, and 5.5 and 6.1 on MUP in Fall 1 and Fall 2 trials, respectively. Generic *E. coli* concentration was statistically different only on MUP in Fall 1 trial and on TIP in Fall 2 trial at Day 3. No statistical difference was calculated among all conditions in both trials, and population stayed between 5.3 and 5.7 Log CFU/g at Day 7 ($P > 0.05$). No generic *E. coli* concentrations was statistically the same among all sampling days for both trials except for those in MUP in Fall 1 trial and in TOP in Fall 2 trial ($P < 0.05$).

Fall Trials – GCREC

Generic *E. coli* behaved parallel in fecal pats on TOP and TIP and similar to those on MDP and MUP during both Fall 1 and Fall 2 trials at NFREC as shown in Figure 6-3 B and Figure 6-3 D. Concentrations in the fecal pats on mulch always stayed higher than those on tomatoes at all conditions except for Day 0. All means with standard deviations for generic *E. coli* concentration in the fecal pats on tomatoes and plastic mulch were shown for fall season at Table 6-3. Initial concentrations at Day 0 were close and between 5.6 and 5.8 Log CFU/g at all conditions in Fall 1 trial, and ranged from 5.0 to 5.8 in Fall 2 trial. At Day 1, generic *E. coli* concentration in fecal pats decreased to 4.8 Log CFU/g from 5.8 on TOP and to 5.1 Log CFU/g from 5.6 on TIP in Fall 1 trial, and dropped to 5.1 and 4.8 Log CFU/g on TOP and TIP with 0.2 reduction in Fall trial 2 ($P > 0.05$). Concentrations on mulch at Day 1 increased up to 0.3 Log CFU/g, and were 5.5 and 6.1 Log CFU/g on MDP and MUP in Fall 2 trial, and 5.8 Log CFU/g on MDP and MUP in Fall 1 ($P > 0.05$). At Day 3, generic *E. coli* concentration (Log CFU/g) in fecal pats were 4.7 on TOP, 3.9 on TIP, 5.0 on MDP, and 5.4 on MUP in Fall 1 trial. No

statistically significant difference was calculated on concentrations ranging from 4.8 to 5.7 Log CFU/g in Fall 2 trial at Day 3 ($P > 0.05$). At Day 7, generic *E. coli* concentration (Log CFU/g) in the cattle feces on tomato (TOP-3.5, TIP-3.4) were not statistically different from those (MDP-4.1, MUP-4.5) on mulch in Fall 1 trial ($P > 0.05$). In Fall trial 2, the differences were statistically significant between concentrations (Log CFU/g) on tomato and mulch and 3.6 on TOP, 4.3 on TIP, 5.1 on MDP, and 5.4 on MUP ($P < 0.05$) at Day 7. No generic *E. coli* concentrations was statistically the same at all sampling days for both trials except for those on MDP and MUP in Fall 2 trial ($P < 0.05$).

Prediction of Pathogen Survival Based on Generic *E. coli* Concentration

Limited fluctuations in generic *E. coli* concentrations in the fecal pats on tomato and mulch led us to combine TIP and TOP, and MUP and MDP survival data to use in prediction of STEC and *Salmonella* populations for the same season. Generic *E. coli* concentration in fecal pats ranged from 4.0 and 6.5 Log CFU/g on mulch for all conditions, sampling days, and trials. Similarly, differences in generic *E. coli* concentrations on tomato samples were limited and less than 2.5 Log CFU/g in most cases at each sampling time for all conditions, and trials. For each prediction plot, all generic *E. coli* concentrations at each location and season were averaged for tomato and mulch separately at each sampling day ($n=24$). For example, survival data from TOP and TIP in Spring 1 and Spring 2 trials were combined to have more representative predictions. Prediction of STEC and *Salmonella* survival had similar trends to generic *E. coli* in the cattle feces (Figure 6-4 and Figure 6-5). Predicted pathogen populations stayed below indicator microorganism concentration except for GCREC trials on tomatoes as shown in Figure 6.4 B and 6.5 D. The concentration differences between generic *E. coli* and predicted pathogen populations in the fecal pats was less than 1 Log CFU/g at each time interval all the time. *Salmonella* population was predicted ≤ 0.7 higher than STEC population on tomato and mulch in both

seasons with the exception of fall season in NFREC (Figure 6-4 C and Figure 6-5 C). The concentration ranges for each microorganism for all conditions and seasons were 3.7 to 6.3 Log CFU/g for generic *E. coli*, 3.9 to 5.4 Log CFU/g for predicted *Salmonella*, and 3.1 to 5.8 Log CFU/g for STEC. Predicted *Salmonella* and STEC concentrations were close to each other and above 4.2 Log CFU/g in the cattle feces on mulch; lower populations were seen in the samples on tomato tested in GCREC than those in NFREC starting from Day 1. In NFREC, predicted pathogen populations in the cattle feces on tomatoes did not go below 4.3 Log CFU/g in any sampling day during both seasons; they decreased as low as 3.1 Log CFU/g in GCREC.

Dry Matter, Temperature, Relative Humidity, Leaf Wetness, and Solar Radiation

Dry matter content of the cattle feces for each trial were as follows: NFREC-Spring 1 - 10.2%, NFREC-Spring 2 - 11.5%, NFREC-Fall 1 - 20%, NFREC-Fall 2 - 18.0%, GCREC-Spring 1 - 14.6%, GCREC-Spring 2 - 10.6%, GCREC-Fall 1 - 10.2%, and GCREC-Fall 2 - 10.2%. All dry matters and related moisture percentages were listed Table 6-1. Daily minimum, average, and maximum temperature (°C), relative humidity (%), leaf wetness, solar radiation, and wet hour changes during the course of experiment are shown in Figure 6-6, Figure 6-7, Figure and 6-8 for all seasons and sampling sites.

Discussion

There are several studies regarding the survival of commonly accepted indicator microorganisms and pathogens in manure and manure slurries for different length of time (Fukushima et al., 1999; Himathongkham et al., 1999; Jiang et al., 2002; Kudva et al., 1998; Meays et al., 2005; Wang et al., 1996). Different STEC strains at 10^5 CFU/g concentrations survived for 18 weeks at 15°C, in the cattle feces (Fukushima et al., 1999). Cattle feces and manure-amended soils had *E. coli* O157:H7 positive for 2 and 6 months, respectively (Jiang et al., 2002; Wang et al., 1996). In manure and manure slurry, *Salmonella* concentrations dropped

six Log CFU/g in 48 days at 37°C (Himathongkham et al., 1999). In sheep manure, *E. coli* O157:H7 survived for 21 months (Kudva et al., 1998). They are mostly performed in laboratory with stable conditions as humidity and temperature. *E. coli* concentration in bovine feces exhibited an initial growth followed by die-off in laboratory and pasture conditions, but die-off was faster in the field compare to laboratory conditions at similar temperatures (Van Kessel et al., 2007). Rather than checking how long a microorganisms survive in animal feces, the aim of this study was to see differences in the survival of generic *E. coli* in the cattle feces at actual and various field conditions that may change in Florida. The worst case scenario, the presence of fecal deposits directly on fruit, was tested with repetitive trials in fall and spring seasons in Central and North Florida for better understanding. This is the first study that attempt to understand the behavior of generic *E. coli* in fecal pats on fruit located inside and outside of plant and on plastic mulch under and distant to plant in tomato fields.

