DETECTION, ISOLATION, AND ENUMERATION OF *SALMONELLA* FROM CENTRAL FLORIDA SURFACE WATERS

By

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I would like to dedicate this work to my Mom, Dad, and Grama,
and to the memory of my Oma, and Opa.

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LIST OF ABBREVIATIONS

APHA	American Public Health Association
BGA	Brilliant green agar
BPW	Buffered peptone water
BSA	Bismuth sulfite agar
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
DI	Deionized
E.	*Escherichia*
EPA	Environmental Protection Agency
FDA-BAM	Food and Drug Administration – Bacteriological Analytical Manual
FSMA	Food Safety Modernization Act
GAPs	Good Agricultural Practices
H	Flagellar antigen
HE	Hektoen enteric agar
IMS	Immuno-magnetic separation
ISO	International Organization for Standardization
LB	Lactose broth
LGMA	Leafy Greens Marketing Agreement
LIA	Lysine iron agar
MS	Moore swab
MMS	Modified Moore swab
mPCR	Multiplex polymerase chain reaction
MPN	Most probable number
NFF	Normal flow filtration

NVSL	National Veterinary Services Laboratory
O	Somatic antigen
ORP	Oxidation reduction potential
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming unit
qPCR	Quantitative polymerase chain reaction
RV	Rappaport-Vassiliadis
S.	*Salmonella*
SC	Selenite cysteine
SF	Selenite-F
spp.	species (plural)
TFF	Tangential flow filtration
T-GAPs	Tomato – Good Agricultural Practices
TSA	Tryptic soy agar
TSAA	Tryptic soy agar supplemented with ampicilin
TSAN	Tryptic soy agar supplemented with nalidixic acid
TSB	Tryptic soy broth
TSBA	Tryptic soy broth supplemented with ampicilin
TSBN	Tryptic soy broth supplemented with nalidixic acid
TSI	Triple sugar iron agar
TT	Tetrathionate
UPB	Universal Pre-enrichment broth
USA	United States of America
VBNC	Viable but not culturable
Vi	Capsular antigen

XLD	Xylose lysine desoxycholate agar
XLT4	Xylose-lysine-Tegertol4

Abstract of Dissertation Presented to the Graduate School
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DETECTION, ISOLATION, AND ENUMERATION OF *SALMONELLA* FROM CENTRAL FLORIDA SURFACE WATERS

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Salmonellosis outbreaks have been linked to consumption of contaminated fresh produce; water has been suggested as a contamination source. Screening agricultural waters for *Salmonella*, or determining another indicator, can aid in avoidance of produce contamination. A method was developed to screen 10L of surface water for *Salmonella* using a combination of tangential flow filtration, overnight enrichment, immuno-magnetic separation, and qPCR detection. Modified Moore swabs performed equally as well as tangential flow filtration for concentration. Monthly samples from 18 Central Florida surface water sites were evaluated for *Salmonella*; 202 samples were collected, all were *Salmonella*-positive. Aerobic plate counts, coliform/*E. coli* MPNs and *Salmonella* MPN were also determined. Air and water temperature, pH, ORP, turbidity, and conductivity were measured. No attribute exhibited a correlation with *Salmonella*. All 562 isolates were grouped based on a previously-described multiplex PCR (mPCR). *Salmonella* were identified as having the same mPCR pattern in 68 samples. Four hundred and ninety four isolates did not match the thirty most clinically common serovar patterns previously reported. All groupings based on mPCR pattern matched groupings based on serogroup; 19 isolates serogrouped according to mPCR suggested serovar. Isolates

were also characterized using serotyping, pulsed-field gel electrophoresis (PFGE), and antimicrobial resistance patterns. Thirty-two different serovars were identified. Three serovars were added at 8 log CFU/100ml to sterile deionized water (DI), sterile surface water, non-sterile surface water, or EPA worst case water at 15, 21, or 28°C. At six months DI, sterile surface water, and EPA worst case water had *Salmonella* ranging between 6.0 and 7.5 log CFU/100ml. *Salmonella* in non-sterile surface waters ranged between 0.9 and 3.8 log MPN/100ml. *Salmonella* is prevalent in Central Florida surface waters. The role of indicators in determining *Salmonella* prevalence in surface water is limited. Direct screening of surface water for *Salmonella* may be necessary where *Salmonella* levels are high and where waters are applied close to harvest. The mPCR method can be used to group environmental *Salmonella* isolates as different or potentially the same, but has limited ability to predict serogroup. *Salmonella* survives extended periods of time in waters, indicating a continued risk for fresh produce contamination.

CHAPTER 1
INTRODUCTION

Outbreaks of salmonellosis have been linked to the consumption of fresh produce. Nontyphoidal *Salmonella* is the second leading cause of foodborne disease in the USA resulting in an estimated 1.0 million cases of illness per annum (Scallan et al. 2011). There have been a wide variety of produce items that have been linked to salmonellosis outbreaks recently including: basil, cantaloupe, lettuce, mangoes, Serrano peppers, spinach, and tomatoes (CDC 2008, Erickson et al. 2010, Klontz et al. 2010, Behravesh et al. 2011).

The sporadic and heterogeneous nature of contamination, indicator organisms, and microbial counts in fresh produce both within and between fields makes direct screening of produce problematic (Kase et al. 2012). It is therefore imperative to reduce the incidence of possible contamination.

Irrigation waters, and other agricultural waters, have been suggested to be the major source of in-field contamination of fresh produce (Jacobsen and Bech 2012). The presence of *Salmonella* in pre-harvest water that comes into contact with, or is in close proximity to, produce production may represent a risk to produce safety. The screening of pre-harvest waters for *Salmonella*, or other biological, chemical, or physical indicator, can aid in the avoidance of produce contamination.

Screening of larger volumes of surface waters will decrease the lower limit of detection and allow for a more representative sample to be analyzed. Current methods of screening for *Salmonella* either allow for the use of 25 – 100 ml samples or do not provide quantitative data, as in the case of Moore swabs which are suspended in a body of water for a given period of time (APHA 1992, US-FDA 1998). Larger sample

volumes would enable to acquisition of quantitative data, which could in turn be used to generate more accurate risk assessments for microbial water quality of waters to be used in fresh produce production.

Tests for indicator microorganisms are less cumbersome and time consuming, as well as typically less expensive, as compared to screening for the presence of *Salmonella*. Thus, the use of indicator microorganisms is a much more economical and practical choice. However, contradictory results have been reported as to the efficacy of indicator microorganism in predicting the presence and/or prevalence of human pathogens, such as *Salmonella*, in surface waters (Burton et al. 1987, Rhodes and Kator 1988, Chandran and Hatha 2005, Chandran et al. 2011). Physicochemical water characteristics also have the potential to correlate with the presence and/or prevalence of *Salmonella* in surface waters (APHA 1992). Physicochemical measurements have the added advantage of being able to be measured rapidly.

Currently, there are no nationally required microbial water quality standards for fresh produce production. The US produce industry relies on testing 100 ml of water for indicator organisms, specifically, generic *E. coli*, as a means to monitor water quality used for edible horticultural crop production (LGMA 2012, F-DACS 2007). Testing of 100 ml for *E. coli*, and having a geometric mean less than 126 MPN/100 ml, is a requirement only for Florida tomatoes; leafy greens producers may opt to follow this standard as a condition of belonging to the Leafy Greens Marketing Agreement.

Once isolated, the identification of *Salmonella* serovars is important for source tracking and outbreak investigations. The current serotyping procedure requires numerous O and H antisera for the characterization of each serovar (Jean-Gilles

Beaubrun et al. 2012). Pulsed-field gel electrophoresis (PFGE) is used to produce a DNA fingerprint for the purpose of comparing *Salmonella* isolates; this procedure requires five days (Ribot et al. 2006). A more time effective method for comparing and serotyping *Salmonella* isolates would be beneficial. A multiplex PCR method could potentially be used to identify the most clinically common *Salmonella* serovars (Kim et al. 2006). *Salmonella* serovars isolated from surface waters are frequently not those serovars included in the 30 most clinically relevant serovars (Kim et al. 2006, Haley et al. 2009, Patchanee et al. 2010, Rajabi et al. 2011, Strawn et al. 2013), hence alternative methods for serovar identification of clinical isolates need to be validated for those coming from environmental sources.

The duration of survival of *Salmonella* in surface water constitutes a public health concern through direct consumption, indirect consumption (via irrigation of edible crops), and contact during recreation (Greene et al. 2008, Ijabadeniyi et al. 2011, Ge et al. 2012). Minimal work has focused on the duration of *Salmonella* survival in various types of environmental waters. Many previous works have also focused on marine or estuary waters, results from which cannot be entirely relied upon to predict survival in fresh water. Most studies have focused on clinically important serovars, such as *S*. Typhimurium, rather than other less clinically common serovars (Rhodes and Kator 1988, Maki and Hicks 2002, Chandran and Hatha 2005). The use solely of a local water source, sterile or non-sterile, incorporates uncontrolled variables into each set of experiments that in turn make the studies difficult to compare.

This work had four main objectives: (1) compare i) concentration and detection of *Salmonella* in large volumes (10 L) of surface water by tangential flow filtration and

normal flow filtration and ii) concentration of *Salmonella* by tangential flow filtration and Modified Moore swabs; (2) explore the relationships between the presence and/or concentration of *Salmonella* and biological, chemical or physical indicators in Central Florida surface water samples taken monthly for twelve consecutive months; (3) determine i) if a multiplex PCR method is able to verify that all *Salmonella* isolates from a single water sample are identical; ii) if a multiplex PCR method is able to predict the serogroup of environmental isolates; iii) the serovar, PFGE pattern, and antibiotic resistance profile for the *Salmonella* isolates determined to be different via the multiplex PCR ; (4) explore the relationship between the persistence of *Salmonella* spp., either individually or as a cocktail, and biological, chemical, and physical water quality characteristics, representative of surface waters, over six months; the suitability of EPA worst case water as a standard medium for investigating *Salmonella* persistence was also evaluated.

CHAPTER 2
DETECTION, IDENTIFICATION, PERSISTENCE, AND INDICATORS OF SALMONELLA IN SURFACE WATERS OF AGRICULTURAL PURPOSES: A LITERATURE REVIEW

The presence of *Salmonella* in pre-harvest water that comes into contact with, or is in close proximity to, produce production may represent a risk to produce safety. Direct screening of produce items is problematic. It has been repeatedly shown for many produce items that contamination, indicator organisms, and microbial counts are all sporadic and heterogeneously distributed both within and between fields (Kase et al. 2012). Testing one produce sample may not provide an accurate representation of the overall microbial quality of the field. It is therefore imperative to reduce the incidence of possible contamination. It has previously been suggested that irrigation waters, and other agricultural waters, are the major sources of contamination of fresh produce in the field (Jacobsen and Bech 2012). The notion of pre-harvest *Salmonella* contamination of fresh produce elicits several unanswered questions regarding prevalence, and persistence of *Salmonella* in surface waters, which could be used for agricultural purposes. The objective of this review is to discuss the prevalence and persistence of *Salmonella* in surface waters, as well as methods used to determine either the presence/absence or enumeration of *Salmonella* from surface waters.

Salmonella

Salmonella spp. are members of the family *Enterobacteriaceae*; these are mesophillic, facultatively anaerobic Gram-negative rod-shaped bacteria. The genus *Salmonella* comprises two species: *S. bongori* and *S. enterica* (D'Aoust and Maurer 2007). The subspecies *S. enterica* is further differentiated into six subspecies (enterica, salamae, arizonae, diarizonae, indica, and houtenae) among which the *S. enterica*

subspecies enterica is mainly associated with human and other warm-blooded vertebrates. The subspecies are differentiated on the basis of biochemical traits and genomic relatedness. Traditionally members of the genus *Salmonella* are clustered in serovars according to their flagellar (H) and somatic (O) anitgens, some serovars also have capsular (Vi) antigens (Kim et al. 2006). The majority of *Salmonella* are motile via peritrichous flagella; however, nonflagellated variants to occur. Both *S*. Pullorum and S. Gallinarum are nonmotle. The serovar is determined via agglutination with specific antibodies for the O, H, and Vi antigens (Kim et al. 2006, Jean-Gilles Beaubrun et al. 2012). The serogroup is determined by the O antigen. Each subspecies contains numerous serovars. Currently over 2,500 *Salmonella* serovars have been described, but only about 50 serovars, all within the subspecies enterica, are common causes of infections in humans and warm-blooded animals.

Salmonella is currently believed to be a zoonotic foodborne pathogen, which is spread by the fecal-oral-route (D'Aoust and Maurer 2007). This microorganism can enter aquatic environments directly with the feces of an infected human or animal, or indirectly via sewage discharge or agricultural land run off. *Salmonella* is a ubiquitous enteric pathogen with a worldwide distribution that comprises a large number of serovars characterized by different host specificity and distribution. Two serovars, *S.* Typhi and *S.* Paratyphi, are host adapted to infect only humans; these cause typhoid fever. Nontyphoidal *Salmonella* is the second leading cause of foodborne disease in the USA resulting in an estimated 1.0 million cases of illness per annum (Scallan et al. 2011), following only norovirus at 5.5 million cases of illness (Scallan et al. 2011). With an estimated 27% of salmonellosis cases requiring hospitalization, *Salmonella* is the

estimated leading cause of hospitalization due to foodborne disease (Scallan et al. 2011). Nontyphoidal salmonellosis typically results in enterocolitis between 8 to 72 h after consumption. The disease is generally self-limiting with symptoms lasting approximately five days. Symptoms include nonbloody diarrhea and abdominal pain.

Outbreaks

There have been a wide variety of produce items that have caused salmonellosis outbreaks recently including: basil, cantaloupe, lettuce, mango, tomatoes, Serrano peppers, and spinach (CDC 2008, Erickson et al. 2010, Klontz et al. 2010, Behravesh et al. 2011). Fresh produce related outbreaks are typically shorter in duration, when compared to nonperishable food items, and occur with the seasonality of harvest of the particular produce item. Outbreaks can be large or small scale depending on the distribution of the produce item.

Linking environmental sources of *Salmonella* with foodborne outbreaks is very challenging; only two such cases exist. In a 2005 outbreak of *S.* Newport, *Salmonella* strains isolated from a pond on the Eastern shore of Virginia used for irrigation of tomato fields matched an outbreak strain recovered during the outbreak investigation (Greene et al. 2008). The same strain caused a 2002 outbreak as well (Greene et al. 2008), which indicates a possible long-term environmental survival of *Salmonella* as well as suggesting the irrigation pond was a source of contamination for the tomatoes.

A second outbreak where the same *S.* Saintpaul was isolated from an on-farm source was the Serrano pepper outbreak in 2008, when the matching isolate was able to be isolated from an irrigation pond on a farm where the epidemiologically-linked Serrano peppers could have been produced (Klontz et al. 2010).

Although only two outbreaks have been conclusively linked back to *Salmonella* contamination from irrigation water many more instances potentially exist. Changes in microbial populations between incidence of contamination and outbreak investigation, heterogeneous distribution of *Salmonella* within the water source, or a lack adequate sampling could account for why so few outbreaks have conclusively been linked to contamination through irrigation water. Often trace back to the farm has occurred in outbreaks involving produce, while the definitive identification of the mode of contamination is left unknown.

Contamination through Agricultural Waters

It is widely accepted that contaminated agricultural waters are able to cause produce contamination. The percentage of pathogen that is able to remain on, or internalize with the various produce items is not yet well established; however it is thought to occur at low levels.

Evidence of pathogens internalizing in the edible portions of plants is inconsistent. When irrigating greenhouse tomatoes for 14 days with *S*. Montevideo (7 log CFU/ml in 350 ml) in the irrigation water that was applied directly to the soil, no *Salmonella* was found in the red-ripe tomatoes after harvest (Miles et al. 2009). Another study irrigating with *S*. Newport at 7 log CFU/ml once every seven days, found internalization did occur, but at a low incidence (Hintz et al. 2010).

During an in-field study performed in South Africa irrigation water from the irrigation canal was positive for *Salmonella* in 33% (4/12) of 25 ml samples and 11% of broccoli and cauliflower, the crops grown in the irrigated field, were positive for *Salmonella* (Ijabadeniyi et al. 2011). Ge et al. (2012) noted that lettuce was able to internalize *S.* Typhimurium from soil irrigation; higher rates of internalization resulted

when drought conditions preceded irrigation. Another pathogen, *E. coli* O157:H7, can internalize into spinach leaves after being spray irrigated, but only at high concentrations (6 log CFU/ml) in the irrigation water (Erickson et al. 2010).

Antimicrobial Resistance

Concern regarding the emergence of *Salmonella*, or other bacterial pathogens, resistant to antimicrobials is increasing. Such resistance can complicate treatment of both human and animal cases of the disease. The environmental occurrence of antimicrobial resistant serovars of *Salmonella* has been the topic of several investigations.

In Morocco, a monthly survey of coastal seawater isolated nine *S.* Senftenberg isolates; six of these isolates were resistant to ampicillin (Setti et al. 2009). A Tunisian survey of clams harvested from coastal waters, isolated *S*. Typhimurium, all of which were resistant to ampicillin, erythromycin, rifampicin, and tetracycline (El Mejri et al. 2012).

Sixty-three *Salmonella* isolates were obtained from various environmental samples from tomato farms in the Mid-Atlantic Region of the U.S., all of these isolates were susceptible to amikacin, ceftriaxone, ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, trimethoprim/sulfamethoxazole and streptomycin (Micallef et al. 2012). Three isolates were multidrug resistant; these three isolates came from all different farms and included two *S*. Newport and one *S*. Tennessee (Micallef et al. 2012).

Environmental Presents

The data provided in Table 2-1 demonstrates the ubiquitous nature of *Salmonella* in environmental waters. Salmonellae have been detected in different geographic regions throughout the world, in very diverse water sources, ranging from pristine to

those heavily impacted by anthroprogenic activities. Detection frequencies were extremely variable in surface waters.

A surface water sampling survey has previously been performed in North Florida in the Suwannee River watershed (Rajabi et al. 2011). Here the researchers isolated *Salmonella* from 96% (106/110) of water samples when three-by-five MPN method was used (1, 10, 50, 100, 500 ml) (Rajabi et al. 2011). *Salmonella* populations ranged from <18 MPN/100 ml to 5400 MPN/100 ml, the highest concentration obtained in February when samplings were performed January through April (Rajabi et al. 2011).

Another geographically nearby surface water sampling survey for *Salmonella* to has been performed in the state of Georgia. Here, *Salmonella* was found in 79.2% (57/72) of surface water samples when a five-by-three MPN method was used (1, 10, 100 ml) (Haley et al. 2009). *Salmonella* populations ranged from <1.8 to 68.6 MPN/L, with the highest levels being obtained in August (Haley et al. 2009). In Northeast Georgia pond water has been sampled and had *Salmonella* concentrations ranging from below detectable limits (<0.015 MPN/L) to 3.4 MPN/L (Jenkins et al. 2008). Sampling was conducted in August, October, December, March, and May; again, the largest concentration of *Salmonella* was obtained in August (Jenkins et al. 2008).

Many parameters could potentially influence the presence of *Salmonella* in surface waters. Such parameters as water quality, type of water (fresh, estuary, or marine), the soil surrounding the water source, as well as the biotic sources that come near or contact the surface water could influence the fate of *Salmonella*.

Chemical and Physical Water Quality Parameters

Various water quality parameters may, potentially correlate with the microbial quality of the water source. A diverse array of physical and chemical water quality parameters have been considered as means to predict microbial water quality.

While numerous survey and microcosm studies exist, the wide array of parameters considered and recorded often makes multiple studies difficult to compare. The most commonly reported parameter is water temperature. Water temperature has been shown to correlate significantly with microbial water quality, and specifically with *Salmonella* presence and/or persistence. In marine bathing waters, *Salmonella* had an inverse relationship to water temperature when the recorded temperatures were grouped into the following categories: <20.5°C, 20.6 to 21.9°C, 22.0°C to 22.5°C, and >22.6°C (Serrano et al. 1998). Water temperature also had a significant correlation with aerobic plate count, aerobic spore formers, anaerobic spore formers, and intestinal *Enteroccocus* (Ijabadeniyi et al. 2011). In microcosm experiments, temperature has repeatedly been an important factor for the survival of *Salmonella* in environmental samples (Jacobsen and Bech 2012); in general, higher temperatures result in an increased rate of *Salmonella* die-off (Jacobsen and Bech 2012).

Other parameters that have been reported, and correlated with higher *Salmonella* prevalence include: overcast skies, low or outgoing tides, rough seas, and intense turbidity (no values given) (Serrano et al. 1998). Exposure to sunlight (UV) may also affect *Salmonella* survival: incubating microcosms in the dark has been shown to slow the rate of inactivation, changing the two log reduction time from 122 h to 144 h for *S*. Typhimurium (Bae and Wuertz 2012). Indicator organisms correlate with chemical oxygen demand (COD) (fecal and total coliforms), pH, turbidity, and rainfall (aerobic

plate count, aerobic spore formers, anaerobic spore formers, and intestinal *Enteroccocus*) (Ijabadeniyi et al. 2011).

The use of turbidity as a means of predicting *Salmonella* presence or prevalence may be either flawed or not applicable to all situations. At 20°C, in filter sterilized surface water streptomycin-resistant *S*. Typhimurium remained detectable by pour plating to bismuth sulfite agar for up to 41 days; suspended particles (clay, silt, sand, organic flocculent) did not significantly affect the survival of *S*. Typhimurium (Maki and Hicks 2002). In this experiment it was noted that the *Salmonella* decreased in a non-linear fashion, regardless of particle size or concentration (Maki and Hicks 2002). This would indicate that turbidity might not be a good indicator of pathogen presence in water.

Fresh Water

Fresh water has <0.5 ppt dissolved salts. Fresh surface water sources include: irrigation canals, streams, rivers, ponds, and lakes. In attempts to elucidate the prevalence as well as the parameters that affect survival of *Salmonella* in fresh water sources numerous surveys and microcosms have been reported along with some select parameters.

It can be suggested that *Salmonella* has a relatively high prevalence in surface waters throughout the world, especially in warmer climates. A South African 12-month survey of an irrigation canal and the two rivers that feed into it, determined 42% (15/36) of their 25 ml samples were positive for *Salmonella* when screening the samples using the ISO 6579 method (Ijabadeniyi et al. 2011).

During an environmental survey in North Carolina, 25 ml surface water samples taken from crop-agricultural areas were 50% (6/12) positive for *Salmonella* via cultural

methods based on the ISO 6579 method (Patchanee et al. 2010). Similarly, this survey also found surface water from a residential/industrial area to have a 58.8% (20/34) positive for *Salmonella*, and 57.1% (16/28) of surface water samples were positive for *Salmonella* from a forested area (Patchanee et al. 2010). This study also reported on the diversity of serovars found in each area; the highest diversity of serovars came from the residential/industrial area (eight serovars), followed by crop agriculture (five serovars); the least came from forestry (four serovars) (Patchanee et al. 2010).

Another US-based survey was performed in the Mid-Atlantic Region. Fourteen tomato farms (nine large scale, and five small scale) were sampled (irrigation pond water and sediment, groundwater, irrigation ditch water, soil, leaves, and tomatoes) periodically. *Salmonella* was isolated using a culture based method, which was based on the FDA-BAM method; 250 ml of water was filtered through 0.45 µm, 47 mm mixed cellulose ester filter, the filter pre-enriched in 30 ml lactose broth, 1 ml aliquot to 15 ml Tetrathionate Hajna broth with iodine, streaked to XLT4 and R & F *Salmonella* Chromogenic Plating Medium; isolates were confirmed by PCR amplification of *Salmonella*-specific enterotoxin (*stn*) gene. *Salmonella* was isolated on 29 % (4/14) of the tomato farms; four of the large-scale farms, and none of the small-scale farms (Micallef et al. 2012). Ponds on the small-scale farms were typically small, artificially created and disconnected from other water ways or wooded areas. Ponds on the large-scale farms were typically large, deep and supported more wildlife (frogs and snakes), and received run-off from wooded areas and were connected to other bodies of water (Micallef et al. 2012). Any or none of these factors could explain or partially explain the difference in *Salmonella* prevalence at the two distinct farm sizes. No *Salmonella* was

isolated from the groundwater, leaves, or tomatoes (Micallef et al. 2012). *Salmonella* was isolated from 7.7% (3/39) of irrigation pond water samples, while irrigation pond sediment only yielded 1.8% (2/111) positive samples (Micallef et al. 2012). Irrigation ditch water yielded *Salmonella* in 15.4% (2/13) of samples (Micallef et al. 2012). Isolates from water or sediment samples included: *S*. Newport, *S*. Braenderup, *S*. Javiana, *S*. Lille, and *S*. Tennessee (Micallef et al. 2012). Multiple serovars were identified from the same water sample; however, detection of various or multiple serovars in one water sample was not consistent (Micallef et al. 2012). The authors note the abundant presence of goose and deer feces in several of the fields sampled, suggesting possible biotic sources of the *Salmonella*. Four of the six serovars isolated from the tomato farms have previously been associated with outbreaks from the consumption of raw tomatoes in the US and Canada (Micallef et al. 2012).

It cannot be assumed that all surface waters in warm climates will have detectable *Salmonella* when using cultural methods. Water samples (1 to 100 ml) taken from Spring Lake, the headwaters of the San Marcos River, TX from just below the surface and 50 cm of depth; all samples taken were negative for *Salmonella* when pre-concentrated by centrifugation followed by ISO 6579 cultural method (Gaertner et al. 2008). The number of samples screened during this survey was not reported. It also should not be assumed that no *Salmonella* were present in these waters; one cannot forget the possibility of viable-but-non-culturable state (VBNC), as well as the possibility that the *Salmonella* were present, but in numbers less than 1 CFU/100 ml, as would have been the detection limit.

Estuary Water

An estuary is a transition zone between rivers and ocean waters; depending on water flow the salinity varies within and among estuaries. Findings from *Salmonella* survival studies in estuarian waters are often difficult to compare due to the many and widely varying water parameters that make up these water bodies.

A microcosm-based study on the survival of *S*. Typhimurium in estuarian water observed a 3 log CFU/100 ml decrease in two days when the *Salmonella* was inoculated into raw estuarian water and held at either 20 or 30°C (Chandran and Hatha 2005). The raw water in their experiment contained 5-6 log CFU/ml heterotrophic bacteria, 2 log PFU/ml bacteriophage predating *Salmonella*, 3 log PFU/ml coliphage, and had protozoans detected by qualitative microscope observation (Chandran and Hatha 2005). Alternately, *S*. Tennessee held in diffusion chambers in the estuarian waters of Chesapeake Bay displayed growth during the first three days in raw water (Rhodes and Kator 1988). The microbial quality of the raw water used in the Chesapeake Bay study was not reported, but can be assumed to be a significant factor in the observed multiplication of the *Salmonella*. The difference in serovars used in the respective studies many also explain some of the difference in the observations.

Marine Water

Marine water, while of little agricultural use, will be discussed here because of the numerous studies reported and possibility of the findings within being applicable to waters of agricultural significance. The salinity of marine waters varies from 3.1 to 3.8% (31 to 38 ppt). Much of the *Salmonella* in marine water studies that have been carried out center around bathing waters, as this is a primary form of contact (and thus health risk) related to *Salmonella* in marine waters.

Bathing waters in Spain were sampled and screened for *Salmonella* over a 10-week period using the ISO 6579 method (Serrano et al. 1998). Three beach areas were sampled and ranged between 0.8 to 8.5% (of 356 samples) positive for *Salmonella* (Serrano et al. 1998). In Northwestern Spain, 2.5% (18/707) water samples were positive for *Salmonella* using the ISO 6579 method (Martinez-Urtaza et al. 2004). This study was performed over four years and was able to discern a seasonal pattern; an increased incidence of *Salmonella* existed from October to December (Martinez-Urtaza et al. 2004). It is also noted that weather data (wind, rainfall, temperature, and hours of sunshine) from the previous day correlated with *Salmonella* isolation frequency (Martinez-Urtaza et al. 2004). When conditions were correlated with specific serovars, stronger correlations were obtained for *S*. Seftenburg and *S*. Agona (the two most frequently isolated serovars) (Martinez-Urtaza et al. 2004).

A survey performed in Southern Morocco found *Salmonella* in 4.1% (10/243) of their monthly seawater samples when 100 ml was screened using the ISO 6579 method (Setti et al. 2009). The region screened was noted to be populous. The same survey noted a higher prevalence in sediment from the same sampling locations; 6.8% (19/279) of 25 g sediment samples were positive for *Salmonella* (Setti et al. 2009). These authors found a positive correlation (linear relationship) to rainfall and the presence of *Salmonella* (Setti et al. 2009); a seasonal variation was also noted, with higher prevalence in the autumn and winter, however, no average temperature is given. This survey was unusual in that only two serovars were obtained (*S*. Blockley and *S*. Senftenberg) throughout the 24-month survey (Setti et al. 2009).

Four strains of *S*. Typhimurium were isolated from clams harvested from the Tunisian coast in December 2005 and January 2006 (El Mejri et al. 2012). These four strains, along with an isogenic wild type (*S*. Typhimurium C52) were individually inoculated into sterile sea water microcosms and held in the dark at ambient temperatures (authors do not state actual temp). The culturability of these isolates on TSA decreased progressively throughout the 30 days that they were held (El Mejri et al. 2012). The time for one log reduction (28, 27, 27, and 30 h) determined for each of the strains originally isolated from clams taken from the coast of Tunisia were all greater than the time for one log reduction obtained for the *S*. Typhimurium C52 strain used as a control (25 h) (El Mejri et al. 2012).

Biotic Sources

Numerous biotic sources of *Salmonella* exist, including various reptiles, cows, horses, cats, goats, emus, dogs, elk, coyotes, ground squirrels, raccoons, chipmunks, and various birds (Moore et al. 2003). Shedding of *Salmonella* in feces is thought to contribute to the pathogen load of watersheds by runoff from adjacent lands.

Reptiles are common inhabitants both in and around surface waters in Florida. Reptiles (snakes, lizards, chameleons, and turtles) have a 50.5% *Salmonella* prevalence, when cultural methods are used to test fecal samples (Corrente et al. 2004). Of the reptiles screened by Corrente et al. (2004), snakes had the highest prevalence (83.3%), lizards a 50% prevalence, and turtles a 10.3% prevalence. Chameleons had 100% prevalence, however, this is reflective of only two samples taken (Corrente et al. 2004). Serovars isolated include: Aqua, Gaminara, Gbadago, Give, Havana, Minnesota, Muenster, Newport, Oldenburg, Poona and Senftenberg (Corrente et al. 2004).

Wild turtles (including common musk turtles, red-eared sliders, Texas cooters, snapping turtles, and Guadalupe spiny soft-shell, n=52) have been *Salmonella* positive via carapace or cloacal swabs using the ISO 6579 method (Gaertner et al. 2008). The most common serovar associated with these turtle was *S*. Rubislaw; *S*. Gaminara, *S*. Newport, and *S*. Thompson were also isolated (Gaertner et al. 2008). Water samples from the water source where these turtles were captured tested negative for *Salmonella* when up to 100 ml was screened (Gaertner et al. 2008), indicating the presence of *Salmonella* on/with turtles in the environment does not necessarily predict the presence of *Salmonella* in the water habitat.

American alligators have been a source of both fecal coliforms and *Salmonella* when cloacal swabs were taken from wild alligators (Johnston et al. 2010); captive American alligators have fecal coliforms at a level of 9 log CFU/g (wet wt) of feces (Johnston et al. 2010).

Haley et al. (2009) suggested that the dominance of *S. enterica* subspecies arizonae (40% of isolates) in the Little River rural watershed (in Georgia USA) indicate the local reptile populations are the likely source of the *Salmonella* isolated from the Little River watershed.

Other wild, non-reptilian animals can also be carriers of *Salmonella*. Raccoons have been noted to be asymptomatic carriers of *Salmonella*, at rates of up to 65% of individuals in urban areas or 27% of individuals in rural areas (Jardine et al. 2011). Serovars isolated from raccoon feces include both those that have and those that have not caused human outbreaks (Jardine et al. 2011); *S*. Typhimurium the most prevent

serovar in rural, and *S.* Newport the most common serovar in urban raccoons (Jardine et al. 2011).

Birds are another potential biotic source of *Salmonella*. Seagulls along the western Mediterranean coast were 17% (31/182) positive for *Salmonella* when fecal samples were screened using ISO 6579-2002 method (Ramos et al. 2010). In another study performed in the Oldman river watershed (Alberta, Canada) only the serovar Rubislaw could be detected in the collected water samples; this serovar was also detected in wild bird feces, but never in the analyzed human sewage or domestic animal feces of the area (Jokinen et al. 2011).

Insects are capable of being carriers of *Salmonella* from one location to another. Flies are known to transmit *Salmonella*, typically via their sponging mouth parts, body and leg hairs, the sticky parts of their feet, fecal deposition, or vomitus (Wang et al. 2011). Flies captured near pig farms carry *Salmonella* at a rate of 26.4% (58/220); predominate serovars included Anatum, Choleraesuis var. kunzendorf, and Derby (Wang et al. 2011). These same serovars, with matching PFGE patterns, were also isolated from pig feces on the nearby farms (Wang et al. 2011), thus suggesting flies as a vector. Midges can act as a carrier for *S.* Typhimurium DT104 in the laboratory, becoming infected with *Salmonella* and transfer it to the next, otherwise sterile, aqueous environment (Moore et al. 2003).

Fate Outside Host

The duration of survival of *Salmonella* in the aquatic environment may influence the potential risk to human health; as duration of survival increases so does the time for human contact with the contaminated water source. Studying the duration of survival of *Salmonella* in the aquatic environment is problematic because there are numerous

variables that could be affecting their survival. These variables are too numerous to completely account for in microcosm-based studies. *In-situ* studies do not allow for control of variables. Environmental samplings do not account for re-contamination, rather than solely duration of survival, when attempting to discern the fate of *Salmonella* in aquatic environments.

During one study on the Red River of the North (between North Dakota and Minnesota), *Salmonella* survived three days (62 river miles) from the point of suspected contamination (sewage treatment plant) during the month of November; the survival time increased to four days (73 river miles) during January, when water temperatures are noted as extremely cold and ice cover was present (Spino 1966). From this study it is not known, however, if the *Salmonella* was capable of surviving for a longer duration or if they were being diluted out to lower than the limit of detection, yet still surviving. To more accurately determine duration of survival in various types of surface water, microcosm-based studies are required to control certain parameters, as well as avoid the *Salmonella* from merely being diluted out beyond the limit of detection due to environmental events (river flows, rainfall, etc.) and thus not being detected.

Microcosm and Survival Time

A microcosm is a 'small world' designed to mimic certain parameters of the real world. This allows for the effect of one parameter to be studied whilst the others are held constant. Duration of *Salmonella* survival in surface waters can be evaluated using this means as it can be assured that no dilution effect or additional contamination events are occurring.

