DISCRETE STORM IMPACTS ON THE LOADING OF *SALMONELLA* AND CAMPYLOBACTERS WITHIN A SOUTH GEORGIA RURAL WATERSHED

by

GORDON MARTIN

(Under the Direction of Erin K. Lipp)

ABSTRACT

Salmonella and campylobacters are among the top enteric food and waterborne disease causing organisms in the United States. The Georgia South Health District 8-1 in the South Georgia coastal plain, spanned by the Little River Watershed (LRW), consistently reports among the highest salmonellosis rates in the state and country along with high levels of campylobacteriosis infections. Environmental transmission, possibly from surface waters, is suspected in many cases, given the sporadic and seasonal nature of reported outbreaks coupled with no identified contaminated food source. A previous study in the watershed demonstrated a significant relationship between increased precipitation and increased levels of *Salmonella* and fecal contamination. In this study, we investigated whether storms are in fact significant drivers in microbial contamination of environmental waters. Higher bacterial levels were observed during storm events compared to baseline events in both pathogens studied; *Salmonella* levels and serotype variation were higher ($p = 0.007$ and $p = 0.006$); as were campylobacter levels ($p < 0.001$). We demonstrate that these organisms are ubiquitous in environmental waters, can persist under harsh conditions, including drought, and finally, increased microbial contamination of surface waters follow storm events.

INDEX WORDS: *Salmonella* spp., Campylobacters, Storm events, Drought, Microbial loading, Climate, Waterborne pathogens, Little River Watershed, Environmental exposure

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DEDICATION

This is dedicated to all those that shared this trip with me, and supported me; as well as those that shook their head and said “Gordon, what are you getting into now?”

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter 1

Introduction

Salmonella and Campylobacters are among the top enteric food and waterborne disease causing organisms in the United States. Several rural counties in the South Georgia coastal plain, spanned by the Little River Watershed, were identified as consistently reporting the highest salmonellosis rates in the state, and further, continually exceeds the national average. The Georgia South Health District reported a 10-year salmonellosis case rate of 69.3 per 100,000 people; while the state of Georgia reported a 10-year average of approximately 22.0 cases per 100,000 people; and the reported 10-year national average was approximately 15.1 per 100,000 people. During the past ten years, salmonellosis case rates in this health district have ranged from a "low" of 45.2 per 100,000 in 1996, to a high of 105.2 per 100,000 in 2003 (Marcus, Rabatsky-Ehr et al. 1998-2000; L. Amanda Ingram, David Blythe et al. 2003-2006; CDC 2004; USDA-ARS-SEWRL 2008a). Environmental transmission, possibly from contaminated surface waters, is suspected in many of these cases due to their seasonal and sporadic nature and no identified contaminated food source. Here, we hypothesized that microbial loading during storm events may contribute significantly to surface water contamination. The primary objective of this study was to observe the effects of discrete storm events on microbial loading of the Little River Watershed, which also included a severe drought, during the last half of 2007 (Holcomb and & Couch 2007; National Oceanic and Atmospheric Administration 2007). The Little River Watershed received 23 cm less rain (84.3cm, a 28.8% deficit) than the previous 10 year average for this area (118.4cm) (USDA-ARS-SEWRL 2008a).

This thesis is an investigation into the impact that discrete (or individual) storm events have on the microbial loading of fecal indicator bacteria, *Salmonella* spp. and campylobacters into the Little River Watershed. Chapter 1 introduces the study and provides some general background, Chapter 2 takes a

more in depth look at the organisms, their ecology, and the environments they are exposed to and the repercussions this has on the individual organisms and potentially the entire species or genus of these pathogens as a whole. Chapter 3 details this study, its objectives, methods and findings, and Chapter 4 discusses the conclusions and observations of this study.

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Chapter 2

Literature Review

Background: Climate, Environment, and Waterborne Disease

In 2004, the WHO reported that microbiological contamination of water led to an estimated 200 million diarrheal episodes and 2.1 million deaths from diarrhea worldwide (Ford 1999; Fewtrell and Bartram 2001). In the United States, an annually estimated 7.1 to 9 million cases and 2100 deaths are attributed to waterborne illnesses (Morris and Levin 1995; Rose, Epstein et al. 2001). In 2004, the Centers For Disease Control (CDC) reported that during 2001-2002, approximately 100 waterborne disease outbreaks (WBDO) occurred in the United States (Blackburn, Craun et al. 2004a; Blackburn, Craun et al. 2004b). While outbreaks do have an impact on overall illness rates, for many of the pathogenic waterborne microbes, such as *Salmonella* spp. and *Campylobacter* spp., the majority of illnesses are attributed to sporadic cases (WHO 2000; WHO and USEPA 2004; WHO 2005). With both outbreaks and sporadic cases, gastroenteritis constitutes approximately half of the symptoms of waterborne illnesses (Fewtrell and Bartram 2001).

Accurately estimating waterborne disease incidence has proven to be problematic due to significant underreporting along with large numbers of gastrointestinal diseases of unknown etiology (Ford 1999). There is a wide range of reasons why cases may remain unreported including the short duration of symptoms, the self-limiting nature of the illness in healthy individuals (Rose, Epstein et al. 2001), asymptomatic cases, or problems with the records themselves. Morris and Levin (1995) found cases that sought treatment but did not receive a diagnosis and / or no information on the exposure route was given and cases that simply were not reported by the medical staff were among the primary contributors to underreporting.

Microbial waterborne diseases are caused by the contamination and spread of pathogens through drinking or environmental waters, where human exposure may occur. Human exposure pathways include ingestion, inhalation, and dermal contact (Rose, Epstein et al. 2001). There are many routes of environmental water contamination, such as effluent from waste water treatment plants (WWTPs), contaminated storm water runoff from urban areas or agricultural lands, runoff from Confined Animal Feeding Operations (CAFOs), inefficient or overloaded septic systems, and fecal contamination from local fauna (Rollins and Colwell 1986; Fewtrell and Bartram 2001; Rose, Epstein et al. 2001; Kistemann, Claßen et al. 2002; Borchardt, Chyou et al. 2003; Blackburn, Craun et al. 2004a; Blackburn, Craun et al. 2004b; Ahn, Grant et al. 2005; Callaway, Keen et al. 2005). Numerous studies have demonstrated a positive correlation between precipitation events and increased microbiological contamination of environmental waters (Rose, Epstein et al. 2001; Kistemann, Claßen et al. 2002; Lipp, Huq et al. 2002; Ahn, Grant et al. 2005; Haley, Cole et al. 2009). Moreover, other studies have reported a correlation between seasonality and water borne disease case rates (Curriero, Patz et al. 2001; Rose, Epstein et al. 2001; Lipp, Huq et al. 2002), suggesting climate may have an influence on waterborne disease incidence, through seasonal changes in weather such as precipitation and temperature (Srikantiah 2004).

Climate and weather are influenced by numerous and complicated factors. Large-scale atmospheric phenomena are known to play a significant role in weather variability, from global to the local level. The El Nino Southern Oscillation [ENSO] (Rankin, Aceto et al.) and the North Atlantic Oscillation (NAO) are two such phenomena that affect interannual and interdecadel temperature and rainfall variability in the Southeastern United States (and elsewhere) and could influence microbial loading of pathogens in environmental waters (Lipp, Luther et al. 2001 ; Lipp, Huq et al. 2002). ENSO events tend to lead to cooler and wetter winters in the southeastern U.S. At least one study has demonstrated a strong link between ENSO events and cholera illness rate variability in Bangladesh (Rodo`, Pascual et al. 2002).

Waterborne Pathogens

Numerous microbial pathogens are associated with waterborne diseases, including *Salmonella* and campylobacters (Ford 1999; Leclerc, Schwartzbrod et al. 2002; Schaffter and Parriaux 2002; Arnone and & Walling 2007). While these zoonotic agents are also associated with foodborne illness, historically, both have been among the top bacterial causes of waterborne disease outbreaks worldwide and in the United States (Kramer 1996; Olsen, Bishop et al. 2001; Sails, Bolton et al. 2002), and can be readily found in environmental waters contaminated by human or animal feces (Rosef, Kapperud et al. 1987; Callaway, Keen et al. 2005). Agricultural runoff, sewage and wastewater pollution from human or urban activities (combined sewage overflows), and feces from local fauna may all contribute to environmental water contamination with these pathogens (Rollins and Colwell 1986; Rose, Epstein et al. 2001). Several studies have demonstrated that fecal indicator bacteria are not accurate in predicting even the presence or absence of pathogens, such as *Salmonella*,(Cherry 1972; Angulo, Tippen et al. 1997; Polo, Figueras et al. 1998; Ahn, Grant et al. 2005; Anderson, Whitlock et al. 2005; Brands, Inman et al. 2005; Callaway, Keen et al. 2005; Benham, Baffaut et al. 2006; Berney, Weilenmann et al. 2006; Sampson, Swiatnicki et al. 2006; Arnone and & Walling 2007).

Watershed Studies

The body of scientific literature concerning the ecology of environmental pathogens is growing. Studies conducted at the farm level have found that surface waters near agricultural and livestock farms that utilize manure as fertilizer or that had received irrigation water contaminated with pathogens such as *Salmonella* or campylobacters potentially pose a long term environmental contamination hazard to nearby surface waters (Islam, Morgan et al. 2004; Kemp, Leatherbarrow et al. 2005; Jensen, Dalsgaard et al. 2006). *Salmonella* from contaminated fertilizers and irrigation water can survive on vegetables and the surrounding soil for months, causing a potential threat to public health and environmental contamination

(Islam, Morgan et al. 2004). Molecular epidemiological evidence has linked campylobacters originating from a farm environment with those causing disease in a nearby community (Fitzgerald, Stanley et al. 2001). Additionally, studies show that these pathogens adhere to sediments or manure and persist until being washed into local streams (Pachepsky, Sadeghi et al. 2006).

Watershed level studies give us a view on a broader scale, of the fate and transport of pathogens once they have been introduced into the larger environment. There appear to be some outstanding common factors which seem to influence microbial contamination across many watershed-scale studies such as rainfall and surrounding land use (Baxter-Potter and Gilliland 1988; Mallin, Williams et al. 2000; Ferguson, Husman et al. 2003; Jamieson, Gordon et al. 2004; Hose, Gordon et al. 2005; Benham, Baffaut et al. 2006; Ferguson, Davies et al. 2007).

Water Quality Criteria and Waterborne Pathogens

Current water quality criteria, designed to assure safe water under the Clean Water Act, rely on a few fecal indicator bacteria, such as *E. coli* and enterococci (U.S.EPA 1986), in part because it would not be economically feasible to test for every possible waterborne pathogen. Theoretically, the presence of these bacteria indicates potential fecal contamination, and therefore, an increased risk of disease due to the presence of pathogens that were not tested for. However, several studies have reported a lack of accurate quantitative correlation between levels of these fecal indicator organisms and levels of enteric pathogens such as *Salmonella* and campylobacters (Carter, Pacha et al. 1987; Polo, Figueras et al. 1998; Jones 2001b; Lemarchand and Lebaron 2003; Winfield and Groisman 2003; Brands, Inman et al. 2005). Given the lack of accuracy in predicting pathogen levels from fecal indicator bacteria in surface waters, including public and recreational waters, the potential for human infection is difficult to quantify with any accuracy (El-Shaarawi, Esterby et al. 1981; Hood, Ness et al. 1983; Morris and Levin 1995; Baudart, Grabulos et al. 2000a; Fewtrell and Bartram 2001; Rosef, Rettedal et al. 2001; Anderson, Whitlock et al. 2005; Chandran and Hatha 2005; Shehane, Harwood et al. 2005; Sampson, Swiatnicki et al. 2006) .

Never-the- less, fecal indicator bacteria have continued to play a major role in the public health sector of many states, for example, when their levels rise above specific Environmental Protection Agency (EPA) thresholds, public beaches may be closed and No Swimming advisories may be given (Burton, Gunnison et al. 1987; Carter, Pacha et al. 1987; Buswell, Herlihy et al. 1998; Curriero, Patz et al. 2001; Auld, MacIver et al. 2004; Blackburn, Craun et al. 2004a; Ahn, Grant et al. 2005; Anderson, Whitlock et al. 2005; Chandran and Hatha 2005; Benham, Baffaut et al. 2006)

Salmonella spp.

The genus *Salmonella* is composed of facultative anaerobic, gram negative, motile, rod shaped bacteria with peritrichous flagellae, belonging to the Family *Enterobacteriaceae*. *Salmonella* nomenclature is complicated and is widely debated. Currently, there are two nomenclature schemes in use to describe *Salmonella*. For simplicity's sake, the current system used by the Centers for Disease Control and Prevention (CDC) will be used here. The genus is split into two species, *Salmonella enterica,* and *Salmonella bongori*. Each species is further subdivided into multiple serovars or serotypes. *Salmonella enterica* includes subspecies I, II, IIIa, IIIb, and VI and *Salmonella bongori* includes subspecies V (Brenner, Villar et al. 2000). Currently, there are over 2000 known *Salmonella enteric* serovars, all of which are considered to be potentially pathogenic to humans (Baggesen, Sandvang et al. 2000).

Salmonella is one of the top enteric disease causing organisms in the United States and throughout the world (WHO 2005), and the etiologic agent of salmonellosis and typhoid fever. Typical symptoms of salmonellosis are fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food or beverage. The illness is self-limiting, usually lasting 4 to 7 days, and most persons recover without antibiotic treatment (CDC 2005b). However, the diarrhea can be severe, and occasionally requires hospitalization. The elderly, infants, and those with impaired immune systems may be prone to more severe illness (Darwin and Miller 1999; CDC 2005b; Jones, Ingram et al. 2006). In these patients, the infection may spread from the intestines to the blood stream, and then to other body

sites and can cause death unless prompt treatment with antibiotics is given. Persons with diarrhea usually recover completely, although it may be several months before their bowel habits are entirely normal. A small percentage of people will develop chronic pains in their joints, irritation of the eyes, and painful urination. This is called Reiter's syndrome and can last for months or years. Reiter's Syndrome can develop into chronic arthritis, which is difficult to treat. Antibiotic treatment does not make a difference in whether or not the person later develops arthritis (Lake, Hudson et al. 2002; CDC 2005b). Though serious side effects tend to be rare, the economic burden of these diseases is real. It has been reported that non-typhoidal *Salmonella* foodborne disease results in an estimated total of 1,341,873 cases, 15,608 hospitalizations, and 553 deaths (approximately 30.6% of total foodborne deaths) in the U.S. annually (Mead 1999). The resulting economic impact results in productivity losses estimated to be in the billions of dollars (Heinitz 2000).