Populations of naturally present generic *E. coli* in the cattle feces under different field conditions in the shade and not in the shade stayed high and in similar concentrations (4 to 6.5 Log CFU/g) almost in all trials in both spring and fall season for a week period of time. Generic *E. coli* population did not go below 4 Log CFU/g in over 90% of all conditions and trials. one gram of the cattle feces on tomatoes inside or outside of plant had as high as *E. coli* concentrations in 10 g of the cattle feces on mulch located under or distant from plants in a week. This supports that fecal deposits in the field should be treated the same in tomato fields without considering the condition and amount of the deposit. Similar results were obtained in a study that looked at the effect of solar radiation on survival of naturally found generic *E. coli* in fecal pats in south-central interior of BC in Canada for a week in summer (Meays et al., 2005). There were no significant difference among 0, 20, 40, 80, and 100% shade treatment on *E. coli* concentration

in 2.5 kg cattle manure in a week, but shade treatment affected the survival of *E. coli* after Day 17, and to the last sampling at Day 45. (Meays et al., 2005). In another study, shaded cowpats (600 g) had significantly slower *E. coli* die-off and 2.2 times higher *E. coli* concentrations than non-shaded cowpats over 27 days of sampling (Van Kessel et al., 2007).

Effect of seasonality was seen on generic *E. coli* population in the cattle feces over the 7 days of sampling. No general trend in *E. coli* survival in fecal pats on mulch samples was seen in trials at different conditions performed during spring over the 7 day of sampling. A slow decline in *E. coli* concentrations in the cattle feces on mulch was observed with a maximum 1.5 Log CFU/g change in a week in fall trials in both Central (GCREC) and North Florida (NFREC). In the spring trial, *E. coli* concentration in the fecal pats on TOP, TIP, MDP, and MUP showed fluctuations up to 3 logs between sampling days. Particularly, the decline in generic *E. coli* concentration in the cattle feces reached up to 3.2 Log CFU/g on tomato in one of Spring 2 trial in GCREC. Differences in generic *E. coli* population behavior between fall and spring trials can be explained with changes among the exposure amount of solar radiation and proportionally temperature as the major affecting factors in the field. During spring, overall solar radiation average over the incubation period was around two times more than that was in fall season. Overall temperature averages were up to 3 and 7°C higher during spring in both North and Central than fall, respectively. Relative humidity did not differ as great as solar radiation and solar radiation during spring and fall seasons in GCREC (80 and 79%) and NFREC in (80 and 84%). Leaf wetness did not show any trend or stability in any trial and season over 7 day sampling periods. Even though temperature and proportionally solar radiation did not reduce *E. coli* concentrations in the cattle feces in tested conditions during fall trials, important increase and decreases occurred between sampling days in spring trials during 7 days of sampling.

Generic *E. coli* concentration at all conditions mainly increased in the first day of experiment almost all the time in fall trials, whereas they declined or increased in spring trials unpredictably. In previous studies, fecal coliform and *E. coli* populations in cattle manure and amendments showed a trend of growth up to a week followed by decline (Howell et al., 1996; Thelin and Gifford, 1983; Wang et al., 1996; Wang et al., 2004; Van Kessel et al., 2007). Various growth phase observation in previous studies proves the importance of several trials at different conditions in here. In stable conditions, such as in the laboratory, survival of target microorganisms was decided from internal factors of feces. However, environmental factors play important role in both internal factors of feces and microorganism of interest.

In Florida, the use of pathogens and any inoculated material is not allowed in the field studies, so indicator microorganisms or surrogates are commonly used for prediction of pathogens. In this study, previously developed models in Chapter 5 were used for prediction of STEC and *Salmonella* survival on tested conditions. Based on models, survival of pathogens is similar to generic *E. coli* with small differences. Risk of contamination with pathogen due to fecal deposits is high, and cannot be ignored. The exclusion of animals from fields and removal of fecal deposits are important with remedial animal intrusion treatment, especially for crops grown close to the ground.

The California Leafy Greens Marketing Agreement requires the establishment of 5-foot radius around fecal matter for all type of feces in leafy green fields (LGMA, 2013). Since the amount of fecal deposits and different conditions including shade or sunny do not affect the survival of human enteric microorganisms in fecal pats for short time, this remedial action required by LGMA should be applied immediately upon animal intrusion. Practices and risk assessment may change based on type of crop and field setup. In tomato fields, risk of fecal

deposits will be different (probably lower) than leafy greens depending on location and distance of fecal deposit to plants.



Table 6-1. Dry matter and moisture content of the cattle feces used for each trial.

Place	Trials	Dry Matter (%)	Moisture (%)
NFREC	Spring 1	10.2	89.8
	Spring 2	11.5	88.5
	Fall 1	20	80
	Fall 2	18	82
GCREC	Spring 1	14.6	85.6
	Spring 2	10.6	89.4
	Fall 1	10.2	89.8
	Fall 2	10.2	89.8

Table 6-2. Comparison of generic *E. coli* population means (Log CFU/g) with standard deviation in the cattle feces during spring trials.

Place	Trials	Time (Days)	Generic <i>E. coli</i> Population (Log CFU/g)			
			Tomato- Outside (TOP)	Tomato- Inside (TIP)	Mulch- Distant (MDP)	Mulch- Under (MUP)
NFREC	Spring 1	0	5.6 ± 0.2 A ¹ ,a ²	4.5 ± 0.3 C,c	5.0 ± 0.4 BC,ab	5.4 ± 0.4 AB,a
		1	6.0 ± 0.2 A,a	5.8 ± 0.2 AB,a	5.9 ± 0.7 A,a	5.1 ± 0.4 B,a
		3	4.6 ± 0.4 AB,b	5.1 ± 0.4 A,bc	4.2 ± 0.5 B,b	5.2 ± 0.5 A,a
		7	4.6 ± 0.5 A,b	5.2 ± 0.6 A,ab	5.1 ± 0.5 A,a	5.1 ± 0.5 A,a
	Spring 2	0	6.1 ± 0.2 A,a	5.9 ± 0.3 AB,a	4.7 ± 0.3 C,b	5.3 ± 0.6 BC,a
		1	4.4 ± 0.3 B,b	4.6 ± 0.4 B,b	6.1 ± 0.7 A,a	5.0 ± 0.5 B,a
		3	4.5 ± 0.4 C,b	5.5 ± 0.5 AB,a	5.9 ± 0.3 A,a	5.2 ± 0.4 B,a
		7	4.8 ± 0.7 B,b	4.5 ± 0.6 B,b	5.7 ± 0.3 A,a	5.1 ± 0.4 AB,a
GCREC	Spring 1	0	5.9 ± 0.4 A,a	5.7 ± 0.2 AB,a	5.6 ± 0.1 AB,a	5.4 ± 0.2 B,c
		1	3.5 ± 0.8 B,c	4.3 ± 0.6 B,b	4.1 ± 0.6 B,b	5.9 ± 0.3 A,b
		3	5.7 ± 0.9 AB,ab	5.0 ± 0.4 B,ab	5.7 ± 0.7 AB,a	6.5 ± 0.2 A,a
		7	4.7 ± 0.7 A,b	4.8 ± 0.7 A,b	4.5 ± 0.6 A,b	5.2 ± 0.4 A,c
	Spring 2	0	5.1 ± 0.4 A,a	5.6 ± 0.7 A,a	5.2 ± 0.2 A,a	5.1 ± 0.1 A,ab
		1	3.4 ± 1.0 B,b	3.9 ± 0.5 B,b	4.0 ± 1.1 B,b	5.9 ± 0.6 A,a
		3	2.5 ± 0.8 B,b	2.4 ± 0.5 B,c	5.0 ± 0.5 A,ab	4.6 ± 0.7 A,b
		7	2.9 ± 0.9 B,b	3.9 ± 0.6 AB,b	4.6 ± 0.7 A,ab	5.1 ± 0.9 A,ab

¹Means with different letter within each row within each trail are significantly different ($P < 0.05$).