The longest duration of survival reported for *Salmonella* in a water microcosm is seventeen years (Amel and Amina 2008). This microcosm contained sterile seawater

into which the *Salmonella* had been inoculated, this was held at ca. 20°C and allowed to dry to salt crystals. To culture the *Salmonella* from this microcosm an extended, three-month, resuscitation in nutrient broth was required (Amel and Amina 2008). This lengthy duration of survival has not been repeated, however many microcosm experiments simply stop after a given period of time (typically months) and do not undertake such extensive resuscitations in nonselective enrichment media.

In the same article, a steady decline in *Salmonella* population in sterile seawater, held at 20°C, from 8 log CFU/ml to 1.5 log CFU/ml over a 15-month time frame (Amel and Amina 2008). *Salmonella* Typhimurium multiresistant DT-104 has survived at culturable levels up to 54 days in 23°C sterile well water (Moore et al. 2003), although detection was intermittent between days 21 and 54 (when sampled every other day) (Moore et al. 2003). At 20°C, in filter sterilized surface water streptomycin-resistant *S*. Typhimurium remained detectable by pour plating to bismuth sulfite agar for up to 41 days; suspended particles (clay, silt, sand, organic flocculent) did not significantly affect the survival of *S*. Typhimurium (Maki and Hicks 2002). In this study, *Salmonella* decreased in a non-linear fashion, regardless of particle size or concentration (Maki and Hicks 2002), indicating turbidity might not be a good indicator of pathogen presence in water.

Microcosms established using sterile water do not accurately represent the real world situation. *Salmonella*, in real world surface waters, are subject to predation by protozoa, competition for resources from other bacteria, and attack by bacteriophages. While microcosms established using filter-sterilized water would still be expected to contain bacteriophages, most of the competing bacteria, and all of the predacious

protozoa are removed. Sterilizing via autoclaving would inactivate protozoa, bacteria, and bacteriophages, additionally the heat exposure would also be expected to alter some of the nutrients in the original water sample.

At 20°C, in filter sterilized surface water streptomycin-resistant *S*. Typhimurium remained detectable by pour plating to bismuth sulfite agar for up to 41 days; non-filter sterilized surface water, with organic flocculent, showed a significantly higher decrease in *Salmonella* overtime, remaining culturable for 16 days; this is speculated to be due to both predation and competition with the native microflora (Maki and Hicks 2002).

A microcosm study that investigated the survival *S*. Typhimurium in estuary waters focusing on the effect of biological factors, dissolved organic and inorganic substances, and sunlight on the inactivation of these bacteria. Sunlight was the most important factor for inactivation, followed by biotic factors. An overall 2.5 log CFU/ml reduction in *Salmonella* is seen over four days at 20°C in the non-sterile water; a greater decrease (3.5 log CFU/ml) was observed when the temperature was 30°C (Chandran and Hatha 2005). The biotic factors were determined to comprise of a combination of predation by protozoa and bacteria, bacteriophage lysis, and competition with autochthonous microbes. Dissolved organic substances present in estuarine water, in the absence of the native biota, promoted survival and growth of *S*. Typhimurium (Chandran and Hatha 2005).

Possible Correlating Bacteria (Coliforms and *E. coli*)

The direct detection of pathogenic bacteria, such as *Salmonella*, is costly and time consuming, requiring well-trained labor. It is these factors that lead to the concept of indicator organisms for detecting fecal contamination, and thus potentially human pathogens. In 1914 the coliform group was adopted by the US Public Health Service as

an indicator of fecal contamination (APHA 1992). There are both total coliforms and fecal coliforms.

Total coliforms are a group of bacteria belonging to the *Enterobacteriaceae*; these include both aerobes and facultative anaerobes, they are Gram-negative, non-spore forming, rod shaped bacteria that ferment lactose with gas production within 48 h at 35°C. Included in this group are: *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp. Fecal coliforms are thermotolerant coliforms that ferment lactose at 44.5°C; these include *E. coli* and *Klebsiella* spp.

Generic *E. coli* are also used as indicator bacteria. *Escherichia coli* can be differentiated from other fecal coliforms by the absence of urease and the presence of β-glucuronidase. It is these differences in metabolism that products such as Colilert and Colisure (IDEXX, Westbrook, Maine) are based on. In Colilert, *β*-galactosidase from the total coliforms is used to metabolize ortho-nitrophenyl *β*-galactoside (ONPG) and change it from colorless to yellow; while *E. coli* uses *β*-glucuronidase to metabolize 4-methylumbelliferyl- *β*-D-glucuronide to *β*-D-glucuronide and 4-methylumbelliferone which is fluorescent under UV light. Colisure uses the same enzyme reaction for *E. coli*, but uses chlorophenolred-ß-D-galactopyranoside as the substrate for the total coliforms' *β*-galactosidase that then has the color change going from yellow to magenta.

Ideally, an indicator organism fits the following criteria: associated with the intestinal tract of warm-blooded animals; present when pathogens are present, and absent from uncontaminated samples; present in greater numbers than the pathogen; be at least equally resistant as the pathogen to environmental factors and disinfectants; does not multiply in the environment; be detectable by easy, rapid, and inexpensive

methods; and be non-pathogenic. Many commercial products, such as Colisure and Colilert discussed above, have been developed to make coliforms and *E. coli* easy, rapid, and inexpensive to detect. It is the 'present at greater numbers than pathogens' and 'equally resistant to environmental factors' criteria that require more investigation for coliforms and *E. coli* as indicator organisms for use in Florida surface waters. The correlation between coliforms or *E. coli* and *Salmonella* still needs to be better understood.

Understanding the correlation between *Salmonella* and indicator organisms is an important for setting scientifically valid Good Agricultural Practices (GAPs). Currently, Tomato Good Agricultural Practices (T-GAPs) are the only regulatory requirement for microbial water quality of use on edible horticultural crops (FL5G-6). The Leafy Greens Marketing Agreement (LGMA) also contains water quality standards for waters used on edible leafy greens, however, the LGMA standards are voluntary (F-DACS 2007, LGMA 2012). The T-GAPs regulation contains two microbial water standards, one for foliar applied waters applied near the time of harvest, and one for non-foliar applied waters.

Under T-GAPs, foliar applied waters are required to meet potable water quality standards; these standards are contained in 40 CFR 141.63. That is, no more than 5.0% of 40 samples may be positive for total coliforms; when fewer than 40 samples are collected in a month, no more than one sample per month may be total coliform positive. Fecal coliform or *E. coli* positive samples are not permitted.

The regulation for non-foliar waters uses the US EPA standards for recreational waters, 40 CFR Part 131.41(c); no single sample may contain greater than 235 generic *E. coli* per 100 ml and the geometric mean of all samples taken must be below 126

generic *E. coli* per 100 ml. These requirements were originally designed for bathing waters, and have not been validated for indirect consumption, as would be the case when the water is used to irrigate edible horticultural crops. The LGMA, a voluntary grower group, also relies on these standards for irrigation waters. With these standards in mind, the EPA assessed 33% of US waters in the year 2000 and found that 40% of streams, 45% of lakes, and 50% of estuaries were not clean enough to bathing water quality standards (Jacobsen and Bech 2012). This may be problematic when looking for acceptable water sources for use on edible horticultural crops.

While other indictor organisms do exist and are used, including *Enterococci*, fecal *Streptococci*, various bacteriophages, etc., this review will focus on *E. coli* and coliforms due to their significance in agricultural regulations and guidance documents. The American Public Health Association (APHA) suggests monitoring heterotrophic plate counts, as well as indicator organisms, such as total coliforms, fecal coliforms, and *E. coli* to determine microbial water quality (APHA 1992). The remainder of this section will focus on *E. coli* and coliforms, and their ability to predict the presence or prevalence of *Salmonella*.

Several studies have reported coliforms and/or *E. coli* being acceptable indicators of *Salmonella* presence or prevalence. Microcosm-based studies have shown *Salmonella* dies-off at a greater rate than *E. coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* when a variety of sediment types were included in the microcosms (Burton et al. 1987). This would indicate good potential as indicator organisms because the slower die-off rate would result in the indicator organisms being present at greater numbers than the *Salmonella*; this is a requirement for an ideal

indicator organism. Another microcosm-based study looked at the survival of *S*. Typhimurium in estuarian water. The authors used a starting inoculum of 6 log CFU/ml when inoculating *Salmonella* into the microcosms. A rapid, approximate 3 log CFU/ml, inactivation of the *Salmonella* in raw estuarian water was observed over a two day period when held at either 20 or 30°C (Chandran and Hatha 2005). It is noted that *Salmonella* declined at a faster rate than the *E. coli* in the same experimental conditions (Chandran and Hatha 2005). When the biotic factors were removed by filter sterilization, enhanced survival of *Salmonella* and *E. coli* was observed under the otherwise identical conditions; however, *E. coli* remained at a higher concentration than *Salmonella* (Chandran and Hatha 2005), however this is not significantly different. The raw water in their experiment contained 5-6 log CFU/ml heterotrophic bacteria, 2 log PFU/ml bacteriophage predating *Salmonella*, 3 log PFU/ml coliphage, and had protozoans detected by qualitative microscope observation (Chandran and Hatha 2005). Although these examples both suggest that coliforms and/or *E. coli* is capable of acting as an indicator organism for *Salmonella*, the findings must be carefully considered because the microcosm conditions will not be perfect representations of real world occurrences, and thus may miss some parameters that could change the survival rate of either bacteria. It is therefore important to also consider results obtained from real-world samplings.

Samplings performed in South Africa have consistently high correlations between both fecal coliforms and total coliforms and the presence of *Salmonella* (Ijabadeniyi et al. 2011). In cases of known human fecal contamination both fecal and total coliforms can be used as indicator organisms for *Salmonella*. In an example going back to 1966,

sewage outfall was monitored 22 miles downstream from initial outfall in the Red River, ND fecal coliforms were enumerated at 2,850 CFU/100 ml, total coliforms were enumerated at 47,600 CFU/100 ml at the furthest location where *Salmonella* was able to be isolated (Spino 1966). This would suggest that either set of indicator organisms could be used for *Salmonella*, however, this study is limited in that few samples were taken, and only takes into account an obvious source of human fecal contamination. Observations may not hold true when there is no obvious source of human fecal contamination, or when the bacteria are previously from other, less hospitable, sources.

Numerous examples also exist where coliforms and/or *E. coli* was not a good indicator of *Salmonella* presence or prevalence. Microcosm studies have also been able to show a lack of correlation and a greater die-off of *Salmonella*, compared to *E. coli*, when temperatures of 10°C were investigated (Rhodes and Kator 1988). Sampling of ornamental fountains, which could be interpreted as microcosms in the real-world, in Guadalajara City, Mexico showed a correlation between the presence of coliforms and generic *E. coli*, however, a correlation was not seen in the presence / absence of *Salmonella* in the same water samples (Escartin et al. 2002). The lack of correlation could be explained by the difference in sampling methods for the two sets of organisms; coliforms and *E. coli* were determined by MPN (10, 1, 0.1 ml), whereas *Salmonella* was determined through enrichment of a 24 h Moore swab (Escartin et al. 2002), intuitively this samples a larger volume of water. Both of these examples not exact representations of the real world, and can thus only be considered with careful prior thought.

There are real world sampling examples that also demonstrate the potential lack of correlation between coliforms and/or *E. coli* with *Salmonella*. A study in Georgia found *E. coli* in all surface water samples, with concentrations varying significantly by site; however, *E. coli* was not found to be able to correlate with levels of *Salmonella* determined for the same surface water samples (Haley et al. 2009). In a pond water study in Northeastern Georgia when *Salmonella* populations were at their highest level, both generic *E. coli* and fecal enterococci were not detected from the same water sample (Jenkins et al. 2008). At other sampling times in the same study *E. coli* was detectable, but did not correlate with *Salmonella* populations. Furthermore, mineral water from Portugal allowed for *S*. Typhimurium to persist up to 20 days longer than *E. coli* (Ramalho et al. 2001). This aqueous environment provided a very low nutrient level with a background microflora level of approx. 6 log CFU/ml (Ramalho et al. 2001). This last example is especially concerning because of the long duration during which *Salmonella* was able to survive and *E. coli*, the indicator organism, died-off much sooner; an ideal indicator organism must be able to persist in the environment for a greater duration than the predicted pathogen.

Some attempts to identify parameters that can predict whether a specific indicator can be useful have been carried out. One study looked at *E. coli* in sediment and water microcosms and the effect of temperature (Craig et al. 2004). Sediment type was found to affect the survival of *E. coli*, with mainly sandy soil allowing for less survival than others with higher silt, clay, and organic carbon content (Craig et al. 2004), difference in *E. coli* concentration was noted at day zero sampling of sediment. Water column *E. coli* concentrations remained relatively constant for the initial sampling period

(days zero and one) (Craig et al. 2004). *E. coli* persisted better at 10°C, than at 20 and 30°C (Craig et al. 2004). In another study, *Salmonella*, originally isolated from sewage, survived over 56 days in static sterile creek water microcosms (Fish and Pettibone 1995). The *Salmonella* population increased from the initial 6.7 log CFU/ml to 8.1 log CFU/ml within the first 10 days; the population decreased to back to 6.7 log CFU/ml after 56 days (the end of the experiment). There was no significant difference between the plate counts (spread plated on nutrient agar) and direct counts or direct viable counts (Fish and Pettibone 1995), demonstrating a lack of stressed cells. *Escherichia coli* followed a similar pattern in this experiment, with a starting population of 6.3 log CFU/ml a maximum population was reached at 10 days (7.6 log CFU/ml), then remained relatively constant through the remaining experiment (56 days) (Fish and Pettibone 1995). This study does demonstrate that the *E. coli* and *Salmonella* follow similar survival patterns in the surface water microcosm; however, it should be noted that in each case the bacteria were in otherwise sterile conditions (i.e., no competition or predation), a situation that is not accurately reflective of real world situations. In this case the *E. coli* does not demonstrate to be an ideal indicator organism because it was not consistently present at greater numbers than the *Salmonella*.

Considering the plethora of conflicting data on the ability of coliforms or *E. coli* to act as indicator organisms for *Salmonella*, it may be suggested that the ability to predict *Salmonella* presence needs to be established for individual sets of parameters (geographic location, sources of contamination, water and sediment characteristics) under which one wishes to implement the use of indicator organisms, rather than selecting and accepting one or one set of indicator organisms universally.

Possible Correlating Factors (Chemical and Physical Water Parameters, and Environmental Conditions)

In addition to microbial indicators of water quality, several chemical and physical water parameters have been suggested as a possible correlating data point to indicate the presence or prevalence of *Salmonella*, or other pathogens. The major advantage to using a chemical or physical water quality parameter is the general ability to obtain immediate results for these parameters, rather than the typically long time delay in obtaining microbial results.

The APHA states in its Standard Methods for the Examination of Water and Wastewater that physicochemical factors, such as turbidity, pH, salinity, temperature, assimilable organic carbon, dissolved oxygen, biochemical oxygen demand, and ammonia may provide useful information about contamination or the potential of water to support bacterial growth (APHA 1992). During a twelve-month sampling of Georgia surface waters no significant correlations could be found between *Salmonella* populations and any measured water parameters, these included conductivity, pH, DO, turbidity, ORP, fluorescence, and chlorophyll a (Haley et al. 2009). The only factor that the APHA recommends monitoring that did have a significant correlation with *Salmonella* populations was water temperature (Haley et al. 2009). It should be noted, however, that this is the only measured parameter that varied significantly over the study. It is perhaps due to the lack of variability of these parameters in the sampling location that resulted in the lack of correlations.

Through the use of microcosm-based experiments, numerous studies have determined sunlight to decrease the survival time in a variety of surface waters (Ramalho et al. 2001, Chandran and Hatha 2005, Bae and Wuertz 2012,). This should

be expected due to the effect that UV from sunlight has on DNA; bonds between adjacent thymine base-pairs are broken and some bonds form between neighboring base pairs. Repair mechanisms do exist, however, many cells are irreversibly inactivated from this damage.

Besides sunlight, rainfall has also been recognized as a possible correlating environmental condition for *Salmonella* populations. In Georgia a 12-month survey found the daily rainfall volumes for three days prior to sampling had a strong positive correlation with *Salmonella* populations (Haley et al. 2009). Rainfall from beyond three days was not found to correlate with *Salmonella* populations. It is possible that rainfall can also result in a decrease in bacterial populations in surface waters; this decreasing effect is hypothesized to result from a dilution effect that could be expected when large volume rainfall events occur. This has been suggested as a reason why fecal coliforms have been found to have no correlation with rainfall when it is known they are commonly found in tropical sediments and re-suspension would be expected during rainfall events (Santiago-Rodriguez et al. 2012). Rainwater itself is not typically thought of as a source of contamination, although a study performed in Singapore, found rainwater to be (21/50) positive for *E. coli* when 100 ml samples were screened with qPCR (Kaushik et al. 2012).

Many parameters and environmental conditions can affect the ability of *Salmonella* to persist in surface waters. It is likely that these have synergistic effects and that one parameter cannot be considered on an individual basis as a predictor of *Salmonella* presence of persistence. It is therefore imperative that more complex models be built using greater amounts of data that is still to be gathered.

Sample Collection

Collecting surface water samples must utilize either nonreactive borosilicate glass or plastic bottles that have been cleansed, rinsed, and sterilized (APHA 1992). The sampling container must be large enough to allow for a minimum of 2.5 cm headspace to remain above the sample; this allows for mixing the water sample before examination (APHA 1992).

To establish the time of day water should be sampled the APHA suggests peak bathing hours when the waters are expected to be used for recreational purposes (APHA 1992). No other suggestion of time of day is given. This concept is reinforced by a study performed in Spain where sampling at 10:00, 13:00, and 16:00 h was performed in bathing waters and the highest level of *Salmonella* was detected at 16:00 h (6.7%), whereas at 10:00 and 13:00 h *Salmonella* positives were 3.3% and 4.4%, respectively (Serrano et al. 1998). An opposite pattern was observed for all other indicator microorganisms enumerated during the same study. While this sampling strategy may allow for *Salmonella* to be detected more readily, the decrease in indicator microorganism may result in a lower level of risk being assumed if only indicator microorganisms were to be enumerated.

When manual sampling is being performed the APHA suggests the bottle should be held by the base and plunged, neck downward, below the surface (APHA 1992). The bottleneck should then be turned up slightly and into the current, and thus allowed to fill. If sampling by hand is not possible a weight may be attached to the bottle, and this lowered into the water source (APHA 1992).

Cultural Methods

Current protocols for enrichment culture of *Salmonella* from foods or water rely on a primary nonselective enrichment broth to recover stressed cells followed by secondary enrichment in selective broths. A solid medium is then required to obtain isolated colonies, which can then be biochemically, serologically, or genetically confirmed as *Salmonella*. These methods are laborious and require several days to complete; this is due to *Salmonella* being a biochemically diverse genus, thus multiple media types containing different selective and differentiating agents are required. Even with the use of selective broths and selective and differential agars, *Citrobacter* and *Proteus* are both capable of causing false-positive results in *Salmonella* enrichments (Gorski and Liang 2010). Multiple organizations have developed their own standard protocols which all follow the same general format; in the US standard *Salmonella* protocols have been developed by the APHA, the US Food and Drug Administration (US FDA), and the International Organization for Standardization (ISO).

American Public Health Association

The APHA suggested methods for *Salmonella* detection from water consist of a concentration step, enrichment, selective growth, and biochemical confirmations (APHA 1992). The concentration techniques suggested for use include swabs, diatomaceous earth, large volume samplers, and membrane filter technique (APHA 1992). The swab technique uses sterilized cheesecloth, which is folded and wrapped, or gauze pads being suspended in the water to be sampled for a desired (unspecified) length of time. The swab acts to concentrate particulate matter and microorganisms from the water as it flows through and over the swab (APHA 1992). The diatomaceous earth technique requires diatomaceous earth to be added to a funnel above an absorbent pad (which

hold the diatomaceous earth in place); the water sample is then vacuum filtered through the plug (APHA 1992). The APHA states that this method should be capable of filtering up to 2 L of sample water. The large volume sampler technique uses a borosilicate glass microfiber bonded with epoxy resin as the filter through which the APHA states larger sample volumes can be passed through (a specific volume is not reported) (APHA 1992). The membrane filter technique, recommended for low-turbidity waters, requires the use of 0.45 µm pore size filter at either 142 mm diameter or using multiple 47 mm diameter filters (APHA 1992). For higher turbidity waters, it is suggested that this method may still be used if the filter is pre-coated with diatomaceous earth by filtering a suspension of the fore-mentioned (APHA 1992). In either case the filter, or filters, are homogenized via blending into the enrichment medium.

After the selected filter concentration method has been performed the concentrated sample needs to be selectively enriched in a growth medium that will suppress the growth of coliform bacteria (APHA 1992). The APHA recommends selecting two of the following enrichment media and running them in parallel: selenite cysteine (SC) broth, selenite-F (SF) broth, or tetrathionate (TT) broth (APHA 1992). Selenite cysteine broth will inhibit Gram-positive and nonpathogenic enterobacteria while allowing for recovery of most species of *Salmonella*, including *S.* Typhi (APHA 1992). It is suggested that after incubation turbid tubes be streaked to solid media daily, for five days, in order to optimally obtain all serovars that may be present (APHA 1992). Selenite-F broth is stated to allow for optimum recovery of most *Salmonella*, including *S*. Typhi; however, this increased recovery is accompanied by a decrease in selectivity (*E. coli* growth is not inhibited) (APHA 1992). Again, repeated streaking daily for five days

after incubation is recommended to better obtain all serovars present. Tetrathionate broth inhibits coliforms and Gram-positive bacteria, while allowing for the growth of most *Salmonella* including *S*. Typhi (APHA 1992). The APHA states that TT broth is more selective for *Salmonella* than the selenite-based broths, especially when incubated at 43°C for 48 h (APHA 1992). Unfortunately, this broth allows for the optimum growth of *Proteus mirabilis*; without the addition of brilliant green *Proteus* and *Citrobacter* can also grow (APHA 1992). The addition of brilliant green and incubation at 43°C will inhibit the growth of some species of *Salmonella* including *S*. Typhi (APHA 1992).

Following enrichment, a secondary differentiation on selective solid media is required. The following solid media are suggested for use: brilliant green agar (BGA), bismuth sulfite agar (BSA), xylose lysine desoxycholate agar (XLD), and xylose lysine brilliant green agar (APHA 1992). Brilliant green agar will cause *S.* Typhi and a few other *Salmonella* to grow poorly because of the brilliant green dye content this can be aided by incubating plates for 48 h (APHA 1992). Slow lactose-fermenters, such as *Proteus*, *Citrobacter,* and *Pseudomonas*, will produce colonies that resemble *Salmonella* (APHA 1992). On BGA, non-lactose fermenting colonies can be masked by lactose-fermenting ones in close proximity (APHA 1992). Bismuth sulfite agar allows for much growth of most *Salmonella* (APHA 1992). These plates also require 24 and 48 h incubations to better obtain the slow growing serovars. After 48 h of incubation, typical *Salmonella* colonies may be masked by numerous bordering colonies (APHA 1992). *Proteus* and some coliforms can also develop the typical black color of the *Salmonella* colonies due to H_2S production (APHA 1992). Xylose lysine desoxycholate agar can be slightly toxic to fastidious *Salmonella* (APHA 1992). Coliforms, *Proteus*, and some

Enterobacter can produce yellow colonies on this agar; depending on the starting sampling this many cause difficulties in isolating *Salmonella* colonies (APHA 1992). Xylose lysine brilliant green agar is recommended for marine samples as it inhibits the *Proteus*, *Enterobacter*, and *Citrobacter* (APHA 1992). After isolation on selective media, biochemical followed by serological confirmations are suggested (APHA 1992). Any combination of these methods can be used to perform a five-by-three MPN in order to obtain quantitative results (APHA 1992).

Food and Drug Administration – Bacteriological Analytical Manual

The Food and Drug Administration – Bacteriological Analytical Manual (FDA BAM) method contains specific instructions for the preparation of a variety of food types for the isolation of *Salmonella* (US FDA 1998). This method follows the common protocol of pre-enrichment, selective enrichment, colony isolation, and colony confirmation as *Salmonella*. From sampling to final result, this method requires five days. A detection limit of 1-2 log CFU/25 g has been established for this protocol (Delibato et al. 2011).

In this method the pre-enrichment media is lactose broth, universal pre-enrichment broth, buffered peptone water, trypticase soy broth, or Dey-Engley broth. In each case only one of these pre-enrichment broths is to be used; the recommended broth is based on the composition of the food item as well as the expected background microflora. The typical pre-enrichment conditions require 24±2 h at 35±2°C.

Selective enrichment for low microbial load foods uses both Rappaport-Vassiliadis (RV) medium and TT broth are to be used. From the pre-enriched broth 0.1 ml is transferred to 10 ml RV and 1 ml is transferred to TT broth (US-FDA 1998); these are incubated 24±2 h at 42±0.2°C and 24±2 h at 35±2°C, respectively. Foods with

higher microbial loads require the TT broth to be incubated at 24±2 h at 43±0.2°C. Only when the product is Guar gum, or *S*. Typhi is expected is the use of SC broth recommended to replace the RV.

Colony isolation is performed on BSA, XLD, and Hektoen enteric (HE) agar (US-FDA 1998). Plates are incubated 24±2 h at 35±2°C. Typical colonies for each media are identified and picked; it is recommended two or more colonies from each selective agar plate be used. If no typical colonies are available, atypical *Salmonella* colonies should be picked. Picked colonies are inoculated to lysine iron agar (LIA) and triple sugar iron agar (TSI); tubes are incubated 24±2 h at 35±2°C. *Salmonella* typically produce alkaline (red) slant and acid (yellow) butt, with or without the production of H_2S (blackening of agar) in TSI. In LIA, *Salmonella* typically produce alkaline (purple) reaction in the butt of the tube; most will produce H_2S in LIA. Once presumed to be *Salmonella* from the reactions of the slants, isolates are confirmed biochemically and serologically.

International Organization for Standardization 6579

The ISO is the world's largest developer of international standards. The standard ISO method has a detection method equal to the 1-2 log CFU/25 g of the FDA-BAM method (Delibato et al. 2011) and also requires five days. The ISO method for *Salmonella* enrichment is number 6579.

ISO 6579:2002 method uses a 25 g sample in 225 ml Buffered peptone water (BPW) for 16-20 h at 37±1°C; 0.1 ml to 10 ml Rappaport Vassiliadis broth with soy peptone (RVS) 24 h at 41.5±1°C and 1 ml in 10 ml Muller Kauffman tetrathionate broth with Novobiocin (MKTTn) 24 h 37±1°C; plating to XLD and a Chromagar of the laboratories choice 24 h at 37±1°C; one presumptive colony per plate is then picked and

streaked to solid nutrient agar, incubated 24 h at 37±1°C, this is then used for serological and biochemical confirmation (Schonenbruecher et al. 2008).

Variations on Standard Methods

Salmonella are a metabolically diverse genius of bacteria, and it is thus difficult to ensure all species and subspecies will be isolated by a particular method. This fact is reflected in the numerous differences within the standard methods for *Salmonella* isolation. To add to this difficulty, variations in sample composition and background microflora also add to the complexity of *Salmonella* isolation. For these reasons many variations in cultural methods for *Salmonella* isolation can be found in the literature.

The pre-enrichment broth can be BPW, lactose broth (LB), universal pre-enrichment broth (UPB), or tryptic soy broth (TSB). Buffered peptone water as a medium for pre-enrichment may yield a higher number of positive results as compared to us of LB or straight enrichment with TT broth (Thomason et al. 1977). When looking at 100 soil and water samples 29 were positive for *Salmonella* when using BPW as a pre-enrichment before TT broth, LB as a pre-enrichment method gave only 24 positives, 26 were positive when pre-enrichment was avoided and the sample inoculated straight into TT broth (Thomason et al. 1977). Additionally, *S. enterica* grows equally well in both UPB and BPW (Mafu and Sirois 2009). When comparing TSB, BPW, and UPB for the detection of *S*. Baildon, *S*. Cholerasuis, *S*. Poona, *S*. Saintpaul, and *S*. Typhimurium from either green leaf lettuce or Roma tomato pre-enrichments, TSB allowed the greatest *Salmonella* detection when the final real-time PCR was completed (Gorski and Liang 2010). Fewer aerobic mesophilic bacteria grew in BPW, than in TSA or UPB at the common 35°C incubation temperature (Gorski and Liang 2010).

After the pre-enrichment, a selective enrichment must be performed prior to colony isolation on solid media. Beginning enrichment with the use of Rappaport's medium does not allow some *S*. Arizona strains to multiply, these are often associated with reptiles; especially when the *Salmonella* injured from environmental conditions (Madsen 1994). Use of RVS in isolating *Salmonella* from artificially inoculated (10 CFU/25 g) pork samples gave 100% recovery when a 24 h enrichment was used (Schonenbrucher et al. 2008).

When screening environmental samples from a zoo, both animal enclosures and guest areas, significantly higher number of positives samples were obtained when the selective enrichment was performed in either RVS or MKTTn as compared to SCSC broth (Bauwens et al. 2006). For each selective enrichment, HE provided the highest number of positives, followed by XLD; BGA and semisolid Rappaport medium gave the fewest positive results (Bauwens et al. 2006). When isolating *Salmonella* from reptile feces, no significant difference has been noted between use of Rambach and XLD after use of either SC broth or RV broth (Corrente et al. 2004).

XLD and xylose lysine Tergitol 4 (XLT4) have been seen to have false-positives from *Citrobacter freundii*, and false-negatives from *S.* arizonae and *S.* Senftenberg (Schonenbrucher et al. 2008); BGA has been shown to give false-positives for *C. freundii* and *Pseudomonas aeruginosa* (Schonenbrucher et al. 2008). *Salmonella* Senftenberg can be a weak producer of hydrogen sulfide. *Citrobacter. freundii* is H2S-positive (Schonenbrucher et al. 2008). While these false-positives are not noted for the chromogenic agars, *S*. Dublin and *S*. Derby can give false-negatives (Schonenbrucher et al. 2008). Bismuth sulfite agar remains a medium of choice for isolating *Salmonella*

because, in addition to a high level of selectivity, it responds solely and most effectively to the production of extremely low levels of hydrogen sulfide, eliminating the problem of missing the biochemically atypical lactose and/or sucrose positive *Salmonella*.

Due to the numerous options for isolating *Salmonella*, it is advised that each type of sample have the isolation method optimized prior to sampling projects being performed. Sample physicochemical characteristics and background microflora, as well as expected serovars need to be hypothesized prior to optimizing the isolation protocol.

Water Filtration

Water filtration can be used as a concentration technique prior to pre-enrichment or enrichment. When concentrating bacteria by water filtration, filters can either be used to trap the bacteria within the filter matrix, or as a barrier to push the excess water through, while retaining the bacteria on the retentate side, resulting in a smaller sample volume to move forward with. The most important advantage of filtration as a means of concentration of bacteria from water samples is the ability to handle much larger sample sizes, thus decreasing the final limit of detection.

Filters possess complex and irregular structures; the pore shape, tortuosity, and connectivity will determine the dimensions of bacteria that are able to pass through the filter. Filter pore sizes are measured based on physical measurements and mathematical extrapolations. For example the bubble point is the force necessary to extrude air through the capillary network of a wet filter. Although test bacteria are larger than 0.2 µm, some, such as *Listeria monocytogenes* and *Hydrogenophaga pseudoflava*, may penetrate 0.2-µm rated filters (Lee et al. 2010). Some autochthonous aquatic bacteria are able to pass through the standard 0.2 µm pore size commonly used for filter sterilization (Ramalho et al. 2001).

Extremes of process fluid composition (osmolarity, ionic strength, and viscosity) can impact bacterial passage through a filter. Osmotic shock is known to cause either shrinkage or swelling of bacterial cells, which presumably impacts filter passage. Fluid composition also impacts the membrane itself, for example charge shielding can occur. Some membrane materials, including aluminum oxide, cellulose acetate, and polyethersulfone, are not significantly impacted by extreme process fluid physicochemical characteristics (Lee et al. 2010). Both nitrocellulose acetate and polysulfone are affected more significantly (Lee et al. 2010).

Use of filters to concentrate bacteria can result in reductions of the bacteria; this is may be due to caking of the bacteria on the filter membrane (Lee et al. 2010). At lower starting concentrations in the starting fluid, lower reductions have been observed after filtering (Lee et al. 2010).

Various types of filters exist for water filtration. Two filter categories are normal filtration, and tangential flow filtration. These will be discussed in the sections below, along with Moore swabs (MS), a specialized type of filtration used for water.

Normal Filtration

The term normal filtration comes from mathematics, in that the water (vector) is hitting the filter (plane) at a right (90°) angle. The water can be pulled through the filter either by gravity or use of a vacuum, which is pulled under the filter membrane. A major disadvantage to normal flow filtration is that a limited volume of water can be passed through the filter, especially in cases of high turbidity, before other components become entrapped in an on the filter causing the filter to clog. The APHA uses normal flow filtration in their standard water testing methods; 100 ml is passed through a sterile 0.45 µm pore size, 47 mm diameter filter and the filter is applied directly to an appropriate

agar plate (APHA 1992). The APHA has normal flow filtration methods for coliforms or *Salmonella,* as well as other bacteria; in both cases 100 ml of sample water is collected and filtered through a normal flow filter as a means of concentrating the target organism. If a larger volume of water is to be analyzed, the APHA states that sterile diatomaceous earth may be place on top of an absorbent pad over the filter, in order to act as a filter aid (APHA 1992).

The normal flow filtration method has been used numerous times to survey environmental waters for *Salmonella*. In the mid-Atlantic region of the USA, up to 250 ml of ground, pond, or irrigation ditch was passed through a 0.45 µm, 47 mm cellulose ester filter; these filters were then processed through an enrichment method (Micallef et al. 2012). This enabled *Salmonella* to be isolated from 7.7% (3/39) of the pond water samples, and 15.4% (2/13) of the irrigation ditch water samples; none of the 25 ground water samples were *Salmonella*-positive using normal flow filtration (Micallef et al. 2012). The same normal filter specifications were used to as a pre-concentration step before using cultural methods to screen seawater (100 ml) from coastal waters of Agadir Morocco for *Salmonella*, finding 4.1% (10/243) *Salmonella*-positive samples (Setti et al. 2009). The limited volumes of water, 100 – 250 ml, that have been reported to have been screened for *Salmonella* using normal flow filtration may be a contributing factor to the lower percentage of *Salmonella*-positive samples.