Salmonella Host Specificity and Dose Response

The *Salmonella* infectious dose in humans is widely variable and dependant on factors such as serovar and host status (Latimer, Jaykus et al. 2001). *Salmonella* pathogenicity, virulence, and host adaptation also varies not only between serotypes but also among the same serotype. *Salmonella enterica* serovar Typhi, is highly virulent and pathogenic to only humans and higher primates (Uzzau, Brown et al. 2000). *S.* Typhimurium lies on the other end of the spectrum. It is traditionally considered the "prototypical" broad-host-range serotype, virulent to humans, domestic fowl, livestock, rodents, and birds. However, *S.* Typhimurium variant Copenhagen, Phage Type 99, is found almost exclusively in pigeons and has been found to be of very low virulence to humans (Rabsch, Andrews et al. 2002; Pasmans, Van Immerseel et al. 2003; Pasmans, Van Immerseel et al. 2004). The virulence and pathogenicity of the more rare serovars is unknown. Therefore, formulations of concrete dose-response formulae are difficult because of the number of different strains, each strain potentially differing in pathogenicity, virulence, and host adaptation. For example, an oral dose of at least 10^5 *S.* Typhi cells are

needed to cause typhoid fever in 50% of human volunteers, whereas at least 10^9 *S.* Typhimurium cells are needed to cause gastroenteritis. Over all of the *Salmonella* serovars, a dose of 1 X 10^6 cells is usually cited as an average infectious dose (Haas, Rose et al. 1999; FAO/WHO 2000; Latimer, Jaykus et al. 2001; CDC 2005b).

Salmonella excretion among humans may continue months after symptoms have abated (Angulo, Tippen et al. 1997; Borchardt, Chyou et al. 2003; Ashbolt 2004a; Ashbolt 2004b; Blackburn, Craun et al. 2004b; Ekdahl, de Jong et al. 2005; Gradel 2007). Asymptomatic carriers can continue to shed the bacteria and potentially infect others. Antibiotics are usually ineffective on *Salmonella* carriage (even if salmonellae are susceptible to them) because the site of carriage may not allow penetration by the antibiotics (CDC National Center for Immunization and Respiratory Diseases: Division of Bacterial Diseases 2005b).

Salmonella and Environmental Surface Waters

Salmonella is traditionally associated with food, but has also been shown to be among the top three bacterial pathogens causing waterborne disease outbreaks (Kramer 1996; WHO and USEPA 2004). *Salmonella* has long been known to be ubiquitous in fresh and marine environmental surface waters (Cherry 1972; Heinitz 2000). Contamination may come from many different routes such as fecal contamination from human or animal sources, effluent from Waste Water Treatment Plants (WWTP), contaminated runoff from urban or agricultural areas, overburdened septic systems, or even local and migratory fauna (Baird-Parker 1990; Heinitz 2000; Borchardt, Chyou et al. 2003; Brands, Inman et al. 2005; Callaway, Keen et al. 2005). Contamination of environmental waters with *Salmonella* may be of a greater public health concern than previously thought due to the ability of it to persist and in some cases reproduce outside of a host organism (discussed below) (Cherry 1972). This characteristic increases the probability of survival between hosts (Winfield and Groisman 2003). The environment, including surface waters, can be considered as a potential part in the lifecycle of *Salmonella*.

Salmonella, like many other bacteria in an aquatic environment, are able to persist for an extended period of time (over a month) within the water column under certain conditions (Arnone and & Walling 2007). Several studies have shown that pathogenic species of *Salmonella* (*S. enterica*) may persist for significantly longer periods, even up to a year, within aquatic sediments as opposed to the water column itself, creating the opportunity for re-suspension and increased water contamination during storms (Burton, Gunnison et al. 1987; Moore, Martinez et al. 2003). *Salmonella* have adapted many strategies that allow it to exploit the environment itself in order to survive. For example, *Salmonella* cells attach to soil particles and have the ability to aggregate, forming niches more conducive to survival. In this way, they are able to utilize organic nutrients from the surrounding matrix and cells that lyse after death (Winfield and Groisman 2003). Some *Salmonella* serotypes have been shown to exploit microbial fauna. *S.* Thompson cells have the ability to be ingested by protozoa of the *Tetrahymena* spp., thus gaining protection from an inhospitable external environment (Brandl, Rosenthal et al. 2005). *Salmonella* have also been shown to have the ability to enter a "viable but not culturable state" (VBNC) which may help them survive environmental stressors (Caro 1999; Brandl, Rosenthal et al. 2005), though there is still debate on the potential infectivity and thus potential threat to public health of VBNC cells.

Campylobacters

The family *Campylobacteriaceae* includes the genera of both *Campylobacter* and *Arcobacter* spp. However, the term "campylobacters" is often used to refer to a broader group of closely related organisms including *Helicobacter* and *Sutterella* spp. (van-Vliet and Ketley 2001; Newell 2002). Though the term "campylobacter" encompasses many closely related organisms, the thermophilic species *Campylobacter jejuni* and *Campylobacter coli* account for approximately 99% of human campylobacter infections. *C. jejuni* and closely related *C. coli* are the etiologic agents of campylobacteriosis, which is recognized as the most common cause of acute diarrheal illness in many industrialized countries (WHO 2000).

Most campylobacters are characterized as fastidious gram-negative, rod shaped, thermophilic, micro-aerophilic, non-spore forming, motile spiral shaped organisms with a polar flagellum at one or both ends of the cell. Campylobacters colonize in the intestinal mucosa of most warm-blooded animals and many birds and are ubiquitous in the environment (van-Vliet and Ketley 2001; Newell 2002; Snelling, Matsuda et al. 2005a). While *Campylobacter* spp. have adapted the ability to colonize a wide range of organisms, some species, such as *C. jejuni* and *C. coli* preferentially colonize the avian gut (Newell 2002; Waldenstrom, Broman et al. 2002)

Campylobacteriosis

Campylobacter jejuni and closely related *Campylobacter coli* are the etiologic agents of campylobacteriosis, which is recognized as the most common cause of acute diarrheal illness in many countries (Koenraad, Rombouts et al. 1997; WHO 2000; Rosef, Rettedal et al. 2001). *C. jejuni* and *C. coli* account for approximately 99% of human campylobacter infections. It has been estimated that an infective dose of *Campylobacter* from food or water is very small (approximately 500 cells), which makes this pathogen a public health concern (Black, Levine et al. 1988; Snelling, Matsuda et al. 2005a). Common exposure sources are undercooked poultry, contaminated milk and water, and animal contact (Louis, Gillespie et al. 2005). After ingestion, the incubation period of ranges from 1 to 7 days followed by acute abdominal pain, fever, and general malaise. Symptoms progress to profuse diarrhea that later contain mucous and blood. The disease is self-limiting and usually ends after 5 to 8 days, however, the bacteria may continue to be shed in the feces after symptoms have cleared (van-Vliet and Ketley 2001).

In industrialized countries, typical symptoms of campylobacteriosis are inflammatory diarrhea with severe cramping. In these countries, infection rates are associated with warmer seasons and affect mostly young children and young adults. In developing countries, there is a much higher rate of asymptomatic infection with clinical symptoms tending to be milder. In these countries, infection appears to be restricted to children and infection rates appear to show no seasonality. This is thought to be a result

of multiple exposures to Campylobacters at a young age and acquiring local immunity. Development of Guillain-Barré syndrome (GBS), a neurological disorder, has been associated with infection from certain strains of *C. jejuni* (Allos 1997; van-Vliet and Ketley 2001).

Campylobacters and Environmental Waters

Campylobacteriosis is considered a foodborne illness; however, in many clinical cases, a food source can not be identified. Several studies have reported that illness rates exhibit seasonal patterns, with higher incidence in the warmer seasons (Rollins and Colwell 1986; Carter, Pacha et al. 1987; Talibart, Denis et al. 2000; Jones 2001b; Newell 2002; Louis, Gillespie et al. 2005).

Thermophilic campylobacters (optimal growth between 34˚C and 44˚C) are ubiquitous in the environment and are associated with human, animal, or avian feces (Koenraad, Rombouts et al. 1997; Wesley, Wells et al. 2000; Newell 2002; Waldenstrom, Broman et al. 2002; Hubbard, Newton et al. 2004; Abulreesh, Paget et al. 2005). As campylobacters cannot multiply outside of a host and typically do not survive as long in the environment, the fecal contamination is usually thought of as recent (Wesley, Wells et al. 2000; Jones 2001b). Once campylobacters have been introduced into environmental waters, many factors such as temperature, UV radiation, nutrients, oxygen content, and pH influence their persistence and culturability (Butler, Lund et al. 1987; Obiri-Danso, Paul et al. 2001; Snelling, Matsuda et al. 2005a; Boyle, Sichel et al. 2008).

In order to survive environmental stresses, campylobacters have been shown to enter a VBNC state, in which the microbe exhibits decreased metabolic activity and a change in morphology. VBNC cells are difficult to detect using standard laboratory detection methods, such as incubation at 37˚C followed by spread plating, and likely contributes to the difficulty in recovering campylobacters from the suspected water sources during outbreaks (Jones 2001b). Resuscitation of campylobacters into a culturable state has been demonstrated only under certain conditions, presumably similar to conditions inside of a host (Rollins and Colwell 1986; Talibart, Denis et al. 2000; Newell 2002).

The Little River Watershed

Both *Salmonella* spp. and campylobacter are associated with human and animal feces, and are prevalent in livestock, poultry, migratory birds, and other wildlife (Atabay and Corry 1997; Hudson, Quist et al. 2000; Wesley, Wells et al. 2000; Newell 2002; Waldenstrom, Broman et al. 2002; Colles, Jones et al. 2003; Hald, Pedersen et al. 2004; Callaway, Keen et al. 2005). Georgia leads the nation in poultry production, is a large producer of livestock, and has large agricultural areas which use poultry litter and manure for fertilizer (Kramer 2005; USDA-ARS-SEWRL 2008b). Poultry farms and agricultural lands are prevalent in the coastal plain of Georgia (Kramer 2005; USDA-ARS-SEWRL 2008b).

The Little River Watershed spans the Georgia South Health District #8-1, in the rural south central Georgia Coastal Plain, and comprises the headwaters of the upper Suwannee River Basin (**Figures 2.1** and **2.2**). The watershed crosses Tift, Turner, and Worth Counties and drains a 334 km^2 area. The region is characterized by areas of broad floodplains, river terraces, and gently sloping ridges. The bottomlands are level and most of the ridge slopes are less than 5%. Only 7% of the land within the watershed has slopes in excess of 7% (Suttles, Vellidis et al. 2003). The primary surface soil matrix of the LRW is composed of mixtures of loamy sand, sandy loam, sandy clay, and gravelly sand mixed with sandy clay with grain size increasing the further downstream. After periods of precipitation, this porous sandy horizon effectively connects surface waters with shallow, seasonal unconfined ground waters (surficial aquifer underlying the watershed) which range in thickness from 2 to 10 meters. The surficial aquifer overlays a limestone cap which bounds the Floridian aquifers. Since the limestone is much more impervious to water penetration than the sandy soils above, penetrating water from the surface is shunted laterally back to surficial stream channels completing a shallow groundwater cycle (Bosch, Lowrance et al. 2003; Suttles, Vellidis et al. 2003; Sullivan, Batten et al. 2007; Bosch, Sheridan et al. 2007a). Throughout the watershed stream channels are bordered by riparian buffer zones and surface waters are used primarily for irrigation and recreation, including fishing, swimming and boating. Recharging of farm

ponds, limited irrigation, and the domestic water supply come from the shallow groundwater, while the deeper artesian aquifer supplies water for irrigation, industry, and municipal and rural domestic water supplies (Suttles, Vellidis et al. 2003).

Subwatershed land use varies within the LRW. Overall, the LRW is characterized as mostly wooded, with 44% - 50% forested, 25% - 31% cultivated (primarily row crops like peanuts and cotton), 10% - 15% pasture, 13% wetlands, and 3% urban (Suttles, Vellidis et al. 2003; Sullivan, Batten et al. 2007). Subwatershed agricultural land use varies from 25% to 60% (Suttles, Vellidis et al. 2003). Within the LRW, there are approximately 7,700 head of cattle (around 240 are dairy cattle, many of which are located in the experimental farm adjacent to a study site in this research), about 500 swine, and two broiler houses producing approximately 440,000 chickens per year (Suttles, Vellidis et al. 2003).

The LRW climate is humid subtropical, with temperatures ranging from 11˚C in January, to 27˚C in July and August, giving a yearly mean temperature of 19.2˚C (Suttles, Vellidis et al. 2003) and average long-term annual precipitation levels of 120cm. The mild climate conditions coupled with high average yearly precipitation are conducive to a long growing season, hence intensive agricultural activities are common to the area (Bosch, Sheridan et al. 1999).

The main watershed is divided into 7 nested “subwatersheds”, each has been outfitted with a monitoring station to collect data such as rainfall, stream flow, and stream height, by the United States Department of Agriculture – Agricultural Research Station [USDA-ARS] (Bosch, Sheridan et al. 1999; Sullivan, Batten et al. 2007; Bosch, Sheridan et al. 2007a).

Campylobacteriosis rates within this health district consistently fall close to the reported state average incidence level and below the national average. In 2005, the reported incidence of campylobacteriosis within the South Health District was 7.6 cases per 100,000 people (GDPH-NDS 1997 - 2008), while the reported state and national averages were 6.5 per 100,000 and approximately 20 per 100,000 respectively (GDPH-NDS 1997 - 2008; CDC 2006b). However, The Little River Watershed

comprises the headwaters of the Upper Suwanee River basin which has historically reported among the highest campylobacteriosis rates in the state (Onifade 2005).

