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EFFECTS OF EXTREME FLOODING ON WATER QUALITY IN AREAS OF DENSE FOOD ANIMAL PRODUCTION

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Effects of Extreme Flooding on Water Quality in Areas of Dense Food Animal Production

Abstract

Storm events are expected to increase with climate change, with the potential to adversely impact environmental quality and public health. This study assessed impacts of Hurricane Florence on water quality in areas of dense food animal production. Twelve surface water sites in rural, eastern North Carolina were sampled before and after Hurricane Florence to assess storm effects and duration of impacts. Concentrations of fecal indicator bacteria *Escherichia coli* were not significantly different in our first sampling event, conducted 10 days after Hurricane Florence. Instead, the longitudinal data show that first runoff events, defined as rainfall following a dry period, are associated with increased concentrations of fecal indicator bacteria in these watersheds. Additionally, watersheds with larger commercial hog operations (CHOs) and CHOs closer to sampling sites, as well as sites with larger number of households closer to sampling sites had increased *E. coli* concentrations**.** Unlike *E. coli* concentrations, we found that mean ranks of microbial source tracking markers associated with swine wastes (pig-2-bac) and human wastes (HF183) were higher immediately after Florence (Mann Whitney U p=0.009, p=0.0003 respectively). The swine MST marker was markedly elevated at several sites in watersheds with CHOs, while the human MST marker suggested diffuse human contamination across study sites. Antimicrobial resistance among *E. coli* isolated from study waters was also higher in watersheds with CHOs. Resistant *E. coli* was detected in 46% (n=147) of samples collected downstream of CHOs compared to 22% (n=94) of samples collected in watersheds without CHOs, resulting in a relative risk of 1.47 (95% CI: 1.21, 1.78). Bacteria with multiple antibiotic resistance was isolated in 15 of the CHO-associated samples and 1 background sample. Occurrence of AMR does not appear to be driven by precipitation, suggesting other dynamics such as spray events or antibiotic use practices may better explain contributions to resistant bacteria to surface waters. These results help clarify the effects of extreme flooding on microbial contamination of surface waters and can inform strategies for waste management, antibiotic use, and one health surveillance. Ultimately, this work contributes information to build more resilient agricultural systems and communities better prepared to weather the storms that frequent the North Carolina coast.

Acknowledgments

This study would not have been possible without previous support from a student fellowship from WRRI to Dr. Elizabeth Christenson-Diver (project #16-14-W) that allowed us to study water quality in small watersheds in eastern NC, establishing baselines from which the impacts of Hurricane Florence could be investigated. The study also would not have been possible without funding from the NC Policy Collaboratory, who provided funds that enabled rapid mobilization for sample collection after the hurricane.

Sample collection involved a large, team effort of graduate student researchers who dropped their individual projects to help collect and archive samples. Students involved in sample collection and analysis after the hurricane included (in alphabetical order): Collin Knox Coleman, David Holcomb, Alyssa Grube, Kara Kochek, Connor LaMontagne, and Corinne Wiesner. Additional lab support was provided by Sage Smelnik, Megan Miller, and Nikhil Kothegal. Data analysis and reporting were completed by Nikhil Kothegal, Elizabeth Christenson-Diver, Marc Serre, and Jill Stewart.

Hurricane Florence caused devastating flooding in North Carolina. We would like to acknowledge all those impacted by the storm, and to thank the response and recovery workers.

Introduction

Hurricane Florence made landfall in North Carolina (NC) in September 2018, and the slow-moving cyclone delivered rainfall exceeding 30 inches in some locations. Massive flooding was reported throughout southeastern NC, raising concerns about near-term and long-term impacts on water quality and transport of contaminants. Previous research has established that storms events can be major transporters of microbial contaminants (Ahmed et al. 2019). Loading of microbial contaminants to waterbodies tends to be higher during storm events than during low or baseflow conditions (McKergow and Davies-Colley 2009; Stumpf et al. 2010). Also, increased loading of pathogens during precipitation events can lead to non-compliance in recreational waters and increased waterborne disease (Cann et al. 2013; Lee et al. 2019). During Hurricane Florence, there were also concerns about release of contaminants associated with commercial hog operations (CHOs).

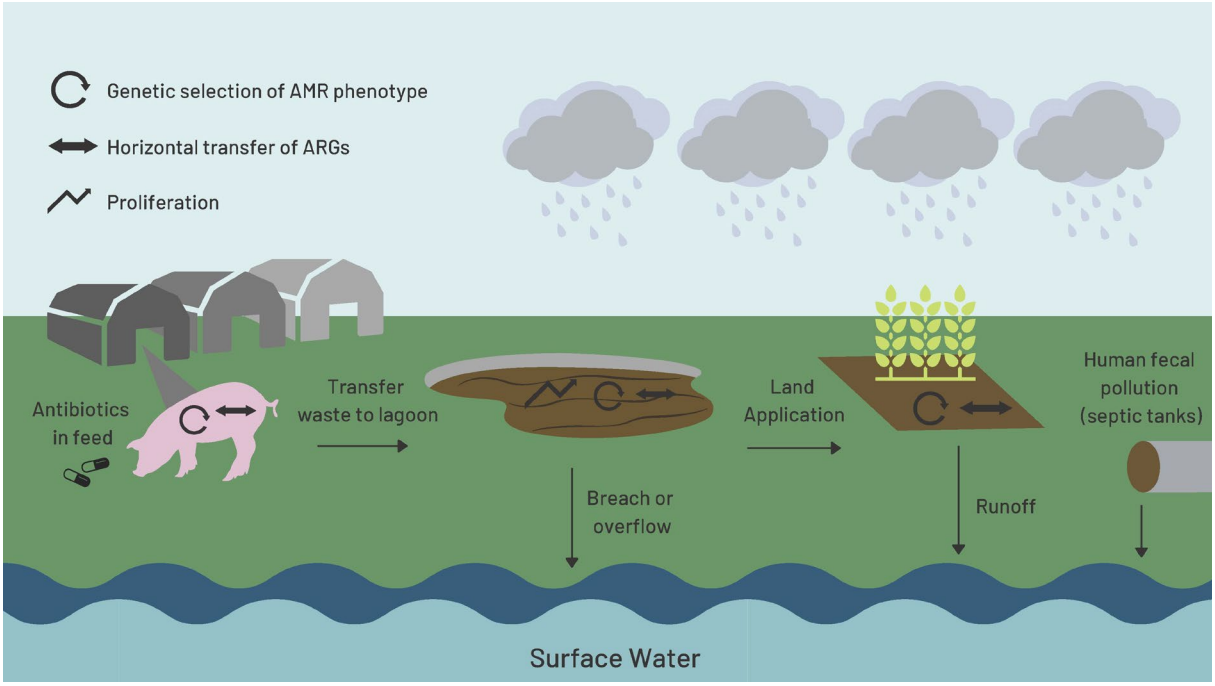
Hog production in NC and across the US has transformed in the past several decades from diversified small farms to fewer, larger industrial facilities capable of producing large numbers of animals (Taylor 2001). Over 10 million hogs are raised in NC CHOs, and the industry produces approximately 14% of US pork (NCAGR 2009). At these CHOs, antibiotics are added to the feed of animals to treat and prevent diseases (GAO 2017; Pew Charitable Trusts 2008; FDA 2013). Much of the antibiotics consumed by livestock are not absorbed but are excreted via feces and urine (Bolan et al. 2008; Chee-Sanford et al. 2009; Makridis et al. 2012; Poulsen 1998). In NC, this waste is typically flushed into outdoor, open-air lagoons and eventually sprayed onto nearby fields. However, during storms these lagoons can leak, overflow, or rupture, spilling waste containing fecal pathogens and antibiotic resistant bacteria into the surrounding environment. During Hurricane Florence, there were multiple reports of lagoon breaches and overflows, as well as reports of animal carcasses in the floodwaters. Contaminants from hog waste are currently unmonitored and poorly understood. It is similarly unclear how human and animal wastewater might contribute to environmental antibiotic resistance (environmental AMR), important because the environment is increasingly being recognized for its role in the spread of antibiotic resistance (Bolan et al. 2008; Burkholder et al. 1997; Makridis et al. 2012).

Due to logistical challenges and other constraints, environmental research following extreme weather events is often fractured and reactive with little if any integration and coordination. However, our team was uniquely positioned to measure impacts of extreme flooding in the wake of Hurricane Florence. Leveraging a student fellowship funded earlier by WRRI, we were able to revisit locations where we had a history of data collection. We mobilized field crews to collect water at selected sites ten days after the hurricane, the earliest date that roads were passable, and followed with three additional sampling events over the next year to investigate duration of impacts. This systematic approach allowed us to evaluate hurricane-related impacts and to compare levels of contaminants in watersheds with and without CHOs.

Using a One Health framework that recognizes that human, animal, and environmental health are inextricably linked, the objective of this study was to assess impacts of Hurricane Florence

on water quality in areas of dense food animal production (Figure 1). Specific objectives were to (1) measure microbial water quality at sites proximal to commercial hog operations and control sites, (2) compare levels of environmental antimicrobial resistance (environmental AMR) at study sites, and (3) model spatial and temporal distributions of contaminants in surface waters. Results of this research help improve scientific knowledge regarding effects of extreme weather events, and informs environmental health issues important to North Carolina and globally.

Figure 1. The goal of this study was to examine the impact of large-scale flooding on water quality and environmental antimicrobial resistance. *(Figure courtesy of Dr. Alyssa Grube)*



Methods

Site Selection

This study leverages a field project funded previously by WRRI (project #16-14-W) assessing water quality in small, agricultural watersheds in eastern NC. Longitudinal data collected before Hurricane Florence have been reported previously (Christenson 2019). After Hurricane Florence, a subset of 12 sites were selected for continued sampling (Figure 2). These sites comprised the "southern route" described in Christenson's dissertation and included seven sites downstream of at least one commercial hog operation (CHO) and five background sites that did not have a CHO in the watershed. Watersheds were small with similar land use variables (Table 1) to reduce confounding on measured indicators. All sites were selected to avoid proximity to permitted discharge sites (e.g. wastewater treatment plants).

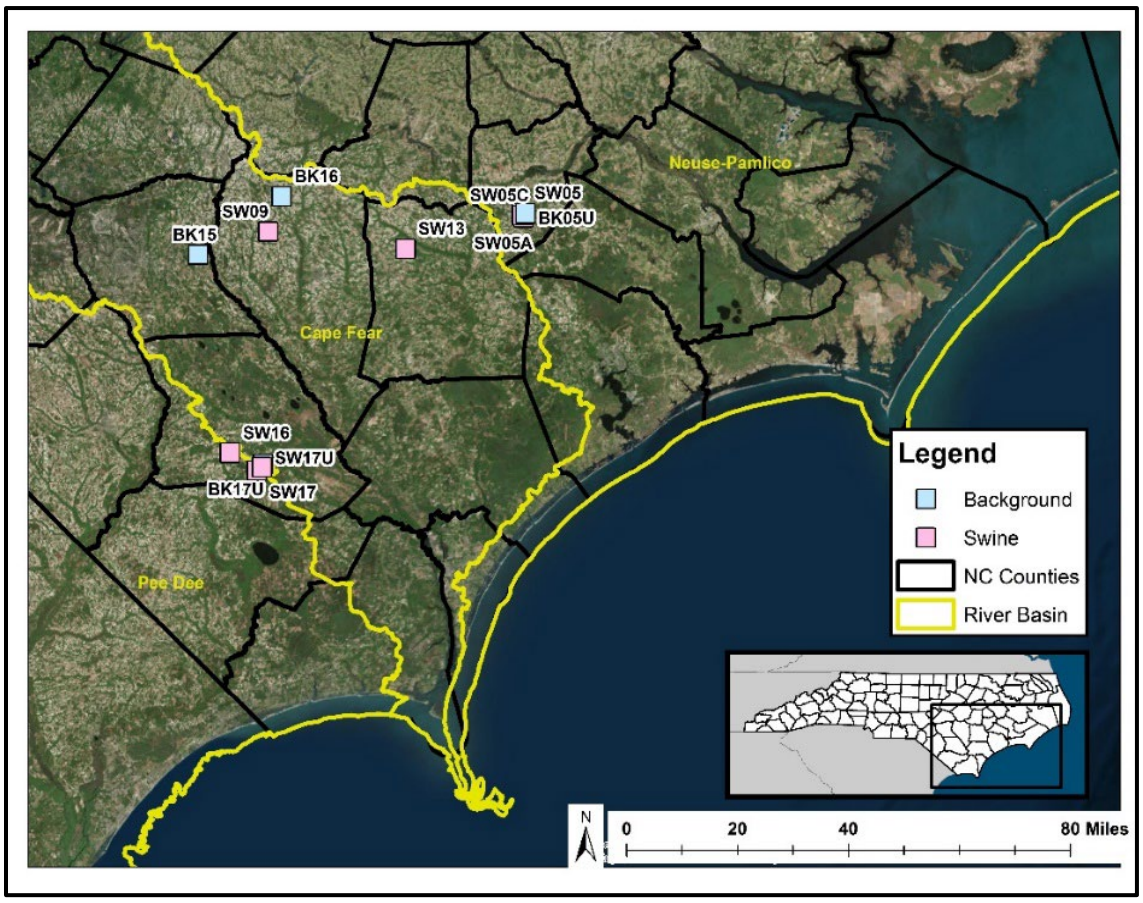


Figure 2. Selected study sites revisited after Hurricane Florence made landfall in North Carolina.

Prior to Hurricane Florence, 22 sites were sampled nine times between August 2016 and September 2017. After Hurricane Florence, sampling continued between September 2018 and September 2019 at a subset of 12 sites. These sites were sampled four times over the course of the year. In total, the dataset includes 242 total sampling events, including samples collected before and after the Hurricane. Counting only the subset of sites revisited after Hurricane Florence, the dataset includes a total of 153 sampling events.

Table 1. Evaluation of comparability of watershed variables in background (n=9) and sites downstream of CHOs (n=13); table copied from Christenson (2019).

