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Storm loads of culturable and molecular fecal indicators in an inland urban stream

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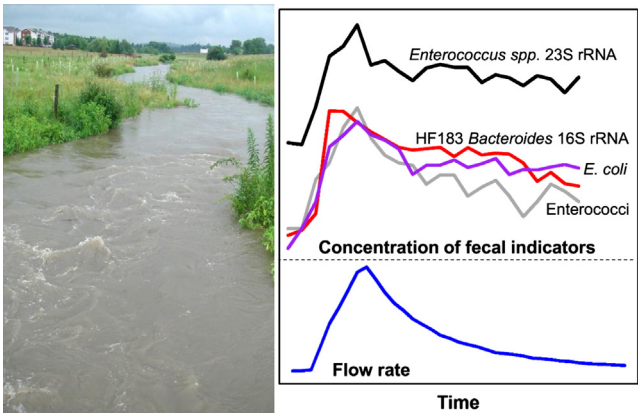
HIGHLIGHTS

- High-frequency intra-storm samples were taken in an instrumented urban stream.
- Concentrations and loading rates of four fecal indicators were quantified.
- Culturable and molecular general fecal indicators correlate significantly.
- Source-specific and general fecal indicators show different patterns.

GRAPHICAL ABSTRACT



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ABSTRACT

Elevated concentrations of fecal indicator bacteria in receiving waters during wet-weather flows are a considerable public health concern that is likely to be exacerbated by future climate change and urbanization. Knowledge of factors driving the fate and transport of fecal indicator bacteria in stormwater is limited, and even less is known about molecular fecal indicators, which may eventually supplant traditional culturable indicators. In this study, concentrations and loading rates of both culturable and molecular fecal indicators were quantified throughout six storm events in an instrumented inland urban stream. While both concentrations and loading rates of each fecal indicator increased rapidly during the rising limb of the storm hydrographs, it is the loading rates rather than instantaneous concentrations that provide a better estimate of transport through the stream during the entire storm. Concentrations of general fecal indicators (both culturable and molecular) correlated most highly with each other during storm events but not with the human-associated HF183 *Bacteroides* marker. Event loads of general fecal indicators most strongly correlated with total runoff volume, maximum discharge, and maximum turbidity, while event loads of HF183 most strongly correlated with the time to peak flow in a hydrograph. These observations suggest that collection of multiple samples during a storm event is critical for accurate predictions of fecal indicator loading rates and total loads during wet-weather flows, which are required for effective

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watershed management. In addition, existing predictive models based on general fecal indicators may not be sufficient to predict source-specific genetic markers of fecal contamination.

1. Introduction

Fecal pollution from agricultural operations, human-derived sources, domestic animals and wildlife are often transferred to receiving waters during intermittent storm events, potentially leading to the downstream dissemination of pathogens (Ford and Colwell, 1996; Rose et al., 2000; Krometis et al., 2007; Collier et al., 2012). This represents a considerable public health concern and challenge in watershed management. An increase in the frequency and magnitude of storm events is predicted given anticipated climate change, with the most dramatic effects expected in urban areas due to their altered landscapes and hydrology. Consequently, efforts to better understand sources, transport processes, and accompanying risks associated with fecal indicator bacteria (FIB) in urban stormwater runoff continue to receive substantial attention (Gaffield et al., 2003).

The majority of previous studies have relied upon the collection and analysis of single grab samples of surface waters during high-flow events to understand relationships with major environmental factors such as land use and hydro-meteorological variables (Reeves et al., 2004; Gentry et al., 2006; Vidon et al., 2008; Liao et al., 2014). However, the samples collected at single time points do not account for concentration changes during the course of a storm and therefore provide limited information regarding how loading rates might fluctuate during storm events. Pollutant loading information is critically important for watershed managers, as the current regulatory strategy informing remediation efforts is the Total Maximum Daily Load (TMDL) program under the U.S. Clean Water Act's sections 305(b) and 303(d), which quantifies target reductions in contamination per source in terms of loads rather than concentrations (Benham et al., 2011). Recent studies that have analyzed multiple discrete samples per storm event have noted that a single storm in an urban watershed could transport the same FIB loads as multiple years' of dry-weather loads, further emphasizing the need to focus on reductions of stormwater inputs of fecal contamination to reduce downstream impacts and exposure risks (Krometis et al., 2007).

Previous estimates of stormwater microbial loads have focused exclusively on levels of culturable FIB (e.g., *Escherichia coli*, enterococci) (Surbeck et al., 2006; Hathaway et al., 2010; McCarthy et al., 2013), so little is known regarding stormwater loading rates of emerging microbial source tracking (MST) indicators. Focusing on the transport of culturable FIB remains important given current regulatory standards and programs that list maximum permissible culturable FIB concentrations in terms of coliform forming units (CFU) per 100 mL. However, it appears likely that quantitative polymerase chain reaction (qPCR) will eventually supplant culture-based methods as the monitoring mechanism of choice. Compared to culture-based methods, qPCR is advantageous in situations that require quick regulatory decisions (e.g., beach closures) (Griffith et al., 2009) or quantification of relative contributions from different pollutant sources in order to prioritize remediation efforts (Sauer et al., 2011). Given that MST markers generally target genetic material rather than viable organisms, their transport dynamics during stormflows may be different. This represents an important knowledge gap in developing improved approaches to watershed management and research is needed to investigate the relationships between the two method endpoints and understand associations with environmental variables.

The goal of this study was to quantify the transport of culturable and molecular fecal indicators in an inland urban stream during storm events. The specific objectives were to 1) contrast the intra-storm patterns of concentrations and loading rates of culturable and molecular fecal indicators throughout each storm; 2) estimate the total event load of each fecal indicator during each storm; and 3) investigate how storm-induced event loads of different fecal indicators relate to a variety of hydro-meteorological and physicochemical factors. High-frequency samples were collected during six summer storms in 2013, and analyzed using multiple methods to generate concentrations of culturable *E. coli* (EC), culturable enterococci (ENT), *Enterococcus* spp. 23S rRNA gene copies (ENT-23S) (Haugland et al., 2005), and the human-associated HF183 *Bacteroides* 16S rRNA gene copies (HF183) (Seurinck et al., 2005) for each sample. It is important to note that these indicators were selected based on the primary objective to examine relative changes of different types of fecal indicators commonly used in urban watersheds during storm events; although a widely used indicator of human pollution was one of these targets, the goal of this work was not to definitively quantify or detect specific sources of fecal inputs in the watershed.