Tangential Flow Filtration

Tangential flow filtration (TFF) has the water flowing along the filter membrane; the filter membrane is formed in a tube shape inside which the water sample flows. The water sample is pumped through the TFF unit, and is re-circulated numerous times until the desired final volume has been obtained. The difference between the pressure on

the retentate side and the permeate side is referred to as the transmembrane pressure. The transmembrane pressure is what causes the water to move through the filter and to the permeate side. The tangential flow of the water sample across the filter membrane acts to push the filter cake along and off the filter membrane, thus increasing the amount of sample that can be passed through the filter. The material that the filter membrane is constructed will play a major role in the volume of sample that can pass before the filter is irreversibly clogged. Ideally, the material should have low-protein binding properties; polyethersulfone and cellulose acetate have low-protein binding tendencies (Gibson and Schwab 2011).

Although not used in any standard methods, TFF has been used in used in numerous situations to concentrate bacteria from large volumes of water. A cellulose triacetate TFF has been used to recover *Enterococcus faecalis*, *E. coli*, and *Clostridium perfringens* spores from 100 L surface water samples with a recovery efficiency of 70.5% (Gibson and Schwab 2011). Using TFF with a dead-end (no recirculation of sample) coupled with a culture-based final detection, recovers up to 93% of *Enterococcus feacalis* when 3 log CFU where inoculated into 100 L of tap or surface water when the turbidity was low (0.29 NTU) (Smith and Hill 2009); a higher recovery rate (78%) was seen with higher turbidity (4.3 NTU) (Smith and Hill 2009). TFF, coupled with continuous centrifugation and immuno-magnetic separation followed by qPCR can detect *E. coli* O157:H7 at 50 MPN per 40 L of surface water with a rate of 100% detection (Mull and Hill 2009). Occasionally, studies using TFF obtain >100% recovery efficiencies, this is hypothesized to be due to disaggregation of bacterial clumps during

the filtration process (Smith and Hill 2009). This could also explain the relatively high recovery rate obtained when using TFF as a concentration method.

Potential additional benefit of TFF is the passage of several PCR-inhibiting compounds to the permeate side and thus removed from the sample. TFF using a polysulfone membrane has been combined with conventional PCR to detect human-associated *Bacteroidales* (inoculation from sewage, actual concentrations not given), without problems of PCR inhibition, due to compounds regularly found in either sewage or other waters, from both fresh and estuarine waters (Leskinen et al. 2010). The use of TFF gave the ability to sample larger volumes of water than normal filtration (due to fouling) (Leskinen et al. 2010).

Moore Swabs

In 1948, Moore introduced the use of cotton gauze swabs for isolating *Salmonella* from sewage-contaminated waters. The cotton gauze was used at various locations in the sewage system in a small town in England to trace the source of paratyphoid to a single carrier. More recently, MS have been used to isolate *Salmonella* from surface waters and ornamental fountain waters (Spino 1966, Sears et al. 1984, Thomas et al. in press). In Santiago Chile, MS have been used to isolate *Salmonella* from 11% (8/76) of samples used in rivers and irrigation canals, known to have received raw sewage (Sears et al. 1984). *Salmonella* was isolated from 12.4 % (18/145) of MS used in ornamental fountains in Guadalajara City, Mexico (Escartin et al. 2002). Moore swabs were used in Southern Ontario to isolate *Salmonella* from 78.4% (91/116) river water samples collected from rivers in one watershed which was influenced by numerous recreational and agricultural activities (Thomas et al. in press). When placed in drainage lines, MS have been used to isolate *E. coli* O157:H7 from soil

and irrigation water (Ogden 2001). Moore swabs allow for a higher percentage (44/181 vs. 13/181) of positive samples, compared to 4 L of the same water being passed through a 0.45 µm pore size membrane filter, when used to concentrate *Campylobacter* from river water in Chile (Fernandez et al. 2003). The APHA standard method for qualitative isolation of *Salmonella* from waters uses the MS, which is to be suspended below the surface for one to three days (APHA 1992). Their method states that a longer duration of swab exposure will not increase final isolation efficacy (APHA 1992).

A modification to the MS concept has also been reported. A MS is a rolled cotton gauze tied and suspended in a water sample for a minimum of 24 h, a Modified Moore swab (MMS) uses the same concept of the rolled cotton gauze, but fits it into a pipe. The water sample is pumped through the gauze in the pipe, and the bacteria are trapped within. Modified Moore swabs have been used to isolate *Escherichia coli* O157 and *Salmonella* at levels down to 1 log CFU/100 ml from 10 L of irrigation water when coupled with cultural detection methods (Bisha et al. 2011).

Major advantages of the MS, and MMS, is that they are inexpensive, simple to use, can be used in the field rather than collecting and returning large volumes of water to the laboratory, and require minimal operator prerequisite skill-base and training. The major disadvantage to MS is the inability to give quantitative results (APHA 1992); however, this can be somewhat overcome by pumping a known volume through the MMS.

Immunology Based Methods

Rapid methods for *Salmonella* detection often require one to three days because a pre-enrichment step is still required. Cultural-based enrichment can be substituted by or combined with biospecific purification and concentration procedures, such as

immuno-magnetic separation (IMS), in order to decrease analysis times (Roda et al. 2012). IMS involves the use of polyclonal or monoclonal antibodies bound to paramagnetic particles/beads to bind target molecules or cells and remove them from the sample matrix by use of a magnet (Warren et al. 2007). The beads are paramagnetic, meaning that they only exhibit magnetic properties when exposed to a magnetic field. Without exposure to a magnetic field the beads remain in suspension with the sample. Conventionally, these are referred to as microbeads, however, they are in the nanoparticle size range and are neither round nor bead-like in shape.

The main advantage of immuno-based methods is that they are able to remain highly specific even in complex matrices (Roda et al. 2012), which can include environmental samples. IMS can be used to reduce time for analysis completion, while enhancing selectivity and sensitivity (Notzon et al. 2006). Disadvantages of immuno-based methods include: lower sensitivity than molecular-based detection methods, usually requiring a pre-enrichment step; specificity and sensitivity are largely dependent on the quality of the antibodies; possible difficulties in obtaining high performance antibodies for certain strains; antibody ability can be compromised if changes in expression or denaturation of target antigen occurs; sample matrix can make the antibody unstable; and viable and non-viable cells are not distinguished (Roda et al. 2012). Two commercially available IMS kits are available for *Salmonella* concentration: Dynabeads (Invetrogen, Grand Island, NY, USA), and Pathatrix (Matrix MicroScience, Golden, CO, USA). Both require a sample pre-enrichment, but can replace selective enrichments, thus decreasing total time for sample analysis.

Dynabeads

Dynabeads are produced by Invetrogen (Grand Island, NY, USA) and can accommodate a 5 ml total sample volume. The initial start-up costs are generally low, as only a Dynal BeadRetreaver is required (in addition to consumable supplies).

Dynabeads have been coupled with qPCR to produce an assay capable of detecting *Salmonella* at 10 CFU/25 g from meat samples using only a 6 h pre-enrichment time (total 12 h analysis time) (Notzon et al. 2006). Fat and other food debris can lead to a loss of magnetic beads during procedure (Notzon et al. 2006). *Citrobacter freundii*, *Pseudomonas* spp. and *Proteus* spp. may interfere with *Salmonella* binding to IMS beads (Colman et al. 1995).

Rough forms of *Salmonella* may not bind to Dynabeads, theoretically due to the loss of their O-specific antigen structure (Notzon et al. 2006). Additionally, *Salmonella* Putten, Worthington (serogroup G), Minnesota (L), and Urbana (N) will not bind with Dynabeads (Leon-Velarde et al. 2009), and result in false-negatives when further analysis is completed.

Pathatrix

The Pathatrix system is an IMS method that functions as a flow-through immunocapture; the initial purchase of the required equipment can be rather costly. However, this set-up allows for much larger sample volumes to be analyzed; previously 250 ml sample homogenates have been processed through this system (Warren et al. 2007). In the Pathatrix system the sample is continuously pumped around inside sterile tubing system for a pre-set 30 min time period. During this the sample continuously passes a magnet where the IMS beads are immobilized. Sample debris and other

potentially inhibitory substances and cells are removed during a washing step, which occurs prior to the retrieval of the IMS beads.

Studies using the Pathatrix system to isolate *Salmonella* from surface water, or other water types, have not previously been reported. However, numerous studies have used the Pathatrix system to isolate *Salmonella* from food or environmental samples, including green tomatoes, ground beef, potato salad, poultry meats, and swabs from poultry houses (Warren et al. 2007, Leon-Velarde et al. 2009, Taban and Aytac 2009).

Molecular Methods

The use of molecular detection methods has numerous advantages over cultural techniques. These include: decreased time to results, lower detection limits, as well as the ability to detect VBNC cells. Culture-dependent methods to measure and/or detect *Salmonella* or fecal indicator bacteria require 18 h to five days for results to become available. The use of rapid methods, which include molecular-based methods, reduces the time required for results to become available. This decrease in testing time, could reduce human health risks associated with contaminated food items or use of contaminated water for edible horticultural crop production as risk management decisions could be made in a more timely manner.

When screening water samples for *Salmonella,* there is a risk of underestimating the contamination level when using culture-based methods because bacterial cells may enter viable but non-culturable state (Jacobsen and Bech 2012). The bacteria are still present and able to cause disease, but are not capable of being grown in the laboratory. The true risk level of using the water source would thus be underestimated. The disadvantages to these types of techniques are that they often require specialized

training for laboratory personnel, and typically require costly equipment and supplies. Additionally, molecular-based methods can detect both live and inactivated target organisms (Bae and Wuertz 2012). In these methods a particular molecular target is detected as a means of determining organism presence, it is possible for the DNA of inactivated cells to remain intact enough to be detected by some molecular-based methods (Bae and Wuertz 2012). False-negative results can occur because of the high specificity of these methods, if a mutation in the target organism has caused a mutation in the target molecule negative results will be obtained, when in fact the target organism is present (Malorny et al. 2003).

PCR

The polymerase chain reaction (PCR) is an example of a molecular-based method. PCR allows the specific and exponential synthesis of a targeted DNA region through the use of specifically designed primers (oligonucleotides). PCR often required pre-enrichment prior to *Salmonella* detection (Malorny et al, B. 2003, Gorski, and Liang 2010).

Selection of the pre-enrichment broth much be carefully considered. When comparing TSA, UPB and BPW for use as a pre-enrichment broth prior to PCR detection of *Salmonella* from either lettuce or tomato samples, TSA had the least effect on PCR efficiency (Gorski and Liang 2010). *Salmonella* was not detected in any of the UPB samples in any of the trials; *Salmonella*-positive samples were seen from BPW the target (Gorski and Liang 2010). UPB contains iron (III) and this is a known PCR inhibitor (Gorski and Liang 2010).

The *inv*A gene, which encodes a protein in the Type III secretion system required for invasion of epithelial cells by *Salmonella* during infection, has been used previously

for the detection of *Salmonella* from water samples (Gaertner et al. 2008). The *inv*A gene has been suggested as the international standard to be used for the detection of *Salmonella* (Malorny et al. 2003). Using the proposed standard primers, results in a 284 base pair amplicon. *inv*A primers have previously been used to detect *Salmonella* from lettuce and tomato enrichments without producing detectable amplicons from non-*Salmonella* bacteria (Gorski and Liang 2010). Molecular detection targets must be specific enough not to share DNA sequences with phylogenetically near neighbors. The selection of targets must take into account the fact that the microflora of enrichment culture may affect detection, and that bacterial cultures differ depending on the source of inoculum.

A disadvantage associated with PCR-based detection methods is the inability to distinguish between DNA associated with live bacteria, and DNA remaining from destroyed cells. There are two ways to ameliorate this situation: use reverse-transcriptase PCR (RT-PCR) or quench DNA not inside a non-compromised cell membrane. RT-PCR uses RNA as the target, RNA is less stable than DNA and will not remain around after the cell is dead. Propidium monoazide is a DNA-intercalating dye with an azide group and will penetrate only the membrane of dead cells with compromised cell walls/membranes and then bind their DNA for attach to free DNA. Upon exposure to bright visible light, the photoactive azide group on the dye is converted to a nitrene radical, which is readily attached to a carbon atom of the DNA through a C-H insertion reaction, resulting in an inhibition of amplification of DNA from dead cells or free DNA (Bae and Wuertz 2012).

Real-Time PCR

Real-Time PCR (qPCR) requires less time to results than does conventional PCR, this is because the amount of PCR product formed is measured as the reaction cycles progress. During the PCR reaction cycles, real-time fluorescence data are collected cycle-by-cycle for each individual reaction (Delibato et al. 2011, Balachandran et al. 2011). Positive signals show an increase of the target-specific fluorescence signal while the fluorescence of negative signals remains below the threshold of detection. The reaction remains in one closed tube during the entire PCR amplification and detection, thus significantly reducing the potential for cross-contamination and false-positives (Mafu and Sirois 2009).

While technical expertise is still required, the use of commercial kits can reduce the amount of personnel training required. Using a commercially available kit in which all the reaction components are lyophilized into a bead ready for use in the reaction tube can reduce technical error due to excessive pipetting (Balachandran et al. 2011). The lyophilized format can also allow for a greater volume sample (up to 30 µl) to each reaction tube allowing for increased sensitivity (Balachandran et al. 2011). Additionally, these kits contain an internal positive control that allows for monitoring for the presence of PCR inhibitors, which reduces the occurrence of false-negatives. Detection from environmental samples can often be problematic because of the presence of PCR inhibitors (e.g., humic acid, bacterial debris, complex polysaccharides, and metal ions) in qPCR assays; it is imperative to have an internal positive control in each reaction tube (Gibson and Schwab 2011).

In previous studies to validate the MicroSEQ real-time PCR kits 45 samples for each of 10 food matrixes (dry dog food, ground black pepper, raw ground beef, raw

chicken wings, shrimp, cantaloupe, brie cheese, dry infant formula, chocolate, or shell eggs) were spiked with *Salmonella* (Anatum, Newport, Typhimurium, Saintpaul, Infantis, Senftenberg, Enteritidis, Paratyphi A, or Poona) and enriched in BPW, with no false-negatives being reported (Balachandran et al. 2011). Additional work in the same study showed that this real time PCR kit was able to detect 100 different *Salmonella* serovars at a 3 log CFU/ml limit of detection; when screening for exclusivity 30 non-*Salmonella* organisms were tested and only *Serratia marcescens* created a false-positive result (Balachandran et al. 2011). *Citrobacter freundii*, which is commonly found giving false-positives in cultural based *Salmonella* detection methods (Schonenbruecher et al. 2008), did not generate a positive response (Balachandran et al. 2011).

Real-time PCR has been shown to correlate with standard culture methods when at least 3 log CFU/ml are present in the samples; when *Salmonella* counts are lower, qPCR is more sensitive (Mafu and Sirois 2009). It is thus possible to decrease the limit of detection in an experiment via the use of qPCR rather than cultural methods.

Multiplex PCR

Multiplex PCR consists of multiple pairs of primers being used in one PCR reaction in order to target more than one gene. The genes can be from multiple organisms, such as those multiplex PCRs used to detect numerous pathogens in one reaction, or the genes can be from one organism and be used to differentiate between subspecies, such as the multiplex PCR reported by Kim et al. (2006) for the serotyping of *Salmonella*.

Kim et al. (2006) has suggested a multiplex PCR method for serotyping *Salmonella*; this method consists of two five-plex PCR reactions and one two-plex PCR reaction. The entire method is based upon six genetic loci from *S. enterica* serovar

Typhimurium and four loci from *S. enterica* serovar Typhi. This method is reported to be sensitive, reproducible, and cost-effective (Jean-Gilles Beaubrun et al. 2012). The Kim et al. (2006) multiplex PCR is designed to be able to identify the 30 most common serovars of clinical importance.

The same Kim et al. (2006) multiplex PCR was confirmed against 30 serovars from an FDA reference collection, using two or three isolates per serovar; an exact band pattern match to the patterns reported by Kim et al. (2006) was found for 29 of the serovars reported (Jean-Gilles Beaubrun et al. 2012). *Salmonella* Oranienburg could not be matched. The same study noted serotypic variations in at least one of the isolates tested from the following serovars: Montevideo, Newport, Muenchen, Hadar, Infantis, Paratyphi, Derby, and Berta. Banding pattern variations included missing expected genes or amplification of serovar-specific PCR products not typical for their serotypic banding pattern (Jean-Gilles Beaubrun et al. 2012). The authors suggest that these maybe results of SNPs, insertions, or deletions at the gene loci.

When using the same Kim et al. (2006) multiplex PCR reactions to serovar isolates from tomato farms, in the Mid-Atlantic region of Virginia, samplings the multiplex banding patterns were not able to type 23.8% (15/63) of their isolates (Jean-Gilles Beaubrun et al. 2012). The 15 isolates that could not be typed were not completely reported, however, the authors do mention they were outside of the 30 most common serovars and give the examples of *S*. Lille and *S*. Tennessee (Jean-Gilles Beaubrun et al. 2012). Of the 48 isolates from the tomato farm samplings, the typable serovars included multiple isolates of: *S*. Typhimurium, *S*. Newport, *S*. Javiana, *S*. Thompson, and *S*. Braenderup (Jean-Gilles Beaubrun et al. 2012). These authors also

report some isolates from food-associated outbreaks had different banding patterns than those reported by Kim et al. (2006); these included: *S*. Montevideo, *S*. Seftenberg, and *S*. Tennessee (Jean-Gilles Beaubrun et al. 2012).

The use of a multiplex PCR for determining serovar for *Salmonella* has the advantage of decreased time requirement and decreased labor costs, as fewer steps are required.

Risk Analysis

Previous attempts to assess the human health risk for surface waters contaminated with *Salmonella* have been hampered by a lack of quantitative data, such as concentration, as well as large enough sample volumes to be truly representative samples. It is currently unknown what is an acceptable microbial quality for waters that are to be used on edible horticultural crops. Many variables surround such a knowledge gap. For example, should one water quality standard be applied to all waters to be used on edible horticultural crops, or are separate standards for irrigation, frost-protection, and foliar applications, more appropriate? Do various modes of irrigation necessitate different microbial water quality standards? Furthermore, it has previously been suggested that a 'zero tolerance' for pathogens in the pre-harvest setting in not realistic (Doyle and Erickson 2012). This is mostly due to the understanding that there exists a widespread distribution of foodborne pathogens in the farm environment (Doyle and Erickson 2012). This then raises yet another question, if 'zero tolerance' is not attainable then what is an acceptable level of risk, or even can there be an acceptable level of risk? Currently the Food Safety Modernization Act (FSMA) has directed the US FDA to establish science-based minimum standards for the production of fruits and vegetables that may be consumed raw, so that the standards would minimize risks of

foodborne disease (Harris et al. 2012). Agricultural waters can act as sources for contamination of fruits and vegetables, so the risk analysis type questions posed here are in imminent need of investigating.

In 1989, the US EPA set a goal that all water from surface sources should not pose a risk of infection from waterborne pathogens greater than 1:10,000 per year (Stine et al. 2005). With this as a set goal, a risk assessment was performed by Stine et al. (2005) to determine the microbial water quality required for irrigation water to obtain the same rate of infection from fresh produce consumption, specifically looking at *Salmonella*. The horticultural items considered included: cantaloupe, iceberg lettuce, and bell peppers. These authors determined that an annual infection risk of 1:10,000 could result from furrow irrigation waters containing *Salmonella* at levels as low as 2.5 CFU/100 ml when harvest occurred the day after irrigation (representing a worst case scenario) (Stine et al. 2005).

A greater understanding of risk associated with the use of surface waters on edible horticultural crops could be established if larger, more representative, samples could be quantitatively analyzed. To ease the labor-burden on producers, indicator organisms or physicochemical indicators of *Salmonella* presence should be established.

Of additional importance is an understanding of which serovars of *Salmonella* are predominating in surface waters relevant to edible horticultural crop production, such as the surface waters of Central Florida, and the duration of the *Salmonella* survival once contaminating the surface water.

Table 2-1. *Salmonella* isolated from surface waters

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Anatum	Fresh	Georgia	Meandering streams in one watershed	333 ml	1/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Bareilly	Fresh	Georgia	Weather pools	Moore swab 5 days in river	29/100	Enrichment in peptone, then TT broth	(Thomas et al. in press)
Bareilly	Fresh	Georgia	Meandering streams in one watershed	333 ml	6/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Blockley	Fresh	North Dakota	River, ca. 22 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 1/6 and 0/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Blockley	Marine	Southwestern Morocco	Coastal, Tourism and shellfish	100 ml	1/243 samplings	ISO 6579-1993	(Setti et al. 2009)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Braenderup	Fresh	North Dakota	River, ca.7, 12 and 22 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 3/6 and 0/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5˚C	(Spino 1966)
Braenderup	Fresh	Mid-Atlantic Region	Tomato farms, pond sediment	250 ml	1/39	0.45µm filter, lactose broth, XLT4 and R & F *Salmonella* Chromogenic plating medium	(Micallef et al. 2012)
Braenderup	Fresh	Georgia	Meandering streams in one watershed	333 ml	12/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Braenderup	Fresh	North Florida	Suwannee River	1,983 ml	2/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Brandenburg	Fresh	North Carolina	River, Crop agriculture area	25 ml	6/12 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Chester	Fresh	North Dakota	River, ca. 7, 12 and 32 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 0/6, and 3/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5˚C	(Spino 1966)
Cubana	Fresh	North Florida	Suwannee River	1,983 ml	1/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Enteritidis	Fresh	North Dakota	River, ca. 12, 32, and 62 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 0/6, and 3/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Gaminara	Fresh	North Carolina	River, forest area	25 ml	16/28 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Gaminara	Fresh	North Carolina	River, crop agriculture area	25 ml	6/12 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Gaminara	Fresh	Georgia	Meandering streams in one watershed	333 ml	3/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Give	Fresh	North Carolina	River, forest area	25 ml	16/28 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Heidelberg	Fresh	North Dakota	River, ca. 12 and 62 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 2/6 and 1/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
I 4,[5]:b	Fresh	Georgia	Meandering streams in one watershed	333 ml	4/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
I 47:z4z23	Fresh	Georgia	Meandering streams in one watershed	333 ml	1/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Infantis	Fresh	North Dakota	River, ca. 13 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 1/6 and 0/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Inverness	Fresh	North Carolina	River, forest area	25 ml	16/28 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Inverness	Fresh	North Carolina	River, Crop agriculture area	25 ml	6/12 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Inverness	Fresh	North Florida	Suwannee River	1,983 ml	9/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Ituri	Fresh	Democratic Republic of the Congo, Ituri region	Duck feces (normal adult)	Not reported	Not reported	Not reported	(Kauffmann and Fain 1953)
Javiana	Fresh	Mid-Atlantic Region	Tomato farms, Pond sediment	250 ml	2/39	0.45µm filter, lactose broth, XLT4 and R & F *Salmonella* Chromogenic Plating Medium	(Micallef et al. 2012)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Johannesburg	Fresh	North Florida	Suwannee River	1,983 ml	1/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Kasenyi	Fresh	Democratic Republic of the Congo, Ituri region	Duck feces (normal adult)	Not reported	Not reported	Not reported	(Kauffmann and Fain 1953)
Kentucky	Fresh	North Dakota	River, ca. 0 and 12 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	2/3, 0/6, and 0/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Lille	Fresh	Mid-Atlantic Region	Tomato farms, irrigation ditch	250 ml	1/39	0.45µm filter, lactose broth, XLT4 and R & F *Salmonella* Chromogenic Plating Medium	(Micallef et al. 2012)
Liverpool	Fresh	Georgia	Meandering streams in one watershed	333 ml	6/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Miami	Fresh	North Carolina	River, Crop agriculture area	25 ml	6/12 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Mikawasima	Fresh	Georgia	Meandering streams in one watershed	333 ml	12/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Montevideo	Fresh	Georgia	Meandering streams in one watershed	333 ml	1/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Montevideo	Fresh	North Florida	Suwannee River	1983 ml	1/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Muenchen	Fresh	North Carolina	River, forest area	25 ml	16/28 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Muenchen	Fresh	Georgia	Meandering streams in one watershed	333 ml	28/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Muenchen	Fresh	North Florida	Suwannee River	1,983 ml	3/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Newport	Fresh	North Carolina	River, Crop agriculture area	25 ml	6/12 samplings	Buffered peptone water, RV broth, XLT4	(Patchanee et al. 2010)
Newport	Fresh	Mid-Atlantic Region	Tomato farms, pond water	250 ml	4/39	0.45µm filter, lactose broth, XLT4 and R & F *Salmonella* Chromogenic plating medium	(Micallef et al. 2012)
Newport	Fresh	North Florida	Suwannee River	1,983 ml	1/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)
Not determined	Fresh	Zimbabwe	Nile crocodiles, concrete pond, 29 to 33°C		4.6 log CFU/ 100 ml (DPC) 2.6 log 100 ml (MPN)	Spread plate to XLD, slide agglutination and biochem rxn MPN starting with RV	(Madsen 1994)
Not determined	Marine	San Sebastian, Spain	Bathing beaches	Not reported	0.8, 4.2, and 8.5% at each of three sites sample number not reported	ISO 6579 (buffered peptone – RVS & MKTTn)	(Serrano et al. 1998)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Oranienburg	Fresh	North Dakota	River, ca. 7 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 0/6, and 1/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Reading	Fresh	North Dakota	River, ca. 7, 12 and 13 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 4/6 and 0/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Rubislaw	Fresh	Georgia	Meandering streams in one watershed	333 ml	26/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Rubislaw	Fresh	North Florida	Suwannee River	1983 ml	6/30 isolates	5x3 MPN with buffered peptone water enrichment, TT selective enrichment, XLD agar	(Rajabi et al. 2011)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
S. enterica subsp. *arizonae*	Fresh	Georgia	Meandering streams in one watershed	333 ml	109/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Saintpaul	Fresh	North Dakota	River, ca. 7, 12, 22, 32, 62, and 73 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	1/3, 0/6 and 5/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Saintpaul	Fresh	Georgia	Meandering streams in one watershed	333 ml	9/197 isolates from 72 samples	5x3 MPN with buffered peptone water enrichment, RV selective enrichment, XLD agar	(Haley et al. 2009)
Senftenberg	Marine	Northwestern Spain	Ria	25 ml water		ISO 6579	(Martinez-Urtaza et al. 2004)
Senftenberg	Marine	Southwestern Morocco	Coastal, Tourism and shellfish	100 ml	9/243 samplings	ISO 6579-1993	(Setti et al. 2009)
Tennessee	Fresh	Mid-Atlantic Region	Tomato farms, irrigation ditch	250 ml	1/39	0.45µm filter, lactose broth, XLT4 and R & F *Salmonella* Chromogenic plating medium	(Micallef et al. 2012)

Table 2-1. Continued

Serovar	Water Type	Source Area	Environmental conditions	Volume	Concentration	Detection or isolation method	Reference
Thompson	Fresh	North Dakota	River, ca. 7, 32, 62 and 73 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 0/6, and 4/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)
Typhimurium	Fresh	North Dakota	River, ca. 7 and 12 miles downstream from sewage treatment plant	Moore swab 5 days in river, just below surface	0/3, 1/6 and 1/5 sampling locations	Moore swab held in water 5 days, tetrathionate or SBG-sulfa enrichment broth to Brilliant Green Agar, all incubated at 41.5°C	(Spino 1966)

CHAPTER 3
DETECTION OF *SALMONELLA* SPP. FROM LARGE VOLUMES OF WATER BY MODIFIED MOORE SWABS AND TANGENTIAL FLOW FILTRATION

Reliable screening of fresh produce for the presence of foodborne pathogens, including *Salmonella*, is difficult for multiple reasons. Challenges include collecting representative but manageable volumes of water for analysis, how and where to collect representative samples, and the time required for culture-based conformations of pathogens (i.e., *Salmonella*, up to seven days; US FDA 1998). This interval may represent a significant portion of the shelf life of often highly perishable commodities, making test and hold screening financially and logistically untenable. Broadly viewed, the occurrence of pathogen contamination on produce items has been determined to be sporadic and heterogeneously distributed (Kase et al. 2012), rendering even a single composite sample an unreliable representation of the entire lot.

Testing pre-harvest water for *Salmonella* or *E. coli* O157:H7 that comes into contact with, or is in close proximity to, produce production may be one component of an effective strategy to implement a risk-based approach to enhance produce safety. Large volume water samples can be concentrated and screened pre-season and pre-harvest, and may provide information for management decisions to reduce the risk of initial contamination. Current microbial water quality standards in the US produce industry rely on testing 100 ml of water for generic *E. coli* (F-DACS 2007, LGMA 2012) rather than pathogen populations. It is likely that pathogen contamination of surface water occurs at low rates (Madsen 1994, Rhodes and Kator 1988, Escartin et al. 2002, Haley et al. 2009, Ijabadeniyi et al. 2011); large volume samples may be required to provide a more realistic representation of the risk of common pathogen presence in the body of water at levels relevant to the mode of application.

Various filtration methods can be used to concentrate large-volumes of water. Normal flow filtration (NFF) is the standard water testing method of the American Public Health Association (APHA, APHA 1992), where 100 ml is passed through a sterile filter, and the filter applied directly to an agar plate for enumeration of coliforms. Alternative filtration methods include Tangential Flow Filtration (TFF) where water flows across a filter membrane, Moore swabs (MS), where rolled cotton gauze is tied and allowed to sit in a body of water for at least 24 h, and Modified Moore swabs (MMS) where pieces of pipe are filled with rolled cotton gauze which traps bacteria as water is pushed through the pipe. Previously, up to 100 L of surface water has been concentrated using TFF (Mull and Hill 2009, Gibson and Schwab 2011) and MS have been used to isolate *Salmonella* (Spino 1966, Sears et al. 1984, Escartin et al. 2002), *E. coli* O157 (Ogden 2011), *Campylobacter* (Fernandez et al. 2003), and *Vibrio cholera* (Barrett et al. 1980). MMS have been used to isolate *E. coli* O157 and *Salmonella* (Bisha et al. 2011).

Screening for the presence of pathogens is preferred to enumeration of indicator organisms in surface waters as no strong correlations between the presence and/or concentration of pathogens and indicator or index organisms have consistently been documented (Burton et al. 1987, Chandran and Hatha 2005, Pachepsky et al. 2011). The use of PCR-based methods for foodborne pathogen detection reduces testing times compared to cultural-based methods (Mafu and Sirois 2009). Real-Time PCR (qPCR) has the added advantage of increased speed, sensitivity and minimizing post-PCR contamination (Balachandran et al. 2011; Delibato et al. 2011).

The objectives of this study were to compare i) concentration and detection of *Salmonella* in large volumes (10 L) of surface water by TFF and NFF and ii) concentration of *Salmonella* and *E. coli* O157:H7 by TFF and MMS.

Materials and Methods

Bacterial Strains and Preparation of Inoculum

Two different *Salmonella* cocktails were used in the experiments. NFF and TFF optimization was done using *Salmonella* serovars Saintpaul (BAC 133); Newport (C4.2); Anatum (LRB6802); Montevideo (LJH0654) and Typhimurium (LJH0738) resistant to naladixic acid (50 mg/l). For the comparison of MMS and TFF identical strains to Bisha et al. (2011) were used; including *Salmonella* serovars Montevideo (MDD 22 pGFP), Poona (MDD 237 pDsRed) and Newport (MDD 314 pAMCyan) and three *Escherichia coli* O157:H7 stains (PVTS 016 pGFP, PVTS 087 pDsRed, and PVTS 088 pAMCyan) all transformed to express a differential fluorescent protein moiety and selectable ampicillin resistance at 20 mg/l, as previously described (Bisha et al. 2011). All isolates are available from the culture collection of Dr. Michelle D. Danyluk.

Frozen stocks, stored at -80°C in Tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) with 15% glycerol, were streaked onto TSAN or 20 mg/l ampicillin (TSAA; Sigma), and incubated 24±2 h at 35±2°C. An isolated colony was transferred into TSB supplemented with nalidixic acid (TSBN) or ampicillin (TSBA), and incubated 24±2 h at 35±2°C. A 10 µl loop of the culture was transferred to TSBN or TSBA and incubated 24±2 h at 35±2°C. Cultures were combined in equal parts and diluted in 0.1% peptone water (Difco, Becton Dickinson). Following addition to the 10 L water sample, the inoculum was distributed through the water by swirling in a 10 L carboy in a 30 cm circle for 30 s.

Inoculum concentrations were determined by serial dilutions in 0.1% peptone water, plating onto TSAN or TSAA and incubation 24±2 h at 35±2°C. Target concentrations were 10^0 – 10^3 CFU/L for NFF and TFF optimization, 10^2 – 10^5 CFU/L for MMS and TFF comparison, and 10^0 CFU/L for MMS validation.

Collection of Water Samples

For NFF and TFF optimization and MMS validation, surface water (10 L) was collected from Lake Swoope, in the town of Lake Alfred, FL, USA and stored for no longer than 24 h at 4°C prior to use. For MMS and TFF comparison 10 L of non-sterile 0.85% saline was used to emulate the methodology of Bisha et al. (2011).

Normal Filtration

Vacuum filtration was used to attempt passage of 10 L of water, through a 0.45 µm pore-size filter (Millipore, Billerica, MA, USA). Whatman Grade 41(Fisher Scientific, New Jersey, USA) filter paper and diatomaceous earth (1 g; Fisher Scientific) were evaluated separately as filtration aids.

Tangential Flow Filtration

The tangential flow filter used was a Mini Kros Plus Tangential Flow Filter Module (Spectrum Labs, Rancho Dominguez, CA, USA) composed of polyethersulfone with a nominal pore rating of 0.2 µm and a surface area of 1,050 cm^2. Filter and tubing sets were sterilized prior to each experiment. All tubing was L/S 17, constructed of silicone with platinum coating (Cole-Palmer, Vernon Hills, IL, USA). A KrosFlo Research II Pump was used in combination with Masterflex easy-load pump head (Spectrum Labs). The inlet flow rate was standardized at 1,000 ml/min. The TFF was run at a trans-membrane pressure of 67 Pa. The initial 10 L of water was concentrated to a final retentate volume of ca. 250 ml.

Modified Moore Swabs

The MMS was constructed out of PVC pipe (12 by 4.4 cm diameter; Home Depot, Winter Haven, FL) with 2.54 cm PCV connector to a 2.54 cm male-to-male coupler on each end. Inside the PVC pipe, was pre-sterilized 80 by 22 cm cheesecloth (Fisher Scientific; Grade #90) folded lengthwise and rolled. The 10 L water sample was passed through the MMS at a flow rate of 300 ml/min. The use of MMS here was introduced as a proof of concept of another concentration means for pathogens, *Salmonella* and *E. coli* O157:H7, from water; further experiments were not performed using *E. coli* O145:H7.

