ECOLOGY OF *SALMONELLA* SPP. IN A SOUTHEASTERN WATERSHED

by

BRADD JOSEPH HALEY

(Under the Direction of Erin K. Lipp)

ABSTRACT

Salmonella is primarily considered a zoonotic foodborne disease. However, outbreaks from contaminated water and produce-associated foodborne salmonellosis have increasingly been reported. These waterborne disease outbreaks may be caused by sewage or wastewater pollution due to human activities or urban and agricultural runoff. Moreover, storm events and climate variability have been shown to increase pathogen loads in surface waters, decrease water quality and precede disease outbreaks. In this study we investigated the seasonality of *Salmonella* densities and *Salmonella* serotype diversity in a southeastern watershed (Little River watershed). Our results show that salmonellae are ubiquitous in natural waters and their concentrations fluctuate seasonally and may be influenced by rainfall. We further demonstrate that *Salmonella* serotypes in natural waters demonstrate differential persistence which may influence the variability in environmental exposure routes and the observed seasonality of salmonellosis in this region.

INDEX WORDS: *Salmonella*, *Salmonella* Muenchen, *Salmonella* Typhimurium, environmental transmission, salmonellosis, waterborne pathogen, water quality, climate, weather

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DEDICATION

To my family.

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CHAPTER 1

INTRODUCTION

Salmonella species are the etiological agents of salmonellosis and typhoid fever and are among the leading causes of gastroenteritis worldwide. Serotypes of both *Salmonella* species are believed to cause disease in humans, but typically infections are caused by relatively few serotypes [1]. The majority of *Salmonella* infections in humans and warm-blooded animals are caused by serotypes of *Salmonella enterica* subsp. *enterica* (I). *S*. Typhi and *S*. Paratyphi A, B, and C are the etiological agents of typhoid fever and are transmitted directly from human-to-human [6]. Other serotypes of *Salmonella enterica* subsp. *enterica* are rarely transmitted directly between humans but rather transmitted through exposure to contaminated foods, water, soils, and contact with infected animals such as reptiles and amphibians.

Although salmonellosis case rates are decreasing in the United States, trends in human serotype infections have been changing in the past decade [1]. Clinical isolation rates of known foodborne serotypes, such as *S*. Entiriditis, are declining; while clinical isolation rates of serotypes whose transmission routes are not well defined such as *S*. Javiana and *S*. Muenchen, are increasing [1]. Moreover, animal host species of these serotypes are not defined and may include animals that are not generally consumed by humans [8]. Furthermore, their presence in natural waters has been well documented [9], suggesting that this medium may play a role in salmonellosis transmission.

As with many other enteric diseases, human and animal salmonellosis case rates typically increase in warmer summer months and decrease during winter months [4] [7]. For many cases

food sources have not been identified and human-to-human transmission would not account for this observed seasonality. Recent research has suggested that precipitation events may play a role in the transmission of waterborne diseases [3] [2] and that the presence of clinically relevant *Salmonella* serotypes in natural waters is related to precipitation [5].

This thesis is an investigation into the environmental parameters that may govern the presence and persistence of *Salmonella* serotypes of public health significance in a high salmonellosis case-rate region of the southeastern United States. The second chapter of this thesis provides an overview of the literature concerning the presence of clinically relevant *Salmonella* serotypes in natural waters. The third chapter presents the research and findings of the field study. The fourth chapter presents the research and findings of the *in vitro* study. The final chapter, Chapter 5, presents the findings and conclusions of the study.

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CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

The *Salmonella* genus consists of over 2,500 known serotypes, all of which are considered potential human pathogens [8]. Serotypes of *Salmonella enterica* subsp. *enterica* (I) account for approximately 99% of all *Salmonella* infections in warm-blooded animals [20]. Although these serotypes are capable of infecting and causing illness in a wide range of animals, epidemiologic evidence suggests that some serotypes may have host-specific virulence genes and cycle through populations of specific species by direct transmission [88]. Nontyphoidal *Salmonella* serotypes are not host-adapted to humans and must therefore be introduced into human populations from exposure to contaminated foods, soil, water, or animal contact. Most cases of human salmonellosis have been linked to the consumption of contaminated foods, but environmental exposure to salmonellae is now being considered a significant transmission route [128]. The ubiquitous nature of *Salmonella* and its widespread occurrence in both fresh and marine waters suggests that transmission in the aquatic environment from water consumption, recreation, or the consumption of food treated with or harvested in contaminated water is probable [87] [128] [106].

TAXONOMY OF *SALMONELLA*

Salmonella refers to a genus of motile, rod-shaped, Gram-negative facultative anaerobic bacteria of the family Enterobactericeae. The DNA base composition of *Salmonella* is 50-52 mol% G+C, which is similar to other enterobacters including *Escherichia*, *Shigella*, and

Citrobacter [85]. Members of this genus were first isolated in 1885 by Daniel Salmon and Theobold Smith from swine believed to have been ill with swine plague [123].

Salmonella nomenclature is complex and has been debated widely in recent decades [20]. Several nomenclature systems exist for this genus but uniformity is required to facilitate communication between scientists and health officials. Originally *Salmonella* serotypes were categorized by Kauffman and White [109] by serology and were considered separate species. Crosa et al. [40] observed by DNA-DNA hybridization that all serotypes from serologically classified subgroups I, II, IIIa, IIIb, IV were related at the species level. *Salmonella enterica* serotypes from subgroup V were shown to comprise a separate species known as *Salmonella bongori* [40].

Currently the genus *Salmonella* is comprised of two species: *Salmonella enterica*, the type species, and *Salmonella bongori*. Members of *S. enterica* are typically the causative agents of salmonellosis in warm blooded animals. *S. enterica* is further divided into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* also known as subspecies I, II, IIIa, IIIb, IV, and, VI respectively) which are differentiated biochemically and by genomic similarity [40] [113]. These subspecies are commonly referred to by both a Roman numeral and a name (Tbl. 1). Fifty percent of the more than 2,500 known *Salmonella* serotypes are grouped as *S. enterica* subspecies *enterica* (I) [109]. Cumulatively this subspecies encompasses the causative agents for approximately 99% of all *Salmonella* infections in warm-blooded animals [20].

Members of *S. bongori* and *S. enterica* subspecies are composed of individual serotypes (also referred to as serovars) that are based on the similarities in their flagellar H-antigens. Originally serotypes were named based on host specificity or geographic location of its first

identification. Given the complexity of the serological nomenclature scheme, the Centers for Disease Control and Prevention (CDC) has suggested that the original serotype names remain and that serotypes be referred to simply by their genus name and serotype name. For example, *Salmonella enterica* subspecies *enterica* serotype Typhimurium is referred to as *Salmonella* Typhimurium.

SALMONELLOSIS

Salmonella, a zoonotic bacterium, is readily transmissible between vertebrate animals and humans. *Salmonella* spp. have a broad host-range and can therefore cause infections in many species [12]. *Salmonella* serotypes that are commensal with reptiles, such as members of *Salmonella enterica* subsp. *arizonae*, are capable of causing potentially fatal infections in humans [148]. Similarly, *S*. Typhimurium can infect and cause illness in many species such as humans, cattle, poultry, rodents, sheep, swine [12]. Zoonotic diseases can be transmitted to humans by consumption of contaminated foods, direct contact with infected animals, and exposure to contaminated environmental media such as water and soil.

Salmonella infections, known as salmonellosis, may result in gastroenteritis in humans and animals or typhoid disease in humans only. Although in the past decade rates of salmonellosis have dropped by 5%, *Salmonella* remains one of the leading causes of gastroenteritis in the United States [27]. Risk factors for salmonellosis include but are not limited to extremes of age, recent antibiotic usage, diabetes, rheumatological disorders, and immunosuppression [70].

The causative agents of typhoid disease are *S.* Typhi and *S.* Paratyphi A, B, and C. This disease is most common in developing nations. Nontyphoidal gastroenteritis may be caused by several other *S. enterica* serotypes. The two most commonly isolated serotypes from human

clinical cases are *S.* Typhimurium and *S.* Enteriditis [27]. These serotypes accounted for approximately 34% of human *Salmonella* infections in the United States in 2004 [27]. This is a dramatic decrease from 1980 when they accounted for a combined 75% of the total human *Salmonella* infections [103]. Other serotypes commonly isolated from human clinical cases include *S*. Newport, *S*. Javiana, *S*. Heidelberg, *S*. Montevideo, and *S*. Muenchen. Clinical isolation rates of *S*. Newport and *S*. Javiana increased by 99% and 228%, respectively, from 1994 to 2004 [27].

Salmonella infections can cause mild to serious disease, including death, in humans. The most vulnerable subpopulations are the very young, elderly, pregnant, and immunocompromised individuals [51]; however, for most people infections with nontyphoidal *S. enterica* serotypes are self-limiting and are usually associated with diarrhea, cramping, fever, nausea and muscle pain. Few cases will experience vomiting and headaches and even fewer will experience dysentery [135]. Septicemia only occurs in about 1% of total cases with the exception of infection with *S*. Dublin and *S*. Choleraesuis in which septicemia has been shown to occur in 25% and 75% of patients, respectively [138]. Infections with *S*. Typhi and *S*. Paratyphi A, B, and C are more severe. Symptoms include fever, malaise, septicemia, headaches and myalgia; however, vomiting and diarrhea are infrequent. Incubation times are generally 8-14 days [88]. If treated appropriately symptoms last for 3-4 days. If untreated, symptoms may persist for several weeks [88]. It is unknown whether protection against *Salmonella* is mediated by cellular or humoral immunity [79]. Exposure to or vaccination with a *Salmonella* serotype will elicit cross-immunity only against serotypes of the same serogroup.

Salmonellosis affects all demographics, but distribution of the disease among age groups and gender is not equal. In the United States most reported cases of salmonellosis occur in

children less than 5 years of age [27]. In 2004, 27% of all reported cases occurred in this age group [27]. 10% of cases occurred in people ages 5 to 9 years old [27]. 10% of cases also occurred in each of the following age groups: 10 to 19, 20 to 29, 30 to 39, and 40 to 49 years [27]. After the fifth decade of life case rates steadily decrease [27]. Male infants and children are more commonly reported to have salmonellosis than female infants and children; however this trend reverses after the second decade of life when adult women are more frequently reported to have salmonellosis than adult men [27].

Transmission of *Salmonella* to humans traditionally has been attributed to contaminated animal-product foods, but epidemiological studies have demonstrated that cases are sporadic and may more likely involve environmental sources than previously thought. *S.* Enteriditis is most commonly transmitted by egg consumption [63], but transmission routes for other commonly isolated serotypes are unclear. It has been suggested that contaminated soils, sediments and water as well as wildlife may play a significant role in *Salmonella* transmission [60] [126] [127]. Moreover, geographic clusters of cases in which no verifiable food source have been determined, such as those recently caused by *S.* Javiana in the southeastern U.S., do not follow the same geographic patterns as cases which have been linked to a known food source but rather mimic amphibian distribution patterns [134]. *S.* Typhi, which is only transmitted from human to human, is most common in developing nations where access to safe drinking water may be limited and waste disposal and treatment may be inadequate [16] [94] [140].

EVOLUTION OF PATHOGENICITY IN *SALMONELLA*

The *Salmonella* genus and *Escherichia* genus are believed to have diverged from a common ancestor in the family Enterobactericeae about 100 million years ago [97]. The two share many similar genes including those that allow for interactions with animal cells, including

those that regulate the biosynthesis of nutrients that are scarce in host tissues, code for proteins that allow for defense against antimicrobial molecules produced by the host, and code for proteins that regulate the repair of damaged DNA [55]. Non-pathogenic *E. coli* share these genes with all members of both *Salmonella* species, all of which are believed to be potentially pathogenic to humans, although infections from *Salmonella* subspecies II-IV are rare. Thus, the pathogenicity of *Salmonella* is due to the acquisition of unique virulence gene clusters [55]. These *Salmonella*-specific virulence genes clusters, known as pathogenicity islands and pathogenicity islets, are found in several locations on the *Salmonella* chromosome and allow for specific interaction between *Salmonella* cells and host tissues [55].