2. Materials and methods

2.1. Study area description

Samples were collected at a sampling bridge at the Virginia Tech Stream Research, Education, and Management Laboratory (StREAM Lab) along Stroubles Creek in Blacksburg, VA, USA (Fig. 1) (Thompson et al., 2012). The 15-km creek, classified as a recreational water, runs from northeastern Blacksburg, through the Virginia Tech campus, and then into the New River, which has been the region's main source of drinking water supply and home to various recreational activities since the 1950s. A segment of the creek is instrumented with real-time monitoring capacity for a variety of hydro-meteorological and physicochemical variables as described in previous studies (Thompson et al., 2012; Liao et al., 2014). The watershed that drains to the sampling site encompasses an area of 14.4 km^2 currently classified as 84% urban/residential, 13% agricultural, and 3% forested (Fig. 1). The average annual precipitation is 103.9 cm (40.9 in.) with 30% of this precipitation occurring during the summer (June–August). The average temperature in summer is 20.9 °C (69.7 °F), with a minimum of 14.7 °C (58.4 °F) and a maximum of 27.2 °C (80.9 °F).

Growth and urbanization is rapidly changing the Stroubles Creek watershed and stream hydrology (VADEQ and VADCR, 2006). The complete watershed above the sampling bridge is serviced by a sewer system, and storm and sanitary sewers are entirely separate. A Total Maximum Daily Load (TMDL) implementation plan was established in 2006 to address violation of the general biological integrity clause of the Clean Water Act; reduced benthic macro-invertebrate abundance and diversity was assumed to be mainly the result of excessive sediment loads (VADEQ and VADCR, 2003, 2006). In 2008, the 11.6-km segment of the creek (Fig. 1) was added to the state's 303(d) impaired waters list due to elevated *E. coli* levels, with a TMDL specifying reductions in reducing bacteria loads impending (VADEQ, 2010).

2.2. Sampling strategy

Discrete water samples were collected automatically via a Teledyne 6712 ISCO sampler (Isco, Inc., Lincoln, NE, USA) during six summer storms (June 26, June 27, June 30, July 2, July 10, and July 21, 2013). Prior to an anticipated storm event, the ISCO was loaded with 24 pre-sterilized sampling bottles and programed for equal-volume (i.e. 750 mL) sampling at constant intervals (i.e. 15 min or 30 min). The ISCO was triggered manually based on direct observations of rainfall within the watershed. The samples were kept on ice and retained for analysis if discharge and turbidity values (continuously monitored in

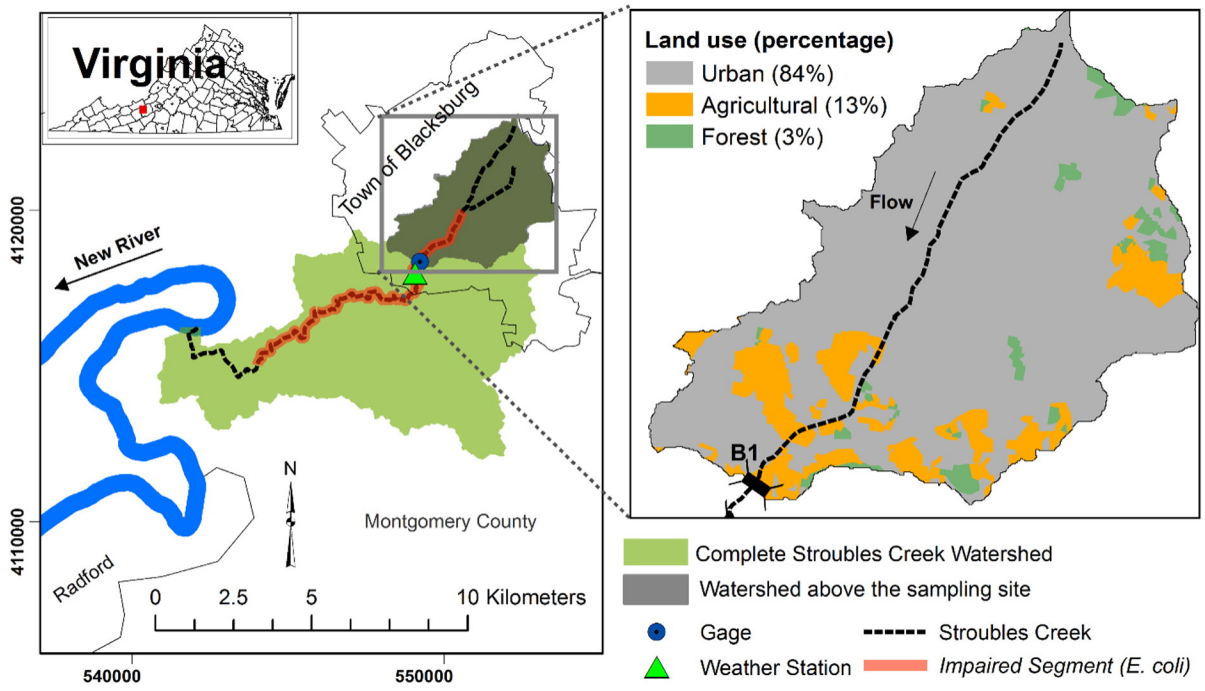

Fig. 1. Location and land use distribution of upstream watershed above the sampling bridge — B1 (map projection: NAD83/UTM zone 17 N; land cover data source: NLCD 2006 http://nationalmap.gov/).

real-time at the StREAM Lab) were higher than the observed average dry-weather values. In addition, three grab samples were collected during dry-weather conditions, defined as no rainfall for the previous 24 h (June 25, July 9, and July 16, 2013). These samples provided baseline FIB concentrations to enable comparison with wet-weather FIB concentrations. All samples were preserved on ice during transport and stored at 4 °C for less than 24 h in the Water Microbiology Lab of Virginia Tech prior to analysis.

