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Seasonal Loading and Concentration Patterns for Fecal *Bacteroidales* qPCR Markers and Relationships to Water Quality Parameters at Baseflow

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Abstract Bacteria belonging to the Order *Bacteroidales* predominate the intestines of warm-blooded animals, and monitoring of these bacteria can indicate fecal pollution impacts to a waterbody. Differences in seasonal concentrations and loadings for *Bacteroidales* and their relationship with physicochemical water parameters were investigated in temperate, inland streams. Seasonal samples ($n = 321$) were collected during baseflow in three central Tennessee, USA, watersheds. To estimate total fecal bacteria in receiving streams, general *Bacteroidales* 16S rRNA gene targets were analyzed by quantitative PCR and reported as concentration and loadings for individual and combined watersheds. In most cases, *Bacteroidales* marker concentrations were highest during spring/summer and loading values were highest in the spring. *Bacteroidales* concentrations were positively correlated with temperature and total suspended solids and negatively with dissolved oxygen, while no consistent correlations were found between loadings and abiotic factors. Temperature, total suspended solids, and dissolved oxygen are likely drivers influencing seasonal patterns for *Bacteroidales* concentrations. Researchers and water quality stakeholders should carefully consider measurement type (concentration versus loading), season, and water quality parameters as elements that could impact results when developing fecal monitoring projects.

Keywords Fecal indicator bacteria · *Bacteroidales* · qPCR · Season · Water quality parameters

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1 Introduction

Fecal pollution in freshwater ecosystems throughout the world is known to negatively impact both ecological functions and human health. For instance, excessive fecal inputs from concentrated agricultural feeding operations cause an increase in nutrient concentrations and subsequent eutrophication (Mallin and Cahoon 2003), while elevated fecal bacteria concentrations in recreational waters have been associated with increased swimmer illness rates (Prüss 1998; Wade et al. 2008). Sources of fecal pollution include direct inputs, such as sewer overflows, or from indirect contributions like stormwater runoff carrying domestic animal waste. In freshwater ecosystems, fecal pollution and associated pathogens responsible for causing

human illness are typically monitored at stream baseflow by culturing *Escherichia coli*, fecal indicator bacteria (FIB) pervasive in warm-blooded animals' gastrointestinal tracts (U.S. EPA 1986, 2001).

Fecal bacteria species within the Order *Bacteroidales* are recognized as alternative FIB (Fiksdal et al. 1985; Kreader 1995; U.S. EPA 2005) that can provide complementary information to *E. coli* monitoring. *Bacteroidales* are obligate anaerobes that are specific (Dick et al. 2005) or selective (Layton et al. 2006) within the digestive system of their host making them particularly useful to determine both the sources and amount of fecal contamination. Rapid culture independent quantitative polymerase chain reaction (qPCR) assays for both general *Bacteroidales* 16S rRNA markers and those specific for human or other animal hosts have been developed (Bernhard and Field 2000a, 2000b; Dick et al. 2005; Seurinck et al. 2005; Layton et al. 2006; Okabe et al. 2007; Shanks et al. 2009). The *Bacteroidales* are considered to be good indicators of recent fecal inputs because they have generally low survival rates in the environment (Fiksdal et al. 1985; Dick et al. 2010), although recent research indicates that long-term persistence outside of a gut is possible (Weidhaas et al. 2015).

Once deposited onto a watershed and transported into a waterbody, fecal bacteria encounter fluctuations in physicochemical water quality parameters (e.g., pH, dissolved oxygen, temperature, total suspended solids) and flow that can be season-dependent or not. Understanding how *Bacteroidales* genes may persist in the environment will benefit users of fecal source-tracking applications, assist water quality managers in watershed management plans using alternative indicators, and raise awareness to water quality regulators considering these organisms as risk-based fecal indicator bacteria supplementary to traditional FIB, such as *E. coli*. Most research to understand how environmental abiotic factors may influence the fate of *Bacteroidales* has been conducted in laboratory microcosm experiments and to a more limited extent in field situations. Using conventional PCR in a laboratory microcosm study using river water, Kreader (1998) found that DNA of *Bacteroides distasonis* was detectable for significantly longer at 4 °C compared to higher temperatures (14, 24, 30 °C). Subsequent laboratory and field microcosm research using qPCR has supported the finding that *Bacteroidales* 16S rRNA genetic targets tend to be detected more frequently at lower temperatures (Okabe and Shimazu 2007; Balleste and Blanch 2010; Bell et al. 2009). The effects of other variables including salinity, dissolved oxygen, and sunlight on survival of members of the *Bacteroidales* have also been investigated (Baughn and Malamy 2004; Okabe and Shimazu 2007; Walters and Field 2009; Bae and Wuertz 2009; Balleste and Blanch 2010).

