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Landscape and seasonal factors influence *Salmonella* and *Campylobacter* prevalence in a rural mixed use watershed

Ethell Vereen Jr.[a,b,1], *R. Richard Lowrance*[b], *Michael B. Jenkins*[c], *Paige Adams*[d,†], *Sreekumari Rajeev*[e], *Erin K. Lipp*[f,*]

[a] *University of Georgia, Odum School of Ecology, Athens, GA 30602, USA*
[b] *USDA-Agricultural Research Service, Southeast Watershed Research Unit, Tifton, GA 31793, USA*
[c] *USDA-Agricultural Research Service, National Sedimentation Laboratory, Oxford, MS 38655, USA*
[d] *University of Georgia, College of Agriculture and Environmental Sciences, National Environmentally Sound Production Agriculture Laboratory, Tifton, GA 31793, USA*
[e] *University of Georgia, College of Veterinary Medicine, Veterinary Diagnostic and Investigational Laboratory, Tifton, GA 31793, USA*
[f] *University of Georgia, College of Public Health, Dept. of Environmental Health Science, 206 Environmental Health Science Bldg, Athens, GA 30602, USA*

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ABSTRACT

Salmonella and *Campylobacter* prevalence in stream networks of the Satilla River Basin (SRB) were monitored monthly from August 2007 to August 2009 to study relationships between these pathogens and land use, presence of poultry houses and wastewater treatment plant (WWTP) discharge. *Salmonella* and *Campylobacter* were detected at all 10 stream sites and the three sites at the sole wastewater treatment plant (WWTP) in the study area. In all, 43% (129/299) and 62% (96/156) of samples were positive for *Salmonella* and *Campylobacter*, respectively, with detection frequency increasing in downstream sites with more poultry production and influence of WWTP discharge. Both *Salmonella* and *Campylobacter* detection frequencies were positively associated with the number of poultry houses in the subwatersheds, but agricultural land use as a proportion of the watershed was not a significant predictor of either pathogen. Fecal indicator bacterial levels were assessed and evaluated for their ability to predict the presence of pathogens. Of those examined, enterococci was most predictive; of the 129 samples positive for *Salmonella*, 88% (113/129) were detected when enterococci were above EPA single sample threshold (61 CFU 100 ml^{-1}); and of the 96 samples positive for *Campylobacter*, 90% (86/96) were detected when enterococci levels exceeded this level. Comparatively, *Escherichia coli* concentrations were above EPA single sample thresholds in 38% (49/129) of the positive *Salmonella* samples. Detection of the pathogens throughout the watershed indicated that there was potential for waterborne transmission especially in downstream areas that were more likely to have recreational users.

* *Corresponding author*. Tel.: +1 706 583 8138; fax: +1 706 542 7472.
E-mail address: Elipp@uga.edu (E.K. Lipp).
[1] Current address: Emory University, Rollins School of Public Health, Atlanta, GA 30322, USA.
[†] Deceased.

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1. Introduction

The production of chickens and other domesticated birds for food is a billion dollar industry and is expanding worldwide due to demand for poultry meat ("broilers") and eggs ("layers") (Smith et al., 2010). In the U.S., which is the largest producer globally, Georgia produces more broilers than any other state (USDA/NASS, 2007). There are an estimated 5000 poultry farms in the state, with each producing up to 200 tons of poultry litter (a mix of manure, soiled bedding and spilled feed) each year (Dunkley et al., 2011). In the Coastal Plain region of Georgia, and elsewhere, much of this litter is applied to local pastures and crop land as a soil amendment (Edwards and Daniel, 1992; Endale et al., 2002). However, the practice of spreading livestock manures to land presents an obvious and well-described mode of contamination of nearby surface and ground waters with nutrients and bacteria (Giddens and Barnett, 1980; Jenkins et al., 2008; Mishra et al., 2008; Soupir et al., 2006). If these animal wastes contain zoonotic bacteria such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Escherichia coli* O157:H7 and the protozoan pathogens *Cryptosporidium* and *Giardia*, then transport of pathogens to downstream water resources would increase exposure risks for humans (Atabay and Corry, 1998; Hoar et al., 1999; Hutchison et al., 2004; Mawdsley et al., 1995). Given the high likelihood of poultry manure as a source of *Salmonella* and *Campylobacter*, in particular (Koenraad et al., 1997; Keener et al., 2004; Berghaus et al., 2012), the widespread application of poultry litter to agricultural fields may lead to an increased occurrence of pathogens contaminating surface waters.

Although food is considered to be the typical route of exposure, water is also directly and indirectly associated with *Salmonella* and *Campylobacter* transmission (e.g., Clark et al., 2003; Levantesi et al., 2012; MacRitchie et al., 2013). Both *Salmonella* and *Campylobacter* have been frequently detected from a variety of water sources including coastal waters, well waters and inland streams associated with a variety of contamination sources (Koenraad et al., 1997; Jones, 2001; Horman et al., 2004; Martinez-Urtaza et al., 2004; Vereen et al., 2007; Haley et al., 2009; Rajabi et al., 2011; Viau et al., 2011; Walters et al., 2011). Recent studies have also shown varying influence and association of these pathogens with different land use types (Walters et al., 2011; Wilkes et al., 2011).

The primary objectives of this study were to examine the effect of specific land-use characteristics, density of poultry production, and stream physico-chemical characteristics at the sub-watershed level on the relative distribution of fecal indicator bacteria (FIB), and the enteric bacterial pathogens, *Campylobacter* and *Salmonella*. The secondary objective was to assess the utility of traditional indicator bacteria as proxies for these pathogens among flowing inland waters of the Coastal Plain.

