Evidence of Genetic Fecal Marker Interactions between Water Column and Periphyton in Artificial Streams

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ABSTRACT: Periphyton is a complex mixture of algae, microbes, inorganic sediment, and organic matter that is attached to submerged surfaces in most flowing freshwater systems. This natural community is known to absorb pollutants from the water column, resulting in improved water quality. However, the role of periphyton in the fate and transport of genetic fecal markers suspended in the water column remains unclear. As application of genetic-based methodologies continues to increase in freshwater settings, it is important to identify any interactions that could potentially confound water quality interpretations. A 16 week indoor mesocosm study was conducted to simultaneously measure genetic fecal markers in the water column and in the associated periphyton when subject to wastewater source loading. Treated wastewater effluent was pumped directly from a treatment facility adjacent to the experimental stream facility. Inflow and outflow surface water grabs were paired with the collection of periphyton samples taken from the mesocosm substrates on a weekly basis. Samples were analyzed with three genetic fecal indicator quantitative real-time polymerase chain reaction assays targeting Escherichia coli (EC23S857), enterococci (Entero1), and Bacteroidales (GenBac3), as well as, two human host-associated fecal pollution markers (HF183 and HumM2). In addition, periphyton dry mass was measured. During wastewater effluent loading, genetic markers were detected in periphyton at frequencies up to 100% (EC23S857), 59.4% (HF183), and 21.9% (HumM2) confirming sequestration from the water column. Mean net-flux shifts in water column inflow and outflow genetic indicator concentrations further supported interactions between the periphyton and water column. In addition, positive correlations were observed between periphyton dry mass and genetic marker concentrations ranging from \( r = 0.693 \) (Entero1) to \( r = 0.911 \) (GenBac3). Overall, findings support the notion that genetic markers suspended in the water column can be trapped by periphyton, further suggesting that the benthic environment in flowing freshwater systems may be an important factor to consider for water quality management with molecular methods.

INTRODUCTION

Periphyton is a complex biological matrix that mainly includes algae, cyanobacteria, fine inorganic sediment, microbes, and organic detritus that are attached to submerged surfaces in most flowing freshwater systems. Periphyton is typically found in shallow areas of lakes, ponds, streams, and rivers and is a major food source for many invertebrates and fishes. It is also known to sequester many pollutants, such as inorganic nutrients, heavy metals, and even Escherichia coli cells, originating from sewage. This natural process can help improve water quality, but recent studies also suggest that sequestered pollutants can become concentrated in periphyton and resuspended into the water column under certain conditions.

Advances in environmental science now offer genetic methods to characterize the level and sources of fecal pollution in natural settings. These technologies are more rapid than traditional cell-based fecal indicator bacteria (FIB) cultivation approaches, and some can even discriminate human waste from other animal pollution sources. However, it remains unclear whether these genetic fecal markers can interact with periphyton communities in a similar fashion as other pollutants. Previous studies report genetic marker interactions with beach sands and submerged aquatic vegetation, but little is known about periphyton.

In this study, indoor mesocosms at the U.S. EPA Experimental Stream Facility (ESF) were used to simultaneously measure FIB (E. coli, enterococci, and Bacteroidales) and human-associated genetic markers in the water column and associated periphyton when subjected to treated wastewater point source loading. Paired measurements were analyzed to characterize the capacity of the periphyton to sequester fecal genetic markers from the water column. In addition, water column inflow and outflow...
genetic marker concentrations were investigated for evidence of a biofiltration effect, as well as, genetic marker resuspension events under test conditions. Overall, findings support the notion that genetic markers in the water column can be absorbed by periphyton, and in some instances resuspended, suggesting that the benthic environment in flowing freshwater systems is an important factor to consider for water quality management with molecular indicators.