Research to investigate fecal bacteria relationships with abiotic factors in a field setting has focused primarily on stormwater monitoring approaches using conventional culturable FIB, but not *Bacteroidales*. For instance, it has been shown that FIB are often increased during storm conditions in inland watersheds (Rowny and Stewart 2012) and tidal creek headwaters (Stumpf et al. 2010), indicating that runoff is a factor for increased FIB concentrations instream. Little research in regard to relationships between genetic targets and factors has been conducted on inland streams at baseflow conditions. However, recent research conducted in northeast Georgia, USA, at baseflow conditions found that the prevalence of *stx2* gene from Shiga toxin-producing *E. coli* (STEC) was negatively correlated with water temperature, pH, and conductivity (Bradshaw et al. 2016).

Seasonal changes and dynamic flow and water quality parameters could modulate the presence of *Bacteroidales* markers, yet literature is sparse on this topic. Nonetheless, no seasonal fluctuations of *Bacteroidales* markers were found in a coastal study near Monterey Bay, California (Schriewer et al. 2010), while other FIB, such as *E. coli*, have been detected in higher concentrations in the spring and summer months with lower concentrations in the cooler seasons (Stallard et al. 2016; Traister and Anisfeld 2006). Flow-based *E. coli* loading measurements, which are important components of stormwater management programs (e.g., total maximum daily loads (TMDLs)), were found to be higher during the rainy, spring season at baseflow conditions in inland watersheds (Stallard et al. 2016) and during November for a coastal stormwater study (Converse et al. 2011).

The objective of the present study was to examine relationships between general *Bacteroidales* genetic markers and abiotic factors from a concentration and flow-based (loading) measurement standpoint in three central Tennessee, USA, watersheds at baseflow. Specific objectives were to (1) determine seasonal trends for *Bacteroidales* concentrations and loadings and (2) examine relationships between *Bacteroidales* concentrations/loadings and water quality parameters (temperature, dissolved oxygen, conductivity, total suspended solids, and pH) both in individual and grouped watersheds. These findings will fill in data gaps

for understanding how environmental factors could play a role in *Bacteroidales* presence for temperate, inland waters at baseflow stream conditions. This information will be useful for water quality stakeholders considering a comprehensive approach to conducting water quality activities (e.g., fecal-tracking studies, water quality monitoring, watershed management planning) instead of sampling solely for a single FIB.

2 Materials and Methods

2.1 Study Design

During 2009–2012, sites within three pathogen-impaired watersheds (Browns, Richland, Mill; Tennessee Department of Environment and Conservation [TDEC] 2014) located in central Tennessee were sampled and analyzed for fecal bacteria presence during summer (June, July, August), fall (September, October, November), winter (December, January, February), and spring (March, April, May). There was a major flood event in May 2010, and these samples were omitted from analysis. The number of yearly samples, combined by season for each watershed, ranged from 12 to 31 with a total of 321 samples. Data was pooled seasonally across multiple years to account for a wide range of site, seasonal, and yearly variation, thus providing a very robust estimate of FIB. Sample sizes are presented within figures and tables.

2.2 Study Site Characterization

The three watersheds under study fall within the Outer Nashville Basin Level IV ecoregion (TDEC 2000) and exhibit a mild climate with an annual mean temperature of 15 °C (https://ag.tennessee.edu/climate/Pages/climatedatatn.aspx). The ecoregion consists of non-cherty limestone bedrock, deciduous forests land cover, and low to moderate gradient streams; however, the selected watersheds are heavily developed and may not conform to ecoregion characteristics. There is no direct influence of wastewater treatment plants or concentrated animal feeding operations within the watersheds. Each of the three watersheds is approximately 5000 ha, and land uses within the watersheds are largely similar, with approximately 50% residential, 10–20% commercial, and 25% open area. However, the imperviousness of Mill watershed is 40%, which is 10–20% higher than the other two watersheds in the study (a more detailed description of the watersheds was previously given in Stallard et al. 2016).