Watershed Variable	Background Mean (95% CI)	CHO-associated Mean (95% CI)	p-value[a]	Shapiro-Wilkes
Watershed area (mi^2)[b]	4.5 (1.5, 7.5)	3.2 (1.9, 4.5)	0.95	0.81
% Wetland	20 (14, 27)	18 (16, 20)	0.50	0.68
% Forest	23 (15, 31)	22 (16, 29)	0.91	0.24
% Cultivated	38 (27, 49)	37 (28, 46)	0.87	0.85
% Wetland, 50m Buffer	36 (20, 51)	49 (40, 58)	0.18	0.52
%Forest, 50m Buffer†	12 (6, 17)	14 (9, 19)	0.39	0.72
% Cultivated, 50m Buffer	36 (15, 57)	18 (8, 27)	0.23	0.008*
% Hydrological Soil Class A	54 (35, 72)	48 (36, 61)	0.56	0.02*
% Hydrological Soil B	28 (14, 41)	31 (21, 41)	0.72	0.37
% Hydrological Soil C	14 (0, 30)	13 (2, 23)	0.52	< 0.001*
% Hydrological Soil D	2 (0, 5)	3 (0, 6)	0.85	< 0.001*
Population†	300 (2, 599)	128 (81, 174)	0.97	0.97
Population Density (Population/mi^2)	51 (30, 71)	55 (24, 87)	0.99	0.99

[a]t-test or *Mann-Whitney rank sum test for non-normal distributions when Shapiro Wilkes p<0.1
[b] log10-transformed for t-test

Sample Collection

Grab samples were collected in a single day for each sampling event. Approximately 1 L of water was collected in sterile, plastic bottles that were rinsed with surface water immediately prior to sample collection. At the time of sampling, a handheld YSI Pro Professional Plus meter was used to assess water temperature, specific conductance, dissolved oxygen, and pH of the sample. Samples were transported on ice to the laboratory at UNC and stored at 4°C until processing. All samples were processed within 24 hours of sample collection.

Escherichia coli Concentrations

For samples collected after Hurricane Florence (n=48), *E. coli* concentrations were enumerated using the IDEXX Colilert-18 kit (Maine, United States) according to the manufacturer's instructions. A raw sample (100 mL) and 1:10 dilution were prepared for each sampling site. Samples were incubated for 18 hours at 35°C. Resulting concentration estimates for total coliforms and E. coli were averaged between the raw and diluted sample and reported as most probable number (MPN)/100 mL. In cases where estimations exceeded the limit of detection in the diluted sample, the value >24,196 MPN/100 mL was recorded instead of the average. For downstream analyses, values below the limit of detection were assigned a value of 1 MPN/100 mL while values above the limit of detection were assigned a value of 24,196 MPN/100 mL.

For all samples, standard membrane filtration was conducted to determine thermotolerant *E. coli* concentration from each water sample collected (U.S. EPA (Environmental Protection Agency) 2005). Volumes of 50 mL, 25 mL, 5 mL, and 1 mL sample were vacuum filtered through 0.45 µm, 47 mm mixed cellulose ester filters (MilliporeSigma, Burlington, MA) and aseptically placed onto selective M-TEC ChromoSelect agar (Sigma-Aldrich, St. Louis, MO). The plates were inverted and incubated at 37 ^{o}C for 2 h followed by 44^{o}C for 22 h (+/- 2 h) then colonies with purple morphological characteristics of *E. coli* were counted.

To determine concentrations of colony forming units (CFUs) per 100 mL, dilution plates with *E. coli* counts between 20 and 80 colonies were normalized to 100 mL and averaged. Samples with all dilution plates below 20 colonies were considered to be at the lower limit of quantification (LLQ) and the plate with the highest colony count was used to determine CFU/100mL. Samples with all dilution plates with a count above 80 were considered to be at the upper limit of quantification (ULQ) and the plate with the lowest colony count was used to determine CFU/100mL. Samples with zero colonies identified were determined to be at the lower limit of detection (LLD) and set to one CFU/100mL. Samples with plates too numerous to count (TNTC) were considered to be at the upper limit of detection (ULD) and samples set to the highest colony count observed per 100mL. When *E. coli* count by membrane filtration was not conducted, and if IDEXX value was available, IDEXX was substituted.

Following membrane filtration of surface water samples, up to six presumptive *E. coli* colonies per sample were isolated, purified, and confirmed as *E. coli* through biochemical testing including indole production using Kovac's reagent. Isolates were taken from different dilution plates when available to reduce possibility of selecting clones. All isolates were archived in tryptic soy broth with 15% glycerol solution at -80^{o}C for additional characterization including antimicrobial resistance testing.

Microbial Source Tracking Marker Concentrations

Samples were analyzed for microbial source tracking markers including the Pig-2-bac (Mieszkin et al. 2009) marker associated with hog waste and the HF183 marker (Green et al. 2014) associated with human waste. In total, 194 samples collected before Hurricane Florence at 22 sites were assessed using ddPCR (Christenson 2019) for human and swine MST markers. Two sampling events after Hurricane Florence comprising 24 samples at 12 sites were assessed for human and swine MST markers using qPCR.

For samples collected after Hurricane Florence, sample preparation consisted of 100 mL sample filtration through 0.4 µm polycarbonate membranes (MilliporeSigma, Burlington, MA, USA), which were aseptically folded into DNEasy PowerBead tubes (Qiagen Inc, Germantown, MD) and immediately stored at -80°C until further analysis. We extracted DNA from sample filters using the DNEasy PowerSoil kit (Qiagen). Immediately prior to DNA extraction, we added 120 or 125 ng salmon testes DNA (Sigma-Aldrich, St. Louis, MO) to each thawed bead tube as a sample processing control (SPC) and to an additional tube in each extraction batch containing a clean

membrane to serve as a negative extraction control (NEC) (Haugland et al. 2005). For qPCR analyses we lysed samples on a Mini-Beadbeater (BioSpec, Bartlesville, OK, USA) for two minutes and completed the PowerSoil extraction procedure on the lysate according to the manufacturer protocol. Purified DNA was eluted with 100 µL elution buffer and immediately stored at -80 °C in 25 µL aliquots.

Samples were analyzed by qPCR for the human fecal marker HF183/BacR287 (Green et al. 2014) and the swine fecal marker Pig-2-Bac (Mieszkin et al. 2009). We screened samples for qPCR inhibition using the Sketa22 assay targeting the salmon testes DNA SPC (Haugland et al. 2010, 2012). Samples with Sketa22 quantification cycle (Cq) values more than three cycles higher than the mean Sketa22 Cq of the NEC reactions were considered inhibited and diluted 1:5 with nuclease-free water (NFW) for further analysis by qPCR (Haugland et al. 2012). Each 25 µL reaction consisted of 5 µL DNA template and 12.5 µL 2x TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers were used at 1000 nM final concentration and probes at 80 nM final concentration. Reactions were performed on a CFX96 Touch thermocycler (Bio-Rad, Hercules, CA) with an initial 10-minute incubation at 95 °C followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. Samples were analyzed in duplicate and each instrument run included three no-template control (NTC) reactions and a seven-point standard curve in triplicate. We constructed standard curves from ten-fold serial dilution series of artificial plasmids (gBlocks, Integrated DNA Technologies, Skokie, Il, USA) that were commercially synthesized with the expected PCR product sequence for each assay, representing $10^1 – 10^7$ copies of the target sequence per reaction. Standard curves were estimated by simple linear regression using $\log_{10}$ copy numbers as the independent variable and observed Cq values as the dependent variable. Cq values were calculated for a 100 RFU threshold using the baseline subtraction method in the CFX Manger software (Bio-Rad) (Cao et al. 2012; Layton et al. 2013) We considered a sample positive for a given target if any individual reaction was positive and considered reactions with Cq values >39 to be negative (Odagiri et al. 2015). Target concentrations were calculated from standard curves using the simple mean Cq of all the positive reactions for each sample and multiplied by 20 and divided by 100 to obtain the concentrations in terms of copies/mL surface water.

Below detect values for ddPCR analyses of MST markers were set to half the detection limit for all MST outcomes. As such, since the detection limit for ddPCR was 1.4 copies/mL, non-detects for HF183 or pig-2-bac were set to 0.7 copies/mL. Samples below the detection limit for qPCR analyses were set to 5.5 copies/mL.

Antimicrobial Resistance (AMR) Testing of *E. coli* Isolated from Surface Waters

Antimicrobial resistance testing was conducted on all archived, confirmed *E. coli* isolates using standard Kirby-Bauer disc diffusion methods and following guidelines from the Clinical Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute 2014). Isolates were tested for resistance to eleven antibiotics comprising nine antibiotic classes (Table 2).The panel including antibiotics used primarily in industrial agriculture (U.S. Food and Drug Administration 2015) and antibiotics used primarily in human medicine (U.S. Food and Drug

Administration 2012) with risk assessment priority levels assigned based on WHO criteria (World Health Organization 2017). Multi-drug resistance was defined as resistance to three or more classes of antibiotics.

Table 2. Panel of antibiotics included in antimicrobial resistance testing of surface water *E. coli*

Antibiotic	Antibiotic Class	Conc (ug)	Veterinary Use	Human Use	WHO Priority
Amoxicillin-Clavulanate Acid	Penicillin	20/10	Yes	Yes	High Priority Critical
Ampicillin	Penicillin	10	Yes	Yes	High Priority Critical
Cefoxitin	Cephalosporin	30	No	Yes	Highly Important
Ceftriaxone	Cephalosporin III	30	No	Yes	Highest Priority Critical
Chloramphenicol	Amphenicol	30	Yes	Yes	Highly Important
Ciprofloxacin	Fluoroquinolones	5	No	Yes	Highest Priority Critical
Gentamicin	Aminoglycosides	10	Yes	Yes	High Priority Critical
Imipenem	Carbapenem	10	No	Yes	High Priority Critical
Levofloxacin	Fluoroquinolones	5	No	Yes	Highest Priority Critical
Sulfamethoxazole-Trimethoprim	Sulfas	24/1	No	Yes	Highly Important
Tetracycline	Tetracyclines	30	Yes	Yes	Highly Important

Data Analyses

Statistical Tests for *E. coli* and MST Concentrations

The Mann-Whitney U Test was used to evaluate the difference in mean ranks of concentrations of *E. coli* and MST markers. To determine whether Hurricane Florence impacted *E. coli* or MST marker concentration, the concentration was compared between the sampling event ten days after Hurricane Florence to all other sampling events at those sites. To determine if different precipitation regimes impacted *E. coli* concentration, *E. coli* concentration was compared between samples with and without runoff events, and samples with and without first runoff events. Runoff events were defined as precipitation over 10 mm (0.39 inches) within 48 hours of the sampling date. First runoff events were defined as a runoff event with a preceding dry 5-day dry period that did not receive over 10 mm (0.39 inches). To determine whether site type impacted *E. coli* concentration, *E. coli* concentration was compared between samples collected at background sites and samples collected at sites downstream of CHOs.

Modeling *E. coli* Concentrations

To determine how geospatial characteristics, land use, or environmental factors affected *E. coli* concentration, we updated a multiple linear regression model from Christenson (2019) with additional data collected after Hurricane Florence.

A stepwise multivariate linear regression was developed and validated in Christenson (2019) predicting log10 *E. coli* using all *E. coli* measurements prior to September 2018 (n=177). This

report presents results of similar multivariate linear regression updated to include *E. coli* measurements from 2018-2019 and measured hydrological variables. After running the same multivariate linear regression, a stepwise regression was conducted to select the variables that best predicted *E. coli.*

In brief, 22 non-exposure independent variables and sets of exposure variables were considered for inclusion in the multiple linear regression predicting *E. coli* concentration. Non-exposure variables included precipitation variables (such as prior 24-hour precipitation, runoff event, and first runoff event), measured hydrological variables (such as conductivity and dissolved oxygen), and land use variables (such as percent wetland) (Table 3). To represent hypothesized fecal sources from wildlife, human septic, and CHOs, exposure variables were also constructed (Table 4).

To reduce collinearity, groups of similar independent variables were assessed for collinearity using principal component analysis and the variance inflation factor. Model development resulted in three multivariate general linear models each with a set of predicting independent non-exposure variables and each with a different set of predicting exposure variables. We report the results of two stepwise regression models with the same set of non-exposure variables and different sets of exposure variables: (1) homogenous and (2) sum of exponential decay. Model 1 corresponds to the homogenous and model 2 corresponds to the sum of exponential decay with distance interaction (SED-int) in Christenson (2019) but with additional sampling data.

Each model was then re-assessed using a forward stepwise regression to improve model performance and remove variables that did not contribute to the model's performance. Model performance metrics reported include the adjusted R2 and Akaike information criteria (AIC) for predictive capability.

Table 3. Non-Exposure Independent variables (copied from Christenson, 2019).

Variable Type	n	Variable	Description	Source
Watershed Land Use	1	% Wetland in 100m and 50m buffer	Land use 2016 CDL wetland within 100m or 50m of all perennial streams within watershed	2016 CDL; NHD flowline
	2	% Forest in 100m and 50m buffer	Land use 2016 CDL forest within 100m or 50m of all perennial streams within watershed	2016 CDL; NHD flowline
	3	% Cultivated in 100m and 50m buffer	Land use 2016 CDL cultivated within 100m or 50m of all perennial streams within watershed	2016 CDL; NHD flowline
	4	% Wetland	% Watershed area with wetland (defined as herbaceous wetlands, woody wetlands, and wetlands)	2016 CDL

	5	% Forest	% Watershed area forest (defined as deciduous forest, evergreen forest, forest, mixed forest)	2016 CDL
	6	% Cultivated	% Watershed area cultivated	2016 CDL
	7	Soil type A	% Watershed area with hydrological soil class A	SSURGO
	8	Soil type B	% Watershed area with hydrological soil class B	SSURGO
	9	Soil type C	% Watershed area with hydrological soil class C	SSURGO
	10	Soil type D	% Watershed area with hydrological soil class D	SSURGO
Environmental	11	Inches precipitation – prior 24 hours	Precipitation determined for sampling location using gridded observed precipitation data for 24 hours prior to 7AM of sampling date	National Weather Service
	13	Inches precipitation- prior 48 hours	Aggregated precipitation determined for sampling location using gridded observed precipitation data for 48 hours prior to 7AM of sampling date	National Weather Service
	14	Precipitation – prior 7 days	Aggregated precipitation determined for sampling location using gridded observed precipitation data defined as precipitation for 7 days hours prior to 7AM of sampling date	National Weather Service
	15	Runoff event	Greater than 10mm rain within 48 hours prior to 7AM sampling date	National Weather Service
	16	First runoff event	Runoff event within 48 hours prior to 7AM of sampling date and no runoff event prior to 48 hours of sampling event through 7 days prior to sampling event	National Weather Service
Measured Hydrological	17	Water pH	pH	Field measurement
	18	Water Temperature	Degrees Celsius	Field measurement
	19	Conductivity	uS/cm	Field measurement
	20	Dissolved Oxygen	mg/L	Field measurement
	21	pig-2-bac concentration *	Gene copies/mL of swine-feces specific microbial source tracking marker	Laboratory measurement

	22	HF183 concentration *	Gene copies/mL of human-feces specific microbial source tracking marker	Laboratory measurement

* MST marker concentration variables were removed as independent variables for this report

Exposure variables were constructed to approximate exposure of surface water to *E. coli* from fecal sources due to overland flow/surface runoff and surface water flow transport from fecal sources: wildlife, human septic tanks, CHO lagoons, and CHO sprayfields. We report here on two sets of exposure variables: homogenous and the more complex sum of exponential decay variables that take into account intensity and distance (Table 4, Christenson 2019).