2.3. Laboratory analyses

Concentrations of culturable *E. coli* and enterococci in each sample were measured via the Colilert® and Enterolert® Defined Substrate Technology® kits, respectively (IDEXX Laboratories, Inc., Westbrook, ME, USA). All samples were diluted with reagent-grade water by 1:100 prior to analysis following the manufacturer's instructions. Both tests used the Quanti-Tray®/2000 for enumeration of cells, and the number of positive wells were converted to most probable number (MPN) per 100 mL water via the MPN generator software (IDEXX, 2015). An additional 50 mL from each sample was filtered through a Millipore™ Isopore™ Polycarbonate Membrane Filter (0.4 um pore size, 47 mm diameter; Fisher Scientific, Pittsburgh, PA, USA) and stored in 2-mL sterile cryotubes at −80 °C for future qPCR analyses. DNA was extracted from the filters via the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. Filters were cut into smaller fragments with sterile razor blades prior to addition to the PowerBead Tubes, a technique frequently employed in environmental studies to maximize DNA removal and recovery (Sauer et al., 2011). Extracted DNA concentrations were quantified using the Qubit® dsDNA BR Assay kit (Life Technologies, Grand Island, NY, USA), following the manufacturer's instructions.

Molecular assays were performed using previously published methods for the *Enterococcus* spp. 23S rRNA genetic marker (Haugland et al., 2005), and the human-associated HF183 *Bacteroides* 16S rRNA genetic marker (Seurinck et al., 2005), adhering to the guidelines outlined in Bustin et al. (2009). Briefly, qPCR reaction mixtures to detect ENT-23S consisted of 12.5 μL of 2× iTaq™ universal probes supermix (BioRad, Hercules, CA, USA), 500 nM each primer, 400 nM probe, 2 mg/mL bovine serum albumin (Fisher Scientific, Pittsburgh, PA, USA), and 5 μL DNA template (sample DNA extracts containing 15 ng total DNA, or 10 to 10^7 copies of plasmid standards). The total reaction volume was adjusted to 25 μL with PCR-grade water (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The reaction mixtures for HF183 contained 12.5 μL of 2× iTaq™ universal SYBR Green supermix (BioRad, Hercules, CA, USA), 300 nM each primer, and 5 μL DNA template (sample DNA extracts containing 15 ng total DNA, or 10 to 10^7 copies of plasmid standards), in a total reaction volume of 25 μL adjusted by PCR-grade water. All qPCR reactions were performed in triplicate in 96-well plates using an Eppendorf Mastercycler® ep realplex instrument (Eppendorf North America, Hauppauge, NY, USA). A no template control (i.e. replacing DNA template with PCR-grade water) was included in each 96-well plate run. The amplification efficiency (E) was estimated from the slope of the log standard curve as $E = 10^{-1/\text{slope}} - 1$. Data from each run were only used if efficiency was between 0.9 and 1.1, the correlation coefficient value was above 0.99, and the no template controls did not amplify. Concentrations of molecular markers were reported as copy number (CN) per 100 mL as described previously (Yun et al., 2006).

2.4. Data analysis and statistics

Colilert and Enterolert samples above the maximum detection limit were assigned the highest value within the limits of detection (241,960 MPN/100 mL) and samples below the detection limit were assigned the lowest value within the limits of detection (100 MPN/100 mL), as in similar studies (Converse et al., 2011). Only 4 out of 130 total samples (3%) measured via the Colilert and Enterolert techniques fell into either of these categories. All qPCR results were within the dynamic range of the standards.

For each monitored storm event, two key parameters were calculated using previously published methods: 1) event load (EL), which is the total pollutant load during a storm event (Eq. (1)) (Chong et al., 2011a; McCarthy et al., 2012, 2013); and 2) equivalent background period (EBP), which is the ratio of a storm event load to a dry-weather load

Table 1
Hydrometeorological characteristics of the six storm events during which water samples were collected for this study.

Storm ID (sample size)	S-1 (n = 23)	S-2 (n = 18)	S-3 (n = 24)	S-4 (n = 24)	S-5 (n = 19)	S-6 (n = 22)
Sampling date	June 26	June 27	June 30	July 2	July 10	July 21
Sampling frequency (min)	15	15	15	30	30	15
Event rainfall depth (mm)	16	6	17	17	12	7
Event duration (h)	12	7	13	19	23	6
Time to peak flow (h)	3	1	3	11	3	1
Event runoff volume (m^3)	58,000	8100	57,000	37,000	70,000	12,000

during an equivalent time period (Eq. (2)) (Krometis et al., 2007). Because the 15-min intervals of continuously monitored streamflow monitoring data did not always strictly overlap with the water sampling time, the instantaneous loading rates were calculated by multiplying the fecal indicator concentration with the streamflow recorded (or linearly interpolated) at the time of sample collection. The maximum capacity of 24 sampling bottles of the ISCO sampler allowed us to obtain samples from the majority of each storm hydrograph (i.e. rising limb, peak, and partial falling limb of a storm), but not the entire recession to baseline. Therefore, in order to estimate the event load during a storm, average FIB concentrations measured during three dry-weather conditions were used as the FIB concentration corresponding to the end of each storm event (Fig. 4), in keeping with previous studies (Krometis et al., 2007).

$$EL = 10^4 \sum_{i=1}^{N} Q_i C_i \Delta t \quad (1)$$

$$EBP = EL/DL \quad (2)$$

where, C_i = *i*th discrete fecal indicator concentration (MPN/100 mL); Q_i = *i*th discrete discharge (m^3/s); N = total number of discrete concentrations measured; Δt = sampling frequency (s); DL = mean total dry-weather loads in the same duration of the storm event (MPN)

Principal component analysis (PCA) is often used to identify relevant information from environmental data by reducing a complex dataset to a lower, and more easily visualized, number of dimensions (Vialle et al., 2011). In this study, by resolving multiple variables into lower dimensional principle components, PCA was used to 1) identify and visualize relationships between measurements of concentrations of culturable *E. coli*, culturable enterococci, the *Enterococcus* spp. 23S rRNA genetic marker, and the human-associated HF183 *Bacteroides* 16S rRNA genetic marker; and 2) investigate relationships between the event loads of each fecal indicator and various environmental variables available from ongoing in-stream monitoring efforts at the StREAM Lab and an extensive literature review of previous studies (Table A.1) (McCarthy et al., 2013). The environmental variables that had statistical correlations with event loads of at least one of the fecal indicators (Pearson's correlation analysis of log-transformed data, $p < 0.1$) were selected for PCA analysis. During the PCA analysis, in order to eliminate the effect of different units associated with different variables, all variables were

