Characterizing Relationships Among Fecal Indicator Bacteria, Microbial Source Tracking Markers, and Associated Waterborne Pathogen Occurrence in Stream Water and Sediments in a Mixed Land Use Watershed

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ABSTRACT

Bed sediments of streams and rivers may store high concentrations of fecal indicator bacteria (FIB) and pathogens. Due to resuspension events, these contaminants can be mobilized into the water column

and affect overall water quality. Other bacteria indicators such as microbial source tracking (MST) markers, developed to determine potential sources of fecal contamination, can also be resuspended from bed sediments. The primary objective of this study was to predict occurrence of waterborne pathogens in water and streambed sediments using a simple statistical model that includes traditionally measured FIB, environmental parameters and source allocation, using MST markers as predictor variables. Synoptic sampling events were conducted during baseflow conditions downstream from agricultural (AG), forested (FORS), and waste water pollution control plant (WPCP) land uses. Concentrations of FIB and MST markers were measured in water and sediments, along with occurrences of the enteric pathogens *Campylobacter*, *Listeria* and *Salmonella*, and the virulence gene that carries Shiga toxin, *stx₂*. Pathogens were detected in water more often than in underlying sediments. Shiga toxin was significantly related to land use, with concentrations of the ruminant marker selected as an independent variable that could correctly classify 76% and 64% of observed Shiga toxin occurrences in water and sediment, respectively. FIB concentrations and water quality parameters were also selected as independent variables that correctly classified Shiga toxin occurrences in water and sediment (54% to 87%), and *Salmonella* occurrences in water (96%). Relationships between pathogens and indicator variables were generally inconsistent and no single indicator adequately described occurrence of all pathogens. Because of inconsistent relationships between individual pathogens and FIB/MST markers, incorporating a combination of FIB, water quality measurements, and MST markers may be the best way to assess microbial water quality in mixed land use systems.

Keywords: Fecal Indicator Bacteria; Microbial Source Tracking Markers; Recursive Partitioning; Waterborne Pathogens; Sediment

1. Introduction

Several species of bacteria such as *Escherichia coli* and enterococci are commonly used as indicators of fecal contamination in surface waters because they are abundant, easy to culture, and widely available in the intestinal flora of warm-blooded vertebrates. They may not directly cause human gastrointestinal illness, but occurrence of illness often correlates with high concentrations of fecal indicator bacteria (FIB) (USEPA, 1984; 1986). The waterborne pathogens that directly cause illness are generated by a variety of hosts and are notoriously difficult to quantify due to low numbers in the environment or the presence of viable but non-culturable (VBNC) bacteria (Bridle, 2013). To manage pathogens within watersheds, it is critical to know their level of occurrence and their sources. Pathogens are more difficult to detect or predict than FIB, however, due to their complex, variable occurrence in the environment, and which leads to the additional expense of pathogen-specific assays (Wilkes et al., 2009).

Fecal bacteria enter surface water through various pathways on a continual basis or episodically during storm events; their sources can include direct discharges from wastewater treatment plant outfalls and direct defecation by livestock and wildlife into streams. Fecal bacteria contamination is typically higher during storms due to upland runoff from areas containing manure fertilizers, depositional manure (e.g., grazing pastures), combined stormflow systems, and failed onsite wastewater treatment systems. Elevated counts of *E. coli* have also been found in fresh water beaches during prolonged dry-weather periods (McLellan and Salmore, 2003) which potentially increase human health risk for recreational and drinking water uses when there has not been recent rainfall.

Naturalized communities of FIB are found in soils and stream bed sediments (Ishii et al., 2006; Litton et al., 2010; Surbeck et al., 2010), but there is mixed evidence about whether the same is true of waterborne pathogens (Burton et al., 1987; Gordon et al., 2002; Hendricks, 1971; Whitman et al., 2006). Fecal bacteria can accumulate and survive for extended periods in stream sediments (Alm et al., 2003;

Burton et al., 1987; Davies et al., 1995; Hendricks, 1971; LaLiberte and Grimes, 1982); they are resuspended in the water column during high flows and by mechanical disturbance of the sediment (Cervantes, 2012; Droppo et al., 2009; McDonald et al., 1982; Muirhead et al., 2004; Yakirevich et al., 2013). The mechanisms for increased survival in stream sediments include protection from grazing protozoa (Davies et al., 1995), protection from UV light (Garzio-Hadzick et al., 2010), and increased availability of nutrients and soluble organic matter in near-surface sediments (Cho et al., 2010; Jamieson et al., 2005a; Jamieson et al., 2005b).