Table 4. Exposure variables considered for model 1 (homogenous) and model 2 (sum of exponential decay; SED). Table adapted from Christenson (2019).

Fecal Source	Exposure Variable	Intensity	Distance
Wildlife	Watershed Area	Homogenous (i.e. none)	none
Human Septic	Pop. Density	Homogenous (i.e. none)	none
	Household Exposure SED	Number of Households	Euclidean and Surface Water Flow
CHO Lagoon	Manure Density	Homogenous (i.e. none)	None
	Lagoon Exposure SED	Manure Produced, weighted by number of lagoons per CHO	Euclidean and Surface Water Flow
CHO Sprayfield	Manure Density	Homogenous (i.e. none)	None
	Sprayfield Exposure SED	Manure Produced, weighted by sprayfield area	Euclidean and Surface Water Flow

To compare effects of independent variables on *E. coli* concentration, we present results of each univariate prediction as well as interquartile range ratios for variables selected in the stepwise regressions. A univariate model shows strength of the relationship between *E. coli* concentration and each independent variable included in two final stepwise regression models without accounting for other variables. We also report the interquartile range ratio for selected variables as the measure of effect on *E. coli* concentration. The association of each independent variable with E. coli concentration was assessed by weighting the variable's regression coefficient, B, with the variable's interquartile range (as described in Christenson 2019). The IQR is a measure of the independent variable's range for the middle 50% of the data from the 25^{th} to 75^{th}. Multiplying B by the IQR yields an IQR ratio for the percent increase of *E. coli* concentration associated with one increase in the independent variable's IQR. The IQR ratio is helpful when comparing effect sizes across variables with different units of measurement.

Statistical Tests for Antimicrobial Resistance

Antibiotic resistance was defined at the sample level as resistance to at least one antibiotic class and categorized into a binary variable for presence or absence of resistance. Resistance to antibiotic classes was summed across each sample to determine the maximum number of classes to which a sample was resistant. For statistical analysis, samples were also categorized into two additional binary variables: resistance to at least two classes of antibiotics and resistance to at least three classes of antibiotics.

Bar charts were constructed to visualize the frequency of sample resistance to maximum number of antibiotic classes by site type (background compared to CHO-associated), prior rain, runoff event, and first runoff event. Prior rain was defined as aggregated precipitation for a sampling location in the 7 days preceding the sampling date. Runoff events and first runoff events were defined as above. Box plots were also constructed to visualize the distribution of antibiotic resistance to number of antibiotic classes by date of sampling event and by site.

To determine if different precipitation regimes impacted antibiotic resistance, prevalence of resistance to number of antibiotic classes and to the tetracycline antibiotic class was compared between site type (CHO-associated and background samples), samples with and without prior rain, samples with and without runoff events, and samples with and without first runoff events. A measure of effect size was included when comparing prevalence outcomes by calculating a relative risk (RR) and corresponding 95% Confidence Interval (CI) using the standard errors.

The impact of precipitation regimes on prevalence of antibiotic resistance to at least one antibiotic class and to tetracycline was also assessed according to sample site type (among CHO-associated sites only or among background sites only), with RRs and 95% CIs calculated as a measure of effect.

To determine whether Hurricane Florence impacted antibiotic resistance, prevalence of resistance to number of antibiotic classes and to tetracycline was compared between the sampling event ten days after Hurricane Florence (September 24, 2018) to all other sampling events at those sites in three ways: across all Southern Route samples, CHO-associated samples in the Southern Route, and background samples in the Southern Route. RRs and 95% CIs were calculated as a measure of effect.

Modeling Antibiotic Resistance

Logistic regression was performed to fit a regression model to two binary response variables: resistance to at least one class of antibiotics and resistance to tetracycline. Independent predictor variables for site type, precipitation (24-hour prior precipitation, 48-hour prior precipitation, precipitation in the prior 7 days, runoff event, and first runoff event), exposure variables (see Table 4) including density metrics for each fecal source location (population density, manure density, and sprayfield acres), and fecal exposure (lagoon, sprayfield, and household) were included in univariate logistic regression models to assess each variable's relationship to resistance to at least one class of antibiotics and resistance to tetracycline.

Models were assessed for strength of relationship between the outcome and independent variables using odds ratios (ORs) and 95% CIs.

Multinomial logistic regression was subsequently conducted from a subset of the variables from the univariate regressions that best predicted antimicrobial resistance to at least one class of antibiotics and resistance to tetracycline using model metrics of residual deviance, Akaike Information Criterion (AIC), and by assessing model performance by comparing the accuracy of predicted outcome probabilities to actual values. Variables were assessed for multicollinearity using correlation plots and the VIF.

Results and Discussion

This report summarizes measures of water quality and environmental AMR from samples collected before and after Hurricane Florence including areas of dense hog production. Of the collected 242 sampling events, we could not determine *E. coli* count for 17 sampling events across two sampling dates (1/30/2017; 6/5/2017) due to an incubation error in the laboratory. Additionally, *E. coli* counts for 12 sampling events in September 2019 were imputed using IDEXX count values rather than colony forming units. All *E. coli* concentrations reported here represent 225 sampling events.

Escherichia coli Concentrations

E. coli concentrations collected ten days after Hurricane Florence were not significantly different compared to concentrations prior (p=0.7) (Figure 3). Seven of twelve *E. coli* concentrations collected ten days after Hurricane Florence were within the interquartile range of *E. coli* concentration seen in prior data at the same sampling sites. Three of twelve *E. coli* concentrations were below the interquartile range and two of twelve *E. coli* concentrations were above the interquartile range. No concentrations were outliers (Figure 4). The overall lower levels of *E. coli* immediately following Hurricane Florence may be due to dilution associated with extreme flooding.

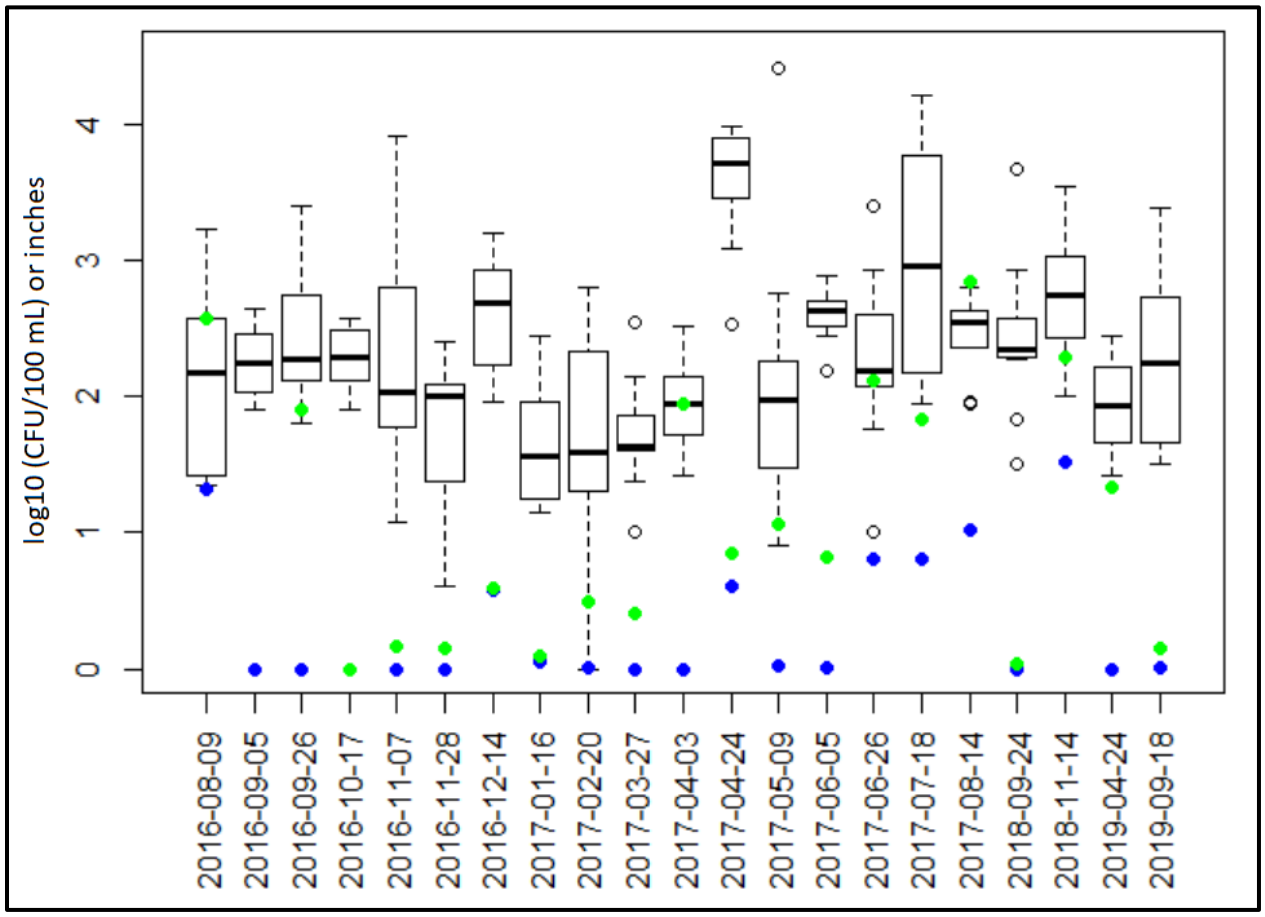


Figure 3. Boxplots for log_{10}-transformed *E. coli* concentration for full dataset including all (n=225) sampling events between 2016 and 2019. In each boxplot the thick black line represents the median value, the box represents the interquartile range between the 25th and 75th percentile, and the dotted lines extend up to 1.5 times the interquartile range. Circles represent statistical outliers. Blue dots represent average prior 48 h precipitation in inches; green dots represent average prior one week precipitation in inches. The first sampling event ten days after Hurricane Florence was 9/24/18.

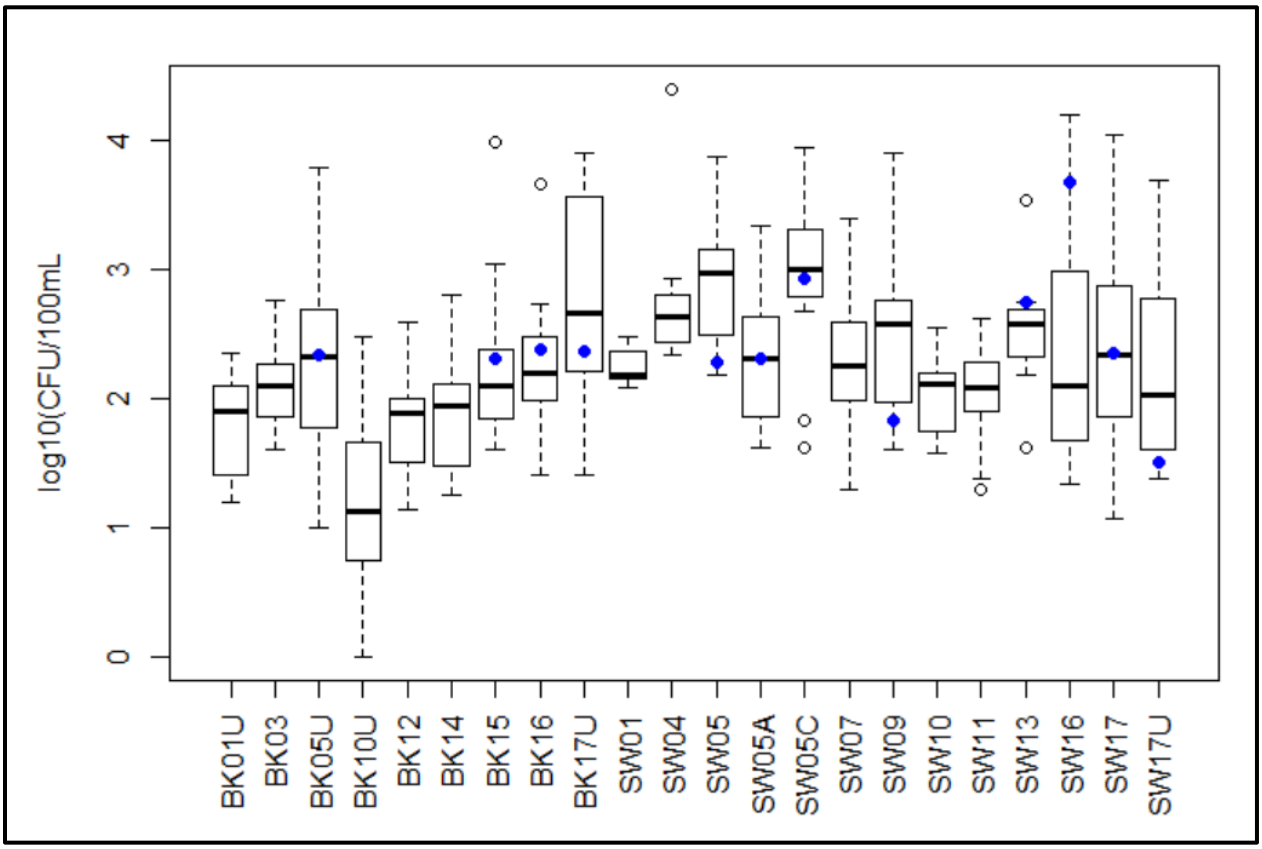


Figure 4. Boxplot for log_{10}-transformed *E. coli* concentration for each site among all (n=225) events with background (BK) sites listed before CHO-associated sites (SW). In each boxplot the thick black line represents the median value, the box represents the interquartile range between the 25th and 75th percentile, and the dotted lines extend up to 1.5 times the interquartile range. Circles represent statistical outliers. Blue dots represent the first sampling event ten days after Hurricane Florence 9/24/18 at the subset of twelve sites.