²Means with different letter within each column are significantly different ($P < 0.05$).

Table 6-3. Comparison of generic *E. coli* population means (Log CFU/g) with standard deviation in the cattle feces during fall trials.

Place	Trials	Time (Days)	Generic <i>E. coli</i> Population (Log CFU/g)			
			Tomato- Outside (TOP)	Tomato- Inside (TIP)	Mulch- Distant (MDP)	Mulch- Under (MUP)
NFREC	Fall 1	0	5.3 ± 0.2 A ¹ ,b ²	5.2 ± 0.2 A,c	5.1 ± 0.2 A,b	5.0 ± 0.5 A,a
		1	6.5 ± 0.3 A,a	6.2 ± 0.3 A,a	5.6 ± 0.2 B,a	5.4 ± 0.2 B,a
		3	6.3 ± 0.2 A,a	6.2 ± 0.3 AB,a	5.9 ± 0.2 AB,a	5.6 ± 0.7 B,a
		7	5.6 ± 0.4 A,b	5.7 ± 0.3 A,b	5.7 ± 0.2 A,a	5.3 ± 0.5 A,a
	Fall 2	0	6.1 ± 0.4 A,a	6.0 ± 0.3 A,ab	5.6 ± 0.5 A,bc	5.6 ± 0.4 A,bc
		1	6.0 ± 0.4 A,a	6.5 ± 0.6 A,a	6.2 ± 0.2 A,a	6.5 ± 0.4 A,a
		3	5.6 ± 0.7 AB,a	5.1 ± 0.4 B,c	5.9 ± 0.1 A,ab	6.0 ± 0.2 A,ab
		7	5.3 ± 0.8 A,a	5.7 ± 0.4 A,bc	5.4 ± 0.3 A,c	5.3 ± 0.4 A,c
GCREC	Fall 1	0	5.8 ± 0.4 A,a	5.6 ± 0.3 A,a	5.6 ± 0.2 A,ab	5.7 ± 0.2 A,a
		1	4.7 ± 0.2 B,a	4.9 ± 0.5 B,ab	5.8 ± 0.3 A,a	5.8 ± 0.1 A,a
		3	4.7 ± 1.1 AB,a	3.9 ± 1.0 B,bc	5.0 ± 0.4 AB,b	5.4 ± 0.9 A,ab
		7	3.5 ± 1.0 A,b	3.4 ± 0.9 A,c	4.1 ± 0.5 A,c	4.5 ± 0.6 A,b
	Fall 2	0	5.3 ± 0.2 AB,a	5.0 ± 0.2 B,a	5.5 ± 0.3 AB,a	5.8 ± 0.4 A,a
		1	5.2 ± 0.4 BC,a	4.8 ± 0.4 C,a	5.5 ± 0.3 AB,a	6.0 ± 0.3 A,a
		3	5.0 ± 0.7 A,ab	4.8 ± 0.4 A,a	5.1 ± 0.5 A,a	5.7 ± 0.8 A,a
		7	4.3 ± 0.4 B,b	3.6 ± 0.6 C,b	5.1 ± 0.4 A,a	5.4 ± 0.2 A,a

¹Means with different letter within each row within each trail are significantly different ($P < 0.05$).

²Means with different letter within each column are significantly different ($P < 0.05$).

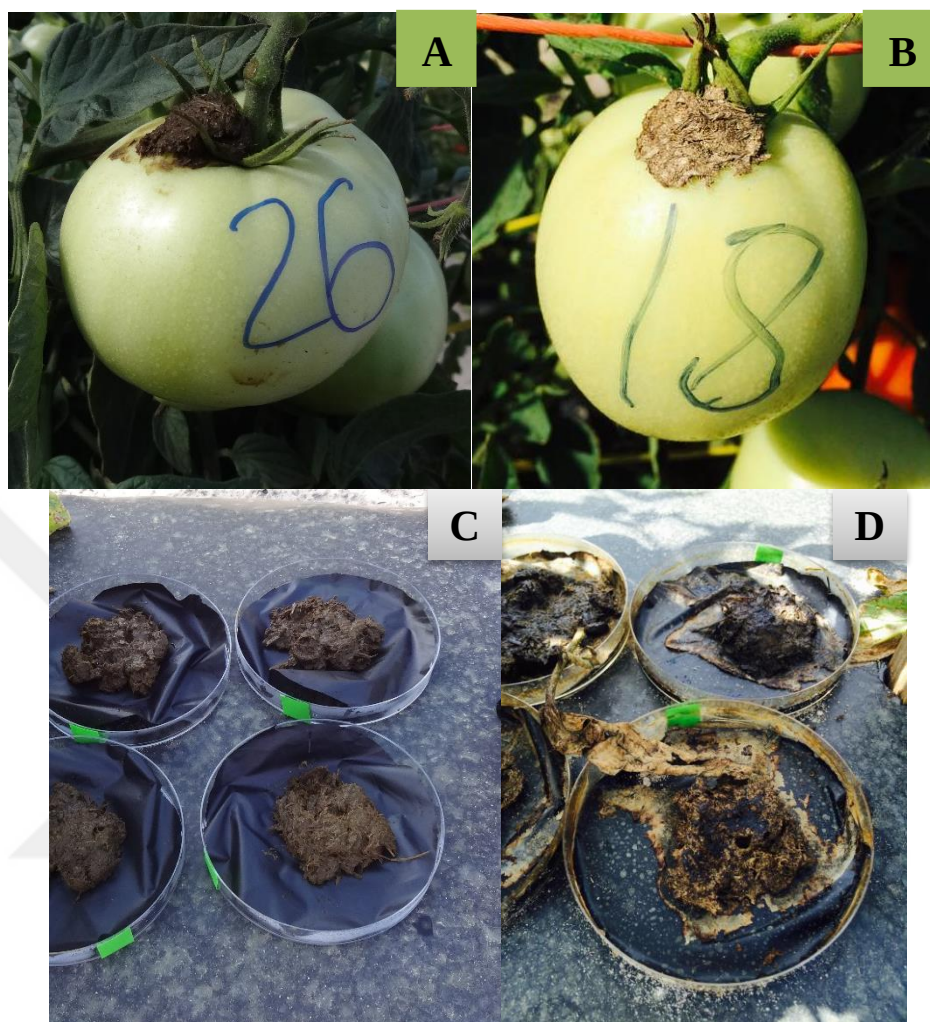


Figure 6-1. Field setup sample picture for 1 g fecal pats on tomato and 10 g on plastic mulch at different sampling time. A) Fecal pats on tomato at Day 0, B) Fecal pats on tomato at Day 7, C) Fecal pats on plastic mulch at Day 0 and D) Fecal pats on plastic mulch at Day 7.