Recent studies have demonstrated that bed sediments can be an additional FIB source to the water column during storms, as reported by Cho et al. (2010), Garzio-Hadzick et al. (2010), and Yakirevich et al. (2013). Re-entrainment of bed sediments typically occurs during a few storm events each year when the critical shear stress threshold of the stream bottom sediments is exceeded and bedload movement activated (Cho et al., 2010; Jamieson et al., 2005b). For example, Cho et al. (2010) showed that streambed *E. coli* “hotspots” were resuspended during artificial high flows and propagated along a 630-m section of stream without substantial attenuation. Resuspension also occurred during lower flow with physical disturbance of the sediment such as cattle crossings (Grant et al., 2011; Muirhead et al., 2004). Longer survival times in sediments, coupled with hydrological and mechanical processes that re-entrain sediment-associated fecal contaminants, can increase human health risk from waterborne pathogens stored in sediments.

Microbial source tracking (MST) methods have been developed to improve the ability to detect microbes that are specific to an animal host in water and sediments and thus, unlike FIB, can discriminate between different sources of fecal contamination. In a review of 41 MST methods conducted by multiple laboratories, Boehm et al. (2013) listed the human (HF183 and HF183SYBER), ruminant (Rum-2-Bac), and cow (CowM3) markers as being among the most sensitive, specific assays developed to date for detecting target and non-target markers in fecal samples. Strong correlations

between *E. coli* and the general (Dick and Field, 2004; Mulugeta et al., 2012) and ruminant-specific (Frey et al., 2013; Oladeinde et al., 2014) *Bacteroidales* MST markers have also been shown, which supports their use as indicators for sources of fecal bacteria. *Bacteroidales* are anaerobic bacteria commonly found in fecal sources; presence of *Bacteroidales* MST markers in aquatic environments reflects repetitive fecal inputs since they have limited survival under aerobic conditions (Bae and Wuertz, 2009; Lee et al., 2008), making them ideal for microbial fate and transport investigations.

The primary objective of this study was to predict occurrence of waterborne pathogens in water and streambed sediments during dry-weather periods, when the probability that people will be recreating in a body of water is higher, using a simple statistical model that includes traditionally-measured FIB, environmental parameters, and source allocation using MST markers as predictor variables. To address this objective, we (i) determined prevalence of four important waterborne pathogens in water and sediments: *Campylobacter* spp., *Listeria* spp., *Salmonella* spp., and *stx₂*, a toxin carried by the virulence gene of a prophage in shiga toxin producing *E. coli* (STEC); (ii) quantified FIB in water and stream sediments using culture-based methods and qPCR assays; (iii) quantified three MST markers (cattle, ruminant, and human) in water and sediments; and (iv) examined relationships between pathogens and easily measured water quality variables, FIB concentrations, and MST marker concentrations in water and sediments. We used classification analyses (i.e., recursive partitioning) and logistic regression to determine if relationships between FIB, fecal source, and water quality parameters could adequately predict occurrence of pathogens in water and sediments during dry-weather conditions.

2. Materials and methods

2.1. Study locations

Water and sediment samples were collected from 15 locations in the South Fork Broad River (SFBR) watershed located in northeast Georgia (Fig. 1). Sites were selected by determining their

predominant land use with the National Land Cover Database (NLCD) (Fry et al., 2011) and on-the-ground surveys. Six sites were designated as agricultural (AG); seven sites as forested (FORS); and two sites as impacted by water pollution control plants (WPCP). The WPCP sites receive discharges from National Pollutant Discharge Elimination System (NPDES)-permitted facilities immediately upstream of the sampling point. The NLCD land cover distribution for each sampling location is summarized in Table 1. Water drains from North to Southeast in the SFBR watershed.

2.2. *Water and sediment collection*

Water and sediment samples were collected every other week over a four-week period in October and November, 2012; and every other week over a six-week period in May and June, 2013; and November and December, 2013. Each site was sampled eight times. Sediment samples were collected by pressing a sterile acrylic tube (i.d. 5 cm) approximately 15 cm into the sediment, while maintaining suction on the tube's top with a gloved hand as the core was pulled from the streambed. Water above the sediment core was carefully poured from the tube and the top 5 cm of the core was placed in a sterile bowl. This procedure was repeated eight to ten times throughout the channel cross-section to obtain a representative sample. Once all cores for the cross-section were collected, sediments were homogenized with a sterile spoon and homogenized samples were transferred to a sterile 1L container and placed on ice until delivered to the laboratory within 6 h.