The South Health District also reports significantly higher salmonellosis incidence rates than both the state and national levels. In 2006, the South Health District reported 76.75 salmonellosis cases per 100,000 (GDPH-NDS 1997 - 2008) while the state reported 19.65 per 100,000 (GDPH-NDS 1997 - 2008; CDC 2006b). The most recent obtainable national salmonellosis data was from 2004 in which the reported incidence was 14.2 per 100,000 (CDC 2004; CDC 2005a). For nationwide campylobacter analysis, data from the CDC's FoodNet Program, which monitors 10 states, is commonly used as a proxy for national campylobacteriosis rates (CDC 2004; Voetsch, Van Gilder et al. 2004). For perspective, the 10 year average for reported salmonellosis cases in the Valdosta Health District is 69.28 /100,000 people; ranging from a low of 49.80 / 100,000 to a high of 104.76 / 100000 people. The 10-year state and FoodNet averages (national proxy) are 22 and 15.07 respectively (GDPH-NDS 1997 - 2008; CDC 2008a; CDC 2008c). In many of the cases from the South Health District, infection from a food source has not been confirmed. This suggests the possibility of infection from other sources such as the environment. This possibility of environmental exposure is further supported by analysis of the *Salmonella* serotypes recovered from human infections in this health district from 2005 to 2006 (provided by Dr. Dana Cole), compared against environmental *Salmonella* isolates collected from the Little River Watershed during this same time period (Haley 2006; Haley, Cole et al. 2009). In 2004, the top three serotypes reported to the CDC that were isolated from humans were, in descending order, *S. Typhimurium, S. Enteritidis,* and *S. Newport* (CDC 2005a). Over the entire state of Georgia, the top three serotypes reported, in descending order, were *S.* Newport*, S.* Typhimurium*,* and *S.* Javiana (CDC 2004). The majority of Human *S.* Javiana infections have been reported to be geographically restricted to the southeastern United States (Srikantiah 2004). However, comparison of the human and environmental *Salmonella* isolates collected from the South Health District in 2006 do not follow the same state wide or national trends in terms of serotype prevalence. Many of the top ten human isolates were among the top 10 serotypes isolated from the

environment during the same month within the health district in 2006 (Haley 2006; Haley, Cole et al. 2009). During this study in 2007, we observed a similar trend. Many of the most commonly isolated serotypes nationwide are not among those most commonly isolated from human infections in the Georgia South Health District 8-1 (CDC 2005b), and further, some of the most common serotypes isolated from humans in this health district were also frequently found in local environmental surface waters (**Table 2.1**). Additionally, *S.* Typhimurium*,* one of the most commonly isolated serotypes from food exposures, was only detected once during this study (**Table 3.5**). This strengthens the notion that environmental exposure may play a more important role in human infections in this health district.

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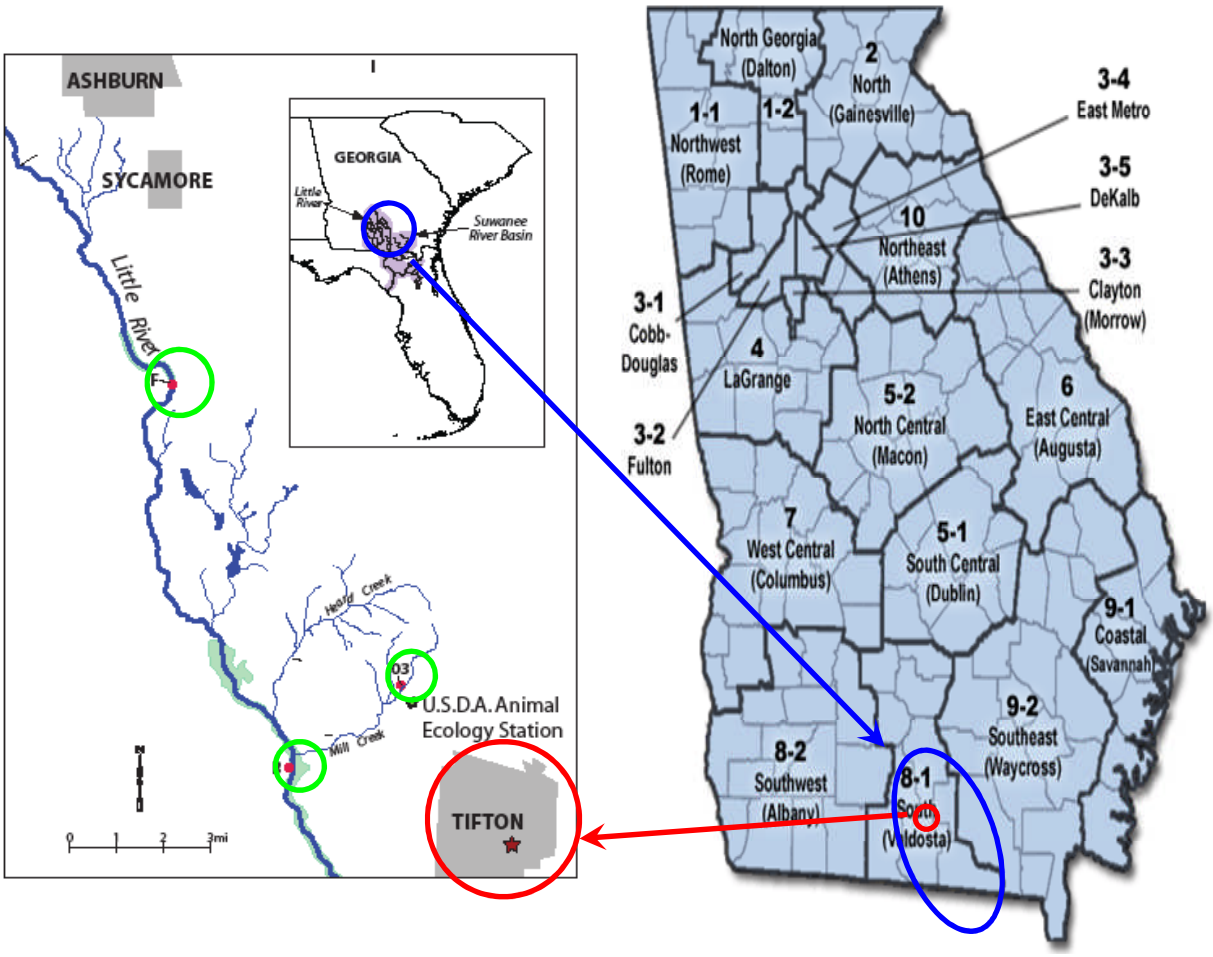

Figure 2.1 The Little River Watershed and surrounding Health Districts. Sampling stations are circled in green.

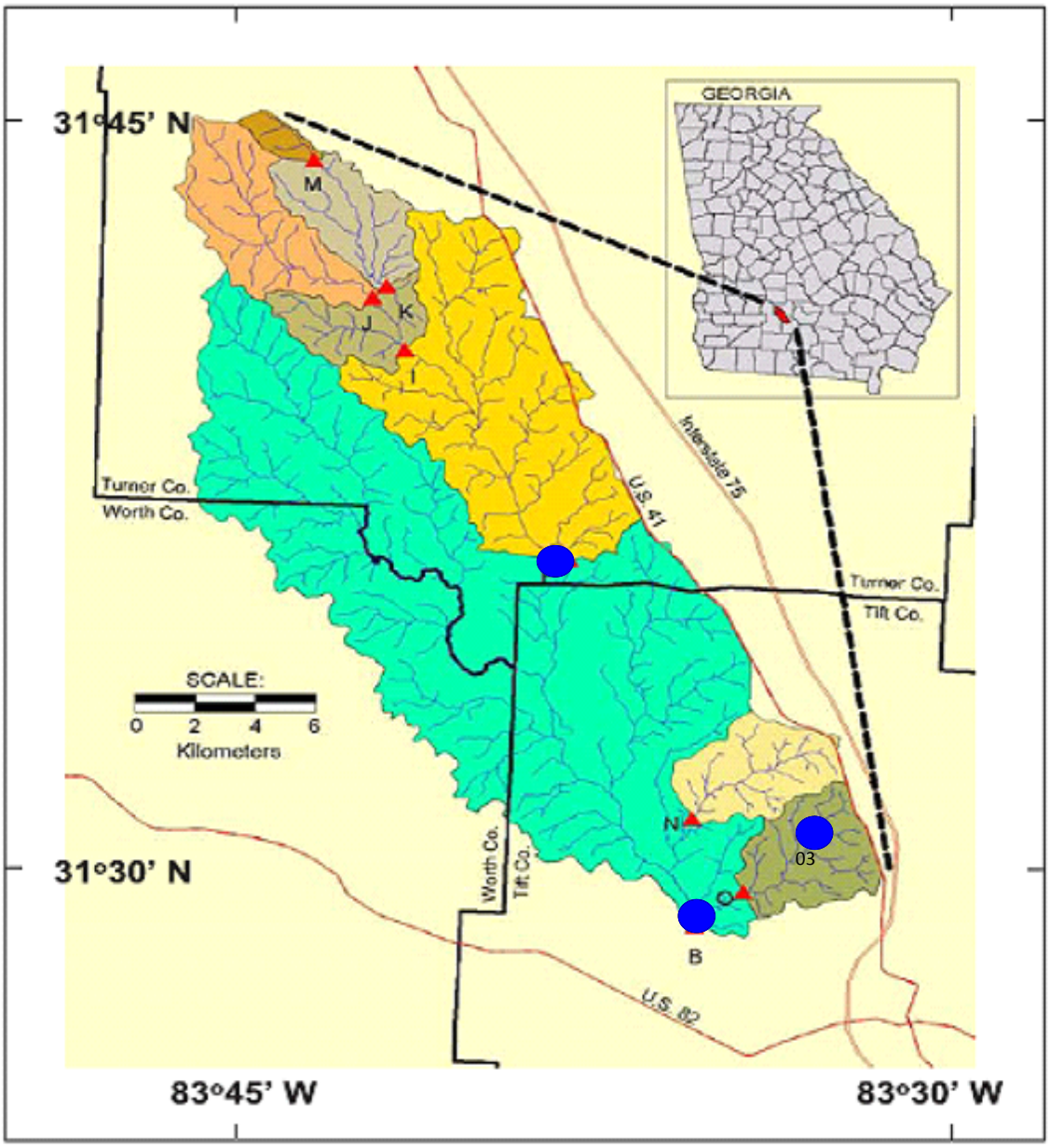

Figure 2.2 The Little River Watershed and Subwatersheds. Sampling Stations are labeled with blue circles

Table 2.1 Serotype Burden.

* Foodborne Disease Active Surveillance Network. Available at www. cdc.gov/foodnet/factsandfigures/6.pdf

** Courtesy of Dr. Dana Cole and the Public Health Lab, Atlanta, GA.

Top 10 *Salmonella* serotypes of 2007						
	Location					
Rank	U.S.* Serotype	Health District 8-1** Serotype	U.S. Total Reported Cases	District 8-1Total Reported Cases	U.S. Reported Cases/100,000	District 8-1 Reported Cases/100,000
1	Enteritidis	Newport	1,062	41	2.33	16.96
2	Typhimurium	Javiana	1,006	25	2.21	10.34
3	Newport	Typhimurium	656	12	1.44	4.96
4	I 4,[5],12:i:-	Unknown	358	10	0.79	4.14
5	Javiana	Saint Paul	347	7	0.76	2.90
6	Heidelberg	Group C2	243	7	0.53	2.90
7	Montevideo	Muenchen	211	5	0.46	2.07
8	Muenchen	Enteritidis	194	< 5	0.43	< 2.07
9	Tennessee	Montevideo	140	< 5	0.31	< 2.07
10	Saint Paul	Heidelberg	117	< 5	0.26	< 2.07

Chapter 3

DISCRETE STORM IMPACTS ON THE LOADING OF *SALMONELLA* AND CAMPYLOBACTERS WITHIN A SOUTH GEORGIA RURAL WATERSHED[1]

[1] Gordon Martin, Erin K. Lipp, Dana Cole. To be submitted to *Journal of Water and Health*

Abstract

Normally associated with food, *Salmonella* and campylobacters both are also among the top waterborne diseases that cause gastroenteritis. In the Little River, a positive relationship between increased environmental pathogen levels and rainfall has been previously demonstrated, consistent with other watershed studies. In this study, we investigated the impacts that individual storm events had on microbial loading of pathogens in the Little River Watershed. We hypothesized that contamination in this watershed would be higher during storm events when compared against the baseline samples. Between January and December 2007, 5 storm samples and 5 baseline water samples were collected from three stations within the LRW. Storm samples were collected when at least 1.3 cm (one-half inch) of consistent rain had fallen over the watershed. Baseline samples were collected during periods when no rain had fallen for at least seven days prior to sampling. During this study, one-hundred one *Salmonella* isolates, representing sixteen *S. enterica* subsp. enterica serotypes, and one-hundred eighty nine campylobacter isolates were recovered and identified, including six *Salmonella* serotypes commonly isolated from humans in Health District 8-1 during 2007 (*S.* Bareilly, *S.* Java, *S.* Muenchen, *S.* Montevideo, *S.* Rubislaw, and *S.* Typhimurium). Significantly higher *Salmonella* and campylobacter levels were consistently detected in storm samples compared against baseline samples ($p = 0.007$ and $p < 0.001$, respectively). Individual monthly *Salmonella* levels ranged from non-detectable (< 1.8 MPN L^{-1}) to a high of 350 MPN L^{-1}, recovered from Site 03 during a June storm sample. Storm samples also reflected significantly higher *Salmonella* serotype diversity compared to baseline samples ($p = 0.006$). Campylobacter levels ranged from non-detectable (< 5 CFU ml^{-1}) to approximately 15,700 CFU ml^{-1}, collected during a July storm sample from site 03. Given the high pathogen levels observed after storm events and finding clinically relevant *Salmonella* strains in the environment, ambient waters may be an underappreciated source of *Salmonella* or campylobacter exposure.

INDEX WORDS: *Salmonella*, Campylobacters, Little River Watershed, Storm events, Microbial loading, Environmental exposure, Climate, Waterborne pathogens, Drought

Introduction

Salmonella and campylobacters are among the top enteric food and waterborne disease causing organisms in the United States. *Salmonella* is separated into two species, *Salmonella enterica* and *Salmonella bongori*. Each species is further subdivided into multiple serovars or serotypes. Currently, there are over 2500 hundred known *Salmonella* serovars, all of which are considered to be potentially pathogenic to humans, though the majority of human infections are limited to relatively few *Salmonella enterica* subsp. *enterica* (Baggesen, Sandvang et al. 2000). The family *Campylobacteraceae* includes the genera of both *Campylobacter* and *Arcobacter* spp. However, the term "campylobacters" is often used to refer to a broader group of closely related organisms including *Helicobacter* and *Sutterella* spp. (van-Vliet and Ketley 2001; Newell 2002). Though these species have been found to be ubiquitous in the environment, the improper handling and consumption of poultry is considered one of the primary sources of infection (Atabay and Corry 1997; GDPH-NDS 1997 - 2008; Engberg, On et al. 2000; van-Vliet and Ketley 2001; Newell 2002; Diergaardt 2003; Brown, Christensen et al. 2004; CDC 2008b; CDC 2008c), and it is estimated that at least 1% of the American population becomes infected with campylobacteriosis annually (Wassenaar and Newell 2000).

Several rural coastal plain counties in South Georgia, U.S.A., consistently report the highest salmonellosis rates in the state, and consistently exceeds the national average. The Georgia South Health District 8-1 reported a 10-year mean salmonellosis case rate of 69.3 per 100,000 people (GDPH-NDS 1997 - 2008); and the state of Georgia reported a 10-year average of approximately 22.0 cases per 100,000 people (GDPH-NDS 1997 - 2008; CDC 2005a; CDC 2005b; CDC 2006b) The reported national average for the past 10-years was only 15.1 per 100,000 people (CDC 2005a; CDC 2005b; CDC 2006b).