Microbial Source Tracking Marker Concentrations

Results showed that mean ranks of pig-2-bac and HF183 concentrations were higher immediately sampling after Hurricane Florence ($p=0.009$, $p=0.0003$ respectively) compared to all sampling events before. This was interesting because the more general fecal indicator marker of *E. coli* was not significantly different immediately after the hurricane. While it is possible that the difference is due to methodology since methods used ddPCR before Hurricane Florence and qPCR afterwards, we did not find systematically different values between qPCR and ddPCR samples across all sites. The biggest difference among qPCR and ddPCR methods was that ddPCR had a lower limit of detection than qPCR methods.

For HF183 concentrations, four of twelve sites were below the detection limit after Hurricane Florence. When HF183 concentrations were detected, only one sample was over 1 copy per mL. This samples had the highest observed HF183 concentration observed at all sites in the study, with a concentration of 79 copies per mL (See Figure 5, BK17U, maximum for site).

For pig-2-bac concentrations, after Hurricane Florence five of twelve sites were below the detection limit. All background sites did not have detectable pig-2-bac concentrations. Among the CHO-associated sites, three sites had pig-2-bac concentrations that were maximum values and statistical outliers compared to samples collected the year before Hurricane Florence (See Figure 6, SW05A, SW13, SW16). These sites had much higher pig-2-bac concentrations than surrounding locations.

It appears that high outlier values for MST markers after Hurricane Florence are indicative of possible infrastructure leaks or longer persistence of the marker compared to *E. coli* concentrations. The sampling also supports prior findings that pig-2-bac is detected more often in CHO-associated sites compared to background sites, while HF183 is detected among both site types.

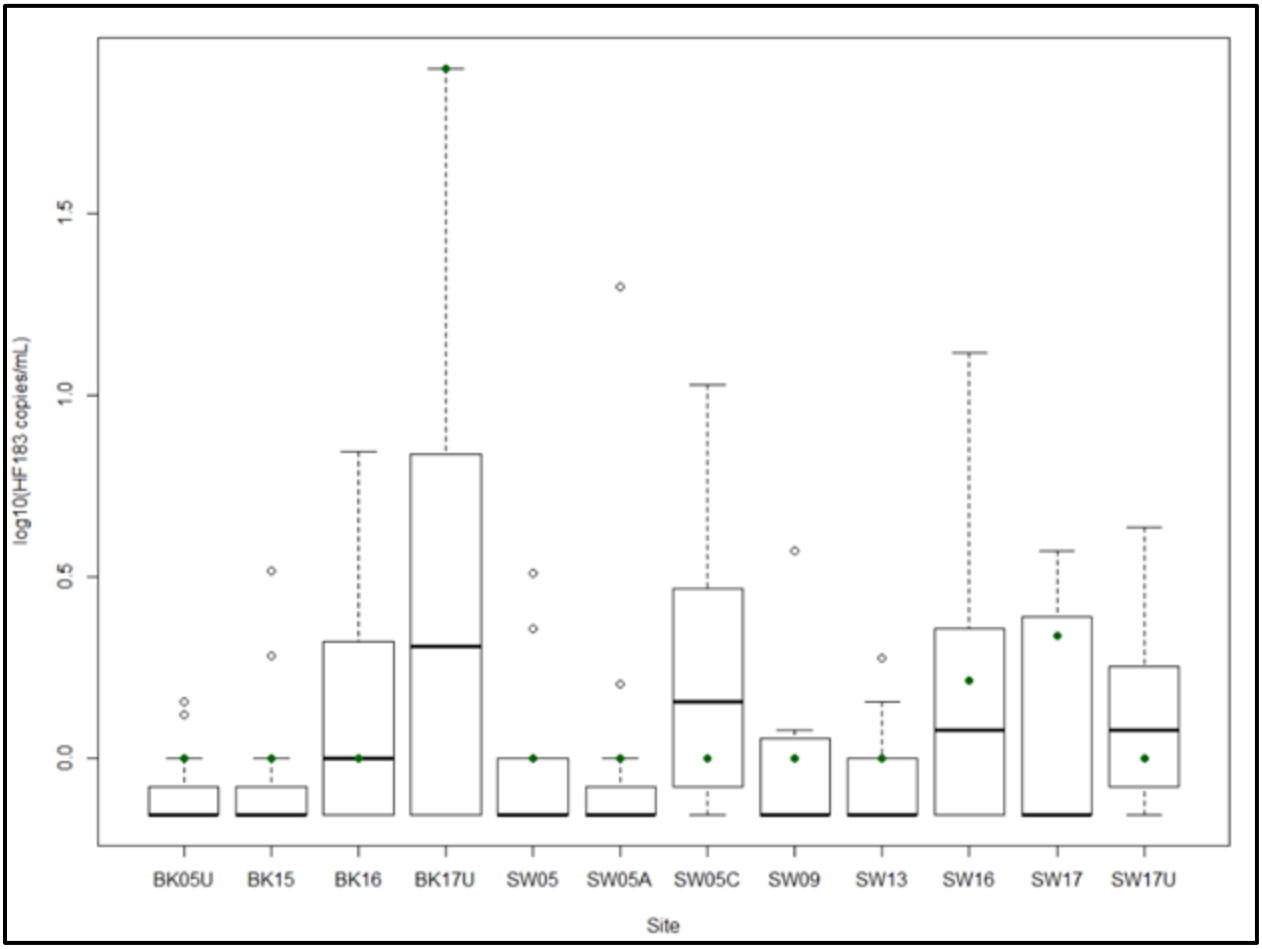


Figure 5. Boxplot for log_{10}-transformed HF183 concentration for each site among (n=129) events among subset of twelve sites with background sites listed first (BK) followed by CHO-associated sites (SW). In each boxplot the thick black line represents the median value, the box represents the interquartile range between the 25th and 75th percentile, and the dotted lines extend up to 1.5 times the interquartile range. Circles represent statistical outliers. Green dots represent immediate post-Florence 9/24/18

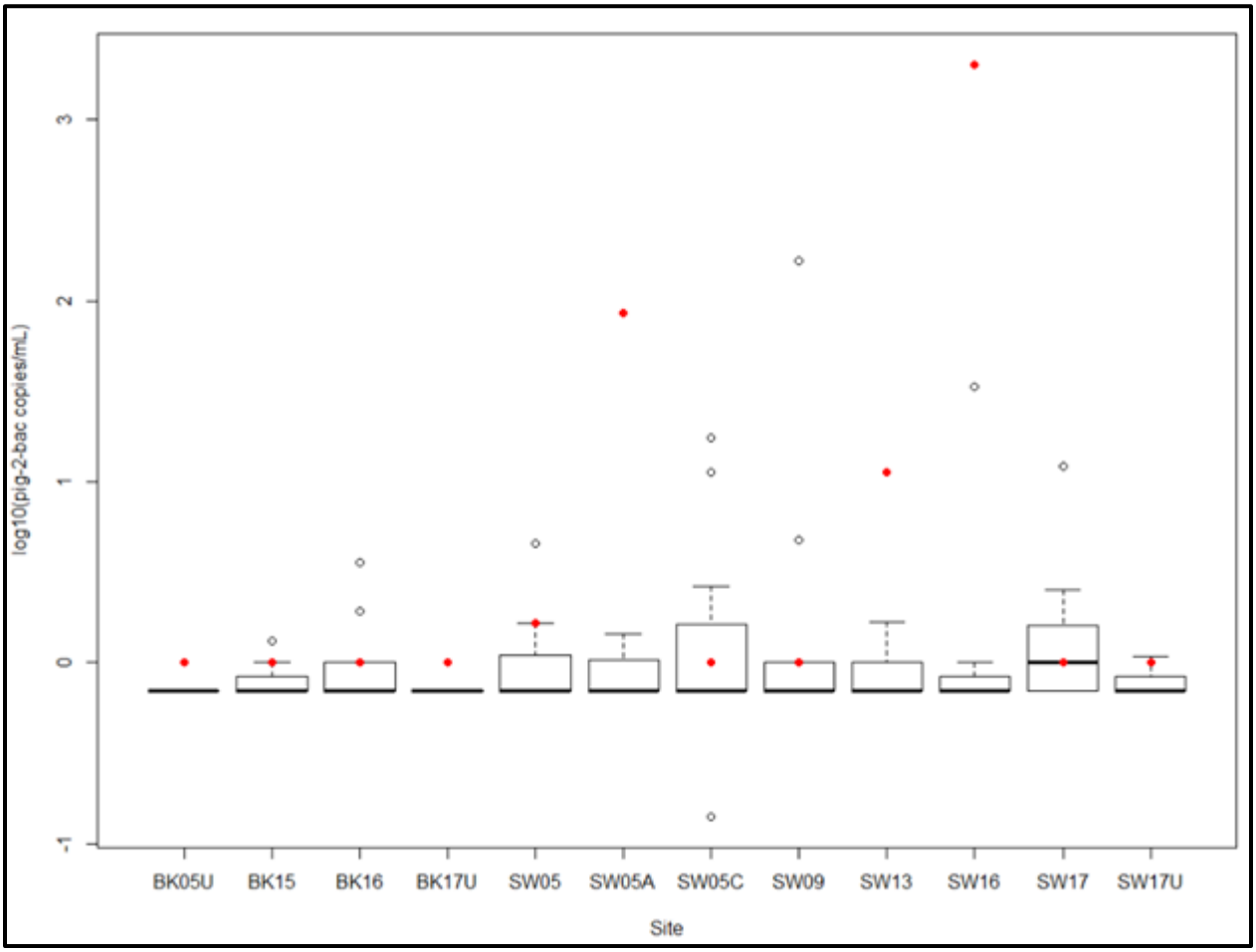


Figure 6. Boxplot for log_{10}-transformed pig-2-bac concentration for each site among all (n=129) events among subset of twelve sites with background sites listed first (BK) followed by CHO-associated sites (SW). In each boxplot the thick black line represents the median value, the box represents the interquartile range between the 25th and 75th percentile, and the dotted lines extend up to 1.5 times the interquartile range. Circles represent statistical outliers. Red dots represent immediate post-Florence 9/24/18

Effects of Different Precipitation Regimes on *E. coli* Concentrations

Of the 225 sampling events, 58 events were precipitation events defined as having greater than 0 inches rain in the prior 24 h period. Of the precipitation events, 57 events were runoff events and 21 events were first runoff events.

First runoff events were more strongly associated with higher concentration of *E. coli* compared to runoff events and prior 24-hour precipitation (Figure 7). First runoff events had significantly higher (p=0.0000002) *E. coli* concentrations (mean=1276 CFU/100 mL; 95% CI: 701, 2321 CFU/100 mL) compared to events that were not first runoff events (mean=168 CFU/100 mL; 95% CI: 136, 207 CFU/100 mL) (Figure 8C). Similarly, runoff events had significantly higher (p=0.00000003) *E. coli* concentrations (mean=549 CFU/100 mL; 95% CI: 367, 824 CFU/100 mL) compared to events that were not runoff events (144 CFU/100 mL, 95% CI: 115, 181 CFU/100 mL) (Figure 8B).

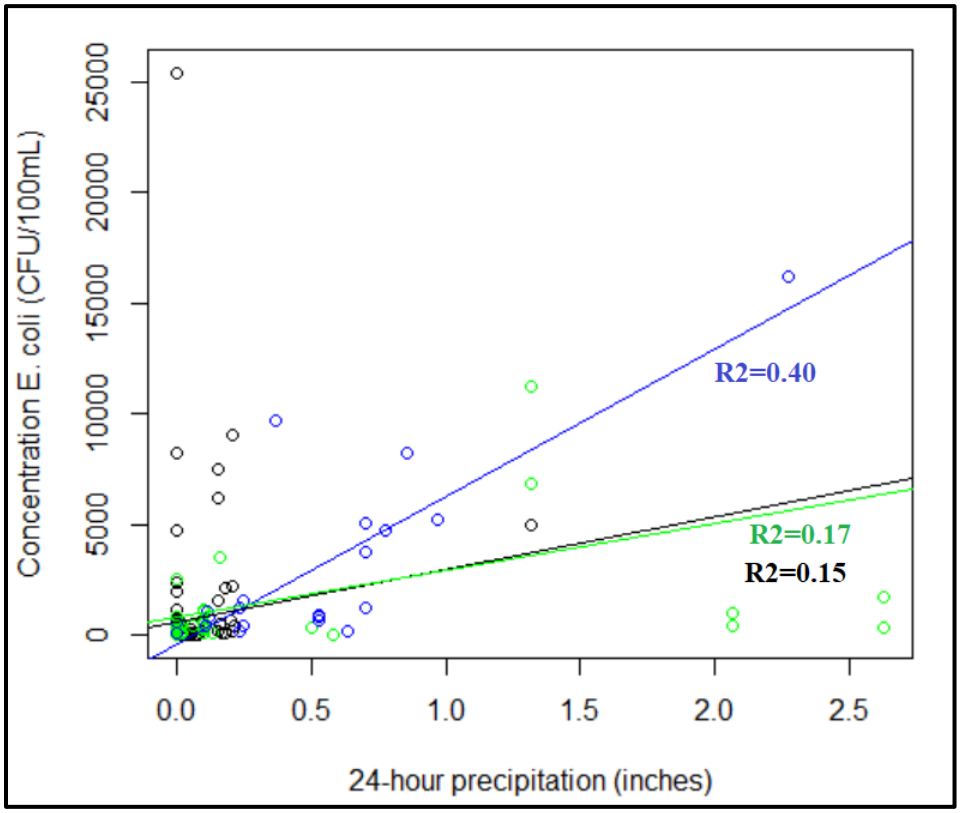


Figure 7. Correlation of log10 *E. coli* concentration predicted by 24-hour prior precipitation (black) any runoff event (green), or first runoff event (blue)

E. coli concentrations were significantly higher (p=0.00005) among CHO-associated sites compared to background sites with a mean of 122 CFU/100 mL (95% CI: 87, 172 CFU/100 mL) for background sites and 283 CFU/100 mL (95% CI: 218, 367 CFU/100 mL) for CHO-associated sites (n=225). See Figure 8A.