Water samples were collected in the center of the stream by dipping a sterile 1 L bottle into the stream following Standard Method 1060 (APHA, 1998); a total of 4 L of water was collected and placed on ice until delivered to the laboratory within 6 h. Conductivity, pH, dissolved oxygen (DO), and temperature of the water were measured in-situ with a YSI multi-parameter meter (YSI Inc., Yellow Springs, OH).

2.3. *Laboratory methods*

Water samples were analyzed for *E. coli* using the defined substrate methodology, as described in the IDEXX Colilert assay (IDEXX Laboratories, Westbrook, ME). They were analyzed for enterococci by membrane filtration following USEPA 1600 (USEPA, 2006). Concentrations of FIB in water samples were reported as MPN 100 mL⁻¹ and cfu 100 mL⁻¹ for *E. coli* and enterococci, respectively. Duplicate analyses were performed on 10% of the dilutions for the *E. coli* assay and on each dilution for the enterococci assay. The detection limit for *E. coli* and enterococci assays was 1 MPN or CFU 100 mL⁻¹. To extract FIB from sediment samples, 100 g of wet sediment was mixed with 100 mL of sterile water to make a sediment slurry (1:1 m/v), shaken for 5 min, then poured through sterile cheesecloth to filter out debris. A second 100 mL of sterile water was added to the sediment, shaken for 5 min, and poured through cheesecloth. *E. coli* was measured on the sediment slurry using the MPN-based IDEXX Colilert assay and enterococci was measured using USEPA 1600. Duplicate analyses were performed on all sediment dilutions. Sediment dry weight (d.w.) was determined gravimetrically by drying 10 g of sediment overnight at 110°C; FIB concentrations were reported as MPN 100 g⁻¹ d.w. and cfu 100 g⁻¹ d.w. for *E. coli* and enterococci, respectively.

Total suspended solids (TSS) were measured on water samples and sand content was measured on composite bed sediment samples. The samples were enriched and analyzed for presence of *Campylobacter*, *Listeria*, *Salmonella*, and the virulence gene that carries Shiga toxin (*stx*₂). Markers for FIB (*E. coli*, enterococci, and *Bacteroidales*) and MST markers for cattle, ruminants, and humans were also quantified (Table 2). See “Supplementary Methods” for a detailed description of these procedures. Pathogen occurrences were evaluated in water and sediment samples (n = 90 to 120) collected between October 2012 and December 2013. We attempted to quantify *Salmonella* directly in water and sediment but were unable to detect the pathogen. To avoid false negative determinations, an enrichment protocol was implemented for all pathogens. Enrichments for *Salmonella* in water began during the third week of sampling in November 2012 (n = 105) and *Salmonella* in sediment during the first week of

sampling in May 2013 (n = 90). We added the analysis for stx2 on the first week of sampling in May 2013 (n = 90 for water and sediment, respectively). Water quality affects the viability and survivability of fecal indicator bacteria and waterborne pathogens, so TSS, DO, temperature, pH, and conductivity measurements were collected to supplement other indicator variables in statistical models for predicting pathogen occurrence.

2.4. Statistical methods

Concentrations of FIB and MST markers were log-transformed by taking the base-10 logarithm. Non-detects for FIB were given a log transformed value of 0 (i.e., the detection limit of 1 cfu or MPN 100 mL⁻¹). Markers that were undetected or below the limit of quantification (LOQ) were assigned the value of LOQ/√2 (see Supplemental Materials for details). Quantification limits for MST markers are shown in Table 2. Significant differences among land uses were tested for using a Kruskal-Wallis test and a significance level of $\alpha = 0.05$. Bonferroni post-hoc analysis was used for pairwise comparisons between land use categories ($\alpha = 0.05$). Pathogens were assigned binary values (present (1) or absent (0)). Contingency tables were generated in R (R Core Team, 2012) using the “gmodels” package (Warnes, 2013) to determine overall occurrence of pathogens from the sample population and relative to upstream land use. Fisher’s exact test was then used to test pathogen dependence on land use category. Correlations between pathogens, FIB, and MST markers were investigated by Spearman’s rank correlations, using R with the “Hmisc” package (Harrell Jr. and Dupont, 2013).