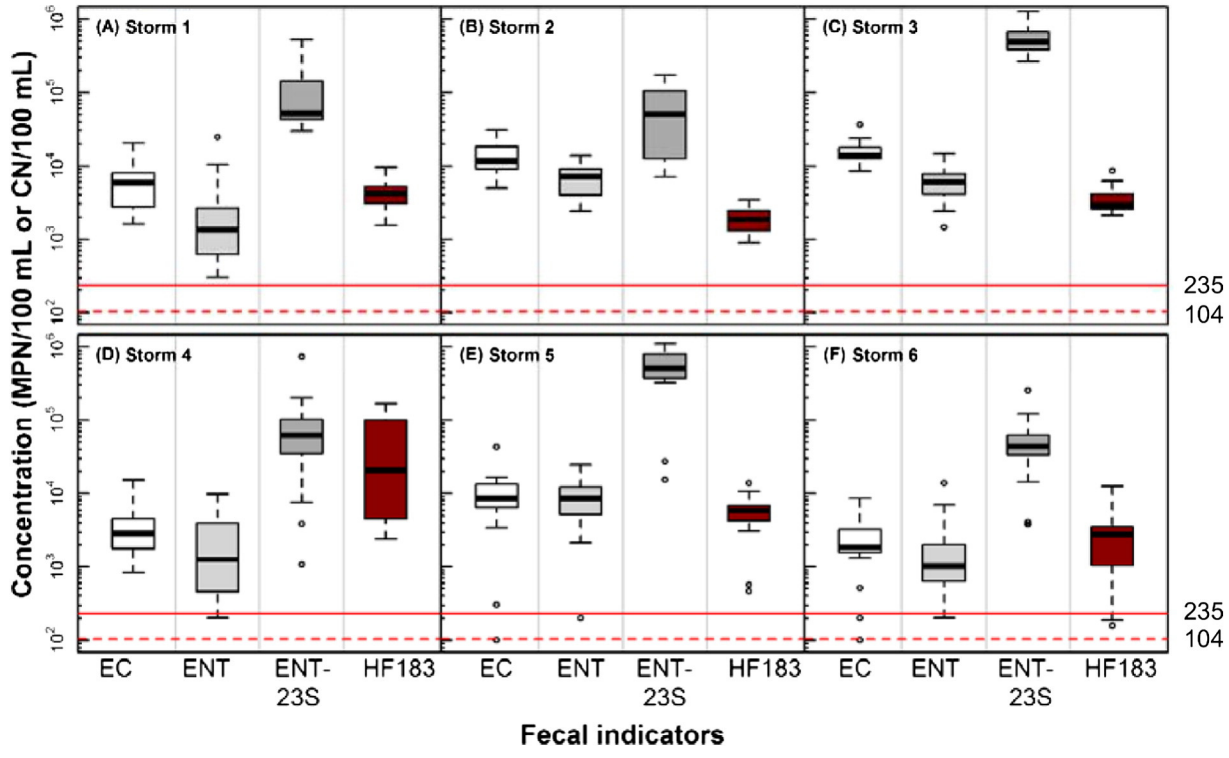

Fig. 2. Box-and-whisker plots of fecal indicator concentrations observed during each storm. The upper and lower whiskers represent the 90th and 10th percentile, respectively; the box shows 75th, 50th, and 25th percentile; circles represent outliers. The solid red line represents the Virginia recreational water single sample standard for *E. coli* (235 MPN/100 mL) and the dotted red line represents a single sample enterococci standard (104 MPN/100 mL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

standardized to have zero means and unit variance, and eigenvalues and eigenvectors were calculated using the Spearman's rank correlation matrix. The magnitude of EL and EBP associated with each storm was compared using Wilcoxon signed-rank text. All statistical analyses in this study were performed in R version 3.1.1 (R Development Core Team, 2013).

3. Results and discussion

3.1. Summary of storm events and statistics of concentrations of each fecal indicator

The six observed storm events varied considerably in terms of duration, rainfall depth, and total runoff volume (Table 1), reflecting a wide spectrum of wet-weather flow conditions. Single sample measures of fecal indicator concentrations during storms were uniformly high. Concentrations of culturable *E. coli* and enterococci were 5 to 50 times higher than the current Virginia surface water quality standards (SWCB, 2010). Concentrations of the *Enterococcus* spp. 23S rRNA genetic marker were 10 to 100 times higher than corresponding culturable enterococci concentrations (Fig. 2).

The human-associated HF183 marker was detected in all samples, with median concentrations for each storm ranging from 1000 to 30,000 CN/100 mL (Fig. 2). There is no regulatory standard for HF183, but these values would be classified as moderate (1000–5000 CN/100 mL) to high (>5000 CN/100 mL) according to a recent study of leaking sewer infrastructure in coastal urban areas (Sauer et al., 2011). The HF183 marker was selected as a study target based on the results of a recent multi-laboratory study of the performance of many of the MST markers proposed for water quality management, which suggested the HF183 marker is one of the most sensitive and specific genetic markers of human fecal contamination (Boehm et al., 2013; Harwood et al., 2014). However, a few studies have recorded cross-reactivity of HF183 marker with fecal samples from animals (i.e. dog, chicken, duck and deer) (Staley et al., 2012). Given that source-tracking was not the primary goal of this study, the potential for cross-reactivity in this specific watershed was not explored. Despite the potential for some cross-reactivity, the consistently high levels of the HF183 marker in the stream is surprising for a watershed with wholly separate storm and sanitary sewers that is so heavily urbanized (>80%). It also agrees with several recent source-tracking studies of urban areas that suggest sewage intrusion into storm sewers and urban streams is generally ubiquitous due to aging infrastructure (Marsalek and Rochfort, 2004;