Salmonella Pathogenicity Island 1 (SPI-1), is present in both *Salmonella bongori* and *Salmonella enterica* and allows for invasion of host cells. SPI-1 encodes two proteins, InvF and HilA, as well as a type III secretion system, known as Inv/Spa. Inv/Spa allows for cell-surface appendages that appear during cellular contact with the host [50]. Inv/Spa secretes proteins that elicit the cellular invasion of *Salmonella* into host cells [50]. Inactivation of Inv/Spa prevents this internalization [50]. SPI-1 also allows for the induction of apoptosis in *Salmonella*-infected macrophages [33] [92]. The base composition of this pathogenicity island is only 42% G+C while the *Salmonella* genome has a 52% G + C composition [50]. Moreover, SPI-1 contains genes that have sequence and functional homologues in other invasive enteric bacteria such as the *Shigella* spp. [98]. There are no similarities in structure or function to the invasion genes utilized by *Yersinia* or *Listeria*. The presence of this pathogenicity island in all subspecies of *Salmonella* suggests SPI-1 was acquired before the diversification of this genus [54].

The SPI-2 pathogenicity island is present in all members of *Salmonella enterica* but absent in members of *Salmonella bongori* suggesting a more recent acquisition than SPI-1 [67]

[98]. The acquisition of this pathogenicity island has been considered an evolutionary step towards colonization of warm-blooded hosts [11] [55]. SPI-2 encodes another type two secretion system allows for the translocation of effector proteins by intracellular *Salmonella* into the host cells. This type two secretion system protects *Salmonella* in the *Salmonella*-containing vacuole from the innate immune system of the host. It is also believed that SPI-2 was acquired by *S. enterica* by horizontal gene transfer. This island has a lower base composition (45% G+C) than the *Salmonella* genome [129] and is located near tRNA genes, similar to many pathogenicity islands and prophages in other species [98] [32]. These pathogenicity islands have provided the *Salmonella* genus new abilities that have expanded its ecological niche [99].

SALMONELLA SEROTYPE HOST-SPECIFICITY

Several *Salmonella* serotypes appear to be host-specific, but host specificity for most serotypes is unclear and is a current topic of research [88]. Two explanations for this apparent host-specificity exist: environmental factors and genetic differences [88]. An increase in environmental exposure to certain serotypes may account for the increased prevalence of those serotypes in affected host species [88]. Genetic differences between *Salmonella* serotypes may also exist, allowing some serotypes with host-specific adaptations to persist within specific niches found in certain host species and not others [88].

The majority of host-specificity information has been derived from epidemiological surveys. Serotypes which most frequently cause diseases in certain species of animals are thought to be adapted to that host species. For example, *S*. Choleraesuis is most commonly associated with illness in swine and is known to circulate in swine populations [88]. It is therefore suggested that *S*. Choleraesuis is host-adapted to swine [79]. However *S*. Typhimurium

circulates and causes illness in many species of animals and is therefore suggested to have a broad range of host adaptations [79].

To be considered to have a species-specific host adaptation, a *Salmonella* serotype must circulate and cause disease in a population of a specific species [79]. Many *Salmonella* serotypes cause disease in a wide range of animals, but the disease frequency may be low therefore preventing circulation through a population of a specific species [79].

Exposure studies have shown evidence of host-specificity in several *Salmonella* serotypes. These studies have compared the virulence of different serotypes in different species of animals. A *Salmonella* serotype must be virulent in a species to be considered adapted to that species [79]. *S*. Typhimurium, *S*. Dublin, and *S*. Choleraesuis are all virulent in cattle, pigs, sheep, poultry, and mice while *S*. Abortusovis and *S*. Gallinarum are both avirulent in these species [104] [143] [144]. Likewise *S*. Typhi is only virulent in humans and other primates [88]. Moreover, different virulence factors are expressed when one *Salmonella* serotype infects different animal species, suggesting that some virulence genes are host-specific [91] [139].

Infections with these serotypes in humans are most likely introduced into a human population from outside sources, such as food or water. There is no compelling evidence of circulation of nontyphoidal *Salmonella* serotypes through human populations.

SEASONALITY OF SALMONELLOSIS AND *SALMONELLA* POPULATIONS

Human and animal salmonellosis demonstrate a distinct seasonal trend in the United States and other developed nations [27] [101] [42] [46]. This trend is similar to other bacterial infections of humans where infections peak in the warmer summer months and then decline as temperatures decline [130] [108].

Explanations for this marked seasonality in salmonellosis cases include seasonal eating and food preparation habits such as holding food at insufficiently cool temperatures in warmer months and cross-contamination, infection trends in food animals and wildlife [101], and increased concentrations of *Salmonella* in the environment during warmer and/or wetter months [87] [19].

Salmonella carriage rates in animals are typically higher in summer months. Poultry broiler flocks were shown to carry *Salmonella* with greater frequency during summer months in Denmark [145]. Similarly *Salmonella* infections in populations of the non-domesticated Quokka marsupial in Australia were shown to peak during summer months [62]. *Salmonella* densities have been shown to increase in environmental media, such as water, as ambient air temperatures and precipitation increase suggesting that climate and other extrinsic environmental parameters influence *Salmonella* prevalence in the environment [87]. Precipitation and runoff have been shown to mobilize *Salmonella* from land-based sources to marine and fresh waters [87].

SALMONELLA IN AQUATIC ENVIRONMENTS

Salmonella has been shown to be present and persist in the environment. Although previously considered solely a foodborne disease, environmental salmonellae are now considered a significant source of *Salmonella* infections [128] [127]. Potential reservoirs of *Salmonella* in the environment include water, and soil. Humans may be exposed to these reservoirs through primary contact or ingestion. These reservoirs may account for cases of salmonellosis in which there is no known food source [128]. Studies of non-foodborne *Salmonella* have primarily focused on its presence in aquatic environments. *Salmonella* has been readily isolated from sewage effluents, fresh water, and marine waters [78] [121] [122] [136] [82] [10] [86] [106] [21] [19] [10] [65] [87] [34] [25] [107] [93] [13] [57] [125] [141] [34] [65] [107] [47] [44].

Transport of Salmonella *from land to aquatic environments*

Salmonella is commonly found in the large intestines of humans and animals and can be excreted in numbers as high as 10^8 to 10^{11} cells g^{-1} feces [58]. Studies have demonstrated a high prevalence of *Salmonella* in the feces of domesticated farm animals [22] [72] [111]. Confined animal feeding operations (CAFOs) are a major source of pathogenic bacteria disseminated into the environment. Studies have shown that animal feeding operations generate 100 times as much manure as sewage sludge generated by municipal waste treatment facilities in the U.S. [52]. *Salmonella* may be a potential contaminant of the manure produced in CAFOs and has been shown to survive in liquid swine wastes for 26-85 days [105] [68]. Swine farms in eastern North Carolina were shown to be a significant source of *Salmonella* in stream waters in this area [37]. Runoff originating from these swine operations was implicated in the contamination of these streams.

Feces from animals infected with *Salmonella* may be applied directly to agricultural plots as fertilizer. Manure that is properly treated may be used safely and effectively, but untreated manure may contaminate water supplies [36]. *Salmonella* was shown to persist in animal manure used as fertilizer for up to 63 days after application to agricultural plots [73]. Runoff may subsequently carry microbial contaminants in this fertilizer into surface waters. *Salmonella* originating from agricultural lands was shown to survive in surface waters for up to 45 days [124].

Other studies have shown that non-domesticated animals such as birds, marsupials, amphibians, and reptiles demonstrate a high prevalence of *Salmonella* in natural ecosystems [62] [71] [29] [114]. Infected animals may shed *Salmonella* onto soils or directly into surface waters. Thomason et al. [137] isolated *S*. Bareilly in rain water pools on the crest of Stone Mountain,

Georgia, suggesting contamination of this water source by birds, the local rabbit population, or other species living on this mountain. Furthermore, gulls were implicated as the contamination source in a human salmonellosis outbreak from ingestion of surface waters in Norway in 1999 [115].

Salmonella can be recovered readily from soil ecosystems [137] [1] [137] [136] where they may survive and multiply for up to one year [41] [137]. Soils act as microecological niches for free-living bacteria by providing substrates to form aggregate structures. Bacteria attach to sites that are favorable for replication. As replication occurs exopolysaccharides are formed that trap clay particles creating an aggregate of bacteria colonies and clay particles. These aggregates protect bacteria from predation, allow for nutrient cycling and concentration, and alteration to more a suitable soil pH [84]. Enhanced survival of *Salmonella* in soil environments has also been attributed to survival within soilborne protozoa. *S*. Thompson was shown to survive internally in soilborne *Tetrahymena* species and subsequently released inside its vesicles in a soil environment, allowing for enhanced survival and dissemination into surface waters [18]. The main mechanisms of bacterial transport through or over a soil matrix include movement via infiltrating water or surface water runoff [112]. Precipitation events create runoff which may transport *Salmonella* from animal wastes that are deposited onto terrestrial soils into proximal surface waters.

Humans may contribute to the environmental *Salmonella* load through excretion of contaminated feces into onsite wastewater treatment systems (septic systems) or centralized municipal wastewater treatment plants. Improperly constructed or poorly maintained septic systems can be a significant source of *Salmonella* into ground waters and possibly surface waters. *Salmonella* has been shown to survive in septic tanks for up to 15 days [102].

Pathogens, such as *Salmonella*, can subsequently be transported by groundwater from these waste disposal systems to surface waters [77]. Several studies have also shown that *Salmonella* serotypes of public health significance may survive treatment at secondary and tertiary municipal sewage treatment plants and can be ultimately introduced via treated discharge into aquatic environments [78] [121] [122]. *Salmonella* serotypes that are frequently isolated in clinical samples, including *S*. Newport and *S*. Enteriditis, were found in the final effluent of a tertiary municipal wastewater treatment plant in southern California [78]. Similarly solid and liquid sludge deposited onto agriculture plots as fertilizer may contain salmonellae which have survived sewage treatment and may subsequently be transported into aquatic environments by runoff [121] [122].

Waterborne Salmonella

Salmonella is commonly detected in natural surface waters [44]. The presence of this pathogen in water sources aids in the transmission of *Salmonella* to humans and animals [49]. Many studies have demonstrated the presence of serotypes of public health significance in aquatic ecosystems [136] [82] [10] [86] [106] [21] [19] [10] [65] [87] [34] [25] [107] [93] [13] [57] [125] [141]. Typically these field studies have isolated a range of 17-20 different serotypes from environmental waters [25] [86] [141] usually reflecting the range of serotypes that predominate in the local human and animal populations [7] [10] [101]. Moreover, *Salmonella* serotypes isolated from environmental waters demonstrate similar antimicrobial resistance patterns as those that are isolated from humans and terrestrial animals [7].

Efficacy of Fecal Indicators for Predicting Salmonella *Densities*

Fecal indicator bacteria, traditionally used as a measure of water quality, have not been shown to correlate with or predict *Salmonella* densities [116] [82] [30] [10] [106] [107] [25].

While *E. coli* has typically been used as a proxy for *Salmonella* contamination of aquatic ecosystems [147], several studies have shown that these bacteria do not demonstrate similar survival patterns in water [116] [30]. *Salmonella* was shown to persist longer than *E. coli* in a brackish water microcosm at temperatures less than 10ºC [116]. Yet a second study suggested that *E. coli* persists longer than *Salmonella* at all environmentally realistic temperatures in brackish waters [30]. Field studies have yet to demonstrate a consistent or significant correlation between fecal indicators and *Salmonella* densities. *Salmonella* serotypes of public health significance have been detected in waters with little or no total Coliform bacteria, fecal Coliform bacteria, and fecal streptococci [106] [107]. Similarly, fecal Coliform bacteria were not detected in marine harvested oysters that contained *Salmonella* [19].

