Water Research 144 (2018) 424–434

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Determination of wild animal sources of fecal indicator bacteria by microbial source tracking (MST) influences regulatory decisions

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ARTICLE INFO

Article history:
Received 13 March 2018
Received in revised form
5 July 2018
Accepted 14 July 2018
Available online 17 July 2018

Keywords:
Microbial source tracking
Fecal indicator bacteria
Water body management
Water quality
Recreational water
TMDL

ABSTRACT

Fecal indicator bacteria (FIB) are used to assess fecal pollution levels in surface water and are among the criteria used by regulatory agencies to determine water body impairment status. While FIB provide no information about pollution source, microbial source tracking (MST) does, which contributes to more direct and cost effective remediation efforts. We studied a watershed in Florida managed for wildlife conservation that historically exceeded the state regulatory guideline for fecal coliforms. We measured fecal coliforms, enterococci, a marker gene for avian feces (GFD), and a marker gene for human-associated *Bacteroides* (HF183) in sediment, vegetation, and water samples collected monthly from six sites over two years to: 1) assess the influence of site, temporal factors, and habitat (sediment, vegetation, and water) on FIB and MST marker concentrations, 2) test for correlations among FIB and MST markers, and 3) determine if avian feces and/or human sewage contributed to FIB levels. Sediment and vegetation had significantly higher concentrations of FIB and GFD compared to water and thus may serve as microbial reservoirs, providing unreliable indications of recent contamination. HF183 concentrations were greatest in water samples but were generally near the assay limit of detection. HF183-positive results were attributed to white-tailed deer (*Odocoileus virginianus*) feces, which provided a false indication of human sewage in this water body. FIB and GFD were positively correlated while FIB and HF183 were negatively correlated. We demonstrated that birds, not sewage, were the main source of FIB, thus avoiding implementation of a total maximum daily load program (TMDL). Our results demonstrate that the concomitant use of FIB and MST can improve decision-making and provide direction when water bodies are impaired, and provides a strategy for natural source exclusion in water bodies impacted by wild animal feces.

1. Introduction

The sources of fecal pollution in environmental waters influence human health risk and remediation strategies (Stoeckel and Harwood, 2007; Yan and Sadowsky, 2007; Soller et al., 2010; Harwood et al., 2014). Fecal pollution of water bodies can come from many sources, including livestock and wild animals, but the original fecal indicator bacteria (FIB) paradigm assumed that the majority of FIB originate from human sewage (Field and Samadpour, 2007; Harwood et al., 2014). However, FIB, including fecal coliforms, enterococci, and *Escherichia coli*, inhabit the gastrointestinal tract of most endo- and ectotherms (Gordon and Cowling, 2003), e.g. turtles (Harwood et al., 1999), deer (Kuntz et al., 2004), dogs (Wright et al., 2009), alligators (Johnston et al., 2010), and agricultural animals (e.g. cattle, swine, and poultry) (Sinton et al., 2007; USEPA, 2010).

Persistence of FIB also differ among environmental matrices, which we define broadly as sediment, vegetation, and water. For example, FIB have been found in beach sand (Alm et al., 2003; Beversdorf et al., 2007; Bonilla et al., 2007; Yamahara et al., 2007; Eichmiller et al., 2014) and sediment (Lee et al., 2006; Ishii et al., 2007). Decaying vegetation (i.e. wrack) near marine shores can also serve as a FIB source (Weiskel et al., 1996). Moreover, evidence suggests that submerged aquatic vegetation harbors higher concentrations of FIB compared to sediment and water (Badgley et al. 2010, 2011). These factors have led to uncertainty about best management practices to reduce levels of these indicators of fecal contamination.

In spite of the issues cited above, recreational water quality is regulated in the United States using FIB, such as fecal coliforms,

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https://doi.org/10.1016/j.watres.2018.07.034
0043-1354/

enterococci, or *E. coli* (USEPA, 2012; FDEP, 2016). In Florida, for example, fecal coliform concentrations should not exceed a monthly average of 200 colony forming units (CFU) per 100 mL water, nor exceed 400 in 10% of the samples, nor exceed 800 on any one day (FDEP, 2016). Similarly, enterococci should not exceed a monthly average of 130 CFU per 100 mL water, nor exceed 130 in 10% of the samples (USEPA, 2012). If water bodies consistently exceed state and/or USEPA water quality guidelines, they are placed on an impaired water body list and management organizations are required to develop Total Maximum Daily Load (TMDL) programs, which must contain a plan to decrease FIB levels. However, the planning and implementation of TMDL programs is costly, e.g. a 2001 USEPA report stated that the national average cost of developing TMDL programs per water body is $52,000 but can range from under $26,000 to over $500,000. The USEPA estimated that $17.3 million would be needed annually for TMDL monitoring across the country (USEPA, 2001).

Because FIB are ubiquitous in human and non-human sources, management organizations have argued for natural source exclusion (NSE), the idea that FIB from "natural" sources should be treated differently for TMDL calculations than those that originate from human sewage or high-risk animal sources, such as cattle and poultry. The USEPA has provided technical support for developing site-specific water quality criteria, which can rely on alternative methods and tools to define impaired waters. Use of an alternative indicator(s) that is as protective of public health as the conventional FIB water quality standards can provide monetary savings and advantages in terms of ease of use (USEPA, 2014). An alternative indicator or method must first be vetted for performance characteristics, and then correlated with an existing EPA method, or with human health outcomes (USEPA, 2014). Achieving NSE status can be cost-effective because remediation practices and TMDL programs may not be required.

While testing for pathogens would seem inherently preferable and provide a more straightforward interpretation of human health risk, monitoring for all waterborne pathogens is unrealistic due to the diversity and low concentration of pathogens found in most environmental waters (Harwood et al., 2014). The growing need to discriminate between fecal sources led to the development of microbial source tracking (MST), which aids in host identification for polluted water bodies. MST markers target genes of host-associated bacteria that are specific to the gastrointestinal (GI) tract of the host species (specificity) and ubiquitous within individuals of the host species (sensitivity). A variety of human-associated markers, such as HF183 (Layton et al., 2010; Seurinck et al., 2005), the pepper mild mottle virus (PMMoV) (Rosario et al., 2009), and human polyomaviruses (HPyVs) (McQuaig et al., 2009) have been developed, in addition to markers for non-human sources, such as cattle (Shanks et al., 2008), poultry (Weidhaas et al., 2010), ruminants (Bernhard and Field, 2000), shorebirds (Lu et al., 2008), and general avian species (Green et al., 2012). MST markers can help identify major sources of fecal pollution, which may result in more directed and cost effective remediation efforts for management organizations as alternatives to implementing a TMDL program (Stoeckel and Harwood, 2007). MST is not yet widely used in regulatory applications, although the USEPA is considering adoption of human-associated markers (Harwood et al., 2017).