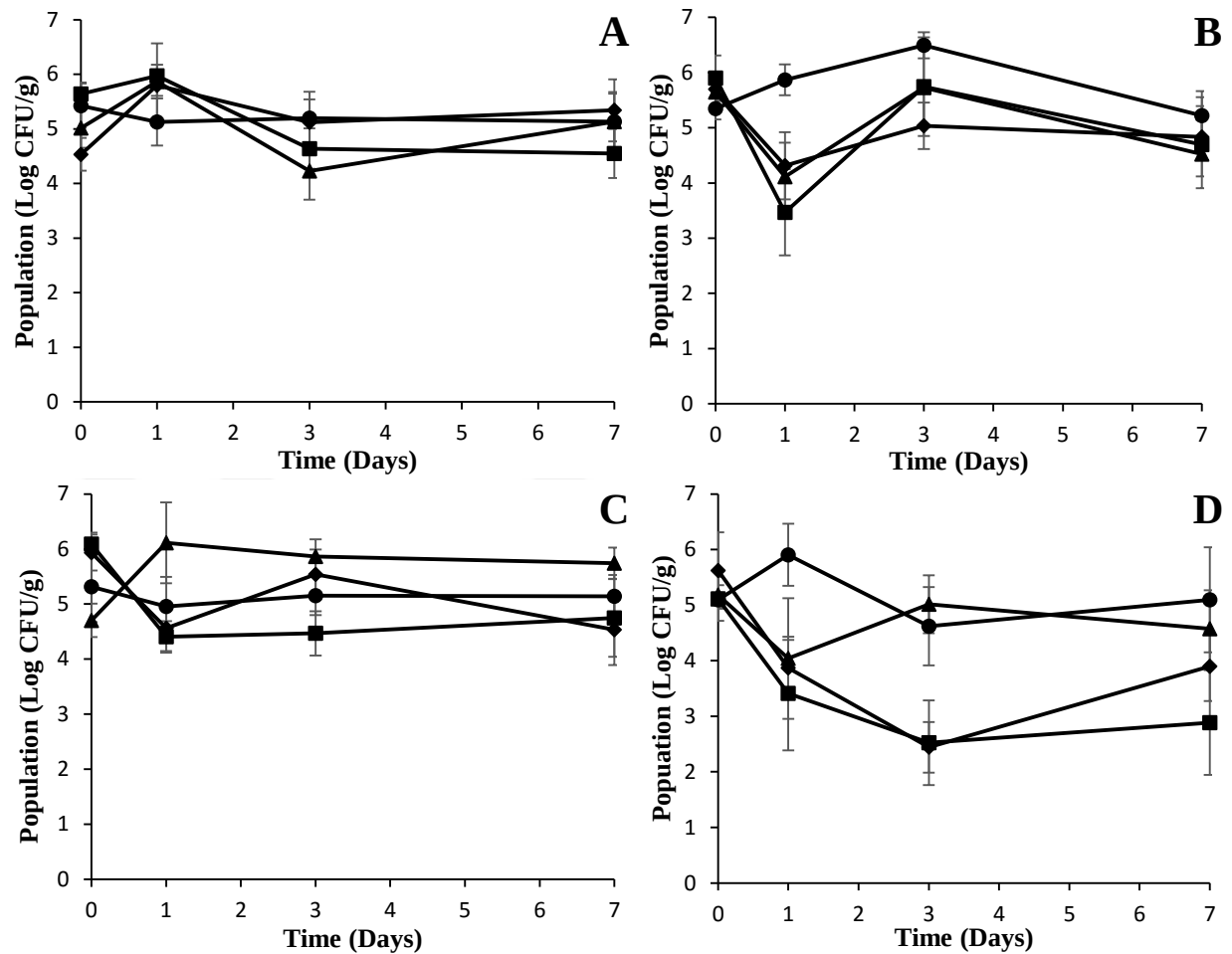


Figure 6-2. Survival of generic *E. coli* in the cattle feces on: (■) tomatoes outside of plants, (◆) tomatoes inside of plants, (▲) mulch under plants, (●) mulch distant from plants at the field conditions in the spring 2015 (A) NFREC-Trail 1, (B) GCREC – Trail 1, (C) NFREC – Trail 2, (D), GCREC – Trail 2 (Mean $\pm$ standard deviation, n=6).

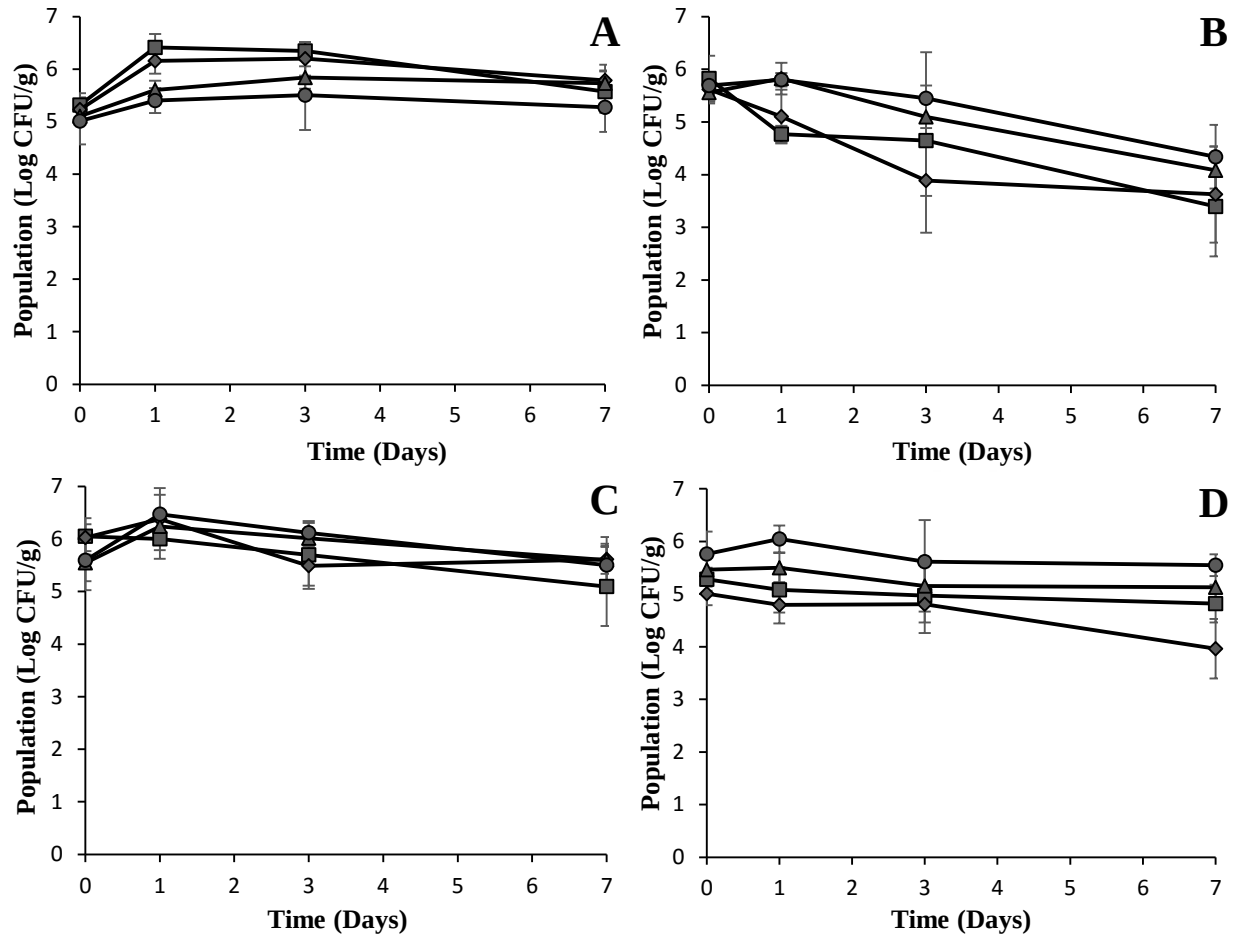


Figure 6-3. Survival of generic *E. coli* in the cattle feces on: (■) tomatoes outside of plants, (◆) tomatoes inside of plants, (▲) mulch under plants, (●) mulch distant from plants at the field conditions in the fall 2015 (A) NFREC – Trail 1, (B) GCREC – Trail 1, (C) NFREC – Trail 2, (D), GCREC – Trail 2 (Mean $\pm$ standard deviation, n=6).