Recursive partitioning was used to develop classification trees that predict pathogen presence with independent variables; the R “rpart” package was used in this study (Therneau et al., 2013). Recursive partitioning is a nonparametric data-mining method that splits dependent variables into nodes (presence and absence), based on independent variable splitting criteria. All possible independent variables can be included in the initial setup of the model and the algorithm will choose independent variables that best describe the data. Independent variables are assigned a threshold value (i.e., the

splitting criteria) in which splits “greater than” or “less than” a specific value lead to a final prediction of pathogen presence or absence. The “rpart” default Gini data-splitting method was used. When a split occurs, the Gini method puts the largest dependent variable class into the purest node and the rest in the other. An improvement score associated with each split defines how pure the split was, with the highest purity scores corresponding to the most homogeneous grouping of data into a node.

To determine the odds ratio for each variable, logistic models were fit to the independent variables from the best recursive partition model for each pathogen using the R “stats” package (R Core Team, 2012). The Variance Inflation Factor (VIF) was computed for each model to check for multicollinearity (Fox and Weisberg, 2011).

3. Results

3.1 Pathogen detections

Waterborne pathogens were widely detected in both water and sediments, but more often in water. Pathogen detection rates for each land use are shown in Table 3. Shiga toxin, *Listeria*, and *Salmonella* were detected in 61%, 70%, and 76% of water samples and in 43%, 68%, and 51% of sediment samples, respectively; *Campylobacter* was detected in 33% of water samples and 8% of sediment samples. *Campylobacter* and Shiga toxin occurrences in water varied by land use ($p < 0.05$), but *Listeria* and *Salmonella* were ubiquitous in both water and sediment samples in all three land uses.

3.2 FIB and MST marker concentrations in water and sediments

Average culturable and molecular FIB concentrations were generally higher in water and sediment in AG land uses, except for culturable enterococci in sediment (Fig.2). High concentrations of *E. coli* in sediment may indicate persistence of viable, culturable *E. coli* in streambed sediments. Low concentrations of culturable enterococci in sediment may indicate that viable, culturable enterococci do not persist in sediment (Li et al., 2014).

Overall, average cattle and ruminant MST markers were higher in AG land uses; average MST marker concentrations for each land use are shown in Fig. 3. Average Rum-2-Bac MST markers (Fig. 3a) in water and sediment were significantly different between all three land uses. Average CowM3 concentrations (Fig. 3b) were significantly higher in water and sediment in AG sites, compared to FORS and WPCP sites. Average HF183/HF183MGB concentrations (Fig. 3c) were not significantly different in water or sediment among the three land uses.

3.3 Spearman rank correlations with pathogen occurrence

3.3.1 FIB correlations

FIB may indirectly indicate a potential risk of exposure to pathogens, but poorly indicate the presence of specific pathogens which is partially supported by this study because enterococci and *E. coli* were not always significantly correlated with pathogen occurrences (Supplemental Table 1). *Bacteroidales* in water were also not significantly correlated to any measured pathogens, however, culturable *E. coli* in water were positively correlated with two pathogens: Shiga toxin occurrence in water and *Campylobacter* occurrence in sediment. The number of culturable *E. coli* in sediment was significantly positively correlated with only *Campylobacter* occurrence in sediment. Culturable enterococci in sediment were negatively correlated with Shiga toxin occurrence in water. *Bacteroidales* in sediment, however, were positively correlated with Shiga toxin in water and sediment, *Salmonella* in water, and *Campylobacter* in sediment.

3.3.2 MST marker correlations

MST markers may indicate upland sources of potential pathogens. The Rum-2-Bac and CowM3 markers used in this study were positively correlated with occurrence of Shiga toxin in both water and sediment. *Campylobacter* in water was also positively correlated with the CowM3 and Rum-2-Bac markers in sediment, and *Campylobacter* in sediment was correlated with the CowM3 marker in sediment. The HF183 marker in water did not significantly correlate with any pathogens detected in

water and sediment, but the HF183 marker in sediment was positively correlated with occurrence of *Listeria* in sediment.

3.3.3 Stream water quality measurements and percent bed sand correlations

Spearman correlations between water quality parameters and pathogens were used to investigate potential relationships. Water temperature was negatively correlated with occurrences of *Campylobacter* in water and Shiga toxin in water and sediment, but positively correlated with occurrence of *Salmonella* in water. *Campylobacter* in water was the only pathogen correlated with TSS. Shiga toxin in water was negatively correlated with pH and conductivity, and positively correlated with DO; Shiga toxin in sediment was also negatively correlated with pH. And finally, Shiga toxin was positively correlated with percentage of sand in the streambed.