Persistence of Salmonella *in Aquatic Systems*

Salmonella has a relatively high rate of survival in aquatic ecosystems compared to other enteric bacteria [31] [43]. It has been shown to outlive *Staphylococcus aureus* and *Vibrio cholerae* in freshwaters [43]. Based on microcosm studies, *Salmonella* can remain viable in aquatic environments for up to 365 days [59].

Prolonged persistence of *Salmonella* in aquatic environments is attributed to metabolic and physiological changes that this pathogen undergoes when exposed to unfavorable conditions [75]. In aquatic environments, *Salmonella* cells become exposed to changes in pH and temperature, as well as nutrient limitations and osmotic stress. Persistence outside of the host is in part due to periplasmic D-Ala D-Ala dipeptidase and a gene known as *PcgL*, which allows *Salmonella* to utilize D-Ala D-Ala as a carbon source [95]. Thus *Salmonella* in the environment may persist for extended periods by metabolizing D-Ala D-Ala from the cell walls of dead bacteria [95]. Furthermore, in response to prolonged exposure to unfavorable conditions

Salmonella cells have been shown to transition into a viable but nonculturable (VBNC) state [24] [119] [131] [61]. It is hypothesized that the VBNC state allows gram-negative cells to remain viable for extended periods of time outside of the host [151] [120] [90]. VBNC cells undergo a decrease in metabolic activity as well as a morphological change to a small coccoidal shape (approximately 0.3 µm) in order to increase their surface area to volume ratio [151] [119]. Although *Salmonella* has been isolated from most water types, this pathogen demonstrates different survival patterns and dynamics in different aquatic ecosystems.

Salmonella *in fresh surface waters*

Clinically relevant *Salmonella* serotypes have been readily recovered from fresh surface waters that were both influenced by urban and agricultural runoff and waters that were relatively unpolluted [34] [65] [107] [47] [44]. *Salmonella* prevalence in fresh waters has been linked to human and animal fecal pollution [37]. In a study in rural eastern North Carolina *Salmonella* densities were twice as high in rivers that were impacted by human and swine presence than those that were removed from human and animal influences [37]. This study also demonstrated a distinct seasonality in waterborne *Salmonella* densities. Higher *Salmonella* densities were observed during warmer and wetter summer months (June-August) than colder and drier winter months. Dondero et al. [44] isolated *Salmonella* from pristine fresh surface waters in upstate New York. This study demonstrated the presence of several *Salmonella* serotypes of public health significance, such as *S*. Typhimurium and *S*. Entiriditis, in a region of relatively low human and animal salmonellosis cases. *S*. Typhimurium was readily detected in streams that were relatively unpolluted suggesting that local fauna may harbor and shed this pathogen or that long-distance transport and long-term survival of this pathogen contributes to its widespread dissemination in fresh surface waters [44].

Salmonella was shown to multiply in fresh surface waters by Hendricks and Morrison [66]. In this study *S*. Seftenberg multiplied in dialysis bags above and below a sewage outfall in the Poudre River, suggesting that *Salmonella* may survive as a free-living organism in freshwater ecosystems but other studies have failed to demonstrate *Salmonella* re-growth in freshwaters far from sewage outfalls [74] [150].

Salmonella *in groundwater*

There has been limited research on the presence of *Salmonella* in groundwater. These water sources are becoming increasingly contaminated with human and animal pathogens and disease outbreaks caused by the consumption of polluted groundwater have recently occurred [4] [39]. Although most disease outbreaks from contaminated groundwater have been caused by viruses, the presence of *Salmonella* in septic systems [102] and the ability of *Salmonella* to persist in this environment [35] [48] [89] [15] suggest that groundwater contamination with *Salmonella* is a credible health hazard. Microbial contaminants can enter groundwater though faulty septic tanks, infiltration through soils, and faulty sewer lines [83]. Soil column studies have shown that *Salmonella* may persist in groundwater for 2 to 30 days and longer than both *E. coli* and [15] Coliform bacteria [89]. Presence of *Salmonella* in groundwater presents a health hazard to those who use well water for consumption or supply, as most groundwater is not treated if drawn by private wells. Further research must be conducted to fully understand the persistence patterns and risks presented by *Salmonella* in regions where groundwater is used as a source of potable water.

Salmonella *in marine and brackish waters*

Salmonella is commonly found in marine and brackish waters [136] [82] [10] [86] [106] [21] [19] [10] [65] [86] [34] [25] [107] [93] [13] [56] [125] [141]. *Salmonella* may be

transported to the marine environment by rivers or directly by agricultural and urban runoff [10] [10] [87]; *Salmonella* densities and diversity in river waters are higher than those in the marine environments that they feed into [141]. *Salmonella* persistence in marine water is low in comparison with its persistence in freshwater [141] [23]. As in freshwater, the provenance of *Salmonella* in the marine environment are primarily the fecal wastes of terrestrial animals [147]. In various locations wastewater containing *Salmonella* may also be directly introduced into coastal ecosystem from combined sewer overflows (CSOs) during periods of extreme precipitation. These untreated sewage outputs have been shown to contain human pathogens [76] [6] and the frequency of CSOs have been associated with illness [38]. Similarly, cruise ships often directly discharge raw human waste into the marine environment thereby introducing pathogens such as *Salmonella* directly into these ecosystems.

Clinically relevant *Salmonella* serotypes are commonly found in coastal waters that are used for recreation or shellfish harvesting. *S*. Typhimurium and *S*. Enteriditis, the two most commonly isolated clinical serotypes, are frequently recovered from coastal waters [86] [141]. Other commonly isolated serotypes coincide with those serotypes found among local human and animal populations [152]. Martinez-Urtaza et al. [87] was able to isolate *Salmonella* from shellfish harvested in the coastal water column and shellfish that inhabit the coastal sediments. Approximately 3% of water column shellfish were positive for *Salmonella* while only 0.7% of the bottom dwelling shellfish were positive for *Salmonella*. This marked difference in *Salmonella* incidence may be associated with salinity gradients between the ocean surface and marine bottom as the less-dense contaminated freshwater may remain near the sea surface [87].

Salmonella *Association with Marine Fauna*

Salmonella are often closely associated with fauna in marine environments. Venkateswaran et al. [141] isolated *Salmonella* from 100 µm marine plankton fractions and surrounding waters in coastal Japan. At several sites in this study *Salmonella* serotypes that were associated with zooplankton were not isolated from surrounding waters. These findings suggest that *Salmonella* persistence may be enhanced by its association with zooplankton. *Salmonella* has also been frequently isolated from shellfish, particularly oysters. Oysters are filter feeders and tend to concentrate bacteria such as *Salmonella* [87] and harbor them for extended periods of time [87]. Estimates of oysters contaminated with *Salmonella* in the United States ranged from 1.2-8% [64] [146]. If consumed raw or undercooked, these oysters may pose a health threat to humans [19].

WATERBORNE OUTBREAKS OF SALMONELLOSIS

Although *Salmonella* densities in water sources may be relatively low compared to contaminated foods, infectious dose studies suggest that infection by ingestion of contaminated waters is possible especially among population subgroups that are more susceptible, such as the elderly, young, and immunocompromised [81] [117] [118]. Rose et al. [117] estimated a 1% infection probability from the ingestion of 4.3 *Salmonella* cells using a beta-Poisson dose-response model. Using an exponential dose-response model Rose et al. [118] estimated a 1% infection probability from the ingestion of only 1.3 *Salmonella* cells. *Salmonella* concentrations in aquatic environments are comparable to concentrations of cells in these dose-response studies suggesting that *Salmonella* infections from environmental exposures are possible.

Although the majority of salmonellosis outbreaks have been linked to the consumption of contaminated foods [26], *Salmonella* is considered a leading cause of waterborne disease

outbreaks [80]. The U.S. waterborne salmonellosis caseload has been estimated at 60,000 cases per year [14]. In the summer of 1965, 16,000 residents of Riverside, California presented with salmonellosis due to contamination of local water supplies by *S*. Typhimurium [17]. In 1993, a community outbreak of *S*. Typhimurium from a contaminated municipal water supply occurred in Gideon, Missouri affecting 650 people and resulting in death for 54% of those that were hospitalized from infection [5] [80].

CONCLUSIONS

Although most *Salmonella* infections are caused by the consumption of contaminated animal-product foods, cases of waterborne infection have been reported [14] [17] [5]. Given that most cases of waterborne gastrointestinal infections go unreported [9], salmonellosis cases from environmental exposures are probably much higher than reported. *Salmonella*, found primarily in the large intestines of warm-blooded animals, is introduced into the environment through excretion. *Salmonella* cells can be subsequently transported into fresh waters, marine waters, and groundwater and studies have shown clinically relevant *Salmonella* serotypes to be common and highly persistent in natural waters. Furthermore, the association of *Salmonella* with marine fauna, such as plankton and oysters, may increase their persistence while establishing yet another exposure route by which humans may be infected. Waterborne *Salmonella* recovery rates increase after periods of precipitation and during summer months, which is consistent with clinical trends [86], and suggests that environmental exposures may be a significant factor in the overall salmonellosis disease burden.

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TABLES

Table 1. Taxonomy of the *Salmonella* genus.

Genus	Species	Subspecies
Salmonella	*S. enterica*	*S. enterica* subsp. *enterica* (I)
		S. enterica subsp. *salamae* (II)
		S. enterica subsp. a*rizonae* (IIIa)
		S. enterica subsp. *diarizonae* (IIIb)
		S. enterica subsp. *houtenae* (IV)
		S. enterica subsp. *indica* (VI)
	S. bongori	N/A

CHAPTER 3

SEASONALITY OF *SALMONELLA* SPP. IN A SOUTHEASTERN WATERSHED (USA)[1]

[1] Haley, B., D. Cole, and E.K. Lipp. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Salmonella is primarily considered a zoonotic foodborne disease, but outbreaks from contaminated water and non-animal origin foodborne salmonellosis have been reported. In this study, we investigated the seasonality of total *Salmonella* densities and frequencies of *Salmonella* serotypes isolated from surface waters in a rural coastal plain watershed in a high case-rate public health district in Georgia (U.S.A.) (>50/100,000 in 2004). Water samples were collected monthly over a 12 month period and analyzed for *Salmonella* densities using a 3-step Most Probable Number (MPN) assay. Presumptive *Salmonella* isolates were subsequently identified to the serotype level by serological analysis. One-hundred three *Salmonella* isolates from 13 serotypes of *S. enterica* subsp. *enterica* were isolated during the study. Eight of these, including the three most commonly isolated serotypes, are associated with human disease in the study region. Over the 12 month study period, average *Salmonella* densities for the watershed ranged from 5.9 MPN 1 L^{-1} in winter months to 19.6 MPN 1 L^{-1} in summer months. *Salmonella* densities differed significantly between seasons and were highest in the wetter summer months as was the frequency of detection for most serotypes. Ten different serotypes were recovered in summer, 9 in winter, 7 in fall, and 5 in spring. Total rainfall for the 24 hours and 48 hours preceding sample collection were significantly associated with *Salmonella* densities ($r=0.77$, $p=0.0012$ and $r=0.68$, $p=0.014$, respectively) in the watershed. The results of this study suggest that *Salmonella* densities in natural waters are seasonal and may be influenced by rainfall.