2. Methods and materials

2.1. Watershed and sample site description

The U.S. Geological Survey (USGS) has divided the Satilla River Basin (SRB) into three sub basins or 8-digit Hydrologic Unit Codes (HUCs): Satilla River (HUC 03070201), Little Satilla River (HUC 03070202) and the Cumberland-St. Simons (HUC 03070203). This study was conducted in the upper portions of the Satilla River HUC. The largest city in the study area is Douglas, GA with a population of 11,589 (2010 Census). The micropolitan area, centered on Douglas, which includes Coffee and Atkinson Counties, had an estimated census population of 48,708 in 2008. Douglas is home to a broiler chicken processing plant that delivers wastewater to the municipal wastewater treatment plant (WWTP). The WWTP uses primary and secondary treatment methods to remove pollutants followed by exposure to banks of ultraviolet lights for disinfection. Discharge from the WWTP moves into a small holding pond and then enters the Seventeen Mile River (a major tributary of the Satilla River) through a small stream. The broiler chicken processing plant is supported by over 112 poultry producers and over 440 poultry houses; the processing plant waste contributes to as much as 50% of the flow into the municipal WWTP (personal communication, plant operator). Poultry houses are operated on a variety of farms and given the large amount of crop land in the upper SRB the majority of the poultry litter from the poultry houses is used within the watershed. Most litter is used on pastures or cotton crops, where it is applied in the spring before the crop is planted (B. Bannister, USDA-NRCS personal communication). Crop year data for cotton (2009) were obtained from the USDA Farm Service Agency (FSA) to map the spatial distribution of cotton in the SRB. These data showed that cotton, the main crop receiving poultry litter, was grown in all of the agricultural watersheds.

In addition to poultry, the main animal production in the watershed is cattle (mainly cow/calf operations) and swine. Based on 2007 Census of Agriculture data, the four primary counties in the watershed (Atkinson, Bacon, Coffee, and Ware) had 603 farms with cattle/calves and 40,032 total animals. The same four counties had 64 farms with swine and 10,481 total animals. Three facilities account for over 90% of the estimated swine in the four county area (USDA/National Agricultural Statistics Service, 2007b).

For this study, 13 sampling sites were established (Fig. 1; see also Supplemental Material, Table S.1 for watershed characteristics), including 10 in-stream (sites 1–10) and 3 within the municipal WWTP (sites 11–13). Among the in-stream sites differences among land cover and land use types were determined, using 1998 land use classification data (Georgia GIS clearinghouse) and normalized by the total sub-watershed area for each site to obtain percent agricultural, forest, urban, and other (includes open water, transportation, utility, clear-cut/sparse vegetation and golf course) land use (see Table S1). The number of poultry houses and the number of poultry producers within each sub-watershed of each site was provided by the broiler chicken processing plant. Seven sites were located upstream of the WWTP discharge point and 3 were located downstream.

2.2. Physico-chemical water quality measurements

From August 2007 to August 2009 monthly samples were collected from all sites. No sampling was conducted during

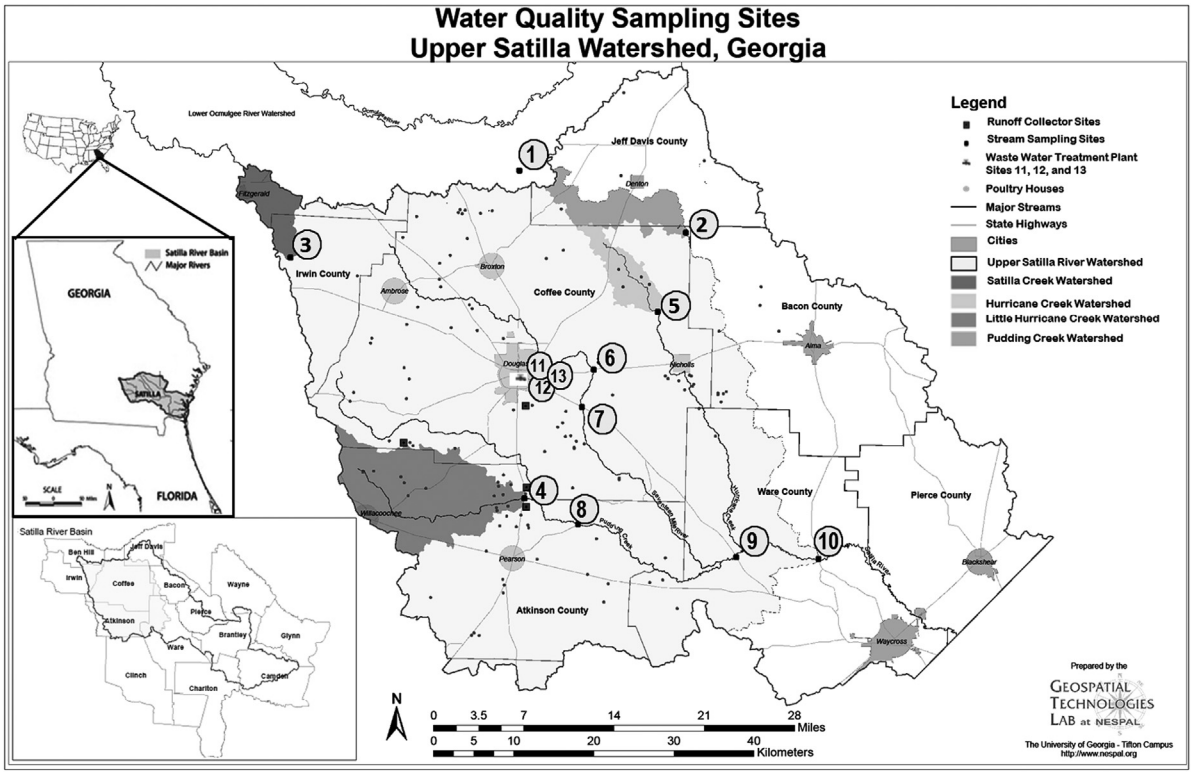

Fig. 1 – Map of sample sites in the Satilla River Basin (numbered). Small gray circles indicate poultry production farms (multiple houses per farm) within the watershed(s).