We studied a watershed managed for wildlife conservation in central Florida where ambient water samples consistently exceeded state regulatory guidelines for fecal coliforms in Class III freshwaters. Given the limitations of FIB as a sole indicator of fecal contamination and the varied levels of FIB in environmental matrices, we chose to monitor fecal coliforms, enterococci, a general avian marker (GFD), and a human-associated marker (HF183) in sediment, vegetation, and water. Our objectives were to follow USEPA recommendations for developing site-specific criteria, which we achieved by: 1) quantifying FIB and MST markers in sediment, vegetation, and water, 2) determining if avian feces and/or human sewage contributed to FIB levels, 3) assessing whether FIB and MST marker concentrations were influenced by matrix, year, month, and sample site, and 4) testing for correlations among FIB and MST markers independent of matrix, year, month, and sample site.

2. Methods

2.1. Sample sites and collection

We studied a watershed managed for wildlife conservation in central Florida that was placed on Florida's Department of Environmental Protection 303(d) (water body impairment) list in 2010. Land use varied from suburban to natural, and the stream and associated trees served as a nesting site for various avian species.

Six sites within the water body were used for the duration of the study. Samples from three environmental matrices, sediment, vegetation, and water, were collected monthly from January 2013 to September 2015. Water samples were collected at each sampling event, but sediment and vegetation samples were not collected during events when water levels were too high or when vegetation was scarce. Samples from each environmental matrix were collected by boat and transferred to sterile containers. Vegetation was collected at the surface and stored in gallon Ziploc bags, sediment was collected at exposed shoreline areas with 50-mL Falcon centrifuge tubes (Corning Science, Reynosa, Mexico), and water samples were collected at the surface with 1000-mL Nalgene containers (Thermo Fisher Scientific, Waltham, MA).

Water samples for FIB enumeration were processed by the management organization at the water body, while water samples for MST analysis, sediment, and vegetation samples were processed on the University of South Florida (USF) campus. All samples were processed within 6 h of collection. Given the known cross-reactivity of HF183 with deer feces (Layton et al., 2013), 12 discrete Rusa deer (*Rusa timorensis*) fecal samples from Australia and five white-tailed deer (*Odocoileus virginianus*) fecal samples from the watershed were collected in January 2014 and processed for DNA extraction at USF.

2.2. Fecal indicator bacteria (FIB)

Fecal coliforms and enterococci were enumerated for each environmental matrix. The management organization processed water samples using Standard Method 9221E (APHA, 2012) and IDEXX Enterolert (IDEXX Laboratories, Inc., Westbrook, Maine) for fecal coliforms and enterococci. Quantities were reported as most probable number (MPN) per 100 mL water. At USF, sediment and vegetation were processed using modified membrane filtration methods on selective media (USEPA, 2002; APHA, 2012). 30g wet weight of sediment or vegetation was placed into 1000-mL Nalgene containers (Thermo Fisher Scientific, Waltham, MA) with 300 mL of sterile phosphate buffer solution (pH 7.1 ± 0.1). The mixtures were shaken by hand for two minutes to detach particles and then processed in volumes ranging from 0.1-25 mL for membrane filtration. Sample concentrations were standardized as colony forming units (CFU)/100g wet weight. At the end of the experiment, sample sizes for fecal coliforms ($n = 477$) and enterococci ($n = 470$) were unequal because some enterococci plates were uncountable due to interference from the environmental matrix.

Positive and negative controls for the selective media (Difco mFC and Difo mEI, Becton Dickinson, Sparks, MD) were used in each sampling event. For positive controls, pure cultures were

streaked on the selective media plates (*E. coli* 9637 for fecal coliforms on mFC agar, *Enterococcus faecalis* 19433 for enterococci on mEI agar), and incubated at respective optimal temperatures with the samples. For negative controls, pure cultures of *E. coli* 9637 were streaked on mEI agar and *E. faecalis* 19433 was streaked on mFC agar and incubated with all other samples.

2.3. MST sample processing and DNA extraction

Five hundred mL water samples were filtered through 47 mm, 0.45 μm-pore-size nitrocellulose filters (Fisher Scientific, Waltham, MA) for MST analysis. Ten to 25 mL of supernatant from agitated sediment and vegetation samples were filtered as described in the previous section. Filters were folded, placed in PowerBead tubes (MoBio Laboratories, Carlsbad, CA), and stored at −80 °C until later processing with the PowerWater DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) using the manufacturer's instructions. White-tailed deer fecal samples were processed immediately upon arrival or were stored at −80 °C until DNA extraction. For DNA extraction, 0.3g of deer feces was added directly to a PowerBead tube and processed using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). All samples of final eluent from DNA extraction were diluted 1:5 (volume/volume) in nuclease-free water to assess inhibition. An extraction blank (PowerBead tube with nothing added) was subjected to all steps in the extraction protocol and was used as an extraction control.

2.4. QPCR analysis of MST markers

DNA extracted from all environmental matrices was tested for MST markers that identify avian and human fecal sources using qPCR. A general avian marker (GFD) (Green et al., 2012) and human-associated *Bacteroides* (HF183) marker (Bernhard and Field, 2000; Seurinck et al., 2005) were used. For all qPCR analyses, samples were run in duplicate and quantities were interpolated by comparison to a standard curve. The standard curve was constructed from plasmid DNA containing the target sequence at ten-fold dilutions ranging in concentration from 10^6 to 10^1 gene copies per reaction. In addition, negative controls for the qPCR reaction and for the extraction process (no template added) were included on each qPCR plate run. Gene copy quantities were calculated as the mean concentration of duplicate reactions and reported as per 100g wet weight of sediment and vegetation or per 100 mL of water. All assays were run using an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) at USF.

QPCR of GFD was based on a previously published method (Green et al., 2012). Samples were prepared for qPCR in 25 μL reactions containing 12.5 μL of GoTaq Mastermix (Promega, Madison, WI), 7 μL of nuclease free water, 0.25 μL of 10 μM forward primer (5′-TCG GCT GAG CAC TCT AGG G-3′), 0.25 μL of 10 μM reverse primer (5′-GCG TCT CTT TGT ACA TCC CA-3′), and 5 μL of DNA template. The qPCR thermocycler conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 57 °C, 15 s at 95 °C, 1 min at 57 °C, 30 s at 95 °C, and 15 s at 57 °C.