INTRODUCTION

The *Salmonella* genus consists of over 2,500 known serotypes, all of which are considered potential human pathogens [6]. Serotypes of *Salmonella enterica* subsp. *enterica* (I) account for approximately 99% of all *Salmonella* infections in warm-blooded animals [11]. Nontyphoidal *Salmonella* serotypes are not host-adapted to humans and must be introduced into human populations from exposure to contaminated foods, soil, water, or animal contact [36]. Although salmonellosis traditionally has been thought of as an animal-origin foodborne disease, many cases and outbreaks have not been linked to any verifiable food source [20] [2] [30] [29]. In addition, recent outbreaks resulting from water and produce contaminated with *Salmonella* confirm environmental sources of *Salmonella* contribute to human illness [14] [15] [16]. Consequently, environmental sources of *Salmonella* are now being increasingly investigated as a potentially significant reservoir of *Salmonella* transmission [48]. The ubiquitous nature of *Salmonella* and its widespread occurrence in both freshwaters and marine waters suggests that transmission in the aquatic environment from water consumption, recreation, or the consumption of food treated with or harvested in contaminated water is probable [48] [34] [10].

Understanding the environmental parameters that influence *Salmonella* loading into aquatic ecosystems is important in predicting and preventing waterborne transmission of this pathogen. Here we investigate the seasonality of total *Salmonella* presence as well as the temporal distribution different *Salmonella* serotypes known to cause disease in southern Georgia (USA). We further investigate those seasonal environmental parameters which may influence

Salmonella prevalence in this watershed along with the efficacy of fecal indicators in predicting *Salmonella* concentrations.

MATERIALS AND METHODS

Sampling Area

Water samples were collected within the 334 km^2 Little River watershed spanning Tift, Turner, and Worth counties and located within the Suwannee River Basin of the coastal plain of south central Georgia (USA) (Fig. 1). The topography is generally flat with meandering streams [12]. This watershed is located in a humid subtropical climate [49] and receives approximately 120 cm of rainfall each year [50]. Precipitation is generally highest in summer months but streamflow is at its lowest during this time of year due to high levels of evapotranspiration from croplands [9]. Soils in the region range from loamy sands to sandy loams [50]. Surface water and shallow groundwater (an unconfined surficial aquifer that is present throughout the southeastern coastal plain) were described by Bosch [9] as being "substantially interconnected," especially during and after periods of precipitation. The surficial aquifer ranges in thickness from 10 m to 2 m in the Little River watershed and is underlain by the relatively impermeable Hawthorne Formation which prevents the downward movement of surficial groundwater [4] [5]. Surface water in this watershed is used for irrigation and recreation and shallow groundwater is used for domestic water supply and irrigation [50]. Recreational activities in the Little River include fishing, swimming, and boating. The area is 44% forested, 25% cultivated, 15% pasture, 13% wetlands, and 3% urban [50]. The watershed contains intensive row-crop corn, soybeans, peanuts and cotton farms [22] as well as approximately 7,700 head of cattle, 500 swine and two poultry houses that produce approximately 440,000 broiler chickens per year [50].

Samples were collected from 6 stations along the Little River (Fig. 1). Sites I and F were located in Turner County near the city of Ashburn, which has a population of approximately 9,000, with 4,000 housing units, 1,500 septic tanks (38% of all households), and one wastewater treatment plant [51]. Sites N, B, 03 and O were located in Tift County near Tifton, which has a population of approximately 38,000, with 15,000 housing units, 5,000 septic tanks (33% of all households), and one wastewater treatment plant [51]. Site I is the most upstream sampling location and site O is at the mouth of the watershed. All sampling sites were influenced by both agriculture and minimal urban development [50]. Site 03 was proximal to a University of Georgia research farm containing dairy cattle and crops that are fertilized with liquid dairy cattle manure from a pivot irrigation system. Pivot irrigation systems are common throughout the watershed.

Sample Collection

Water was collected monthly from April 2005 – April 2006 as grab samples (2 L) in sterile polypropylene bottles. Water was analyzed *in situ* for conductivity (mS cm^{-1}), temperature (^{o}C), pH, dissolved oxygen (DO, mg L^{-1}), turbidity (Nephelometric Turbidity Units [NTU]), oxidation reduction potential (ORP, mV), fluorescence, and chlorophyll *a* (µg L^{-1}) with a YSI® 6600 Multiparameter Sonde (Yellow Springs, OH) by the USDA Southeast Watershed Research Laboratory (Tifton, GA). The YSI probe was placed into the deepest section of the stream to record physicochemical parameters at the time of sample collection. Samples were placed on ice immediately and kept at 5^{o}C until microbiological analyses were completed.

Microbiological Analyses

Within 24 hours of collection, samples were screened for *Escherichia coli* by membrane filtration using modified mTEC agar (Becton Dickson, Franklin Lakes, NJ) following USEPA

Method 1603 [52]. 10 ml and 1 ml were passed, in duplicate, through 0.45 µm pore size cellulose acetate membranes and placed on modified mTEC agar plates. Plates were initially incubated at 35ºC ± 0.5ºC for 2 ± 0.5 hours, and then transferred to a waterbath at 44.5ºC ± 0.2ºC for 22 ± 2 hours. *E. coli* were enumerated by counting the red and magenta colored colonies. Samples were also screened for enterococci by membrane filtration using mE agar (Becton Dickson) with indoxyl β-D-glucoside (Sigma-Aldrich, St. Louis, MO) following USEPA Method 1600 [53]. Plates were incubated at 41ºC ± 0.5ºC for 24 ± 2 hours. Enterococci were enumerated by counting all colonies with a blue halo. All bacterial fecal indicators were recorded as CFU 100 ml^{-1}.

Salmonella spp. were detected using a 3-step most probable number (MPN) assay involving pre-enrichment, enrichment and selection. Five replicates of three 10-fold dilutions were pre-enriched with a 1% buffered peptone water solution (pH 7.2 ± 0.2) and incubated at 37ºC for 18-24 hours. One-hundred microliter aliquots of the overnight enrichments were then added to individual 10 ml aliquots of Rappaport-Vassiliadis (RV) broth (Beckton Dickson, Franklin Lakes, NJ) and incubated at 43ºC for 24 ± 2 hours for selective enrichment of salmonellae. Ten microliters of the RV enrichment were then streaked onto xylose-lysine-deoxycholate (XLD [Becton Dickson]) agar for isolation at 35ºC for 24 ± 2 hours. Colonies that appeared black were presumptively identified as *Salmonella*. Positive (*Salmonella enterica* serotype Berta) and negative (no inoculate) controls were used to ensure the quality of culture results. Suspected *Salmonella* colonies were identified to the genus level using Enterotube™ II (Becton Dickson) assay. Colonies that were positively identified as *Salmonella* were grown overnight in 10 ml of tryptic soy broth, and then amended with glycerol (LabChem, Pittsburgh, PA) (20% final concentration), and dispensed into 1 ml aliquots before freezing at -80ºC.

Replicate cultures from each isolate were shipped to the *Salmonella* Reference Center (SRC, University of Pennsylvania New Bolton Center) for serotyping (SRC, University of Pennsylvania New Bolton Center).

Weather Data

Precipitation and streamflow data for each site were obtained from the USDA Southeast Watershed Research Laboratory precipitation archives (http://www.tifton.uga.edu/sewrl/archived_data.htm). Each station, except site 03, was equipped with a rainfall and streamflow gauge that collected daily precipitation and streamflow readings for the duration of the study. Site 03 precipitation and streamflow data were estimated from site O data. Precipitation data were gathered for the daily precipitation total for the seven days prior to sample collection, the two, three, and four week totals, and the four week daily average prior to sample collection for each site and then pooled to estimate the mean for the watershed. Streamflow data were gathered for the daily average flow and the maximum instantaneous flow for each day preceding sample collection.

Statistical Analyses

Data were analyzed using Statistical Package for Social Sciences (SPSS) for Windows, release 13.0 (SPSS., Chicago, IL). The MPN procedure estimates Colony Forming Units (CFUs) per sample volume. All estimated densities were standardized to CFUs 1 L^{-1} and were subsequently log-transformed to approximate a log-normal microbial density distribution. Pearson linear correlation coefficients were determined to describe the relationships between microbial densities and environmental parameters and the relationships between *Salmonella* and fecal indicator densities. Differences in mean microbial densities and environmental parameter means were determined using the Repeated Measures Analysis of Variance (RMANOVA). We

evaluated differences in the frequencies of recovered serotypes among sampling sites and seasons with a χ^2 test. Relationships between the presence or absence of serotypes and various environmental parameters and fecal indicators were determined by binary logistic regression analyses. For seasonal analyses, December, January and February were designated as winter; March, April and May as spring; June, July and August as summer; September and November as fall (water was not collected during the month of October). For all measures of association, p values ≤ 0.05 were considered significant.

RESULTS

Spatial Variability

In total 12, samples were collected from each site between April 2005 and April 2006. *E. coli* concentrations differed significantly by site (Fig. 2, Tbl. 1) ($p < 0.05$). The highest *E. coli* concentrations were consistently observed at site 03 (mean 412 CFU 100 ml^{-1}) while the lowest concentrations were observed at site B (mean 52 CFU 100 ml^{-1}). Enterococci concentrations also varied significantly by site (Fig. 2, Tbl. 1) ($p < 0.05$), with highest levels at site 03 (1170 CFU 100 ml^{-1}) and the lowest concentration at site B (97 CFU 100 ml^{-1}).

Salmonellae were detected at similar levels at all sites in the Little River (Fig. 2, Tbl. 1). The highest mean density was observed at sites N and I (13.7 MPN 1 L^{-1}). The lowest mean density was observed at site F (8.0 MPN 1 L^{-1}). *Salmonella* was detected 75% of the time at sites 03, N, I, and F; 83% of the time at site O and 100% of the time at site B.

Conductivity was the only water quality parameter that varied significantly by site ($p < 0.05$) and was significantly higher at site 03 than at all other stations.

Seasonal Variability

Total precipitation for summer, winter, and spring were significantly higher than total precipitation for fall ($p<0.05$) (Tbl. 2). Water temperatures ranged from 8ºC in December to 27ºC in September and were significantly higher in summer and fall than in winter and spring ($p< 0.05$) (Tbl. 2), but spring water temperatures were significantly higher than winter water temperatures ($p< 0.05$) (Tbl. 2). Streamflow varied significantly by season ($p<0.05$). Streamflow in winter, spring, and summer were significantly higher than streamflow in fall. The highest observed streamflow was 1.20 $cm^3\ s^{-1}$ in winter and the lowest was 0.0 $cm^3\ s^{-1}$ observed in fall (Tbl. 2).

E. coli levels did not vary significantly by season. The highest *E. coli* levels were 357 CFU 100 ml^{-1} in the summer and the lowest *E. coli* levels were 281 CFU 100 ml^{-1} in the fall (Tbl. 2). Enterococci levels were significantly higher in summer than in all other seasons ($p<0.05$). The highest mean seasonal enterococci concentration was 1068 CFU 100 ml^{-1} in summer. Enterococci levels also varied significantly by month ($p< 0.05$), with the highest mean concentration during August (1133 CFU 100 ml^{-1}) and the lowest mean concentration during January (134 CFU 100 ml^{-1}).

Salmonella densities were significantly higher in summer (19.6 MPN 1 L^{-1}) than winter (5.9 MPN 1 L^{-1}) and fall (6.9 MPN 1 L^{-1}) ($p< 0.05$) (Tbl. 2). For all stations, the highest *Salmonella* densities were observed in August, excluding site N where the highest densities were observed in late winter. One-hundred percent of stations were positive for *Salmonella* in summer months, while only 50% were positive during fall months. Eighty three percent of all stations were positive during spring and 78% were positive in winter months. Mean pathogen densities ranged by month from 4.6 MPN 1 L^{-1} in February to 39.3 MPN 1 L^{-1} in August (Fig. 3).