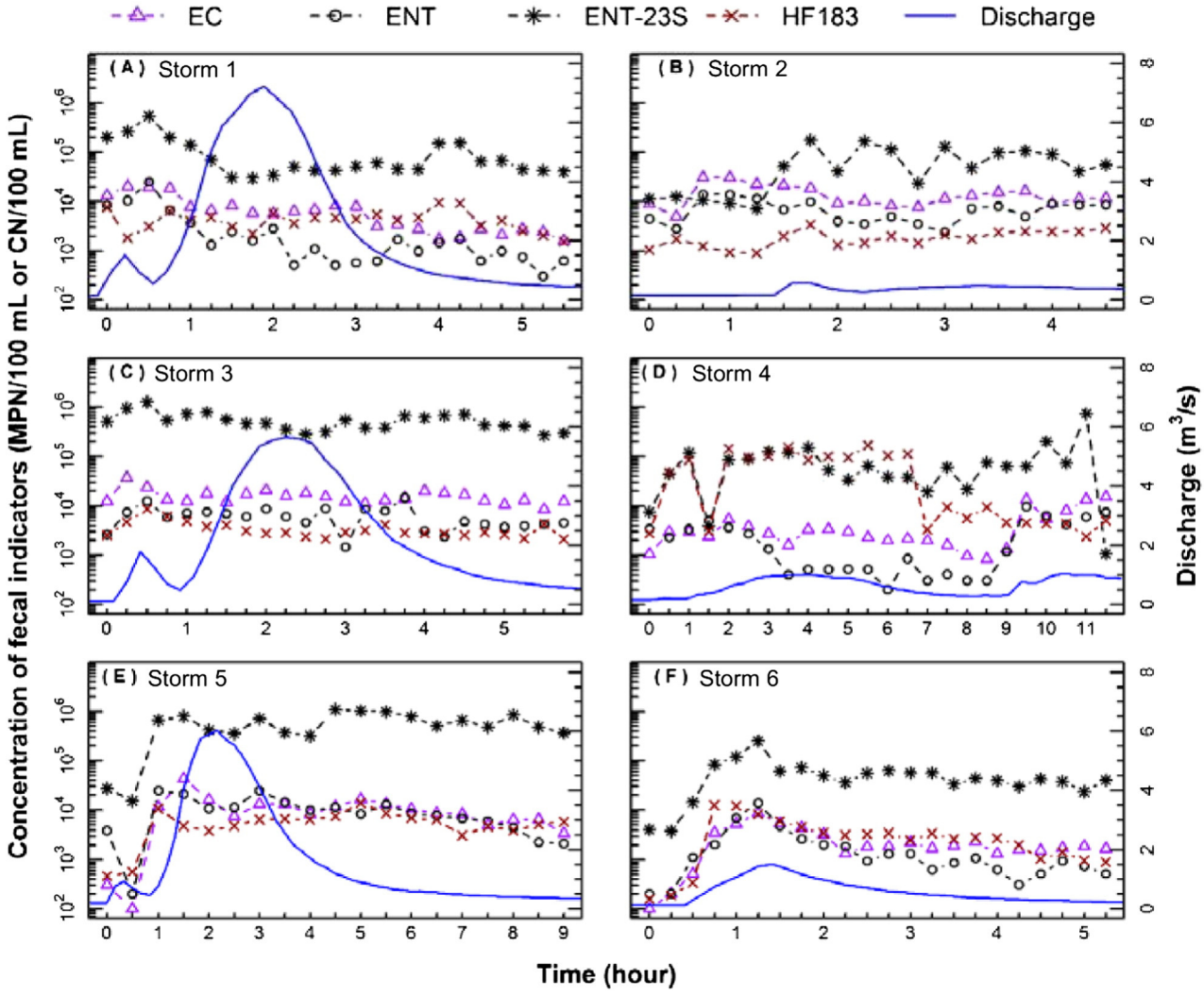

Fig. 3. Intra-storm patterns of concentrations of each fecal indicator. X-axis represents hours passed since the beginning of each storm event; Y-axis represents concentrations of *E. coli* (EC) and enterococci (ENT) in MPN/100 mL water samples, as well as *Enterococcus* spp. 23S rRNA genetic marker (ENT-23S) and the human-associated HF183 *Bacteroides* 16S rRNA genetic marker (HF183) in CN/100 mL water samples.

Sauer et al., 2011; Guérineau et al., 2014). For the purposes of TMDL implementation, more rigorous source tracking efforts in this watershed would be recommended to confirm the presence of human fecal contamination and identify possible sites of sewage intrusion into the storm sewer system.

3.2. Intra-storm patterns of concentrations and loading rates

The intra-storm patterns of the *Enterococcus* spp. 23S rRNA marker were quite similar to culturable enterococci in terms of both concentrations and loading rates (Figs. 3–4). This agrees with previous studies that have observed strong correlations between culturable and molecular indicators of *Enterococcus* spp. concentrations at both coastal (Converse et al., 2011) and inland urban watersheds (Krometis et al., 2007; Chong et al., 2011b). While estimates of numerical concentrations using the two methods can be quite different as qPCR detects nonculturable and nonviable cells in addition to culturable organisms, overall differences in the intra-storm patterns between the two indicators were generally quite small (Spearman's rank $r = 0.53$, $p < 0.001$). Intra-storm patterns of the HF183 appeared less predictable, or perhaps more sensitive, to stormflow patterns. For storms with a single peak, the concentrations of HF183 increased rapidly at the beginning of each storm, but did not decrease as rapidly as stream discharge during the falling limb. For storms with a double peak (e.g. Fig. 3, storm 4), the concentrations of HF183 decreased at the end of the first peak of the storm, and remained low during the second peak of the storm. This may be the result of different sources (e.g. faster depletion of the HF183 from storm sewers) or different transport properties. However, it is important to note that only one storm followed this pattern in the current study and that it also had a relatively low peak discharge. Therefore, it is difficult to know whether this pattern is generalizable. In fact, it is in contrast to a previous study that observed increasing concentrations of *Bacteroides* spp. gene copies over the course of a storm at coastal sites (Converse et al., 2011). This difference may be due to the variation in the climate and landscape, which affected hydrologic responses. For example, the storms in this study lasted less than 24 h, while the storms in the study of Converse et al. (2011) lasted approximately 80 h with four or five peaks in each hydrograph.