E. coli concentration was not significantly different between background and CHO-associated sites ten days after Hurricane Florence (September 24, 2018) or 30 days after Hurricane Florence on November 14, 2018 (p>0.5).

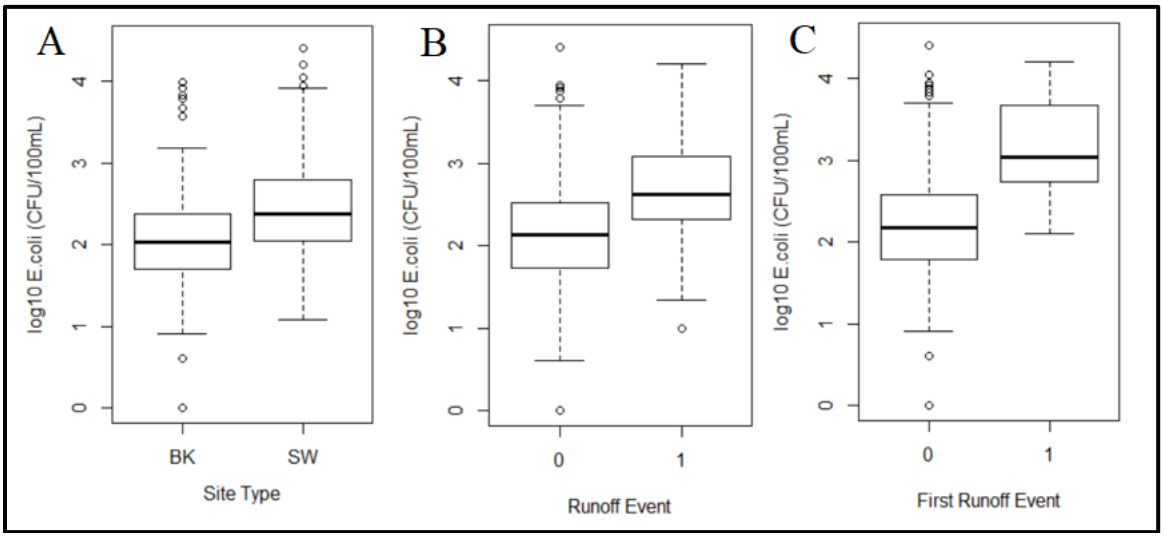


Figure 8. Boxplot for log_{10}-transformed *E. coli* concentration (n=225) comparing (A) background (BK) to CHO-associated (SW) sites; (B) Runoff events (1) compared to non-runoff events (0); and (C) first runoff events (1) compared to non-first runoff events (0). In each boxplot the thick black line represents the median value, the box represents the interquartile range between the 25th and 75th percentile, and the dotted lines extend up to 1.5 times the interquartile range. Circles represent statistical outliers.

For precipitation-related variables, a univariate model shows that when not controlling for other variables, 24-hour prior precipitation, 48-hour prior precipitation, runoff events, and first runoff events are all significantly associated with higher *E. coli* concentrations ($p<0.001$) (Table 5). Among precipitation-related variables, first runoff event was associated with twice the increase in *E. coli* concentration compared to runoff events (IQR ratio of 7.62 compared to 3.81, respectively) (Table 5).

For land-use related variables, a univariate model shows that when not controlling for other variables, the variables, household exposure, lagoon exposure, and sprayfield exposure are also significantly associated with higher *E. coli* concentration in streams ($p<0.001$). The exposure variable with the highest IQR is the lagoon exposure variable indicating a one IQR increase in lagoon exposure is associated with a 2.1 times higher concentration of *E. coli.*

Table 5 lists the variables included in both regression models highlighting that model 1 includes manure density, sprayfield acres, and population density to represent CHO and human household sources while model 2 includes sum of exponential decay exposure variables for lagoon and household exposure.

Table 5. Univariate model performance predicting $\log_{10}$ *E. coli* concentration.

Variable	Model 1 (includes homogenous exposure variables)	Model 2 (includes SED exposure variables)	Adjusted R2	B	p	IQR	IQR Ratio
24 hr prior precipitation	x	x	0.15	0.70	***	0.05	1.09
48 hr prior precipitation			0.13	0.43	***	0.39	1.47
Runoff	x	x	0.12	0.58	***	1.00	3.81
First Runoff	x	x	0.13	0.88	***	1.00	7.62
Water Temperature	x	x	0.01	0.01		9.10	1.32
Conductivity	x	x	0.02	0.00		67.00	1.33
DO %	x	x	0.01	0.00	.	38.10	0.75
Watershed Area	x	x	0.00	-0.01		3.93	0.91
Wetland %	x	x	0.00	-0.66		0.08	0.88
Forest %	x	x	0.00	-0.04		0.16	0.99
Buffer 50 m Forest	x	x	0.02	1.34	*	0.11	1.41
Soil D	x	x	0.00	-0.76		0.03	0.96
Lagoon Exposure		x	0.07	0.00	***	3577340	2.12
Household Exposure		x	0.11	0.10	***	2.16	1.63
Sprayfield Exposure			0.07	0.00	***	4271849	1.51
Manure density	x		0.01	0.00	.	202754	1.27
Sprayfield Acres	x		0.03	0.00		176.72	1.61
Population density	x		0.01	0.00	.	45.00	1.21

***p<0.001, **p<0.01, *p<0.05, .p<0.1

The results of the multiple linear regression model show that model 2 outperforms model 1 by having higher adjusted R2 and lower AIC (Table 6). As such, exposure variables that incorporate intensity and distance metrics improve model fit compared to exposure variables that are based on measures of density.

Table 6. Model descriptive and performance parameters including number of variables included in the stepwise model, maximum and average variance inflation factor (VIF), adjusted R2, and AIC for two models predicting $\log_{10}$ concentration of *E. coli* using different sets of variables to approximate exposure to CHO and human sources.

Model Parameter	Model 1	Model 2
n Variables	9	8
Maximum VIF	1.48	1.3
Adjusted R^2	0.3235	0.3592
AIC	406	392

Figure 9 displays the actual compared to the predicted *E. coli* concentrations using Model 2.

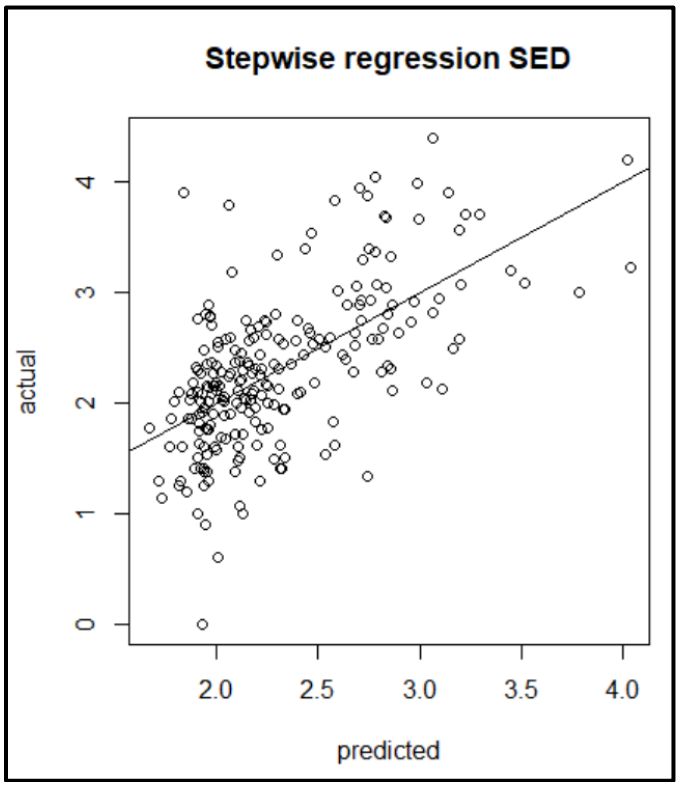


Figure 9. Actual vs. predicted $\log_{10}$ *E. coli* concentration for 225 sampling events for Model 2.

Modeling results suggest that the largest predictors of *E. coli* concentration are antecedent dry conditions followed by rainfall, distance to a rural household, and distance and/or intensity of a commercial hog operation lagoon compared to other variables such as prior precipitation, land cover, soil type, water temperature, conductivity, and dissolved oxygen. Table 7 displays the effect sizes, using interquartile range ratios, for variables identified in the stepwise models. Modeling results support that moderate rainfall (> 10 mm, 0.4 inches) over 48 h after a five-day period without rain is a significant mechanism for transporting *E. coli* to surface water in agricultural, small watersheds with or without CHOs. First runoff events were significantly associated with higher *E. coli* concentrations ($p<0.001$) and had the highest IQR in both models finding that *E. coli* concentrations were 6.90 or 6.99 times higher after first runoff events compared to events that were not first runoff events (Table 7). While other precipitation variables were also investigated in the models, moderate rainfall that did not occur after a dry period (I.e. runoff events) was not selected in the stepwise model indicating that it did not sufficiently add "value" to the model's predictive capacity. Additionally, while prior 24-hour precipitation was included in the models and was significantly associated with higher E. coli concentrations ($p<0.01$), the IQR ratio was much lower than the first runoff event's IQR ratio. This model supports the findings that we did not find high *E. coli* concentrations sampling ten days after Hurricane Florence, which was neither a first runoff nor runoff event.

The modeling results also find that both CHOs and human households are significantly associated with higher concentrations of *E. coli* even when controlling for precipitation. That is, whether precipitation occurs or not, presence of CHOs and households are associated with higher *E. coli* concentrations downstream. Specifically, for Model 1 (Table 7), an increase of 45 people/square mile contributes an additional 28% *E. coli* concentration downstream and an increase of 177 sprayfield acres contributes an increase of 59% *E. coli* concentration downstream. For Model 2 (Table 7) considering variables taking into account CHO and human household distance and size, one IQR increase of lagoon exposure was associated with a 39% increase in *E. coli* concentration and an IQR increase of human exposure was associated with a 40% increase in *E. coli* concentration.

While this research was not designed to assess the impact of households on fecal bacteria in surface water, the findings suggest that there may impacts on fecal bacteria in surface water from septic systems, which is commonly used in the rural, agricultural watersheds where we sampled to contain and treat human wastes.

Table 7. Interquartile range (IQR) ratios and associated p-values for variables included in stepwise models. Greyed out variables denote variables not chosen in the stepwise model. Model 1 considered homogenous exposure variables while Model 2 considered sum of exponential decay exposure variables.

Variable			Model 1 Homogenous		Model 2 SED	
Type	**Description**	**IQR**	**IQR ratio**	**p**	**IQR ratio**	**p**
Precipitation	Prior 24 hours	0.05	1.06	***	1.05	***
	Runoff Event	1.00[1]				
	First Runoff Event	1.00[1]	6.90	***	6.99	***
Land Use Variables	% Wetland	0.08	0.78	*		
	% Forest	0.16				
	%Wetland, 50m buffer	0.28				
	%Forest, 50m buffer	0.11	1.22			
	Soil type B	0.23				
	Soil type D	0.26				
Measured	Water Temp.	9.10	1.31		1.30	
	Conductivity	67.0	1.27	<0.1	1.27	*
	Dissolved Oxygen %	38.1	0.73	*	0.73	*
Exposure Variables	Watershed Area	3.93				
	Pop. Density	45	1.28	*	n/a	
	Sprayfield Acres	177	1.59	**	n/a	
	Manure Density	202754			n/a	
	Lagoon Exposure	3577340	n/a		1.39	*
	Household Exposure	2.16	n/a		1.40	***

***p<0.001, **p<0.01, *p<0.05; [1]IQR for binary variables fixed at 1

Antibiotic Resistance

Antibiotic resistance among tested *E. coli* isolates was analyzed at the sample level (N=241). A sample was defined as resistant to an antibiotic class if any *E. coli* isolate from that sample showed resistance to that class. In this report, resistance to antibiotic classes for the following antibiotics was analyzed: amoxicillin-clavulanate acid, ampicillin, cefoxitin and ceftriaxone, chloramphenicol, ciprofloxacin and levofloxacin, gentamycin, tetracycline, and sulfamethoxazole-trimethoprim (Table 2). Based on previous findings from analyses in Christenson (2019), we report here on sample resistance to number of antibiotic classes (maximum, at least one, at least two, and at least three classes) and on the tetracycline class specifically, as resistance to this class was observed more frequently than to other antibiotic classes (n=79).

Resistance to at least one antibiotic class was observed in 89 (36.9%) of 241 total samples, with 68 (46.3%) of CHO-associated samples and 21 (22.3%) of background samples showing resistance (RR=1.47; 95% CI: 1.21, 1.78) (Table 8). CHO-associated samples also had higher observed resistance to at least two (RR=1.61; 95% CI: 1.37, 1.89) and to at least three (RR=1.60; 95% CI: 1.35, 1.89) antibiotic classes compared to background. Overall, *E. coli s*amples ranged from being resistant to zero antibiotic classes to a maximum of five antibiotic classes. CHO-associated sites represented higher frequencies of samples resistant to multiple classes of antibiotics (especially to three, four, and five classes). Background sites did not have any samples that were resistant to five classes of antibiotics and only one sample resistant to four classes, while CHO-associated sites had one sample resistant to five classes and six samples resistant to four classes of antibiotics (Figure 10). Observed resistance to the tetracycline class was higher in CHO-associated sites compared to background sites (RR=1.50; 95% CI: 1.24, 1.80) (Table 8).

Table 8. Number, percent, and relative risk of *E. coli* samples with observed resistance to number of antibiotic classes and tetracycline by site type.