Over all samples collected, *Salmonella* densities ranged from < 0.03 MPN 1 L^{-1} (at least once at each station excluding site B) to 68.6 MPN 1 L^{-1} (site I, August 2005).

Mean *Salmonella* densities were positively correlated with the total precipitation for the 24 hours (r=0.77, p=0.0012) and 48 hours (r=0.68, p=0.0014) preceding sample collection. Significant associations between *Salmonella* densities and precipitation were lost when rainfall exceeding 48 hours prior to sample collection was used at individual stations. *Salmonella* densities also were significantly correlated with water temperature (r=0.44, p<0.05), which is consistent with its seasonal and monthly distribution. There were no significant correlations between streamflow and *Salmonella* densities (r=0.01 p=0.48).

Salmonella *Serotype Distribution*

In total, 189 *Salmonella* isolates, representing 13 different serotypes, were recovered from the Little River watershed during the 12 month sampling period (Tbl. 3). 86 of the 189 (45%) *Salmonella* isolates were identified as *Salmonella enterica* subsp. *arizonae* by biochemical analysis. The remaining 103 isolates were identified to the serotype level in *Salmonella enterica* subsp. *enterica* as listed in Table 3. *S*. Rubislaw (26 isolates, 14%), *S*. Muenchen (24 isolates, 13%), *S*. Mikawasima (12 isolates, 6%) were the three most frequently isolated serotypes from this watershed.

Serotype diversity did not vary by site or position in the watershed, but did vary by season. However, serotype diversity did not follow the seasonal prevalence of the *Salmonella* densities. Nine serotypes were isolated during winter months, 5 during spring months, 10 during summer months, and 7 during fall months (Tbl. 3). Serotype frequency also varied seasonally with *S*. Mikawasima most frequently detected in winter months, and *S*. Liverpool, *S*. Anatum, *S*. I 47:z4z23 only being detected in winter months. Members of *Salmonella enterica* subsp.

arizonae were most frequently isolated in spring months. *S*. Muenchen, *S*. Rubislaw, *S*. Braenderup, *S*. Saint Paul, *S*. Bareilly, and *S*. Gaminara were most frequently isolated in summer months. *S*. Montevideo was only isolated during the summer. *S*. Pullorum was most frequently isolated during the fall. *S.* I 4,[5]:b was only detected in the fall.

Using binary logistic regression, there was a 91% and 82% correct prediction percentage between the presence of *S*. Gaminara and *S*. Pullorum, respectively, and precipitation totals for the 48 hours prior to sample collection ($p<0.05$). *Salmonella* densities were not significantly correlated with either *E. coli* or enterococci concentrations, however, there was a 90% and 89% correct prediction percentage between the presence of *S*. Braenderup and *E. coli* and enterococci densities, respectively, as determined by binary logistic regression ($p<0.05$). There were no other significant predictors of *Salmonella* serotype presence.

Water Quality Parameters

The USEPA single-sample maximum for *E. coli* (235 CFU 100 ml^{-1}) and enterococci (61 CFU 100 ml^{-1}) in freshwater [24] were exceeded in 39% and 93%, respectively, of samples. Neither indicator provided predictive value, with respect to the presence of total *Salmonella*, which was detected 77% and 75% of the time when *E. coli* and enterococci levels were below the USEPA single-sample maximum. Neither indicator provided significant predictive value by binary logistic regression when the presence of all *Salmonella* serotypes was pooled and serotypes not commonly isolated from humans in the region were removed (*Salmonella arizonae*, *S*. Pullorum, *S.* I 4,[5]:b, *S*. Mikawasima, and *S*. 47:z4z23,). *E. coli* varied inversely with oxidation reduction potential ($r= -0.32$, $p< 0.05$) while enterococci were positively correlated with conductivity ($r= 0.54$, $p< 0.05$). *E. coli* and enterococci concentrations were not significantly correlated with each other or rainfall.

Few physicochemical parameters had a significant association with *Salmonella* levels, especially over the entire study area; however, *Salmonella* levels did vary inversely with DO at site O ($r = -0.84$, $p < 0.05$).

DISCUSSION

Salmonella is an enteric pathogen that can be readily isolated from environmental sources including surface waters [18] [19] [35]. The presence and persistence of *Salmonella* in environmental waters may be governed, in part, by extrinsic environmental parameters and may vary spatially and temporally. Furthermore, *Salmonella* serotypes may vary in their ability to persist in the environment and some may be better suited for survival during different seasons [35] [42] [56]. Our objective in this study was to investigate the seasonality of total *Salmonella* presence as well as the presence of *Salmonella* serotypes known to cause disease in humans in a south Georgia (USA) watershed. We further investigated those seasonal environmental parameters which may influence *Salmonella* prevalence in this watershed along with the efficacy of fecal indicators in predicting *Salmonella* loads.

Efficacy of Fecal Indicators

Fecal indicators are used to assess the quality of surface waters. Theoretically, the presence of fecal indicator bacteria suggests pollution of surface waters, but their absence does not assure the safety of the water [25]. *E. coli* and enterococci are common indicators of fecal contamination, but studies have shown that *Salmonella* spp. are more resistant to environmental degradation than either indicator [7] [45] [55]. Moreover, others have shown that *Salmonella* spp. are present at low concentrations or in absence of indicators of fecal pollution in surface waters [8] [13] [18] [32] [37] [38] [41] [42]. In this study, neither *E. coli* nor enterococci were significantly correlated with *Salmonella* densities. Neither indicator provided significant

predictive value for the presence of all but one *Salmonella* serotypes of public health significance when analyzed by binary logistic regression. The presence *S*. Braenderup demonstrated a 90% and 89% correct prediction percentage ($p<0.05$) when compared to *E. coli* and enterococci concentrations respectively. Furthermore, the station with the highest indicator densities, station 03, yielded the second lowest *Salmonella* densities.

Seasonal Trends

Seasonal *Salmonella* recovery trends in this watershed corresponded to trends in clinical cases of salmonellosis reported among humans and animals [39]. The higher prevalence of salmonellosis in humans and animals during warmer months [39] may cause an increase in the density of *Salmonella* in surface waters from fecal contamination through sewage outputs or runoff in the summer.

An increase in *Salmonella* densities in the watershed corresponded with an increase in precipitation, which varied seasonally, and suggests that rainfall may influence the transport of *Salmonella* into the watershed by an increase in both agricultural and urban runoff as well as septic tank leakage. Others have also demonstrated that the presence of *Salmonella* in aquatic environments is linked to episodes of rainfall [27] [28] [41].

Higher *Salmonella* densities were also associated with an increase in water temperature, which like rainfall peaked in summer months. While this trend also corresponds to clinical case rates of salmonellosis in the region of study [21], it is unknown how temperature may influence *Salmonella* survival in the aquatic environment. Results of previous microcosm and field studies suggest that water temperature may not directly influence total *Salmonella* densities, but rather reflects overall seasonal changes in environmental parameters in the watershed [45] [1].

While river water was present continuously at all sites, streamflow decreased to 0 cm^3 s^{-1} several times throughout the study (at all sites during September and November 2005). These decreases occurred in fall months when rainfall was low and evapotranspiration from intensive crop agriculture was high. Evapotranspiration in the region often depletes the surficial aquifer which influences streamflow in this watershed [9]. The highest mean *Salmonella* densities (39.3 MPN 1 L^{-1}) and the highest single-sample *Salmonella* density (68.6 MPN L^{-1} at site I) occurred during August 2005 when streamflow was the third lowest of all four seasons (1.08 cm^3 s^{-1}), suggesting that an increase in runoff from high levels of precipitation (13.0 cm) and a decrease in streamflow may lead to a decrease in dilution of *Salmonella* cells. In the winter months precipitation was relatively high (12.4 cm) as was streamflow (1.20 cm^3 s^{-1}) suggesting that *Salmonella* cells were more diluted and may account for lower *Salmonella* densities (5.9 MPN 1 L^{-1}). These data suggest that the seasonality of *Salmonella* densities may primarily be a factor of seasonal carriage rates of *Salmonella* in humans and animals in the study area and changes in loading from precipitation and runoff coupled with a dilution factor from streamflow levels.

Spatial Trends

Salmonella densities did not differ by sampling station in this study. All stations were located within the same rural watershed and were influenced by agriculture (25% cultivated land and 13% pastureland) and limited urban development (3% urban). Station 03 was proximal to a research farm containing cattle and a pivot irrigation system was hypothesized to yield the highest *Salmonella* densities of all the potential sources in the watershed. This station did yield significantly higher concentrations of both *E. coli* and enterococci than all other stations and may have received a higher direct fecal input than other sites; however, mean *Salmonella* densities

were lowest at this site for the 12 month study period suggesting that other sources of *Salmonella*, in addition to agricultural runoff, may exist in this watershed.

Salmonella *Serotype Distributions*

In total, 13 different *Salmonella* serotypes were isolated from our study region. This is consistent with other studies of *Salmonella* spp. in surface waters which have typically found a range of 17-20 different serotypes of the greater than 2,500 known *Salmonella* serotypes [13] [35] [42] [54].

All of the recovered serotypes excluding *S.* I 4,[5]:b and *S*. Mikawasima have been associated with either bovine or poultry infections [17]. The study region contains a high density of both dairy farms and poultry houses (the watershed contains approximately 7,700 head of cattle and 440,000 broilers are produced per year) [50].

Eight of the recovered serotypes have been associated with human disease in Georgia (*S*. Muenchen, *S*. Montevideo, *S*. Braenderup, *S*. Saint Paul, *S*. Bareilly, *S*. Gaminara, *S*. Anatum, and *S*. Liverpool) [17]. *S*. Muenchen, *S*. Montevideo, and *S*. Braenderup, which were all frequently isolated from the Little River watershed, were, respectively, the fifth, sixth, and eighth most frequently isolated serotypes from human clinical cases in the state of Georgia in 2004 [17]. The relatively high incidence of *S*. Muenchen in this watershed is of epidemiological significance due to the increasing isolation frequency of this serotype from human clinical cases in Georgia in the past 10 years (34% increase from 1995 to 2004) [17]. Furthermore, this serotype has been previously linked to non-animal product associated outbreaks of salmonellosis [44] [15].

Studies have demonstrated the presence of *Salmonella* isolates in surface waters that are genotypically related to isolates reported from clinical cases in those areas [31] [3]. The

presence of these serotypes in the surface waters of our study region may be due to both local human and animal fecal pollution. Runoff from nearby cattle operations and poultry production houses as well as contamination from pivot irrigation systems, septic tanks, and the inability of municipal wastewater treatment plants to completely disinfect human waste [47] may all contribute to the presence of these serotypes in these waters.

Some of the more common human and animal clinical serotypes such as *S*. Enteriditis, *S*. Typhimurium, *S*. Heidelberg, and *S*. I 4,[5],12:i- were not recovered during our study period even though common hosts of these serotypes were present in the watershed (cattle, poultry, and swine). The different population structures of *Salmonella* serotypes in both human and animal infections and surface waters may be related to different rates of survival of these serotypes in freshwaters. However, it is currently unknown if there is differential persistence between serotypes in surface waters.

Members of *Salmonella enterica* subsp. *arizonae*, most commonly found in snakes and other reptiles as normal constituents of their intestinal flora [33], contributed heavily to the total *Salmonella* load (42% of all *Salmonella* isolates). The isolation frequency of this subspecies did not differ significantly between stations suggesting that the high prevalence of this subspecies may influence the total *Salmonella* load across the entire watershed. Given that this region has a high density of wetlands, results suggest that local reptile populations may be a significant source of *Salmonella* in this watershed.