## EXPERIMENTAL SECTION

**Experimental Design.** Paired water column and periphyton samples \( (n = 64) \) were collected over a 16 week period (weekly basis) from two mesocosm reaches (Figure S1) housed at the United States Environmental Protection Agency (USEPA) Experimental Stream Facility (Milford, OH), as previously described.\(^{16,17}\) Briefly, mesocosm 1 received water from the East Fork Tributary (EFK), a fifth order tributary draining 1300 km\(^2\) of agricultural dominated lands, and Mesocosm 2 received water from the Heiserman Stream Tributary (HST), a first order headwater draining 1.1 km\(^2\) of mostly forested land. Each mesocosm consisted of a two concatenated 12 m channels made of a head tank, upstream section containing sterilized, unglazed ceramic tiles (tile area = 144 cm\(^2\); 141 tiles/channel), providing standardized substrate for periphyton analysis, downstream section with river gravel, and a tail tank.\(^{17}\) Flow to each mesocosm was regulated via a diaphragm valve controlled by a linear actuator (A300 Poscon Electric Actuator) wired to a Foxboro magnetic flow meter (model 801H-WCT). Stormflow hydrology simulations (see Supporting Information for details) were triggered by local rain events \((\geq 0.01\) mm/min\) measured from a meteorological station located outside the facility. The experiment was divided into two 8 week study periods from June to September including a periphyton colonization period (stream water only for 8 weeks) followed by a wastewater loading phase (stream water + wastewater for 8 weeks) simulating a point source discharge. Treated wastewater effluent was pumped directly to experimental mesocosms at a rate of 2 gal/min of sewage \((13\) gal/min stream baseflow) from the Lower East Fork Waste Water Treatment Plant (Milford, OH) to simulate the discharge of human fecal pollution at a rate typical of wastewater discharge into a receiving stream during summer low-flow conditions (see Supporting Information for more detailed information).

**Water Column and Periphyton Sampling.** For each sampling event, 100 mL of water column samples was collected and processed separately from the head and tail tank of each mesocosm, as previously described.\(^{18}\) Each water sample was filtered through a 0.2 \( \mu \)m Supor-200 filter (GE Healthcare Life Sciences, Pittsburg, PA), placed in a sterile 2.0 mL screw cap tube containing a silica bead mill matrix (GeneRite North Brunswick, NJ), and stored at \(-80\) °C until time of molecular analysis \((<6\) months). For periphyton dry mass (PDM) measurements, the remaining homogenized material \((108\) cm\(^2\)) was weighed, dried at 75 °C for 48 h, and reweighed to determine total dry mass. PDM was reported as an aerial density \( (mg/cm^2) \).

**Water Physiochemical and Periphyton Measurements.** Water properties, including conductivity \((\mu S/cm)\), temperature \((^\circ C)\), dissolved oxygen \((mg/L)\), and pH, were measured every 5 min at the inlet and outlet of each mesocosm (Figure S1), as previously described.\(^{17,19}\)

**Molecular Analysis.** DNA Extraction. Water column sample DNA extractions were performed with the DNA-EZ Kit (GeneRite North Brunswick, NJ), as previously described.\(^{20}\) DNA extraction of 0.1 g of each homogenized periphyton sample was performed using the PowerBiofilm DNA isolation kit (MoBio Carlsbad, CA) according to the manufacture instructions.

**Reference DNA Material Preparation.** Reference DNA materials consisted of plasmid constructs (Integrated DNA Technologies, Coralville, IA) and salmon testes DNA (Sigma-Aldrich, St. Louis, MO). Please refer to the Supporting Information for details on preparation and storage procedures.

**Quantitative Real-Time Polymerase Chain Reaction (qPCR) Amplification.** All DNA extracts were subject to qPCR testing with three FIB assays (GenBac3,\(^{21}\) Entero1,\(^{22,23}\) EC23S857,\(^{24}\) and two human-associated methods (HF183\(^{5}\) and HumM2\(^{6}\)) as well as a sample processing control (SPC) assay (Sketa22). All reaction mixtures contained 1X TaqMan Universal MasterMix (Thermo Fisher Scientific, Grand Island, NY), 0.2 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 1 \( \mu M \) of each primer, 80 nM 6-carboxyfluorescein (FAM)-labeled probe, and 80 nM VIC-labeled probe. Multiplex reaction mixtures contained 10\(^5\) copies (Entero1, EC23S857, HumM2, and HF183) or 10\(^3\) copies (GenBac3) of IAC template combined with either PCR grade water, 10 to 1 \( \times 10^5\) target gene copies of reference DNA standard material, or 2 \( \mu L \) of DNA sample extract in a total reaction volume of 25 \( \mu L \). A minimum of two reactions was performed for each DNA extract using a 7900 HT Fast Real-Time Sequence Detector (Thermo Fisher Scientific, Grand Island, NY). All reactions were performed in MicroAmp Optical 96-well plates with MicroAmp Optical Adhesive Film (Thermo Fisher Scientific, Grand Island, NY). The thermal cycling profile for all assays was 2 min at 95 °C followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The threshold for each assay was manually set to 0.03 (GenBac3, Entero1, HF183, EC23S857, and Sketa22) or 0.008 (HumM2), and quantification cycle \( (C_q) \) values were exported to Microsoft Excel.