Overall, indicator concentrations generally peaked slightly earlier than the hydrograph, suggesting rapid inputs into the stream after the onset of precipitation. Concentrations did not typically decrease with discharge during the duration of the storm, while the intra-storm loading rates generally follow the hydrograph more closely. The difference between these two measures is critical from a watershed management

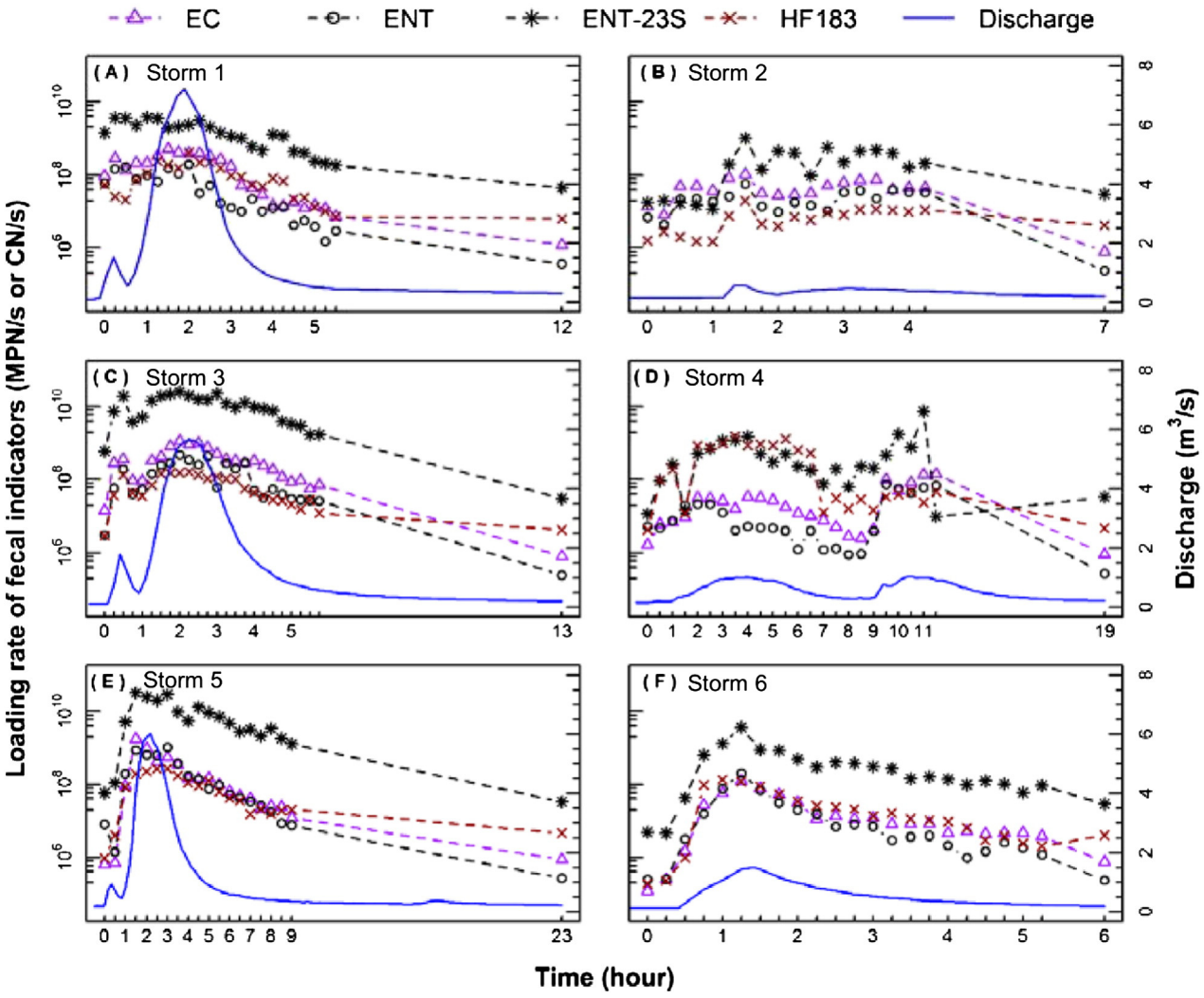

Fig. 4. Instantaneous loading rates of each fecal indicator during storm events. X-axis represents hours passed since the beginning of each storm event; the loading rates of *E. coli* (EC) and enterococci (ENT) were reported in MPN/s, and the loading rates of *Enterococcus* spp. 23S rRNA genetic marker (ENT-23S) and the human-associated HF183 *Bacteroides* 16S rRNA genetic marker (HF183) were reported in CN/s. The average of dry-weather samples were used to estimate the instantaneous loading rate corresponding to the end of each storm.

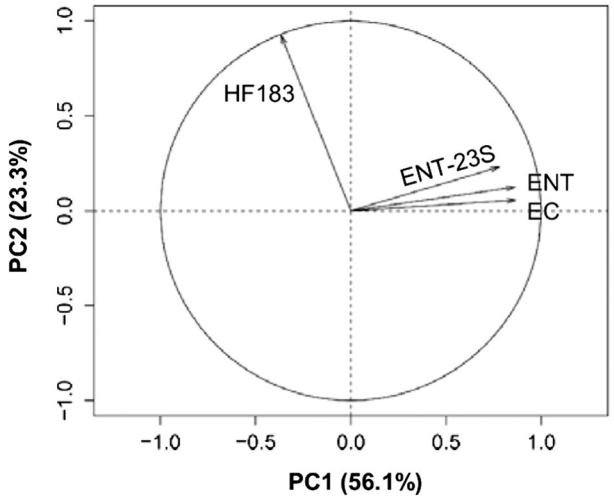

Fig. 5. Variables factor map of PCA on the concentrations of the four fecal indicators.

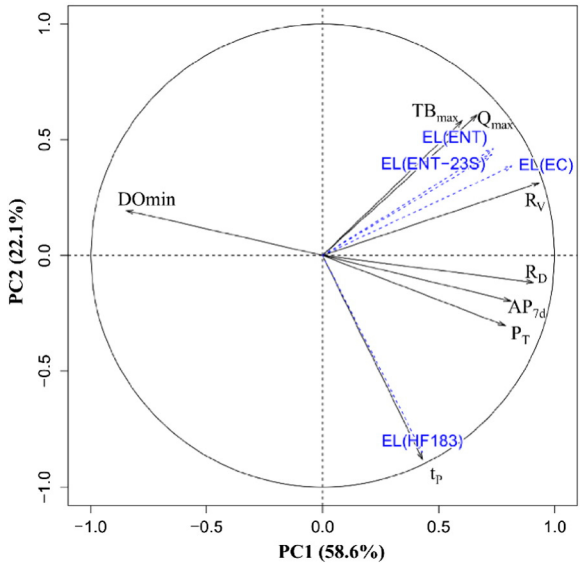

Fig. 7. Variables factor map of PCA on the event loads of the four fecal indicators and the environmental variables that showed statistical significant correlation with event loads of at least one of the four fecal indicators (Table A.2).