2.3 Sample Collection

Samples were collected during baseflow stream conditions (< 0.1 in. rainfall within last 72 h) following TDEC standard protocols (TDEC 2009). For *Bacteroidales* analysis, approximately 110 mL of water was collected from the subsurface at the thalweg of each stream in a sterile polyethylene container, placed on ice, and transported to the laboratory within required hold time (< 6 h) for sample processing and analysis. A 1 L sterile polypropylene bottle was used for the collection of samples for total suspended solids (TSS) and processed by Method 2540D in Standard Methods (APHA 2012). Concurrent with sample collection, stream velocity (ft/s) was taken for most samples by a Swoffer Model 3000 Current Velocity Meter-Flowmeter and used to calculate flow (cubic feet per second, CFS). Overall loadings to assess gene copies/day to a given sampling site were calculated by multiplying *Bacteroidales* concentrations (gene copies/μL) by simultaneous flow measurements. During each sample collection, pH (Hach Sension meter), dissolved oxygen (DO) (mg/L), conductivity (μS/cm), and temperature (°C) (YSI 85) were recorded.

2.4 *Bacteroidales* Analysis

An aliquot of 1.3 mL was transferred from the 110 mL sample volume to a 1.5 mL microcentrifuge tube and stored at − 80 °C until analysis. Storage time before analysis never exceeded six months. The number of *Bacteroidales* 16S rRNA gene copies was determined for stream samples by the AllBac real-time quantitative PCR assay without DNA extraction (Layton et al. 2006; Fode-Vaughn et al. 2001). A total volume of 25 μL per well was used for each qPCR carried out on 96-well plates. Each PCR well contained 2.5 μL of stream sample, standard, or negative control, 11 μL of Brilliant® II qPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA), 400 nM AllBac 296f forward and 412r reverse primers, 300 nM Taqman AllBac 375Bhqr fluorescent probe, and 9.5 μL of sterile HPLC water. Plasmid DNA standards containing the *Bacteroidales* 16S rRNA gene target were used to generate a standard curve with dilutions from 1×10^7 to 10 copies per μL for each run. A 2.5 μL aliquot, taken

from a sewage treatment plant influent sample that served as the positive control, was run in separate wells and confirmed as a positive signal with an average Ct of 27. Filter-sterilized HPLC water was used for the no-template control. All standards, negative, and positive controls were performed in triplicate. Stream samples were initially run in triplicate; however, samples in 2010 and 2011 were processed in duplicate to increase number of samples per plate. The limit of detection (LoD) was determined by running 20 replicates of diluted plasmid standard. The lowest plasmid standard concentration with detections > 95% was 5 copies/μL. Six samples (4 from Browns and 2 from Richland) were below this value, and these were assigned a value of half the detection limit (2.5 copies/μL). An additional well following every sample replicate set was spiked with 2.5×10^5 copies of the plasmid DNA plus sample to evaluate qPCR inhibition of each sample. Percentage of spike recovery was calculated as copy number recovered in the spiked well minus the copy number in an unspiked sample well divided by 2.5×10^5, then multiplied by 100. Only one sample generated a spike recovery of < 70%, indicating qPCR inhibition, and was rerun with a 10-fold dilution. Gene targets were amplified using a CFX 96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The direct qPCR amplification protocol was as follows: 55 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, and 60 °C for 30 s. *Bacteroidales* 16S rRNA gene concentrations in a stream sample are reported as copies/μL, and loadings are presented as copies/day.

2.5 Statistics

One-way Analysis of Variance (ANOVA) tests were performed to assess seasonal differences for concentrations and loadings of *Bacteroidales* for individual and grouped watersheds. When significant seasonal differences were found by ANOVA, Tukey's post hoc tests were carried out to detect differences among seasons. Log_{10} transformations were implemented on bacteria concentrations and loadings if assumptions of normality and equality of variances were not met. A non-parametric Spearman's rank order test was used for each watershed to assess correlations between *Bacteroidales* concentrations and loadings with temperature, DO, conductivity, TSS, and pH. An alpha (α) = 0.05 was used as the significance level for all statistical analyses performed with IBM SPSS Ver 20 (Armonk, NY: IBM Corp.).