QPCR of HF183 was accomplished using a previously published method (Seurinck et al., 2005; Staley et al., 2012), with an appropriate forward primer (5′-ATC ATG AGT TCA CAT GTC CG-3′), the SSHBacR reverse primer (5′-TAC CCC GCC TAC TAT CTA ATG-3′), and SSHBac probe ((FAM)-TTA AAG GTA TTT TCC GGT AGA CGA TG-(TAMRA)) (Harwood et al., 2011). Samples were prepared for qPCR in 25 μL reactions containing 12.5 μL of Taqman Environmental Mastermix 2.0 (Applied Biosystems, Warrington, UK), 2 μL of nuclease free water, 2.5 μL of 2 mg/μL bovine serum albumin (BSA) (Fisher Scientific, Waltham, MA), 3 μL of primer and probe mix, and 5 μL of DNA template. The final concentrations of the primers and the probe were 0.92 μM per reaction and 0.07 μM per reaction, respectively. The qPCR thermocycler conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C, and 1 min at 60 °C.

A qPCR assay for *Vibrio vulnificus* was used to test for inhibition of the qPCR. *V. vulnificus* is an opportunistic human pathogen that is autochthonous to estuarine and marine waters, therefore the probability of its presence in a freshwater system was negligible. Sample DNA (5 μL) was spiked with 2.0×10^4 gene copies of plasmid DNA containing the target sequence for *V. vulnificus* and a SYBR Green qPCR method (Wright et al., 2007; Staley et al., 2013a) was used to determine the copy number recovered from samples after qPCR by extrapolating to a standard curve. The average copy number recovered from spiked nuclease free water (5 μL of nuclease free water spiked with 2.0×10^4 gene copies) was used as a bench mark for all samples tested.

Standard curves for GFD and HF183 were analyzed according to published Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2010; Forootan et al., 2017) and were similar to those reported in previous studies (Green et al., 2014; Ahmed et al., 2016; Symonds et al., 2017). For the duration of the study, the mean slope, R2, and efficiency for GFD was −3.6, 0.989, and 88.8 respectively. The mean slope, R2, and efficiency for HF183 was −3.5, 0.991, and 92.6 respectively. Limits of detection were defined as the lowest concentration of target gene copies that could be reliably detected above the negative control for each qPCR run. Limits of quantification were based on the standard curve and defined as the concentration of target gene copies wherein the standard deviation for all replicates was less than or equal to 2 C_T. Two components were considered for reliability of detection: (1) amplification of both duplicates; and (2) the mean cycle threshold (C_T) value was lower than that of the negative control (blank). Results from qPCR were deemed quantifiable and reported as mean concentrations of the duplicate qPCR reactions if C_T values for the duplicate qPCR reactions fell within 2 standard deviations of one another and they were above the level of the lowest standard. QPCR results deemed non-quantifiable were characterized as non-detectable ("ND") or detected but not quantifiable ("DNQ"). If zero or one well was amplified, the result was deemed "ND" and was designated a value half of the limit of detection in the data set. If both duplicates amplified, the result was deemed "DNQ" and assigned the value of the limit of detection (LOD) in the data set, therefore DNQ values were twice that of ND values.

2.5. Cross-reactivity of HF183

DNA was extracted from five discrete water samples and five white-tailed deer (*O. virginianus*) fecal samples collected on site in January 2014. Samples were tested for HF183 using both conventional (Bernhard and Field, 2000) and qPCR (Seurinck et al., 2005). DNA was also extracted from 12 discrete Rusa deer (*Rusa timorensis*) fecal samples from Australia to compare presence of HF183 across geographic regions. For sequence comparison, a 525 bp region of the *Bacteroidales* 16S rRNA gene was amplified from the water and local deer fecal samples using the conventional PCR primer pair (183F and 708R) and cloned into competent *E. coli* cells using the TOPO® TA Cloning® kit (Invitrogen, Carlsbad, CA). Sequences were aligned using ClustalW (MEGA 6.0) and a phylogenetic tree was constructed using the Neighbor-Joining algorithm. The 16S rRNA gene sequence for *Bacteroides dorei* (GenBank: NR_041351) was included in the analyses along with the sequence of an uncultured *Bacteroidales* strain (GenBank: EF704867.1; source: human gastrointestinal) for comparison. Eight sequences from water samples (two each from sites 1, 4, 6 and one each from sites 2 and 5) and five

sequences from deer fecal samples were deposited in GenBank under accession numbers MG970697, MG970698, MG970699, MG970700, MG970701, MG970702, MG970703, MG970704, MG970705, MG970706, MG970707, MG970708, and MG970709.

2.5.1. Calculating exceedances for FIB and geometric means for MST

Statistical analyses were conducted using R 3.2.4 (Team, 2017). All packages and functions are listed in a supplementary table (Table S1). First, different transformations were tested (logarithmic, square root, and fourth root) to comply with statistical assumptions of normality and homoscedasticity (Bartlett, 1937; Royston, 1982). The log10(x+1) transformation was retained because it had the greatest performance and accounted for non-detect values (Table S1).

Because management organizations sample water but not sediment and vegetation, we initially calculated the frequency of exceedance of regulatory guidelines for FIB (i.e. fecal coliforms and enterococci) across years and sites in water samples (Table S2). Regulatory levels were considered 400 CFU/100 mL and 130 CFU/100 mL for fecal coliforms and enterococci, respectively (USEPA, 2012; FDEP, 2016). Sediment and vegetation are not subject to regulatory guidelines, therefore exceedances were not calculated for those samples. Next, we calculated the geometric means and frequency of detection for GFD and HF183 across environmental matrices, years, and sites to compare their concentration and environmental persistence (Table S4).

2.5.2. ANOVA and mixed-effects ANOVA

We used an ANOVA to assess FIB concentrations in water as a function of year, month, and site. The variance attributed to these predictors was quantified using an adjusted R^2 value. Mixed-effects ANOVA were then used to assess the relationships among FIB and MST markers (i.e. fecal coliforms, enterococci, GFD, and HF183) as a function of matrix (i.e. sediment, vegetation, and water) while accounting for the spatial and temporal dependency of observations (Gelman and Hill, 2007; Zuur et al., 2009; Emi Fergus et al., 2011; Nakagawa and Schielzeth, 2013). For each FIB and MST marker, we compared models with matrix as a fixed effect and all possible combinations of year, month, and site as categorical random effects (Table 1). Akaike Information Criterion (AIC), a relative measure of performance for a statistical model (Zuur et al., 2009; Emi Fergus et al., 2011), was used to compare all mixed-effects ANOVA models. Required normality and homoscedasticity assumptions were confirmed by visually inspecting plots of the fitted values versus the residuals of the mixed-effects ANOVA (Zuur et al., 2009).