perspective. The bulk of water quality literature has focused solely on concentrations of indicators because the primary historical concern with regard to microbial water quality has been public health risks related to direct contact between swimmers and surface waters at recreational sites (e.g., beaches) (Schoen and Ashbolt, 2010; Soller et al., 2010). However, it is important to note that in small urban streams, the primary concern is typically not direct human contact with water; instead, it is their role in transporting fecal contaminants to larger water bodies downstream where human exposure risks are more likely. In this case, or any time water quality management is considered from a whole watershed or system perspective, consideration of loads rather than concentrations is critical in understanding system behavior (Badgley et al., 2011). To improve the accuracy of load estimations, multiple samples spanning the whole hydrograph with higher frequency during the rising limb should be employed if possible, instead of existing single grab sampling at regular (e.g., monthly or seasonally) frequencies. Calculating loading rates of fecal indicators, rather than simply reporting concentrations, is critical to identifying the most prominent sources across all spatial and temporal scales in a watershed, and to determining the magnitude of source reductions required of remediation efforts.

3.3. Relationships between the FIB concentrations measured by different methods

Analysis via PCA of the complete dataset of concentrations of the four fecal indicators (EC, ENT, ENT-23S and HF183, n = 137) resulted in most of the data variance (79.4%) being contained in the first two components, PC1 and PC2 (Fig. 5). The correlation circle describes the correlations between each variable and the two components, with the angle between two arrows representative of the correlation of the respective variables; there is no linear dependence if the angle is 90°, while an angle greater than 90° indicates a negative correlation

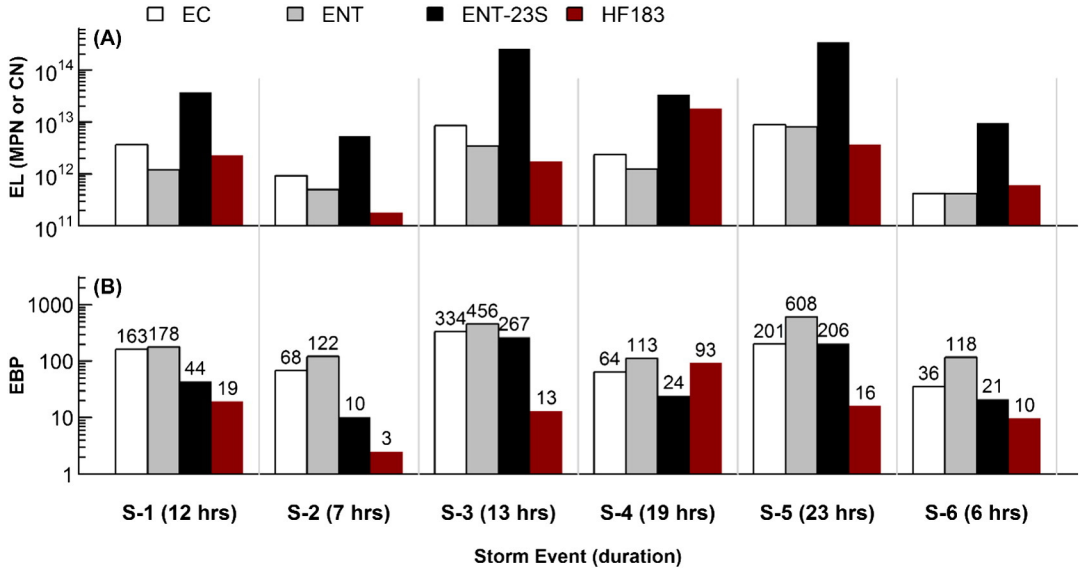

Fig. 6. Measures of (A) event load (EL) and (B) equivalent background period (EBP) for each fecal indicator during each storm event (the event load of EC and ENT were reported in MPN, and the event load of ENT-23S and HF183 were reported in CN).

(Husson et al., 2014). EC, ENT and ENT-23S were most important variables for PC1 ($r = 0.88, 0.86, 0.78$ respectively; $p < 0.001$), while HF183 was the most important variable for PC2 ($r = 0.93$; $p < 0.001$). EC, ENT and ENT-23S were closely correlated with each other as indicated by the narrow angles between each arrows, while showing weak correlations with HF183 (Fig. 5). This is not surprising given that EC, ENT, and ENT-23S are associated with all fecal sources in the watershed, while HF183 is associated predominantly with human sources. These observations agree with data from coastal areas that demonstrated weak or no correlations between culturable FIB concentrations and qPCR measures of the human *Bacteroidales* genetic marker (Sauer et al., 2011; Gonzalez et al., 2012). The observation of differences between concentrations of general fecal indicators and the human-associated HF183 marker indicates that the existing watershed-scale models based on culturable *E. coli* may not be sufficient to predict downstream fluctuations of source-specific genetic markers of fecal contamination. As emerging MST markers are more thoroughly integrated into water-quality monitoring efforts, additional research on the ecology of these targets in natural environmental systems will be necessary to understand and predict their performance.

3.4. Event load, equivalent background period, and association with key environmental variables

Event load (EL) represents the cumulative magnitude of total fecal inputs associated with a storm event, while the equivalent background period (EBP) represents the number of time periods equal to the duration of the storm that would be required to transport the same load during dry-weather. Overall, the event loads of the *Enterococcus* spp. 23S rRNA marker were significantly higher than the event loads of culturable enterococci (Wilcoxon test, $p < 0.05$, Fig. 6A), which was expected. However, EBPs associated with the qPCR measures were significantly smaller than those based on the culturable measure (Wilcoxon test, $p < 0.05$, Fig. 6B), indicating that the ratio of culturable to molecular indicators increases during storm events. For example, stormwater concentrations of the *Enterococcus* spp. 23S rRNA marker averaged 130-times higher than corresponding culturable enterococci measures in dry weather, but only 60-times higher during storm events. This differential response to storm events suggests a potentially important difference in the transport dynamics between the two types of marker. We hypothesize that during wet-weather flows, a substantial amount of fresh fecal material is washed from the watershed surfaces into the stream, resulting in a higher proportion of newer cells that can still be captured on culture media. In contrast, during dry weather, exposure to environmental stresses such as temperature and solar radiation since the last storm results in a higher relative proportion of dead or viable but nonculturable cells. Values for the EBPs of HF183 were generally even lower (except for storm 4, Fig. 6B), which may indicate a more substantial dry-weather source (e.g., constant sewage intrusion) that only modestly increased during storm events (e.g., flushing of storm sewer system). If these data truly represent human fecal contamination, these results are consistent with what would be expected from sewage intrusion, which would predominate in dry weather and be flushed during storm events. For all markers, the results of this work echo previous observations that total microbial loads from individual storms can be equivalent to loads transported during several months to a year of dry-weather (Krometis et al., 2007). Therefore, monitoring efforts aiding in remediation plans such as the TMDL program should preferentially target storm events whenever possible in addition to dry-weather monitoring.