3 Results and Discussion

3.1 Seasonal *Bacteroidales* Concentrations

Bacteroidales concentrations were highest in the warmer months of spring and/or summer in two of the three watersheds (Browns $F_{3,121} = 3.457$, $p < 0.05$; Richland $F_{3,117} = 16.518$, $p < 0.01$), as well as when the data from the three watersheds were grouped ($F_{3,317} = 8.449$, $p < 0.01$) (Fig. 1). Interestingly, in the Mill watershed, there were no significant differences among the seasons for *Bacteroidales* concentrations ($F_{3,71} = 1.286$, $p = 0.286$).

The pattern of higher *Bacteroidales* concentrations in the warmer seasons vs lower in the winter that was seen in the grouped watersheds and two out of three individual watersheds was similar to that seen in a prior study from our laboratory measuring *E. coli* concentrations (Stallard et al. 2016). In fact, there is a significant, but weak correlation between *E. coli* and *Bacteroidales* concentrations for grouped watersheds ($p < 0.05$; $r = 0.113$). These data imply that while *Bacteroidales* (measured as AllBac) and *E. coli* concentrations are related, there are most likely environmental factors which are impacting the two differently. *Bacteroidales* patterns in the present study do not agree with the only other report of *Bacteroidales* seasonal concentrations. Schriewer et al. (2010) reported no seasonal fluctuations of *Bacteroidales* (or fecal coliforms) around Monterey Bay, California, although no statistical comparisons were reported. Perhaps the lack of seasonal trends was due to the specific *Bacteroidales* gene marker targeted (BacUni in Schriewer et al. versus AllBac in the present study). Additionally, streams, rivers, and estuaries were all compiled to assess seasonal trends in Schriewer's study, whereas only freshwater streams with no marine influence were sampled in the present study.

It is plausible that increased activity/movements near riparian zones in warmer months from domestic animals or wildlife could increase fecal inputs both in riparian zones and in-stream. Increases in *Bacteroidales* concentrations during the warmest summer months may be more pronounced because stream flow and water levels are reduced at this time. In the possibility that *Bacteroidales* is surviving in the environment, the concentration patterns seen in the present study may be due to reduced metabolic activity in cooler winter temperatures followed by growth and replication on the watershed in warmer months and transportation to streams by spring rainfall events.