	Antibiotic Resistance	**CHO-associated Sites n (%)**	**Background Sites n (%)**	**Relative Risk (95% CI)**
	n samples	147	94	n/a
Number of Antibiotic Classes	Resistance to >= 1 Antibiotic Class	68 (46.3%)	21 (22.3%)	1.47 (1.21, 1.78)
	Resistance to >= 2 Antibiotic Class	24 (16.3%)	2 (2.1%)	1.61 (1.37, 1.89)
	Resistance to >= 3 Antibiotic Class	15 (10.2%)	1 (1.1%)	1.60 (1.35, 1.89)
Individual Antibiotic	Tetracycline (TE)	62 (42.2%)	17 (18.1%)	1.50 (1.24, 1.80)

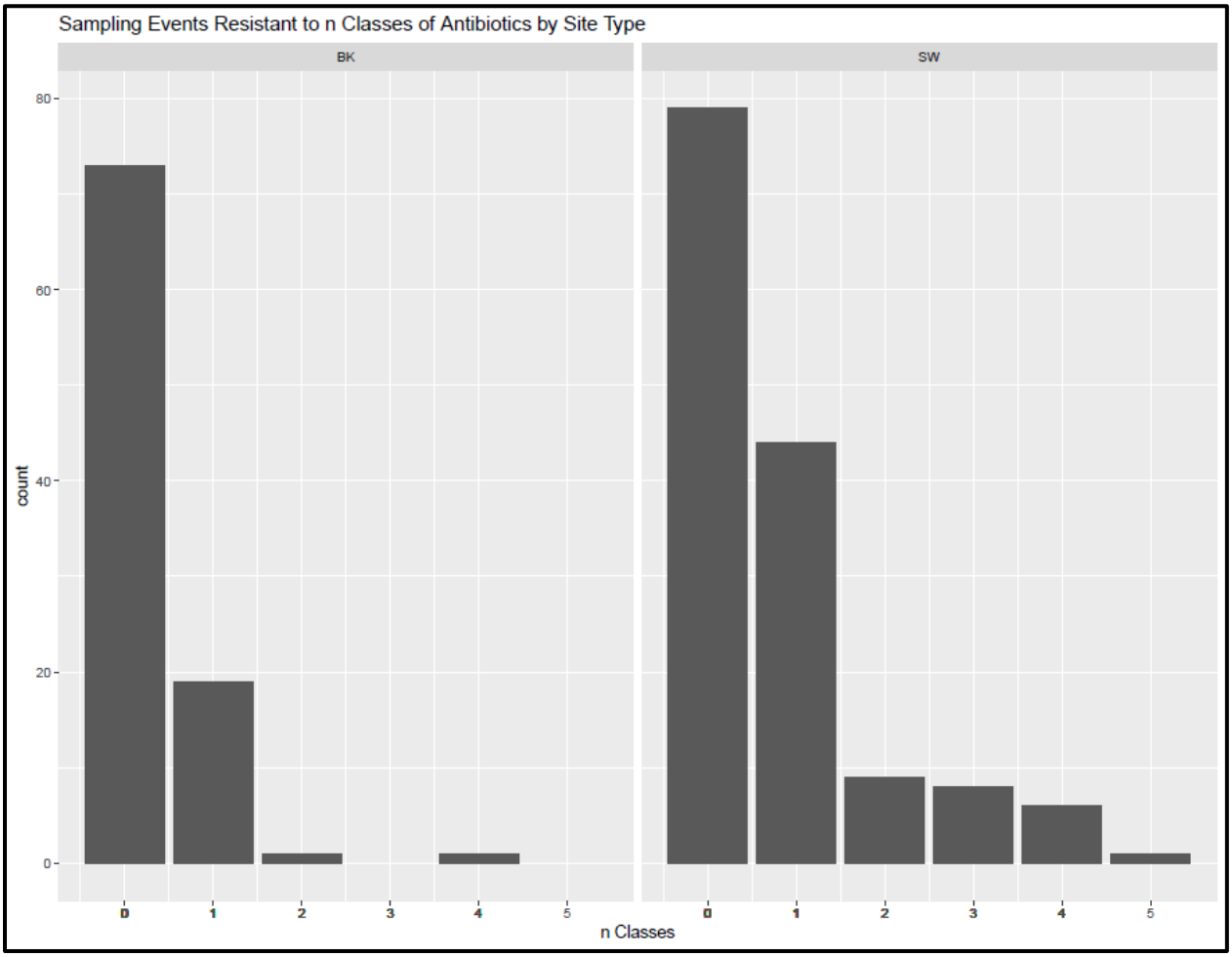


Figure 10. Frequency counts of the total number of antibiotic classes to which a sample was resistant (ranging from zero to five classes) comparing between background (BK) and CHO-associated (SW) sites.

Compared to background sites, CHO-associated sites had a wider spread of samples resistant to multiple antibiotic classes. Four of thirteen CHO-associated sites had a median value of resistant antibiotic classes greater than one. However, only two of these sites had a median value of two or three antibiotic classes resistant (SW04 and SW05, respectively). The remaining CHO-associated sites had median resistance to one or zero antibiotic classes. Site SW7 had one outlier sample resistant to five classes of antibiotics, but its median resistance was to only one antibiotic class. Background sites all had a median resistance to zero antibiotic classes. Site BK15 had one sample resistant to four antibiotic classes, which was the highest number of resistant antibiotic classes in any background site samples (Figure 11).

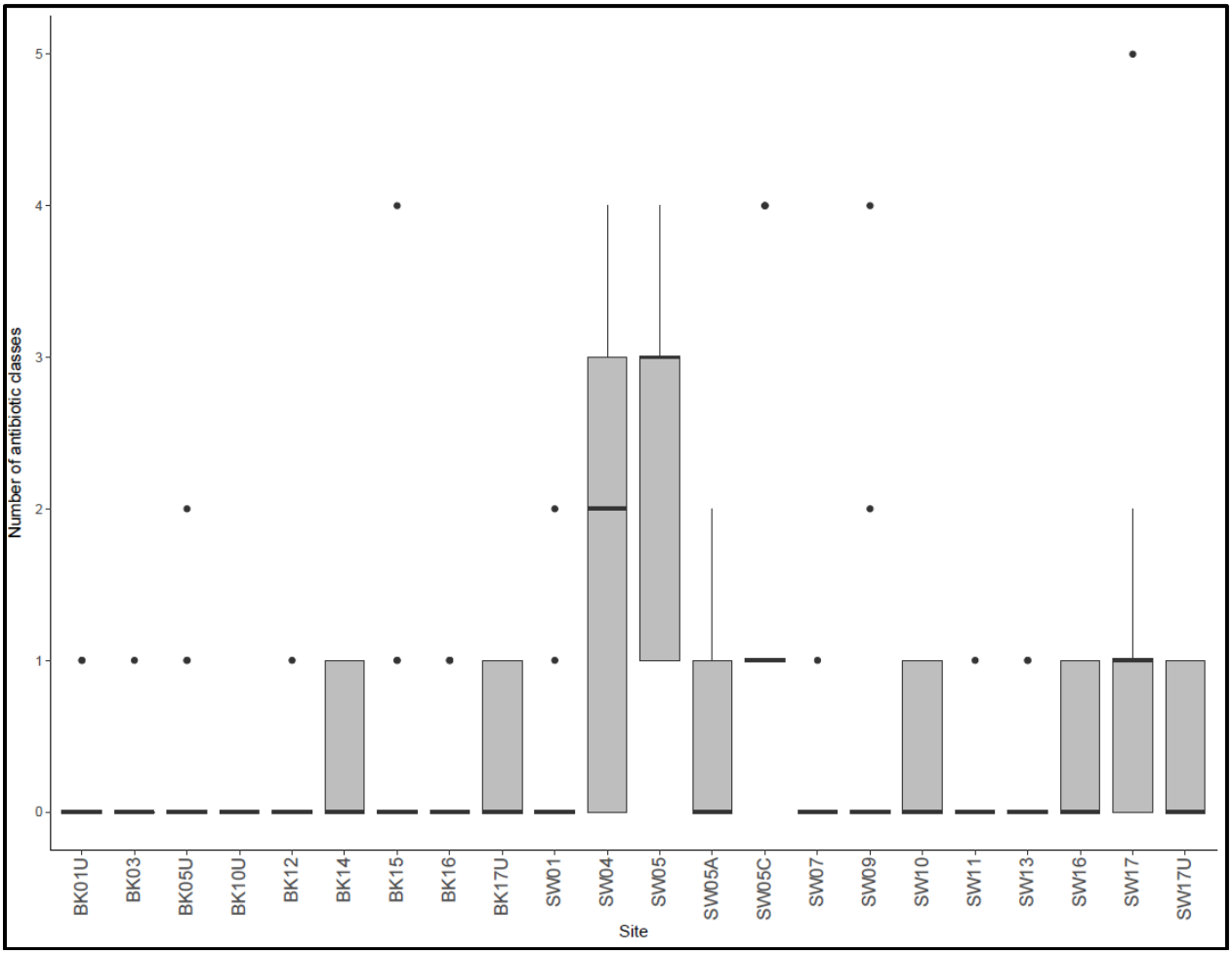


Figure 11. Boxplots of total number of antibiotic classes to which a sample was resistant for each site, with background (BK) sites listed before CHO-associated sites (SW).

Effects of Different Precipitation Regimes on *E. coli* Antimicrobial Resistance

Of 241 samples tested for antibiotic resistance, 219 of the sampling events had any rain in the prior seven days, 60 were runoff events, and 21 were first runoff events (Table 9). Among events with prior rain in the previous seven days, antibiotic resistance was observed in 82 (37.4%) of samples; in events with no prior rain, 7 (31.8%) of samples had antibiotic resistance. Though resistance to at least one antibiotic class was similar between prior and no prior precipitation in the past week, multi-drug resistance was observed in fifteen samples with prior rain, but in only one sample with no prior rain (Table 9). Furthermore, resistance to four and to five antibiotic classes was only observed in events with prior rain (Figure 12).

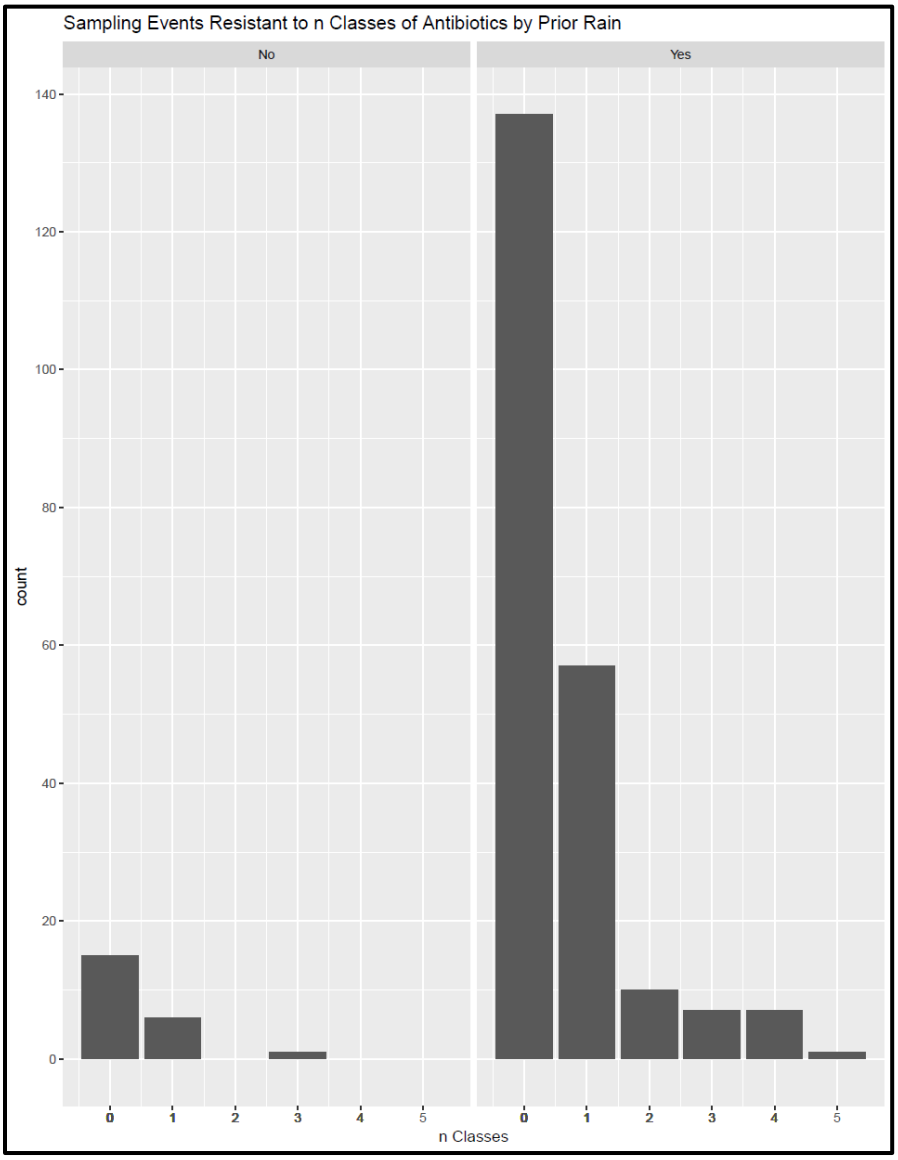
Among samples that were runoff events, 27 (45.0%) showed resistance while 62 (34.3%) of non-runoff events had resistance to at least one antibiotic class. Multi-drug resistance was present in 1.7% of runoff events and 8.3% of non-runoff events (Table 9). Resistance to four and five antibiotic classes was observed only in non-runoff events (Figure 13).