Mixed-effects ANOVA models with the lowest AIC values were retained and one best-fit model was chosen per FIB and MST marker. When AIC were similar (i.e. within three AIC), the model with the least number of random effects was chosen. For all FIB and MST markers, the proportion of the variance explained by fixed effects (i.e. matrix) was quantified using marginal R^2 and the variance explained by both fixed and random effects (i.e. matrix and year, month, and site) was quantified using conditional R^2 (Nakagawa and Schielzeth, 2013; Johnson, 2014) (Table S4). Therefore, the difference between conditional R^2 and marginal R^2 values corresponds to the proportion of variance accounted for by the random effects (i.e. year, month, and site).

The predictive power of best-fit mixed-effects ANOVA for each FIB and MST marker was assessed by cross-validation (jackknife, leave-one-out procedure), which iteratively removes one sample from the data set and predicts the FIB or MST concentration for that sample using the model developed with the remaining data set. This was done until each FIB and MST marker concentration from every sample was predicted. The model's predictive power was quantified by P^2 (Guénard et al., 2013). This value is bound between $-\infty$ and 1, where a P^2 of 1 indicates that all predictions perfectly match the observations, a P^2 above 0 indicates accurate predictions, and a P^2 equal to or below 0 indicates poor model performance. Cross-validation ensured that our models were not over-fitted. When comparing AIC values, over-fitted models can have high R^2 values but subsequently low P^2 values, which indicates the model is specific for the data set and results cannot be applied generally.

2.5.3. Correlations between FIB and MST

Lastly, we assessed associations between fecal coliforms and enterococci, fecal coliforms and GFD, fecal coliforms and HF183, enterococci and GFD, and enterococci and HF183 independent of year, month, and site using Kendall's non-parametric measure of correlation, which allows for departure from normality and for non-linear relationships (Hollander and Wolfe, 1973) (Table S1).

3. Results

3.1. FIB analysis

Water, sediment, and vegetation samples were analyzed for fecal coliforms (FC) and enterococci (ENT) concentrations (Fig. 1). FC and ENT concentrations were significantly greater in sediment and vegetation compared to water (Fig. 1). FC levels in sediment were approximately two orders of magnitude greater than in water, while the corresponding comparison for ENT showed sediment and vegetation levels approximately 1.5 orders of magnitude greater than that in water.

FC and ENT had geometric means of 75 and 168 MPN/100 mL in water, respectively (Fig. 1). Site 6 had significantly lower mean FC concentrations than site 2 (p-value = 0.0294) (Table S3). The highest FC concentrations across years occurred in January and March (p-value = 0.0007 and 0.0445). Year, month, and site accounted for almost ¼ of the variance observed in FC concentration levels (adjusted R^2 = 23.5%). Mean ENT levels were also

Table 1
Modeling the relationship among FIB and MST markers by mixed-effects ANOVA. Matrix was considered a fixed effect. All possible combinations of random effects (i.e., year, month, and site) were compared. AIC and ΔAIC, the difference in AIC from the chosen model, are presented. Lower AIC values indicate better model fit. Chosen models combined a lower AIC value with the fewest number of variables for parsimony, and are indicated in bold.

Random effect	FC		ENT		GFD		HF183	
	AIC	ΔAIC	AIC	ΔAIC	AIC	ΔAIC	AIC	ΔAIC
Year + Month + Site	1196.4	0.0	1218.6	4.9	1608.1	67.1	1022.4	116.6
Year + Month	**1198.3**	**1.9**	**1213.7**	**0.0**	**1541.0**	**0.0**	**905.8**	**0.0**
Year + Site	1205.4	9.0	1221.3	7.6	1623.3	82.3	1055.9	150.1
Month + Site	1218.8	22.4	1236.5	22.8	1642.0	101.0	1064.4	158.6
Year	1224.5	26.2	1221.3	7.6	1621.3	80.3	1054.0	148.2
Month	1235.3	38.9	1234.1	20.4	1633.3	92.3	1021.3	115.5
Site	1217.4	21.0	1234.7	21.0	1640.0	99.0	1076.2	170.4