3.4 Recursive partitioning to classify pathogen occurrence using FIB, MST, and WQ variables as independent variables

3.4.1 Classification trees for pathogens in water

The independent variables shown in Supplementary Materials Table 1 were used as predictors for classifying individual pathogen occurrence in water and sediment. They were divided into four parameter sets: 1) all Parameters (combined at once), 2) culturable FIB and WQ variables, 3) qPCR quantified FIB and WQ variables, and 4) MST host-specific markers. Recursive partitioning analysis was conducted on each pathogen, using each parameter set. It should be noted that log transformation of FIB and MST data is not required for classification analyses, but was done so to more easily present the results. The best models were selected by the prediction score which is the sum of correctly classified pathogen presences (P) and absences (A). Results from the classification analysis on pathogens in water and sediment are shown in Tables 4 and 5, respectively; classifications for pathogens that did not result in splits are not included.

The model with the highest prediction score for Shiga toxin in water split the “all variables” dataset using the Rum-2-Bac concentration in sediment and water temperature as predictors. The model correctly predicted 100% of presence and 54% of absence of Shiga toxin. Shiga toxin was present when the Rum-2-Bac concentration in sediment was $> 4.5 \log \text{TSC } 100 \text{ g}^{-1}$ and temperature was $< 16.3^{\circ}\text{C}$. Models using FIB split the data using temperature and the culturable log *E. coli* concentration (for the culturable FIB independent variable set) and log concentration of GenBac in sediment (for the qPCR quantified FIB independent variable set). Both models predicted 87% of presence and 66% of absence of Shiga toxin. The model using MST host-specific markers split the data, using the log Rum-2-Bac sediment concentration. Shiga toxin was present in water when the Rum-2-Bac concentration in sediment was $> 4.5 \log \text{TSC } 100 \text{ g}^{-1}$.

Salmonella in water had the highest prediction score, using D.O., pH and the log culturable *E. coli* concentration in water from the parameter set that included culturable FIB and WQ variables (Table 4). The best classification model for *Salmonella* correctly predicted 96% of presence and 48% of absence in water. *Salmonella* was present in water in 100% of samples when D.O was $< 11.3 \text{ mg L}^{-1}$. The MST host-specific markers did not minimize the cross-validated error in the recursive partition model for *Salmonella* and, therefore, no splits were made.

The model with the highest prediction score for *Campylobacter* in water was split only when using the CowM3 sediment concentrations in the MST host-specific parameter set. *Campylobacter* was present in 13% of the samples when the CowM3 marker in sediment was $> 6.4 \log \text{TSC } 100 \text{ g}^{-1}$. While *Campylobacter* was correctly classified as absent in 100% of the samples, the false negative rate of 84% indicated poor model performance in predicting occurrence of *Campylobacter*. All other independent parameter sets failed to reduce the model’s cross-validated error and no splits occurred. Similarly for *Listeria* in water, all independent parameter sets failed to reduce the cross-validated error in the respective models and no splits occurred. WPCP sites were in rural areas (Table 1) and included some

upstream agricultural lands (i.e., 39% and 21% AG at WPCP1 and WPCP2, respectively) which likely were the source for cattle and ruminant markers in those systems and potentially, the presence of *Campylobacter*.

3.4.2 Classification trees for pathogens in sediment

The model with the best prediction score for classifying Shiga toxin in sediment split the culturable FIB and WQ parameters using temperature, TSS and the log *E. coli* concentration in sediment, and correctly classified 79% of presence and 80% of absence (Table 5). Rum-2-Bac was an important split parameter for the host-specific MST marker model, indicating ruminants are likely sources of fecal contamination, however, the percentage of correctly classified occurrences decreased 15% when compared to the best model. In the models using all parameters and FIB (qPCR) and WQ parameters, Shiga toxin was present in 54% of samples where the concentration of GenBac in sediment was > 7.1 log TSC 100 g⁻¹.

The models with the highest prediction score for classifying *Salmonella* in sediment used splits on temperature and qPCR *E. coli* and enterococci sediment concentrations; this model correctly predicted 70% and 80% of presences and absences, respectively (Table 5). The model that included culturable FIB and WQ parameters only selected splits based on water temperature and pH, and did not use FIB concentrations in either sediment or water to classify *Salmonella*. The model for predicting *Salmonella* with host-specific MST markers failed to minimize the cross-validated error and no splits occurred. Similarly, all models tested for classifying *Campylobacter* and *Listeria* in sediment failed to minimize cross-validated error and resulted in zero splits.