November of each year due to low flow conditions. Physico-chemical water quality measurements were taken *in situ* with a YSI® 6600 Multiparameter Sonde (Yellow Springs, OH, USA): temperature (°C, YSI probe 6560), conductivity (mS cm^{-1}, YSI probe 6560), pH (YSI probe 6565), dissolved oxygen (DO, mg L^{-1}, YSI probe 6562), turbidity (nephlometric turbidity units, NTU, YSI probe 6136), oxidation reduction potential (ORP, mV, YSI probe 6565) and chlorophyll *a* (mg L^{-1}, YSI probe 6025). All probes were calibrated by a YSI trained technician using recommended techniques before each sampling. The probe was placed at a depth of about 30 cm in the deepest part of the stream channel and used to record instantaneous values after stabilization.

2.3. *Rainfall and streamflow*

The Satilla River (HUC 03070201) has a calibrated USGS continuous streamflow (discharge) monitoring station at the Satilla River, near Waycross, GA. This stream gauge is approximately 13 km downstream from sampling site 10 and the watershed above site 10 encompasses about 91% of the watershed area defined at the USGS gauge. Using discharge measured (m^3 s^{-1}) at this gauge, we calculated the predicted area proportional discharge for all of the water quality monitoring sites as [(drainage area of sample site)/(drainage area of USGS gauge at Waycross, GA)] * (measured discharge at USGS gauge at Waycross, GA). In order to examine the potential impact of discharge we calculated the daily flow (cm day^{-1}), total daily flow 30 days preceding the sampling and average total daily flow 30 days preceding the sampling.

Basin-wide rainfall was estimated using data retrieved from a National Climatic Data Center (NCDC) weather station in Douglas, Georgia (Cooperative Station Identification # 092783) (www.georgiaweather.net). In order to examine the potential impact of rainfall on other study variables, several measures of precipitation were compiled for analyses: daily rainfall, rainfall on the day preceding the sampling, total rainfall in the 7 days before the day sampled and total daily rainfall 30 days preceding the sampling.

2.4. *Sample collection*

All water samples were collected in sterile 3-l polypropylene bottles as discrete surface grabs and transported on ice to the University of Georgia National Environmentally Sound Production Agriculture Laboratory (NESPAL). Sample processing began immediately upon return to the lab.

2.5. *Fecal indicator bacteria*

Concentrations of *E. coli* and enterococci were determined with the Colilert and Enterolert Quanti-Tray systems, using the IDEXX most probable number (MPN) estimation (IDEXX Laboratories, Inc., Westbrook, ME, USA). Trays were inoculated and incubated for 24 h at 35 °C and 41 °C, for *E. coli* and enterococci, respectively, according to the manufacturer's guidelines. Water samples were screened for fecal coliform

bacteria by membrane filtration and growth on mFC agar following Standard Methods (Greenberg et al., 1992). Plates were placed in sealed containers and incubated in a water bath for 24 ± 2 h at 44.5 °C. All blue colonies were counted as fecal coliform bacteria and enumerated as colony forming units (CFU) per 100 ml.

Streams in the SRB study area are classified as designated recreational waters (GAEPD, 2004). Individual samples were scored as exceeding recreational water quality standards if fecal coliform bacteria concentrations were greater than 400 CFU 100 ml^{-1} (GAEPD, 2004), enterococci concentrations were greater than 61 MPN 100 ml^{-1} or *E. coli* were greater than 235 MPN 100 ml^{-1}, as recommended by the US EPA for freshwater (USEPA, 1986, 2006, 2002).

2.6. Salmonella *and* Campylobacter

A 1 L sample was mixed by gentle inversion several times; 250 ml of the sample were then filtered through a 0.45 μm pore size 47 mm diameter nitrocellulose membrane. The filters were aseptically cut in half and placed into 25 ml of buffered peptone water (BPW, 0.1% peptone) and incubated at 37 °C for 24 ± 2 h for non-selective pre-enrichment. One hundred microliters of the BPW pre-enrichment was then transferred to 10 ml of Rappaport-Vassiliadis (RV) broth for selective enrichment at 42 °C for 24 ± 2 h. This was followed by streaking from RV broth onto xylose lysine tergitol (XLT) agar and incubating plates at 37 °C for 24–48 h. Following incubation, 3–5 presumptive *Salmonella* colonies per XLT plate were picked and saved (identified as black colonies). Suspect *Salmonella* colonies were confirmed by O antigen screening and the Sensititre Microplate System (AP 80; Trek Diagnostics, Westlake, OH, USA). Isolates confirmed as *Salmonella* were preserved on ceramic beads (CryoBank beads, Copan Diagnostics Inc., Murrieta, CA) in tryptone soy broth with 15% glycerol (final concentration) in a −80 °C freezer.