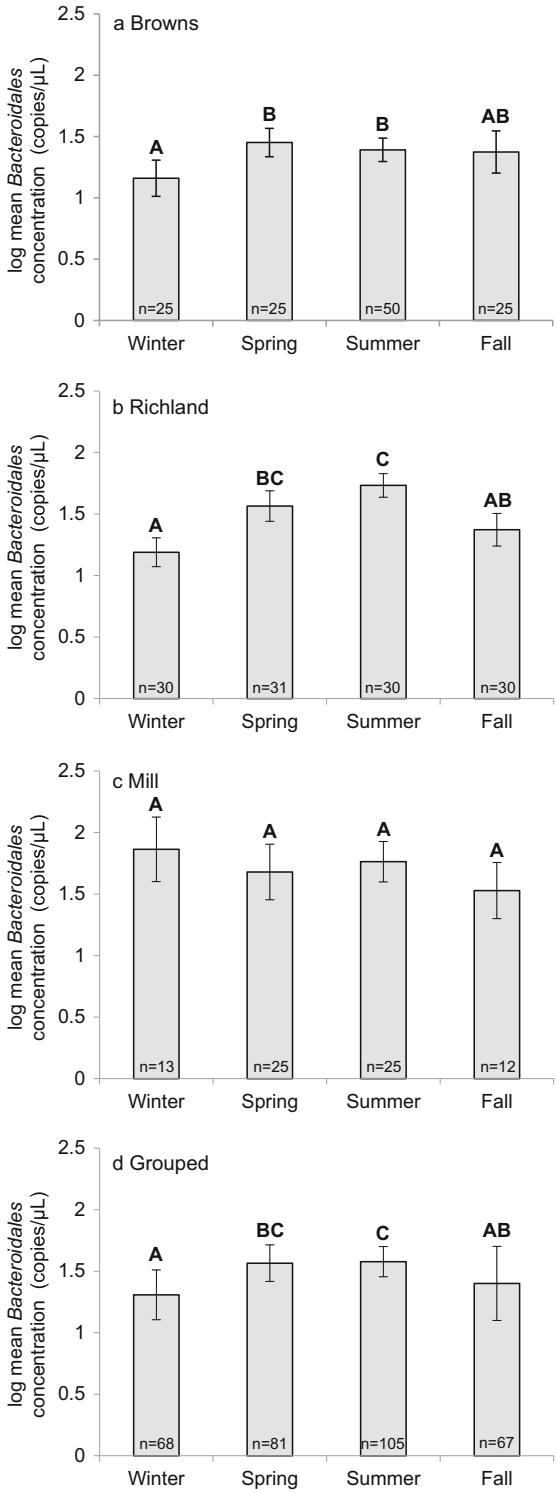

Fig. 1 Seasonal comparisons for *Bacteroidales* concentrations (bars) in Browns (**a**), Richland (**b**), Mill (**c**), and grouped (**d**) watersheds. Seasons not sharing similar letters are significantly different from each other. Data presented as mean ± 95% confidence intervals

3.2 Seasonal *Bacteroidales* Loadings

For grouped watersheds, *Bacteroidales* spring loadings were higher than all other seasons ($F_{3,270} = 16.142$, $p < 0.01$) (Fig. 2). For individual watersheds, *Bacteroidales* loadings were found to be highest in spring for Browns ($F_{3,93} = 9.539$, $p < 0.01$), while for Richland watershed, loadings during spring were significantly higher than summer and fall, but not winter ($F_{3,107} = 6.808$, $p < 0.01$). As with concentrations, loadings in the Mill were similar in all seasons ($F_{3,62} = 2.204$, $p = 0.096$). Also, as with concentrations, there is a significant correlation between *E. coli* and *Bacteroidales* loadings for grouped watersheds ($p < 0.001$; $r = 0.542$) (for complete *E. coli* data, see Stallard et al. 2016).

Loadings for *Bacteroidales* were highest predominantly in the spring even though the highest concentrations occurred typically in the summer. Antecedent rainfall during winter and spring rainy seasons could have increased flow leading to higher loading even during baseflow sampling. Similarly, the increased flow could have agitated and resuspended historically deposited FIB in stream sediments leading to higher loadings. Another potential factor influencing *Bacteroidales* seasonal patterns for loadings in Browns, Richland, and the grouped watersheds could be the integration of FIB into the water, sediment or riparian soil matrix (i.e., naturalization). Vierheilig et al. (2012) found a high level of AllBac *Bacteroidales* marker in pristine alpine soils (10 cm depth) void of fecal pollution or animal activity. This suggests that the *Bacteroidales* may have been from feces deposited at an earlier time that became integrated into soil microbiota or possibly derived from resident soil microorganisms. Likewise, Byappanahalli et al. (2006) have suggested that naturalized *E. coli* in soil from a Great Lakes watershed could be a fecal input to Great Lakes waterbodies. Also, Ishii et al. (2006) reported the concentrations of naturalized soil *E. coli* from three temperate Lake Superior, USA, watersheds were highest in summer and fall and lowest in winter and spring months. High spring loadings in central Tennessee streams in the present study could be originating from naturalized soil FIB that are being transported by runoff during the rainy season. It is probable that the majority of *Bacteroidales* genetic markers seen in the present study are associated with fecal deposition at some point in time because *E. coli* were also detected at high levels at this time in the same watersheds (Stallard et al. 2016) and the concentration