3.5 Logistic regression to determine odds ratios for pathogen presence using independent variables from recursive partition models with highest prediction scores

The odds ratios (OR) for independent variables used to predict pathogen presences in recursive models with the highest prediction scores discussed in section 3.4 are shown in Table 6. OR values > 1

indicate increased odds of pathogen presence, while OR values < 1 indicate decreased odds of pathogen presence. Overall, independent variables with the largest ORs were the log Rum-2-Bac concentration in sediment, the log qPCR and culturable *E. coli* concentrations in sediment, the log CowM3 concentration in sediment, and the log culturable *E. coli* concentration in water, indicating that increasing concentrations of these parameters increases the odds of the respective pathogens' presence. Unit increases in these parameters corresponded to increased odds of the respective pathogen's presence ranging from a factor of 1.27 to 3.10. In some cases, a unit increase in FIB concentration (e.g., enterococci in sediment) corresponded to decreased odds of pathogen presence (e.g., *Salmonella* in sediment), indicating that higher concentrations of FIB do not indicate higher odds of *Salmonella*, and possibly presence of other pathogens'. The VIFs for the independent variables in Table 6 were all < 5 which indicated acceptable collinearity among variables within each model. For MST host-specific markers included in the logistic regression analysis, unit increases in log CowM3 and log Rum-2-Bac concentrations in sediment increased the odds of pathogen presence by factors of 1.34 and 2.29, respectively. Water temperature had mixed effects on the odds of Shiga toxin and *Salmonella* presence; unit increases in water temperature increased the odds of *Salmonella* presence in sediment, but decreased those of Shiga toxin presence in water.

4. Discussion

In agreement with previous research, our study shows that high FIB concentrations do not necessarily indicate presence of specific waterborne pathogens because recursive partitioning thresholds indicated less frequent pathogen occurrence when FIB concentrations were higher, and an OR < 1 for a unit increase in FIB concentrations (Jokinen et al., 2012; National Research Council, 2004; Pachepsky et al., 2006). The MST markers for cattle and ruminants, however, successfully predicted occurrence of shiga toxin in water and sediments, and also indicated the possible source of fecal contamination. In some cases, the suite of independent variables selected for this study did not

adequately describe pathogen presence (e.g., *Listeria* in water and sediment and *Campylobacter* in sediment), indicating that those pathogens were found ubiquitously in the environment (e.g., *Listeria*) or the occurrence of a particular pathogen may be better described by a different set of independent variables such as other MST markers not selected for this study. *Campylobacter* and *Salmonella*, for example, may be better described by MST host-specific markers for poultry and/or wild birds (Ryu et al., 2014). Seasonal factors such as water and air temperature may also be important predictors for certain pathogens and should be further investigated in the region. Wilkes et al. (2011) reported seasonal effects in Canada where higher instances of *Campylobacter* were observed in cooler temperatures, and Liang et al. (2013) reported higher instances of *Salmonella* in warmer water. Overall, pathogen detections in water were higher in this study than in a study by Wilkes et al. (2011) of mixed-use freshwater streams in Canada, but seasonal differences were not assessed.

Shiga toxin has been shown to be strongly associated with pathogenic *E. coli* (i.e., *E. coli* O157:H7) originating from upstream pastures (Wilkes et al., 2011); the positive correlations with cattle and ruminant markers observed in this study may indicate upland pastures containing cattle are also a source of Shiga toxin and potentially pathogenic *E. coli*. Shiga toxin in water also appeared to be strongly dependent on AG land use, with > 80% occurrence in this land class, but there was no correlation between land use and Shiga toxin in sediments. High concentrations of the MST marker for cattle predicted presence of Shiga toxin in both water and sediments and indicated transport of cattle feces to streams. Shiga toxin was absent in sediment when ruminant concentrations were low, present when ruminant concentrations were intermediate, and absent when ruminant concentrations were high. Mechanisms for bovine feces contamination in agricultural streams include upland transport from manure applied and deposited on pastures, and deposited directly in the stream (Lee et al., 2008). In many cases, cattle in this watershed were not fenced out of streams and could directly impact stream water quality during base flow conditions. Absence of Shiga toxin when ruminant concentrations were

high indicates ruminants other than cattle within the watershed contribute to presence of the ruminant marker, but do not carry Shiga toxin.