**Quality Assurance Measures.** This section describes quality assurance measures for molecular analysis, including extraneous DNA controls, calibration model performance, amplification inhibition screening, and SPC. To monitor for false positives, a minimum of four no-template controls and three method extraction blanks were included, where laboratory grade water was substituted for environmental samples for each instrument run and sample batch, respectively. Calibration models generated from repeated testing of reference DNA standard material for Entero1, GenBac3, EC23S857, HF183, and HumM2 assays were evaluated based on linearity \((R^2)\), amplification efficiency \((E = 10^{(−1/slope)} − 1)\), range of quantification (ROQ), and lower limit of quantification (LLOQ). An IAC was used to evaluate the suitability of isolated DNA for qPCR amplification was performed on each DNA extract for Entero1, GenBac3, EC23S857, HF183, and HumM2.
assays. The criterion for concluding no significant amplification inhibition was established for each assay and sample type (water or periphyton) as the mean cycle threshold (Cq) from 27 repeated experiments measuring Cq values from control reactions containing 10^5 (Entero1, EC23S857, and HF183, HumM2) to 10^3 (GenBac3) copies of the respective IAC in laboratory grade water only plus 1.5Cq. Water or periphyton test samples exhibiting amplification inhibition were discarded from the study. To monitor for variability in sample processing efficiency, each water filter and periphyton composite sample were spiked with a fixed concentration of salmon sperm DNA, and the resulting DNA elute was tested with the Sketa22 qPCR assay as previously described.\(^2^3\) Water filter and periphyton sample processing acceptance thresholds were calculated based on repeated control experiments (n = 60 for water; n = 36 for periphyton), where laboratory grade water was substituted for test sample material (water or periphyton). For spiked water sample DNA extracts, Sketa22 Cq measurements greater than 27.3 (24.3 mean Cq + 3) indicated an unacceptable sample processing efficiency. For periphyton DNA extracts, the acceptance threshold was 24.3Cq (21.3 mean Cq + 3). DNA extracts failing the sample processing control were discarded from the study.

**Data Analysis.** Master calibration curves and unknown DNA concentration estimates were determined using a Markov chain Monte Carlo approach on publicly available software WinBUGS, version 1.4.1.\(^2^5\) For quantitative data interpretation, the lower limit of quantification (LLOQ) was defined as the 95% credible interval upper bound from repeated measures (n = 18) of respective 10 copy reference DNA standard. For qualitative data interpretation, a positive detection was defined as any Cq less than or equal to the respective qPCR assay LLOQ. Genetic marker concentrations in water column samples are expressed as mean log_{10} copies/100 mL, whereas periphyton densities are reported as mean log_{10} copies/cm². Student-t tests were used to identify any significant differences (α = 0.05) in PDM between water sources and study period. Potential interactions of FIB in water or periphyton, where laboratory grade water was substituted for test sample material (water or periphyton). For spiked water sample DNA extracts, Sketa22 Cq measurements greater than 27.3 (24.3 mean Cq + 3) indicated an unacceptable sample processing efficiency. For periphyton DNA extracts, the acceptance threshold was 24.3Cq (21.3 mean Cq + 3). DNA extracts failing the sample processing control were discarded from the study.

**Table 2. Detection Frequency of Genetic Indicators in Water Column and Periphyton Biosamples**

| period | indicator | water inflow | water outflow | periphyton |
|--------|-----------|--------------|---------------|------------|
| 1      | GenBac3   | 100          | 100           | 100        |
|        | EC23S857  | 93.8         | 93.8          | 75         |
|        | Entero1   | 100          | 93.8          | 87.5       | 100        |
|        | HF183     | 12.5         | 12.5          | 18.8       | 6.3        | 15.6       | 9.4       |
|        | HumM2     | 0            | 0             | 12.5       | 0          | 6.3        |
| 2      | GenBac3   | 100          | 100           | 100        |
|        | EC23S857  | 93.8         | 100           | 100        |
|        | Entero1   | 100          | 100           | 100        |
|        | HF183     | 81.3         | 87.5          | 100        |
|        | HumM2     | 56.3         | 43.8          | 56.3       | 43.8       | 18.8       | 21.9       |

EC23S858, and Entero1) detection frequencies were consistently high, ranging from 87.5 to 100%, regardless of experiment period or water source (Table 2). The introduction of treated wastewater increased human-associated genetic marker detection frequencies in water samples by at least 68.7% for HF183 and 31.3% for HumM2. The concentrations of FIB and human-associated genetic markers for all samples are shown in Figure 1. No significant difference (p ≥ 0.06) was observed between water sources or experimental periods for FIB genetic markers except GenBac3 (p ≤ 0.001). In contrast, human-associated genetic marker concentrations reached as high as 4.12 log_{10} copies/100 mL (HF183) and 3.54 log_{10} copies/100 mL (HumM2) after the addition of treated wastewater.