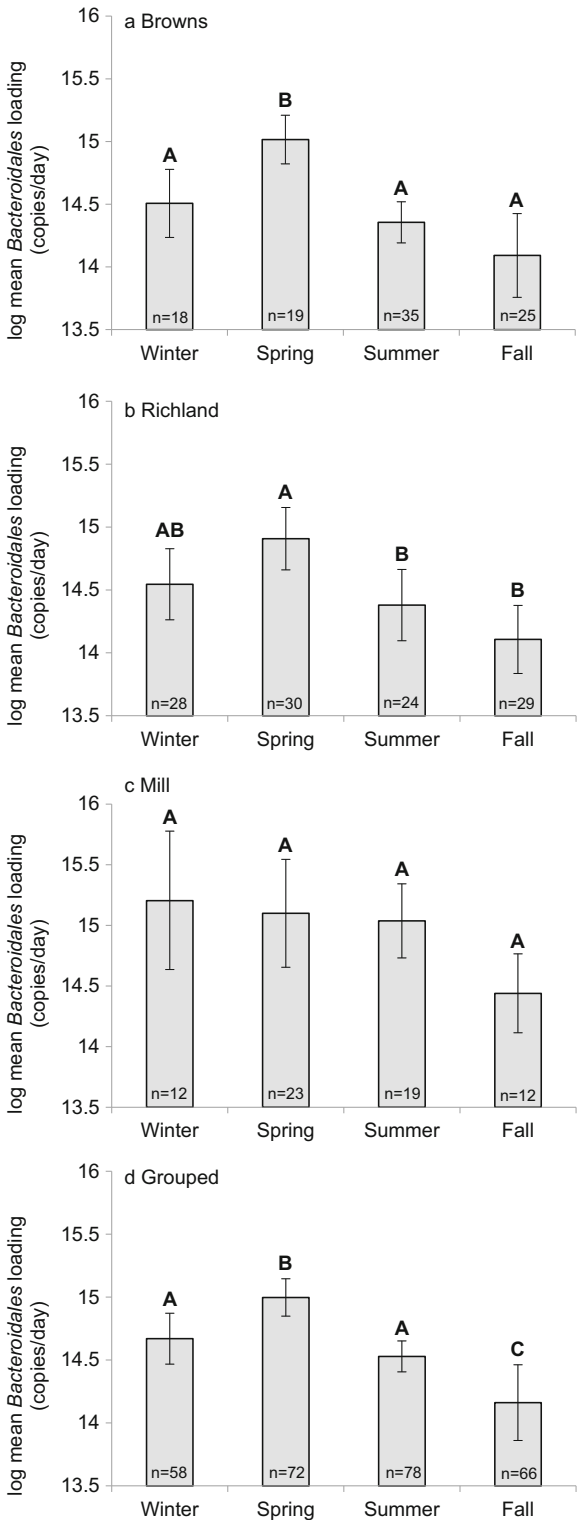

Fig. 2 Seasonal comparisons for *Bacteroidales* loadings (bars) in Browns (**a**), Richland (**b**), Mill (**c**), and grouped (**d**) watersheds. Seasons not sharing similar letters are significantly different from each other. Data presented as mean ± 95% confidence intervals

and loading values for *Bacteroidales* and *E. coli* are correlated.

3.3 Concentrations and Loadings vs Water Quality Parameters

Bacteroidales concentrations consistently showed significant, though weak-moderate, negative correlations with DO and positive correlations with temperature in Browns (DO $r = -0.275$, temperature $r = 0.200$), Richland (DO $r = -0.424$, temperature $r = 0.542$), and the three watersheds grouped (DO $r = -0.201$, temperature $r = 0.255$), but not the Mill watershed individually (Table 1). When investigated individually or grouped, weak-moderate positive correlations were found for TSS and *Bacteroidales* concentrations in the three watersheds under study (r values ranged from 0.195 to 0.334), while concentrations were not correlated with conductivity in any of the watersheds.

Correlations between abiotic factors and *Bacteroidales* loadings were more inconsistent than those with concentrations. Again, Mill watershed was frequently found to be different than the other two in relation to loading correlations. Unlike with concentrations, no significant correlations were revealed for loadings with DO or temperature in either individual or grouped watersheds. However, *Bacteroidales* loadings were correlated negatively with conductivity in Browns ($r = -0.345$), Richland ($r = -0.233$), and the grouped ($r = -0.200$) watersheds and were positively correlated with TSS in Mill ($r = 0.323$). Negative correlations between loadings and pH were seen in Browns ($r = -0.277$) and Richland ($r = -0.329$), while a positive correlation was seen in Mill ($r = 0.446$).