The complete dataset of event loads for the four fecal indicators (EC, ENT, ENT-23S rRNA and HF183) and selected environmental variables was analyzed via PCA. It is worth noting that during the selection of statistically significant environmental variables, multiple comparisons were employed without adjustment. Although the probability of Type I errors may be elevated via this methodology, correction for multiple comparisons (e.g. a Bonferroni adjustment) would elevate the probability of Type II errors (Perneger, 1998, 1999). Minimizing the chance of not finding a true relationship was considered more important in this study. This was in keeping with a number of previous studies (Vidon et al., 2008; Hathaway et al., 2010; McCarthy et al., 2013). Most of the data variance (80.7%) was contained in the first two components, PC1 and PC2 (Fig. 7). The event loads of each of the four fecal indicators show moderate positive associations with runoff duration (R_D), total precipitation in each storm (P_T), and antecedent 7-day precipitation (AP_{7d}), but were negatively associated with minimum dissolved oxygen (DO_{min}). Environmental variables most strongly positively associated with the event loads of general fecal indicators (EC, ENT and ENT-23S) included total runoff volume (R_v), maximum turbidity (TB_{max}), and maximum flow (Q_{max}). In contrast, event loads of HF183 were most strongly positively associated with time to peak flow (t_p) during a storm (Fig. 7). These observations together indicated storm wash-off is a key process controlling the event load of fecal indicators during a storm event, which is not surprising in a flashy stream within a steep watershed. Interestingly, the strong association between t_p and event load of HF183 potentially suggested that mild storms of long duration can introduce more fecal contamination from human sources than storms that had intense rainfall of short duration. This could potentially occur if, for example, the source of human contamination is exfiltration of aging sewage infrastructure into groundwater, compared to surface sources of other (e.g., animal) fecal material. Further research is needed to determine if this finding is generalizable to other watersheds. However, for watershed management implications, t_p could be targeted in predictive models for quick evaluation of the effectiveness of attempts to mitigate human-associated fecal inputs during wet-weather flows.

4. Conclusions

This study highlights the similarities and differences among storm loads of commonly used conventional and emerging fecal indicators during urban wet-weather flows. The results suggest that indicator specificity (general vs. source-specific) rather than type (culturable vs. genetic marker), may be a stronger determinant of transport processes for fecal indicators. Given that human fecal contamination is generally considered to present more severe human health risks than other sources (Soller et al., 2010), and that urbanization, growing populations, and climate change can create more chances for exposure and dissemination, there is a need to improve understanding and prediction of transport processes for source-specific fecal indicators. Ideally, existing water-quality models, such as the Hydrological Simulation Program-Fortran that is most commonly used for watershed management in United States, will be adapted to incorporate these advances. Needed improvements include more accurate identification of non-point sources of fecal contamination via microbial source tracking techniques, improved load estimation of fecal contamination via higher-frequency sampling techniques, and more appropriate fate and transport pathways and parameters for relating upstream to downstream concentrations.

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Appendix A

Table A.1
Hydro-meteorological and physicochemical variables used in correlation analyses.

Variable name	Description
Antecedent climatic variables that can indicate growth and removal on watershed surfaces	
Rad_x (x = 1, 2, 7, 14, 28 days)	Mean net radiation (MJ/m^2) for x days prior to a storm event
AP_x (x = 1, 2, 7, 14, 28 days)	Total precipitation (mm) in antecedent x days of a storm event
T_x (x = 1, 2, 7, 14, 28 days)	Minimum temperature (°C) for x days prior to a storm event
Rainfall variables that can indicate microbial wash-off	
P_T	Total precipitation (mm) in a storm event
P_D	Precipitation duration of a storm event (h)
P_I	Mean precipitation intensity of a storm event (mm/h)
Stream flow variables that can indicate in-stream transport	
R_V	Total runoff volume (m^3)
R_D	Runoff duration of a storm event (h)
Q_{max}	Maximum flow rate (m^3/s)
Q_{mean}	Mean flow rate (m^3/s)
t_p	Time to peak flow (h)
Physicochemical water-quality variables that can indicate source, growth and removal in the stream and watershed surfaces	
TB_x (x = mean, min, max)	Mean, minimum, maximum turbidity during a storm event (NTU)
pH_x (x = mean, min, max)	Mean, minimum, maximum pH during a storm event
DO_x (x = mean, min, max)	Mean, minimum, maximum dissolved oxygen during a storm event (mg/L)

Table A.2
Pearson's correlation coefficient between event loadings of each fecal indicator and environmental variables ($p < 0.1$). All variables were log10-transformed to introduce log-normal distribution.

Environmental variables (log10-transformed)	Event loadings of fecal indicators in log10(CFU)			
	EC	ENT	ENT-23S	HF183
Total precipitation (mm) in antecedent 7 days of each storm event (AP_{7d})	0.71	0.78	0.70	0.81
Total precipitation (mm) of each storm event (P_T)	0.75			0.81
Total runoff volume (m^3) of each storm event (R_V)	0.90	0.85	0.90	0.73
Runoff duration (h) of each storm event (R_D)	0.82	0.85	0.80	0.84
Maximum observed flow (m^3/s) during each storm event (Q_{max})	0.76	0.71	0.80	
Time to peak flow (h) of each storm event (t_p)				0.92
Minimum observed dissolved oxygen (mg/L) during each storm event (DO_{min})	−0.86	−0.73	−0.83	
Maximum observed turbidity (NTU) during each storm event (TB_{max})		0.77	0.75	

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