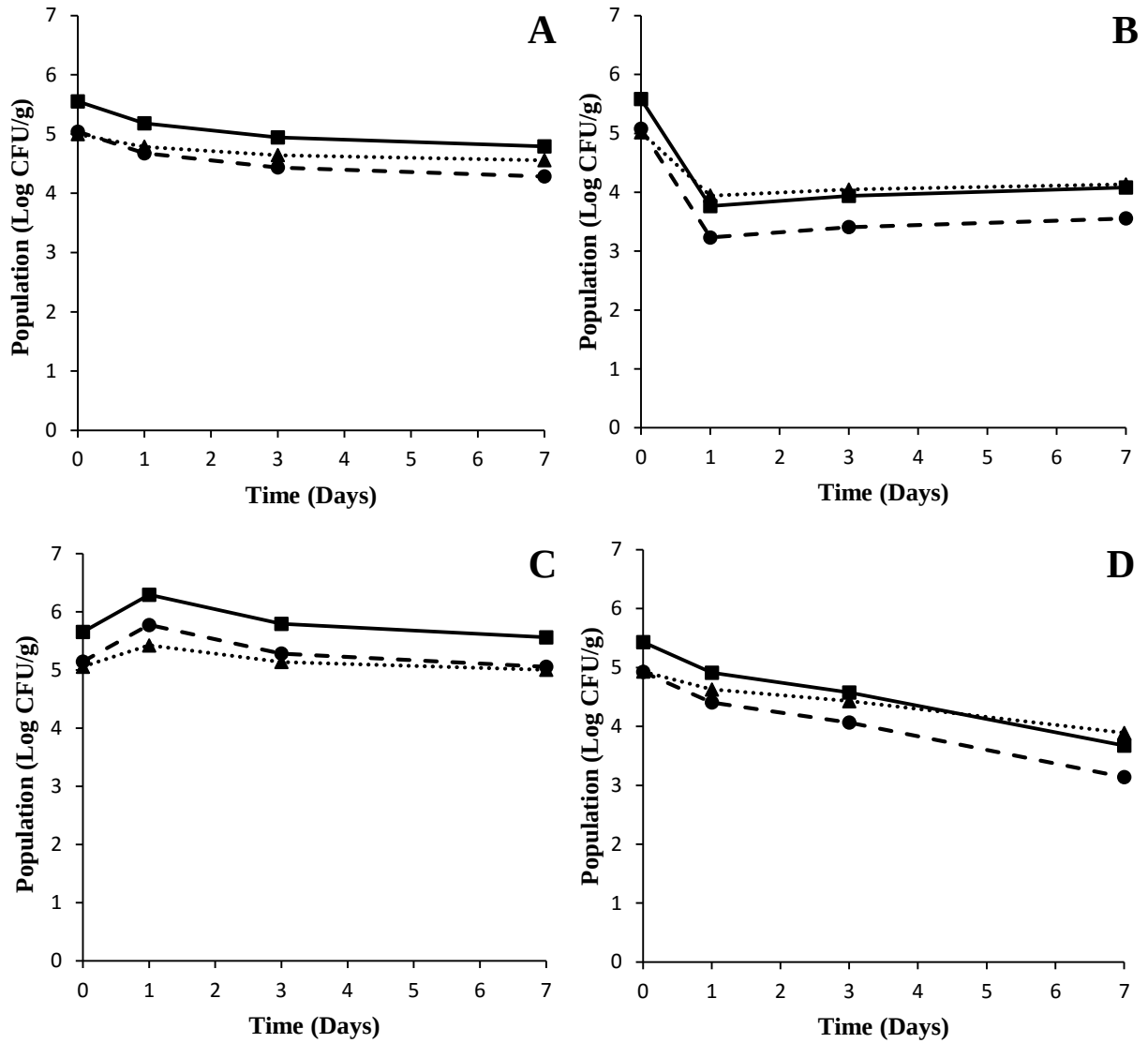


Figure 6-4. Prediction of STEC and *Salmonella* survival on tomatoes based on generic *E. coli* concentration in the cattle feces on: (■) generic *E. coli*, (▲) *Salmonella*, (●) STEC at the field conditions (A) NFREC - Spring, (B) GCREC - Spring, (C) NFREC - Fall, (D), GCREC - Fall (Average generic *E. coli* concentration were calculated by combining all data about tomato in each season, n=24).