Not surprisingly, strong positive associations between *Bacteroidales* concentrations and loadings were exhibited by all individual and grouped watersheds. However, the very strong correlation at the Mill watershed ($r = 0.810$) compared to Browns ($r = 0.457$) and Richland ($r = 0.475$) watersheds again seemed to indicate differences at the Mill.

Seasonal patterns of concentrations and loadings are likely attributable to seasonal variation of associated water parameters, at least in part. Understanding what factors play a role in the presence of FIB would inform water quality regulators to allocate resources for collecting data on such. For *Bacteroidales* concentrations in the present study, two of the three individual as well as the grouped watersheds had positive and

Table 1 Spearman's rank correlation analyses for *Bacteroidales* concentrations and loadings with dissolved oxygen, temperature, total suspended solids, conductivity, and pH (values represent Spearman's *r*, with sample sizes in parentheses)

		Bacteroidales concentration	*Bacteroidales* loading
Dissolved oxygen (mg/L)	Browns	− 0.275** (125)	0.036 (97)
	Richland	− 0.424** (121)	− 0.049 (111)
	Mill	0.096 (75)	0.111 (66)
	Grouped	− 0.201** (321)	0.034 (274)
Temperature (°C)	Browns	0.200* (125)	− 0.094 (97)
	Richland	0.542** (121)	0.012 (111)
	Mill	0.008 (75)	− 0.025 (66)
	Grouped	0.255** (321)	− 0.032 (274)
Total suspended solids (mg/L)	Browns	0.236* (75)	− 0.132 (65)
	Richland	0.334** (91)	− 0.025 (83)
	Mill	0.281* (70)	0.323** (65)
	Grouped	0.195** (236)	− 0.009 (213)
Conductivity (μS/cm)	Browns	0.129 (125)	− 0.345** (97)
	Richland	− 0.060 (121)	− 0.233* (111)
	Mill	− 0.021 (75)	− 0.173 (66)
	Grouped	0.046 (321)	− 0.200** (274)
pH	Browns	− 0.077 (125)	− 0.277** (97)
	Richland	− 0.294** (121)	− 0.329** (111)
	Mill	0.378** (75)	0.446** (66)
	Grouped	0.014 (321)	− 0.054 (274)
Bacteroidales concentration	Browns	1.000 (125)	0.457** (97)
	Richland	1.000 (121)	0.475** (111)
	Mill	1.000 (75)	0.810** (66)
	Grouped	1.000 (321)	0.603** (274)
Bacteroidales loading	Browns	0.457** (97)	1.000 (97)
	Richland	0.475** (111)	1.000 (111)
	Mill	0.810** (66)	1.000 (66)
	Grouped	0.603** (274)	1.000 (274)

**Correlation is significant at the 0.01 level

*Correlation is significant at the 0.05 level

negative relationships with temperature and oxygen, respectively. These temperature and DO associations make sense in conjunction with each other since temperature and DO are negatively correlated in aquatic systems. However, these results were dissimilar to prior laboratory and microcosm studies that revealed *Bacteroidales* gene targets were more persistent in colder temperatures (Kreader 1998; Bell et al. 2009; Schulz and Childers 2011). High temperature was cited as the key factor affecting increased decay of environmental *Bacteroides* strains in both a laboratory study and an on-site river study where sewage-filled dialysis bags were exposed to stream conditions (Balleste and Blanch 2010). Differences between the present study and others may be explained by site-specific differences in abiotic factors and effects

related to study design. For example, in the present study, samples and measurements were taken directly from field sites and no fecal samples were manipulated or introduced into closed systems (e.g., dialysis bags) and attributes of water parameters in laboratory mesocosms may have degraded and caused more rapid decay of bacteria or DNA from dead/non-viable bacteria. It is also possible that there were continuous or intermittent inputs of fecal bacteria in the present study at some locations.