Conventional *Salmonella* Detection from NFF and TFF

A modified conventional *Salmonella* isolation method (US FDA 1998) was used. For NFF, the finished filter was placed into 50 ml lactose broth (Difco, Becton Dickinson), and for TFF, a 1:1 volume of double strength lactose broth was added to the retentate. The inoculated lactose broth was allowed to incubate for 1 h at room temperature, before addition of 50 µg/l nalidixic acid or 20 µg/l of ampicillin and incubation for 24±2 h at 35±2°C. A 1 ml aliquot of the incubated lactose pre-enrichment was transferred to tetrathionate broth (TT broth; Difco, Becton Dickinson) and Rappaport Vassiliadis R10 broth (RV broth; Difco, Becton Dickinson) and incubated at 35±2°C for 24±2 h and 41±2°C for 48±2 h, respectively. Following incubation, 10 µl was streaked onto xylose lysine tergitol-4 agar (XLT4; Difco, Becton Dickinson) and Chromagar *Salmonella* Plus (DRG International Inc, Mountainside, NJ, USA). Both media were incubated for 24±2 h at 35±2°C; colonies were confirmed on lysine iron agar slants (LIA; Difco, Becton Dickinson) and triple sugar iron agar slants (TSI; Difco, Becton Dickinson).

Immuno-magnetic Separation of *Salmonella* during TFF Optimization.

Salmonella was captured on antibody-coated paramagnetic beads using either Pathatrix (Matrix MicroScience, Golden, CO, USA) or Dynabeads (Invitrogen, Grand Island, New York, USA) microspheres. The entire lactose broth pre-enrichment was used in the Pathatrix by replacing the 100 ml conical tube that came with the purchased set up, with a sterile plastic stomacher bag. Either Pathatrix or Dynabeads (50 µl) were added and used according to the manufacturers' instructions on the pre-set 30 min circulation cycle. Following IMS, beads (10 µl, each) were plated in duplicate onto XLT4 and Chromagar *Salmonella* Plus; colonies were confirmed on LIA and TSI as described above.

Detection of *Salmonella* During TFF Optimization

DNA was extracted from the remaining IMS beads (50 µl) using the MoBio UltraClean DNA kit (MoBio, Carlsbad, CA, USA). DNA was used to detect *Salmonella* by PCR (*invA* and *oriC* genes) or by qPCR, using Applied Biosystems' MicroSEQ *Salmonella* spp. Detection Kit (Applied Biosystems, Carlsbad, CA, USA) according to manufacturer's instructions in a BioRad CFX96 RealTime thermocycler.

The *invA* and *oriC* primer sets were as described previously by Malorny et al. (2003). The conventional PCR reaction was optimized using overnight cultures of the *Salmonella* cocktail used in the TFF optimization approach. All reagents were obtained from the Fisher exACTGene Complete PCR kit (Fisher Scientific). Primers were used at a concentration of 20 µM. The PCR reaction mix was as follows: 34.75 µl water; 5 µl 10X PCR buffer; 1 µl of each primer; 0.25 µl Taq DNA polymerase; 5 µl template DNA. The optimized PCR conditions were: 3 min at 94°C melting, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C; a final elongation of 5 min at 72°C. Gel

electrophoresis on a 1.8% agarose gel with 0.5X TBE buffer. DNA was stained with ethidium bromide (1 µg/ml), and amplicons were visualized and imaged under UV light (MultiDoc-It Digital Imaging System; UVP, Upland, CA, USA). PCR amplicon size was determined by comparison to molecular size markers (Hyperladder V; Bioline USA Inc., Randolph, MA, USA).

Detection of Pathogens from MMS

Following MMS filtration, the cheesecloth was removed and placed in a stomacher bag. Excess liquid (ca. 25-35 ml) was wrung out of the cheesecloth and passed through a 0.45 µm pore-size, 47 mm diameter filter using NFF vacuum filtration. The filter was placed on TSAA and incubated for 24±2 h at 35±2°C. Colonies were checked for fluorescence, and confirmed by REVEAL *Salmonella* or *E. coli* O157:H7 lateral flow device (Neogen Corp., Lansing, MI, USA).

Validation of MMS Concentration

MMS and TFF were used to concentrate a 10^0 CFU/L *Salmonella* spiked 10 L water sample, and retentates pre-enriched in lactose broth. The entire pre-enrichment was used in the Pathatrix system with the Pathatrix beads (50 µl); beads (10 µl) were plated to XLT4 and Chromagar *Salmonella* Plus and typical colonies confirmed. Remaining beads (40 µl) were used in DNA extraction and qPCR was performed, all as described above.

Statistical Analysis

Experiments evaluating TFF and detection methods were repeated six times for each dilution reported. Experiments comparing MMS to TFF were repeated in triplicate. Data was analyzed using McNemar's test using GraphPad.

Results

Results of the concentration and detection of *Salmonella* by TFF for surface water samples spiked with the five-strain cocktail of *Salmonella* at varying concentrations are shown in Table 3-1. Detection of 1 CFU/L in 10 L was consistently obtained within two days when using the TFF-immuno capture-Real Time-PCR method. The use of the modified conventional FDA-BAM method following the TFF concentration and overnight pre-enrichment only detected *Salmonella* in 5 out of 6 water samples when spiked at 1 CFU/L in 10 L. Taking the immuno-magnetic beads straight from the Pathatrix and spread plating them onto Chromagar *Salmonella* Plus or XLT4 agar was not able to consistently detect *Salmonella* at spiked levels below 76 CFU/L in 10 L of surface water (Table 3-1). Inconsistent rates of detection were achieved when taking the immuno-magnetic beads to conventional PCR. Switching to the more convenient DynaBeads brand of immuno-magnetic beads also gave inconsistent rates of detection at all concentrations of *Salmonella* attempted. No *Salmonella* was detected when a 0.250 ml aliquot of the permeate was spread plate to TSAN and incubated overnight, ensuring that the filter had not unknowingly ruptured during usage. *Salmonella* detection in the permeate would indicate a failure of the TFF and that the bacteria were not being concentrated.

The 10 L surface water sample was not able to completely pass through the NFF filter. Approximately 500 ml (data not shown) could be passed through the filter before the filter became irreversibly clogged. The addition of filter-aids (Whatman Grade 41 filter paper or diatomaceous earth) did not significantly improve the volume of water filtered prior to irreversible clogging. No further efforts were made to concentrate 10 L surface water samples by NFF.

A comparison of MMS to TFF using non-sterile saline as the starting medium and detecting the pathogen by capturing bacteria from the respective retentates on to a filter membrane and growing overnight on TSA-amp is shown in Table 3-2. MMS is consistently able to detect *E. coli* O157:H7, while the TFF results were more variable, with only the highest concentration (ca. 100,000 CFU/L in 10 L) of *E. coli* O157:H7 detected in all samples. *Salmonella* was detected by TFF in samples containing ≥10,000 CFU/L in 10 L, and in none of the samples at the lowest (>100 CFU/L in 10 L) concentration of *Salmonella.* Only at the highest concentration (>100,000 CFU/L in 10 L) of *Salmonella* was the MMS concentration able to detect *Salmonella* in all the samples.

Due to the poor detection of *Salmonella* at levels below ≥10,000 CFU/L in 10 L in work comparing MMS to TFF, a final set of experiments to compare these concentration protocols using *Salmonella* detection methods described in TFF optimization were carried out. When surface water was inoculated with the *Salmonella* fluorescence cocktail at 2 CFU/L, concentrated using either the MMS or TFF, and coupled with the modified conventional FDA-BAM culture method, immuno-magnetic separation using the Pathatrix beads followed by culture methods or Real Time-PCR, *Salmonella* was detected from all samples (n=6, data not shown). Using more rigorous *Salmonella* detection methods, both MMS and TFF concentration protocols perform equally well in detecting low levels of *Salmonella* (<10 CFU/L) from spiked surface water samples.

Discussion

The public health impact of the use of recreational water quality standards based on testing 100 ml water used in produce production is poorly established. Poor correlation between pathogens presence/absence and indicator organisms (Rhodes

and Kator 1988, Bae and Wuertz 2012) makes it is advantageous to screen for specific pathogens in agricultural waters, rather than enumerating indicator organisms. Screening for the presence of pathogens is a preferential practice over the enumeration of indicator organisms because of differing survival times and population sizes that have been observed to occur in environmental waters (Burton et al. 1987, Chandran and Hatha 2005).

Reproducible detection of *Salmonella* in 10 L of surface water was accomplished within two days using the TFF or MMS concentration, pre enrichment in lactose broth, further concentration by immunocapture and detection by qPCR methods described. Levels of as low as 1 CFU/L in 10 L were reliably detected in spiked surface waters. *Salmonella* populations at levels below 1 CFU/L may be below a produce safety significance threshold level, thus the detection limit described here are believed to be low enough to meet all practical screening purposes for surface waters used in the produce industry. The proposed method detected *Salmonella* more consistently at low levels than the culture-alone based methods of screening for *Salmonella*; this may result fewer false-negative reports when *Salmonella* is present at low levels. An additional benefit of the proposed method is that only two days are required for completion, whereas the current standard culture based methods for *Salmonella* testing requires up to seven days (US-FDA 1998)

The use of NFF for 10 L of the Florida lake surface water was not possible, as the filter irreversibly clogged prior to the sample passing completely through. While NFF is part of the APHA standard methods for screening surface for various pathogens (APHA 1992), the recommended volume for screening is 100 ml. Screening higher

volumes of water may be necessary due the likelihood of pathogens being present at low levels, if and when present (Rhodes and Kator 1988, Madsen 1994, Escartin et al. 2002, Haley et al. 2009, Ijabadeniyi et al. 2011).

TFF has been successfully used to screen large volumes of surface water for the presence of specific pathogens. A major advantage of TFF over NFF is the flowing retentate removes the filter cake during the run; this increases the time before the filter clogs allowing greater volumes of water to be processed. Using 100 L surface water, a recovery efficiency rate of 70.5% for *E. coli* (starting concentration 10 to 100 CFU/L) coupled with a culture-based assay has previously been reported (Gibson and Schwab 2011). Others have used TFF, coupled with continuous centrifugation and immuno-magnetic separation followed by qPCR to detect *E. coli* O157:H7 at 50 MPN per 40 L of surface water with a rate of 100% detection (Mull and Hill 2009). A hollow-fiber ultrafiltration (similar to TFF, but with a dead-end instead of recirculation) method is capable of recovering up to 93% when 3 log CFU of *Enterococcus faecalis* were inoculated into 100 L of tap or surface water when the turbidity was low (0.29 NTU) (Smith and Hill 2009). With higher turbidity (4.3 NTU), the authors found a 78% recovery rate for the same starting concentration of *E. faecalis* (Smith and Hill 2009).

The use of MS is common in water screening (Rhodes and Kator 1998, Madsen 1994, Escartin et al. 2002, Haley et al. 2009, Ijabadeniyi et al. 2011). MS have previously been used to isolate *Salmonella* from surface (Spino 1966, Sears et al. 1984) and fountain water (Escartin et al. 2002), *E. coli* O157 from soil and field water (Ogden 2001), *Campylobacter* from river water (Fernandez et al. 2003), and *Vibrio cholera* from sewage (Barrett et al. 1980). In these examples, the MS was secured it in

the body of water for a duration of time, rather than a specified volume of water run through the swab. Previous studies evaluating agricultural water, and pumping water through MMS demonstrate concentration abilities of MMS. Starting concentrations of 100 CFU/L of either *E. coli* O157:H7 or *Salmonella* were detected when either MMS or continuous flow centrifugation was used to concentrate 10 L starting volume of irrigation water (Bisha et al. 2011). The authors do not state whether they attempted the detection of lower levels. However, as the concentration step was followed by a culture-based method, without enrichment, rather than enrichment followed by PCR-bases methods, the limit of detection may be improved.

MMS was better at concentrating lower levels of both *Salmonella* and *E. coli* O157:H7, than the TFF from saline and detecting by the same culture-based method described by Bisha et al. (2011). This decrease in TFF performance may be due to the use of a culture-based method for detection in that the cells may be damaged from circulating in the TFF system. Using a subsequent immuno-magnetic concentration following pre-enrichment of *Salmonella*, and qPCR to detect the pathogen, both methods of concentration, MMS and TFF, performed equally in detecting *Salmonella* at 2 CFU/L in 10 L of surface water. Replacing the TFF with a MMS does not appear to alter the detection of *Salmonella* spp. in the optimized method. The use of MMS as the concentration method may allow for additional advantages to the produce industry in that MMS are inexpensive, simple to use, can be used in the field rather than collecting and returning large volumes of water them to the laboratory, and require minimal personnel training prior to use.

The method described here for screening large volumes of surface water enables larger, more representative, samples to be screened for *Salmonella* presence. Detection of *Salmonella* within two days following sampling is advantageous in determining food safety risks associated with surface waters and this may be advantageous for the produce industry in avoiding pre-harvest contamination.

Table 3-1. Percent *Salmonella* positive samples of 10 L inoculated surface water, concentrated using TFF and pre-enriched in lactose broth evaluated using different detection techniques (n = 6).

Inoculum Concentration (CFU/L)	Detection Technique[a]						
	Culture	DB/qPCR	DB/PCR	DB/Culture	PTX/Culture	PTX/qPCR	PTX/PCR
1	83%	100%	17%	33%	67%	100%	50%
7.5	100%	100%	33%	33%	67%	100%	17%
76	100%	83%	67%	100%	100%	100%	50%
760	100%	100%	67%	83%	100%	100%	33%
McNemar P value	0.0736	0.0736	0.0009	0.0077	0.0077	Control	0.0003

[a] Detection techniques included: Culture, TT broth and RV broth enrichment, streaking onto XLT4 and Chromagar *Salmonella* Plus; DB/qPCR, DynaBeads for IMS, DNA extraction, and detection by real time PCR (Ct < 35); DB/PCR, DynaBeads for IMS, DNA extraction, detection by conventional PCR (band on agarose gel at expected size; Malorny et al. 2003); DB/Culture, DynaBeads for IMS, beads spread onto XLT4 and Chromagar *Salmonella* Plus; PXT/Culture, Pathatrix used for IMS, beads spread to XLT4 and Chromagar *Salmonella* Plus; PTX/qPCR, Pathatrix beads for IMS, DNA extraction, detection by real time PCR; PXT/PCR, Pathatrix beads for IMS, DNA extraction, detection by conventional PCR.

Table 3-2. Percent *Salmonella* positive samples of 10 L inoculated non sterile saline, pre-concentrated by TFF or MMS and detected by cultural means[a] (n = 3).

Pathogen	Inoculum Concentration (CFU/L)	MMS	TFF
Salmonella spp.	137	67%	0%
	1,366	67%	33%
	13,660	67%	100%
	136,604	100%	100%
E. coli O157:H7	86	100%	33%
	865	67%	67%
	8,652	100%	33%
	86,524	100%	100%

[a]Detection by cultural means involved the liquid retentate being passed through a normal filter, 0.45 µm pore-size, 47 mm diameter, the filter placed on TSAA and incubated for 24±2 h at 35±2°C A positive sample is noted by the presence of a fluorescing colony that also gave positive results from the appropriate REVEAL test.

CHAPTER 4
PREDICTING *SALMONELLA* POPULATIONS FROM BIOLOGICAL, CHEMICAL, AND PHYSICAL INDICATORS IN FLORIDA SURFACE WATER

Water quality can be characterized by three types of analysis; index microorganisms, indicator microorganisms, and other physicochemical water characteristics. Indicator microorganisms are used to suggest or "indicate" the possible presence of fecal contamination (Smith and Schaffner 2004). Index organisms represent the presence and behavior of a pathogen in a given environment (Mossel et al. 1995); one organism can be both an index and an indicator organism. Physicochemical water characteristics include turbidity, temperature, pH, or oxidation-reduction potential (ORP) (APHA 1992). Physicochemical measurements have the advantage of being considerably more rapid than microbial-based measurements, but such measurements may not correlate with microbiological quality.

The US produce industry currently relies on testing 100 ml of water for indicator organisms, specifically, generic *E. coli*, as a means to monitor water quality used for edible horticultural crop production (LGMA 2012, F-DACS 2007). There are a wide variety of bacteria, both groups and species, viruses, and bacteriophages that have been used or proposed for use as indicator microorganisms (Goyal et al. 1977, APHA 1992, Feng et al. 2010, Bae and Wuertz 2012). Coliforms, either total or fecal, are a common choice of indicator organism. Total coliforms are aerobic, facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that produce gas upon lactose fermentation within 48 h at 35°C; fecal coliforms are a sub-set of coliforms that also ferment lactose at 44°C. Generic *E. coli* has been proposed both as an indicator and index microorganism for enterohemorrhagic *E. coli*, *Salmonella* spp., and *Shigella* spp. (Leclerc et al. 2001).

When contaminated agricultural water is used for irrigation, pesticide and fertilizer application, and/or frost protection, the safety of fresh produce items has been affected (Miles et al. 2009, Erickson et al. 2010, Ijabadeniyi et al. 2011). In 2005 a large-scale outbreak of *S.* Newport from tomato consumption occurred, the trace back investigation revealed the same serovar was in the irrigation pond of the farm from which the suspect tomatoes were harvested (Greene et al. 2008). Another large outbreak in 2009 was epidemiologically linked to Serrano peppers, which came from a farm where a matching *S.* Saintpaul was isolated from the irrigation pond (Klontz et al. 2010). Therefore, the quality of water applied to fresh produce is of utmost importance in ensuring food safety.

Contradictory results have been reported as to the efficacy of index or indicator organisms in predicting the presence and/or prevalence of human pathogens, such as *Salmonella*, in surface waters (Burton et al. 1987, Rhodes and Kator 1988, Chandran and Hatha 2005, Chandran et al. 2011). While any specific pathogen may not be present in the sample being tested, the presence of indicator microorganisms is meant to suggest that pathogens have a reasonable likelihood of being present (Smith and Schaffner 2004). The tests for indicator microorganisms are less cumbersome and time consuming, as well as typically less expensive, as compared to screening for the presence of individual pathogens. This makes the use of indicator microorganisms a much more economical and practical choice over screening for individual pathogens.

Physicochemical water characteristics, such as turbidity, temperature, pH, and ORP have all been used to monitor water quality in rivers and lakes (APHA 1992). However, the strength of the correlation is not discussed by the APHA in their methods

manual. Physical measurements like temperature and rainfall (R^2 = 0.317) have been observed to correlate with *Salmonella* isolation frequency in seawater (Martinez-Urtaza et al. 2004). The main advantage of monitoring a physicochemical characteristic is that nearly instantaneous results that can be obtained and used to predict water quality so that a risk management decision can be made in a timely manner.

The relationships between the presence and/or concentration of *Salmonella* and biological, physical or chemical indicators in Central Florida surface water samples taken monthly for twelve consecutive months was explored.

Materials and Methods

Water Sampling

Eighteen surface water sites in Central Florida were sampled monthly for twelve consecutive months starting in August 2010. Sites in rural agricultural areas, away from animal agriculture were selected, and included ponds, creeks, rivers, and canals. Sites 1-6 were located in the center of Central Florida, sites 7-12 were located in Eastern Central Florida, and sites 13-18 were located in Western Central Florida. All sites were in rural agricultural areas, away from animal agriculture. Sampling locations included two lakes, one pond, six creeks, two stream, one river, and six canals. The land-use and catchment watershed for each site is detailed in Table 4-1.

Water was collected in 10 L sterile carboys (Nalgene, Rochester, NY, USA) fitted with 4 kg of lead weights attached to a rope. The rope was used to lower the carboy to a depth of 20 cm into the water source. Samples from all but one location were collected away from shore, using a bridge or other outcropping. Samples were collected on three separate days each month (six samples per day). All samples were taken before solar noon (when the sun is at its highest elevation of the day), from a

shaded area of the water source. Chemical and physical water characteristics were measured immediately after sampling as described below. Samples were transported to the laboratory and stored at 4°C for up to 24 h before microbial testing as described below.

Chemical and Physical Water Characteristics

Turbidity, temperature (air and water), pH, and oxidation-reduction potential (ORP) were measured. Each measurement was repeated in triplicate. Turbidity was measured in Formazin Attenuation Units (FAU) using a portable colorimeter (DR/850, Hach Company, Loveland, CO, USA) as per manufacturer's instructions. Water and air temperatures were measured with a portable temperature probe (SH66A, Cooper Instrument Corporation, Middlefield, CT, USA). The pH value and ORP were measured with a portable pH/ORP meter (pH6 Acorn series, Oakton, Vernon Hills, IL USA).

Total Aerobic Plate Count

Surface water samples were spread plated in duplicate, onto tryptic soy agar (TSA; Difco, Becton Dickinson) following serial dilutions in 0.1% peptone water (Difco, Becton Dickinson, Park, MD, USA) to determine total aerobic counts. Plates were incubated 24±2 h at 35±2°C and all bacterial colonies were enumerated by hand.

Coliform/*E. coli* MPN

Colisure Presence Absence Snap Packs (IDEXX Laboratories, Inc., Westbrook, Maine, USA) were used to determine coliform and *E. coli* Most Probable Numbers (MPN) in a five by three MPN configuration (10, 1, 0.1 ml dilutions). Tubes were incubated 24 h at 35±0.5°C. A yellow color indicated coliforms and *E. coli* was determined by observing fluorescence using a 6-watt UV lamp. The MPN/100 ml was

determined from the table in Standard Methods for the Examination of Water and Wastewater, 18th ed. (APHA 1992).

Salmonella Detection

Salmonella was determined in each water sample as previously described in Chapter 3. Briefly, each water sample was concentrated using tangential flow filtration (TFF) to a final retentate volume of ca. 250 ml. A KrosFlo Research II Pump (vendor, location) was used in combination with Masterflex easy-load pump head (Spectrum Labs, Rancho Dominguez, CA, USA). The inlet flow rate was 1,000 ml/min. A Mini Kros Plus Tangential Flow Filter Module (Spectrum Labs) made of polyethersulfone with a pore rating of 0.2 µm and a surface area of 1,050 cm^2 was used as a tangential flow filter. The TFF was run at a transmembrane pressure of 67 Pa. Double strength lactose broth (250 ml; Difco, Becton Dickinson) was added to the retentate, which was then stored for 1 h at room temperature and incubated 24±2 h at 35±2°C, to pre-enrichment the sample.

Pre-enrichment was followed by an immuno-capture of *Salmonella* using the Pathatrix system (Matrix MicroScience, Golden, CO, USA). The method was modified such that the entire pre-enrichment was placed in a sterile stomacher bag (17.8x30.5 cm; Fisher Scientific, New Jersey, USA; instead of the typical 100 ml conical tube). Pathatrix *Salmonella* capture beads (50 µl) were added to the system and used according to the manufactures' instructions in a pre-set 30 min cycle.

The remaining beads (40 µl) were used in a DNA extraction using the MoBio UltraClean DNA kit (MoBio, Carlsbad, CA, USA). The extracted DNA was used in real time PCR, using Applied Biosystems' MicroSEQ *Salmonella* spp. Detection Kit (Applied

Biosystems, Carlsbad, CA, USA). The real time PCR kit was used according to manufacturers' instructions in a BioRad CFX96 Real-Time thermocycler.

Modified MPN for *Salmonella*-Positive Water Samples

A 50 ml retain sample of each water sample was removed prior to processing. A modified *Salmonella* MPN method was used to determine *Salmonella* concentration (US FDA 1998) for each retained sample. A three-by-three tube MPN was set up and dilutions were as follows: 10 ml in 10 ml double strength lactose broth, 1 ml in 9 ml single strength lactose broth, and 0.1 ml in 9 ml single strength lactose broth. These were incubated for 24±2 h at 35±2°C. Selective enrichment was done by transferring a one ml aliquot to tetrathionate broth (TT broth; Difco, Becton Dickinson) and 0.1 ml to Rappaport Vassiliadis broth (RV broth; Difco, Becton Dickinson); selective enrichment broths were incubated 24±2 h at 35±2°C and 48±2 h at 41±2°C, respectively. Ten µl was streaked onto XLT4 and Chromagar *Salmonella* Plus and incubated for 24±2 h at 35±2°C following enrichment. Colonies displaying typical *Salmonella* phenotypes were confirmed biochemically on LIA and TSI slants as described above.

One representative biochemically confirmed *Salmonella* colony from each plate was transferred to TSA and incubated for 24±2 h at 35±2°C. One colony was transferred to tryptic soy broth and incubated 24±2 h at 35±2°C and DNA extraction using the MoBio UltraClean DNA kit. *Salmonella* was genetically confirmed by PCR of the *invA* and *oriC* genes. The *invA* primers were GTGAAATTATCGCCACGTTCGGGCAA and TCATCGCACCGTCAAAGGAACC, giving a PCR product of 284 bp; the *oriC* primers were TTATTAGGATCGCGCCAGGA and AAAGAATAACCGTTGTTCAC, giving a PCR product of 163 bp. Both primer sets were as described by (Malorny et al. 2003). All reagents were obtained from the Fisher

exACTGene Complete PCR kit (Fisher Scientific, NJ, USA) and primers were used at a concentration of 20 µM. The PCR reaction mix was as follows: 34.75 µl water; 5 µl 10X PCR buffer; 1 µl of each primer; 0.25 µl Taq DNA polymerase; 5 µl template DNA. The optimized PCR conditions were: 3 min at 94°C melting, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C; a final elongation of 5 min at 72°C. Gel electrophoresis was done using a 1.8% agarose gel with 0.5X TBE buffer. Gels were run at 70 V for 90 min.

Weather Data Collection

Rainfall and solar radiation data were collected from the Florida Automated Weather Network (http://fawn.ifas.ufl.edu/). The closest weather recording station to each individual sampling site was determined and the data applied accordingly.

Statistical Analysis

Correlations were determined using JMP Pro 9. Multiple linear regression analysis of *Salmonella* vs. all parameters was done using SAS 9.3 (SAS Institute Inc., NC, USA) software. A p value of < 0.05 was considered significant.

Results

Two hundred and two samples were collected and analyzed over the twelve-month sampling period. Fourteen samples were missed due to laboratory error or inaccessibility of the water source due to unrelated factors (e.g., nearby herbicide application precluded access due to investigator safety). The correlation coefficients for each characteristic vs. *Salmonella* log MPN/100 ml are displayed in Table 4-2. When each R^2 value is calculated for individual sampling sites 152 of the 162 of the R^2 values fall below 0.4 indicating a lack of correlation. Each characteristic will be discussed further in the appropriate section below.

Salmonella MPN

All 202 water samples were positive for *Salmonella* using the TFF-immuno-capture – real-time PCR method. The detection level for the described methodology has a detection limit of ca. 1 CFU/L in 10 L (McEgan et al. 2013); so all 10 L surface water samples collected had greater than 1 CFU/L *Salmonella* spp. in each 10 L sample volume collected and processed.

Salmonella concentrations for each site by sampling date can be seen in Figure 4-1. The median *Salmonella* concentration was 0.79 log MPN/100 ml for all samples taken; the upper quartile was 1.2, the lower quartile was 0.48 log MPN/100 ml; and 139 of 202 samples had *Salmonella* concentrations below 1 log MPN/ 100 ml. The highest concentration of *Salmonella,* 3.0 log MPN/100 ml, was identified in July (Fig 4-1); however, the month of sampling did not exhibit a strong correlation with the concentration of *Salmonella* (R^2=0.20).

Coliform and *E. coli* MPN

Values for coliform and *E. coli* log MPN/100 ml can also be seen in Figure 4-1. Many of the coliform (128/202), and some of the *E. coli* (17/202), MPNs were at or over the upper limit of detection (≥3.2 log MPN/100 ml) for the MPN method used. The median coliform concentration was ≥3.2 log MPN/100 ml, with the lower quartile at ≥2.7 log MPN/100 ml. *E. coli* had a median concentration of 1.7 log MPN/100 ml, an upper quartile of 2.4 and lower quartile of 0.60 log MPN/100 ml. Neither coliform log MPN/100 ml (R^2=0.004; Table 4-2), nor *E. coli* log MPN/100 ml (R^2=0.078; Table 4-2) values were strongly correlated with the *Salmonella* MPN values. However, at sites 11 and 18, *E. coli* log MPN/100 ml resulted in higher correlations, R^2=0.606 and R^2=0.678,

respectively. Interestingly, coliform and *E. coli* log MPN/100 ml were not strongly correlated (R^2=0.364; Table 4-3) with each other.

Aerobic Plate Count

The values for each aerobic plate count for each site can be seen in Figure 4-1. The median aerobic plate count was 3.7 log CFU/100 ml; the upper quartile was 4.0 log CFU/100 ml, and the lower quartile was 3.2 log CFU/100 ml. The two highest aerobic plate counts, 5.2 and 5.5 log CFU/100 ml (Figure 4-1-H and 4-1-D), both occurred during the month of July. The aerobic plate count, in log CFU/100 ml, did not correlate with the *Salmonella* log MPN/100 ml (R^2=0.004; Table 4-2). The aerobic plate count did not strongly correlate strongly with any of the other variables recorded; all R^2 values were <0.212, and p values ranged from 0.8620 to 0.0039.

Air and Water Temperature

Air and water temperatures are displayed in Figure 4-2. Not surprisingly, recorded air and water temperatures showed the strongest correlation, to each other, of any two variables (R^2=0.680; Table 4-3). Neither the air nor water temperature correlated with the *Salmonella* log MPN/100 ml (R^2=0.000 and R^2=0.006, respectively; Table 4-2). However, when calculated on an individual site bases, site 14 (R^2=0.405; Table 4-2) and site 15 (R^2=0.403; Table 4-2) *Salmonella* concentrations showed relatively higher correlation with water and air temperatures, respectively.

pH, ORP, Turbidity, and Conductivity

pH and ORP values by sampling site over time are displayed in Figure 4-3; and turbidity and conductivity by sampling site over time are displayed in Figure 4-4. None of these physical water characteristics correlated with *Salmonella* log MPN/100 ml, as show by the R^2 values in Table 4-2. However, when calculated by site, ORP had a

relatively high correlation with *Salmonella* concentration at site 9 (R^2=0.424) and site 10 (0.555); while turbidity had a relatively high correlation with *Salmonella* concentration at site 4 (R^2=0.691) and site 11 (R^2=0.584); and conductivity had a relatively high correlation with *Salmonella* concentration at site 4 (R^2=0.405) and site 17 (R^2=0.450). pH correlations were below 0.4 at all sites.

Rainfall and Solar Radiation

The average rainfall (in the previous day, week or month) before sampling did not correlate with *Salmonella*, coliform, *E. coli*, or aerobic plate count levels (data not shown). In addition solar radiation was also poorly correlated with *Salmonella,* coliforms, *E. coli* or aerobic plate counts levels (data not shown).

Discussion

Salmonella is present in Central Florida surface waters throughout the year. Surveys of surface waters for *Salmonella* in the South Eastern US have reported similar findings; in North Carolina surface water had a *Salmonella* prevalence of 54.7% (94/172) in 25 ml water samples (Patchanee et al. 2010), and in a Southern Georgia watershed 79.2% of water samples were *Salmonella* positive using a 5-replicate, 3-dilution (100, 10, 1 ml) MPN (Haley et al. 2009). A survey of the Suwannee River in North Florida reported 96% (106/110) of water samples *Salmonella*-positive using a three-replicate, five-dilution (500, 100, 50, 10, 1 ml) MPN method (Rajabi et al. 2011). These findings are also similar to those found by other researchers performing surface water samplings over extended periods of time at the same set of sites in more geographically diverse locations. Two rivers and one creek all in one Southern Ontario, Canada water shed had 78.4% (91/116) *Salmonella*-positive samples when locations were sampled by 5-day Moore swabs twice monthly for 2.5 years (Thomas et al. in

press). Other studies have reported much lower *Salmonella* prevalence in surface waters. Surface waters in the Salinas Valley, CA, surveyed using either 3-day Moore swabs or a 100 ml sample reported 7.1% (18/252) *Salmonella*-positive samples (Gorski et al. 2011). The prevalence of *Salmonella* in the coastal waters of southern Morocco during a 4-year monthly sampling of six sites was reported at 4.1% (10/243) (Setti et al. 2009). Similarly, *Salmonella* in surface waters collected from fruit and vegetable farms in New York state was reported at 11% (16/145) (Strawn et al. 2013). Overall, the presence of *Salmonella* throughout the year in Central Florida surface waters is similar to surface water surveys performed throughout the Southeastern US, especially when considered the increased sample volume used in the current study.

None of the index microorganisms measured strongly correlated with the prevalence of *Salmonella* in the same Central Florida surface water sample. Many of the coliform log MPN/100 ml were at or above the upper limit of detection may be responsible for the lack of correlation between *Salmonella* and coliform populations. In the cases where coliform populations were at or above the upper limit of detection, the population could have in fact been higher and this would not have been able to be reflected in the data point. All water samples in this study with coliform counts above 3.2 log MPN/100 ml, the upper limit of detection, would by the design of the enumeration procedure be recorded as ≥3.2 log MPN/100 ml; this would add error into the calculations of the correlation coefficients.

Numerous publications have described the lack of, or presence of weak correlations between indicator microorganisms and pathogens in surface water (Goyal et al. 1977, Payment and Locas 2011). Moderate correlations were obtained when *E.*

coli or coliform data from the current study were analyzed for correlations with *Salmonella* populations, for individual sites (Table 4-2); however, when the data was aggregated for all sites correlations between *E. coli* or coliforms and *Salmonella* both had $R^2 < 0.1$ indicating that other factors beyond those studied here influence the relationships between pathogens and indicator microorganisms. Studies that report similar results conclude that high correlations between pathogens and indicator microorganisms may be temporal, random, site specific or time specific (Payment and Locas 2011). High correlations between pathogens and indicator microorganisms often occur at point sources where the levels of indicator microorganisms and pathogens are consistently high for a longer period of time (Payment and Locas 2011), such as surface waters impacted by improperly treated sewage or runoffs from livestock farms. Our study was conducted in rural agricultural Florida where the influence of factors such as raw sewage effluent was unexpected. The occurrence of *Salmonella* in the environment may be at a much higher frequency than previously assumed; *Salmonella* may also not be transient in nature within surface waters as was also previously believed (Buchanan 2000).

Not only did our analysis not find strong correlations between *E. coli* or coliform populations and *Salmonella*, both were also not correlated with rainfall or seasons as would have been predicted (Goyal et al. 1977). In our analysis, average rainfall, whether aggregated of 24 h, one week, or one month (prior to sampling), did not correlate with *Salmonella*, *E. coli* or coliform levels. Conflicting results have been reported in several other studies that also attempted to determine a correlation between rainfall and pathogen prevalence. A Southern Ontario surface water study found the lowest

occurrence of *Salmonella* did coincide with the month (February) with the lowest recorded precipitation, air temperature, and water temperature (Thomas et al. in press). A positive correlation between *Salmonella* prevalence and rainfall (mm/day) has been reported, without R^2, during a study in southern Morocco (Setti et al. 2009). The Morocco study analyzed 100 ml of seawater collected monthly at six sites for a four-year duration. In New York, *Salmonella* has been reported to have a higher prevalence in surface waters when measurable precipitation occurred within three days prior to sampling, however, this was only observed in areas of poorly drained soils (Strawn et al. 2013). The differing results could be suggestive that rainfall, does have some effect on *Salmonella* prevalence in surface waters, but not as a direct correlation and perhaps in a multi-factorial way including more characteristics than those noted in the current study.