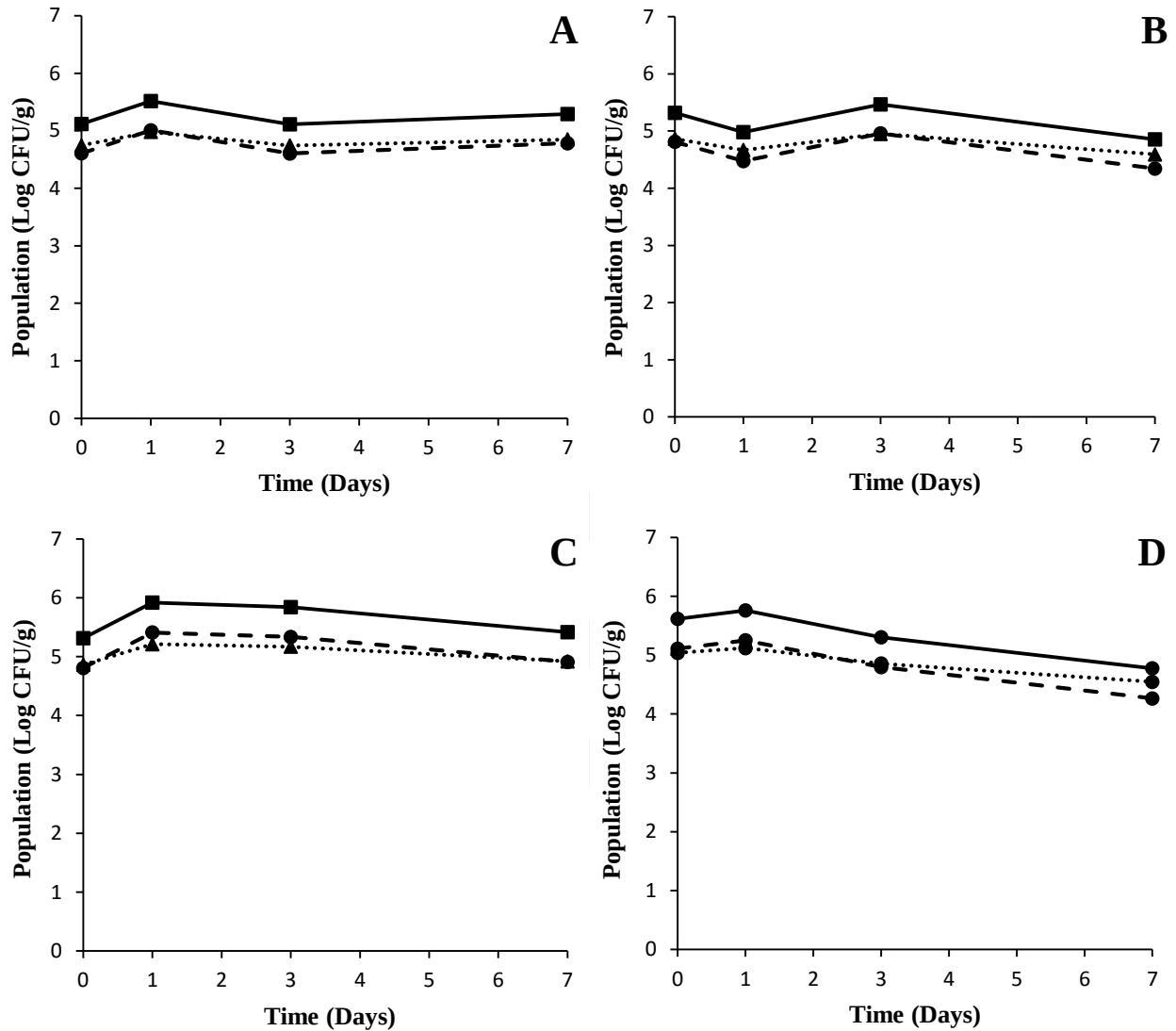


Figure 6-5. Prediction of STEC and *Salmonella* survival on plastic mulch based on generic *E. coli* concentration in the cattle feces on: (■) generic *E. coli*, (▲) *Salmonella*, (●) STEC at the field conditions (A) NFREC - Spring, (B) GCREC - Spring, (C) NFREC - Fall, (D), GCREC - Fall (Average generic *E. coli* concentration were calculated by combining all data about tomato in each season, n=24).

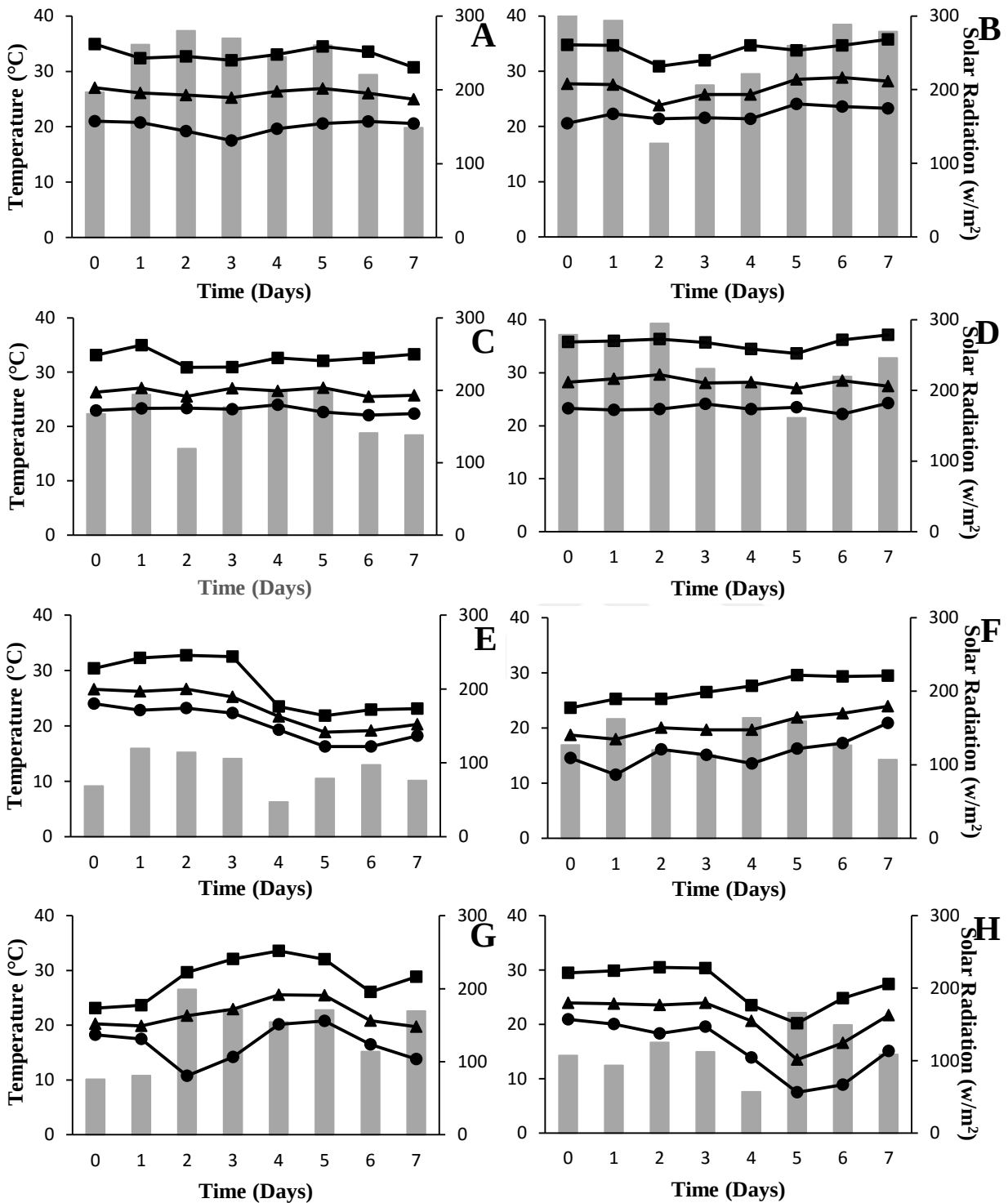


Figure 6-6. Maximum Temperature (■), Average Temperature (▲), Minimum Temperature (●), and Solar Radiation (shaded bars) measured during (A) NFREC – Spring 1 trial, (B) GCREC – Spring 1 trial, (C) NFREC – Spring 2 trial, (D) GCREC – Spring 2 trial, (E) NFREC – Fall 1 trial, (F) GCREC – Fall 1 trial, (G) NFREC – Fall 2 trial, (H), GCREC – Fall 2 trial.