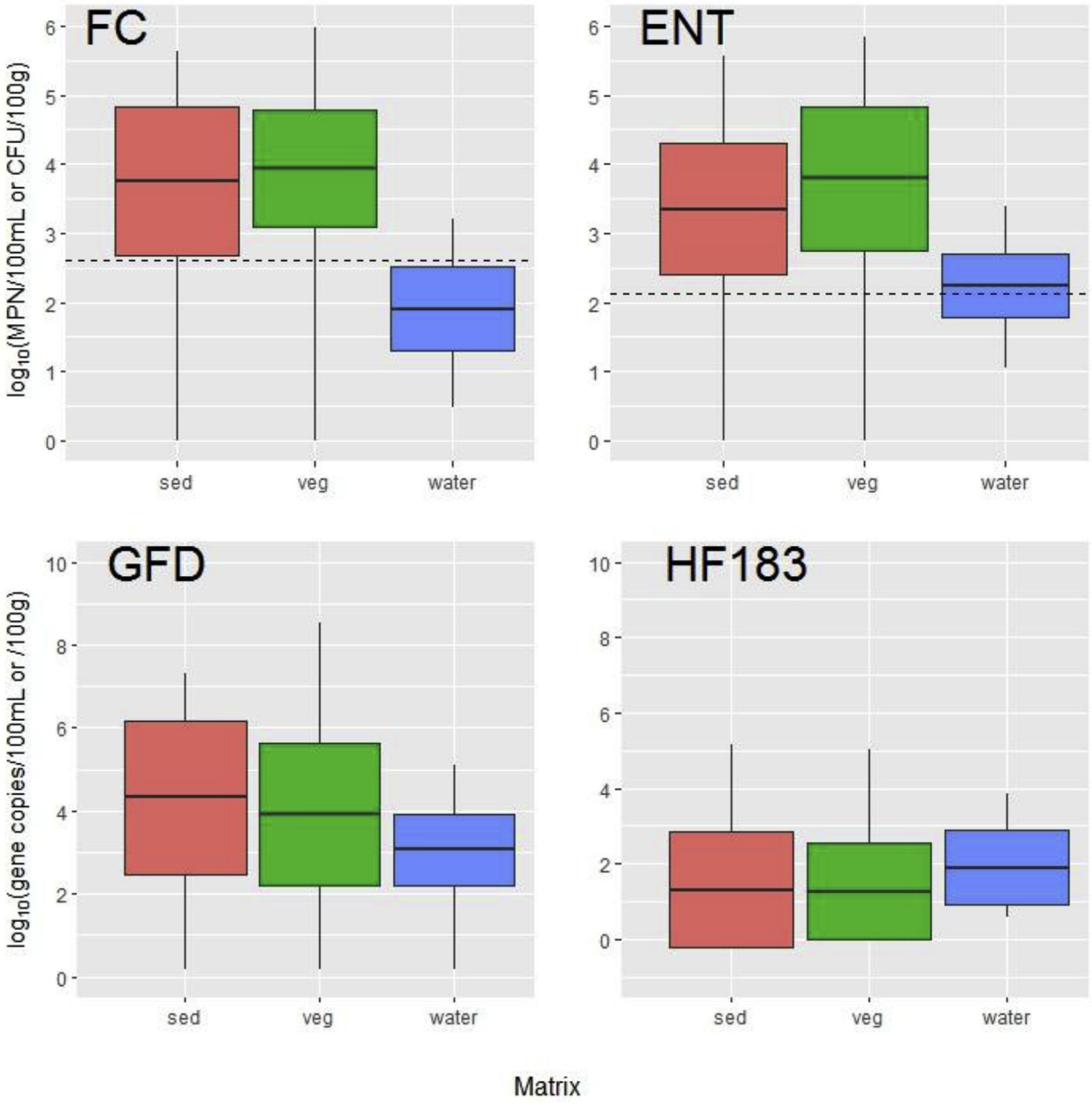

Fig. 1. FIB and MST markers in sediment, vegetation, and water. Boxplots represent the minimum and maximum range of values below and above the mean, respectively, for all sites and years (2013-15). Whiskers display the standard deviation of obtained values for fecal coliforms, enterococci, GFD, and HF183. Dotted lines represent regulatory levels in water for fecal coliforms and enterococci.

elevated in January and March, as well as June across years, and were significantly greater than ENT levels in December (p-values = 0.027, 0.0009, 0.0002, 0.0007) (Table S3). Almost half the ENT variance observed could be attributed to year, month, and site (adjusted $R^2 = 42.7\%$).

FC concentrations exceeded State of Florida regulatory guidelines in 20% of water samples across sites and years. Exceedances were most frequent at sites 1 (24.2%) and 2 (30.3%). ENT concentrations exceeded regulatory guidelines in 63.2% of water samples across sites and years (Fig. 2, Table S2).

3.2. Microbial source tracking

Across all environmental matrices, levels of the general avian marker GFD ($n = 398$) ranged between $10^2 - 10^6$ gene copies/100 mL or/100 g on a $\log_{10}$ scale, while levels of the human-associated marker HF183 ($n = 333$) ranged between $10^0 - 10^3$ gene copies/100 mL or/100 g on a $\log_{10}$ scale (Fig. 1, Table S4). Geometric means for GFD were highest in sediment (3.81×10^3 gene copies/100g), followed by vegetation (2.39×10^3 gene copies/100g), and lowest in water (2.55×10^2 gene copies/100 mL) (Table S4). In contrast, HF183 had overall geometric means of 2.01×10^1 gene copies/100g, 1.82×10^1 gene copies/100g, and 7.68×10^1 gene copies/100 mL in sediment, vegetation, and water, respectively (Table S4). The frequency of GFD detection was 74.7%, 71.6%, and 76.5% in sediment, vegetation, and water samples respectively. While HF183 was detected in 63.4% of water samples, the human-associated marker was rarely detected in sediment (19.2%) and vegetation (22.8%) and levels of HF183 were near the limit of detection in most samples (Table S4, Fig. 1).

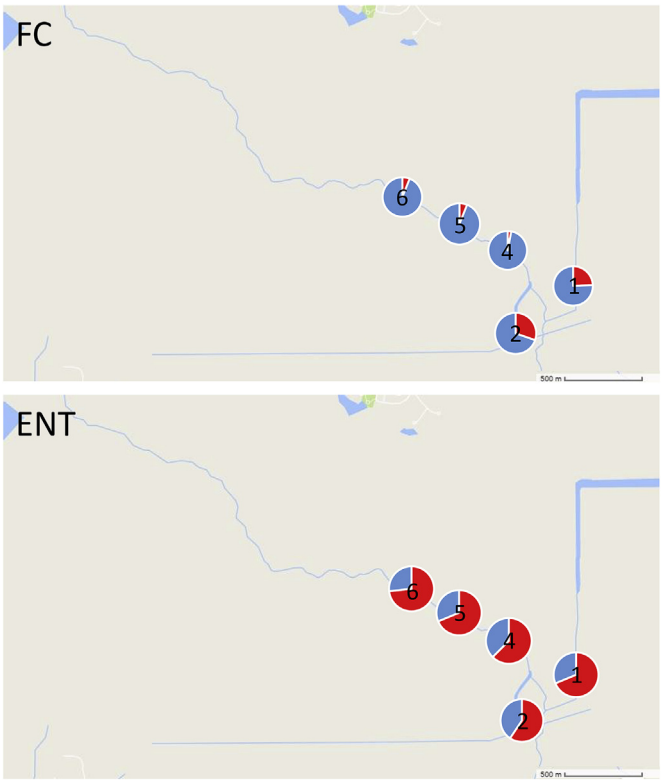

Fig. 2. Frequency of exceedance of regulatory guidelines for fecal coliform and enterococci were calculated across sites and years in water (FIB were not enumerated at site 3). Red signifies the percent of samples at or exceeding regulatory guidelines and blue represents the percent of samples under regulatory guidelines. Following Florida water quality standards and USEPA recreational water quality criteria, regulatory levels were considered 400 CFU/100 mL and 130 CFU/100 mL for fecal coliforms and enterococci, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Cross-reactivity of HF183

DNA extracted from five discrete water samples collected in January 2014 as well as the DNA extracted from five white-tailed deer fecal samples tested positive for HF183 using both conventional and qPCR. HF183 amplicon sequences from water and deer fecal samples demonstrated >99% sequence identity with each other and 95% similarity with the 16S rRNA sequence of *Bacteroides dorei*, a target of many human-source MST assays (Bernhard and Field, 2000; Haugland et al., 2010; Harwood et al., 2014) (Fig. 3). Sequences from water and deer fecal samples were also closely related to the 16S rRNA gene of an uncultured *Bacteroidales* sequence from human feces (GenBank: EF704867.1; source: human gastrointestinal). Primers and probe sequences for the HF183 qPCR assay were 100% identical to the partial 16S rRNA gene sequence of a representative deer fecal sample (data not shown). HF183 was not detected by qPCR in any of 12 Australian Rusa deer (*R. timorensis*) fecal samples.