Serotype diversity fluctuated seasonally with the greatest number of serotypes being isolated during summer months (10) and the fewest serotypes isolated in spring months (5). This result may be due to the high carriage rates of *Salmonella* in animals in humans during warmer months [43] [17] [39] [23] [26]. Prevalence of all serotypes that were isolated during more than

one season, excluding *Salmonella* Pullorum and *Salmonella arizonae*, which are not clinically significant in this region, was highest during the warmer summer and fall months. These results suggest that while human and animal salmonellosis is prevalent year-round, higher carriage rates in summer months coupled with seasonal rainfall patterns influence *Salmonella* densities in this watershed.

Waters contaminated with *Salmonella* may pose a health risk to humans due to exposure from recreation, ingestion, and consumption of produce irrigated with contaminated water [14] [30] [40]. Surface water in the study region is used for irrigation and recreation; and, shallow groundwater is used for domestic water supplies and irrigation [50]. Groundwater and surface water in this watershed have been described as being substantially interconnected due to the presence of loamy sand soils [9]. Thus, the high percentage of septic systems (37% of all households in Turner county and 33% of all households in Tift county), presence of pivot irrigation systems, as well as cattle, swine, and poultry houses throughout the watershed may all be potential sources of *Salmonella* in these surface waters.

Although the majority of salmonellosis outbreaks have been linked to the consumption of contaminated foods [16], *Salmonella* is considered a leading cause of waterborne disease outbreaks [30]. Several large regional outbreaks of salmonellosis have been linked to contaminated water supplies [2] [30] [29]. Moreover, based on epidemiologic evidence, *Salmonella* may be infective at low doses. Using a beta-Poisson model, Rose et al. [46] estimated a 1% probability of infection from ingestion of 4.3 *Salmonella* cells. The low infectious dose of *Salmonella* compared to the *Salmonella* densities observed in this study suggests that ingestion of surface waters may pose a health risk to humans, especially to members of more susceptible populations. Furthermore, seasonal variability in waterborne

Salmonella densities and changes in human activities, such as seasonal variation in the recreational use of the watershed and changes in crop irrigation intensity may contribute to potential *Salmonella* exposure routes and the seasonality of salmonellosis observed in this region.

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TABLES

Table 1: Mean microbial densities for each sampling station. Site I is the most upstream station and site O is the outlet of the watershed.

Site	*E. coli* (CFU 100 ml^{-1})	Enterococci (CFU 100 ml^{-1})	*Salmonella* (MPN 1 L^{-1})
I	169 [a b]	201 [a b]	13.7 [a]
F	173 [a b]	223 [b]	8.0 [a]
N	187 [a b]	344 [b]	13.7 [a]
B	52 [b]	97 [a]	11.1 [a]
03	412 [a]	1171 [c]	9.6 [a]
O	265 [a b]	324 [b]	11.2 [a]

[a b c] Values with same letter are NOT significantly different at the 0.05 (95%) level in the same column

Table 2. Mean seasonal environmental parameters and microbial densities.

Season	Water Temperature (ºC)	Preciptation (cm)	Streamflow (cm^3 s^{-1})	*E. coli* (CFU 100 ml^{-1})	Enterococci (CFU 100 ml^{-1})	*Salmonella* (MPN 1 L^{-1})
Winter	8.9 [a]	12.4 [a]	1.20 [a]	282 [a]	400 [a]	5.9 [a]
Spring	15.7 [b]	10.0 [a]	1.10 [a]	337 [a]	532 [a]	11.1 [ab]
Summer	24.5 [c]	13.0 [a]	1.08 [a]	357 [a]	1068 [b]	19.6 [b]
Fall	25.8 [c]	1.34 [b]	0.00 [b]	281 [a]	270 [a]	6.9 [a]

[a b c d] Values with same letter are NOT significantly different at the 0.05 (95%) level in the same column.

Table 3. *Salmonella* serotype isolation frequencies.

Serotype	Apr-05	May	Jun	Jul	Aug	Sep	Nov	Dec	Jan-06	Feb	Mar	Apr	Total Number of Isolates
S. enterica subsp. *arizonae*	2[a](2.4)[b]	17(20)	7(8.2)	1(1.2)	9(10.6)	5(5.9)	1(1.2)	2(2.4)	11(12.9)	0	14(16.5)	13(15.2)	86
S. Rubislaw	0	2(7.7)	3(11.5)	9(34.6)	2(7.7)	3(11.5)	3(11.5)	2(7.7)	0	0	2(7.7)	0	26
S. Muenchen	0	1(4.6)	1(4.6)	3(12.5)	5(20.8)	6(25)	1(4.6)	1(4.6)	1(4.6)	2(8.3)	3(12.5)	0	24
S. Mikawasima	0	0	2(16.6)	0	5(41.6)	0	1(8.3)	4(33.3)	0	0	0	0	12
S. Branderup	0	1(11.1)	0	3(33.3)	4(44.4)	0	0	0	1(11.1)	0	0	0	9
S. Saint Paul	0	0	0	1(12.5)	4(50)	1(12.5)	0	2(25)	0	0	0	0	8
S. Bareilly	0	2(33.3)	0	0	4(66.6)	0	0	0	0	0	0	0	6
S. Liverpool	0	0	0	0	0	0	0	0	0	5(100)	0	0	5
S. I 4,[5]:b	0	0	0	0	0	0	4(100)	0	0	0	0	0	4
S. Pullorum	0	0	0	0	2(40)	3(60)	0	0	0	0	0	0	3
S. Gaminara	0	0	0	1(33.3)	2(66.7)	0	0	0	0	0	0	0	3
S. Montevideo	0	0	0	0	1(100)	0	0	0	0	0	0	0	1
S. Anatum	0	0	0	0	0	0	0	1(100)	0	0	0	0	1
S. I 47:z4z23	0	0	0	0	0	0	0	0	0	1(100)	0	0	1
Total Number of Serotypes	1	5	4	6	10	5	5	6	3	3	3	1	189

[a] Number of isolates

[b] Percent of isolates of each serotype detected in each month.

FIGURE LEGENDS

Figure 1: Map of the sampling stations in the Little River watershed.

Figure 2: Monthly mean microbial densities.

Figure 3: Mean *Salmonella* densities for the entire watershed by month and total station percent positive.

Figure 4: Precipitation prior to sample collection and mean *Salmonella* densities. Pearson r=0.77, p=0.0012.

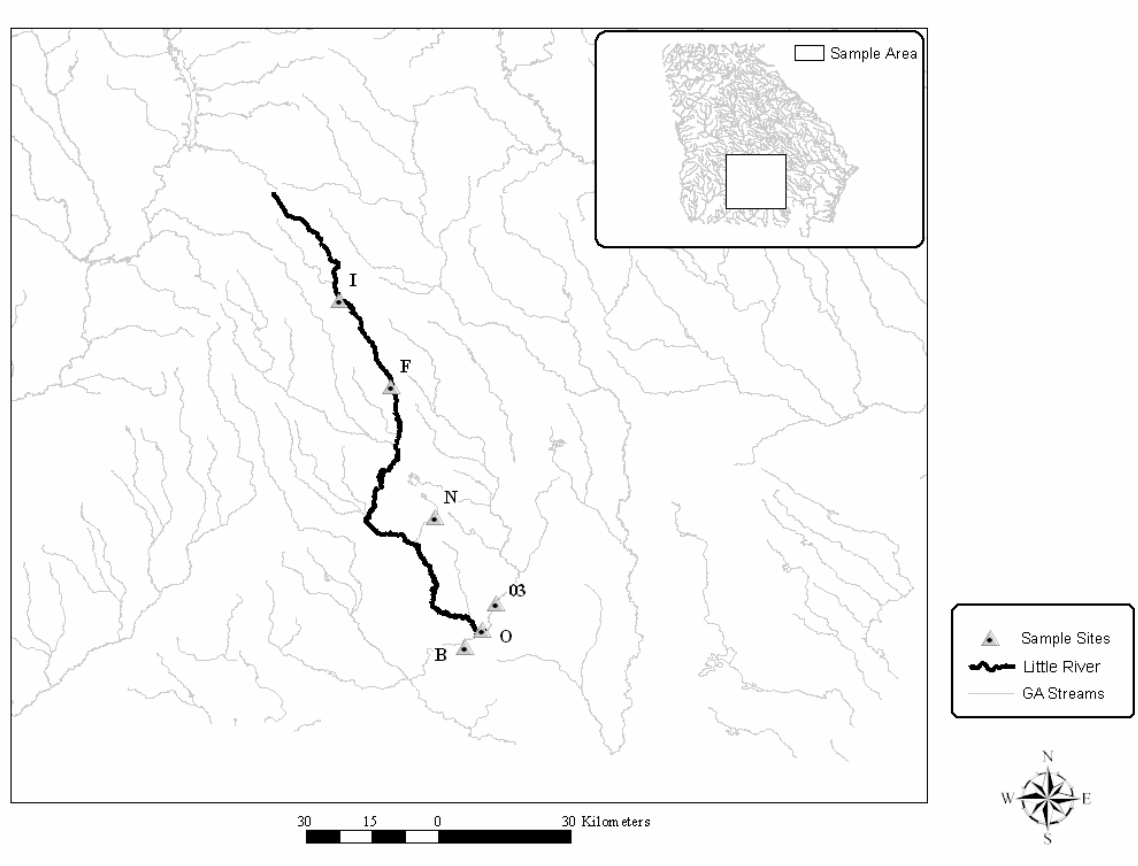
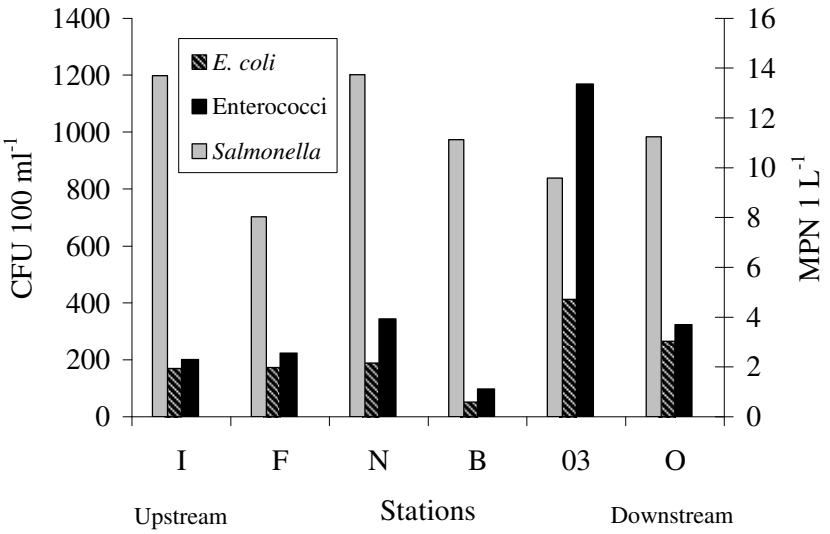

Sample Area
I
F
N
03
O
B
30 15 0 30 Kilometers
Sample Sites
Little River
GA Streams
N
W
E
S

Figure 1.


1400
1200
1000
800
600
400
200
0
CFU 100 ml-1
E. coli
Enterococci
Salmonella
16
14
12
10
8
6
4
2
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MPN 1 L-1
I
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N
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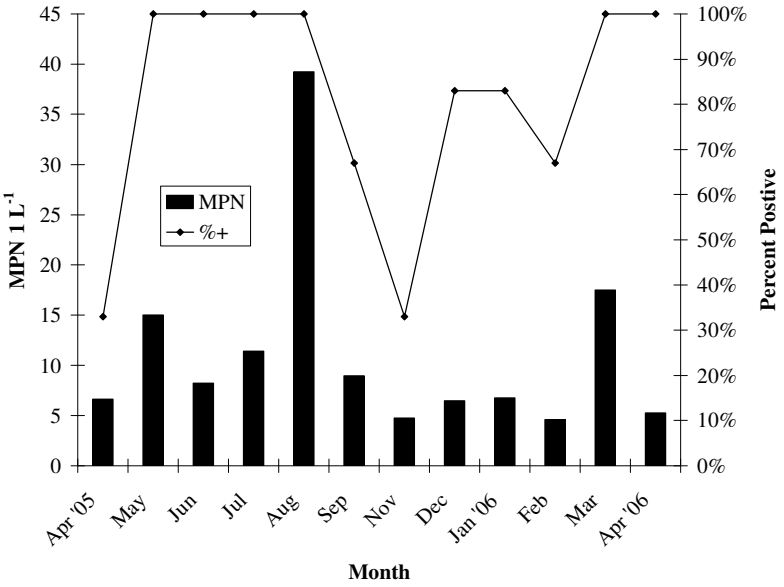
03
O
Upstream
Stations
Downstream

Figure 2.