Campylobacter screening began in year two of the study. Briefly, an additional 250 ml of collected sample was filtered through a second 0.45 μm pore size 47 mm diameter nitrocellulose membrane. The membranes were aseptically cut in half and placed into 10 ml of *Campylobacter* enrichment broth (CEB, Remel, Thermo Fisher Scientific, Lenexa, KS, USA) and incubated at 42 °C for 48 ± 2 h. A 1.5 ml aliquot of each CEB enrichment was used for DNA extraction (MoBio Power Soil DNA Extraction kit, MOBIO Laboratories, Carlsbad, CA, USA). DNA was subjected to PCR targeted at the 23S rRNA gene of *Campylobacter*, allowing for the detection of the four thermotolerant *Campylobacter* species (*Campylobacter jejuni subsp. jejuni, C. coli, C. lari,* and *C. upsaliensis*) by using one primer pair: THERM 1 (5′-TATTCCAATACCAACATTAGT-3′) and THERM 2 (5′-CGGTACGGGCAACATTAG-3′) (Eyers et al., 1993, 1994; Savill et al., 2001). All four thermotolerant *Campylobacter* species yield a 222 bp product when amplified with this primer set.

PCR was performed with a Bio-Rad® thermal cycler (PTC-200, Waltham, MA, USA). The amplification consisted of an initial DNA denaturing step at 95 °C for 1 min 30 s, followed by a 40-cycle reaction (PCR denaturing at 95 °C for 1 min 30 s, PCR annealing at 50 °C for 30 s, and PCR extension at 72 °C for 1 min). The cycling included a final extension step at 72 °C for 5 min to ensure full extension of the product. PCR products were analyzed by electrophoresis at 110 V for 1 h through 1% (w/v) agarose gel. Products were visualized by staining with ethidium bromide under UV light and a 100-bp DNA ladder was used as a molecular weight marker.

2.7. Statistical analyses

Data were analyzed using Statistical Analysis System (SAS) release 9.1 (Cary, NC, USA). Both continuous and binary data were obtained during this study. Among continuous data, FIB levels were not normally distributed (and transformation steps did not result in Gaussian distributions); therefore, the Kruskal–Wallis test (the nonparametric equivalent of a one-way ANOVA) using the NPAR1WAY Procedure of SAS was used to test for significant differences among the measured parameters between sampling sites and dates. Dunn's test was used as a post-hoc test for nonparametric pair-wise comparison. The Spearman's rank correlation coefficient (r_s) was used to determine measures of association of FIB with physical and chemical parameters of the water and environmental variables (e.g., rainfall). When levels were below the limit of detection a value of zero was used for statistical analysis. Only those environmental parameters that showed significant differences or relationships of at least the 0.05 level were reported (p values $\leq$0.05).

Salmonella and *Campylobacter* were evaluated by their frequency of detection (binary variables). Contingency tables were used to evaluate differences in prevalence (i.e., detection frequency). Briefly, multiple comparisons test for proportions was carried out in SAS using PROCFREQ followed by the COMPPROP macro to evaluate pairwise differences (Elliott and Reisch, 2006). Comparisons were made by site and time. Months were pooled into seasons for each sample year, which were defined as Spring (March, April, May), Summer (June, July, August), Fall (September, October, November) and Winter (December, January, February).

Generalized estimating equations (GEE; Hardin and Hilbe, 2008) were used to evaluate the associations of land use, poultry production and other environmental parameters with *Salmonella* and *Campylobacter* detection frequency at the 10 in-stream sites (WWTP sites were excluded from this analysis because detection frequency would be unrelated to these watershed influences). The GEE approach allows for repeated measures (e.g., by sampling site) and has been used in similar studies of pathogen prevalence (e.g., Walters et al., 2011). The tests were carried out using the GENMOD procedure in SAS. Site was held as the repeated class and the model assumed an exchangeable correlation structure. For all measures of association, p values $\leq$0.05 were considered significant. Only those environmental parameters and FIB that showed significant relationships with either *Salmonella* or *Campylobacter* were reported.

3. Results

3.1. Spatial and temporal distribution of fecal indicator bacteria

Fecal indicator bacteria were detected at all stations. At in-stream stations (sites 1–10), *E. coli* were detected at levels up

to 17,329 MPN 100 ml^{-1} with 11 samples where none were observed (<5 MPN 100 ml^{-1}). The mean concentration was 317 MPN 100 ml^{-1} with a median level of 119 MPN 100 ml^{-1}. Enterococci ranged from not detected (<5 MPN 100 ml^{-1}) to 17,329 MPN 100 ml^{-1} and averaged 580 MPN 100 ml^{-1}, with a median value of 193 MPN 100 ml^{-1}. Fecal coliform bacteria concentrations varied between not detectable (<5 CFU 100 ml^{-1}) and 3500 CFU 100 ml^{-1}, with a mean concentration of 229 CFU 100 ml^{-1} and a median concentration of 109 CFU 100 ml^{-1}. Among the 10 in-stream sites, median fecal coliform concentrations did not differ significantly, but median concentrations of enterococci and *E. coli* did show significant differences ($H = 21.47$, 9 d.f., $p < 0.05$ and $H = 18.89$, 9 d.f., $p < 0.05$ for enterococci and *E. coli*, respectively). Median enterococci levels ranged between a low of 72 MPN 100 ml^{-1} at site 1 (the reference site) and a high of 457 MPN 100 ml^{-1} at site 7 (immediately downstream of the WWTP discharge point). *E. coli* levels ranged from a median of 58 MPN 100 ml^{-1} at site 10 (the most downstream station in the study) to 1253 MPN 100 ml^{-1} at site 3 (an upstream station with no poultry houses).