In this study, *Campylobacter* presence appeared dependent on the WPCP land use, with > 50% occurrence in water downstream from wastewater treatment plants. Occurrence of *Campylobacter* in water was not correlated with concentration of the human MST marker, however, but was correlated with concentrations of cattle and ruminant markers. Cattle are an important source of human-pathogenic *Campylobacter* and can shed the bacteria in large numbers (Inglis and Kalischuk, 2003; Inglis et al., 2005; Leatherbarrow et al., 2004); nevertheless, cattle and ruminant markers were also undetected in effluent samples (data not shown). In the recursive partitioning analysis, the cattle marker in sediment was also the only independent variable selected for predicting occurrence of *Campylobacter* in water, although frequency of occurrence was low. Upstream agricultural areas may have been the source for cattle markers in the WPCP streams and not WPCP effluent. Cattle and poultry production are dominant agricultural practices in the watershed. Poultry litter is commonly applied as a fertilizer on cattle pastures and hay fields in Northeast Georgia and can be a significant source of nutrients, FIB, and pathogens affecting water quality (Hartel et al., 2000; Mullen et al., 2011). Wastewaters from poultry processing and poultry manure used for fertilizer are also major sources for *Campylobacter* in the environment (Vereen et al., 2007), but poultry MST markers were not included in this study.

Among pathogens tested, *Listeria* and *Salmonella* were the most abundant, but none of the MST markers were selected as independent variables for predicting pathogen presence. Both pathogens appeared to be ubiquitous within the watershed and likely derived from multiple sources including humans, livestock, domestic animals, and wildlife. The best predictors for *Salmonella* in water included water quality parameters (D.O. and pH), along with *E. coli* in water, which is contrary to a study by Ahmed et al. (2013) that found no relationship between *E. coli* and *Salmonella* presence, using logistic regression analysis. None of the MST markers used in this study were selected in the recursive partition

models for predicting *Salmonella*, which indicated a source other than cattle, ruminants, and humans was responsible for *Salmonella* in the watershed. Markers and pathogens decay at different rates as reported by Ahmed et al. (2013). The prevalence of *Salmonella* reported here is also consistent with synoptic sampling by Meinersmann et al. (2008), in which *Salmonella* was detected in 75% of water samples collected during a single synoptic sampling event of the Oconee River watershed in northeast Georgia, adjacent to the SFBR watershed with similar land use.

Overall, *Listeria* occurred more often in our study (70% of water samples) than in one conducted by Wilkes et al. (2009) in Canada which found the highest prevalence in water during spring (58%). *Listeria* was significantly correlated to the human marker in this study, indicating a possible relationship between its presence and wastewater plants or septic systems; the human marker was not selected as a splitting parameter by the recursive partitioning models, however, suggesting it alone is not adequate to predict *Listeria* presence. Effluent samples were also not tested for *Listeria* so it was difficult to conclude that WPCPs were contributing *Listeria* to the system. Lack of correlation between *Listeria* and the CowM3 and Rum-2-Bac also suggests that cattle and ruminant wildlife (e.g., deer) may not be the dominant sources of *Listeria* in the watershed. A study evaluating wastewater treatment plants in France found *Listeria* in 84% of treated wastewaters and in 89% of raw sludge (Paillard et al., 2005) which supports the notion that wastewater treatment facilities and septic systems can be a source of *Listeria*. In our study, it was difficult to pinpoint wastewater sources as the only contribution because *Listeria* was ubiquitous across waters and sediments in the study area. *Listeria* associated with wastewater sources should be further evaluated due to higher risk associated with human fecal sources (Hagedorn et al., 2011).

Pathogens occurred more often in water and were associated with FIB and MST marker in water and sediments which is similar to other studies evaluating partitioning of microbial contaminants in sediments (Burton et al., 1987; Gordon et al., 2002; Hendricks, 1971). It was not obvious if the source of

pathogens in the sediment was the same as water, but it was assumed that the upstream transport mechanism such as sediment transport contributed to pathogens in the sampled sediments. The mechanism causing high indicator concentrations in sediments is not apparent, but differential survivability and/or preferential partitioning in sediments of FIB, host-specific MST markers and pathogens may be associated with increased nutrient availability and protection from predation (Davies et al., 1995; Garzio-Hadzick et al., 2010). Pathogen presence may also be directly related to upstream land use since some pathogen presence (i.e., Shiga toxin and *Campylobacter*) was significantly related to land use, and there were significant differences in FIB and MST marker concentrations based on land use. In this study, MST marker and FIB concentrations in sediment were selected as the best predictor variables for certain pathogens during baseflow conditions, suggesting that fecal inputs are repetitive and that sediment represents the upstream integration of fate and transport of fecal bacteria in the watershed. Indicator bacteria concentrations in sediment thus may be just as important as those in water. This study also indicated that release of sediment-associated indicators and pathogens (e.g., FIB and MST markers) during resuspension of sediment due to storm events could be an additional source of fecal contaminants to the water column. The impact of resuspension of sediment-associated pathogens should be further evaluated to determine if there is a relationship between pathogen presence and FIB/MST markers during bedload sediment transport.