45
40
35
30
25
20
15
10
5
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MPN 1 L-1
MPN
%+
100%
90%
80%
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40%
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20%
10%
0%
Percent Postive
Apr '05
May
Jun
Jul
Aug
Sep
Nov
Dec
Jan '06
Feb
Mar
Apr '06
Month

Figure 3.

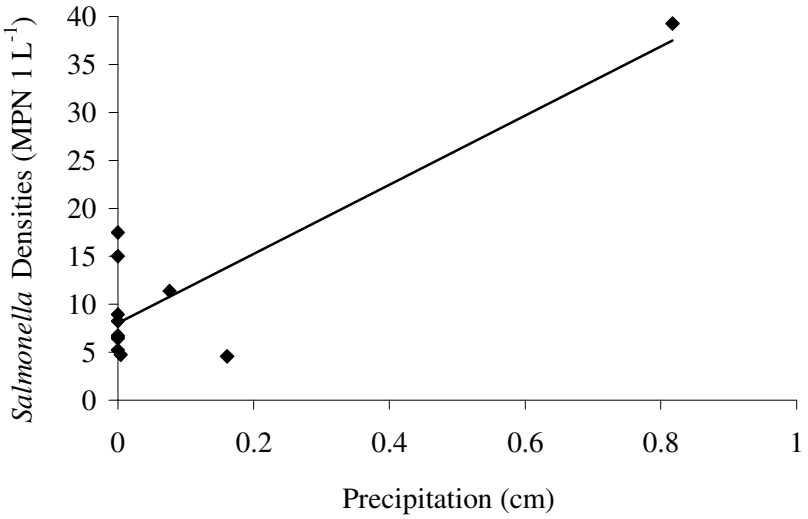
40
35
30
25
20
15
10
5
0
Salmonella Densities (MPN 1 L-1)
0
0.2
0.4
0.6
0.8
1
Precipitation (cm)

Figure 4.

CHAPTER 4

DIFFERENTIAL PERSISTENCE OF *SALMONELLA* SEROTYPES IN AQUATIC ENVIRONMENTS[1]

[1] Haley, B., D. Cole, and E.K. Lipp. To be submitted to *The Journal of Water and Health*.

ABSTRACT

Surface water sources that are impacted by urban and agricultural land use are a significant reservoir of *Salmonella*, which may allow for transmission to humans; however, seasonal temperatures may influence the persistence of *Salmonella* in natural waters. Here we investigated the differential persistence of two *Salmonella* serotypes in a river water microcosm. *S*. Typhimurium and *S*. Muenchen were exposed, in filter-sterilized river water, to a "winter" temperature (5ºC) and a "summer" temperature (30ºC) for 84 days. Water was tested periodically to determine persistence which was measured as culturable cells on TSA, direct viable cells by epifluorescence microscopy, and enriched cells by a 3-step Most Probable Number (MPN) assay. Based on TSA culturable plate counts, *S*. Muenchen was more persistent than *S*. Typhimurium at both temperatures. At 84 days, *S*. Typhimurium lost TSA plate culturability at 5ºC and demonstrated only 0.004% survivability at 30ºC, while *S*. Muenchen demonstrated 0.4% and 0.04% survivability, respectively, at these temperatures. Decay rates on TSA plates were significantly lower for *S*. Muenchen at 5ºC (0.0272) than at 30ºC (0.0388) and *S*. Typhimurium at both temperatures (0.045 and 0.0505). Direct viable counts remained relatively stable throughout the experiment and MPN counts were not estimable. Our results suggest that *S*. Muenchen and *S*. Typhimurium are persistent in river water and that *S*. Muenchen is more persistent than *S*. Typhimurium, especially at lower temperatures. We were also able to demonstrate a transition of *S*. Typhimurium in the VBNC state at low temperatures.

INTRODUCTION

Salmonella, a common causative agent of gastroenteritis, is readily recovered from both fresh and marine waters [11] [12] [22] [7] [19] [27] [26]. Often *Salmonella* serotypes that predominate in the local human and animal populations are among the most frequently isolated serotypes in that region's surface waters, suggesting local human and animal sources [1] [18] [2]; however, serotype isolation frequencies from clinical humans and animal cases do not exactly mimic the frequencies of serotypes found in surface waters [22] [2] [7]. This suggests that serotypes of clinical origin may not be as persistent as environmental strains [27] [37]. It is unknown if differential survival characteristics between *Salmonella* serotypes found in surface waters exist and, therefore, influence the likelihood of waterborne illness with specific serotypes.

The epidemiology of human salmonellosis suggests the presence of different reservoirs for different serotypes [35], and environmental data may support these observations [35] [22]. The extent to which *Salmonella* serotypes are adapted for survival in specific reservoirs (animal hosts and the environment) is unclear [23]. Epidemiologic data suggests that certain serotypes may persist longer in populations of specific species than others [23], suggesting that serotypes may be adapted to specific thermal niches and physicochemical environments and may therefore survive longer in different environments [5]. Moreover, certain serotypes appear to be more widely distributed in animal populations and the environment, such as *S*. Entiriditis, than others, such as *S*. Typhi which is confined primarily to primate hosts [3], suggesting that some serotypes are more tolerant to environmental stresses than others. Field studies suggest that certain serotypes may demonstrate greater persistence than others in natural waters [37], but research

comparing the environmental persistence between serotypes are lacking. *S*. Typhimurium and *S*. Muenchen, two frequently isolated *Salmonella* serotypes from humans [9], are commonly found in surface waters [21] [22] [2] [7] [27] [36]. Both have caused outbreaks of salmonellosis associated with the exposure to a wide range of animal and non-animal products including contaminated waters [17] [28] [8]. *S*. Typhimurium is known to be adapted to the gut of many warm-blooded animals and its host range has been loosely defined as encompassing several species [3]. *S*. Muenchen has been isolated from swine and human wastes suggesting an animal source [14], but its specific host range has not been defined. Although *S*. Typhimurium is the most commonly reported serotype from clinical humans and animals in the southeastern U.S. [9], recent studies have recovered *S*. Muenchen more frequently than *S*. Typhimurium from rivers in this region [13] [16] suggesting natural waters may be a stable reservoir for *S*. Muenchen and a significant source of *S*. Muenchen infections.

It is currently unknown if there are significant differences in persistence between serotypes in natural waters therefore influencing which *Salmonella* serotypes humans are exposed to from the environment. Here we investigated the relative environmental persistence of *S*. Muenchen and *S*. Typhimurium in river water. We conducted a microcosm study to test the hypothesis that differential persistence of *Salmonella* serotypes in environmental waters may contribute to variability in serotype exposures from environment.

MATERIALS AND METHODS

Bacterial strains

Two different *Salmonella enterica* subsp. *enterica* (I) serotypes were used in this study. *S*. Typhimurium (strain RC74) was isolated from a contaminated food source (courteously

provided by Dr. Anwar Huq, COMB, UMB, Baltimore, MD) and *S*. Muenchen (strain BH26) was isolated from the Little River in Tift County, Georgia (USA) [16].

To ensure the integrity of the cultures each serotype was grown overnight in Rappaport-Vassiliadis (RV) broth (Difco [Franklin Lakes, New Jersey]) and then streaked on xylose-lysine-deoxycholate (XLD) agar (Difco) for isolation of pure colonies. One colony was picked for each serotype and transferred to 10 ml of tryptic soy broth (TSB) in two separate tubes and incubated overnight at 35ºC with gentle shaking. The inoculated tubes were removed from the incubator after 18 hours of growth and washed three times in 1X phosphate buffered saline solution (PBS) at 2,400 × *g* at room temperature for 10 minutes.

Survival studies

10 µl of each washed overnight *Salmonella* culture were added to two separate sterile flasks containing 1.2 L of room-temperature, filter-sterilized river water, from the Little River watershed [16], in a room with minimal sunlight. 40 ml of the inoculated river water were then added to each of 24 opaque, sterile polypropylene bottles for each *S. enterica* serotype. Each bottle was covered in aluminum foil to inhibit any possible ultraviolet light interference. For each inocula, 12 bottles were kept static at 5ºC and 12 bottles were kept at 30ºC.

One bottle was removed for each *Salmonella* serotype and temperature at the time of inoculation ($T \approx 0$) and at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 14, 23, 35, and 84 days post-inoculation. At each time point, *Salmonella* densities were determined using culture based and direct methods (described below). A pre-inoculation sample was also tested for bacteria presence using all three enumeration methods.

Culturable counts

Culturable *Salmonella* densities were estimated for each serotype by spread plating 100 µl, in quadruplicate, onto tryptic soy agar (TSA) plates (Difco) followed by incubation at 35ºC for 24 ± 2 hours.

Direct viable counts

Total viable *Salmonella* counts were determined for each serotype by epifluorescence microscopy using a Live/Dead BacLight Kit (Molecular Probes, Eugene, OR). Five-hundred µl aliquots from each time point were stained with 2X Live/Dead staining reagent and then concentrated by filtering onto a 0.2 µm isopore polycarbonate membrane. Total viable direct counts were determined by counting all cells fluorescing green under blue fluorescence at 40X magnification. Final concentrations of viable cells were determined by averaging counts from three random microscopic fields per filter.

Enrichment counts

Salmonella cells were enriched and densities were estimated by employing a 3-step MPN assay involving pre-enrichment with buffered peptone water (Difco), enrichment with RV broth, and selection on XLD agar [25]. Three replicates of a 3-fold dilution ranging from 0.1 ml to 10 ml were used in this assay.

Statistical analysis

Log-linear survival curves were determined for each microcosm and detection assay using Microsoft Excel (Redmond, WA). *Salmonella* densities were log-transformed to approximate a log-normal microbial density distribution. Differences in persistence between serotypes at each temperature, based on plate counts, enrichment counts, and direct viable counts

were compared at each time point using the independent samples t-test and decay functions were compared using the Student's t-test in SAS version 9.0 (Cary, NC).

RESULTS

Thirteen time points, including T ≈ 0, were analyzed for changes in *Salmonella* densities for each serotype at 5ºC and 30ºC. *Salmonella* densities as determined by the MPN assay did not change for either serotype at either temperature throughout the study (>30.75 MPN 1 ml^{-1} at all sampling points). Direct viable counts for both serotypes at both temperatures showed no significant change over the course of the study. After 84 days, direct viable cells demonstrated an 9.3% and 6.6% survival for *S*. Muenchen at 5ºC and 30ºC, respectively and a 3% and a 1.8% survival for *S*. Typhimurium at both temperatures.

Based on TSA culturable plate counts, *S*. Muenchen demonstrated greater persistence than *S*. Typhimurium at both temperatures. After 84 days, 0.4% and 0.04% of the initial *S*. Muenchen population survived at 5ºC and 30ºC, respectively; however no *S*. Typhimurium cells were detected at 5ºC and only 0.004% to this point at 30ºC (Tbl. 1). At 84 days *S*. Muenchen survival at 5ºC was significantly greater than *S*. Typhimurium survival at 5ºC and 30ºC ($p<0.05$). Although *S*. Muenchen kept at 30ºC demonstrated greater persistence than *S*. Typhimurium at both temperatures, this difference was not significant. *S*. Muenchen demonstrated greater persistence at 5ºC than 30ºC ($p<0.05$). There was no differential persistence of *S*. Typhimurium at either temperature.