A study conducted in Puerto Rico attempted to correlate rainfall from 24 h, 48 h, and one week prior to water sampling for fecal coliforms (coliforms that grow at 42°C (APHA 1992); a less inclusive group compared to total coliforms which were measured in the current study) found no correlation between fecal coliforms and precipitation in any of the ten sampling sites (Santiago-Rodriguez et al. 2012). These authors suggest that although fecal coliforms, commonly found in tropical sediments, may be resuspended from preceding precipitation that the rainfall may have a possible dilution effect as well on the fecal coliforms (Santiago-Rodriguez et al. 2012). Some studies have indicated that turbulence caused by rainfall could resuspend pathogens or indicator microorganisms from sediments, raising their levels in the waters above by 100 or 1,000 fold, and that the depth and size of the water body dampen any rainfall

dilution effect on the indicator/pathogen levels (Goyal et al. 1977). Our study did not find a correlation between rainfall and *Salmonella* or *E. coli* and coliform populations. At no point during the water sampling procedure was the sediment disturbed. This could indicate that sediment disturbance is not a major contributing factor to *Salmonella* in Central Florida surface waters.

Correlations were not influenced by season, possibly due to the relatively small temperature changes in the sub-tropical climate. However, in Southern Ontario and New York State, areas with much larger seasonal temperature variations, *Salmonella*-positive samples were not significantly different between seasons (Thomas et al. in press, Strawn et al. 2013). The rise and fall in the levels of biological characteristics was random and did not also coincide with increasing temperatures in the summer and reduced temperatures in the winter. This is contrary to the finding of Gorski et al. (2011), who note a noticeable seasonal trend in the prevalence of *Salmonella* in surface water samples from the Salinas Valley, CA. Further work is required to acquire a better understanding if and over what range water temperature would have an effect on *Salmonella* prevalence and whether geographic location or other climacteric factors also have an effect.

Physicochemical water characteristics have been used to monitor the chemical and microbiological quality of drinking and recreational water. Data from some sites (Table 4-2) showed that turbidity, ORP and conductivity could predict the levels of *Salmonella* in surface water, but when aggregated across all sites none of these parameters produced acceptable correlations. Although R^2 values were low, indicating additional unmeasured factors may be exerting an influence.

When *E. coli* or solar radiation levels increase, these increases are correlated, significantly ($P<0.05$) but with a low R^2, with increases in *Salmonella* levels. The positive correlation of solar radiation with *Salmonella* may be partially explained by *Salmonella* being more resistant to solar radiation than other bacterial pathogens, such as *E. coli*, *Shigella flexneri*, and *Vibrio cholera* (Berney et al. 2006). Potentially leaving fewer bacteria to compete for nutrients after increases in solar radiation, thus *Salmonella* would have access to a greater amount of nutrients as solar radiation increased and inactivated the less resistant bacteria; less nutrient competition as well as nutrients made available through the inactivation of other bacteria may be benefitting *Salmonella* survival.

When conductivity levels increase, there is a statistically significant ($p=0.000$) inverse correlation with *E. coli* concentration. Conductivity of surface water is an indirect measure of salinity and total dissolved solids, these have previously been suggested to be influenced by storm water and runoff (Gonzalez et al. 2012). An inverse correlation between indicator bacteria (*E. coli,* enterococci, and *Bacteroides*) and conductivity or salinity has also been reported for estuary waters in eastern North Carolina (Gonzalez et al. 2012). The inverse correlation noted in the current study is perhaps also due to storm water and runoff effects, in this case the addition of the storm water or runoff in carrying in salinity or other total dissolved solids from the surrounding area, whilst diluting the *E. coli* concentrations. The sampling sites used in the current study were not influenced by seawater. Though these relationships are statistically significant, low coefficient of correlation vales (< 1 or < -1) values indicate that other as yet unknown factors are also involved.

The complex nature of the index or indicator and pathogen relationship, makes predicting the levels of pathogens through index/indicator microorganisms challenging at best; simple, linear relationships cannot be relied upon for predicting pathogen levels from indictor populations. Logistic regression analysis could be used in this case to further develop models to better predict the levels of *Salmonella* using index/indicator microorganisms or physicochemical water characteristics.

The absence of strong correlations (low R^2) between index/indicator microorganisms, chemical or physical indicators with *Salmonella* levels limits their ability to predict the prevalence of *Salmonella*. However significant positive relationships have been shown to occur between *E. coli* and *Salmonella* levels and between solar radiation and *Salmonella* levels. These may offer a fast but qualitative indication of the degree of *Salmonella* risk. However, until a more reliable, and quantitative, index or indicator of *Salmonella* in surface waters can be established, direct screening for *Salmonella* should occur. Finally given the dynamic, heterogonous and complex nature of surface water ecosystems, the challenge remains to find an indicator parameter that has a more intimate relationship besides just being associated with the pathogen and therefore less sensitive to extraneous factors.

Table 4-1. Description of 18 Central Florida surface water sampling sites including water source type, land-use, and watershed as described by local water management district

Site Number	Water source type	Immediate area land-use	Percentage Watershed Agriculture	Percentage Watershed Rangeland	Percentage Watershed Urban/Suburban
1	Lake	Agriculture, Wetlands, Residential	33	<1	29
2	Lake	Agriculture, Wetlands	33	<1	29
3	Pond	Agriculture	33	<1	29
4	Creek	Wetlands	33	<1	29
5	Creek	Wetlands	33	<1	29
6	Stream	Agriculture	32	3	20
7	Creek	Agriculture	26	<1	49
8	Creek	Agriculture	32	12	25
9	Creek	Agriculture	32	12	25
10	Stream	Agriculture	32	12	25
11	River	Wetlands, Agriculture	32	12	25
12	Creek	Agriculture	45	13	10
13	Canal	Agriculture	55	NA*	21
14	Canal	Agriculture	55	NA	21
15	Canal	Agriculture	55	NA	21
16	Canal	Agriculture	55	NA	21
17	Canal	Agriculture	55	NA	21
18	Canal	Agriculture	55	NA	21

*NA – Not Available

Table 4-2. Correlation coefficients (R^2) determined for *Salmonella* log MPN/100 ml enumerated monthly for 12 consecutive months from 18 Central Florida surface water sites vs. each of the physical, chemical, and biological water characteristic recorded at time of samplings, values greater than 0.4 have been bolded

Site	*E. coli* (log MPN /100 ml)	Coliforms (log MPN /100 ml)	APC[a] (log CFU /100ml)	Water Temp (°C)	Air Temp (°C)	pH	ORP (mV)	Turbidity (FAU)	Conductivity (µS/cm)
1	0.108	0.009	0.001	0.000	0.158	0.003	0.007	0.220	0.021
2	0.000	0.059	0.119	0.205	0.235	0.006	0.008	0.011	0.210
3	0.082	0.079	0.178	0.101	0.043	0.123	0.146	0.141	0.033
4	0.149	0.087	0.144	0.099	0.123	0.006	0.027	**0.691**	**0.405**
5	0.034	0.003	0.003	0.069	0.103	0.068	0.080	0.152	0.036
6	0.097	0.254	0.344	0.014	0.127	0.177	0.165	0.195	0.039
7	0.066	0.039	0.023	0.026	0.076	0.009	0.271	0.042	0.265
8	0.263	0.003	0.007	0.031	0.019	0.052	0.240	0.078	0.194
9	0.150	0.001	0.092	0.065	0.016	0.021	**0.424**	0.055	0.315
10	0.278	0.037	0.038	0.037	0.035	0.071	**0.555**	0.359	0.269
11	**0.606**	0.003	0.099	0.005	0.039	0.272	0.070	**0.584**	0.355
12	0.230	0.079	0.020	0.119	0.136	0.204	0.346	0.019	0.024
13	0.372	0.019	0.000	0.153	0.303	0.010	0.005	0.109	0.018
14	0.017	0.322	0.354	**0.408**	0.120	0.191	0.154	0.023	0.030
15	0.016	0.016	0.377	0.138	**0.430**	0.075	0.022	0.010	0.065
16	0.131	0.168	0.069	0.148	0.041	0.013	0.037	0.024	0.054
17	0.107	0.004	0.000	0.022	0.087	0.185	0.102	0.152	**0.450**
18	**0.678**	0.247	0.002	0.001	0.020	0.000	0.101	0.237	0.053
Overall	0.078	0.000	0.004	0.000	0.006	0.076	0.070	0.010	0.015

[a] Aerobic Plate Count

Table 4-3. Correlation coefficients (R^2) with p-values determined between each of the physical, chemical, and biological water characteristics for all sampling sites and months combined

Water Characteristic	Air temp (°C)	pH	ORP (mV)	Turbidity (FAU)	Conductivity (µS/m)	Coliforms[a]	*E. coli*[a]	Aerobic plate count[b]	Solar Radiation (W/m^2)	*Salmonella*[a]
Water temp (°C)	**0.825**[c] (0.0000)[d]	0.208 (0.0031)	0.0117 (0.8800)	**0.144** (0.0418)	0.1300 (0.0707)	-0.0165 (0.8170)	**−0.144** (0.0432)	0.0125 (0.8620)	-0.0330 (0.6410)	0.1210 (0.0946)
Air temp (°C)		0.0127 (0.8580)	0.0254 (0.7450)	0.1410 (0.0473)	**0.225** (0.0017)	0.0307 (0.6680)	-0.0744 (0.2990)	0.0297 (0.6790)	-0.0696 (0.3250)	0.0538 (0.4590)
pH			0.1330 (0.0867)	-0.0686 (0.3370)	**0.423** (0.0000)	-0.0479 (0.5040)	-0.0141 (0.8450)	0.0398 (0.5790)	-0.0101 (0.8860)	0.0506 (0.4880)
ORP (mv)				-0.0209 (0.7880)	-0.0566 (0.4720)	0.0799 (0.3020)	-0.0499 (0.5200)	0.0151 (0.8450)	0.1010 (0.1870)	-0.0578 (0.4640)
Turbidity (FAU)					-0.0878 (0.2210)	**−0.172** (0.0151)	-0.0297 (0.6780)	0.0336 (0.6390)	-0.0819 (0.2460)	0.0824 (0.2570)
Conductivity (µS/m)						-0.0592 (0.4130)	**−0.317** (0.0000)	0.0568 (0.4320)	**−0.273** (0.0001)	-0.1210 (0.0994)
Coliforms							**0.364** (0.0000)	**0.212** (0.0029)	-0.0670 (0.3450)	0.0032 (0.9650)
E. coli								0.0386 (0.5920)	**0.151** (0.0330)	**0.248** (0.0005)
Aerobic plate count									-0.0132 (0.8520)	0.0314 (0.6680)
Solar Radiation (W/m^2)									**0.165** (0.0216)	

[a] *E. coli* , coliforms, and *Salmonella* reported in log MPN/100 ml
[b] Aerobic plate count reported in log CFU/100 ml
[c] coefficients displayed in bold are statistically significant($p < 0.05$)
[d] p-values are displayed in parenthesis

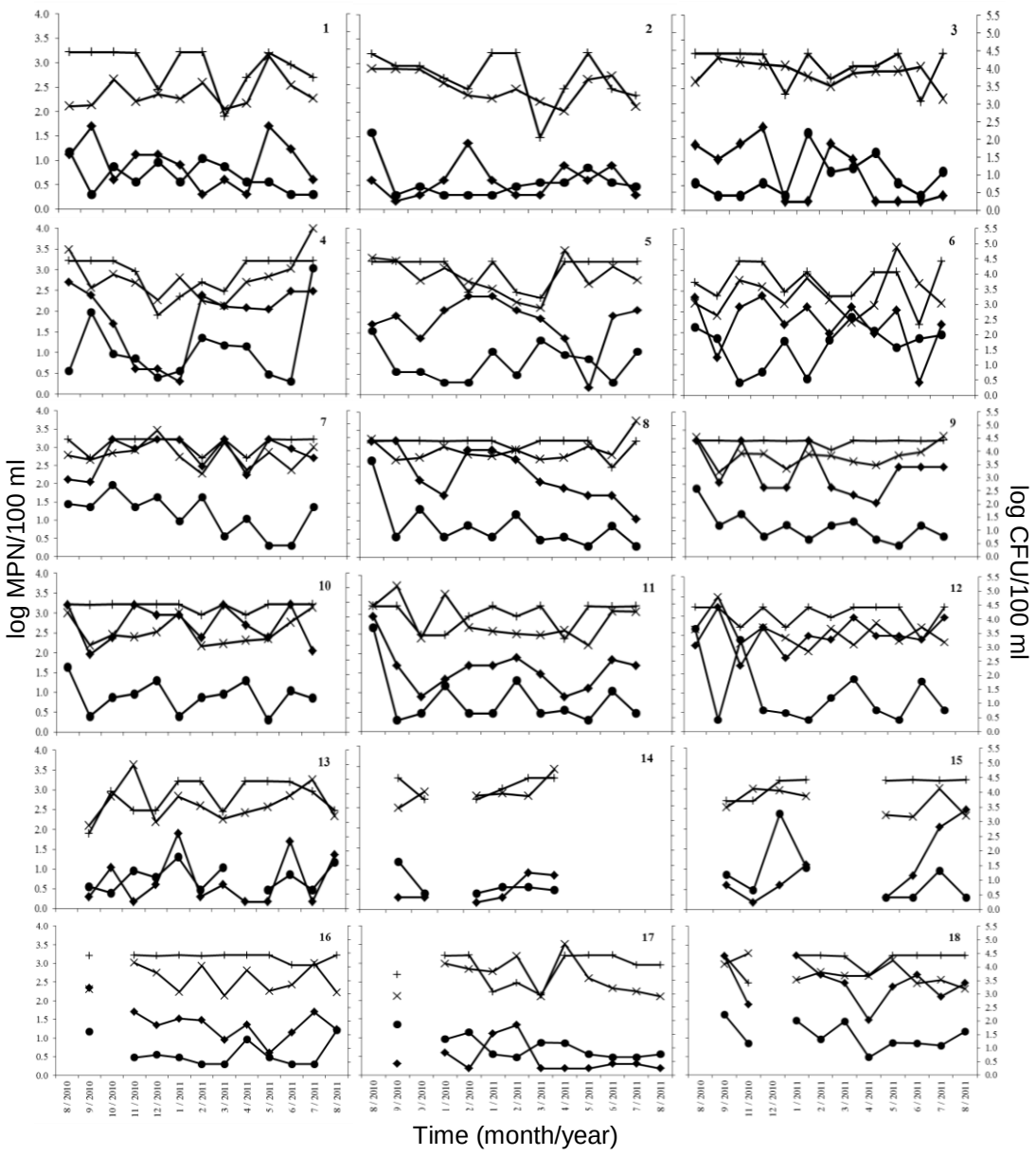

Figure 4-1. Populations of Salmonella enumerated via MPN enrichments in LB, TT broth, and isolation on XLT4, (●), *E. coli* (♦), and coliforms (┼) enumerated via MPN method using Colisure, all reported in log MPN/100 ml (left axis), and aerobic plate counts (×), enumerated on TSA and reported in log CFU/100 ml (right axis), as determined for each of eighteen Central Florida sites sampled monthly for a continuous twelve-month period.

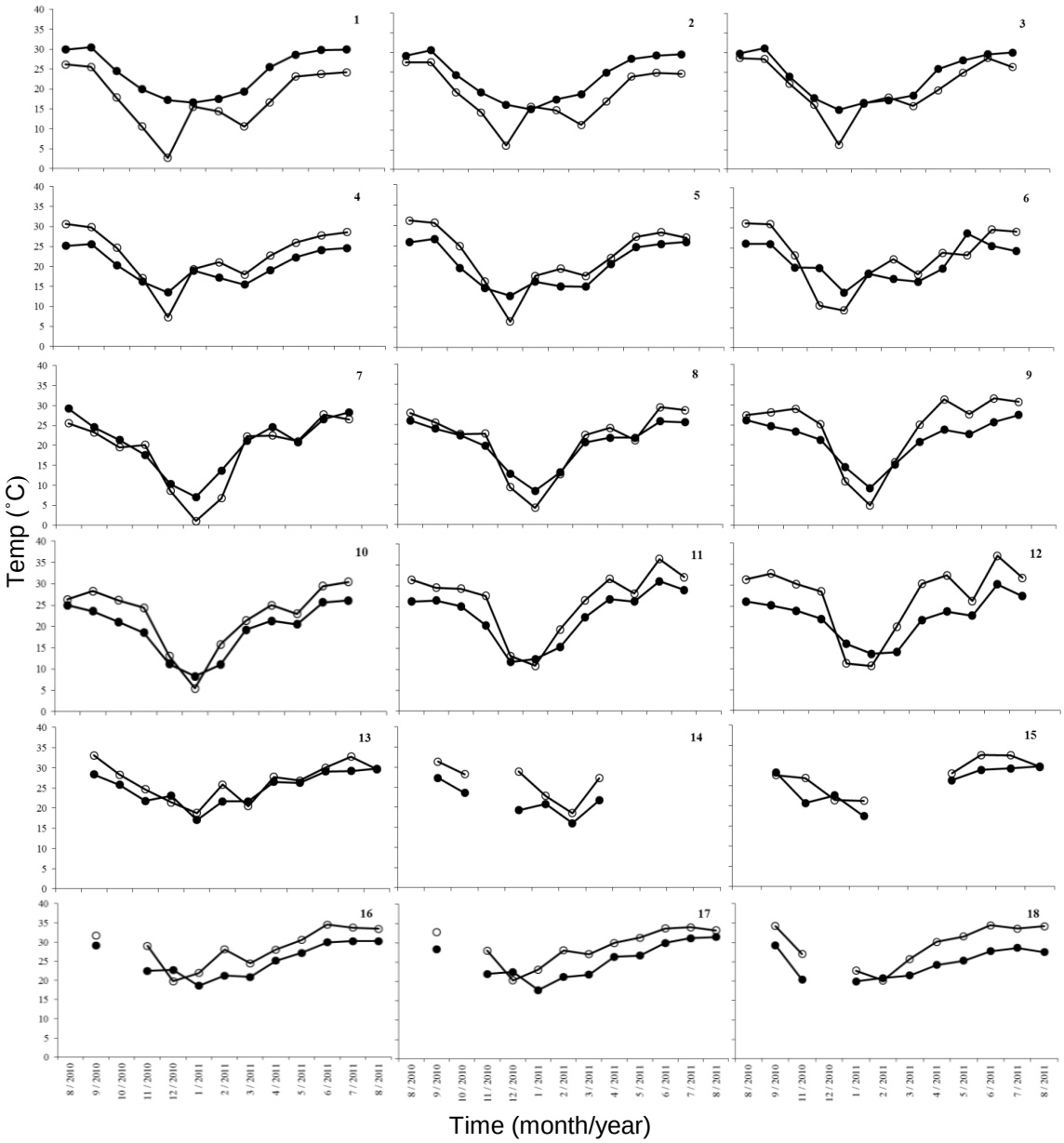

Figure 4-2. Air (○) and water (●) temperatures, reported in °C, measured using a temperature probe at the time of sampling for each of eighteen Central Florida sites sampled monthly for a continuous twelve-month period.

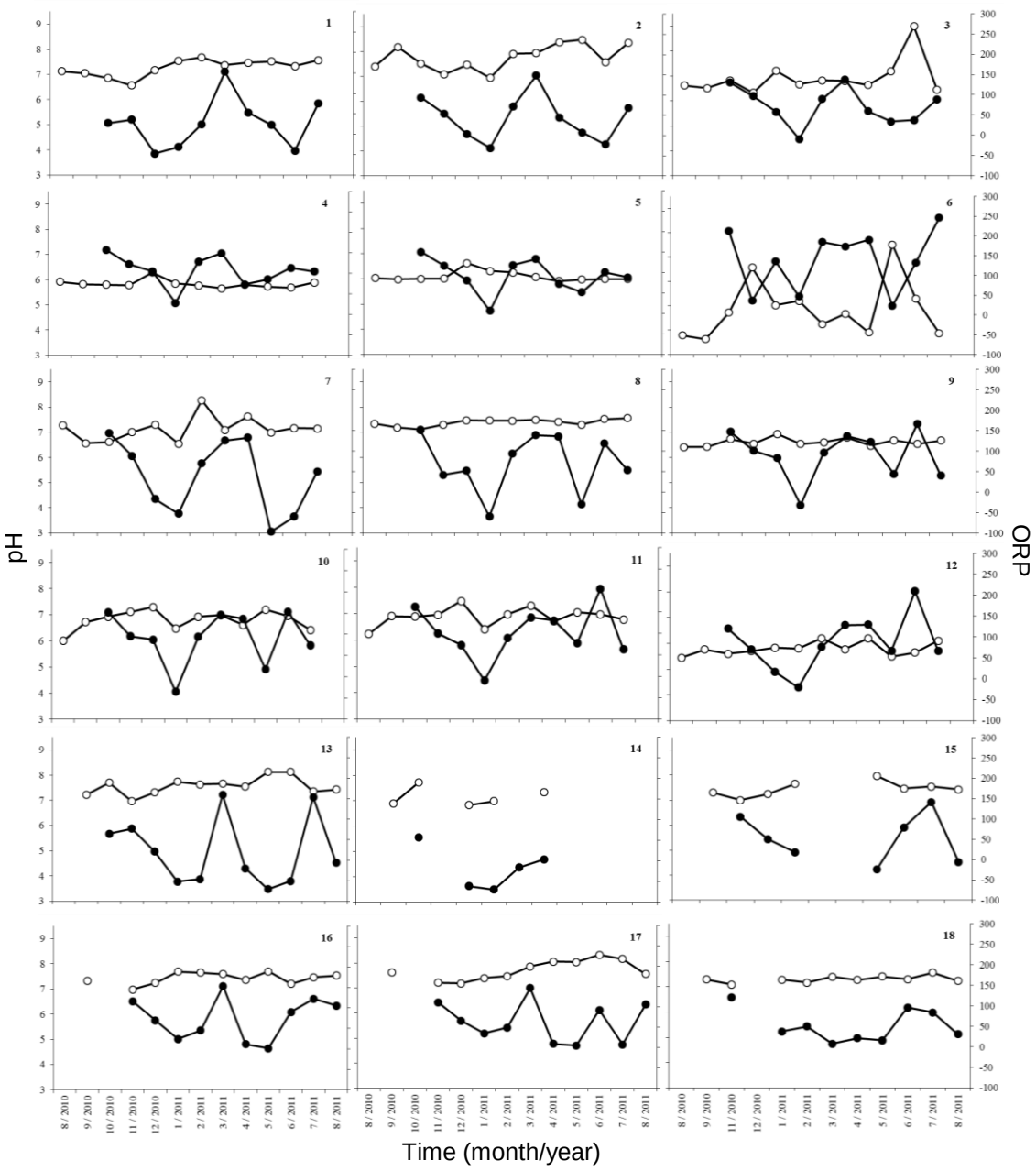

Figure 4-3. pH (○, left axis) and ORP (●, right axis) measured at the time of sampling for each of eighteen Central Florida sites sampled monthly for a continuous twelve-month period.

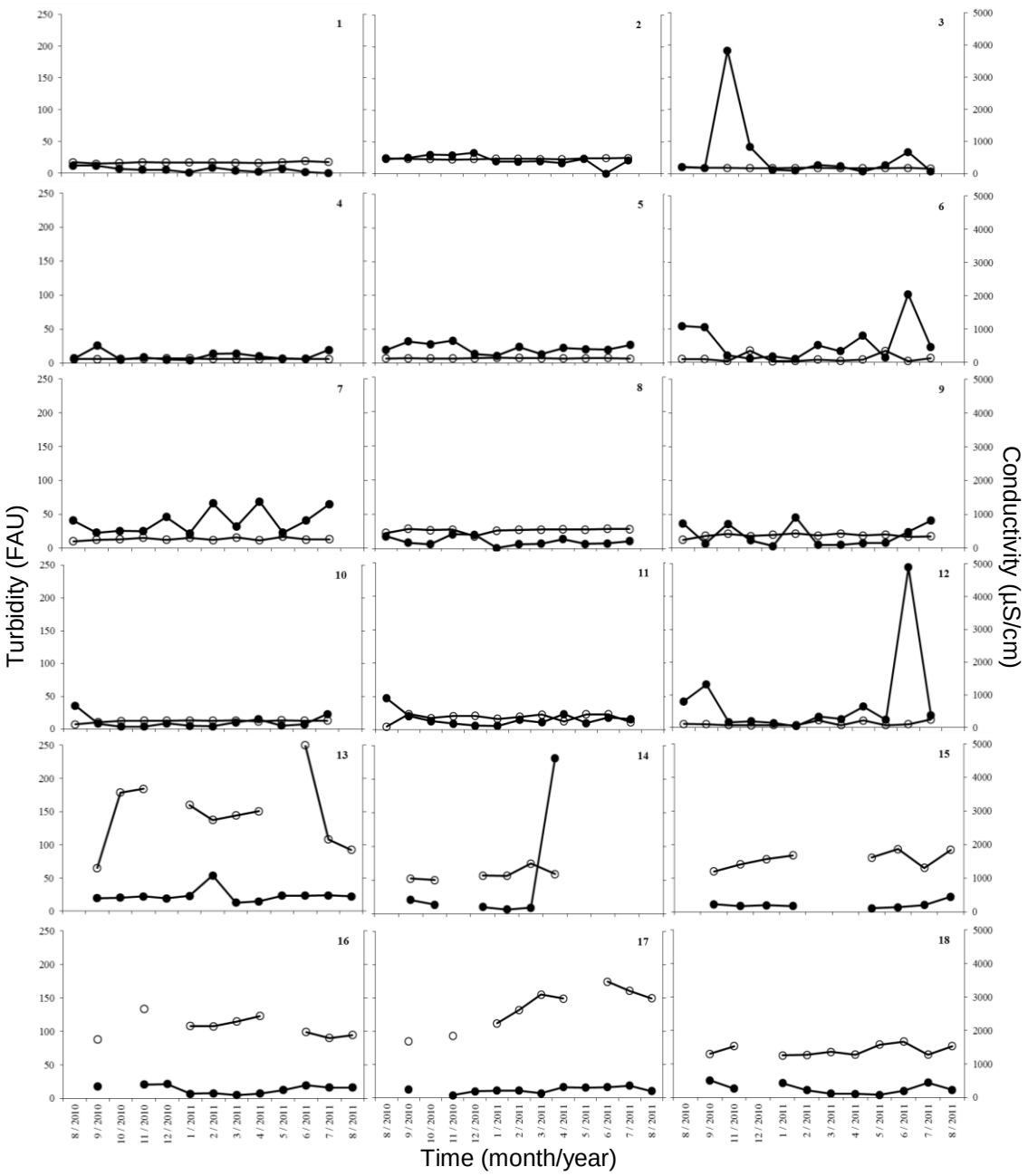

Figure 4-4. Turbidity (●; left axis) and conductivity (○; right axis) measured at the time of sampling for each of eighteen Central Florida sites sampled monthly for a continuous twelve-month period.

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CHAPTER 5
IDENTIFICATION AND CHARACTERIZATION OF *SALMONELLA* FROM CENTRAL FLORIDA SURFACE WATERS

Salmonella is one of the major causes of enteric disease in humans, causing over 40,000 laboratory confirmed cases per year in the US (Scallan et al. 2011). *Salmonella enterica* subspecies *enterica* comprise more than 1,400 non-typhoidal serovars, while only 30 serovars cause the majority of salmonellosis cases in the US (Kim et al. 2006).

The identification of *Salmonella* serovars is important for source tracking and outbreak investigations. The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies. The most common serotyping method for *Salmonella* isolates involves the serological discrimination of surface polysaccharides (O), flagella (H), and capsular (Vi) antigenic properties (Kim et al. 2006). This method requires more than 150 O and H antisera for the characterization of each serovar (Jean-Gilles Beaubrun et al. 2012).

The use of a multiplex PCR method, previously reported by Kim et al. (2006) could be employed to greatly reduce the time and labor required to serotype 30 of the most clinically relevant serovars (Kim et al. 2006). This PCR method consists of two five-plex PCR reactions, and is based on six genetic loci form *S*. Typhimurium and four loci from *S*. Typhi.

Another method used for the typing of *Salmonella* is pulsed-field gel electrophoresis (PFGE). This method relies on a macrorestriction of genomic DNA, producing a DNA fingerprint for comparison to other isolates. PFGE is the CDC ‘gold standard’ method for public health laboratories for comparing *Salmonella* isolates; it has been used in CDC’s PulseNet since 1996 for the comparison of isolates to detect

geographically diverse outbreaks (Ribot et al. 2006). The standardized method has allowed for PFGE profiles from numerous laboratories to be comparable, and thus much data now exists for purposes of comparison. Some disadvantages to PFGE are the time required to results (five days), technical expertise required, and costly equipment.

Previously, *Salmonella* have been isolated from Central Florida surface waters (Chapter 4). Environmental sampling for *Salmonella* has become an important research area in an effort to further understand potential sources of contamination of produce. Irrigation water has been suggested as a potential source of pre-harvest contamination (Miles et al. 2009, Erickson et al. 2010, Ijabadeniyi et al. 2011). Environmental sampling studies could benefit from having a more time and cost-efficient method for typing *Salmonella* isolates.

Salmonella serovars isolated from surface waters are frequently not those serovars included in the 30 most clinically relevant serovars (Kim et al. 2006, Haley et al. 2009, Patchanee et al. 2010, Rajabi et al. 2011, Strawn et al. 2013). Therefore, the present study aimed to determine if the multiplex PCR method, previously reported by Kim et al. (2006), could be used to i) verify that all *Salmonella* isolates from a single water sample are identical; and ii) predict the serogroup or serovar of environmental *Salmonella* isolates. Additionally, *Salmonella* isolates from Central Florida surface waters were characterized by PFGE, serotyping, and antimicrobial resistance profiles.

Materials and Methods

Water-Sample Collection and *Salmonella* Enrichment

Salmonella isolation from Central Florida surface waters is described in Chapter 4. Briefly, 10 L of surface water was collected from eighteen sites in Central Florida sampled for twelve consecutive months starting August 2010. Sites were grouped

generally into three regions based on location (1-6; 7-12; and 13-18). All sites were in rural agricultural areas, away from animal agriculture and included two lakes, one pond, six creeks, two stream, one river, and six canals. The land use and catchment watershed for each site is detailed in Table 4-1 of Chapter 4.

Salmonella isolates were obtained using a modified FDA-BAM *Salmonella* enrichment method (Chapter 4). Up to 36 *Salmonella* isolates were obtained from each sample; *Salmonella* was genetically confirmed by PCR of the *inv*A and *ori*C genes; a total of 562 *Salmonella* were isolated (Chapter 4) and stored in tryptic soy broth (TSB; Difoc, Becton Dickinson, Sparks, MD, USA) supplemented with 15% glycerol at -80°C.

Multiplex PCR Analysis

Frozen (-80°C) isolates were transferred to tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD, USA) and incubated for 24±2 h at 35±2°C. One colony was transferred to TSB and incubated 24±2 h at 35±2°C; DNA was extracted using the MoBio UltraClean DNA (MoBio, Carlsbad, CA, USA). A previously described multiplex PCR reaction (Kim et al. 2006) for *Salmonella* serotype analysis was performed on DNA from all isolates.

The PCR assay uses the amplification of specific gene products that results in the production of a serovar-specific banding pattern for each of 30 top clinically important serovars of *S. enterica* subspecies *enterica* (Kim et al. 2006). This method consists of two five-plex PCR reactions. The primers for each of the reactions are as described by Kim et al. (2006) with the following exceptions: the PCR reagents were all obtained from the Fisher exACTGene Complete PCR kit (Fisher Scientific). The bands are labeled as described by Jean-Gilles Beaubrun (2012).

Gel electrophoresis, using a 2.5% agarose gel, was run for 60 or 120 min for the STM and STY PCR products, respectively, at 0.8 mV cm^2 in 1X TBE buffer. PCR products were stained with ethidium bromide (1 µg/ml), and the gels were imaged under UV light. Amplicon sizes were determined by comparison to Hyperladder V molecular size marker.

Serogrouping

Each isolate was grown on TSA for 24±2 h at 35±2°C. One isolated colony was selected and applied to a glass slide with 5 µl sterile saline. *Salmonella* O Poly Antiserum (Becton Dickinson, Sparks, MD, USA) was applied and autoagglutination was reported after 1 min of rocking the glass slide. All isolates were serogrouped in order to determine if isolates that grouped together based on multiplex PCR banding patterns also belonged to the same serogroup.

Serotyping

Of the 562 *Salmonella* isolates, 217 were selected to represent the multiplex PCR/serogroup groupings within one sampling time/location and sent to the National Veterinary Laboratory Service (NVSL; USDA, Ames, Iowa, USA) for serotyping.

Pulsed-field gel electrophoresis

DNA fingerprinting of *Salmonella* isolates (n=217, the same isolates that were serotyped) was conducted using pulsed-field gel electrophoresis (PFGE) after DNA macrorestriction using *Xba*I restriction enzyme. PFGE was performed according to the standard laboratory protocol recommended by the PulseNet program, Centers for Disease Control and Prevention (Ribot et al. 2006). The PulseNet universal standard strain *Salmonella enterica* Braenderup H9812 was used as the reference marker.

Gel images were transferred to Bionumerics software version 6.6 (Applied Maths, NV, Austin, TX, USA) for cluster analysis. Cluster analysis was performed using the unweighted pair group method with arithmetic averages with 2.0% band position tolerances and 1.5% optimization values. Similarity coefficients were obtained within Bionumerics by calculating Dice coefficients. PFGE banding patterns with a similarity index >80% were grouped with the same genotypic cluster.

Antimicrobial Susceptibility Testing

Salmonella isolates were assayed for susceptibility to 15 antibiotics by the calibrated dichotomous sensitivity method (Bell et al. 2004). Results were interpreted according to test standards. Antimicrobial susceptibility was determined according to test standards. Isolates were grown overnight on TSA. One colony was transferred to 1 ml of 0.85% sterile saline, 330 µl of inoculum was spread plated to Sensitest agar (Oxoid, Ogdensburg, NY, USA). Sensitest agar plates were allowed to dry 30 min before application of antibiotic discs (BBL Sensi-Disc, BD Diagnostic Systems, Sparks, MD). Five antibiotic discs were applied per plate. Zones of inhibition were measured after incubation at 35°C for 18 h. The following antibiotics were tested: amikacin (An), amoxicillin-clavulanic acid (Amc), ampicillin (Am), cefoxitin (Fox), ceftriaxone (Cro), chloramphenicol (C), ciprofloxacin (Cip), gentamicin (G), imipenem (Imp), kanamycin (K), nalidixic acid (Na), streptomycin (S), sulfamethoxazole-trimethoprim (STx), and tetracycline (Te).