**Periphyton.** PDM ranged from 1.60 to 3.05 log_{10} mg/cm² for EFK and from 1.22 to 2.48 log_{10} mg/cm² for HST over the study period (Figure 2). Overall, PDM levels in EFK and HST reaches were significantly different (p < 0.001). In addition, PDM levels in experiment period 1 were significantly lower than period 2 in HST (p < 0.001), but not in EFK (p = 0.1). FIB genetic marker frequencies were always 100%, regardless of experimental period (Table 2). In contrast, human-associated genetic marker detection frequencies increased by at least 43.7% for HF183 and 15.6% for HumM2 in periphyton samples during the treated wastewater loading period. The concentrations of FIB and human-associated genetic markers are shown in Figure 1. Concentrations of FIB genetic markers were not significantly different between water sources (p ≥ 0.973). However, concentrations were always significantly higher (p < 0.0001) in experimental period 2. For human-associated genetic markers, quantifiable levels were only observed during the treated wastewater loading period (Figure 1).

**Water Column and Periphyton Interactions.** A negative net-flux ratio (log_{10} F_{net} < 0) suggests that the periphyton is sequestering genetic markers, whereas a positive net-flux ratio (log_{10} F_{net} > 0) implies the opposite (Table 3). Net-flux log_{10}
copies/min ranged from −0.41 (June 29, HST) to 7.9 (June 1, EFK) for GenBac3, from −6.61 (August 17, HST) to 7.36 (June 1, EFK) for EC23S857, and from −0.15 (June 22, HST) to 7.41 (June 1, EFK) for Entero1. Interestingly, the highest observed positive net-flux ratio for all FIB markers occurred in the EFK mesocosm on June 1st coinciding with the most severe storm event (flow rate = 38.6 L/min) over the course of the study. To further characterize potential interactions, correlations between FIB genetic markers (mean log10 copies/cm²) and PDM (mg/cm²) were determined for each water source (Figure S3). In all instances, a significant correlation was observed ($r \geq 0.762; p \leq 0.003$).

**Quality Assurance Measures.** qPCR calibration model and quality assurance parameters are summarized in Table 4. ROQ was $10^{-10}$ copies/reaction for all assays (range tested). Briefly, $R^2$ values were $\geq 0.989$, and amplification efficiencies ranged from 0.93 (Entero1) to 1.08 (HumM2). Extraction blank and no-template controls indicated absence of contamination in 99.9% of control reactions (699 of 700). A periphyton sample (0.03%) and five water column samples (1.6%) showed amplification inhibition (data not shown). Sample processing controls identified 8.6% (11 of 128) DNA preparations with inadequate extraction recovery. All nonqualified samples were removed from study.

![Figure 1](scatter_plots.png)
**DISCUSSION**

**Evidence for Genetic Marker Interaction between Water Column and Periphyton.** In this study, we measured FIB and human-associated genetic markers in artificial streams to characterize potential interactions between the water column and periphyton when subjected to treated wastewater loading. Findings provide direct evidence for an interaction between DNA targets suspended in the water column and periphyton. Genetic marker sequestration could result from a number of processes ranging from settling to sorption. Periphyton offers ample surface area for interaction with genetic markers as well as protection from sunlight irradiation and predation from indigenous microbes in the water column. Previous studies report prolonged survival of culturable *E. coli* in sands, submerged aquatic vegetation, as well as periphyton. However, it remains unclear that whether genetic markers can survive for a longer period of time once trapped in periphyton, as compared to remaining in the water column. A comparison of water column mean inflow and outflow flux FIB concentrations indicates that periphyton can act as a natural biofilter for genetic markers. This notion is further supported by the increase in detection and concentration of human-associated genetic markers in periphyton during treated wastewater loading (Table 1; Figure 1). However, the partitioning of genetic markers between the water column and periphyton is clearly dynamic and complex. Individual sample event net-flux (log$_{10}$ $F_{\text{net}}$) comparisons show that under certain conditions, periphyton may act as a natural reservoir leading to resuspension of genetic markers into the water column (Table 3). Additional research is warranted to investigate mechanisms, die-off trends, and the influence of factors, such as stress from turbulent flow and periphyton natural growth cycles, on genetic marker sequestration and resuspension.