TSA culturable plate counts and direct viable counts for both serotypes were fit to log-linear decay functions to describe their die-off (Tbl. 1, Fig. 1). In general, survival curves fit TSA culturable plate counts better ($R^2 = 0.83$-0.95) than direct viable counts ($R^2 = 0.45$-0.75) for both serotypes. Decay rates (log-linear die-off slopes) of the TSA culturable plate counts were

significantly higher than the decay rates of the direct viable counts for like-serotypes and between-serotypes at both temperatures as determined by the Student's t-test ($p<0.05$) (Tbl. 1). Based on TSA culturable plate counts the decay rate of *S*. Muenchen was significantly lower at 5ºC than at 30ºC ($p<0.05$). Similarly, the decay rate of *S*. Muenchen at 5ºC was significantly lower than that of *S*. Typhimurium at both 5ºC and 30ºC and the decay rate of *S*. Muenchen at 30ºC was significantly lower than the decay rate of *S*. Typhimurium at 5ºC ($p<0.05$). There were no significant differences between any other decay rates.

DISCUSSION

Our results indicate that *Salmonella* is persistent and remains readily recoverable for extended periods of time in environmental waters at environmentally realistic temperatures. Based on TSA culturable plate counts, we observed a 99.6% and 99.96% die-off of *S*. Muenchen at 5ºC and 30ºC, respectively and a 100% and 99.996% die-off of *S*. Typhimurium at these temperatures, over the course of 84 days. Although TSA culturable plate counts decreased dramatically, direct viable counts and MPN assay counts remained relatively stable over the course of the experiment. Direct viable counts decreased by 90.7% and 93.4% for *S*. Muenchen at 5ºC and 30ºC, respectively, and 97% and 98.2% for *S*. Typhimurium. MPN assay counts did not change over the course of the experiment. Previous studies have demonstrated the ability of *Salmonella* to remain viable for extended periods of time in freshwaters [33] [20], brackish waters [10] [29], and phosphate buffered saline solutions [15]. Gupte et al. [15] demonstrated a loss of culturability of *S*. Typhimurium on TSA plates but directly detected viable cells that were held at 5ºC for 365 days in a phosphate buffered saline solution. These results are consistent with those we report here.

This study also demonstrated a differential persistence of *Salmonella* serotypes in natural waters. *S*. Muenchen was more persistent in river water, especially at 5°C. *S.* Typhimurium, the most common human and animal clinical isolate [9], demonstrated better survival at higher temperatures over a shorter time period (2 and 4 days). However, over the course of the experiment *S*. Muenchen demonstrated better survival. The decay rate of *S*. Muenchen at 5ºC was significantly lower than the decay rate of *S*. Typhimurium at both temperatures and the decay rate of *S*. Muenchen at 30ºC was significantly lower than the decay rate of *S*. Typhimurium at 5ºC. The decay rate of *S*. Muenchen was also significantly lower at 5ºC than 30ºC. These data suggest that *S*. Muenchen may be better adapted to environmental waters than *S*. Typhimurium and may fare better across a wider range of environmentally realistic temperatures, especially at lower temperatures. These data also suggest that river waters may be a stable reservoir for *S*. Muenchen and that the potential for human infections with this serotype from natural waters is probable. *S*. Typhimurium, the most commonly isolated serotype from humans and warm-blooded animals, is adapted to the mammalian gut [3] and has a thermal-niche range of approximately 27.7-39.8ºC [4]. This range is higher than what is observed in most environmental waters and may explain the more rapid decay rate of *S*. Typhimurium at 5ºC than 30ºC.

We demonstrated evidence for a transition into the viable but non-culturable (VBNC) state for *Salmonella*. Direct viable counts and MPN counts were consistently higher than TSA culturable plate counts for both serotypes at both temperatures for the duration of the microcosm. The difference between the TSA culturable plate counts and direct viable counts represents the fraction of *Salmonella* cells that are in the VBNC state [31] [32]. The VBNC fraction was shown to increase as the microcosm progressed and is marked by a significant difference

between the decay rates of the direct viable counts and TSA culturable plate counts (Fig. 1). Moreover, *S*. Typhimurium cells held at 5ºC were unculturable based on spread plating onto TSA at 84 days but remained viable as determined by epifluorescence microscopy and culturable after a nutrient addition and a temperature upshift during the MPN assay. The ability of *Salmonella* to become culturable after enrichment in buffered peptone water and Rapport-Vassiliadis broth suggests that these VBNC cells may become culturable after exposure to conditions that are more favorable to growth. Similarly, Gupte et al. [15] demonstrated culturability of VBNC *S*. Typhimurium DT104 cells after the addition of a nutrient broth and a temperature upshift to 37ºC. Transition into the VBNC state has been suggested as one of several strategies for bacteria to remain viable in unfavorable environmental conditions [31] [24]. Culturable count decay rates of *S*. Muenchen at 5ºC and 30ºC were significantly lower than that of *S*. Typhimurium suggesting that *S*. Typhimurium may transition into the VBNC state more readily. *Salmonella* has previously been induced into the VBNC state in microcosm studies [6] [30] [34]. Caro et al. [6], however, demonstrated a loss of infectivity with a transition into the VBNC state for *S*. Typhimurium cells suggesting that *Salmonella* serotypes that transition into the VBNC state more readily than others may lose infectivity quicker.

Our study suggests that both *S*. Typhimurium and *S*. Muenchen are highly persistent in natural waters at environmentally relevant temperatures and that *S*. Muenchen demonstrates greater persistence than *S*. Typhimurium in natural waters, especially at lower temperatures. The ability of *S*. Muenchen to persist in river water for extended periods of time and its high prevalence in natural waters suggests that waterborne transmission of this serotype is probable. Moreover the ability of *S*. Muenchen to persist in natural waters may be influenced by seasonal changes water temperatures. These findings suggest the need to further investigate the

persistence of other *Salmonella* serotypes of public health significance in surface waters in order to fully assess the health risk presented by these pathogens in the environment.

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TABLES

Table 1. *Salmonella* serotype die-off characteristics.

Serotype	Assay	Temperature (ºC)	Decay Rate	T_{90} (days)	T_{99} (days)	R^2	Survival at 84 days (%)
S. Muenchen	culturable plate counts	5	0.0272 [A]	65.3	71.5	0.95	0.4 [A]
		30	0.0388 [B]	63	69.2	0.92	0.04 [B]
	direct viable counts	5	0.0062 [D]	79.1	102.3	0.45	9.3 [C]
		30	0.0076 [D]	74.5	96.3	0.52	6.6 [C]
S. Typhimurium	culturable plate counts	5	0.045 [C]	41.4	69.1	0.83	0 [B]
		30	0.0505 [BC]	39	66.8	0.83	0.004 [B]
	direct viable counts	5	0.0116 [D]	67.5	101.4	0.7	3 [C]
		30	0.0142 [D]	63.6	95.5	0.75	1.8 [C]

[A, B, C, D] Decay rates that are significantly different do not have the same letter ($p<0.05$).

FIGURE LEGENDS

Figure 1: Culturable plate count and direct viable count die-off curves.

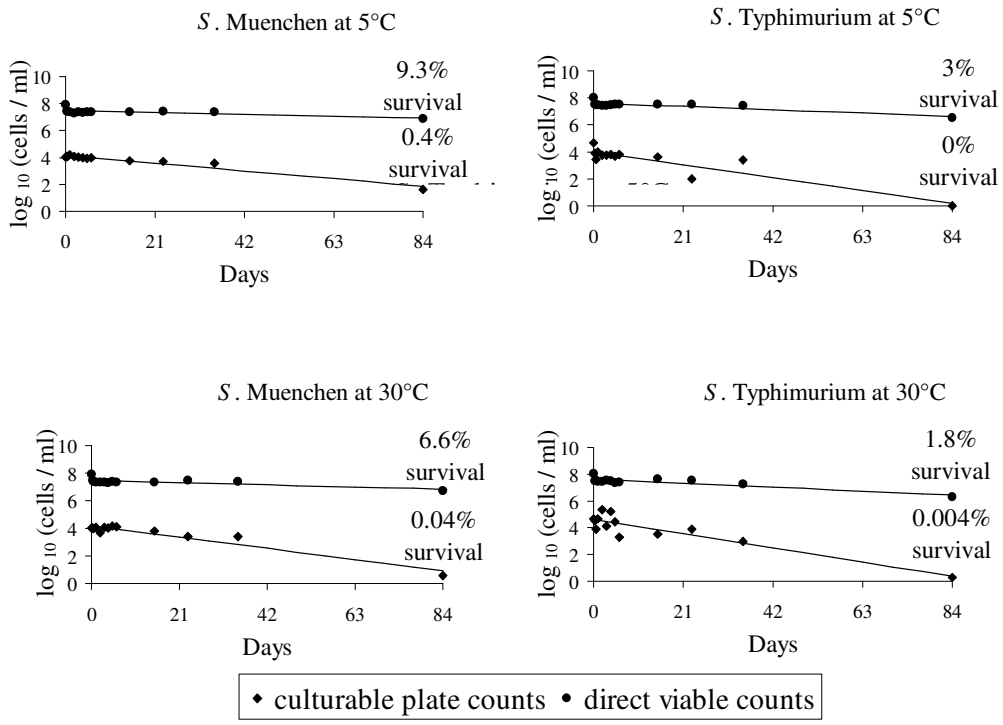
S. Muenchen at 5°C
S. Typhimurium at 5°C
S. Muenchen at 30°C
S. Typhimurium at 30°C
log 10 (cells / ml)
Days
9.3% survival
0.4% survival
3% survival
0% survival
6.6% survival
0.04% survival
1.8% survival
0.004% survival
culturable plate counts
direct viable counts

Figure 1.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Although salmonellosis is primarily considered a foodborne disease, waterborne outbreaks have occurred [5] [1]. Moreover, the presence of clinically relevant *Salmonella* serotypes in natural waters has been well documented [6]. Studies have demonstrated an increase in waterborne Salmonella densities and diversity [4] as well as an increase in waterborne disease [2] [3] after rainfall events. These studies suggest that water may play a significant role in salmonellosis transmission and that climate and weather may influence salmonellosis transmission rates among humans.

The objectives of this study were to investigate the seasonality of clinically relevant *Salmonella* serotypes in the natural waters of a high salmonellosis case rate region of southern Georgia as the environmental parameters that influence the presence of *Salmonella* in the natural waters

The results of this study indicate that while *Salmonella* spp. are ubiquitous in the surface waters of our study region, serotype incidence and diversity fluctuate seasonally. *Salmonella* densities were highest in summer months and lowest in winter months. Serotype diversity was also highest in summer months. In this study, *Salmonella* densities were positively correlated with precipitation. This correlation, however, was not strong suggesting that seasonal variation in human and animal *Salmonella* carriage rates may contribute to the seasonality of *Salmonella* densities in region's surface waters. Our *in vitro* study demonstrated that *Salmonella* serotype persistence may differ at different environmentally realistic water temperatures. *S*. Typhimurium

persisted longer at 30ºC than 5ºC while *S*. Muenchen persisted longer at 5ºC than 30ºC. *S*. Muenchen also persisted longer at lower temperatures than *S*. Typhimurium at both temperatures. These data suggest that seasonal variation in natural water temperatures may play a role in the presence and persistence of clinically relevant serotypes and therefore environmental exposure patterns. Seasonal variations in precipitation and water temperature may therefore contribute to the observed seasonality of salmonellosis in the region.

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