Environmental transmission is suspected in many of these cases due to their seasonal and sporadic nature, compounded by the absence of a positively identified contaminated food source. A study conducted in the same area in 2006 not only demonstrated a positive relationship between rainfall and increased levels of microbial loading in surface waters, but also found that many serotype isolated from

humans were also being recovered from the environment (Haley et al 2009). Here, we hypothesized that microbial loading during storm events may contribute significantly to surface water contamination.

Agricultural runoff, sewage and wastewater pollution from human or urban settings (combined sewer overflows) and feces from local and migratory fauna are all potential contributors to environmental water contamination with enteric pathogens (Rollins and Colwell 1986; Rose, Epstein et al. 2001). Rainfall and climate variability have been shown to significantly increase environmental contamination (Kistemann, Claßen et al. 2002; Martinez-Urtaza 2004) and precede disease outbreaks (Patz, Epstein et al. 1996; Patz, Graczyk et al. 2000; Curriero, Patz et al. 2001; Patz 2001) suggesting the possibility of infection through environmental exposure.

The Little River Watershed, within the Upper Suwanee River Basin in the south Georgia coastal plain, consistently reports the highest rates of salmonellosis and campylobacteriosis in the state (Onifade 2005). In some of these counties, reported human salmonellosis case rates have exceeded 100 infections per 100,000 people, far exceeding the reported national average (CDC 2005a; CDC 2005b; USDA-ARS-SEWRL 2008a). Previously, we have shown a positive relationship between *Salmonella* and campylobacter levels with rainfall in the southeast Georgia coastal plain (Haley, Cole et al. 2009). In this study, the primary objective was to investigate the role discrete or independent storms play on microbial loading into the Little River, and whether other factors such as surrounding land use, or stream order affect any of the driving forces that do load surface waters with pathogenic bacteria, in this study, namely *Salmonella* and campylobacters; and we hypothesize that storm events serve as significant drivers in the loading of these pathogens to surface waters and that influence may vary by surrounding land use.

Materials and Methods

Water samples were collected from the Little River Watershed (LRW), which is located in the headwaters of the Suwannee River Basin in the Tifton Upland of the Southeastern Coastal Plain of Georgia, U.S.A. (**Figures 2.1 and 2.2**). The coastal plain is characterized by broad floodplains gently sloping to uplands used for agriculture (Suttles, Vellidis et al. 2003; Bosch 2008). The LRW drains an

area of approximately 334 km^2 and crosses Tift, Turner, and Worth Counties in the Georgia South Health District 8-1 (Bosch and Sheridan 2007; Sullivan and Batten 2007; Sullivan, Batten et al. 2007; USDA-ARS-SEWRL 2008b).

Subwatershed land use varies within the LRW. Overall, the LRW is characterized as mostly wooded, with 44% - 50% forested, 25% - 31% cultivated (primarily row crops like peanuts and cotton), 10% - 15% pasture, 13% wetlands, and 3% urban (Suttles, Vellidis et al. 2003; Sullivan and Batten 2007; Sullivan, Batten et al. 2007). Subwatershed agricultural land use varies from 25% to 60% (Suttles, Vellidis et al. 2003). Within the LRW, there are approximately 7,700 head of cattle, 500 swine, and two broiler houses producing approximately 440,000 chickens per year (Suttles, Vellidis et al. 2003).

Sampling Site Descriptions

Three sample sites were selected within the LRW representing different stream orders, geographic location within the watershed, and surrounding land use (**Figure 3.1 and 3.2**). Site F, the northernmost upstream site, was located on the Little River in Turner County. It was a 4^{th} ordered stream (Bosch and Sheridan 2007), the second largest stream order used in the study. Site F drains an area of 114.9 km^2 also making it the second largest subwatershed within the LRW (**Table 3.1).**

Site B, the southernmost downstream site, was located on the Little River just south of the confluence of Mill Creek in Tift County, near Tifton. Site B, a 5^{th} ordered stream, is situated at the mouth of the LRW and is the largest ordered stream included in the study. It drains the entire 334.4 km^2 primary drainage area of the LRW (Bosch and Sheridan 2007; USDA-ARS-SEWRL 2008b) (**Table 3.1).**

Site 03, a 1^{st} ordered stream and the lowest stream order in the study, is located in Tift County on Mill Creek, which flows southwest and joins the Little River approximately a half a km north of Site B. Site 03 is adjacent to a University of Georgia research farm, an area of intensive agricultural activity, that contains dairy and beef cattle, and agricultural fields fertilized with liquid dairy cattle manure (**Table 3.1).**

Rainfall Data

Each sampling station, except Site 03, was equipped with gauges which collected 5-minute precipitation and stream flow data throughout the length of the study. These data were accessed from the United States Department of Agriculture–Agricultural Research Service-Southeast Watershed Research Laboratory database (USDA-ARS-SEWRL 2008a). Stream flow calculations for Site 03 were manually calculated from stream height data, which were recorded for all sampling stations in real-time. The correct height-to-flow formula was kindly provided by Dr. David Bosch with SEWRL (Bosch 2008). Two types of rainfall data were collected for each site. First, local rain data were collected from a single rain gauge close to each site from the SEWRL database (USDA-ARS-SEWRL 2008a) to provide a snapshot of discrete precipitation events near each site. The second type of rainfall data was "weighted precipitation", which is a calculation of rainfall for the entire watershed based on several rain gauges located throughout the subwatersheds studied. The weighted precipitation effectively integrated all rain within each station's subwatershed, rather than the simple single rain gauge assessment. Weighted precipitation for Site 03 was estimated from a nearby station, (Site O) because of lack of gauges for this site.

Local and weighted precipitation, mean daily stream flow, mean daily discharge, maximum and minimum instant daily discharge, and flow volume were recorded or calculated for each site. Total and mean values were calculated for the day of sampling, sampling day plus the previous day, the daily totals for sample day plus previous 7 and 30 days, and daily averages for the 2, 7, and 30 days prior to the sampling day.

Sample Collection

Water samples were collected in sterile 1-Liter polypropylene bottles from the three sites between January 2007 and December 2007. Sample collection was targeted at storm events after an average of at least 1.27cm (one-half inch) of consistent rain had fallen over the watershed within a 24 hour period. Baseline samples were collected when at least seven days of either no rain had fallen within the

watershed, or less than the 1.27cm storm threshold level had fallen. Over the study period, five baseline and five storm samples from each station were collected. All samples were collected on the upstream side of the weir, a low concrete v-notched wall that measures stream flow. After collection, water samples were immediately placed on ice and processed for fecal indicator bacteria and bacterial pathogens within 24 hours of collection.

During sample collection, temperature (˚C), conductivity (mS cm^{-1}), salinity (ppt), dissolved oxygen (DO, mg L^{-1}), pH, and oxidation reduction potential (ORP, mV) were measured with a YSI 556 multi-parameter sonde (Yellow Springs, OH).

Microbial Analysis

Fecal Indicator Bacteria (FIB)

Water samples were tested for *E. coli* using EPA Standard Method 1603 (U.S.EPA 2006b). Briefly, duplicate 50 ml, 10 ml, and 1 ml samples were vacuum filtered through 0.45 µm nitrocellulose mixed-ester membrane filters, which were then placed directly onto Modified mTEC agar plates (Becton Dickson, Franklin Lakes, NJ). The plates were incubated for approximately 2h at 35˚C ±0.5˚C followed by water bath incubation at 44.5˚C ± 0.2˚C for about 22h. Enumeration of *E. coli* was performed by counting the red or magenta colored colonies and was reported as colony forming units (CFU) 100 ml^{-1}.

Samples were also tested for enterococci using EPA Standard Method 1600 (U.S.EPA 2006a). Briefly, duplicate 50 ml, 10 ml, and 1 ml samples were vacuum filtered through 0.45 µm nitrocellulose mixed-ester membrane filters, which were then placed directly onto mEI agar plates (mE plates, Becton Dickson, with the addition of indoxyl β-D-glucoside). The plates were incubated for approximately 24h at 41˚C ±0.5˚C. Enumeration of enterococci was performed by counting all colonies that exhibited a blue halo, and were reported as CFU per 100 ml^{-1}.

Enterococcus faecium (ATCC #15336) and *Escherichia coli* (ATCC #15597) were used as positive control strains for fecal indicator bacterial analyses and sterile phosphate buffered saline (PBS) was used as negative controls to ensure quality control during sample processing.

Salmonella

Salmonella detection and enumeration was performed with utilizing a 3-Step Most Probable Number Assay (MPN) involving pre-enrichment, enrichment, and then selection (Fricker 1987; Moriñigo, Martinez-Manzanares et al. 1989; Moriñigo, Munoz et al. 1993). Five replicates of three volume sizes (100 ml, 10 ml, and 1 ml) of river water were analyzed. Briefly, samples from each replicate were vacuum filtered through 0.45 µm nitrocellulose mixed-ester membrane filters and pre-enriched in 50 ml of a 1% buffered peptone water solution (BPW, Becton Dickson, Franklin Lakes, NJ) (pH 7.2 ±0.2) and incubated at 37˚C ± 1˚C for 18 to 24h in sterile conical tubes with the caps loose. 100 µl from each pre-enrichment tube was then aliquoted separately into individual tubes containing 10ml of Rappaport-Vassilaides Broth (RV, Difco Laboratories, MI) and incubated at 43˚C ± 1˚C for 24 ± 2h for selective enrichment of *Salmonella*. Finally, 10µl from each selective enrichment was streaked onto individual Xylose-Lysine-Deoxycholate agar plates (XLD) (Becton, Dickson), and incubated at 37˚C ±1˚C. Colonies exhibiting all black or black with yellow ring morphologies after 24 or 48h were considered presumptive *Salmonella.* One well isolated presumptive *Salmonella* colony from each positive plate was picked, re-isolated three times to ensure a pure culture, and stored in Caso agar (Oxoid, Lenexa, KS) deep stab tubes. The Enterotube™ II (Becton Dickson) biochemical assay was used to confirm each isolate as *Salmonella* spp. All isolates identified as *Salmonella* spp. were serotyped by agglutination at the *Salmonella* Reference Center at the University of Pennsylvania (Kennet Square, PA). Each dilution and replicate that resulted in a confirmed *Salmonella* isolate was scored as positive. Final *Salmonella* levels were reported as MPN L^{-1} (Blodgett and Garthright 2003).

Campylobacters

Enumeration of campylobacter-like organisms (i.e. *Campylobacter* spp. and closely related organisms such as *Arcobacter* spp.) was performed using a direct plating method, without enrichment, described by Rosef et al. (Rosef, Kapperud et al. 1987) and modified by Vereen et al 2007. No

enrichment was performed due to the potential for *Arcobacter* spp. or other microflora to out compete *Campylobacter* spp. (Atabay and Corry 1997; Abulreesh, Paget et al. 2005).

Briefly, duplicate 100 µl aliquots of river water sample from each site were spread directly onto modified charcoal cefoperazone deoxycholate agar plates (mCCDA, Oxoid, Lenexa, KS), as described by Vereen et al (2007). The plates were incubated at 37˚C ± 1˚C for 48 ±2h under microaerobic conditions using non-vented BBL™ (Becton Dickson, Cockeysville, MD) GasPak jar systems with BBL™ (Sparks, MD) CampyPak Plus carbon dioxide and hydrogen generating envelopes with palladium catalyst (5% O_2, 10% CO_2, and 85% N_2). After incubation, colonies exhibiting grey/white to semi-transparent mucoid morphologies were considered presumptive campylobacter-like organism (Fricker 1987; On 1996; Tholozan, Cappelier et al. 1999; Thomas, Hill et al. 1999; Diergaardt 2003; HPA 2007). Randomly selected and well isolated presumptive campylobacter-like colonies were picked from each plate and were re-isolated on mCCDA three times to ensure a pure culture. Pure isolates were grown overnight in Brucella broth at 37˚C for 24 hours under standard microaerophilic conditions and 1 ml aliquots were amended with glycerol (25% final concentration) and stored at -80˚C. Gram negative, spiral shaped isolates that demonstrated positive catalase and oxidase test reactions and that only grew in microaerophilic conditions were described as campylobacter-like, without regard to species. This level of phenotypic testing confirmed the presence of closely related campylobacter-like organisms including *Campylobacter* spp. and *Arcobacter* spp. (On 1996; Engberg, On et al. 2000; Diergaardt 2003; Agency 2005; HPA 2005; Vereen, Lowrance et al. 2007). All campylobacter-like organisms were reported as CFU ml^{-1}.

Thermophilic Campylobacter spp.

All presumptive campylobacter-like isolates were subjected to PCR to determine the presence of thermophilic C*ampylobacter* species, known to contribute to human illness. Additional PCR assays also targeted the four primary species within this group, *C. jejuni* subsp. *jejuni, C. coli, C. lari,* and *C. upsaliensis*. Positive controls for these four thermophilic species, *C. coli* ATCC# 49941, *C. jejuni* ATCC#

49943, *C. lari* ATCC# 35221, and *C. upsaliensis* ATCC# 43954, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Prior to DNA extraction, all isolates and positive controls were grown over night (up to 24h) in Brucella broth at 37˚C under microaerophilic conditions. 10µl of liquid culture was subsequently streaked onto mCCDA plates and incubated 24-48h under microaerophilic conditions. Growth from an isolated colony was suspended in 1ml of Milli-Q water (Millipore Corp., Billerica, MA) in a sterile microcentifuge tube and centrifuged (Eppendorf Centrifuge 5417C, New York, New York) at 2655 x g for 5 minutes to pellet cells for lysing. Cells were lysed at 100˚C for 10 minutes and immediately transferred to a 4˚C cold block for 5 minutes. Tubes were centrifuged at 9,700 x g for 1 minute to pellet cell debris. The supernatant was removed and stored in sterile tubes at -20˚C until ready for use.

Extracted DNA was subjected to PCR targeting the 23S rRNA region of the thermophilic C*ampylobacter* species (**Table 3.2**) (Eyers, Chapelle et al. 1994; Klena, Parker et al. 2004). PCR amplification was performed using 40 pmol of each primer (THERM 1 and THERM 2), in a 50 µl reaction volume, using reagents from a 5-Prime *Taq*Master kit (Gaithersburg, MD). The following was added for each reaction: 4 µl of a 2.5 mM dNTP mix, 5 µl of 10x PCR Buffer with Mg^{2+} (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15mM $Mg(OAc)_2$), 10 µl of 5x PCR enhancer, 0.25 U *Taq* DNA polymerase, an additional 0.5µl of a 25mM $Mg(OAc)_2$ solution, and 24.25µl of PCR grade nuclease free water (Fisher, Fair Lawn, N.J.). Lastly, 2 µl of extracted sample DNA, ranging from 19 ng - 50 ng μl^{-1} was added to each reaction mixture. Each of the four ATCC control strains and a negative control (PCR grade water) were included in all PCR runs for quality control. Thermal cycling conditions consisted of an initial denaturing step of 94˚C for 3 minutes, followed by denaturing at 94˚C for 1 minute, annealing at 54˚C for 1 minute, extension at 72˚C for 1 minute, carried out over 45 cycles, with a final extension step of 72˚C for 8 minutes, using a PTC-200 DNA Engine (MJ Research, Inc., Watertown, MA).