It should be noted that, as with any statistical analysis conducted on environmental concentrations, the treatment of detection limits could affect the interpretation of the analysis. However, the classification analysis used in this study is a non-parametric and non-linear technique and it makes no assumption that the data are normally distributed or that relationships between dependent and independent variables follow any specific linear or non-linear functions. Therefore, we chose a parsimonious approach to censor data at the limits of detection whereby concentrations of MST markers that were undetected or below the LOQ were assigned a value of LOQ/v2 and undetected

culturable FIB concentrations were assigned a value of one prior to log transforming the data. This approach with the detection limit along with pruning of terminal nodes and cross-validation of data sets in the classification method were used to minimize bias in the statistical analysis.

Monitoring that incorporates FIB, water quality measurements, and MST markers, as suggested by Noble et al. (2006), may be the best way to assess microbial water quality in mixed land use systems because of inconsistent relationships between individual pathogens and FIB/MST markers. Further studies in this watershed should use a wider variety of MST markers that may help to describe the source(s) of *Campylobacter*, *Salmonella*, and *Listeria* and improve understanding of how seasonal variability affects the presence of these pathogens in both water and sediments. A combination of classification analysis with the type of monitoring that was conducted in this study is recommended to detect relationships between variables that may otherwise be undetected using parametric statistical methods.

5. Conclusions

- Land use played a clear role in pathogen occurrence as Shiga toxin occurred more frequently in the agricultural watersheds and was related to concentration of the ruminant MST marker.
- Fecal indicator bacteria and water quality variables (e.g., D.O., pH, and temperature) performed better in predicting *Salmonella* occurrence.
- Fecal indicator bacteria alone did not adequately describe pathogen presence, and models showed that the response depends on the independent variables used, which indicates the possible need to use multiple tests for tracking microbial contaminants.
- Concentrations of MST markers and FIB in sediment predicted the occurrence of certain pathogens in water during baseflow, suggesting that bacteria concentrations in sediment represent upstream integration of microbial fate and transport in the watershed; therefore, monitoring fecal indicators in sediment may be just as important as monitoring them in water.

- FIB and MST marker concentrations in sediments were related to pathogens recovered from water. This study suggests that sediments can be an important source for FIB and MST markers in water if they are resuspended by mechanical disturbance or by high flows.
- The source(s) of *Listeria*, *Campylobacter*, and *Salmonella* were not adequately described using the set of MST markers chosen for this study.

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Captions

Fig. 1. NLCD map of the synoptic sampling locations (red circles) within the S. Fork Broad River watershed.

Fig. 2. Mean and standard error of log transformed culturable and qPCR FIB in water and sediment at 15 locations in the S. Fork Broad River watershed (a) *E. coli* in water; b) enterococci in water; c) *E. coli* in sediment; and d) enterococci in sediment. Different lowercase letters indicate significant differences between land uses ($p < 0.05$).

Fig. 3. Mean and standard error of log-transformed MST in water and sediment at 15 locations in the S. Fork Broad River watershed: a) Rum2Bac; b) CowM3; and c) HF183/HF183MGB. Different lowercase letters indicate significant differences between land uses ($p < 0.05$).

Table 1. NLCD land cover characteristics for the 15 sampling sites.

Table 2. Primers and probes used for qPCR assays.

Table 3. Pathogen detections. Dependence of pathogen occurrence on land use was tested with Fisher's exact test.

Table 4. Classification trees for pathogen occurrences (present (P) and absent (A)) in water, using culturable and qPCR quantified FIB, MST host markers, and water quality (WQ) parameters.

Table 5. Classification trees for pathogen occurrences (present (P) and absent (A)) in sediment, using culturable and qPCR quantified FIB, MST host markers, and water quality (WQ) parameters.

Table 6. Logistic regression analysis for predicting pathogens in water and sediment, using parameters from best recursive partitioning models from Section 3.4.

Supplemental Table 1. Spearman rank correlation coefficients (ρ) and associated p-values for correlations between water- and sediment-borne pathogens, fecal indicator bacteria (culturable and markers), water quality parameters, host-associated *Bacteroidales* markers, forest cover, sand content, and antecedent rainfall.

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