Results

Water Sample Collection

A total of 202 water samples were collected and analyzed over the twelve-month sampling period (Chapter 4). *Salmonella* isolates were obtained from 165 (81.7%) of

the 202 water samples analyzed; a total of 562 *Salmonella* isolates were obtained; up to 36 isolates were obtained from one sample.

Multiplex PCR

The 562 isolates were analyzed using the multiplex PCR. *Salmonella* from the same sample (site and time) having the same multiplex PCR pattern, were assumed to be potentially the same; *Salmonella* having different multiplex PCR patterns were assumed to be different. *Salmonella* were identified as having all the same multiplex PCR pattern in 68 samples (33.7%). In 52 (25.7%) of the samples two patterns were identified; in 21 (10.4%) samples three patterns were identified amongst the isolates. The remaining 24 (11.9%) samples had greater than three patterns identified.

Serogrouping

To confirm *Salmonella* that had all the same multiplex PCR pattern from one sample were potentially the same, serogrouping was performed (Table 5-1). When multiple isolates from the same sample had matching multiplex patterns (n=12), all isolates from the same sample matched in serogroup. In all cases where *Salmonella* from the same sample had matching multiplex patterns, the serogroup groupings matched the same groupings; different multiplex PCR patterns did not necessarily mean different serogroup. In each sample, all groupings based on the multiplex PCR pattern matched groupings based on serogroup.

As reported by Kim et al. (2006), the multiplex PCR is able to determine the serovar of the 30 most common clinical *Salmonella* serovars. In the current study, 494 isolates (87.9%) did not match the thirty most clinically common isolates, according to Kim et al. (2006), serovar patterns previously reported. Of the isolates matching reported multiplex patterns, suggested serovars included: Bovismorbificans (22),

Braenderup (9), Chester (1), Derby (1), Hadar (3), Meunchen (4), Newport (2), Ohio (9), Oranienburg (4), Poona (3), Saintpaul (3), Thompson (5), Typhimurium (1). Of these suggested serovars, only 31 (46.3%) serogrouped accordingly. Following serotyping by NVSL, none of the serovars matched the proposed serovar based on the multiplex PCR pattern.

Serotyping

One representative *Salmonella* isolate was sent from each multiplex PCR grouping per sample for serotyping. Of the starting 562 *Salmonella* isolates analyzed using the multiplex PCR method, 217 were sent to the NVSL for serotyping. A total of 31 *Salmonella* isolates were lost during freezer storage and were unable to be analyzed further. The frequency of isolation for each serovar obtained is shown in Table 5-2. Serovars with corresponding multiplex PCR patterns are shown in Tables 5-3. A total of 32 different serovars were isolated during the 12-month sampling from all sampling locations.

The most frequently isolated serovars for all samplings, were *S*. Muenchen (11.5%), *S*. Rubislaw (9.5%), *S*. Anatum (8.8%), *S*. Gaminara (8.8%), *S*. IV_50:z4,z23:- (6.8%). In region one (sites 1-6), serovars Rubislaw, Newport, Muenchen, Gaminara, and IV_50:z4,z23:- were the most frequently isolated; of the 32 serovars isolated, 16 were seen in region one. In region 2 (sites 7-12), serovars Muenchen, Anatum, Florida, Gaminara, IV_50:z4,z23:-, and Paratyphi_B_var.L-tartrate+ were the most frequently isolated; of the 32 serovars isolated, 20 were seen in region 2. In region 3 (sites 13-18), serovars Rubislaw, Muenchen, Gaminara, and Braenderup were the most frequently isolated; of the 32 serovars isolated, 16 were seen in region 3.

Considering isolates from all sampling sites, the greatest number of serovars, either nine or ten, were isolated August to December. All other months had either five or six different serovars isolated. No one serovar was isolated in all months of study from any of the sampling sites.

The number of different serovars isolated from any individual sampling site over the 12-month study ranged from two to 11 different serovars for the duration of the study. The average number of serovars from one site over 12 months was six serovars. The fewest serovars isolated from one site was site 14, a canal in a 55% agriculture land usage watershed where only two different serovars were isolated over the 12 months. The largest number of different serovars were isolated from a single site, were from site 10 (stream in a watershed containing 32% agriculture for land usage), site 4 (creek in a watershed containing 33% agriculture), and site 6 (stream in a watershed containing 32% agriculture); 11, 10, and 9 different serovars, respectively, were isolated from these sites over 12 months. For the three sampling sites with the largest number of serovars isolated: *S*. Anatum, *S*. Gaminara, *S*. Muenchen, and *S*. Newport were common to these three sites.

PFGE Analysis

Isolates sent for serotyping were further analyzed using PFGE *Xba*I macrorestriction banding patterns. Three separate dendrograms were generated, one for each of the geographical groupings of sampling sites, (Figures 5-1 to 5-3). The dendrogram generated for sampling sites 1-6, included 62 isolates and resulted in 10 genotypic clusters with a dice coefficient index cut-off point of 80%. The dendrogram generated for sampling sites 7-12, included 85 isolates and resulted in 17 genotypic clusters with a dice coefficient index cut-off point of 80%. The dendrogram generated

for sampling sites 13-18, included 71 isolates and resulted in 13 genotypic clusters with a dice coefficient index cut off point of 80%.

Antimicrobial Susceptibility Testing

Of the 248 *Salmonella* isolates tested for antimicrobial susceptibility, 24 isolates were susceptible to all antimicrobials. Forty-one *Salmonella* isolates were resistant to streptomycin. Fifty *Salmonella* isolates were resistant to both streptomycin and kanamycin (data not shown).

Eighteen *Salmonella* isolates were resistant to more than five of the antibiotics tested. These antibiotic resistant profiles are shown in Table 5-4. The antibiotic resistance profiles for these *Salmonella* isolates typically (17/18) included resistance to amoxicillin-clavulanic acid and/or cephalothin. Sixteen of the multi-antibiotic resistant *Salmonella* isolates were resistant to ampicillin and/or kanamycin; 15 of these *Salmonella* isolates were also resistant to cefoxitin and/or streptomycin. None of the multi-antibiotic resistant *Salmonella* isolates showed resistance to ceftriaxone or ciprofloxacin.

Ten of the multi-antibiotic resistant *Salmonella* isolates were isolated from sampling sites 1-6; the remaining eight were evenly distributed from sites 7-18. Of the 10 isolates from sampling sites located in sites 1-6, four came were isolated from site 4 and four isolates were isolated from site 5. Three *S*. Braenderup (three out of seven total isolated) and three *S*. Florida (three out of eight total isolated) isolates were multi-anitmicrobial resistant. Two isolates of the following serovars were multi-antimicrobial resistant: *S*. Bareilly (2/2), *S*. Gaminara (2/13), *S*. Hartford (2/5), and *S*. Muenchen (2/17).

Discussion

S*almonella* was isolated from 81.7% (165/202) of water samples using the described MPN methods, this percentage is similar to that reported by Haley et al. (2009) when reporting on *Salmonella* isolated from surface waters in the state of Georgia were 79% (57/72) positive samples. However, the population of S*almonella* may be greater in Central Florida, as a larger water sample volume (555 ml total) that was tested by Haley et al. (2009) compared to our current method that uses 33 ml total in the modified FDA-BAM MPN method. This hypothesis is furthered by a study performed in North Florida where 96% of samples (at 1,000 L total in an MPN) were positive for S*almonella* (Rajabi et al. 2011). In North Carolina, a similarly crop-agriculture dominated area, *Salmonella* prevalence was 50% (6/12; 25 ml sample volume) (Patchanee et al. 2010). The same North Carolina study had samples from residential/industrial areas at 58.8% (20/34) *Salmonella* positive, and a forested area with 57.1% (16/28) *Salmonella* positive (Patchanee et al. 2010).

From each of the *Salmonella* positive samples up to 36 *Salmonella* isolates were obtained, this gave a total of 562 isolates to further identify and characterize. Using the multiplex PCR to group *Salmonella* isolates from each sample as either different or potentially the same decreased the number of isolates to be further characterized to 248. Thus decreasing the number of isolates to less than half of what would have otherwise been required. This grouping of the *Salmonella* isolates was able to be confirmed using serogrouping. Thus, it was demonstrated that the multiplex PCR method described by Kim et al. (2006) can be used to group multiple environmental *Salmonella* isolates from the same sample as different or potentially the same. This can

greatly decrease the cost and amount of time required to screen numerous *Salmonella* isolates resulting from an environmental sampling.

The large number (494) of *Salmonella* isolates from the current study that did not match any of the previously reported banding patterns suggests that many of these *Salmonella* isolates are not included in the 30 most common clinical serovars (Kim et al. 2006). Additionally, this would support the suggestion that the multiplex PCR, as developed, has a limited ability to predict the serovar of some environmental isolates. This notion was further supported by our findings of only 31 *Salmonella* isolates serogrouping according to their predicted serovar, and furthermore by having none of the predicted serovar match the serovar reported by the NVLS.

This is somewhat different from work performed by Jean-Gilles Beaubrun et al. (2012) were they were able to predict the serovar of 76.2% (48/63) of the *Salmonella* isolates obtained from environmental samples taken from tomato farms in the Mid-Atlantic region of Virginia (Jean-Gilles Beaubrun et al. 2012). The 48 successfully serotyped contained *S*. Newport (13 isolates), *S*. Braenderup (10 isolates), and *S*. Typhimurium (4 isolates), these serovars were also isolated in the current study but were unable to be serotyped via the multiplex PCR method. In addition, the Virginia isolates included: *S*. Javiana, and *S*. Thompson; these serovars were not identified in the current study, so the multiplex PCR results cannot be compared. The seemingly increased proficiency of the multiplex-PCR reported for the Virginia isolates may be do to the few (n=5) different serovars identified during their study. In the current study we isolated 32 different serovars, none of which were successfully serotyped using the multiplex PCR method. Of the 32 serovars isolated in the current study, seven had

banding patterns reported by Kim et al. (2006) as members of the thirty top clinical serovars, however, these *Salmonella* isolates did not produce the expected banding pattern for any of these seven serovars.

Differences in banding patterns, including missing expected genes or the amplification of serovar specific PCR products not typical for the serotypic banding pattern, have previously been reported for *S*. Montevideo, *S*. Newport, *S*. Muenchen, *S*. Hadar, *S*. Infantis, *S*. Paratyphi, *S*. Derby, and *S*. Berta (Jean-Gilles Beaubrun et al. 2012). These authors also report more than one banding pattern for *S*. Seftenberg and *S*. Tennessee (Jean-Gilles Beaubrun et al. 2012). Thus there may be genotypic variations in serovars that were not previously expected. This makes the multiplex PCR unreliable as a method for consistently predicting the serovar of environmental *Salmonella* isolates, including those serovars with previously reported banding patterns. This may account for some of the unmatched banding patterns obtained for our *Salmonella* isolates.

The number of different serovars reported in our study is higher than the number of different serovars typically reported during surveys for *Salmonella* in surface waters (Haley et al. 2009, Patchanee et al. 2010, Rajabi et al. 2011). This may be due in part to the greater number of sampling sites included in the current study. A previous study in a crop agricultural area of North Carolina had five different serovars coming from 12 samplings, serovars as reported by the Ohio Department of Health, Columbus, Ohio (Patchanee et al. 2010). The previously discussed South Georgia study reported 13 different serovars being isolated, serovars as reported by *Salmonella* Reference Center, Philadelphia, PA (Haley et al. 2009). Following this trend, the North Florida study

reported eight different serovars, serovars as reported by *Salmonella* Reference Center, Philadelphia, PA (Rajabi et al. 2011). The current study isolated 32 different serovars from 18 sampling sites, serovars as reported by NVSL. The greater diversity of serovars may, in part, be a reflection of the greater distance and numerous watersheds sampled in the current study as compared with the one watershed studied by the other authors in each of which only one watershed was studied.

Surface waters surrounded with crop agriculture in North Carolina isolated *S*. Newport as their most frequently (9/14) isolated serovar; *S*. Brandenburg (n=1), *S*. Gaminara (n=1), *S*. Inverness (n=1), and *S*. Miami (n=2) were also isolated in the North Carolina study (Patchanee et al. 2010). With the exception of *S*. Brandenburg, these same serovars were also isolated in the current study.

Salmonella isolates from South Georgia have previously been reported to contain a high percentage (80/197 isolates) of subspecies 3 (Arizonae), while *Salmonella* isolates from North Florida did not contain any isolates from subspecies 3 (Haley et al. 2009, Rajabi et al. 2011). The current study did isolate serovars from subspecies 3, but at a much lower frequency (6/217). Additionally, the South Georgia isolated seven serovars that were isolated in the current study, these included: *S*. Muenchen (n=28), *S*. Rubislaw (n=26), *S*. Braenderup (n=12), *S*. Saintpaul (n=9), *S*. Bareilly (n=6), *S*. Gaminara (n=3), and *S*. Anatum (n=1) (Haley et al. 2009).

In a 1998-1999 study of the North Florida river, serovars isolated included: *S*. Inverness (n=9), *S*. Muenchen (n=3), *S*. Rubislaw (n=6), *S*. Braenderup (n=2), *S*. Montevideo (n=1), *S*. Newport (n=1), *S*. Johannesburg (n=1), and *S*. Cubana (n=1) (Rajabi et al. 2011). Of these isolates, *S*. Inverness, *S*. Muenchen, *S*. Rubislaw, *S*.

Braenderup, and *S*. Newport were isolated in the current study as well; *S*. Muenchen and *S*. Rubislaw were isolated frequently in the current study, 17 and 14 times respectively.

Salmonella Braenderup, *S*. Gaminara, *S*. Inverness, *S*. Muenchen, *S*. Newport, and *S*. Rubislaw were all isolated in at least two of the other studies described as well as in the current study. This may be suggestive of these serovars having an increased environmental presence as compared with other *Salmonella* serovars. Of these frequently environmentally isolated serovars, only *S*. Braenderup, *S*. Muenchen, and *S*. Newport are also on the CDC top twenty clinical isolates list of 2009 (CDC 2009). For all the serovars identified in the current study, only seven corresponded with the CDC's top twenty list of clinical serovars for the 2009 year. These seven were *S*. Bareilly (2), *S*. Braenderup (7), *S*. Muenchen (17), *S*. Newport (8), *S*. Paratyphi_B_var.L-tartrate+ (5), *S*. Saintpaul (2), and *S*. Typhimurium var 5 (2).

An increased number of different *Salmonella* isolates, determined by PFGE, as compared to other surface water samplings, came from the current study. When using the same analysis parameters, and the same restriction enzyme, the current study resulted in 10, 17, and 13 genotypic clusters in each of the three regions. The previously discussed North Carolina study resulted in seven genotypic clusters using the same parameters (Patchanee et al. 2010). The other surface water sampling studies previously used as a comparison for the current study did not report PFGE profiles.

Most of our isolates were either susceptible to all antibiotics tested or where resistant to kanamyocin and/or streptomycin. This finding could be expected because

both these antimicrobials are secondary metabolites of *Streptomyces* spp., a common soil microbe; thus the finding of resistance in environmental isolates is logical (Turpin et al. 1992, Egan et al. 2001). The higher incidence of multi-antimicrobial resistance in our *Salmonella* isolates may be indicative of *Salmonella* isolates that have come from animal agriculture, as this is more expected in environments influenced by animal agriculture. This can be seen in the study by Patchanee et al. (2010), when an increased amount of antimicrobial resistance was reported for isolates in a watershed known to be influenced by pig farms, as compared to isolates from locations influenced by crop agriculture only.

The continued persistence of *Salmonella* in Central Florida surface waters has been demonstrated. In our twelve-month study we isolated a large number (32) of different serovars of S*almonella*. This wide variety of isolates was also reflected in the DNA fingerprinting performed using PFGE. These *Salmonella* isolates could be grouped as different or potentially the same using a previously reported multiplex PCR (Kim et al. 2006). As evidenced by none of the serotyping results matching the serovar predicted by the multiplex PCR banding patterns, the multiplex PCR could not be used to accurately predict the serovar of the *Salmonella* isolates from Central Florida surface waters.

Table 5-1. Suggested serotypes and corresponding suggested serogroup based on multiplex PCR patterns for *Salmonella* isolated from Central Florida surface waters compared with actual serogroup determined by autoagglutination

Suggested Serovar	Suggested Serogroup	No. Isolates	Actual Serogroup							% Matching
			A	B	C	D	E	F	G	
Bovismorbificans	B	22	13	5			2			23
Braenderup	B	9		7			1		1	78
Chester	A	1			1					0
Derby	A	1		1						0
Hadar	B	3	1				2			0
Muenchen	B	4	2	2						50
Newport	B	2	2							0
Ohio	B	9		7			1		1	78
Oranienburg	B	4	2	2						50
Poona	B	3	1		2					0
Saintpaul	A	3	2	1						67
Thompson	B	5		5						100
Typhimurium	A	1	1							100
Other		494	57	88	46	6	9	5	17	

Table 5-2. *Salmonella* serovars isolated from Central Florida surface waters over 12 consecutive months compared with *Salmonella* serovars on the CDC's list of top 20 most clinically common serovars

CDC Top 20[a]	Serovar	Frequency of Isolation			
		Region 1	Region 2	Region 3	Total Current Study
	Anatum	3	7	3	13
	Baildon			1	1
19	Bareilly	2			2
11	Braenderup	3		4	7
	Florida	1	5	2	8
	Gaminara	4	4	5	13
	Georgia	3			3
	Give	1			1
	Hartford	2	3		5
	Inverness		1		1
	Ituri			3	3
	Lexington			1	1
	Litchfield			1	1
	Miami	2	3	1	6
10	Muenchen	4	8	5	17
3	Newport	5	3		8
	Norwich		2	1	3
15	Paratyphi_B_var._L-tartrate+		4	1	5
	Rubislaw	5	3	6	14
9	Saintpaul		2		2
2	Typhimurium var 5-	2			2
	Weslaco	2			2
	6,8:d:-		1		1
	III_16:z10:e,n,x,z15	1	3		4
	III_44:z4,z32:-		1		1
	III_60:r:z			1	1
	IV Rough O:z4,z23:-		1		1
	IV Rough O:z4,z24:-		1		1
	IV_40:z4,z24:-		2		2
	IV_50:z4,z23:-	4	4	2	10
	Rough O:y:1,5		1		1
	Rough_O:d:1,7			1	1
	Untypable	3	2	2	7

[a] CDC top 20 clinically common serovars, numbers indicate overall placement in top 20

Table 5-3. *Salmonella* serovars isolated over 12 consecutive months from Central Florida surface waters with each different multiplex PCR banding pattern for all serovars

Sampling Month	Sampling Sites	Serovar	Multiplex PCR Patterns
August	3, 5	Bareilly	No bands, H
	7, 8, 9	Florida	D, BEF, CEFGH, No bands
	2, 6, 12	Gaminara	BEGH, D, CEFG, CEFGH, H
	11	III_44:z4,z32:-	I, EI
	12	IV_50:z4,z23:-	CE, BCE
	13, 16	Muenchen	No bands, ABCDIJ, C
	1, 2, 5, 10	Newport	ABCEIJ, No bands, D, CDEIJ
	16	Norwich	CHIJ
	18	Rubislaw	C, No bands
September	18	Braenderup	BDEI
	15, 16	Florida	No bands
	4, 7	Gaminara	ADFG, BEGH
	10	III_16:z10:e,n,x,z15	BC
	18	Ituri	A
	6, 8	Muenchen	ABEIJ, ACDEHI, ACEIJ
	7	Norwich	BCGHIJ
	14	Rough_O:1,7	BCDEFG
	14	Rough_O:d:1,7	BEFG
	5, 6, 13	Rubislaw	H, BGH, ABGH, ACEJ
October	12	50:z4,z23:-	ABEFGHI
	7	Anatum	CH, BCDEG
	5	Florida	No bands, H
	10	Gaminara	BCDEFGH
	8	Hartford	BCDEIJ
	8	Miami	EGHI, CDEGHI
	1	Muenchen	BI, BCDEIJ
	4	Newport	CIJ
	7	Saintpaul	BCDJ
	2, 4	Typhimurium var 5-	No bands, CI

Table 5-3. Continued

Sampling Month	Sampling Sites	Serovar	Multiplex PCR Patterns
November	4, 7	Anatum	BCDE, BDEFGH
	13, 15, 16, 17	Gaminara	ADFGH, F, BCDEGH, No bands, C, J
	3, 4	Georgia	BEI, D
	11	III_17:z10:e,n,x,z15	ABCD, ABCDE
	10	Inverness	ABEJ
	12	IV Rough O:z4,z23:-	ACDEI
	8	IV Rough O:z4,z24:-	CDEI, CEI
	10, 11	IV_40:z4,z24:-	BCDE, ACDE
	9	Miami	ACDEGHI
	1, 6	Rubislaw	ABCDEGH
December	6, 7	Anatum	H, BCE
	17	Baildon	ABDH, ABDEH, BCH, BC, BFG, B, BCDEIJ, ACG, BE, AE
	8, 10	Hartford	BEJ
	6	Miami	BJ
	10	Newport	BCEJ
	7	Paratyphi_B_var._L.tartrate+	BEH, BEHJ
	10	Rough O:y:1,5	BEFG, BEG
	7, 9, 13, 14, 15	Rubislaw	BH, BCD, BC, C, D, No bands
	1, 11	Untypable	BEFGH, CHJ
January	3, 5	IV_50:z4,z23:-	EJ, J, BEI, No bands, BJ
	8	6,8:d:-	ABEI
	7, 18	Anatum	ABCE, ACE, BCDEIJ, CE, BCDE, BCE
	7	Gaminara	BEGH
	9, 11, 18	Muenchen	G, No bands, AE, BE
	17	Litchfield	BCE

Table 5-3. Continued

Sampling Month	Sampling Sites	Serovar	Multiplex PCR Patterns
February	4, 13	Anatum	ACE, No bands
	4	Give	AEG
	18	IV_50:z4,z23:-	E, AE
	18	Miami	EFJ
	10	Newport	BDEIJ
	8	Untypable	BF
March	18	Anatum	BCE, No bands
	3, 4	Hartford	B, BDEIJ, DEIJ
	17, 18	Ituri	No bands, E
	12	IV_50:z4,z23:-	No bands, AE, AEI
	12	Paratyphi_B_var._L.-tartrate+	AEGHI, BEGI
	2	Untypable	J
April	10	Anatum	No bands, ACE
	5	Georgia	C
	6, 8	III_16:z10:e,n,x,z15	BCGH, CI, AB
	2, 6	IV_50:z4.z23:-	CI, BCI, BCDEFI
	3, 6, 10	Miami	C, BCFI, EGHJ
	4	Muenchen	BCE, ABEJ
	4, 6	Weslaco	AC, ACEH
May	15	III_60:r:z	BGH
	17, 18	Muenchen	BDEGI, BDEJ, BEJ, I
	6	Newport	ABCDIJ, GI, BCDEIJ
	13	Paratyphi_B_var.L-tartrate+	CH
	18	Rubislaw	BH, BGH
	1, 17	Untypable	ABC, BD

Table 5-3. Continued

Sampling Month	Sampling Sites	Serovar	Multiplex PCR Patterns
June	10, 12	Anatum	ACE, No bands, A, ABCDE, ABCI
	10	IV_50:z4,z23:-	ACDE
	18	Lexington	BC
	9	Paratyphi_B_var._L.tartrate+	AEHI, ABEHI, DHI
	12	Saintpaul	ACDEGHI, ABCDEJ, CHI
	13	Untypable	CDH
July	2, 4, 5, 18	Braenderup	No bands, BEI, BEHI, BEGI, I BEF
	6, 18	Gaminara	ABFG, FG, F, BEFG, No bands
	5, 7, 9, 10, 11	Muenchen	BEI, ABCEJ, ACEJ, AC, CFIJ
	7	Norwich	BCGHIJ
	6, 7	Rubislaw	BEG, ABGH, ABCGH

Table 5-4. Antibiotic resistance profiles of *Salmonella* isolates from Central Florida surface waters, only isolates resistant to greater than five of the antibiotics screened are listed

Serovar	Site	Month	Antimicrobial Resistance Profile
Anatum	13	February	AmcAmFoxCfSTe
Bareilly	4	August	AmcAmFoxCfKSSTxTe
Bareilly	5	August	AmcAmFoxCfCKNaSSTxTe
Braenderup	2	July	AmcAmCfImpKS
Braenderup	4	July	AmcAmFoxKNaSSTx
Braenderup	5	July	AmcAmFoxCfCKNaStxTe
Florida	4	August	AmcAmFoxCfImpKS
Florida	5	October	AmcAmFoxCfKS
Florida	9	August	AmcAmFoxCfKS
Gaminara	15	November	AmcAmFoxCfCKNaSSTxTe
Gaminara	16	November	AmcAmFoxCfKS
Hartford	3	March	AmcFoxCfKNaSTe
Hartford	4	March	AmcAmFoxCfCKNaSTe
Muenchen	5	July	AmcCfCKNaSTxTe
Muenchen	11	July	AmcAmCfCKNaSTxTe
Rough_O:d:1,7	14	September	AmcAmFoxCfGmS
Rubislaw	7	July	AmFoxCfKSSTxTe
Saintpaul	12	June	AmcAmFoxCfKS

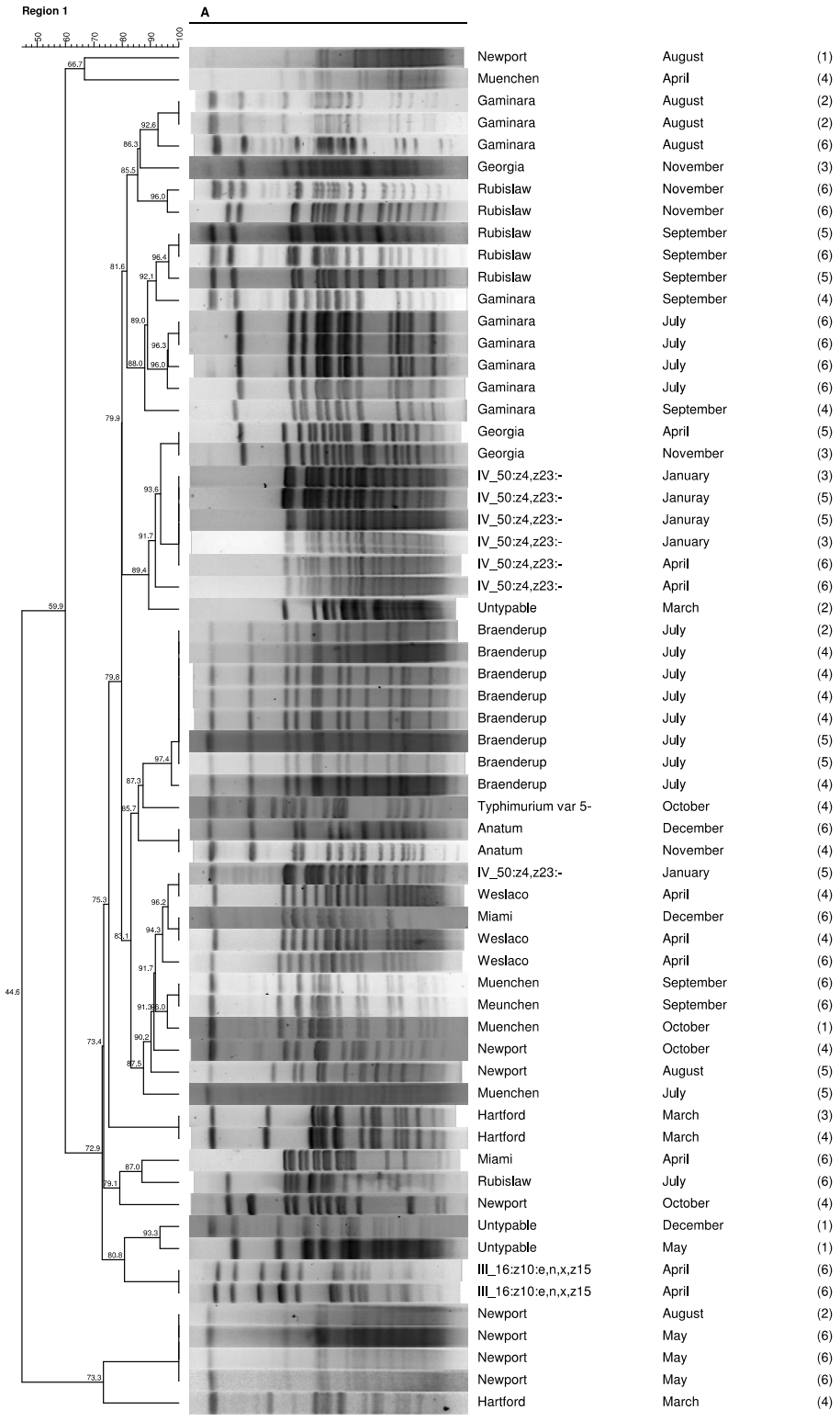

Figure 5-1. Dendrogram of representing PFGE-*Xba*I fingerprinting of *Salmonella* isolates recovered from sampling site 1-6, PFGE, pulsed-field gel electrophoresis

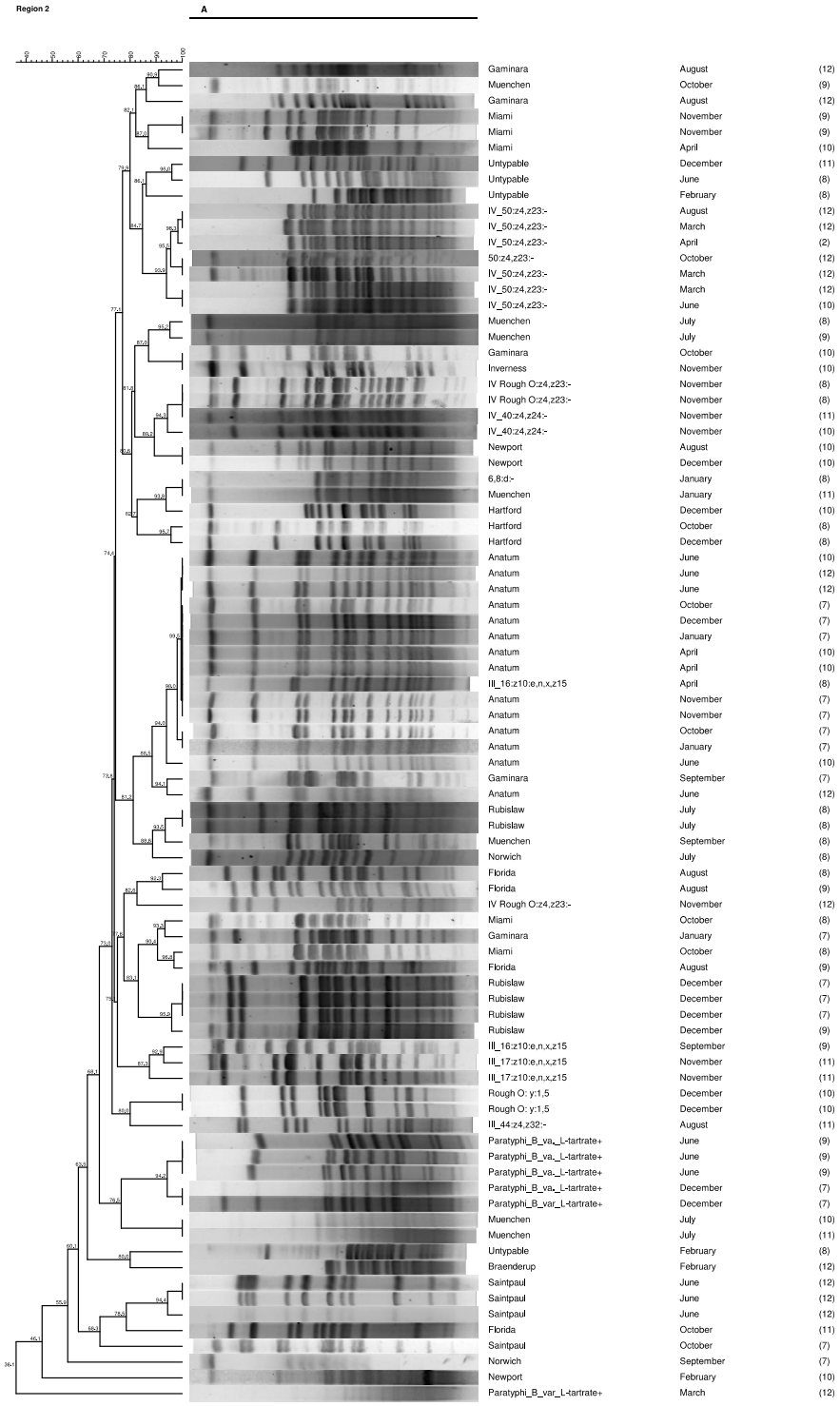

Figure 5-2. Dendrogram representing PFGE-*Xba*I fingerprinting of *Salmonella* isolates recovered from sampling sites 7-12. PFGE, pulsed-field gel electrophoresis

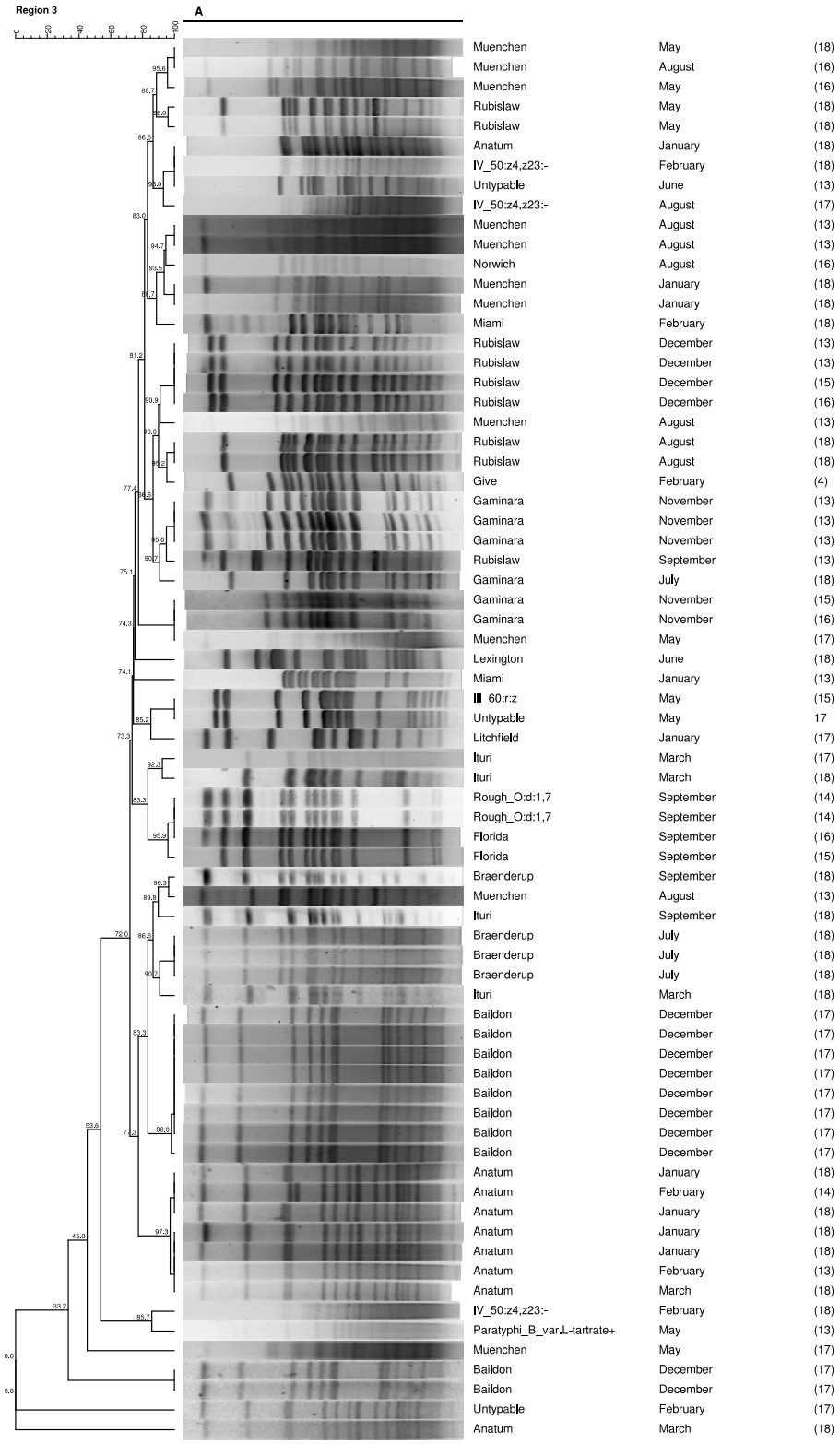

Figure 5-3. Dendrogram representing PFGE-*Xba*I fingerprinting of *Salmonella* isolates recovered from sampling sites 13-18. PFGE. Pulsed-field gel electrophoresis.