Samples that tested positive as thermophilic campylobacters were subjected to further PCR to determine if the isolate(s) belonged to one of the four target thermophilic species. Primers targeted the *lpx*A gene for *C. jejuni, C. coli, C. lari,* and *C. upsaliensis* (Klena, Parker et al. 2004) (**Table 3.2**). In

separate reactions, each isolate was tested for all four species. Additionally, all four species were used as controls in all reactions. Reactions mixtures were the same as described for the thermophilic *Campylobacter* assay.

Thermal cycling conditions for all targets consisted of an initial denaturing step at 94˚C for 1 minute followed by 35 cycles of denaturing at 94˚C for 1 minute, annealing at 50˚C for 1 minute, extension at 72˚C for 1 minute, and a final extension step at 72˚C for 5 minutes.

All PCR amplification products were examined by agarose gel electrophoresis using 1.5% - 1.8% agarose gels subjected to 90V, 400mAmps, for 90 minutes in 1x TAE buffer. Bands were visualized, after staining in ethidium bromide, under UV illumination and photographed using a Kodak Gel Logic Imaging System (Kodak, Inc., Rochester, N.Y.) with Kodak Molecular Imaging Software (Version 4.0).

Statistical Analysis

Data analysis was conducted using Minitab® Release 15.1.1.0 for Windows (Minitab Inc., State College, PA), GraphPad Prism® Prism for Windows 5 (GraphPad Software, Inc., San Diego, CA), and SPSS 16.0.1 (SPSS, Inc., Chicago, Il.). Data were stratified by collection date, site, sample event, and rainfall quartiles. Kruskal-Wallis tests were used to examine differences between pathogen concentrations and sites and FIB levels in relation to U.S. EPA standards. Wilcoxon Signed-Rank Tests were utilized to conduct non-parametric pairwise analysis of baseline versus storm samples at the watershed level and the site level. Due to small sample sizes at the site level (five baseline samples and five storm samples total for each site), p values for the Wilcoxon Signed-Rank Tests and Kruskal-Wallis Tests were derived from Exact Significance 1-tailed tests, which are designed for small sample sizes and do not rely on asymptotic assumptions that the data conform to any particular distribution (Cytel Software Corp. 1996). All p values of ≤ 0.05 were considered significant. *Salmonella,* Campylobacter-like organisms, and fecal indicator bacterial levels that were below the limits of detection (< 1.8 MPN L^{-1}; < 5 CFU 1 ml^{-1}, and < 2 CFU $100ml^{-1}$, respectively) were entered as zero for all statistical analyses.

Results

Precipitation

In 2007, the LRW study area received a total of 84.3cm of rain, a 28.8% deficit compared to the previous 10 year average (118.4cm). This was also the lowest amount of annual rainfall than in any of the previous 10 years. Baseline samples were collected during the months of January, May, October, November and December. Total local rainfall amounts for the seven days prior to the collection of baseline samples ranged from 0 cm – 0.28 cm (7-day daily mean 0.06 cm). Total weighted rainfall for the same period ranged between 0 and 0.25cm of rain (7-day daily mean 0.06 cm).

Storm samples were collected in February, June, July, August, and September. Total local rainfall amounts for the seven days prior to the collection of storm samples ranged from a minimum of 3.10 cm to a high of 7.06 cm (7-day daily mean 4.98 cm). Total weighted rainfall amounts for the same period ranged from 3.10 cm to 6.60cm (7-day daily mean 4.66 cm).

Streamflow

Over all sites during the course of the study, stream flow peaked between February and March 2007. By May 2007, there was virtually no flow at any station; only Site 03 demonstrated intermittent flow after heavy storms (USDA-ARS-SEWRL 2008a). During the summer and fall sampling events (no flow) sampling was restricted to puddles that had collected in the stream bed.

Water Quality Parameters

A total of 30 water samples, 5 baseline samples and 5 storm samples were collected from each of three sites within the LRW between January and December 2007. Water temperatures differed significantly by collection date ($p = 0.001$), from a mean low of 7.42˚C in February 2007 to an average 25.73˚C in August 2007 (N=3). The pH also varied significantly by collection date ($p = 0.003$), with the lowest mean readings from June 2007, (5.48), and the highest mean reading during November (7.85). ORP varied significantly by collection date ($p = 0.026$), with highest readings in February 2007 (mean

241 mV) and lowest in June 2007 (mean 47.17 mV). DO, conductivity, and salinity did not vary significantly by date ($p > 0.05$).

The pH was the only parameter to exhibit any significant difference between baseline and storm samples. The pH levels were significantly lower during storm events (mean 6.45) compared to baseline samples (mean 7.36) ($p = 0.002$).

Fecal Indicator Bacteria (FIB)

Over all samples, *E. coli* and enterococci levels averaged 1,340 and 4,980 CFU 100 ml^{-1}; respectively (N = 30). *E. coli* levels did not differ significantly by site, (N = 10) ($p > 0.05$), however, highest levels were observed at Site 03 (mean 3,174 CFU 100ml^{-1}), and lowest levels at Site B (mean 113 CFU 100ml^{-1}). Enterococci levels differed significantly by site, (N=10) ($p = 0.003$), with highest levels at Site 03, (mean 13,529 CFU 100ml^{-1}), and lowest levels at Site B (mean 174 CFU 100ml^{-1}).

Over the sample area mean levels of *E. coli* during storm events were significantly higher than baseline samples (2,515 and 164 CFU 100ml^{-1}, respectively) (N = 15) ($p = 0.006$). Likewise, overall mean enterococci levels from storm samples were significantly higher than overall mean baseline samples (7,333 and 2,626 CFU 100ml^{-1}, respectively) (N = 15) ($p = 0.015$). The highest mean storm event levels were recorded in February for *E. coli* (5,677 CFU 100ml^{-1}) and September for enterococci (13,013 CFU 100ml^{-1})

Among storm samples both *E. coli* and enterococci showed significant differences between stations ($p= 0.011$ and $p < 0.001$, respectively). Highest mean storm sample *E. coli* and enterococci levels were observed at Site 03 (the smallest stream order and smallest subwatershed) at 6,151 CFU 100ml^{-1} and 20,040 CFU 100ml^{-1}, respectively. The lowest mean levels were recovered at Site B (the largest stream order and subwatershed) with 70 CFU 100ml^{-1} and 265 CFU 100ml^{-1}, N=5 for *E. coli* and enterococci respectively. Storm sample levels at Site F fell between the two with 1325 CFU 100ml^{-1} and 1695 CFU

100ml^{-1}, N=5, for *e. coli* and enterococci respectively. Baseline samples of both *E. coli* and enterococci showed no significant differences between sites (p = 0.949 and p = 0.171 respectively).

Salmonella spp.

Over all months and stations *Salmonella* levels averaged 35 MPN L^{-1} (N = 30). While overall *Salmonella* levels did not differ at a significant level between sites (p > 0.05), *Salmonella* levels increased as stream order decreased (**Figures 3.5** and **3.6**). The highest *Salmonella* levels were consistently detected at Site 03 (mean of 84.4 MPN L^{-1}, N=10), while the lowest levels were consistently recovered from Site B (mean 9.5 MPN L^{-1}, N=10). Site F fell between the two with an overall mean of 11.6 MPN L^{-1}, N=10 (**Figure 3.5**). Throughout the study, Site 03 was positive for salmonellae 80% of the time, 70% of the time at Site B, and 50% of the time at Site F. Individual *Salmonella* counts ranged from non-detectable (< 1.8 MPN L^{-1}), to a high of 350 MPN L^{-1} collected from a storm sample in June at Site 03 (**Table 3.3**). At least once from each site *Salmonella* was not detected in 10 of the 30 total individual samples collected throughout the study (33.3%); 8 of these 10 negative samples were baseline samples (80%). During storm conditions, mean *Salmonella* levels were 63 MPN L^{-1} (N = 15), while baseline samples averaged 7.3 MPN L^{-1} exhibiting a significant difference (p = 0.007) (**Figure 3.1**). When baseline and storm samples of were analyzed by station, *Salmonella* levels were not significantly different between sites (p = 0.054) (**Figure 3.6**). In contrast, samples taken during storm events were significantly different between sites (p < 0.001) (**Figure 3.6**). Mean storm sample *Salmonella* levels were highest at Site 03 (164 MPN L^{-1}, N=5) (**Figures 3.4** and **3.6**), lowest at Site B (2 MPN L^{-1}, N=5) (**Figures 3.2** and **3.6**), with Site F between the two (23.2 MPN L^{-1}, N=5) (**Figures 3.3** and **3.6**).

Over the course of the study, 101 *Salmonella* isolates, representing 16 different serotypes were recovered (**Table 3.4)**. Thirty three of the 101 total recovered isolates were *Salmonella* Braenderup; the most commonly recovered serotype (32.67%). Eleven isolates were each serotyped as *S.* Anatum (10.89%), *S.* Arizona (10.89%), and *S.* Rubislaw (10.89%) (**Table 3.4**). The majority of isolates were

recovered in summer (49 isolates 48.51%), 30 isolates (29.70%) were recovered during the winter, and 20 isolates (19.80%) were recovered in the fall. Only 2 isolates (1.98%) were recovered during the spring. *Salmonella* serotype diversity varied inversely with the size of the sampling site (stream order), with greater levels and variability corresponding to lower stream levels. Fifty nine of the recovered isolates (58.42%) representing 13 of the 16 detected serotypes (81.25%) were recovered from Site 03; twenty six isolates (25.74%) representing 7 of the 16 serotypes (43.75%) were recovered from Site F; and the remaining 16 isolates (15.84%) corresponding to 7 of the 16 serotypes (43.75%) were recovered from Site B (**Table 3.4**). Despite the lack of statistical significance between the number of serotypes and site, many serotypes exhibited definite spatial patterns. 6 serotypes (37.5% of all present serotypes) were only detected at Site 03 (**Table 3.4**). Further, Site 03 contained 84.9% and 80% of two other serotypes. Two serotypes (12.5% of total serotypes), *S.* Gaminara and *S.* Infantis, were only detected at Site F. Site F also contained 72.7% of the *S.* Arizona isolates recovered during the study. 80% of the *S.* Bareilly isolates were recovered from Site B (**Table 3.4)**.

Variation between the number of serotypes recovered from storm samples (mean 2.2 MPN L^{-1}, N=15) and baseline samples (mean 0.73 MPN L^{-1}, N=15) differed significantly ($p = 0.006$) (**Table 3.5**). Of the 16 recovered serotypes, 100% of 7 (43.8%) and 80% or greater of four other serotypes were only recovered during storm events (**Table 3.5**). In contrast, only 100% of 3 serotypes (18.8%) and 80% of another were only recovered from baseline samples (**Table 3.5**). Only two serotypes, *S.* Kentucky, and a monophasic serotype (*S.* 47:z4z23) were approximately evenly distributed between storm and baseline samples.

Campylobacters

The overall mean level of campylobacters observed over the term of the study was 1,585 CFU ml^{-1} (N=30). The highest mean concentration recorded was in June (storm sample, 8,165 CFU ml^{-1}, N = 3). The lowest mean concentration was recovered in December (baseline sample, 16.7 CFU MPN L^{-1},

N=3). Campylobacter levels were significantly different between collection sites, with higher concentrations found at station located within the smallest subwatershed (Site 03) ($p = 0.014$) (**Table 3.3**). The highest mean levels were detected at Site 03 (3,197 CFU ml^{-1}, N=10), followed by Site F (1,486 CFU ml^{-1}, N=10), and the lowest mean levels were detected at Site B (72.5 CFU ml^{-1}, N=10) (**Figure 3.7**).

Campylobacter levels recovered from storm samples (3,122 ml^{-1}, N=15) were significantly higher than those from baseline samples (mean 48 CFU ml^{-1}, N=15) ($p < 0.001$) (**Figure 3.1**). When baseline and storm samples were compared separately between sites, differences in mean levels were more pronounced for the storm events. Mean levels during storm event were highest at Site 03 (6,273 CFU ml^{-1}, N=5), lowest at Site B (137 CFU ml^{-1}, N=5), with Site F falling in between the two (2,956 CFU ml^{-1}, N=5) (**Figure 3.8**).

Over the course of the study, 189 campylobacters isolates were recovered. PCR was performed on all isolates to identify the thermophilic spp. A total of 26 isolates (13.76%) tested positive for thermophilic *Campylobacter* spp. All 26 of these isolates were subjected to further PCR to determine the presence of *C. coli*, *C. jejuni*, *C. lari*, or *C. upsaliensis*. None of the four thermophilic species were detected in the environmental samples. Overall, of the 30 samples collected, 11 (36.67%) tested positive for the presence of thermophilic campylobacters. Thermophilic campylobacter detection did not show any statistically significant relationship with any environmental, seasonal, or water quality variables. 5 of the 11 positive samples were from summer samples (45.45%), 3 were winter samples (27.27%), 2 from fall samples (18.18%), and 1 spring sample (9.09%). No statistically significant difference between baseline and storm sample levels of thermophilic campylobacters was observed. 36.36% of the thermophilic campylobacters were recovered from baseline samples and 63.64% from storm samples.

Pathogens and EPA Water Quality Criteria

Overall, *Salmonella* was detected 100% of the time (8/8) when *E. coli* exceeded the EPA thresholds. However, Salmonella was also detected 45.5% of the time (10/22) when *E. coli* levels were

below EPA thresholds. *Salmonella* was detected 71.4% of the time (15/21) when enterococci levels exceeded EPA thresholds (151 CFU ml^{-1}), however, *Salmonella* was also detected 55.6% of the time (5/9) when enterococci were below EPA thresholds.

Overall, 23.3% of the time, campylobacters were present when *E. coli* exceeded the EPA acceptable levels (7/30), present 56.7% when EPA levels were not passed (17/30), absent 3.3% when *E. coli* did not pass EPA levels (1/30), and absent 16.7% when *E. coli* was absent (5/30). Overall, campylobacters were present 60% of the time when enterococci exceeded EPA limits (18/30), present 20% when EPA limits were not passed (6/30), campylobacters were absent 10% when EPA limits were passed (3/30), and absent 10% when EPA limits were not exceeded (3/30).