3.4. Influence of environmental variables on FIB and MST marker levels

Mixed-effects ANOVA determined whether variations in FIB or MST concentration could be attributed to and predicted by matrix or year, month, and site. The best combination of variables for mixed-effects ANOVA based on AIC values included year and month for ENT, GFD, and HF183. FC had two competing models within 1.9 AIC, so the model with the least number of random effects (i.e. years and months) was chosen (Table 1).

For both FC and ENT, the fixed effect (i.e. matrix) explained more variation than random effects (i.e. years and months). Vegetation had significantly higher concentrations of FC and ENT (p-value = 0.0026 and < 0.0001), followed by sediment (p-value <0.0001 and < 0.0001) and water (p-value <0.0001 and < 0.0001). On an untransformed scale, FC concentrations were 129 and 68 times higher in vegetation and sediment, respectively, compared to water, whereas ENT concentrations were 40 and 12 times higher in vegetation and sediment, respectively, compared to water. The variation in FC explained by environmental matrix corresponded to 53.9% (marginal R^2) and including the effect of years and months only increased the explained variation by 8.3%. Similarly, the variation in ENT explained by environmental matrix was 36.9% (marginal R^2) and including the effect of years and months only increased the explained variation by 8.2% (Table S4). Random effect coefficients (years and months) suggested that concentrations of FC and ENT were larger in 2013, intermediate in 2014, and smaller in 2015. No clear monthly patterns could be identified across years (Fig. S1).

In contrast to FIB results, random effects accounted for more variation than fixed effects for both GFD and HF183. Sediment and vegetation had significantly greater concentrations of GFD than water (p-value < 0.0001). On an untransformed scale, GFD concentrations in sediment and vegetation were eight and three times higher than concentrations found in water, respectively. Conversely, sediment and vegetation had significantly lower concentrations of HF183 than water (p-value < 0.0001). HF183 concentrations in sediment and vegetation were three times lower than concentrations found in water. The variation in GFD explained by environmental matrix was 3.2%, while the effect of years and months accounted for 43.1% of the variation. For HF183, 4.1% of the variation was explained by environmental matrix and the effect of years and months accounted for 50.3% (Table S4). GFD concentrations were larger in 2015, intermediate in 2014, and smaller in 2013, whereas HF183 concentrations were larger in 2014, intermediate in 2013 and smaller in 2015. No clear monthly patterns could be identified across years (Fig. S1).

Similar explanatory (conditional R^2) and predictive power (P^2) indicated that chosen models were not over-fitted and provided robust predictions (Fig. 4, Table S4). In accordance with our marginal and conditional R^2 values for FC and ENT, matrix accounted for much variation in this dataset and environmental matrix clustered by type, whereas the opposite trend was evident for GFD and HF183 (Fig. 4).

3.5. Correlations between microbial variables

We observed positive correlations between FC and ENT (tau = 0.58, p-value < 2.2×10^{-16}), FC and GFD (tau = 0.13, p-value = 0.0009), and ENT and GFD (tau = 0.13, p-value = 0.0007), whereas negative correlations were observed between FC and HF183 (tau = −0.19, p-value = 3.51×10^{-6}) and ENT and HF183 (tau = −0.13, p-value = 0.001).

4. Discussion

We examined a watershed managed for wildlife conservation to identify host types that contributed to elevated FIB levels. Abundant populations of wild birds whose numbers peak in the winter months led us to include the GFD marker, while the upstream presence of urban/suburban development prompted the use of the human-associated HF183 marker. Sediment and vegetation, as well as water, were sampled as previous studies suggested that these

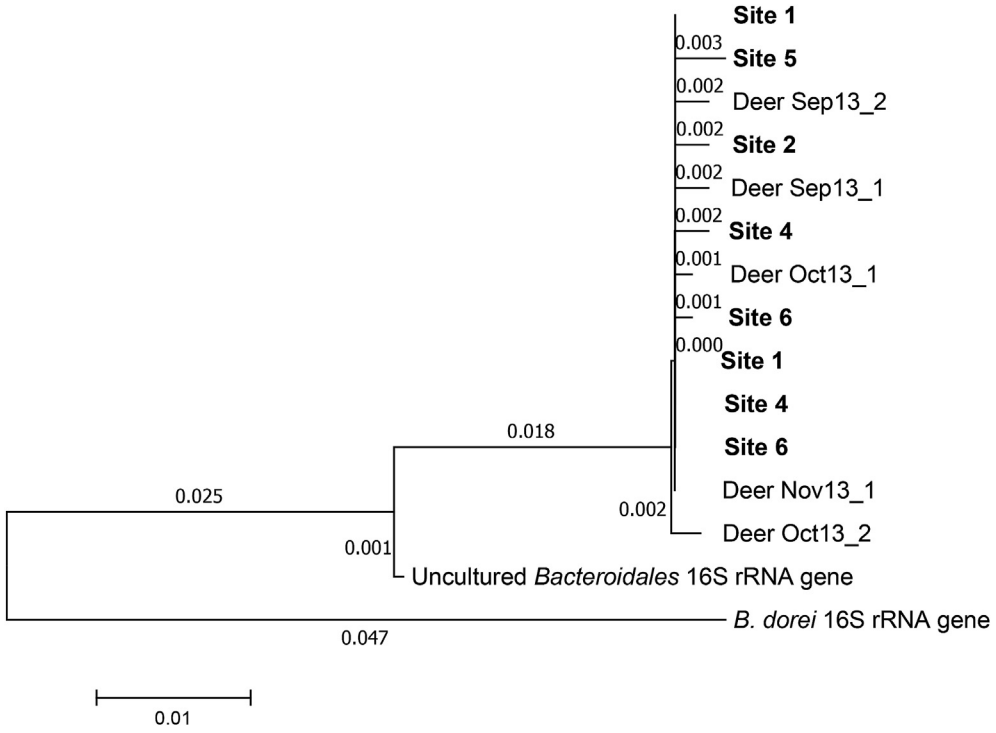

Fig. 3. Genetic relationship of *Bacteroidales* 16S rRNA sequences from white-tailed deer (*Odocoileus virginianus*) feces, water samples from each site, and reference genes that are known targets of the HF183 assay (uncultured *Bacteroidales* and *B. dorei*). Evolutionary distance is shown as the frequency of base substitutions per DNA sequence (i.e. 0.047 for *B. dorei* indicates 47 substitutions per 1000 bases, or 4.7% difference).

matrices can serve as reservoirs of FIB in sub-tropical waters (Badgley et al. 2010, 2011; Chase et al., 2012); however, they have rarely been assessed for MST markers.