When examined over the 2 year study period, the median levels of all three of the FIB differed by collection time ($H = 77.30$, 22 d.f., $p < 0.05$; $H = 71.36$, 22 d.f., $p < 0.05$ and $H = 64.56$, 22 d.f., $p < 0.05$, for *E. coli*, enterococci and fecal coliform bacteria, respectively). The greatest median *E. coli* level was recorded in December 2008 at 688 MPN 100 ml^{-1} while the lowest median level was 21 MPN 100 ml^{-1} (July 2009). Median enterococci levels reach their highest in June 2009 (551 MPN 100 ml^{-1}) and their lowest in March 2008 (20 MPN 100 ml^{-1}). Similar to *E. coli*, the highest median fecal coliform level was 650 MPN 100 ml^{-1} in December 2008 and lowest was 33 MPN 100 ml^{-1} in July 2009. On a seasonal basis, *E. coli* median levels ranged from a high of 192 MPN 100 ml^{-1} in the fall of 2008 to a low of 62 MPN 100 ml^{-1} in the summer of 2009. Median enterococci levels were observed between 467 MPN 100 ml^{-1} in the summer of 2007 and 85 MPN 100 ml^{-1} in the spring of 2008; and fecal coliform bacteria ranged from 200 CFU 100 ml^{-1} in the fall of 2007 to 76 CFU 100 ml^{-1} in the spring of 2008.

Each of the FIB were detected above their respective single sample threshold values at least once from all sites sampled. The percentage of samples above the water quality guidelines ranged from 28% (83 out of 299) for fecal coliform bacteria to 74% (221 out of 299) of the enterococci samples. *E. coli* was detected above the standard in 34% (103 out of 299) of the samples.

3.2. *Spatial and temporal distribution of* Salmonella *and* Campylobacter

Salmonella and *Campylobacter* were detected at all 13 sites monitored in the SRB. Overall, 43% (129 of 299) of all samples were positive for *Salmonella* and 62% (96 of 156) of all samples were positive for *Campylobacter*. In general, prevalence increased downstream of the WWTP discharge point and at sites with more agricultural land use and poultry production (Table 1; see also Supplemental Results, Figure S.1 and 2). Among the 10 in-stream sites, *Salmonella* ranged from 17% positive (4/23) at site 2 (upstream of WWTP with no poultry production) to 61% positive (14/23) at sites 7 and 8 (both downstream of the WWTP discharge and with more agricultural land use and poultry production) ($x^2 = 17.23$, 9 d.f., $p < 0.05$). However, post hoc multiple comparisons were not able to discern statistically significant differences among specific stations. *Campylobacter* detection ranged from 50% positive (6/12) well upstream of the WWTP discharge (site 2) to 75% positive (9/12) at site 6 (immediately upstream of the WWTP). There was no statistically significant difference in detection frequency.

At the WWTP, *Salmonella* was positive in 43% (10/23) of the samples from the influent (site 11), declined to 35% (8/23) in samples collected directly from the effluent discharge (site 12) but increased to 43% positive in the discharge holding pond (site 13). *Campylobacter* was positive in 75% (9/12) of the

Table 1 – Watershed description and prevalence of *Salmonella* and *Campylobacter* by sample site[a]. (Sites 1–6 are upstream of the wastewater treatment [WWTP] discharge point; sites 7–10 are downstream of the WWTP discharge point).

Site	Number of poultry producers	Number of poultry houses	Agricultural land use[b]	% + *Salmonella* N = 23 (# of positives)	% + *Campylobacter* N = 12 (# of positives)
1	0	0	5	35% (8)	58% (7)
2	0	0	22	17% (4)	50% (6)
3	0	0	52	26% (6)	58% (7)
4	12	50	30	57% (13)	58% (7)
5	4	21	20	39% (9)	67% (8)
6	22	98	33	39% (9)	75% (9)
7	22	98	32	61% (14)	67% (8)
8	42	173	32	61% (14)	58% (7)
9	91	368	25	57% (13)	67% (8)
10	112	440	23	48% (11)	67% (8)
11	NA[c]	NA[c]	NA[c]	43% (10)	75% (9)
12	NA[c]	NA[c]	NA[c]	35% (8)	50% (6)
13	0[c]	0[c]	23[c]	43% (10)	50% (6)

a Land cover is based on 1998 land use classification (Georgia GIS Clearinghouse).
b Includes row-crops and pasture land.
c Not applicable; WWTP (influent [site 11], direct effluent [site 12] and effluent holding pond [site 13]).

influent samples (site 11) and declined to 50% (6/12) of the samples from both the effluent discharge (site 12) and the effluent holding pond (site 13). Differences in frequency of detection between the WWTP sites were not significant for either *Salmonella* or *Campylobacter*.

Salmonella prevalence over the two year study ranged from non-detectable (0/13) in April 2009 to 85% (11/13) positive in December 2008 (Fig. 2) ($x^2 = 78.63$, 22 d.f., $p < 0.05$). However, seasonally pooled detection frequency was greatest in the summer of 2009 (69% positive) and winter of 2008 (67% positive), with statistically lower detection frequencies in the summer of 2007 (15% positive) (p values < 0.05). *Campylobacter* were detected in all study months during the one year of collection and ranged from a low of 8% (1/13) positive in August 2009 to 100% (13/13) positive in March 2009 (Fig. 2; $x^2 = 89.10$, 11 d.f., $p < 0.05$). Seasonally, detection frequency was greatest in the spring of 2009 (95% positive) and winter 2008 (87% positive), with statistically lower detection frequencies in the summers of 2008 (23% positive) and 2009 (21% positive) (p values < 0.05).