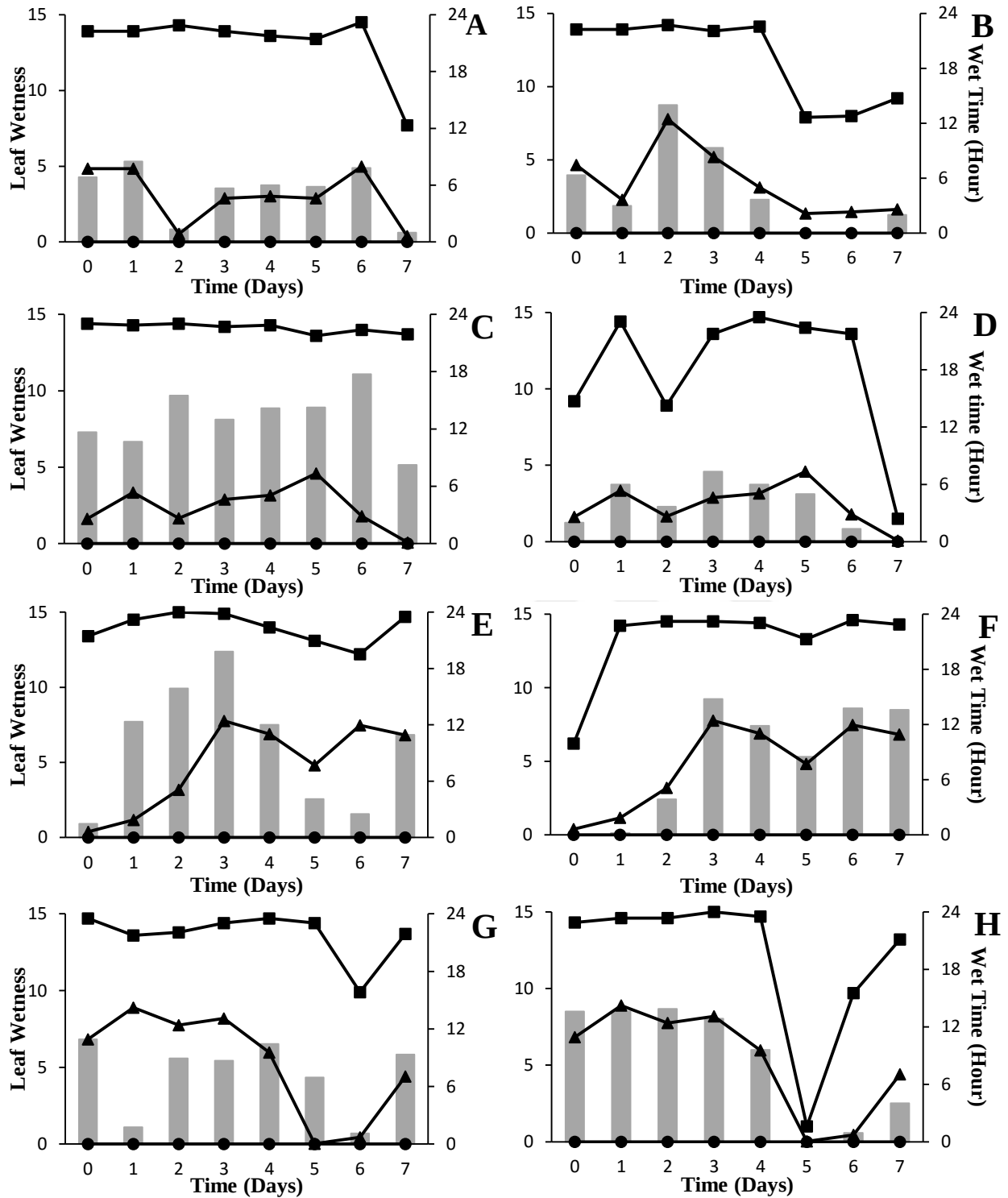


Figure 6-7. Maximum Leaf Wetness (■), Average Leaf Wetness (▲), Minimum Leaf Wetness (●), and Wet Hours (shaded bars) measured during (A) NFREC – Spring 1 trial, (B) GCREC – Spring 1 trial, (C) NFREC – Spring 2 trial, (D), GCREC – Spring 2 trial, (E) NFREC – Fall 1 trial, (F) GCREC – Fall 1 trial, (G) NFREC – Fall 2 trial, (H), GCREC – Fall 2 trial. (Leaf Wetness value range between 0-15, in whole numbers. Any value over 6 is considered ‘wet’).

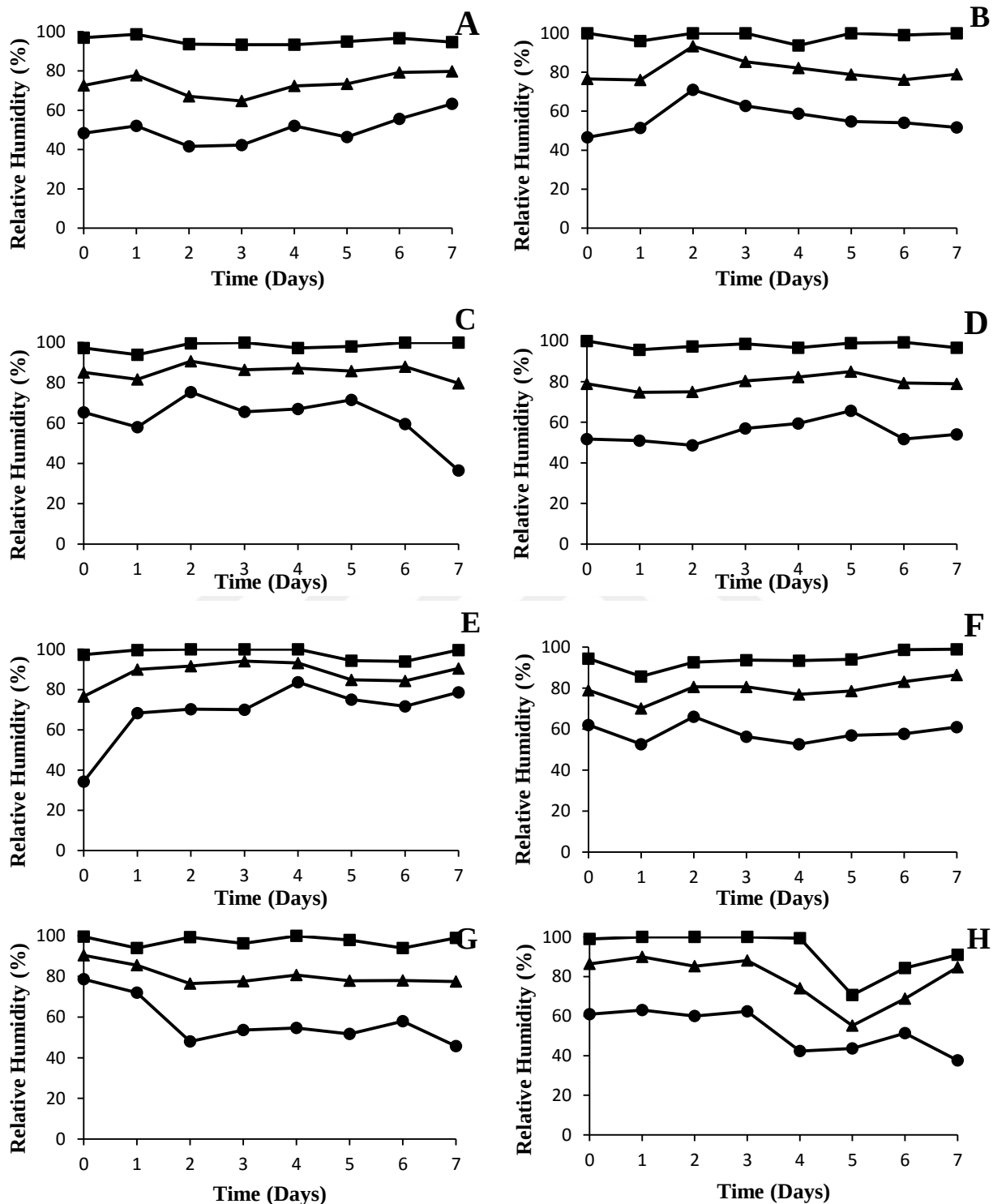


Figure 6-8. Maximum Relative Humidity (■), Average Relative Humidity (▲), and Minimum Relative Humidity (●) measured during (A) NFREC – Spring 1 trial, (B) GCREC – Spring 1 trial, (C) NFREC – Spring 2 trial, (D), GCREC – Spring 2 trial. (E) NFREC – Fall 1 trial, (F) GCREC – Fall 1 trial, (G) NFREC – Fall 2 trial, (H), GCREC – Fall 2 trial.