Discussion

Salmonella and Campylobacters are common waterborne disease causing pathogens that are commonly isolated from environmental surface waters (Abulreesh, 2005; Jones, 2001; Koenraad, 1997; Rollins, 1986; Schaffter, 2002; Srikantiah, 2004; Angulo, 1997; Santo Domingo, 2000). As the body of literature concerning the ecology, fate, and transport of environmental microbial pathogens grows, more credence is lent to the idea that the environment itself may be a significant exposure hazard. The growing numbers of watershed-scale studies similar to this study lend support to the hypothesis that climatic events, such as storms, not only affect the environment around us, but also the ecology of the microbial fauna in the environment.(Rollins and Colwell 1986; Mallin, Williams et al. 2000; Santo Domingo, Harmon et al. 2000; Smith, Wickham et al. 2001; Kistemann, Claßen et al. 2002; Ferguson, Husman et al. 2003; Winfield and Groisman 2003; Jamieson, Gordon et al. 2004; Martinez-Urtaza 2004; Ahn, Grant et al. 2005; Kemp, Leatherbarrow et al. 2005; Benham, Baffaut et al. 2006; Pachepsky, Sadeghi et al. 2006; Vereen, Lowrance et al. 2007; Haley, Cole et al. 2009). What we observed in this study was decreased water quality from samples collected during storm events, as evidenced by significantly higher levels of pathogens and fecal indicator bacteria compared to baseline samples.

The interactions between environmental pathogens, the environment, and human infections are complex. However, relationships between events such as rainfall and increased human illness have been documented (Baxter-Potter and Gilliland 1988; Checkley, Epstein et al. 2000; Curriero, Patz et al. 2001; Auld, MacIver et al. 2004; Reacher, McKenzie et al. 2004; Shehane, Harwood et al. 2005). During the latter half of this study period (2007), Georgia experienced a severe drought. Though it most strongly affected the northern part of the state the southern part of the state containing the study area was also affected (Holcomb and & Couch 2007; National Oceanic and Atmospheric Administration 2007). The Little River Watershed received 23 cm less rain (a 28.8% deficit) than the previous 10 year average for this area (USDA-ARS-SEWRL 2008a).

Overall, 23.3% of the time, campylobacters were present when *E. coli* exceeded the EPA acceptable levels (7/30) present (56.7%) when EPA levels were not passed (17/30), absent 3.3% when *E. coli* levels did not pass EPA levels (1/30), and absent 16.7% when *E. coli* was absent (5/30). Overall, campylobacters were present 60% of the time when enterococci exceeded EPA limits (18/30), present 20% when EPA limits were not surpassed (6/30), and campylobacters were absent 10% when EPA limits were passed (3/30) and absent 10% when the limits were not exceeded.

Studies have demonstrated a relationship between increased stream flow and increased microbial levels, due to factors such as bacterial adherence to re-suspended sediments from storms (Baudart, 2000a; Benham, 2006; Davies-Colley, 2008; Ferguson, 2003; Smith, 1996). The findings of this study generally support the findings of these other watershed scale studies, namely increased microbial levels detected following storm events. However, some of the aspects of this study diverge from the other watershed scale studies, especially regarding the relationship between stream flow and turbidity with increased microbial loading. As is probably true in many other watersheds, in the LRW, typically the summer months, especially June and July, generate the highest precipitation totals via short-term yet frequent and intense storms. However, this is also the lowest stream flow period due to in part to evapotransporation from agriculture (Bosch, Sheridan et al. 1999; Bosch, Lowrance et al. 2003). Stream flow in the two

largest sampling stations in this study, Sites B and F, peaked in April and February respectively. Stream flow ended for both sites within weeks of each other in the beginning of May. At Site 03, stream flow peaked during February and March and constant daily flow ended in late April. Site 03 was the only site to experience any more stream flow, though erratic and only after a significant rainfall. What was observed over the course of this study was that even in the absence of measureable stream flow, high levels of fecal indicator bacteria and the pathogens *Salmonella* and campylobacters continued to be recovered from the sampling sites, sometimes from puddles measuring only a few meters in diameter.

The continued correlation between rainfall and increased microbial contamination of surface waters, even in the absence of normal stream flow illustrates the complexity of the ecology of these pathogens and their potential impact on human health. In the "real world", there are other natural factors potentially leading to increased microbial loading in the absence of stream flow re-suspending bacterial pathogens adhered to sediments. With decreased water available due to evapotransporation, there are less readily available water sources for local fauna, leading to increased possible fecal contamination from indigenous species. There are also other "natural" activities potentially affecting these sites other than just the "normal" agricultural practices. During the May sampling event, someone had butchered a hog and left the head and entrails near the water's edge at Site 03. Also, at Site F, on several occasions, animals, including butchered deer, and what appeared to be a "pit fighting" dog that had been killed, were dumped near the water to be sampled.

The goal of this study was to elucidate the impacts of discrete storm events on microbial loading of surface waters within the LRW. At the watershed and individual site levels, elevated microbial concentrations and pathogen variability were consistently recovered from storm samples compared to baseline samples. Increased levels and variability of pathogens could have a potential impact on human health. For example, 6 of the 16 serotypes (37.5%) recovered from the environmental samples were recovered from human salmonellosis cases from South Health District 8-1, thus further strengthening the link between the environment and human exposure. Additionally, while the findings were not always

statistically significant, trends were observable at the site level. As the stream order increased and proximity to agricultural or livestock activities decreased, recovered microbial levels and variation decreased.

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Table 3.1 Land and Rain Characteristics

Site	Drainage Area	Stream Order	County	Population	Housing Units	% Septic Systems
B	334.4 km2 (1)	5 (1)	Tift	42000 (2,5)	16000 (5)	38% (3,5)
F	114.9 km^2 (1)	4 (1)	Turner	9300 (2,4)	4300 (4)	40% (3,4)
O3	2.4 km^2 (1)	1 (1)	Tift	42000 (2,5)	16000 (5)	38% (3,5)

(1) Bosch, D. and Sheridan, J. 2007c
(2) Georgia Division of Public Health-OASIS; Available online at: http://oasis.state.ga.us/
(3) US Census Bureau Population Division, 2008; Available online at: http://www.census.gov/population/www/
(4) Wynn, M.; Turner County Clerk; Personal Communication, June 26, 2008
(5) Rivera, M.; GIS, South Health District Regional Planning Commission, Personal Communication July 3,2008
(6) NARSAL-GLUT 2005; Available online at: http://narsal.ecology.uga.edu/glut/watershed.php?watershed=19

Table 3.2 Primers and annealing temperatures used in PCR reactions

Primer	Sequence (5'-3')	Annealing Temperature °C	Observed Amplicon Size (bp)	Reference
THERM1	TATTCCAATACCAACATTAGT	54		Eyers et al 2003, 2004;
THERM2	CGGTACGGGCAACATTAG		290	Savill et al 2001
lpx AC coli	AGACAAATAAGAGAGAATCAG	62	400	
lpx AC jejuni	ACAACTTGGTGACGATGTTGTA	64	350	Klena et al 2004
lpx AC lari	TRCCAAATGTTAAAATAGGCGA	64	240	
lpx AC upsaliensis	AAGTCGTATATTTTCYTACGCTTGTGTG	64	210	
Reverse Primer	CAATCATGDGCDATATGASAATAHGCCAT			

Table 3.3 Sample results by collection date and weekly rainfall
* Denotes dead animal carcasses in sampling area
** Denotes "Below Level of Detection"

Site B						
Collection Date	**Sample Event**	**Total Weighted 7-Day rainfall**	***E. coli* CFU 100ml^{-1}**	**Enterococci CFU 100ml^{-1}**	***Salmonella* MPN L^{-1}**	**Campylobacters CFU 100 ml^{-1}**
01/16/07	Baseline	0.762	35	48	< 1.8**	25
02/02/07	Storm	17.63	131	725	< 1.8	15
05/09/07	Baseline	0	37	10	4	< 5
06/03/07	Storm	12.98	119	129	2	75
07/16/07	Storm	12.22	28	56	4	10
08/31/07	Storm	15.7	19	175	< 1.8	35
09/23/07	Storm	11.354	52	240	2	550
10/17/07	Baseline	0.102	11	205	2	< 5
11/13/07	Baseline	0	56	98	2	15
12/14/07	Baseline	0	640	52	79	< 5
% of positive detection by site			100%	100%	70%	70%

Site F						
Collection Date	**Sample Event**	**Total Weighted 7-Day rainfall**	***E. coli* CFU 100ml^{-1}**	**Enterococci CFU 100ml^{-1}**	***Salmonella* MPN L^{-1}**	**Campylobacters CFU 100 ml^{-1}**
01/16/07	Baseline	0.762	165	185	< 1.8	< 5
02/02/07	Storm	17.63	2200	1650	17	55
05/09/07	Baseline	0	5	1	< 1.8	< 5
06/03/07	Storm	12.98	340*	850*	5*	14200*
07/16/07	Storm	12.22	4000*	2350*	22*	270*
08/31/07	Storm	15.7	31	575	23	195
09/23/07	Storm	11.354	52	3050	49	60
10/17/07	Baseline	0.102	235	3500	< 1.8	75
11/13/07	Baseline	0	280	181	< 1.8	< 5
12/14/07	Baseline	0	12	23	< 1.8	5
% of positive detection by site			100%	100%	50%	70%

Site 03						
Collection Date	**Sample Event**	**Total Weighted 7-Day rainfall**	***E. coli* CFU 100ml^{-1}**	**Enterococci CFU 100ml^{-1}**	***Salmonella* MPN L^{-1}**	**Campylobacters CFU 100 ml^{-1}**
01/16/07	Baseline	0.762	700	850	4	35
02/02/07	Storm	17.63	14700	18950	110	125
05/09/07	Baseline	0	37	67	< 1.8	360
06/03/07	Storm	12.98	11200*	12500*	350*	10220*
07/16/07	Storm	12.22	415*	3750*	170*	15700*
08/31/07	Storm	15.7	3400	29250	61	4570
09/23/07	Storm	11.354	1040	35750	130	750
10/17/07	Baseline	0.102	9	12300	< 1.8	115
11/13/07	Baseline	0	53	169	2	45
12/14/07	Baseline	0	188	21700	17	45
% of positive detection by site			100%	100%	80%	100%

Table 3.4 *Salmonella* serotype distribution by site

Serotype	Total Number of Serotypes	Total Isolates Per Serotype By Site		
		B	F	O3
***S.* Anatum**	11	0	0	11 (100%)
***S.* Arizona**	11	3 (27.3%)	8 (72.7%)	0
***S.* Bareilly**	5	4 (80%)	0	1 (20%)
***S.* Braenderup**	33	4 (12.1%)	1 (3%)	28 (84.9%)
***S.* Cambridge**	3	0	0	3 (100%)
***S.* Gaminara**	6	0	6 (100%)	0
***S.* Infantis**	1	0	1 (100%)	0
***S.* Java**	3	1 (33.3%)	0	2 (66.6%)
***S.* Kentucky**	5	0	1 (20%)	4 (80%)
***S.* Montevideo**	1	0	0	1 (100%)
***S.* Muenchen**	5	1 (20%)	2 (40%)	2 (40%)
***S.* Rubislaw**	11	2 (18.2%)	7 (63.6%)	2 (18.2%)
***Salmonella* Rough**	2	1 (50%)	0	1 (50%)
***S.* Typhimurium**	1	0	0	1 (100%)
***S.* 30:-:lw**	1	0	0	1 (100%)
***S.* 47:z4z23**	2	0	0	2 (100%)
Total Number of Serotypes	**101**	**16**	**26**	**59**
Different Serotypes Recovered Per Site		**7**	**7**	**13**
% of the 101 isolates recovered at each site		**15.84%**	**25.74%**	**58.42%**
% of the 16 serotypes present at each site		**43.75%**	**43.75%**	**81.25%**

Table 3.5 *Salmonella* distribution by Event

Serotype	Total Number of Serotypes	Frequency of Serotype by Event	
		Baseline	Storm
***S.* Anatum**	11	0 (0%)	11 (100%)
***S.* Arizona**	11	2 (18.2%)	9 (81.8%)
***S.* Bareilly**	5	4 (80%)	1 (20%)
***S.* Braenderup**	33	4 (12.1%)	29 (87.9%)
***S.* Cambridge**	3	3 (100%)	0 (0%)
***S.* Gaminara**	6	0 (0%)	6 (100%)
***S.* Infantis**	1	0 (0%)	1 (100%)
***S.* Java**	3	0 (0%)	3 (100%)
***S.* Kentucky**	5	2 (40%)	3 (60%)
***S.* Montevideo**	1	0 (0%)	1 (100%)
***S.* Muenchen**	5	1 (20%)	4 (80%)
***S.* Rubislaw**	11	0 (0%)	11 (100%)
***Salmonella* Rough**	2	2 (100%)	0 (0%)
***S.* Typhimurium**	1	1 (100%)	0 (0%)
***S.* 30:-:lw**	1	0 (0%)	1 (100%)
***S.* 47:z4z23**	2	1 (50%)	1 (50%)
Total Number of Isolates by event	**101**	**20**	**81**
Different Serotypes Recovered by Event		**9**	**13**
% of the 101 isolates recovered by Event		**19.80%**	**80.20%**
% of the 16 serotypes present by Event		**56.25%**	**81.25%**