3.3. *Association of fecal indicator bacteria with* Salmonella *and* Campylobacter *prevalence*

Using the GEE approach to account for repeated measures, increasing enterococci concentrations were significantly, but modestly, associated with higher *Salmonella* prevalence ($\beta = 0.0002$, $p < 0.05$). Neither fecal coliform bacteria nor *E. coli* had any significant association with the detection of *Salmonella*. Additionally, Of the 129 samples positive for *Salmonella*, 88% (113/129) were detected when enterococci were above EPA thresholds (61 CFU 100 ml^{-1} for designated water bodies with whole body contact for recreation (USEPA, 1986, 2002, 2006) and only 12% (16/129) were detected when enterococci were below this level (Table 2). *E. coli* and fecal coliform bacteria were less predictive. Sixty-two percent (80/129) of *Salmonella* positive samples were detected when *E. coli* levels were within recommended guidelines (235 CFU 100 ml^{-1} (USEPA, 1986, 2002, 2006); and 67% (87/129) of positive samples were found when fecal coliform bacteria levels were within suggested limits for recreational water (Table 2; GAEPD, 2004).

None of the FIB were significantly associated with *Campylobacter* detection when using the GEE approach. When compared to FIB thresholds, of the 96 samples positive for *Campylobacter*, 90% (86/96) were detected when enterococci levels were above EPA thresholds and only 10% (10/96) of *Campylobacter* were detected when enterococci were below the EPA standard (Table 2). As with *Salmonella* detection, *E. coli* and fecal coliform bacteria were less predictive for *Campylobacter*. Fifty-nine percent (57/96) of the time when *Campylobacter* was detected, *E. coli* levels were within EPA recommendations and 73% (70/96) of *Campylobacter* positive samples were detected when fecal coliform bacteria were within suggested limits for Georgia recreational water quality standards (GAEPD, 2004).

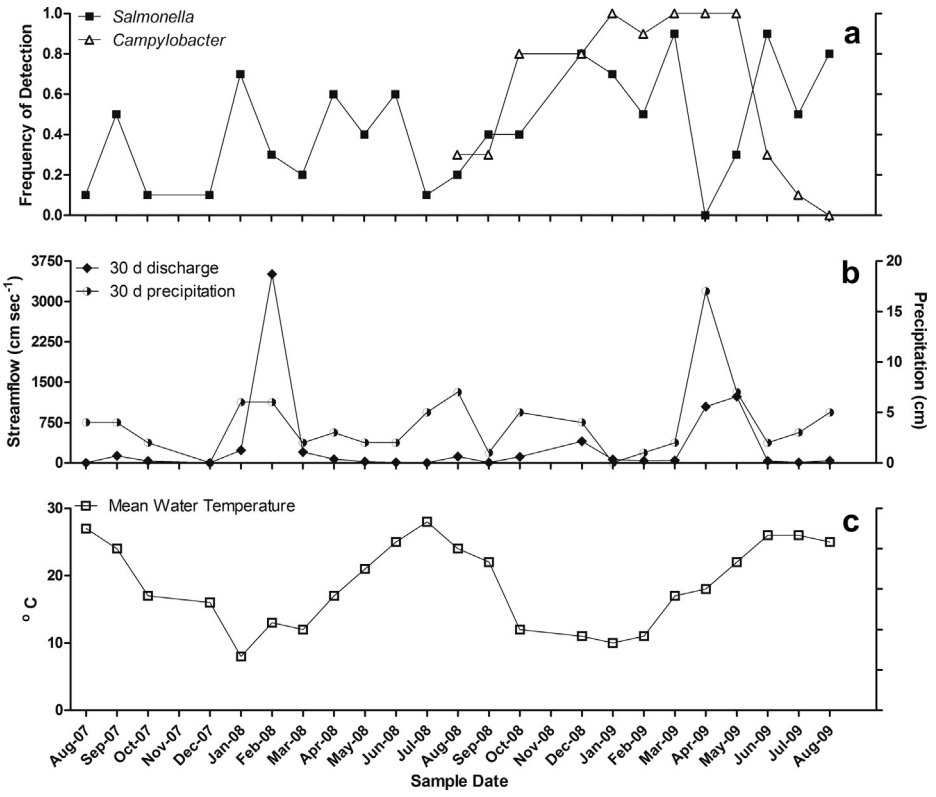

Fig. 2 – *Salmonella* and *Campylobacter* prevalence by month (in-stream stations) (*Campylobacter* surveillance only occurred in year two of the study) (a); mean 30 day antecedent rainfall (cm) and mean 30 day antecedent discharge (cm day^{-1}) (b); and mean water temperature (c).

Table 2 – Frequency (%) of *Salmonella* and *Campylobacter* detection when fecal indicator bacteria were above and below federal[a] and state[b] single sample threshold values for recreational water.

Indicator organism	Single sample maximum (CFU 100 ml^{-1})	*Salmonella* (N = 129) detection		*Campylobacter* (N = 96) detection	
		Above	Below	Above	Below
		Standard	Standard	Standard	Standard
E. coli[a]	235	49 (38%)	80 (62%)	39 (41%)	57 (59%)
Enterococci[a]	61	113 (88%)	16 (12%)	86 (90%)	10 (10%)
Fecal coliform bacteria[b]	400	42 (33%)	87 (67%)	26 (27%)	70 (73%)

a USEPA, 2006.
b GAEPD, 2004.