The water body we studied was placed on the Florida Department of Environmental Protection's 303(d) list in 2010 because ambient water samples from “Site 2” consistently exceeded state regulatory guidelines for FC. This designation was consistent with “Site 2” having the highest frequency of exceedance for FC throughout our study (2013–2015) (Fig. 2). Moreover, January and March had the highest mean FC concentrations across years. Bird count data provided by the management organization (data not shown) coincided with high FC concentrations at “Site 2”. Bird count data at the same site also coincided with high concentrations of ENT during January, March, and June. Migratory bird populations may therefore be the mechanism by which FIB enter the water body. Similar trends have been observed in previous studies (Wright et al., 2009; Wu et al., 2017).

When we included environmental matrix in our models, sediment and vegetation harbored FIB concentrations one to two orders of magnitude greater than concentrations found in water for the entire sampling period (Fig. 1). Here, we would like to note that diverse practices for expressing the concentration of bacteria in sediment (soil particles with interstitial water) and vegetation exist in the literature. For example, Imamura et al. (2011) compared FIB in sand, sediment, and beach wrack with values normalized to dry weight. This methodology cannot be applied to comparisons between solid substrates and water. While we recognize that the comparison is not perfect, we normalized water and sediment values essentially to wet weight (as water weighs 100 g/100 mL). This strategy allows a more realistic comparison than FIB/g dry weight of sediment, which greatly elevates the calculations of FIB in sediments compared to water. This method can also be employed for vegetation, which is relatively rarely sampled in comparable studies. For publications in which this methodology is used, see Lee et al. (2006), Badgley et al. (2011), and Staley et al. (2013b). Overall, observing higher FIB concentrations in sediment and vegetation than water was an expected trend and supports previous research that sediment and vegetation are reservoirs of FIB in water bodies (Alm et al., 2003; Beversdorf et al., 2007; Coulliette and Noble, 2008; Badgley et al., 2011; Byappanahalli et al., 2012).

Accordingly, the best-fit mixed-effects ANOVA in our study confirms the predictive power of environmental matrix for FC and ENT concentrations (Fig. 4, Table S5). Thus, if sediment and vegetation periodically contribute FIB from older fecal sources to the water column, sampling water alone may not give an accurate portrayal of current fecal pollution risk (Lee et al., 2006; Badgley et al., 2010). FIB in sediment and vegetation may provide false indications of recent contamination and could incur costly mitigation efforts in the absence of human health risk.

The GFD bird marker was consistently present at relatively high levels across all sites, indicating the pervasive influence of bird feces on FIB levels. HF183, in contrast, was detected sporadically at low levels. Because both MST markers had non-detects that resulted in left-censored data, we substituted the LOD divided by 2 for these values in both datasets. The use of LOD/2 for non-detectable data points is valid according to standard practices and recommendations as long as it does not create a dataset that violates the assumptions of the statistical methodology employed (USEPA, 2006; Levine et al., 2009; Authority, 2010). Many recent publications have used this method for dealing with non-detect observations, e.g. Chase et al. (2015), (Imamura et al., 2011), Ravaliya et al. (2014), and Schriewer et al. (2015). Alternatives to substitution for left-censored values have been advocated (Helsel, 2005, 2006), including Bayesian and nonparametric analyses (Paulo et al., 2005; Kennedy, 2010; Wangkahad et al., 2017), on the grounds that using a single substituted value for many data points can skew a dataset. Given that