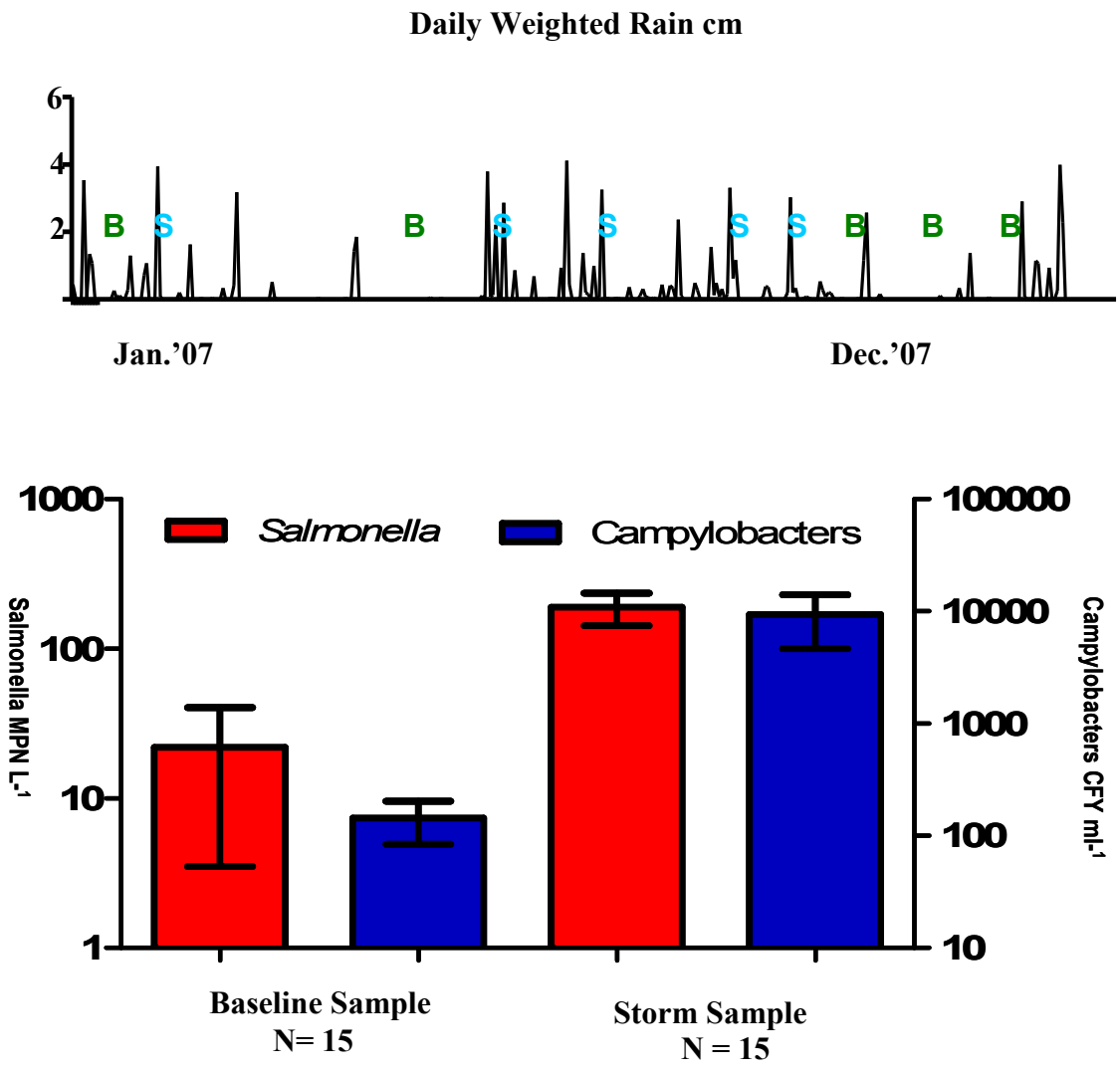

Figure 3.1 Overall Pathogen Levels Baseline vs. Storm Samples Entire Watershed.

Site B

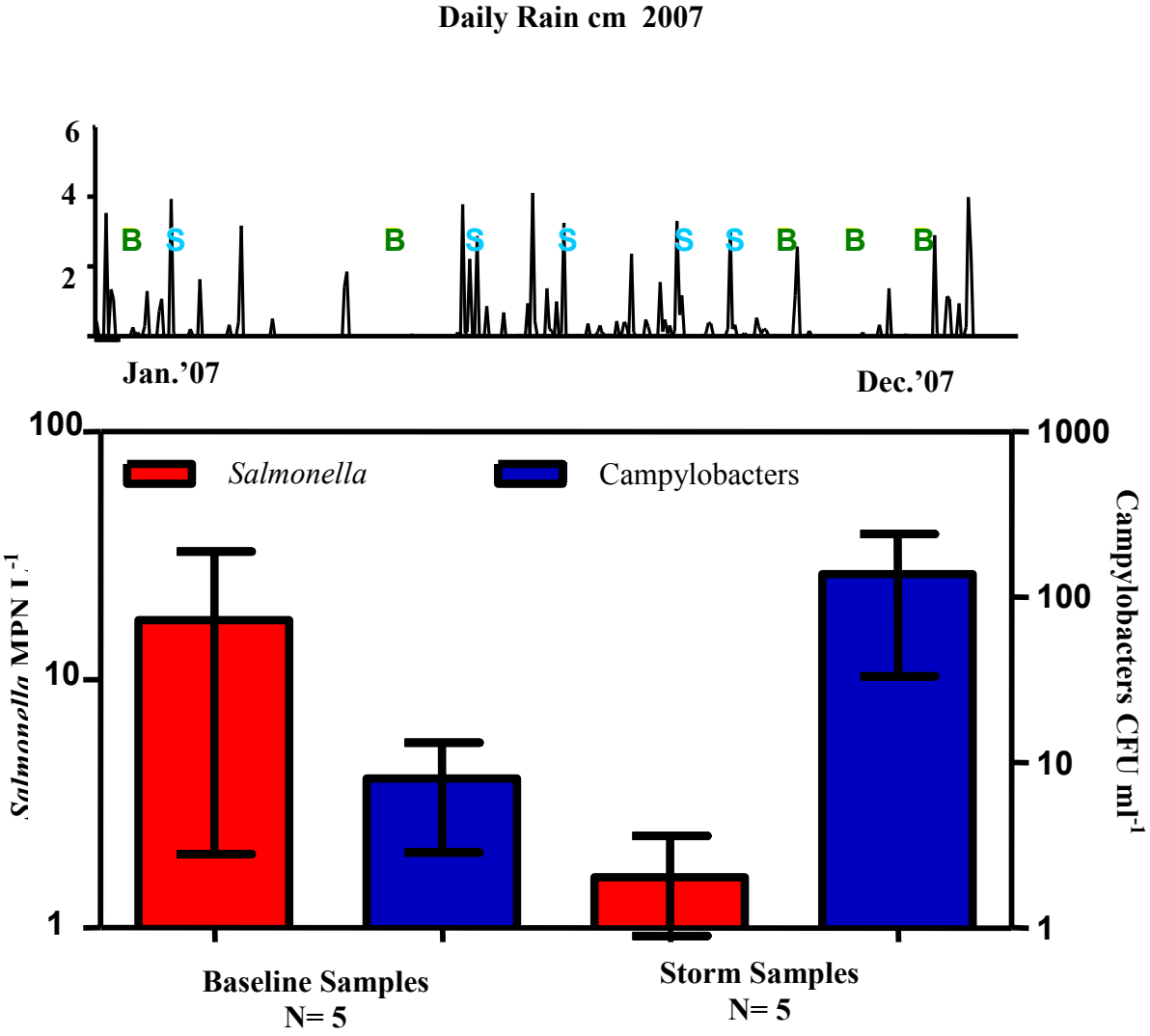

Figure 3.2 Site B Pathogen Levels Baseline vs. Storm Samples.
a. Though the chart seems to demonstrate a significant inverse effect of *Salmonella*, This is due to an outlier. There is no statistically significant difference in means ($p = 0.4432$).
b. The same is true for the campylobacters at this site ($p = 0.0896$).

Site F

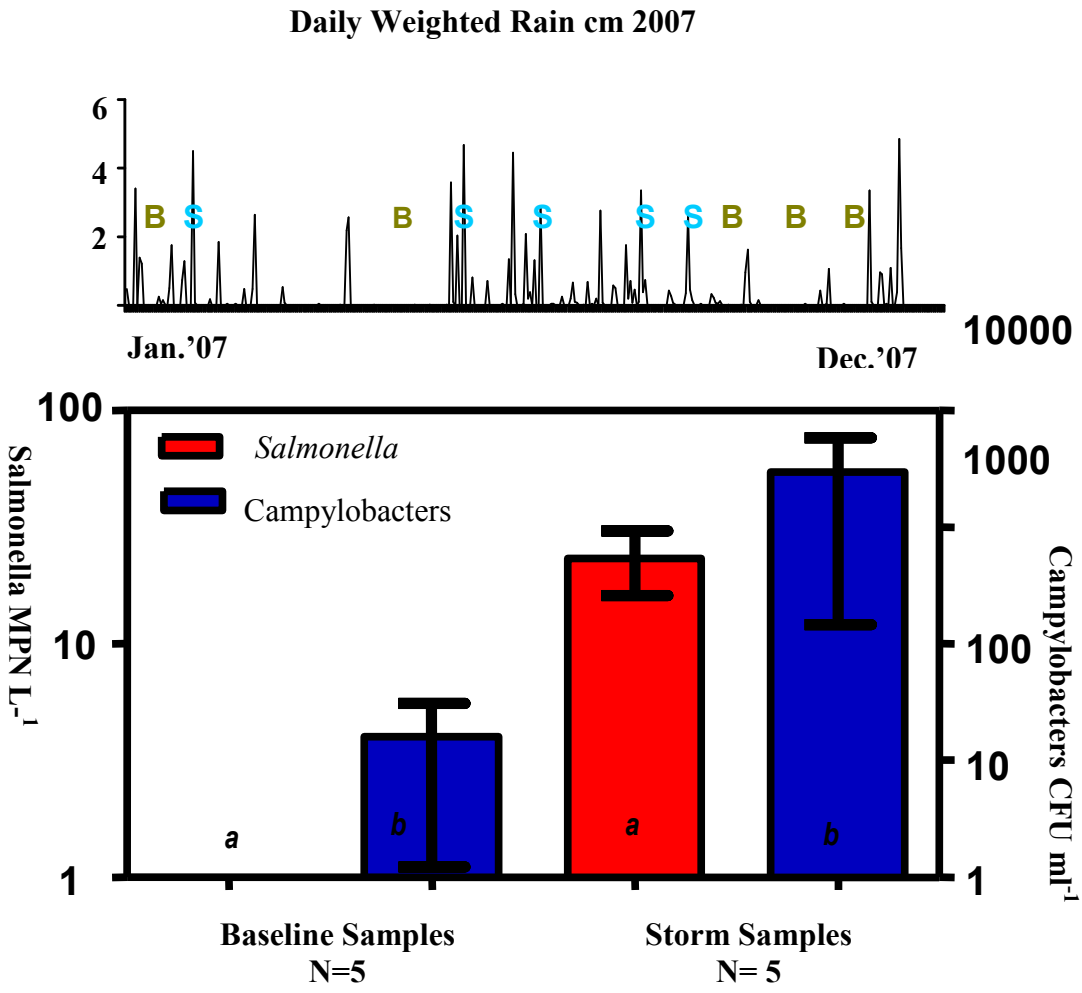

Figure 3.3 Site F Pathogen Levels Baseline vs. Storm Samples
Both *Salmonella* and campylobacter levels were statistically significantly higher following storm events than at baseline ($p < 0.05$). Station F was the most upstream of the sampling sites.

Site 03

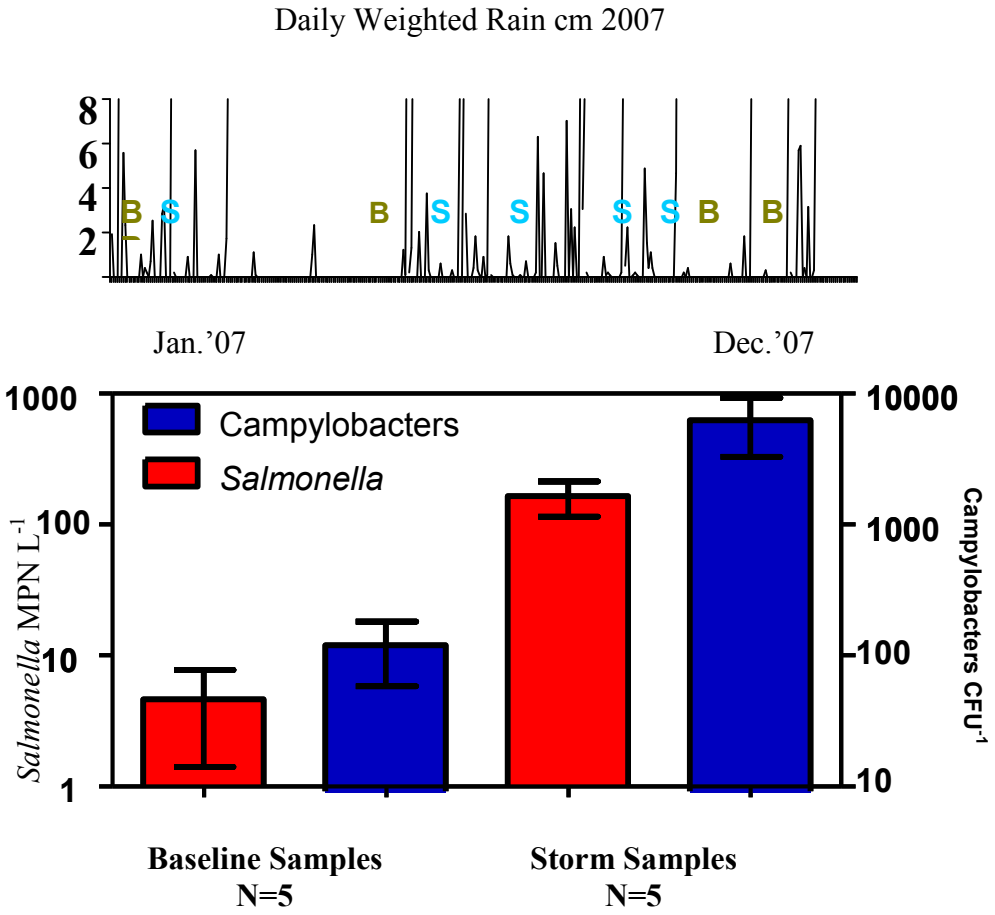

Figure 3.4 Site 03 Pathogen Levels Baseline vs. Storm Samples
a. Salmonella levels were statistically significantly higher than baseline ($p < 0.05$).
b Overall levels of campylobacters were statistically significantly higher in storm samples than Baseline Samples ($p < 0.05$).