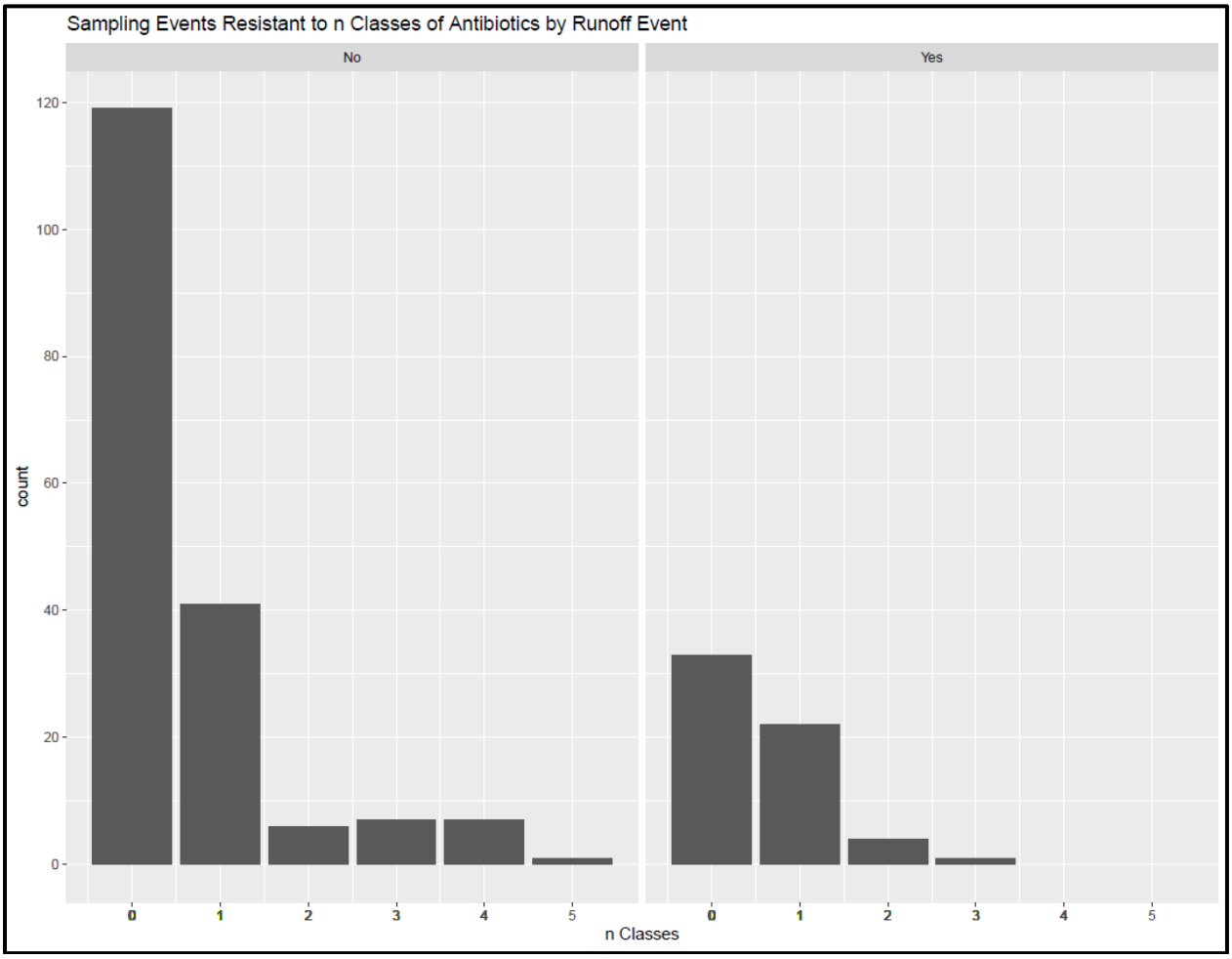
Over half (57.1%) of first runoff events were resistant to at least one antibiotic class, while 35.0% of non-first runoff events had any observed resistance (RR=2.28; 95% CI: 1.00, 5.19). Tetracycline resistance was also higher among first runoff events compared to non-events (RR=2.73; 95% CI: 1.20, 6.22) (Table 9). Similar to runoff events, multi-drug resistance was more frequent in non-first runoff events, especially resistance to four and five antibiotic classes (Figure 14).

Table 9. Number, percent, and relative risk of *E. coli* samples with observed resistance to number of antibiotic classes and tetracycline by prior rain in the last week, runoff event, and first runoff event.

	Antibiotic Resistance	Prior Rain n (%)	No Prior Rain n (%)	Relative Risk (95% CI)	Runoff Event n (%)	No Runoff Event n (%)	Relative Risk (95% CI)	First Runoff Event n (%)	No First Runoff Event n (%)	Relative Risk (95% CI)
	n samples	219	22	n/a	60	181	n/a	21	220	n/a
Number of Antibiotic Classes	Resistance to >= 1 Antibiotic Class	82 (37.4%)	7 (31.8%)	1.02 (0.94, 1.11)	27 (45.0%)	62 (34.3%)	1.40 (0.90, 2.16)	12 (57.1%)	77 (35.0%)	2.28 (1.00, 5.19)
	Resistance to >= 2 Antibiotic Class	25 (11.4%)	1 (4.6%)	1.07 (0.98, 1.16)	5 (8.3%)	21 (11.6%)	0.75 (0.33, 1.71)	3 (14.3%)	23 (10.5%)	1.38 (0.44, 4.36)
	Resistance to >= 3 Antibiotic Class	15 (6.9%)	1 (4.6%)	1.03 (0.90, 1.18)	1 (1.7%)	15 (8.3%)	0.24 (0.04, 1.61)	1 (4.8%)	15 (6.8%)	0.70 (0.10, 4.91)
Individual Antibiotic	Tetracycline (TE)	75 (34.3%)	4 (18.2%)	1.07 (0.99, 1.15)	25 (41.7%)	54 (29.8%)	1.46 (0.95, 2.27)	12 (57.1%)	67 (30.5%)	2.73 (1.20, 6.22)

When restricting to CHO-associated sites, neither antibiotic resistance to at least one class nor tetracycline resistance was substantially different by prior rain in the previous seven days, runoff events, or first runoff events (Table 10). However, in background sites, antibiotic resistance was higher in runoff events (RR=2.23; 95% CI: 1.13, 4.42) and tetracycline resistance was also higher in runoff events (RR=2.42; 95% CI: 1.23, 4.76) compared to non-runoff events. Tetracycline resistance was also substantially higher in first runoff events (RR=4.53; 95% CI: 1.26, 16.33) (Table 10).




Sampling Events Resistant to n Classes of Antibiotics by Runoff Event
No
Yes
count
120
100
80
60
40
20
0
0
1
2
3
4
5
n Classes

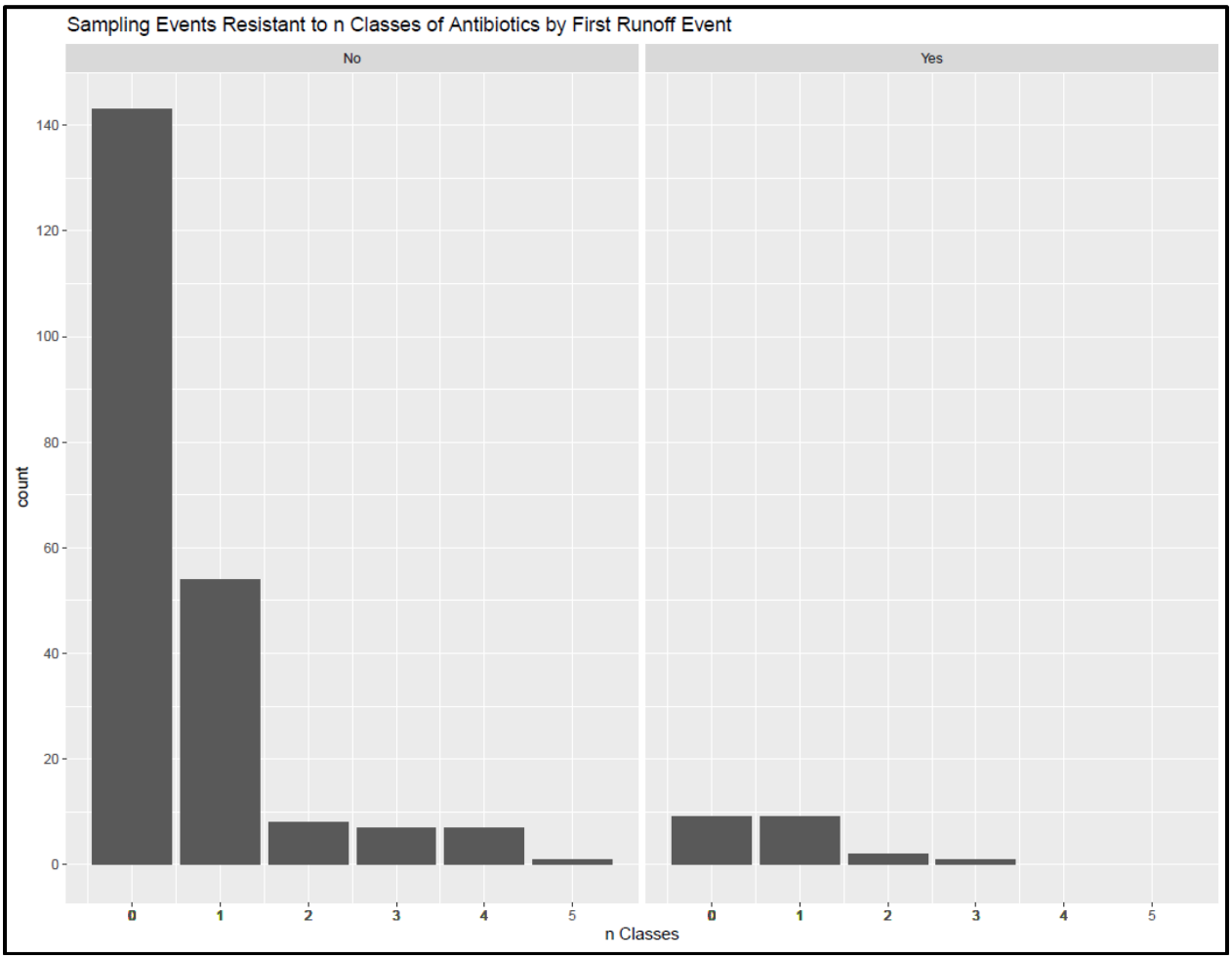

Sampling Events Resistant to n Classes of Antibiotics by First Runoff Event
No
Yes
count
140
120
100
80
60
40
20
0
0
1
2
3
4
5
n Classes

Table 10. Among Site Type (CHO-associated Samples or Background Samples), number, percent, and relative risk of *E. coli* samples with observed resistance to at least one antibiotic class and to tetracycline by prior rain in the last week, runoff event, and first runoff event.

CHO-associated Sites									
Antibiotic Resistance	Prior Rain n (%)	No Prior Rain n (%)	Relative Risk (95% CI)	Runoff Event n (%)	No Runoff Event n (%)	Relative Risk (95% CI)	First Runoff Event n (%)	No First Runoff Event n (%)	Relative Risk (95% CI)
n samples	136	11	n/a	37	110	n/a	13	134	n/a
Resistance to >= 1 Antibiotic Class	64 (47.1%)	4 (36.4%)	1.03 (0.94, 1.13)	18 (48.7%)	50 (45.5%)	1.10 (0.63, 1.92)	8 (61.5%)	60 (44.8%)	1.86 (0.64, 5.42)
Tetracycline (TE)	60 (44.1%)	2 (18.2%)	1.08 (0.99, 1.18)	17 (46.0%)	45 (40.9%)	1.17 (0.67, 2.04)	8 (61.5%)	54 (40.3%)	2.19 (0.75, 6.38)
Background Sites									
Antibiotic Resistance	Prior Rain n (%)	No Prior Rain n (%)	Relative Risk (95% CI)	Runoff Event n (%)	No Runoff Event n (%)	Relative Risk (95% CI)	First Runoff Event n (%)	No First Runoff Event n (%)	Relative Risk (95% CI)
n samples	83	11	n/a	23	71	n/a	8	86	n/a
Resistance to >= 1 Antibiotic Class	18 (21.7%)	3 (27.3%)	0.96 (0.79, 1.17)	9 (39.1%)	12 (16.9%)	2.23 (1.13, 4.42)	4 (50.0%)	17 (19.8%)	3.48 (0.95, 12.73)
Tetracycline (TE)	15 (18.1%)	2 (18.2%)	1.00 (0.82, 1.21)	8 (34.8%)	9 (12.7%)	2.42 (1.23, 4.76)	4 (50.0%)	13 (15.2%)	4.53 (1.26, 16.33)

Sampling events were analyzed by date to explore the impact of Hurricane Florence on antibiotic resistance, comparing twelve samples collected on September 24, 2018 (ten days after the hurricane) with all other sampling dates. Data for this analysis were restricted to a subset of twelve sites on the Southern Route comprising thirteen longitudinal sampling events as these were the only sites sampled both pre- and post-Hurricane Florence.

Ten days after Hurricane Florence, samples across all sites were observed to have resistance to a median number of zero antibiotic classes across sites. This was a slight reduction from the median of the previous sampling date on July 18, 2017, although this was over a year earlier. November 14, 2018, the second sampling date after Florence, also had a median resistance to zero antibiotic classes (Figure 15). These first two sampling dates after Hurricane Florence each had only one sample resistant to two antibiotic classes, which is a lower maximum number of class resistance than all other dates except for January 30, 2017, which had a maximum resistance to one class. This observed reduction in antibiotic resistance in sampling events immediately following Hurricane Florence may be attributable to a dilution effect for fecal indicator bacteria following major storms, at least aggregated across sites.

When comparing the post-Florence sample date to the twelve other sampling dates combined, there was a slight reduction in resistance to at least one antibiotic class following the hurricane (RR=0.89; 95% CI: 0.30, 2.69), but a slight increase in observed tetracycline resistance (RR=1.02; 95% CI: 0.34, 3.07). Among background sites, no samples displayed resistance to any antibiotic classes post-hurricane. However, in CHO-associate sites, there was a small increase in overall resistance (RR=1.48; 95%C CI: 0.37, 5.87) and in tetracycline resistance (RR=1.67; 95% CI: 0.42, 6.61). None of these fluctuations when comparing post-Florence to all other samples were statistically significant (Table 11).

Together, these results suggest that AMR in tested *E. coli* isolates does not appear driven by precipitation, unlike *E. coli* concentrations. Other temporal dynamics such as spray events or antibiotic use practices may better explain contributions of resistant bacteria to surface waters, but more data would be needed to test these associations.