3.4. *Influence of land use and poultry production on* Salmonella *and* Campylobacter *prevalence*

GEE models were used to determine the effect of land use (percent coverage in each sub-watershed) and poultry production on *Salmonella* and *Campylobacter* detection frequencies. Significant collinearity was observed among all of the land use categories (agricultural, forested, urban and other); therefore, to avoid confounding with highly correlated independent variables, percent agriculture alone was used to represent all land use in these analyses. Additionally, poultry production and number of poultry houses were also highly correlated. Number of poultry houses by sub-watershed was used to represent this influence.

Both *Salmonella* and *Campylobacter* detection frequencies were positively, but moderately associated with the number of poultry houses ($\beta = 0.002$, $p < 0.05$ and $\beta = 0.008$, $p < 0.05$, respectively) in the subwatersheds. However, agricultural land use as a proportion of the watershed was not a significant predictor of either *Salmonella* or *Campylobacter*.

3.5. *Influence of physico-chemical measures, rainfall and streamflow on* Salmonella *and* Campylobacter *prevalence*

Using the same GEE approach, *Salmonella* detection frequency was inversely related to the total rainfall in the 30 days preceding sample collection ($\beta = -0.147$, $p < 0.05$). Other measures of rainfall were not predictive, nor were water temperature or streamflow (discharge rates). For *Campylobacter*, detection frequency was inversely related to water temperature ($\beta = -0.242$, $p < 0.05$) and directly associated with total rainfall in the 30 days preceding sample collection ($\beta = 0.089$, $p < 0.05$) and streamflow (discharge rate) ($\beta = 0.013$, $p < 0.05$). Additionally, *Campylobacter* prevalence also increased with DO ($\beta = 0.258$, $p < 0.05$).

Multivariate models were developed for *Salmonella* and *Campylobacter* using those independent variables that were significant in bivariate analyses ($p < 0.05$). Enterococci concentrations ($\beta = 0.0003$, $p < 0.05$) and total daily rainfall in the 30 days preceding the sampling ($\beta = -0.214$, $p < 0.05$) were both retained as significant predictors in the model for *Salmonella* prevalence while the number of poultry houses was no longer significant. Only streamflow (discharge) ($\beta = 0.0001$, $p < 0.05$), temperature ($\beta = -0.274$, $p < 0.05$), and DO ($\beta = 0.398$, $p < 0.05$) were retained in the *Campylobacter* model.

4. Discussion

In mixed-use watersheds, fecal contamination can be of livestock, human, or wildlife origin. Livestock wastes can harbor both bacterial and protozoan pathogens; and, surface and groundwater contamination has been, but is not always, linked to livestock operations and manure spreading (Mawdsley et al., 1995; Hutchison et al., 2004; Rodriguez and Araujo, 2010; Sigua et al., 2010; McAllister and Topp, 2012). In this study *Salmonella* and *Campylobacter* were frequently detected from a majority of sites influenced by varying degrees of agriculture and, in particular, poultry production. The frequency of *Salmonella* detection among the in stream sites from agricultural watersheds with poultry houses (sites 4 and 5) was more than twice that of agricultural watersheds without poultry houses (sites 2 and 3). The prevalence of both *Salmonella* and *Campylobacter* was significantly predicted by the number of poultry houses in the watershed rather than the extent of land classified as agricultural. Additionally, downstream stations that also received inputs from the WWTP (including a significant contribution from poultry processing waste) were also more often positive for these pathogens. These stations also received drainage from both upstream sources and larger subwatersheds, which may also contribute waste (and pathogens) from non-poultry domestic animals and wild animals, which were not accounted for in this study (Abulreesh et al., 2006; Coburn et al., 2007; Benskin et al., 2009; Castillo et al., 2008).

While additional work in this system should begin to monitor the prevalence of *Salmonella* and *Campylobacter* in poultry litter at the time of land application and the potential stream loading from these sources, *Salmonella* detection from areas within and around the poultry house environment has been frequently reported (Craven et al., 2000; Roy et al., 2002; Volkova et al., 2009; Alali et al., 2010; Berghaus et al., 2013). Poultry production is a distinct component of agricultural activity; however, in this system, the percentage of land use devoted to agriculture in general was not a significant predictor of *Salmonella* or *Campylobacter*. Although others have demonstrated that agricultural pressures are highly associated with loading of *Salmonella* and other enteric bacteria (Rodriguez and Araujo, 2010; Sigua et al., 2010), Walters et al. (2011) found that urban sources were significantly correlated with *Salmonella* in coastal California.

Effluent from the WWTP was also a likely source of contamination in the Satilla River. The WWTP has a permitted discharge of 22.71 million liters per day (MLD), of which about

50% was provided by wastewater from the broiler processing plant (plant operator, personal communication). The discharge from the WWTP enters a small stream that flows into a pond behind the plant and then to Seventeen Mile River, a major tributary of the Satilla River. Although, we did not determine specific concentrations, 35% (8/23) of the water samples were positive for *Salmonella* from the direct effluent from the WWTP and increased to 43% positive where the effluent discharged to the holding pond and increased again to 61% (14/23) at site 7, the site immediately downstream of the discharge point to Seventeen Mile River. Similarly, *Campylobacter* was detected in 50% (6/12) of the samples from direct effluent and the effluent pond but increased to a prevalence of 67% at the discharge to the stream (site 7). *Salmonella* and *Campylobacter* have frequently been found in poultry wastewater (Koenraad et al., 1997; Rodriguez and Araujo, 2010) and sewage discharge (Jones, 2001; Moreno et al., 2003; Rodriguez and Araujo, 2010; Simental and Martinez-Urtaza, 2008; Vereen et al., 2007); however, the increasing prevalence moving away from the immediate effluent suggest that there could be some reactivation of these pathogens following disinfection and discharge to the environment (Oliver et al., 2005; Guo et al., 2011) and potential accumulation from local sources (e.g., raccoons, birds or other animals that may visit these water features and possibly carry these pathogens).