CHAPTER 6
FATE OF *SALMONELLA* SPP. IN FLORIDA SURFACE WATERS UNDER LABORATORY CONDITIONS

Salmonella is an important agent of gastroenteritis in humans. *Salmonella* can enter surface water from a variety of sources including: untreated or partially treated wastewaters, agricultural runoff, wastes from domestic and wild animals (Dechesne and Soyeux 2007, Haley et al. 2009, Bolton et al. 2012). The long-term survival of these bacteria in surface water resources constitutes a public health concern through direct consumption, indirect consumption (via irrigation of edible crops), and contact during recreation (Greene et al. 2008, Ijabadeniyi et al. 2011, Ge et al. 2012).

Indirect consumption of *Salmonella* through the use of contaminated irrigation water on edible horticultural crops has previously caused two known outbreaks: one associated with tomatoes and one associated with Serrano peppers. In both outbreaks, the outbreak serovar was isolated from irrigation ponds on the implicated farms (Greene et al. 2008, Behravesh et al. 2011). The causative agent in the tomato-borne outbreak was *Salmonella* Newport (Greene et al. 2008). A previous tomato-borne outbreak, from the same implicated farm, occurred several years prior and involved the same serovar. It is possible that the *S*. Newport survived this whole time in the environment, and possibly in the surface water. A review by Jacobsen et al. (2012) suggests that irrigation water may be one of the major sources of contamination of fresh produce (Jacobsen and Bech 2012).

Currently, only one regulatory microbial water quality standard exits for water used on edible horticultural crops, specifically Florida-grown tomatoes (FDACS 2007). Irrigation water is required to meet recreational water quality standards outlined in 40 CFR 131.41(c), that is water samples must not have *E. coli* populations exceeding a

geometric mean (n=5) of 126/100 ml and a single sample must not have an *E. coli* population exceeding 235/100 ml. However, there is a lack of scientific evidence exists to support the concept that *E. coli* populations can indicate overall water microbial safety.

Minimal work has focused on the duration of *Salmonella* survival in various types of environmental waters. Many previous works have also focused on marine or estuary waters, results from which cannot be entirely relied upon to predict survival in fresh water. Most studies have focused on clinically important serovars, such as *S*. Typhimurium, rather than other less clinically common serovars (Rhodes and Kator 1988, Maki and Hicks 2002, Chandran and Hatha 2005). The use solely of a local water source, sterile or non-sterile, incorporates uncontrolled variables into each set of experiments that in turn make the studies difficult to compare. Chemical, physical, and biological water characteristics may affect *Salmonella* survival.

The use of a standardized, and representative surface water should be included to control chemical and physical water quality characteristics between microcosm-based experiments. There exists a previously described representative surface water developed by the US Environmental Protection Agency (EPA) for the testing of camping water purifiers (US EPA 1987). This water is chemically defined and can be reproducibly prepared in-laboratory using few materials. Use of such a standard medium would make discrete experiments comparable to each other.

Relying solely on sterile water can overlook some potentially important biological variables that would be present in real-world situations. In non-sterile waters *Salmonella* would be exposed to competition from both autochthonous and

anthropogenic bacteria, predation from protozoa, and attack by bacteriophages. These variables need to be included in microcosm-based survival studies in order to more accurately represent the real world.

The relationship between the persistence of *Salmonella* spp., either individually or as a cocktail, and biological, physicochemical water quality characteristics, representative of surface waters, over six months was explored. The suitability of EPA worst case water as a standard medium for investigating *Salmonella* persistence was also evaluated.

Materials and Methods

Waters and Microcosm Conditions

Four waters were used to set up the microcosms: sterile deionized water, sterile surface water, non-sterile surface water, and EPA worst case water. EPA worst case water is a defined medium originally developed for testing point-of-use microbiological water purifiers (US EPA 1987). The EPA worst case water was prepared using deionized water, 1500 mg/l dissolved sea salts (Sigma-Aldrich, St Louis, MO), 10 mg/l humic acid (Sigma-Aldrich), and fine test dust (Powder Technology Inc., Burnsville, MN) to 30 FAU. The final pH of the EPA worst case water was 5.3.

Surface water was collected in a 10 L sterile autoclavable carboy (Nalgene, Rochester, NY, USA) fitted with 4 kg of lead weights all attached to a rope used to lower the carboy into the water source. The carboy was lowered to the water from a manmade structure that protrudes ca. 3 m from the shore; water was collected from approx. 20 cm below the surface. The water source was a fresh water pond in Polk County, Florida that is surrounded with non-animal agricultural lands. All waters listed as sterile were sterilized, in 1 L portions, in an autoclave at 121°C, 15 psi, for 15

minutes. Water was held at 4°C for no more than 24 h, before sterilization and/or being dispensed into the microcosms. Measured as described in Chapter 3, the non-sterile surface water had a turbidity of 9 FAU, and a pH of 7.69; the sterile surface water had a turbidity of 9 FAU, and a pH of 7.37.

To evaluate the effect of protozoal grazing, non-sterile surface water was supplemented with cycloheximide (LeJeune et al. 2001) (20 mg/1 ml; MP Biomedicals Inc, Solon, OH, USA) 24 h prior to the start of the experiment to destroy protozan.

Microcosms, 100 ml, were prepared and stored in 120 ml sterile polypropylene cups (Oxford, Mansfield, MA, USA). A separate cup was prepared for each sampling time to remove the chance of contamination during successive samplings. Three storage temperatures were selected: 15°C, 21°C, and 28°C to represent Central Florida average temperatures during winter, spring/fall, and summer (see Chapter 3). For each temperature, water was stored at the appropriate temperature for 24 h prior to the beginning of the experiment to ensure the water was at the correct temperature before inoculation. The microcosms were held stagnant, protected from light, at the appropriate constant temperature for up to six months. At each sampling time one microcosm was removed and sampled; unused portions were disposed of.

Bacterial Strains and Culture Preparation

Salmonella serovars used were initially isolated from surface water in Central Florida (see Chapter 4); serovars used included: Antatum (MDD 48), Newport (MDD 59), and Gaminara (MDD 50); available from the culture collection of Dr. MichelleD. Danyluk. Serovars were stored at -80°C in Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA), supplemented with 15% glycerol, until time of use. Serovars were streaked to Xylose Lysine Tergitol-4 agar (XLT4; Difco, Becton

Dickinson) and incubated 18 h at 35±2°C. One colony was picked and inoculated into TSB and incubated 18 h at 35±2°C; 10 µl was transferred to fresh TSB and incubated 18 h at 35±2°C. Cells were washed twice in 0.1% peptone water (Difco, Becton Dickinson). Prior to use in experiments cultures were diluted in 0.1% peptone water to ca. 8 log CFU/ml prior to being inoculated into the pre-dispensed water. Serovars were either used separately or prepared as an equal part cocktail. A separate inoculum was prepared for each repetition. Three repetitions were performed for the single-serovar microcosms; six repetitions were performed for the cocktail microcosms.

Microbiological Analysis

One microcosm from each water/temperature/inoculum combination was enumerated at 0, 6, 12, and 24 h, and on days 2, 3, 5, and 7 days. Deionized water, sterile surface water, non-sterile surface water, and EPA worst case water microcosms were additionally enumerated at 14 days, and at monthly increments thereafter until six months.

Salmonella populations were enumerated by removing a one-milliliter aliquot from the microcosm and preparing serial dilutions in 0.1% peptone water. Appropriate dilutions (0.1 ml) were spread plated, in duplicate, onto XLT4. Plates were incubated 24±2 h at 35±2°C and all typical *Salmonella* colonies were counted by hand. If *Salmonella* populations were predicted to be below 2.3 log CFU/ml, one ml was spread plated onto four XLT4 (250 µl per plate) to increase the limit of detection; these plates were incubated and counted in the manner described above.

Once *Salmonella* populations were predicted to be below 1 CFU/ml, a three-by-four tube MPN was used to enumerate cells; dilutions were as follows: 10 ml water in 10 ml double strength lactose broth, 1 ml water in 9 ml single strength lactose broth, and

0.1 ml water in 9 ml single strength lactose broth. The remaining water sample (60 ml) was pre-enriched in 30 ml triple strength lactose broth in the original polypropylene cup. MPN tubes were incubated for 24±2 h at 35±2°C. One ml aliquot of the pre-enrichment were transferred to tetrathionate broth (TT broth; Difco, Becton Dickinson); TT broth was incubated 24±2 h at 35±2°C. Following enrichment, 10 µl was streaked onto XLT4 and incubated for 24±2 h at 35±2°C. Following incubation, colonies displaying typical *Salmonella* phenotypes were confirmed biochemically on lysine iron agar slants (LIA; Difco, Becton Dickinson), and triple sugar iron agar slants (TSI; Difco, Becton Dickinson). MPN values were calculated using the FDA-BAM table (US-FDA 1998).

Coliform and *E. coli* populations were also enumerated from non-sterile waters. A 1 ml aliquot was taken from the selected microcosm and serial dilutions were prepared in 0.1% peptone water. Appropriate dilutions were spread plated, in duplicate, onto Chromagar ECC (DRG, Mountainside, NJ, USA). Plates were incubated 24±2 h at 35±2°C and all typical coliform (mauve) and *E. coli* (blue) colonies were counted by hand. If populations were predicted to be below 2.3 log CFU/ml, one ml was spread plated onto four Chromagar ECC (250 µl per plate) to increase the limit of detection to 1 CFU/ml; these plates were incubated and counted in the manner described above.

Distribution of strain survival in cocktails

Once *Salmonella* populations in cocktail microcosms reached the limit of detection for spread plating, 100 colonies from each temperature/water combination were transferred to tryptic soy agar (TSA; Difco, Becton Dickinson). Plates were incubated 24±2 h at 35±2°C. *Salmonella* serovars in the cocktail were distinguished by serogroup. The serogroup of each of the 100 colonies was determined by scraping a colony from the TSA plate, and placing in 6 µl saline on a clean glass microscope slide.

One drop of *Salmonella* O Antiserum Poly A, B, or C (Difco, Becton Dickinson) was added and the slide rocked back and forth for 1 min; autoagglutination was noted.

Statistical Analysis

Salmonella and coliform enumeration data from each of the replicates on each sampling time from all microcosms were log-transformed and plotted against time. JMP Pro 9.0 (SAS, Cary, NC, USA) was used to determine statistical significances using ANOVA and Tukey's HSD test. The critical *P*-value was set at 0.05.

Results

Cocktail in Each Water and Temperature Combination Over Time

The fate of *Salmonell*a in various surface waters was evaluated over a six-month period. *Salmonella* was inoculated, either as a cocktail or individual serovars, into four types of surface waters and holding at three different incubation temperatures (Figures 6-1 to 6-4). At time zero, no significant differences (P>0.05) between *Salmonella* populations existed between water types or temperatures used in this experiment. At all but one time point (discussed below), there was no difference in the behavior between the individual serovars and the cocktail; only the cocktail data will be discussed in detail. Unless otherwise stated, the data below are in regards to *Salmonella* populations from the cocktail inoculated microcosms.

DI water

When held at 15°C, *Salmonella* populations in DI water were not significantly different from each other for the first 14 days, with an average cocktail population of 7.6±0.2 log CFU/100 ml (Figure 6-1A). Following one month of storage at 15°C, the *Salmonella* population significantly declined, by 0.9 log CFU/100 ml, compared to the previous 14 days; no significant change in *Salmonella* populations occurred between

one and six months in the 15°C DI water. The average *Salmonella* population from one to six months was 6.9±0.5 log CFU/100 ml.

At 21°C *Salmonella* populations in DI water increased between six and 12 h from 7.5±0.3 log CFU/100 ml to 8.0 ±0.1 log CFU/100 ml; this was a slight, but statistically significant increase (Figure 6-1A). *Salmonella* populations then remained statistically the same until a decrease at four months of incubation; at four months *Salmonella* populations were 7.7±0.1 log CFU/100 ml and did not significantly change for the remaining time. The six-month *Salmonella* population level was not significantly different from that at time zero or 6 h of incubation.

At 28°C *Salmonella* populations in DI water remained constant until the one month enumeration with an average population of 8.5±0.2 log CFU/100 ml (Figure 6-1A). At two months of incubation *Salmonella* populations had decreased to 8.1±0.04 log CFU/100 ml; *Salmonella* populations decreased again at three months to 7.2±0.5 log CFU/100 ml, then remained unchanged until the final six-month enumeration.

Sterile surface water

Sterile surface water at 15°C had *Salmonella* populations remain stable for the beginning two months; *Salmonella* populations were at 7.8±0.3 log CFU/100 ml (Figure 6-1B). *Salmonella* populations decreased significantly between two and three months, to 7.6±0.9 log CFU/100 ml, then remained stable through the duration of the experiment. At 21°C *Salmonella* populations remained stable for a longer duration, populations remained at an average of 8.1±0.4 log CFU/100 ml and did not decrease significantly until five months (Figure 6-1B). *Salmonella* populations continued to remain stable at 7.5±0.5 log CFU/100 ml between five and six months. At 28°C *Salmonella* populations in sterile surface water were more variable over time, yet

remained within the range of 8.7±0.1 log CFU/100 ml and 7.3±0.1 log CFU/100 ml throughout the six month incubation (Figure 6-1B). The final six month *Salmonella* population in 28°C sterile surface water was 7.0±0.4 log CFU/100 ml.

Non-sterile surface water

Non-sterile surface water at 15°C *Salmonella* populations remained constant between time zero and one day, averaging 7.8±0.1 log CFU/100 ml (Figure 6-1C). *Salmonella* populations then continuously decreased at each sampling time, requiring MPN enrichment at three months and thereafter. At six months, *Salmonella* populations in non-sterile surface water at 15°C, had reached 2.7±0.8 log MPN/100 ml. *Salmonella* populations behaved similarly at 21°C and 28°C, reaching 3.8±0.7 log MPN/100 ml and 0.9 log MPN/100 ml, respectively, at six months (Figure 6-1C).

Coliform populations started at 7.6±0.8 log CFU/100 ml at all three temperatures of incubation. Coliform populations increased significantly ($P<0.05$) at 12 h to 8.3±0.1 log CFU/100 ml, after this time point the coliform populations continually decreased. At four months of incubation coliforms were undetectable in all three incubation temperatures (limit of detection 1 log CFU/100 ml). No significant difference existed in coliform populations between the different temperatures of storage at any time of enumeration for the six-month experiment (data not shown). *Escherichia coli* was not detected at any point during the experiment.

EPA worst case water

Salmonella populations in EPA worst case water at 15°C had no significant difference from time zero until day three with an average of 7.6±0.3 log CFU/100 ml (Figure 6-1D). At day five an increase to 8.0±0.3 log CFU/100 ml had occurred. *Salmonella* populations did not change again until four months. At four months

Salmonella populations had decreased, significantly, to 7.7±0.1 log CFU/100 ml; *Salmonella* populations in EPA worst case water at 15°C then did not significantly change through to the final six month enumeration.

Salmonella populations in EPA worst case water at 21°C increased from 7.9±0.1 log CFU/100 ml to 8.3±0.1 log CFU/100 ml, at 12 h of incubation (Figure 6-1D). *Salmonella* populations then remained statistically unchanged through three months. At four months *Salmonella* populations had decreased, to 8.0±0.0 log CFU/100 ml, and then remained at this level for the duration of the experiment.

When EPA worst case water was incubated at 28°C, *Salmonella* populations had increased by the first enumeration time point (6 h), from 7.9±0.1 log CFU/100 ml to 8.7±0.3 log CFU/100 ml (Figure 6-1D). *Salmonella* populations then remained statistically not different until a decrease at four months occurred; *Salmonella* populations had deceased to 7.1±1.4 log CFU/100 ml. *Salmonella* populations were not significantly different for the remained of the experiment.

Fate of Individual Serovars Compared to Cocktail Over Time

At time zero, no significant differences ($P<0.05$) between *Salmonella* populations existed between the cocktail or individual serovars used in this experiment (Figures 6-1. 6-2, 6-3, 6-4). The only point where a significant difference existed between serovars was at five months, where *S.* Anatum populations were significantly higher than *S.* Gaminara populations (Figures 6-2 and 6-3); *S.* Newport and the cocktail populations were not significantly different from each other or *S.* Anatum or *S.* Gaminara populations. This significant difference was not seen at the six-month enumeration.

Serovar Survival Within Cocktail Inoculated Waters

In order to evaluate if all *Salmonella* serovars behaved the same in the cocktail inoculated microcosms, or if there was variability amongst the serovars, 100 colonies from the final enumeration by spread plating were serogrouped. The percentage of each serovar in the final *Salmonella* population was determined (Figure 6-5). The last enumerations by plate count for non-sterile surface waters were completed at two months, therefore serogrouping for these samples was performed at this time point. All other water types were able to be enumerated by plating at six months; serogrouping on these samples was performed at this point. The percentage of each serogroup is shown in Figure 6-5. At two months non-sterile surface water at 28°C, *S.* Anatum, *S*. Newport and *S*. Gaminara represented 48% (±6.5), 49% (±10), and 3% (±3.8) of colonies recovered, respectively (Figure 6-5A); at 21°C *S*. Anatum, *S*. Newport, and *S*. Gaminara represented 71% (±13), 25% (±11), and 4 (±3) of colonies recovered, respectively (Figure 6-5B); at 15°C *S*. Anatum, *S*. Newport, and *S*. Gaminara represented 32% (±26), 42% (±20), and 25% (±20) of colonies recovered, respectively (Figure 6-5C).

At six months, *Salmonella* serovars Anatum or Gaminara dominated the recovered colonies in DI water at 28°C (Figure 6-5A); three repetitions had *Salmonella* populations that were predominately (100-89%) *S*. Gaminara, and three repetitions had predominately *S*. Anatum (100-89%). Only one colony from DI water at 28°C serogrouped as *S*. Newport. In DI water at 21°C, *Salmonella* populations were predominately (74%±20) *S*. Gaminara (Figure 6-5B); the remaining colonies were *S.* Anatum (11% ±10) and *S*. Newport (15% ±14). In DI water at 15°C *S*. Gaminara was predominate in all but one repetition (Figure 6-5C), in which *S.* Anatum dominated

(84%). With the one *S*. Anatum-dominated microcosm removed, the recovered colonies from DI water at 15°C were 15% (±12) *S*. Anatum, 16% (±15) *S*. Newport, and 50% (±34) *S*. Gaminara.

At six months in sterile surface water at 28°C, *Salmonella* populations were 70% (±14) *S*. Anatum, 15% (±10) *S*. Newport, and 16% (±14) *S*. Gaminara (Figure 6-5A). At 21°C sterile surface water, *Salmonella* populations consisted of 61% (±13) *S*. Anatum, 32% (±14) *S*. Newport, and 5% (±7) *S*. Gaminara (Figure 6-5B). At 15°C sterile surface water *Salmonella* populations consisted of 29% (±32) *S*. Anatum, 20% (±25) *S*. Newport, and 50% (±32) *S*. Gaminara.

At six months EPA worst case water at 28°C had *Salmonella* populations predominated by *S*. Anatum (80% ±16); the other recovered colonies were 14% (±16) *S*. Newport and 7% (±7) *S*. Gaminara (Figure 6-5A). At 21°C, EPA worst case water *Salmonella* populations were predominately (63% ±19) *S*. Anatum, the other recovered colonies were *S*. Newport (27% ±9), and *S*. Gaminara (11% ±14; Figure 6-5B). At 15°C EPA worst case water *Salmonella* populations consisted of 57% (±10) *S*. Anatum, 23% (±10) *S*. Newport, and 19% (±12) *S*. Gaminara colonies (Figure 6-5C).

Effect of Water Type and Temperature on the Fate of *Salmonella*

DI water

Over six-months, *Salmonella* populations in DI water, EPA worst case water, and sterile surface water were either not significantly different or significantly different at only one time point, then returning to not significantly different at the subsequent sampling times (Figure 6-1). At day three, *Salmonella* populations in non-sterile surface water were significantly lower than those in DI water (Figure 6-1A and 6-1C); *Salmonella*

populations in non-sterile surface water remained significantly lower than those in DI water for the remainder of the study.

In DI water, *Salmonella* populations at 15°C and 21°C were significantly lower than those samples incubated at 28°C (Figure 6-1A). Over day ones through seven, the lowest *Salmonella* populations in DI water were at 15°C; after day seven no difference existed between 15°C and 21°C. At 12 h, and there after, DI water at 28°C had statistically lower *Salmonella* populations than the other water types at the same temperature.

Sterile surface water

Salmonella populations in sterile surface water were not significantly different from *Salmonella* populations in DI water or EPA worst case water for the first five days (Figure 6-1B). At day three, *Salmonella* populations in sterile surface water were significantly higher than those in non-sterile surface water. From day seven to three months, *Salmonella* populations in EPA worst case water and sterile surface water were not significantly different from each other, but were higher than *Salmonella* populations in either DI water or non-sterile surface water. After four months, sterile surface water *Salmonella* populations were not significantly different from EPA worst case water or DI water *Salmonella* populations.

Salmonella populations in sterile surface water at 15°C and 21°C were significantly lower than those incubated at 28°C (Figure 6-1B). From 12 h to seven days, *Salmonella* populations were lowest at 15°C for sterile surface water; after seven days there was no significant difference in *Salmonella* populations in sterile surface water between 15°C and 21°C.

Non-sterile surface water

Salmonella populations in non-sterile surface water were not significantly different from those in DI water or sterile surface water up to 12 h (Figure 6-1); *Salmonella* populations in non-sterile surface water were significantly higher than those in EPA worst case water at 6 h. At day three of incubation, and there after, the non-sterile surface water had significantly lower populations of *Salmonella* compared to the other water types.

From 12 h to day two, *Salmonella* populations were lowest at 15°C incubation for non-sterile surface water (Figure 6-1C). At day three and beyond, the lowest *Salmonella* populations occurred in non-sterile surface water at 28°C or 21°C, followed by non-sterile water at 15°C.

EPA worst case water

EPA worst case water had the lowest populations of *Salmonella*, of all the water types, at 6 h and 12 h (Figure 6-1D). Between 24 h and seven days, EPA worst case water *Salmonella* populations were not significantly different from those in DI water or sterile surface water. By day three, *Salmonella* populations in EPA worst case water were significantly higher than those in non-sterile surface water. At day seven, EPA worst case water and sterile surface water had *Salmonella* populations not significantly different from each other, but were higher than *Salmonella* populations in either DI water or non-sterile surface water; this same pattern existed to, and including, three months. After three months, EPA worst case water *Salmonella* populations were not significantly different from sterile surface water or DI water.

Salmonella populations at 15°C and 21°C were significantly lower than those samples incubated at 28°C (Figure 6-1D). At 12 h through to, and including, seven

days, *Salmonella* populations were lowest at 15°C incubation for EPA worst case water. At 14 days and beyond, no significant difference existed between *Salmonella* populations at 15°C and 21°C.

Effect of Eukaryotes in Non-Sterile Surface Water

At time zero there was no difference between *Salmonella* populations in sterile surface water, non-sterile surface water, or non-sterile surface water with cycloheximide added, at any of the temperatures used in this experiment, 15°C, 21°C, or 28°C. Figure 6-6 displays the survival of *Salmonella* in sterile surface water, non-sterile surface water, and non-sterile surface water with cycloheximide added over seven days. At 6 h all water samples held at 28°C had *Salmonella* populations statistically higher than those at the other two temperatures. Non-sterile surface water with cycloheximide had *Salmonella* populations of 7.2±0.2 log CFU/100 ml at 28°C, 6.7±0.2 log CFU/100 ml at 21°C, and 6.3±0.1 log CFU/100 ml at 15°C. At 12 h the waters held at 21 or 28°C were not significantly different; microcosms held at 15°C had statistically lower ($P<0.05$) *Salmonella* populations. Non-sterile surface water with cycloheximide had *Salmonella* populations of 7.1±0.1 log CFU/100 ml at 28°C, 6.9±0.1 log CFU/100 ml at 21°C, and 6.3±0.2 log CFU/100 ml at 15°C.

Differences based on water types did not develop until one day of incubation, at this time sterile surface water had statistically lower *Salmonella* populations at 21°C and 15°C; no significant difference existed for waters incubated at 28°C. At day one, sterile surface water had *Salmonella* populations of 7.6±0.1 log CFU/100 ml at 28°C, 6.0±0.2 log CFU/100 ml at 21°C, and 6.1± 0.3 log CFU/100 ml at 15°C; non-sterile surface water with cycloheximide had *Salmonella* populations of 7.5±0.1 log CFU/100 ml at 28°C, 7.4±0.1 log CFU/100 ml at 21°C, and 7.0±0.1 log CFU/100 ml at 15°C; non-sterile

surface water had *Salmonella* populations of 7.1±0.3 log CFU/100 ml at 28°C, 7.3±0.3 log CFU/100 ml at 21°C, and 6.8±0.1 log CFU/100 ml in 15°C. The same differences based on water type and temperature continued to exist for two and three days of incubation.

At five days of incubation, non-sterile surface water microcosms had statistically lower populations of *Salmonella*; at this time the sterile surface water and the non-sterile surface water with cyclohexmide were not statistically different. At five and seven days incubation microcosms held at 28°C had lower *Salmonella* populations than microcosms at 21 or 15°C, which were not significantly different.

These microcosms were held for a total of seven days. By the seventh day sterile surface water at the three incubation temperatures had the statistically highest *Salmonella* populations; sterile surface water had *Salmonella* populations of 8.2±0.2 log CFU/100 ml at 28°C, 7.7±0.7 log CFU/100 ml at 21°C, and 7.2±1.0 log CFU/100 ml at 15°C. These were followed by non-sterile surface water with cycloheximide at 15°C with *Salmonella* populations of 6.5±0.2 log CFU/100 ml, and non-sterile surface water at 15°C and 21°C with *Salmonella* populations of 6.1±0.4 log CFU/100 ml and 5.7±0.6 log CFU/100 ml, respectively. The lowest *Salmonella* populations were from the non-sterile surface water with cycloheximide at 28°C and 21°C with *Salmonella* populations of 4.5±0.5 log CFU/100 ml and 5.0±0.2 log CFU/100 ml, and non-sterile surface water at 28°C with *Salmonella* populations of 5.2±0.6 log CFU/100 ml.

Coliforms in non-sterile water with or without cycloheximide at all three temperatures were not different at time zero; coliform populations started at 6.4±0.1 log CFU/100 ml. No *E. coli* was detected at time zero, or at any time during this

experiment. By 12 h of incubation at 15°C coliform populations decreased to 6.3±0.1 log CFU/100 ml; while those held at 21 or 28°C had an increase in coliform populations, averaging 7.0±0.1 log CFU/100 ml. After 24 h there was no difference in coliform populations for any of the variables attempted, coliform populations averaged 6.6±0.3 log CFU/100 ml. Coliform populations remained not significantly different from each other throughout all the variables until day five. At day five non-sterile surface water with cycloheximide incubated at 15°C was significantly lower, 4.8±0.3 log CFU/100 ml, than non-sterile surface water with cycloheximide incubated at 28°C, which had a coliform population of 4.7±0.0 log CFU/100 ml; all other variable combinations were not different from each other. The same difference existed at seven days (the final enumeration of this experiment).

Overtime, the coliform populations in both non-sterile water types incubated at 21°C significantly decreased ($P<0.05$) between day zero and day seven; by day seven coliform populations in non-sterile surface water were 5.2±0.2 log CFU/100 ml and coliform populations in non-sterile surface water with cycloheximide were 5.3±0.5 log CFU/100 ml. Coliform populations in both non-sterile water types incubated at 28°C, had a significant decrease ($P<0.05$) by day 2; by day two, coliform populations in non-sterile surface water were 5.8±0.3 log CFU/100 ml and coliform populations in non-sterile surface water with cycloheximide were 6.0±0.1 log CFU/100 ml. Coliform populations in both non-sterile water types at 15°C experienced no significant difference over the seven day incubation; by day seven, coliform populations in non-sterile surface water were 5.7±0.5 log CFU/100 ml and coliform populations in non-sterile surface water with cycloheximide were 6.5±0.6 log CFU/100 ml.

Discussion

The three microcosm temperatures were selected to represent average winter, spring/fall, and summer temperatures in Central Florida. Previous work has elucidated that in Central Florida, air temperature can be used to predict surface water temperatures (Chapter 4). Ten year average air temperatures at 60 cm from three local weather-recording stations maintained by the Florida Automated Weather Network (http://fawn.ifas.ufl.edu) were used to determine the three microcosm temperatures used in this study. January, February and March temperatures were used to represent winter; April, May, October, November, and December to represent spring/fall; June, July, August and September were used to determine summer. These month groupings gave the smallest standard deviations for the average temperatures (data not shown). The beginning enumeration time points, up to five days, 15°C incubation temperature supported the lowest *Salmonella* populations. During winter months in Florida air temperatures can go below freezing, when this occurs it is common practice to spray crops with water in order to prevent freezing of the crops. This practice is known as frost-protection. As 15°C is representative of the winter months in Central Florida, this can be interpreted as beneficial for a decrease in risk of using surface waters for frost-protection of edible horticultural crops. Although this practice should still be limited because *Salmonella* has been demonstrated to survive for a long duration of time in such waters.

EPA worst case water should be considered a suitable standard medium for investigating *Salmonella* persistence in surface waters. The inclusion of a standard medium in microcosm-based experiments would aid in allowing multiple studies to be compared more fairly than when the test medium is not standardized. EPA worst case

water was evidenced here to support both individual serovars and serovars within a cocktail at the same level or better than sterile surface water at all three temperatures evaluated. EPA worst case water was not different from other water types until three days at which point the non-sterile water had lower *Salmonella* populations. An interesting addition to EPA worst case water as a standard for water-based microcosm work would be the development a standard background biota (protozoa, bacteria, and bacteriophages). However, much work would need to be performed and evaluated to determine a standard biota.

At five days of incubation EPA worst case water was not different from the other sterile waters used in this experiment. After seven days EPA worst case water was supporting a higher *Salmonella* population than all the other waters. Growth of *Salmonella* Tennessee has previously been reported in diffusion chamber based experiments performed using estuary waters at or above 18°C, in both with and without natural biota present (Rhodes and Kator 1988). Additionally, *Salmonella* (not serotyped), originally isolated from sewage, has been observed to increase from 6.7 log CFU/ml to 8.1 log CFU/ml in sterile creak water within 10 days, and continue to survive up until 56 days which marked the end of the experiment (Fish and Pettibone 1995). This would suggest that EPA worst case water is, in fact, representing the worst case in terms of *Salmonella* survival. At four months EPA worst case water incubated at 21°C had the highest *Salmonella* populations, of all the variable combinations, this is disadvantageous in terms of risk assessment as 21°C was the average temperature established to represent spring and fall water temperature; spring and fall are the seasons in which most Florida produce is grown.

For the six-month experiment, in the non-sterile surface waters coliform populations decreased, from 7.5±0.9 log CFU/100 ml at time zero which was not significantly different from time zero *Salmonella* populations, to below the detection limit of 1 CFU/ml at four months; this occurred prior to the *Salmonella* populations decreasing to this level. The coliform population becoming undetectable before *Salmonella* populations would suggest that coliforms do not make for good indicator organisms for *Salmonella* in surface waters representative of Central Florida surface waters. This is especially true when the contamination event is expected to have occurred months prior to sampling being performed. In this event, testing for total coliforms would classify the water as meeting potable water standards and the water permitted for use for foliar application near time of harvest for tomato plants.

The serovars chosen for these experiments were *S.* Anatum, *S*. Newport, and *S*. Gaminara. Each of these isolates were originally isolated from Central Florida surface waters (Chapter 5). Each serovar classifies into a separate serogroup so that colonies identified at final enumeration can be distinguished based on serogroup. *Salmonella.* Anatum was selected because of its prevalence of detection in the 12 month Central Florida surface water survey (Chapter 5). *Salmonella* Newport was selected as it has been associated with multiple tomato-borne outbreaks where *S.* Newport was isolated from an irrigation pond on the implicated tomato farm (Greene et al. 2008). *Salmonella* Gaminara was selected because, although it has caused few produce-borne outbreaks, it was associated with a fresh orange juice outbreak borne outbreak in the state of Florida.