CHAPTER 7

CONCLUSIONS AND FUTURE WORKS

Four pre-harvest level produce safety related objectives were examined with field and laboratory based studies in Florida. Agricultural water quality and animal intrusion was the focus of these investigations. A three-year microbiological water quality survey was conducted for six agricultural ponds located in Central Florida. This was followed by an examination of the survival of generic *E. coli* and STEC in ponds with the greatest and poorest water quality at different temperatures. Domestic and wild animal feces was inoculated with *Salmonella* and STEC cocktails and stored at room temperature for one year. Models were developed to predict *Salmonella* and STEC concentrations based on generic *E. coli* population in different type of feces. Fresh cattle feces was placed on tomatoes and plastic mulch at different conditions in tomato fields in North and Central Florida to compare effect of environmental factors on survival of generic *E. coli* in the cattle feces. Developed models were used to predict the behavior of pathogens in the same field conditions.

Microbiological quality of agricultural surface water may change continuously and dramatically because it is open source and exposed to many types of environmental factors. Microbial water quality indicators: total coliform, enterococci, and generic *E. coli* followed similar trends and fluctuations in Central Florida surface waters throughout the growing season. The concentration of these indicators spiked episodically after rain in all six ponds within a four miles radius area; however, high correlations between precipitation and all tested microbial indicators ($4.0 \geq R^2 \geq 2.2$) was observed in only one pond. Correlations between water attributes (pH, conductivity, turbidity) and microbial water indicators remained limited and under values for consideration of microbial water quality comparison. MWQP values for all ponds met the standards required by final Produce Safe Rule published on November 13, 2015 (FDA, 2015),

whereas *Salmonella* and STEC genes were detected in 150 mL water samples from all ponds. Fourteen different *Salmonella* serotypes were isolated. In total, the *invA* gene was detected in 4.8% of all samples. *Salmonella* has been isolated in Central Florida surface water (100%) and North Florida (28.2%) where larger water volumes were sampled, previously (Luo et al., 2015; McEgan et al., 2013). Based on the findings in this study, pathogens are likely to be present in Central surface agricultural waters in Central Florida even though water quality pass the current quality standards based on generic *E. coli* concentration.

There is no killing step in produce processing for many fresh product that are consumed raw. It is important that growers should use irrigation methods that minimizes the contact with produce and agricultural surface waters. For other agricultural purposes, growers may use alternative water sources as ground and municipal water or treated surface water. A detailed agricultural water survey for North and South Florida would provide better understanding for agricultural water quality profile for all Florida. Also, a model for prediction of pathogens in surface water may be developed by using a three-year microbial indicator survey in this study to help for risk assessment of produce contamination.

Persistence of pathogen and indicator microorganisms in surface waters may change depending on internal and external factors. Generic *E. coli* and STEC survived for more than five months in Florida surface waters, and followed similar survival trends in different water sources and temperatures. While declines in population were different between STEC and generic *E. coli* during the first two weeks after inoculation, long term, generic *E. coli* is a good survival predictor for STEC. Pond water sources from different locations did not affect the survival. Waiting time option for agricultural waters with poor water quality in Final Produce Safety Rule works effectively for Florida surface waters. Similar to previous studies evaluating the survival

of microorganisms in agricultural water sources, this study was conducted in stable conditions in the laboratory. This situation is the most common drawback in survival studies. Environmental factors may change survival of microorganisms in open water sources (Pachepsky et al., 2015). EPA Worst Case Water responded survival study great in here and can be used as reference in microcosm water survival studies for comparison. More pathogens with different environmental stipulations can be evaluated for Florida surface waters. Further and detailed works are needed about the effect of protozoa and viruses in survival of indicator and STEC population.

Domestic and wild animal intrusion represents a significant route of pathogen introduction during produce production; limited data have been published regarding the survival capabilities of pathogens in animal feces. Limited decline (≤ 2.4 Log CFU/g) was observed in both *Salmonella* and STEC strains in the cattle and deer feces over the 364 days of storage. These animals are considered as the most important potential sources of *E. coli* O157:H7 along with goat, sheep, and swine (Jay-Russell, 2013). Ruminant animals are natural reservoirs for *E. coli* O157:H7 and other STEC serotypes (Ferens and Hovde, 2011). Pathogens survived less than two months in the raccoon feces and for several months in the waterfowl and pig feces. Wild animal feces including pig and deer have been implicated as the source of outbreaks (CalFERT, 2007; Laidler et al., 2013). Based on survival capabilities of *Salmonella* and STEC in wild animal feces, animal intrusion and fecal deposits in the fields should be treated the same for all types of feces as in LGMA (2013). The safety of agricultural surface water sources that are open to fecal contamination by animals should be reconsidered for fields that use foliar applications. Also, approved mitigation strategies to prevent animal intrusion should be practiced by growers. These strategies include use of poison baits, removal of non-crop vegetation, removal or abandonment of water source conservation practices, co-management practices (Bianchi and

Lowell, 2016). Models developed here to predict *Salmonella* and STEC concentrations in correlation with generic *E. coli* population may help growers and professionals to understand risks better and to improve their risk assessments. Determining appropriate risk mitigation strategies following animal intrusion can be improved with a better understanding of pathogen survival in animal feces.

The fate of indicator microorganisms and pathogens in animal feces may be different in actual field conditions than stable laboratory environment. Limited data exist on relative risks associated with the presence and fate of pathogens and microbial indicators in animal feces on crops. Generic *E. coli* concentration in fecal pats stayed high on tomatoes inside and outside of plat and on mulch under and distant from plant for a week. Up to three Log CFU/g fluctuations without any consistent trends were observed in *E. coli* concentrations in fecal pats on tomatoes during both spring and fall trials in Central Florida. More stable and lower reductions of *E. coli* populations were detected in fecal pats on tomatoes in North Florida. On mulch, reduction reached a maximum 1.5 Log CFU/g over a week in both seasons and locations. In some trials, an increase in generic *E. coli* population was observed in fecal pats on both tomatoes and mulch. Location affected generic *E. coli* concentration in fecal pats probably due to the amount of sun exposure. Declines in *E. coli* population in fecal pats on tomatoes were more than those on mulch, possibly because of less protection from environmental factors. In general, generic *E. coli* concentrations stayed high in the cattle feces under field conditions for a week, and different conditions did not impact survival dramatically. Further studies are required to understand survival of generic *E. coli* in feces on fruits in filed conditions for long term. Particularly, effect of sunlight and native flora on fruit needs to be examined. Direct placement of fecal material can be compared with survival after touch of fruit with animal feces.

Along with the increase in consumption, produce safety calls researchers' and Government's attention to expand science based explanations for minimization of contamination risks and improvement of remedial approaches starting in the field. Agricultural water and animal intrusion can contaminate produce, and pathogen can persist on fruit and vegetables until consumption. Potential risk may change depending on location, climate, and environment. Florida has high risk due to sub-tropical climate and various and intense animal presence in all over the state. The minimization of pre-harvest contamination risks in produce field is essential with proper mitigation strategies.

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BIOGRAPHICAL SKETCH

Zeynal Topalcengiz is a PhD student in department of Food Science and Human Nutrition Department at the University of Florida, and studying under Dr. Michelle Danyluk. He earned his BE in food engineering from Inonu University in Turkey and MSc degree in Food Science from University of Florida (August 2012). His master project consisted of the estimation of thermal death time parameters (*D-value* and *Z-value*) of acid adapted and non-adapted foodborne pathogens in orange juice during processing. He studied on validation of pre-harvest food safety metrics used during produce production for his PhD. He also received a minor in Soil and Water Science. Prior to his graduate studies, he worked in two main sectors of the food industry: dairy, catering. He has been honored with several scholarships, including scholarship for the master degree by the Republic of Turkey the Ministry of National Education. After graduation, Zeynal would like to stay in academia and become a food safety specialist.