Salmonella and *Campylobacter* infections among humans generally peak in summer months but environmental studies often show varied seasonal peaks for these pathogens, with some suggesting higher prevalence in summer months (e.g., Vereen et al., 2007; Haley et al., 2009) and others in winter months (e.g., Obiri-Danso and Jones, 1999; Simental and Martinez-Urtaza, 2008). These discrepancies may be due to regional differences in seasonal influences including both temperature and rainfall patterns (Simental and Martinez-Urtaza, 2008; Haley et al., 2009; Walters et al., 2011). In the current study, *Salmonella* and *Campylobacter* detection was more variable with regards to seasonal prevalence. *Salmonella* prevalence peaked in the winter of 2008 and summer of 2009 and was highly variable across months. *Campylobacter* prevalence peaked in the winter of 2008 and spring of 2009; however, *Campylobacter* detection remained above 80% between October of 2008 and May of 2009, with much lower prevalence in the summer months, reflecting an inverse association with temperatures. GEE models also suggested that monthly rainfall was inversely related to *Salmonella* detection and directly related to *Campylobacter* detection. The study region experienced both significant drought conditions (e.g., summer and fall of 2007) and intense rain during the collection period. In April 2009, coincident with our collections, a series of extreme rainfall events produced the highest 30-d antecedent rainfall (42.67 cm, 22-April 2009) during our sampling period. This rainfall subsequently resulted in the largest daily streamflow event (1192.14 m^3 sec^{-1}; 05-April 2009) recorded in the entire record period (1939-present) on the Satilla River (USGS, 2013). Despite a 90% prevalence in the preceding month (March 2009), in April 2009, following this rain and flow event, *Salmonella* could not be detected from any of our sample sites, suggesting that dilution effects from such a large event may have masked possible dispersal from run-off. Had samples been captured from the full hydrograph, it may have revealed an initial peak in *Salmonella* (i.e., first flush) followed later by dilution; however, our samples were collected well after the first flush. Interestingly, *Campylobacter* was detected at 92% of the stations in April 2009, suggesting that despite similar sources, in-stream loading or response may vary between these two pathogens. Periodic drought, such as experienced in the summers of 2007 and 2008, which led to no-flow periods in many of the streams studied, could also help to explain the infrequent detection of both *Salmonella* and *Campylobacter* during these periods. Overall, variable rainfall intensity, including sporadic and heavy rainfall events interrupted by periods of no-flow within the stream system may have contributed to detection patterns that were anomalous to other studies and compared to periods with typical seasonal rainfall and streamflow patterns.

Risk of transmission of *Salmonella* and *Campylobacter* to humans may occur through contact with surface waters in this area. These stations are frequently visited year-round for outdoor leisure activities such as fishing, hunting, canoeing/kayaking, picnicking and other forms of nature-based recreation (Brown, 2009). To protect public health, designated recreational waters, such as these, are tested for compliance with microbial water quality standards based on FIB (e.g., fecal coliform bacteria, *E. coli* and enterococci). These bacteria are used as a proxy for human health risks (i.e., enteric illnesses) associated with exposure to fecal contaminants; however, for a variety of reasons FIB may not always be effective surrogates for the presence of bacterial pathogens, including *Salmonella* and *Campylobacter*. In the present study, *E. coli* and fecal coliforms were less predictive than enterococci for the presence of *Salmonella* and *Campylobacter*. Additionally, *Salmonella* and *Campylobacter* were much more likely to be detected in these waters when single sample threshold levels for enterococci were exceeded. Data from this study suggest that enterococci may be the preferred FIB for flowing streams in this Coastal Plain region; this is an area where additional research is warranted.

Recently, litter utilization surveys distributed at UGA Cooperative Extension meetings held for crop production in 64 counties across South Georgia also revealed that 50% ($N = 165$) of crop producers surveyed in South Georgia use poultry litter (Dunkley et al., 2011), indicating opportunities exist for more application, with more litter being applied to land on which cotton or other row crops were grown. Given the increasing likelihood of both drought and high intensity rain events with climate change in the southeast (Seager et al., 2009; Wang et al., 2010), additional work should investigate the effects of variable rainfall intensity and extreme storm events on the occurrence of zoonotic pathogens in these Coastal Plain streams arising from litter application in the watersheds.

5. Conclusions

While *Salmonella* and *Campylobacter* are typically thought to be primarily foodborne pathogens, increasing evidence suggests that environmental exposure, including exposure to both drinking and surface waters, may be an underappreciated source of non-outbreak associated illness. Both pathogens were detected throughout the Satilla River Watershed indicating the potential for transmission to humans. Detection

levels of both pathogens were positively associated with the number of poultry houses in the watershed. In addition, *Campylobacter* detection was positively associated with stream discharge, dissolved oxygen concentration, and the amount of rainfall 30 days prior to sampling, while *Salmonella* detection was negatively associated with the amount of rainfall 30 days prior to sampling. No association with percent agricultural land use was observed for either pathogen. Given the opposite patterns of association between rainfall patterns for *Salmonella* and *Campylobacter* dispersion to streams, as well as the differences in pathogen survival under low dissolved oxygen and high temperature conditions, more research is needed to develop a robust model to predict the risk of non-outbreak associated illness. While fecal coliform bacteria and *E. coli* were found to be poor proxies for the presence of *Salmonella* and *Campylobacter*, enterococci may be a better candidate for monitoring of water quality in these inland flowing streams.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.07.028.

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