No significant difference existed between the serovars of *Salmonella* used in the inoculum or in the enumeration of *Salmonella* populations originally from single serovar inoculums compared to the cocktail prepared from equal volumes of each serovar. When colonies from the final six-month enumeration of *Salmonella* populations in sterile surface water or EPA worst case water, held at either 21 or 28°C, *S.* Anatum was the dominate serovar. A more even distribution was obtained when these waters were held at 15°C. The predominance of *S*. Anatum is not entirely surprising because of its high level of isolation during the Central Florida surface water survey (Chapter 5). The predominance, in some cases >80% of the colonies, of one serovar may suggest that surface waters, under set conditions, are able to support a certain level of *Salmonella*, regardless of the serovar, but when mixed serovars are present one will predominate the population.

Colonies isolated from DI water, at all three temperatures, were predominantly *S*. Gaminara, with very poor *S*. Newport survival. This may suggest that *S*. Newport does not compete well with other *Salmonella* present, but is equally capable of survival in no-nutrient water (as is evidenced by the lack of significant difference between single serovar microcosms). The survival of all the serovars from this experiment surviving up to, and including, six months in the DI water is comparable to the a previous study were *Salmonella* Typhimurium DT-104 survived 54 days in 23°C sterile well water, after which no more enumerations were attempted (Moore et al. 2003).

Colonies isolated from non-sterile surface water were serogrouped at two months, the last enumeration via spread plating. All serogrouping was performed on colonies from spread plating, and not the enrichment MPNs, as either the pre-

enrichment or selective-enrichment could conceivably select for one serovar over the others, and thus skew the serogrouping results. At two months at both 28 and 21°C, microcosms had a low percentage of colonies serogroup as *S*. Gaminara; *S*. Anatum and *S*. Newport percentages were not significantly different from each other. The difference in the trend of serovar recovery is likely due to one of two causes or a combination: bacteriophage present that are better able to attack *S.* Gaminara, and/or *S*. Gaminara are grazed upon at a greater rate by protozoa. The phage hypothesis could be supported further by the lack of difference in *Salmonella* population levels for the first 24 h in non-sterile water that was then followed by a continual decrease in population level over the remaining six months. The delay in population decrease could suggest the attachment and infection phase, prior to cell lysis by bacteriophage attack. We cannot suggest that if bacteriophage were present, that they were only capable of attacking *S*. Gaminara, due to the lack of significant differences among the *Salmonella* populations in the individual serovar microcosms. A cocktail of three bacteriophages, isolated from sewage, has been used to reduce *Salmonella* Kentucky, *S*. Anatum, and *S*. Zanzibar populations in treated wastewaters (Turki et al. 2012). A significant decrease in *Salmonella* after 8 h in treated wastewater with the bacteriophage cocktail was achieved, however, incubation was at 30°C or 37°C. and a phage titer of 4 log PFU/ml (Turki et al. 2012). The higher incubation temperatures may explain some of why the current study would find a significant decrease only after 24 h where the highest incubation temperature was 28°C. In EPA worst case water bacteriophages have significantly reduced *Salmonella* populations at 6 h when incubated at 35°C (McLaughlin et al. 2008); this again is a higher temperature than used in the current

study. Stationary phase cells, those in a 'starved' state, are inactivated by bacteriophage lysis at a significantly slower rate (McLaughlin et al. 2008). This could aid in explaining the slower and prolonged (six months) potential phage inactivation in the current study non-sterile surface water.

To further elucidate which biotic factors were playing major roles in determining *Salmonella* populations in non-sterile surface water cycloheximide was added to the non-sterile surface water to inhibit eukaryotic cells, thus inactivating protozoa. By day five of incubation all non-sterile surface water microcosm had significantly lower *Salmonella* populations than did the non-sterile surface water with cycloheximide; sterile surface water had significantly higher *Salmonella* populations than either of the non-sterile waters. This would suggest that protozoa grazing accounted for only part of the decrease in *Salmonella* populations in the non-sterile surface water microcosms.

Similar results were obtained by Chandran et al. (2011) when comparing *S*. Paratyphi survival in non-sterile and autoclaved lake water. With a starting concentration of 8 log CFU/ml in both water types, a decrease to 1 log CFU/ml in non-sterile lake water was obtained, while autoclaved lake water resulted in a 6 log CFU/ml *Salmonella* population after the same 27 day period (Chandran et al. 2011). Similarly, streptomycin-resistant *S*. Typhimurium in filter sterilized surface water at 20°C for 41 days, while the same water used non-sterile had *S*. Typhimurium surviving 16 days; as determined by pour plating to bismuth sulfite agar (Maki and Hicks 2002).

In the seven day experiment comparing non-sterile surface water with and without cycloheximide coliform populations were not significantly different for any combination of water type and temperature at time zero. After 12 h a decrease in

coliform populations was noted in waters stored at 15°C, while increases in coliform populations were noted for waters incubated at 21 or 28°C. Both of these events suggest that coliforms may not be a suitable indicator organism for *Salmonella* in surface waters; at 15°C a significant decrease was noted in coliform populations prior to a significant decrease in *Salmonella* populations, and at 21 and 28°C an increase in coliform populations was observed. The increase in coliform populations is suggestive of growth of coliforms in the surface waters, and the ideal indicator organism should not be capable of growth in the medium to be evaluated. Growth of *E. coli* has previously been noted in sterile creak water microcosms 10 days into the incubation (Fish and Pettibone 1995).

In conclusion, our results indicate that *Salmonella*, either individually or as a cocktail, are able to survive long-term, up to six months, in various types of surface waters. The use of sterile waters in microcosms has been over estimating the survival duration of *Salmonella* in surface waters. EPA worst case water is a suitable standard medium for studying the survival of *Salmonella* in surface waters. Further investigations should be performed to determine if it can be used as a standard 'worst case' medium for other human pathogens in water.

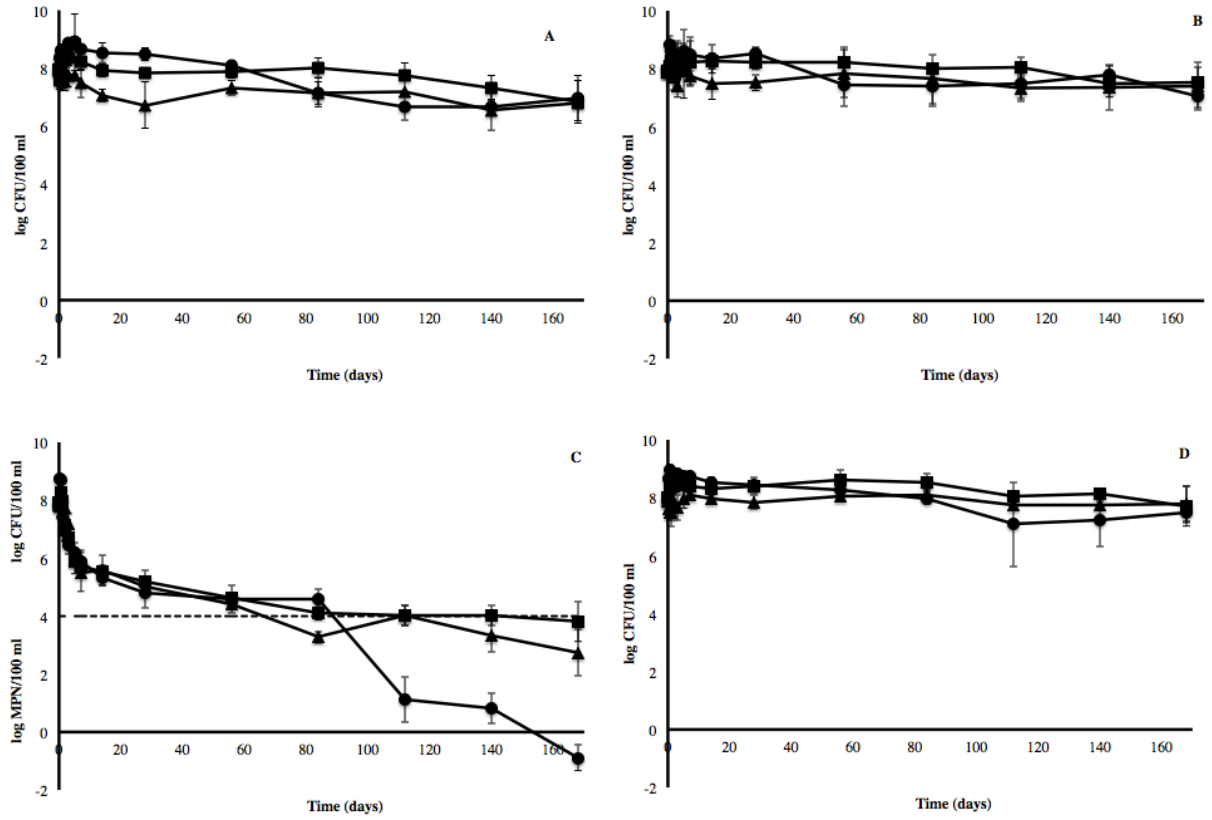

Figure 6-1. Survival of *Salmonella* cocktail in surface waters at (●) 28°C, (■) 21°C, and (▲) 15°C (mean ± standard deviation, n=6). (A) DI water. (B) Sterile surface water. (C) Non-sterile surface water. (D) EPA worst case water.

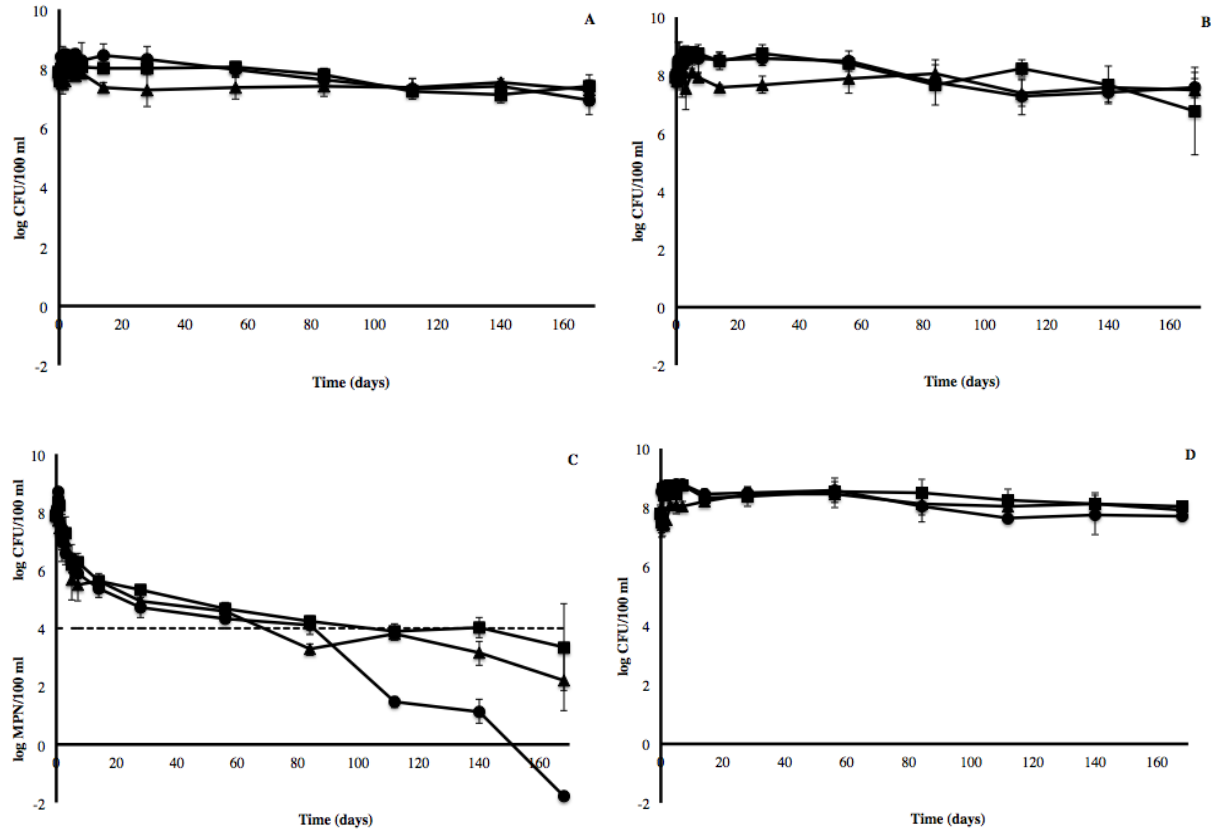

Figure 6-2. Survival of *Salmonella* Anatum in surface waters at (●) 28°C, (■) 21°C, and (▲) 15°C (mean ± standard deviation, n=3). (A) DI water. (B) Sterile surface water. (C) Non-sterile surface water. (D) EPA worst case water.

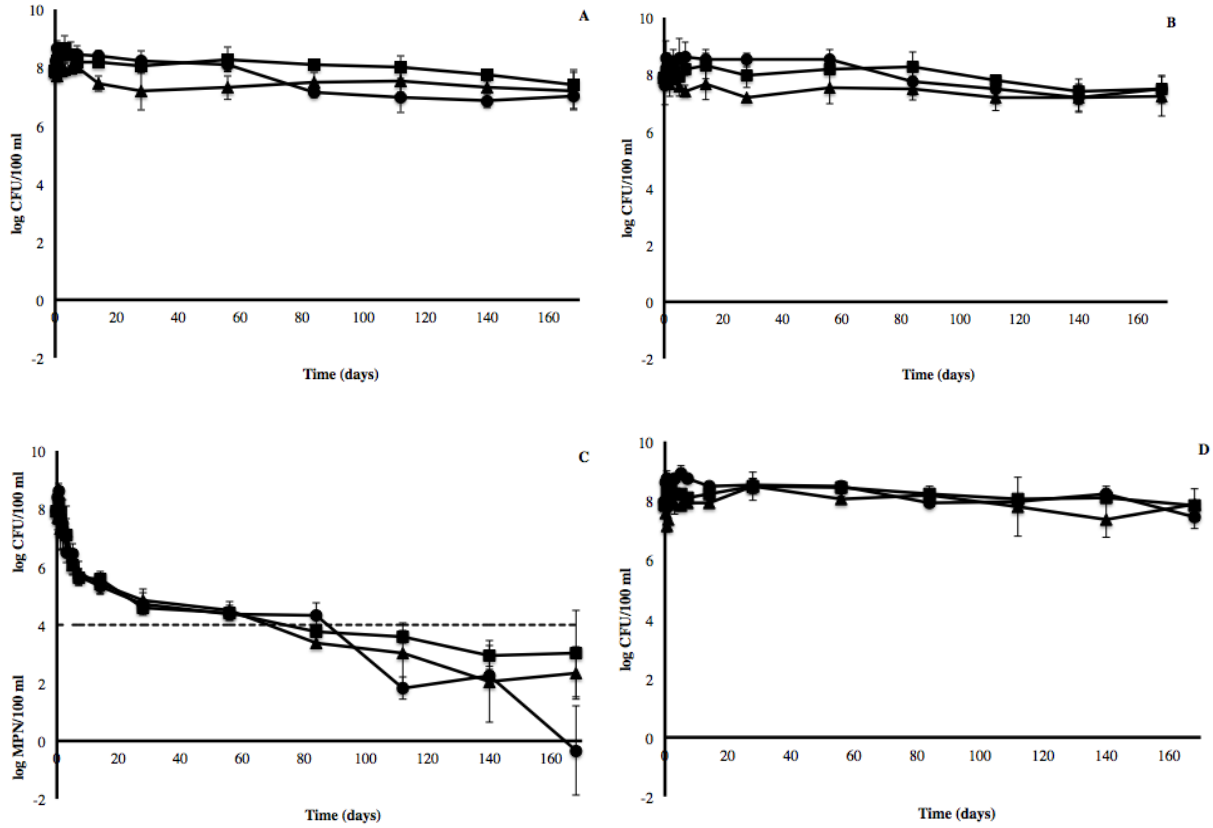

Figure 6-3. Survival of *Salmonella* Gaminara in surface waters at (●) 28°C, (■) 21°C, and (▲) 15°C (mean ± standard deviation, n=3). (A) DI water. (B) Sterile surface water. (C) Non-sterile surface water. (D) EPA worst case water.

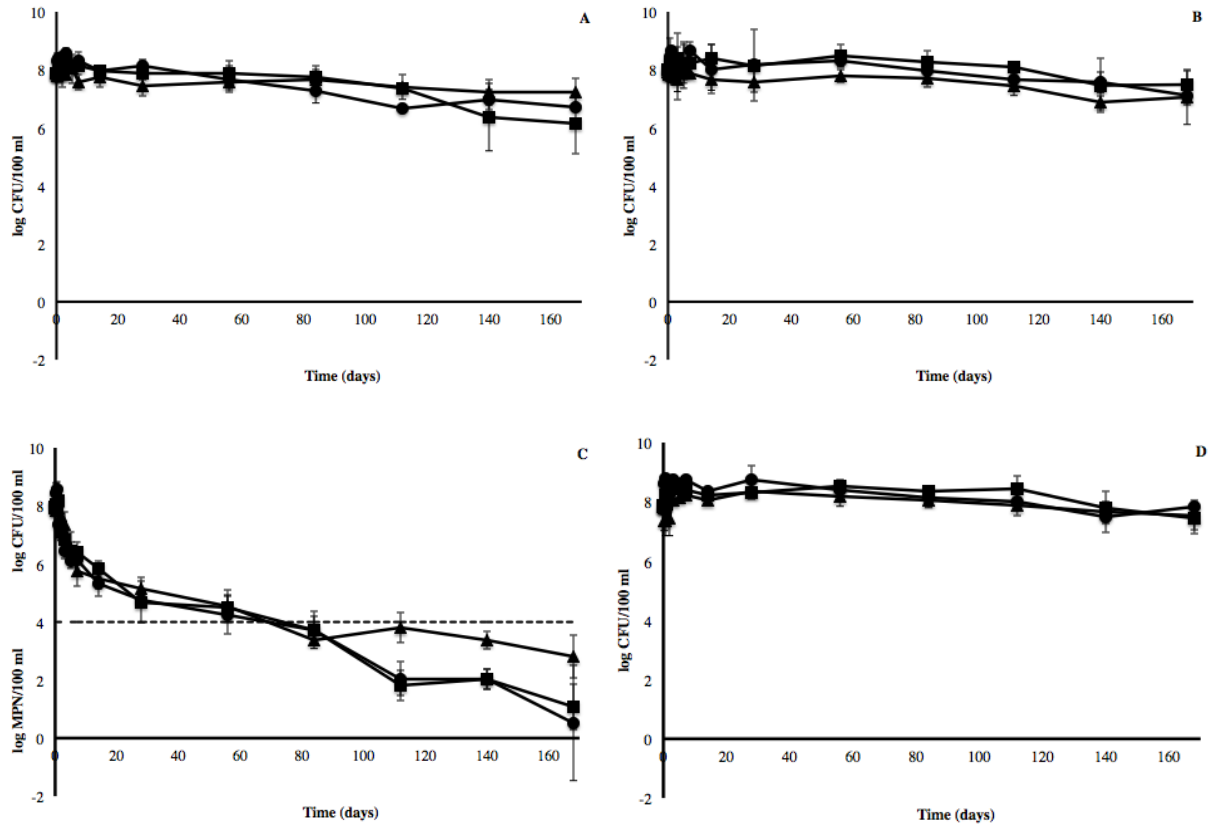

Figure 6-4. Survival of *Salmonella* Newport in surface waters at (●) 28°C, (■) 21°C, and (▲) 15°C (mean ± standard deviation, n=3). (A) DI water. (B) Sterile surface water. (C) Non-sterile surface water. (D) EPA worst case water.

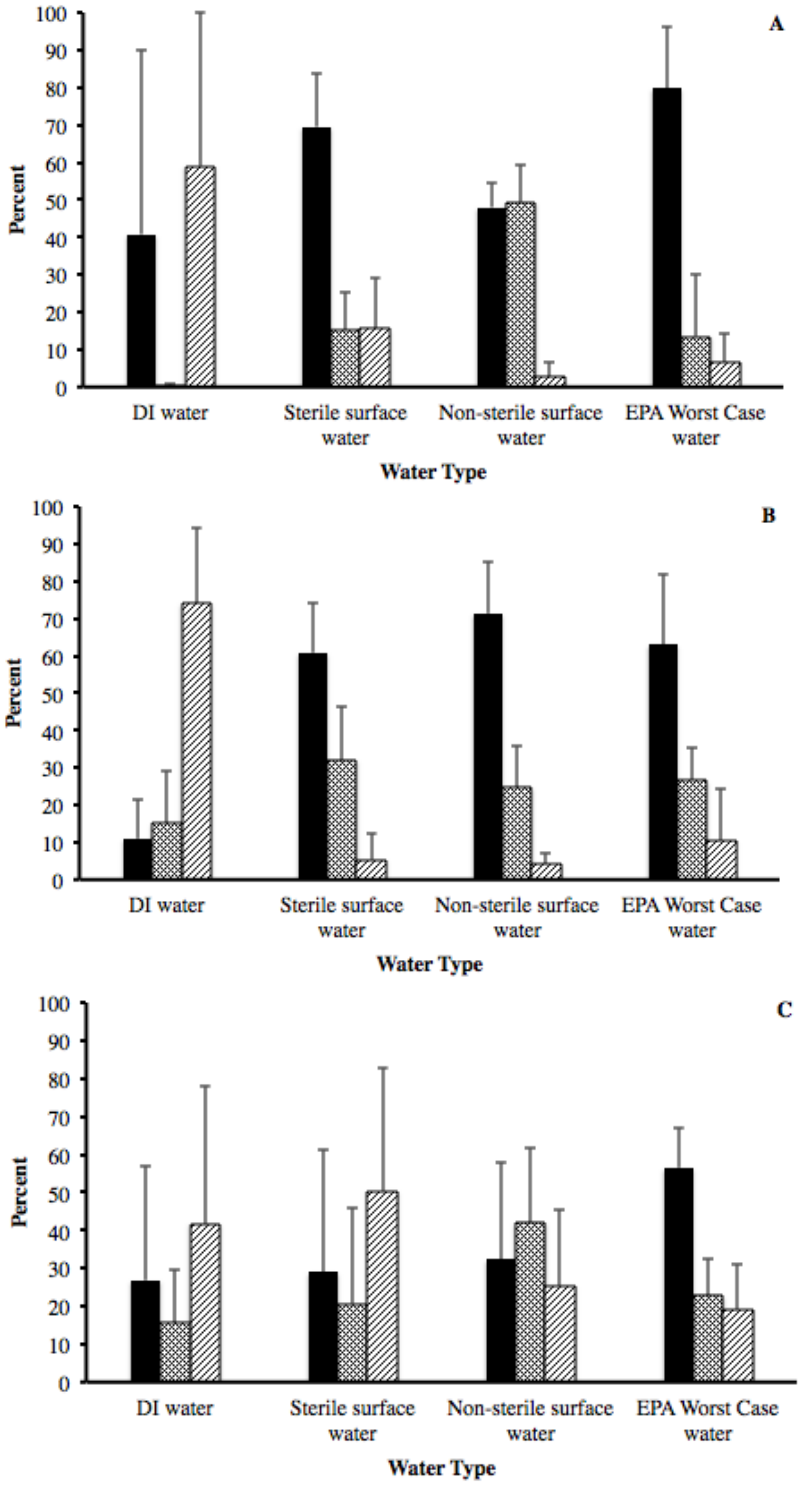

Figure 6-5. Composition of *Salmonella* cocktail in surface waters at final enumeration by spread plating as determined by serogrouping 100 colonies, *Salmonella* serovar Anatum (solid), Newport (crosshatched), and Gaminara (striped) (mean ± standard deviation, n=6). (A) 28°C. (B) 21°C. (C) 15°C.

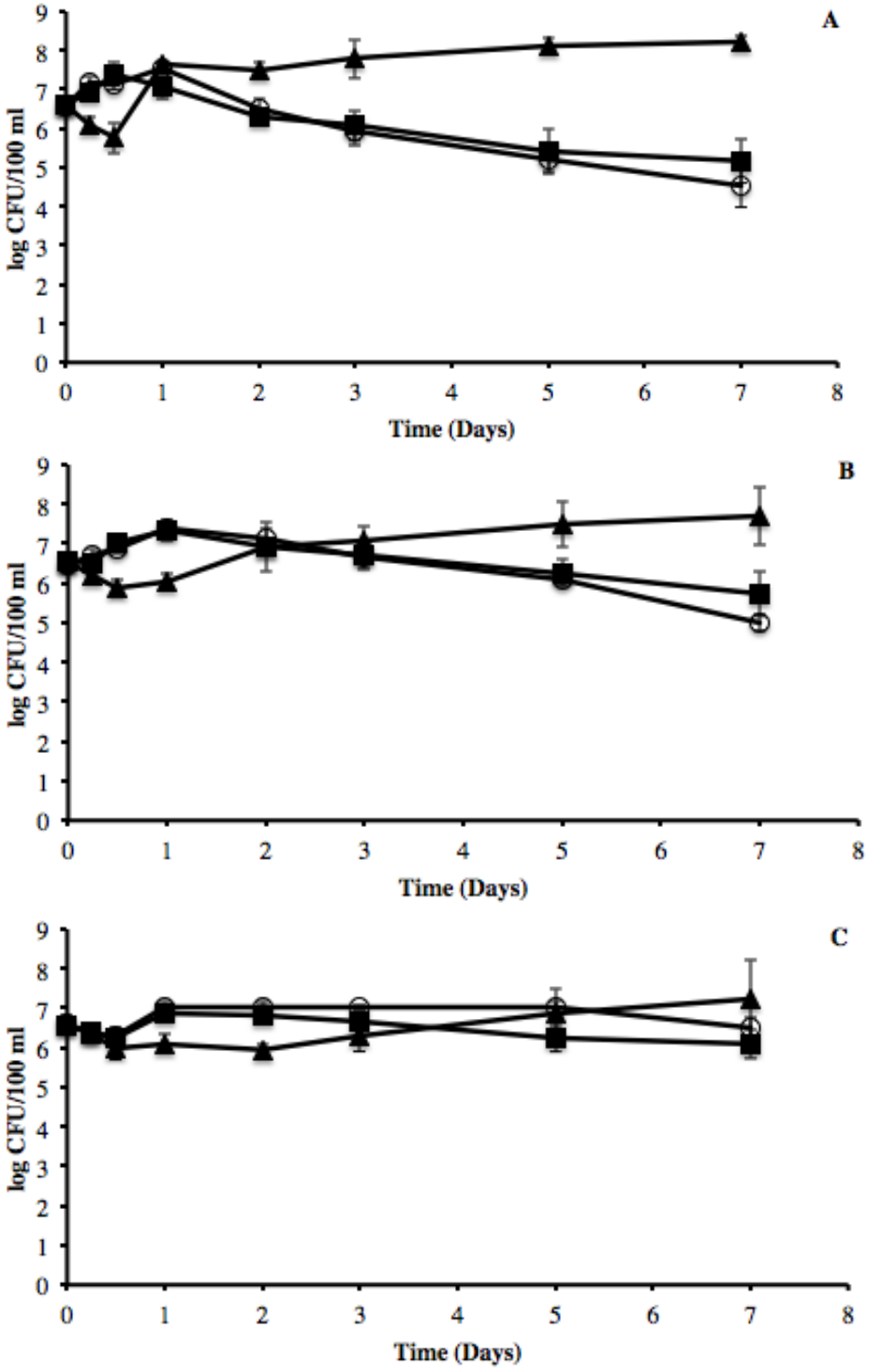

Figure 6-6. Survival of *Salmonella* cocktail in sterile surface water (▲), non-sterile surface water (■), and non-sterile surface water with cycloheximide (○) (mean ± standard deviation, n=6). (A) 28°C. (B) 21°C. (C) 15°C.

CHAPTER 7
CONCLUSIONS AND FUTURE WORK

The work presented here establishes that *Salmonella* is prevalent in Central Florida surface waters. First, a method to screen large volumes of surface water for *Salmonella* was established. Using this method, water samples from various surface water sources throughout Central Florida were screened for *Salmonella*. To better understand the *Salmonella* serovars present in Central Florida surface waters, isolates from this study were identified and characterized using a variety of procedures.

The method to screen 10 L of surface water for the presence of *Salmonella* uses a combination of tangential flow filtration, overnight enrichment, immuno-magnetic separation, and qPCR detection. This method had a limit of detection of 1 CFU/L in 10 L of surface water within two days. Modified Moore swabs performed equally as well as tangential flow filtration for concentration. The use of normal flow filtration was eliminated due to irreversible fouling of the filter prior to the entire sample volume being passed through the filter. The development of this method is significant in that it is now possible to screen large volumes of surface water for the presence of *Salmonella* in a more time efficient manner. The two days time requirement is an important improvement in that risk management decisions can now be made based on more timely data.

The monthly use, for 12 consecutive months, of the developed method to evaluate surface water samples from 18 sites throughout Central Florida demonstrated the continued presence of *Salmonella* in Central Florida surface waters. All 202 water samples collected were *Salmonella*-positive using the developed concentration and detection method. When making risk management decisions, especially in relation to

irrigation, crop protection, and other foliar applications, the likely presence of *Salmonella* in the surface waters should be taken into account. An alternative water source, such as ground water, could be considered. However, a substantial survey of *Salmonella* in Central Florida ground water should be conducted prior to this practice being put into place. Furthermore, the fate of *Salmonella* once being sprayed onto horticultural crops should be further investigated with attempts to replicate real-world conditions as close as possible.

After the 10 L water samples were determined to be *Salmonella*-positive, *Salmonella* MPNs were carried out to provide quantitative data. This data is important for the accurate generation of risk assessments. Of the 202 water samples, 165 (81.7%) yielded MPN values above the lower limit of detection, and thus *Salmonella* isolates for further analysis. This quantitative data should be used to generate risk assessments for the potential use of surface waters on or near edible horticultural crops. Current 'zero tolerance' policies for *Salmonella* may preclude the use of Central Florida surface waters on edible horticultural crops. This presents a potential feasibility issue for producers in Central Florida, especially during times when frost protection is required.

At time of sampling, air and water temperature, pH, ORP, turbidity and conductivity were recorded. In addition to *Salmonella*, each water sample was also analyzed for aerobic plate count, and *E. coli*/coliform MPNs. None of the water characteristics had a strong correlation with *Salmonella* prevalence. In the Central Florida area, indicator or index organisms may not be able to be reliable indicators of potential *Salmonella* presence in surface waters. The present study does not support

the current practice of relying on *E. coli* as an indicator of acceptable microbial water quality, as is currently required by the Tomato-Good Agricultural Practices and suggested by the Leafy Greens Marketing Agreement (F-DACS 2007, LGMA 2012). Hence, direct screening for the presence of *Salmonella* is recommended for surface waters that are to be used in the production of edible horticultural crops that are not expected to undergo a kill-step. Risk assessments should be generated to determine which uses of, and at what times, surface waters represent substantial risk to consumer health when present as a source of contamination.

A previously reported (Kim et al. 2006) multiplex PCR reaction was capable of determining if all *Salmonella* isolates from one surface water sample were likely the same or different; the same multiplex PCR was not able to accurately predict the serogroup or serovar of the *Salmonella* isolated from surface water. This will reduce the time required to screen numerous *Salmonella* isolates from one surface water sample, but will not aid in the identification of which serovars are present in the water sample. Conventional serotyping and Pulsed Field Gel Electrophoresis should continue to be used for serovar analysis.

In microcosm-based experiments, *Salmonella* Anatum, Gaminara, and Newport (all previously isolated from surface water) were able to survive six months in all water types and temperatures evaluated. This extended duration of survival needs to be considered when determining the likelihood of a particular body of water being contaminated with *Salmonella*. With this evidence it should now be considered that once a body of water has been found to have *Salmonella*, the *Salmonella* will be present for an extended duration. The effect of flowing water on the duration of

Salmonella survival should also be examined. If it is now know that *Salmonella* can survive in surface waters for up to six months, investigations into how far surface water could potentially travel in that time and the potential sources of contamination it would encounter along the way.

EPA worst case water provided a standard 'surface water' that was able to support *Salmonella* survival equally well, or better than sterile surface water. EPA worst case water should be used in further microcosm-based studies on the survival of *Salmonella*, so that future studies are more readily comparable.

As demonstrated by the significantly lower survival of *Salmonella* in non-sterile surface waters, compared to other sterile waters, the other microflora present has a significant effect on the survival of *Salmonella* in surface waters. This work was not able to determine cause of this decreased survival. It is believed that the decreased survival was not due to protozoa grazing, as inactivating eukaryotic cells did not increase *Salmonella* survival in non-sterile surface waters. Further work is required to determine the cause of decreased survival. Through better understanding of why the decrease in *Salmonella* populations occurred in non-sterile surface waters could aid in determining potential methods of *Salmonella* inactivation within surface waters.

A variable not examined during this set of experiments is the potential effect of sediments, and their disturbance, to the survival of *Salmonella* in surface waters. Further experiments should be conducted to include a standardized sediment within the microcosms.

The extended survival time, along with the continued presence, of *Salmonella* in Central Florida surface waters indicates that *Salmonella* may be a geonotic, rather than

zoonotic, organism. This is especially relevant in the Southeastern US, where several studies have also reported high levels of *Salmonella* persistence (Haley et al. 2009, Patchanee et al. 2010, Rajabi et al. 2011). As no seasonal or temperature correlations were obtained in the present study, further work is required to better understand why *Salmonella* presence is greater in the Southeastern US than in other geographic areas which typically report ca. 10% *Salmonella*-positive water samples (Gorski et al. 2011, Strawn et al. 2013, Thomas et al. in press).

If *Salmonella* is considered a geonotic organism, implying that it is understood to be present or potentially present, in all surface waters in the Southeastern US 'zero tolerance' policies for *Salmonella* should be re-evaluated. As an alternative risk assessments need to be generated, using quantitative data, to determine best practices for fresh produce production. These risk assessments then need to be used to make appropriate risk management decisions to determine best practices for fresh produce food safety. This is through the use of accurate data and, risk assessments that could best minimize the potential of more fresh produce borne salmonellosis outbreaks from occurring.

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BIOGRAPHICAL SKETCH

Rachel was born and raised in Ontario, Canada. She completed her B.Sc. in nutrition and food sciences, with distinction, at the University of Alberta and her M.Sc. in food science at the University of Guelph. Rachel was awarded the Alumni Fellowship to pursue a Ph.D. in food science at the University of Florida. Rachel served for two years on the Student Professional Development Board for the International Association for Food Protection, and has been the University of Florida's Institute of Food Technologists College Bowl Team Captain.

Previously, Rachel has been employed as an Allergen Program Coordinator for Nestle, and has been an Adjunct Lecturer for Conestoga College, Canada.

Rachel's research interests centers around food safety in the pre-harvest environment. She has specialized in *Salmonella* in the surface water environment, but wishes to extend her research to understanding *Salmonella* in other pre-harvest environments.

Upon completion of the Ph.D. program Rachel intends to seek a teaching position at the community college level.