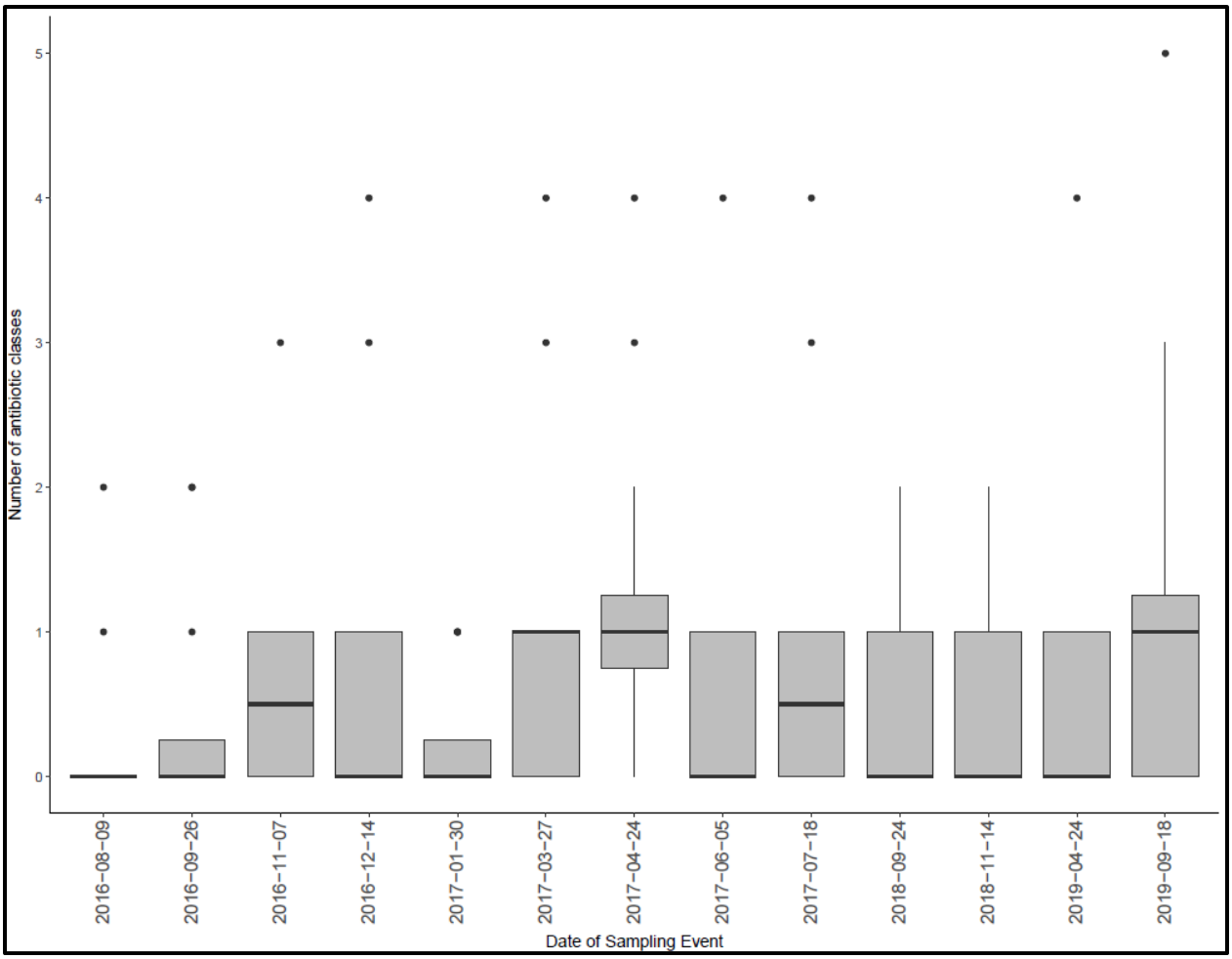


Figure 15. Boxplots of total number of antibiotic classes to which a sample was resistant for each sampling date. Sampling events are restricted to Southern Route sites where samples were collected both pre- and post-Hurricane Florence. The first sampling event ten days after Hurricane Florence was September 24, 2018.

Table 11. Among the subset of sites sampled after Hurricane Florence, number, percent, and relative risk of *E. coli* samples with observed resistance to at least one antibiotic class and to tetracycline, by Site Type (CHO-associated Sites or Background Sites), and by Sampling Date (immediately post-Florence or all other dates).

	CHO-associated Sites Southern Route			Background Sites Southern Route			All Southern Route Sites		
Antibiotic Resistance	Post-Florence (9/24/2018) n (%)	All other sampling dates n (%)	Relative Risk (95% CI)	Post-Florence (9/24/2018) n (%)	All other sampling dates n (%)	Relative Risk (95% CI)	Post-Florence (9/24/2018) n (%)	All other sampling dates n (%)	Relative Risk (95% CI)
n samples	8	94	n/a	4	47	n/a	12	141	n/a
Resistance to >= 1 Antibiotic Class	5 (62.5%)	49 (52.1%)	1.48 (0.37, 5.87)	0 (0.0%)	14 (29.8%)	n/a	5 (41.7%)	63 (44.7%)	0.89 (0.30, 2.69)
Tetracycline (TE)	5 (62.5%)	46 (49.0%)	1.67 (0.42, 6.61)	0 (0.0%)	12 (25.5%)	n/a	5 (41.7%)	58 (41.1%)	1.02 (0.34, 3.07)

Modeling Antibiotic Resistance

Individual predictors for precipitation, fecal exposure measured using the sum of exponential decay with distance interaction (lagoon, sprayfield, and household), and intensity metrics for each fecal source location (population density, manure density, and sprayfield acres) were individually included in a univariate logistic regression model predicting resistance to at least one antibiotic class. Variables for site type (CHO-associated sites), 24-hour prior precipitation, manure density, and all three exposure variables were statistically significant (Table 12). Without controlling for other variables, the odds of resistance to at least one antibiotic class were 2.99 times in sites associated with CHOs, 2.27 times in samples with 24-hour prior precipitation, and 1.46 times in samples with human septic (household) exposure (Table 12).

Table 12. Univariate Logistic Regression for Resistance to at least One Antibiotic Class.

Variable	Odds Ratio (95% CI)	Std. Error	Z value	Pr(>|z|)
CHO-associated site	2.99 (1.67, 5.36)	0.30	3.68	2.33E-04***
24-hr prior precipitation	2.27 (1.06, 4.82)	0.39	2.12	0.03*
48-hr prior precipitation	1.34 (0.87, 2.06)	0.22	1.35	0.18
Precipitation prior 7 days	0.94 (0.77, 1.14)	0.10	-0.66	0.51
Runoff event	1.57 (0.87, 2.84)	0.30	1.49	0.14
First runoff event	2.48 (1.00, 6.14)	0.46	1.96	0.05
Population density	1.01 (1.00, 1.01)	3.09E-03	1.94	0.05
Manure density	1.00 (1.00, 1.00)	7.33E-07	2.23	0.03*
Sprayfield Acres	1.00 (1.00, 1.00)	1.14E-03	1.35	0.18
Lagoon exposure	1.00 (1.00, 1.00)	6.49E-08	5.00	5.61E-07***
Sprayfield exposure	1.00 (1.00, 1.00)	2.99E-08	4.51	6.48E-06***
Household exposure	1.46 (1.28, 1.66)	0.07	5.67	1.45E-08***

***p<0.001, **p<0.01, *p<0.05

Variables from the univariate logistic regression models were pulled into a multiple logistic regression model for resistance to at least one antibiotic class. The variable for 24-hour prior precipitation was correlated with the variables for 48-hour prior precipitation, precipitation in the prior seven days, and runoff events, so these three variables were not included in the multiple logistic regression model. Manure density, sprayfield acres, and sprayfield exposure were removed due to their correlation with lagoon exposure, and population density was removed due to its correlation with household exposure. Although a first runoff event was not statistically significant in the univariate model, it was considered an important environmental variable for precipitation and included in the final multiple logistic regression model predicting resistance to at least one antibiotic class. The variable accounting for site type (CHO-associated vs background site) was statistically significant in the univariate model, however when incorporated with other predictors, it was no longer significant and resulted in a higher AIC value.

The final model includes variables for 24-hour prior precipitation, first runoff event, lagoon exposure, and household exposure. The AIC value for this multiple logistic regression model is 269.9, which is lower than when incorporating additional predictors from the univariate models, suggesting this is a more parsimonious model. In the model where first runoff event was removed, the AIC value slightly increased to 270.68, suggesting worse model fit than the model including this predictor. When assessed for predictive accuracy, the final model predicts resistance to at least one antibiotic class with 76.76% accuracy.

Results from the model demonstrate that human septic (household) exposure significantly contributes to antimicrobial resistance in surface water in agricultural watersheds with and without CHO impacts. The odds of resistance to at least one antibiotic class in samples with human septic (household) exposure is 1.38 times the odds in unexposed samples (Table 13).

Table 13: Multiple Logistic Regression for Resistance to at Least One Antibiotic Class.

Variable	Odds Ratio (95% CI)	Std. Error	Z value	Pr(>|z|)
24-hr prior precipitation	1.67 (0.72, 3.88)	0.43	1.192	0.23343
First runoff event	2.43 (0.86, 6.88)	0.53	1.676	0.09377
Lagoon exposure	1.00 (1.00-1.00)	0.00	3.23	0.00124**
Household exposure	1.38 (1.20, 1.59)	0.07	4.507	6.58E-06***

***p<0.001, **p<0.01, *p<0.05

Next, univariate logistic regression models were run to predict resistance to the tetracycline class. Individual predictors for precipitation, fecal exposure measured using the sum of exponential decay with distance interaction (lagoon, sprayfield, and household), and intensity

metrics for each fecal source location (population density, manure density, and sprayfield acres) were individually included in a univariate model. Variables for 24-hour prior precipitation, first runoff events, population density, and all three exposure variables were statistically significant (Table 14).

Without controlling for other variables, the odds of resistance to the tetracycline class were 2.71 times in samples with 24-hour prior precipitation, 3.04 times for first runoff events, and 1.48 times in samples with human septic (household) exposure (Table 14).

Table 14: Univariate Logistic Regression for Resistance to Tetracycline Class.

Variable	Odds Ratio (95% CI)	Std. Error	Z value	Pr(>|z|)
CHO-associated site	3.30 (1.78, 6.13)	0.3853	2.122	0.0338
24-hr prior precipitation	2.71 (1.23, 5.96)	0.40	2.48	0.01*
48-hr prior precipitation	1.49 (0.96, 2.30)	0.22	1.80	0.07
Precipitation prior 7 days	0.92 (0.74, 1.13)	0.11	-0.82	0.41
Runoff event	1.68, (0.92, 3.07)	0.31	1.68	0.09
First runoff event	3.04 (1.22, 7.57)	0.46	2.40	0.02*
Population density	1.01 (1.00, 1.01)	3.11E-03	2.17	0.03*
Manure density	1.00 (1.00, 1.00)	7.41E-07	2.20	0.03*
Sprayfield acres	1.00 (1.00, 1.00)	1.16E-03	1.57	0.12
Lagoon exposure	1.00 (1.00, 1.00)	6.60E-08	5.33	9.91E-08***
Sprayfield exposure	1.00 (1.00, 1.00)	2.96E-08	4.58	4.75E-06***
Household exposure	1.48 (1.30, 1.69)	0.07	5.98	2.23E-09***

***p<0.001, **p<0.01, *p<0.05

Variables from the univariate logistic regression models were pulled into a multiple logistic regression model for resistance to tetracycline. The final model includes variables for 24-hour prior precipitation, first runoff event, lagoon exposure, and household exposure. The AIC value for this multiple logistic regression model is 248.07, which was preferable to alternatives when

adding additional predictors into the model. When assessed for predictive accuracy, this final model predicts tetracycline resistance with 81.33% accuracy.

Results from the model demonstrate that human septic (household) exposure significantly contributes to tetracycline resistance in surface water in agricultural watersheds with and without CHO impacts. The odds of resistance to the tetracycline class in samples with human septic (household) exposure is 1.41 times the odds in unexposed samples (Table 15).

Table 15: Multiple Logistic Regression for Resistance to Tetracycline Class.

Variable	Odds Ratio (95% CI)	Std. Error	Z value	Pr(>|z|)
24 hr prior precipitation	1.98 (0.83, 4.96)	0.44	1.54	0.12
First runoff event	3.07 (1.06, 8.85)	0.54	2.08	0.04
Lagoon exposure	1.00 (1.00, 1.00)	7.32E-08	3.47	5.17E-04***
Household exposure	1.41 (1.22, 1.62)	0.07	4.72	2.34E-06***

***p<0.001, **p<0.01, *p<0.05

Conclusions and Recommendations

- Major storm events can increase levels of human and animal fecal contamination in surface waters depending on proximal land use. This is a concern in rural North Carolina where wastewater lagoon systems are prevalent, and aging or malfunctioning septic systems may be an unrecognized issue. Efforts are needed to fortify wastewater infrastructure against storms.
- Fecal contamination measured by *E. coli* concentrations occurs across a range of precipitation events, with elevated risks of contamination following first runoff events and not necessarily proportional to amount of rainfall for very large events. Major storms such as Hurricane Florence may be associated with dilution effects for general fecal indicator bacteria such as *E. coli*. Over time, *E. coli* contamination was driven by first runoff events and by higher levels of exposure from CHOs and households.
- Presence of commercial hog operations (CHOs) are associated with increased *E. coli* concentrations in surface water. Additionally, watersheds with larger CHOs and CHOs closer to sampling sites, as well as sites with larger number of households closer to sampling sites, had increased contamination. Distance between CHOs and streams, and households and streams, appear important in spatial models.
- High concentrations of microbial source tracking markers after hurricane events may be indicative of infrastructure repair needs. Detection of general and specific fecal indicators suggest potential presence of pathogens that could risk human or animal health in floodwaters.
- Antimicrobial resistance (AMR) is increased in *E. coli* isolated from surface waters downstream from commercial hog operations compared to background, and the trend does not appear to be driven by precipitation, unlike *E. coli* concentrations. Other temporal dynamics such as spray events or antibiotic use practices may better explain contributions of resistant bacteria to surface waters, but more data would be needed to test these associations.
- The most common antibiotic to which resistance was observed was tetracycline, which is used in animal and human medicine. Resistance was less commonly observed to antibiotics deemed highest priority critical by WHO guidelines (e.g. fluoroquinolones), Resistance to these antibiotics was rarely observed in sites downstream from CHOs and not at all in background sites. Care should be taken in the antibiotic stewardship of these and all antibiotics to reduce spread of antimicrobial resistance.
- The role of the environment in the spread of antibiotic resistance needs to be better studied, particularly downstream of animal production and other healthcare or industrial facilities that use or produce antibiotics. The growing threat of antimicrobial resistance provides an extra and urgent incentive to reduce fecal contamination of environmental waters and to decrease exposures to antibiotic resistance bacteria. Research is also needed to characterize risks of environmental AMR to human and animal health.

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