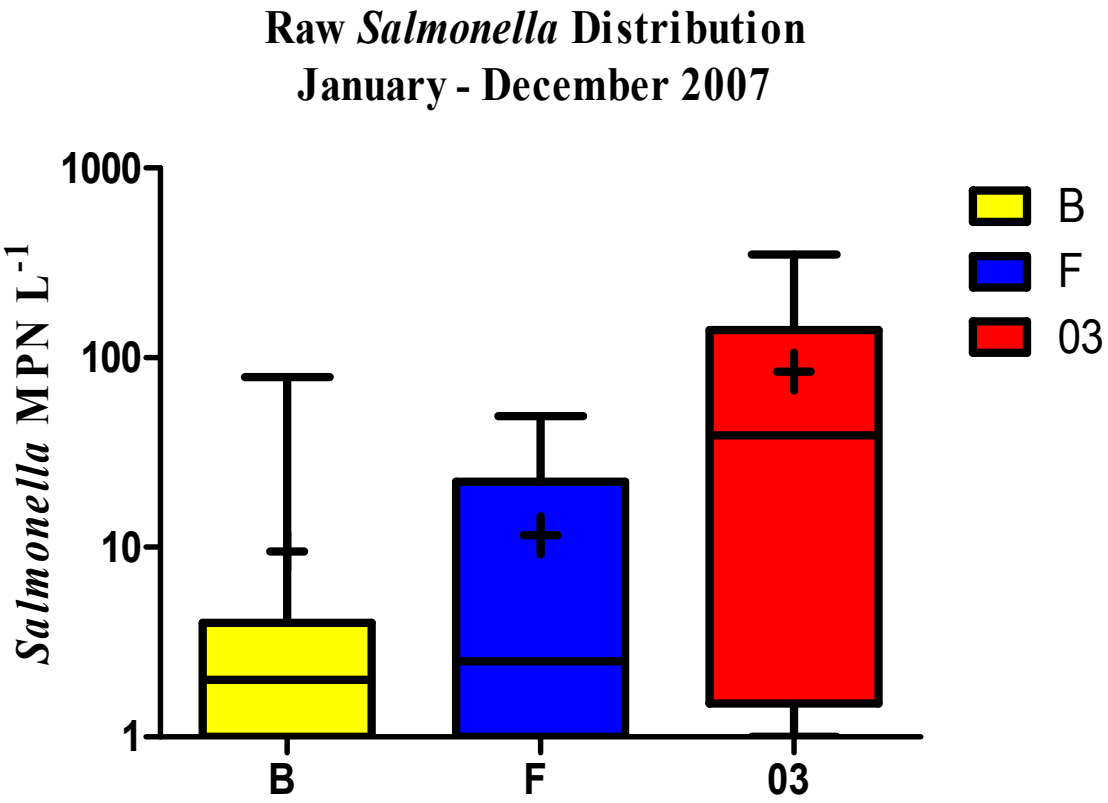

Figure 3.5 Raw *Salmonella* distribution.
(The "+" marks denote the total site mean.)

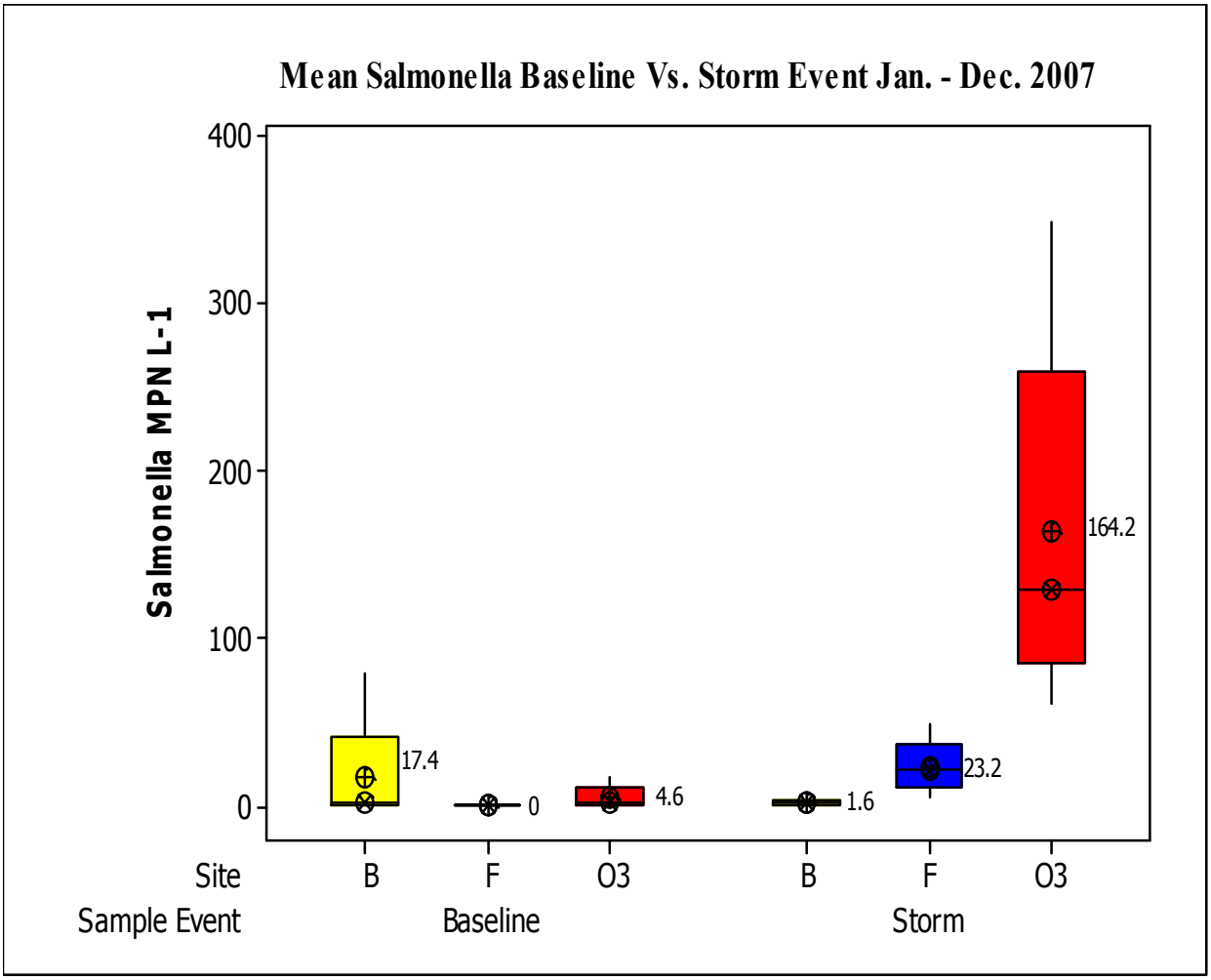

Figure 3.6 Mean *Salmonella* levels Baseline vs. Storm Samples

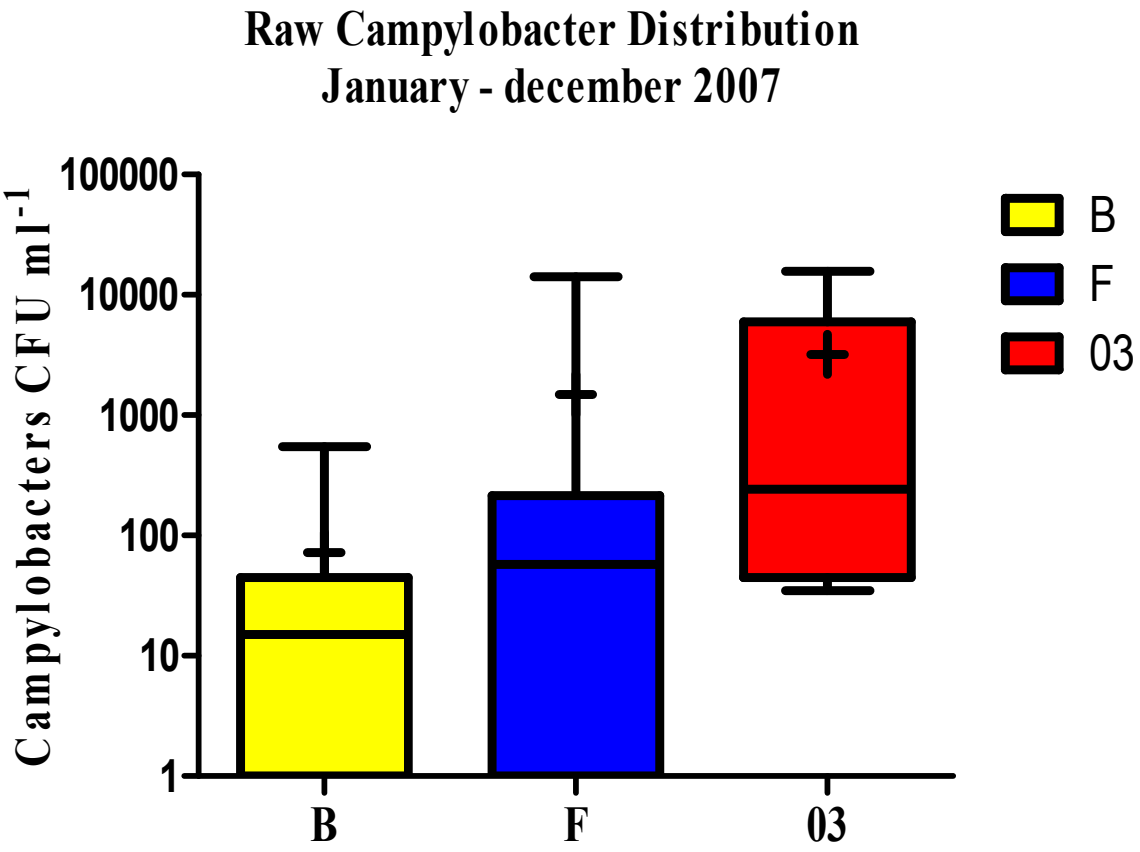

Figure 3.7 Raw campylobacter distribution.
(The "+" marks denote total site mean.)

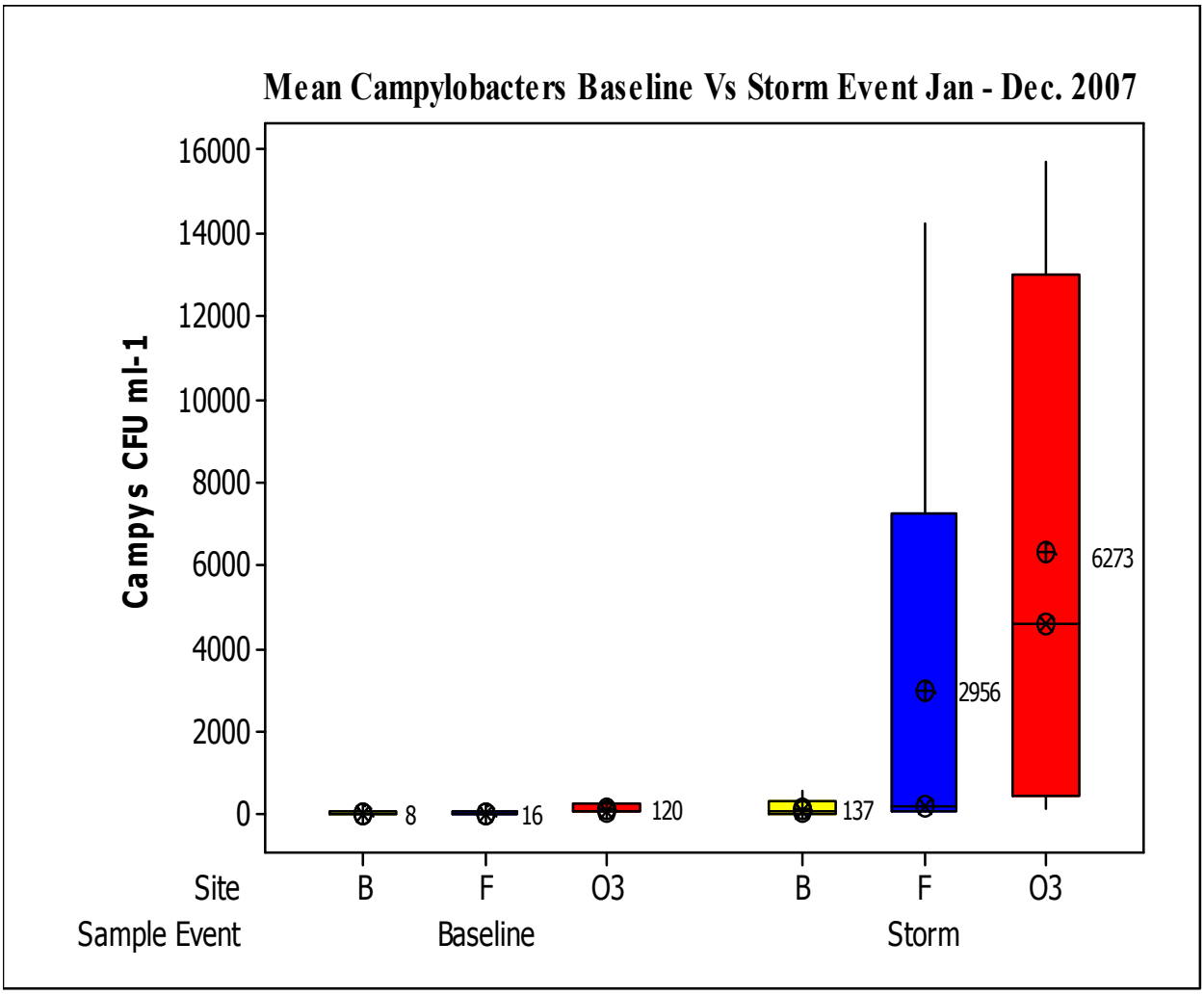

Figure 3.8 Mean campylobacter levels Baseline Vs Storm Samples

Chapter 4

Conclusion

Salmonella and campylobacters are ubiquitous in environmental waters and are leading causes of waterborne disease in humans (WHO 2000; WHO 2005). The results of this study are consistent with others, demonstrating strong positive relationships with microbial loading during storm events, (Islam, Morgan et al. 2004; Kemp, Leatherbarrow et al. 2005; Jensen, Dalsgaard et al. 2006; Haley, Cole et al. 2009). However, this study also demonstrates that rainfall may not be the most significant factor in the fecal contamination of our surface waters due to the high levels observed during both drought conditions, and during at least one baseline sample. Almost contrastingly, this study also was consistent with studies that have found that drought conditions, such as experienced in the study area, also have contributing factors which may lead to potentially increased levels of pathogenic microbes in environmental surface waters, potentially resulting in a human health threat.

The Georgia South Health District 8-1, on the eastern border of the LRW, has consistently reported *Salmonella* infection rates many times that of the state and national average, and the infections are from serotypes not commonly associated with foodborne outbreaks, but with serotypes that are commonly found in the environment (GDPH-NDS 1997 - 2008). In 2007, 6 of the known 33 *Salmonella* serotypes (18.2%) recovered from humans in the Georgia South Health District 8-1 were recovered from environmental samples (*S.* Muenchen, *S.* Bareilly, *S.* Montevideo, *S.* Java, *S.* Rubislaw, and *S.* Typhimurium).

During the course of this study, we observed higher concentrations and serotype variability of *Salmonella* ($p = 0.007$ and $p = 0.003$ respectively) and campylobacters ($p = 0.001$) in storm samples compared against baseline samples. Our findings are also consistent with studies that have found fecal indicator bacteria are not accurate indicators of the presence or absence of other pathogens. The

observations from this study, along with the correlation between serotypes recovered from the environment and from humans, demonstrate the importance of further investigation. Tracing the contamination to a source, which in most cases of non-point source pollution is difficult, would be a logical next step.

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