**Implications for Water Quality Management.** Periphyton biofilms have rapid colonization rates, short life cycles, and are sensitive to a wide array of pollutants making them valuable indicators of short-term water quality impacts. A recent study even suggests that periphyton can influence chlorophyll-$a$ levels helping to regulate harmful algal blooms in eutrophic lakes. Here, we demonstrate that genetic markers from fecal pollutants initially suspended in the water column are readily trapped in periphyton. This has potential ramifications for water quality management. For example, molecular analysis of periphyton may reveal information about recent fecal pollution contamination events. Indeed, human-associated genetic markers were identified in periphyton samples only after wastewater was introduced into mesocosms (Table 1; Figure 1). Another important consideration is the potential ability of periphyton to act as a natural reservoir of genetic markers. It is possible that genetic markers could be released from periphyton potentially biasing future water quality tests, especially after large shifts in stream natural flow rates. It is also important to note that if genetic markers from FIB and human-associated bacteria can be trapped in periphyton, then it is likely that disease causing pathogens could too. Other secondary habitats, such as beach sands, have been shown to harbor pathogens with potential public health risk implications suggesting that a similar scenario may be possible for periphyton. Future studies are needed to

| sampling date | GenBac3 | Entero1 | EC23S857 | GenBac3 | Entero1 | EC23S857 |
|---------------|---------|---------|----------|---------|---------|----------|
| 6/1/2010      | 7.99    | 7.41    | 7.36     | −0.12   | 0.02    | 0.03     |
| 6/8/2010      | 0.03    | 0.12    | ND       | −0.13   | *       | 6.52     |
| 6/15/2010     | 0.06    | 0.05    | −0.15    | 0.13    | −0.08   | 0.08     |
| 6/22/2010     | −0.27   | −0.15   | −0.09    | −0.09   | 0.05    | 0.30     |
| 6/29/2010     | −0.41   | 0.25    | −0.21    | 0.07    | 0.08    | −0.14    |
| 7/6/2010      | 0.34    | 6.60    | *        | 0.26    | *       | *        |
| 7/13/2010     | 0.01    | 0.23    | 6.90     | 0.16    | *       | *        |
| 7/20/2010     | 0.12    | 0.41    | −6.50    | 0.38    | *       | *        |
| 7/27/2010     | 0.12    | 6.99    | 6.73     | 0.35    | *       | *        |
| 8/3/2010      | 0.02    | 0.14    | 0.19     | 0.42    | *       | 6.54     |
| 8/10/2010     | −0.21   | −0.05   | 6.74     | −0.06   | 0.00    | −0.19    |
| 8/17/2010     | 0.18    | −0.03   | 6.34     | −0.03   | 0.01    | −6.61    |
| 8/24/2010     | −0.06   | 0.07    | −0.20    | 0.25    | 0.23    | 0.49     |
| 8/31/2010     | 0.20    | −0.11   | −0.21    | −0.11   | *       | 0.00     |
| 9/7/2010      | ND      | ND      | ND       | 0.43    | 6.57    | 6.94     |
| 9/14/2010     | 0.18    | 0.11    | 0.13     | 0.24    | −0.02   | 0.22     |

*indicates no change ($F_{\text{in}}$ and $F_{\text{out}}$ values below respective LLOQ). ND represents no data available due to quality assurance screening.
characterize what constituents in fecal pollution are sequestered in periphyton, and whether fecal indicators and pathogens only accumulate or can proliferate potentially confounding future water quality monitoring efforts.

CONCLUSIONS

Experiments demonstrate the ability of stream periphyton to interact with FIB and human-associated genetic markers in the water column. The capacity to trap genetic markers appears to be a function of periphyton mass. In addition, the presence of human-associated genetic markers was apparent only after the addition of wastewater suggesting that periphyton testing can reveal information about recent fecal contamination episodes. These trends represent the first steps toward understanding the role periphyton plays in natural freshwater systems during and after a fecal pollution event. Future studies will likely reveal additional information on periphyton genetic marker biofiltration and resuspension dynamics leading to interaction models that can predict potential impacts on water quality monitoring.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01785. Experimental Stream Facility (ESF) mesocosm study design; molecular method procedures; plots comparing FIB genetic marker concentrations with periphyton dry mass (PDF)

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Notes

The authors declare no competing financial interest.

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