Though several studies have investigated sediment as a secondary habitat for *Bacteroidales*, there has been little exploration of TSS as an influential factor for *Bacteroidales* persistence. Turbidity, a parameter which detects the amount of suspended solids in a water column by measuring scattered light instead of a concentration like TSS, was found to have a positive correlation with *Bacteroides* loads (kg/day) and a stronger positive correlation was seen when sampling from headwaters to tailwaters in an eastern Tennessee watershed (Gentry et al. 2007). In the present study, among all watersheds, there was a consistent positive correlation of TSS with *Bacteroidales* concentrations, suggesting that suspended solids may play an overlooked role in gene persistence by a variety of mechanisms. For instance, the suspended particles could serve as infrastructure for attachment of *Bacteroidales* (viable or non-viable) and increase overall densities of *Bacteroidales* gene copies. The solids could provide interstitial spaces for growth and act as a habitat for these anaerobic bacteria during baseflow; however, additional research needs to be conducted to verify if these gene copies are from viable cells.

In the present study, conductivity displayed no relationship with *Bacteroidales* concentrations for all watersheds. This is interesting because TSS and conductivity typically have a positive relationship with each other, but did not in this study (data not shown). The suspended solids measured in the present study may contain chemical properties less likely of carrying an electrical charge and could have confounded the possibility of conductivity having a relationship with *Bacteroidales* concentrations. A negative correlation was found for *Bacteroidales* loadings and conductivity in the Browns and Richland, but no correlation was found in the Mill watershed, again highlighting that the Mill does not follow patterns of the other watersheds. The measurement of pH did not produce a clear pattern for *Bacteroidales* concentration or loadings. These data are similar to those from a watershed study in eastern Tennessee that reported conductivity and pH to not be associated with FIB (*E. coli*) concentrations (Gentry et al. 2006).

3.4 Importance of Differences in the Mill Watershed

In the Mill watershed, the finding of no patterns for seasonal *Bacteroidales* concentration and loadings, as well as lack of correlations with temperature and DO, is quite important and could signify a watershed with a constant fecal input. In fact, there have been a number of documented intermittent sewer overflows at various places in the Mill watershed (Metro Nashville Water Services, personal communication). Perhaps, unique watershed characteristics at the Mill, such as high imperviousness and subsequent increased runoff, may contribute to higher concentrations or loadings of FIB, thus minimizing a seasonal pattern. A human-associated fecal *Bacteroidales* marker (HuBac) was also analyzed and was slightly above detection in the Mill watershed on a few occasions (data not shown). Water quality managers responsible for improving impaired recreational waterbodies should take note of patterns contradictory with baseline data from within the watershed and from other watersheds as a reason to prioritize a watershed for further investigation.

4 Conclusions

The present study demonstrates seasonal patterns for *Bacteroidales* concentrations and loadings in a freshwater system at baseflow and highlights the fact that temperature, TSS, and dissolved oxygen are repeatedly significant in these patterns. Because regulatory water quality sampling or fecal-tracking studies can occur at infrequent intervals, and since peaks for *Bacteroidales* concentrations and loadings can happen in different seasons, water quality managers must carefully select the type (concentration vs loading) and time (season) of sampling that best fit water quality goals (i.e., source reduction or human health protection). For example, winter sampling might be best for detection of sewer overflow or septic tank seepage because fecal indicators are generally lower in the colder months and any spike in concentrations might be indicative of a recent direct input. Also, a priority ranking for monitoring, fecal

source-tracking, and remediation should be considered for sites, such as the Mill watershed in the present study, which do not follow typical patterns for FIB concentrations associated with seasonal and water parameters, as this could indicate a constant fecal input.

Once baseline data have been gathered in a watershed that shows the AllBac marker to be correlated with traditional fecal indicator bacteria, such as *E. coli*, it would be beneficial to utilize the AllBac qPCR assay because of its faster turnaround time compared to conventional *E. coli* methods. However, when AllBac and *E. coli* patterns do not correlate or the AllBac patterns do not match typical patterns for traditional FIB, stakeholders would need to investigate to explain this phenomenon and/or deem the utility of the marker limited. This would be warranted because of the possibility that the lack of correlation is being caused by an input of *Bacteroidales* from non-fecal sources. Continued field studies to reveal how *Bacteroidales* and *E. coli* behave under the same environmental influences will be imperative going forward when considering *Bacteroidales* markers as a surrogate or supplement to traditional indicators.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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