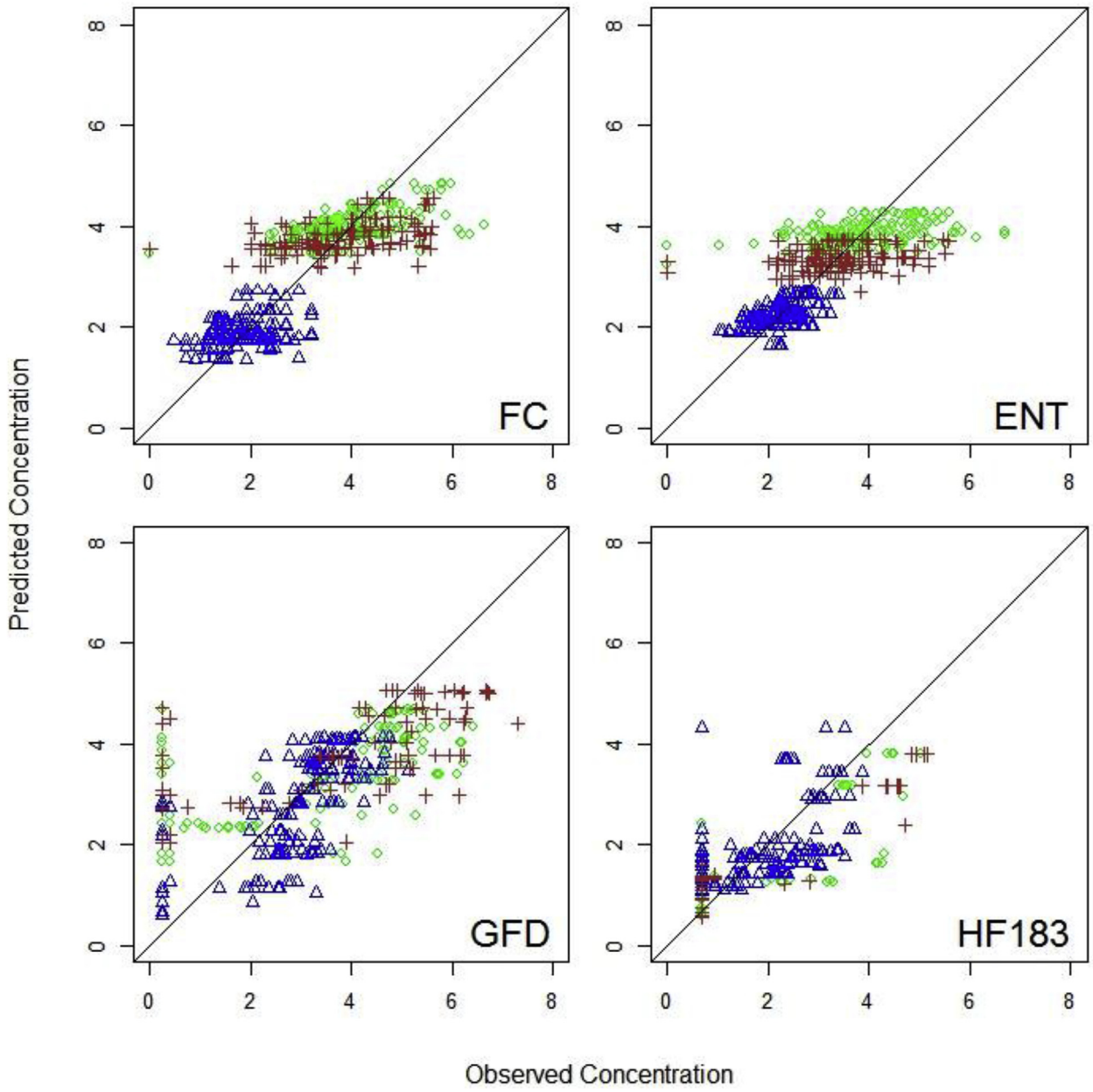

Fig. 4. The predictive power of each mixed-effects ANOVA was assessed by cross-validation (jackknife, leave-one-out procedure) for fecal coliforms, enterococci, GFD, and HF183. Predicted concentrations are plotted against observed concentrations on a $\log_{10}(x+1)$ scale for water (blue triangles), sediment (brown crosses), and vegetation (green circles), with a best-fit line showing 100% model prediction. Predicted and observed concentrations are $\log_{10}$(MPN/100 mL or CFU/100g) for FIB and $\log_{10}$(gene copies/100 mL or/100g) for MST markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

we had large datasets for fecal coliforms ($n = 477$), enterococci ($n = 470$), GFD ($n = 398$), and HF183 ($n = 333$) and all assumptions for ANOVA and mix-effects ANOVA were met, i.e. data normality and homoscedasticity, normality of the residuals of the models, no relationship between residuals and predicted values after data transformation (i.e. log10(x+1)), and independency of observations, we chose to employ the LOD/2 value for non-detect samples.

The relatively weak persistence of HF183 in sediment (Zimmer-Faust et al., 2017) offers one potential explanation of why the human-associated marker was detected more frequently in water than sediment. According to Zimmer-Faust et al. (2017), HF183 exhibited decay rates 2–5 times faster than culturable FIB in sediment. Similar experiments to compare decay rates of FIB and MST markers in vegetation have not been conducted, but our results and those of previous studies suggest that a similar trend may hold. *E. coli*, for example, readily grow on shoreline wrack (Nevers et al., 2016) and seaweeds enhance survival of *E. coli* in beach sand (Quilliam et al., 2014). Enterococci have also been found to grow on vegetation (Badgley et al., 2010).

Confirmation of cross-reactivity of HF183 with white-tailed deer feces in this water body supported our interpretations of the MST results, as detection of HF183 may well have been due to contamination by deer feces, rather than sewage. Consequently, the HF183 marker was not a reliable indicator of human fecal contamination in this water body. Subsequent testing using alternative indicators of human fecal contamination, the sewage-associated pepper mild mottle virus (PMMoV) (Rosario et al., 2009) and human polyomaviruses (HPyVs) (McQuaig et al., 2009), yielded negative results

across all environmental matrices, years, months, and sites (data not shown). Small differences between the DNA sequences analyzed in this study offer an opportunity to design qPCR assays that could discriminate between deer and human sources of *Bacteroides*. An improved HF183 qPCR assay was recently developed by Green et al. (2014), who optimized the HF183 assay by pairing the same forward primer (HF183) used in this study with a new reverse primer (BacR287). This increased the target gene length from 80 bp (this study) to 126 bp (HF183/BacR287). The sensitivity and specificity of the HF183/BacR287 assay was determined to be similar to or better than the HF183/BFDrev assay. Overall, the detection of HF183 in Florida white-tailed deer feces but not in Australian Rusa deer feces indicates geographic variability of the marker and supports the importance of testing for MST marker specificity on local fecal samples.

FC and ENT were positively correlated, as expected. Additional positive correlations between both FIB and GFD provided evidence that elevated levels of FIB were likely due to birds and not humans. By quantifying both FIB and MST markers, we identified birds as a major contributor of fecal pollution in this water body. Moreover, FIB were negatively correlated with HF183 concentrations, which has also been found in a previous study (Sauer et al., 2011), adding to the weight of evidence indicating that sewage was not an important contributor to FIB in the watershed. These results demonstrate the importance of a “toolbox” approach to assessing water quality and human health risk.

The data collected in this study allowed the management organization to meet its designated use as a wildlife conservation site. Natural source exclusion (NSE) status was obtained and the water body was removed from the 303(d) impaired water body list by the Florida Department of Environmental Protection. If regulatory agencies adopt methods similar to those used in this study, funds could more effectively be allocated towards maintaining and improving water quality instead of implementing unnecessary mitigation efforts for water bodies that do not pose a human health risk. Overall, our results lend credence to the growing body of evidence that using culture-based methods for FIB alone provides an incomplete indication of microbial water quality and human health risk (Harwood et al., 2005; Schoen et al., 2011; Kirs et al., 2017), while monitoring both FIB and MST markers over a spatiotemporal scale is more informative of possible sources of fecal pollution (Harwood et al. 2011, 2017; McQuaig et al., 2012; Riedel et al., 2015).

5. Conclusion

- Low levels and intermittent detection of HF183, coupled with consistent detection of GFD, supported our hypothesis that elevated FIB levels were due to bird and not human fecal contamination.
- Cross-reactivity of the human-associated HF183 marker with local deer feces, and a plentiful deer population, explained the intermittent presence of HF183 in the water body.
- Predictive modeling poses a novel method for informing management organizations of likely factors influencing fecal pollution intensity and how to mitigate future fecal pollution events.
- Our study provides an example of how quantifying both FIB and MST markers in different environmental matrices can improve water quality assessment and help tailor TMDL programs for impaired water bodies in the future.

Acknowledgements

We thank Jacob Senkbeil for assistance with sampling, data generation and reporting. We thank Katrina V. Gordon for her early work on this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.07.034.

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