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Factors Influencing the Persistence of Fecal *Bacteroides* in Stream Water

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Laboratory microcosm experiments were used to assess the effects of environmental parameters on the persistence of the *Bacteroides* 16S rRNA genes derived from equine fecal samples in stream water to investigate the utility of *Bacteroides* spp. as fecal indicator organisms. Quantitative real-time polymerase chain reaction (qPCR) was used to measure gene concentrations over time with treatments designed to compare filtered vs. unfiltered stream water, fecal aggregate size, initial fecal concentrations, and water temperatures. Comparison of *Bacteroides16S* rRNA genes/mL in microcosms constructed with unfiltered stream water and filtered stream water indicated that stream water filtration to remove indigenous microorganisms followed by temperature had the largest effects on gene persistence. First-order exponential decay functions were fitted to the data from each microcosm constructed using unfiltered stream water, and the decay constants (k) ranged from 0.0071 h^{-1} in the microcosms incubated at 5°C to 0.0336 h^{-1} in a set of microcosms incubated at 25°C. Analysis of k calculated from the 10 experimental treatments indicated that k is more highly correlated to temperature than initial *Bacteroides* 16S rRNA gene starting concentrations. The equation resulting from graphing k (as the dependent variable) vs. temperature (as the independent variable) best fit a peak, Gaussian, 3 parameter function with a maximum decay at 30°C, a r^2 of 0.83 and all parameters were significant ($P < 0.0015$). Thus this data suggest that factors that reduce biological activity, such as physical removal of stream microorganisms by filtration and low temperature, result in slower *Bacteroides* 16S rRNA gene decay.

Fecal contamination in surface water is typically determined by measuring fecal indicator bacteria such as *Escherichia coli* and *Enterococcus*. Since the 1990s, several studies have suggested that *Bacteroides* species may also serve as fecal indicator bacteria due to their relatively high degree of fecal host-specificity (Bernhard and Field, 2000; Bernhard et al., 2003), predictable concentration in fecal matter (Layton et al., 2006), and ability to be measured quantitatively using qPCR (Dick and Field, 2004; Kildare et al., 2007; Layton et al., 2006; Okabe and Shimazu. 2007; Savichtcheva et al., 2007; Seurinck et al., 2005). A primary drawback in the use of *Bacteroides* as a fecal indicator in surface waters is the lack of information regarding the persistence of its genes.

Understanding the persistence of the *Bacteroides* 16S rRNA genes over time with respect to concentration, particle size, and temperature is important in determining the impact of nonpoint sources such as pasture runoff, which may be more variable than point sources such as wastewater treatment plants with constant or near constant effluent discharge. A number of both biotic and abiotic environmental parameters have been shown to affect survival and persistence of enteric bacteria (Artz and Killham, 2002; Barcina et al., 1997; Gonzalez, 1995; Nwachuku and Gerba, 2008; Peiffer et al., 1988). Thus biological conditions influencing survival of enteric bacteria such as grazing by protozoa, infection by bacteriophage, cell death, and biochemical breakdown of the DNA may also influence the decay of *Bacteroides* genes in stream water. In turn abiotic factors such as temperature, initial concentration, and aggregate size may influence these biological processes.

Previous research into the persistence of *Bacteroides* in surface waters focused on lab microcosm and in situ field studies using culturing methods and presence/absence PCR detection. An early study of *Bacteroides* survivability found that *Bacteroides* viability decreased more rapidly than *E. coli* viability, but that the *Bacteroides* could be detected for at least 8 d using nonculturable techniques (Fiksdal et al., 1985). Studies using standard presence/absence PCR also reported persistence times that varied from 1 d to 3 wk at different temperatures (Kreader, 1998). More recently *Bacteroides* persistence, as measured by qPCR (Seurinck et al., 2005, Okabe and Shimazu, 2007), also found that *Bacteroides* persisted longer at lower temperatures and higher salinities. However, these previous studies did not examine the possible influence

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Abbreviations: AllBac, total *Bacteroides* real-time PCR assay; qPCR, quantitative real-time polymerase chain reaction; k, decay rate.

of starting fecal concentrations and particle size, both of which may influence biological decay of *Bacteroides*.

The objectives of this study were to evaluate the influences of water filtration, fecal aggregate size, fecal concentration, and temperature on the persistence of *Bacteroides* 16S rRNA markers using qPCR. A secondary objective was to model decay rates associated with measurable variables such as temperature and concentration to determine appropriate models to improve the use of *Bacteroides* measurements as used in other water quality studies (Gentry et al., 2007).

Materials and Methods

Microcosm Persistence Experiments

Fresh equine fecal samples (still warm from the horse [*Equus caballus*]) were collected from a single healthy horse for all experiments which were performed over a 6-mo period. Samples were collected within 8 h of the beginning of each experiment and stored on ice while the microcosms were being prepared. The starting *E. coli* concentration measured in one fecal sample was $8.1 \pm 1.7 \times 10^3$ CFU/g fecal sample (wet weight) as determined using the ColiBlue24 assay (MEL/MF total coliform lab; Hach Company, Ames, IA). This value is in the range of fecal coliform concentrations reported in equine feces (Cox et al., 2005). Stream water samples were collected from Second Creek in Knoxville, TN before the beginning of each experiment and transported directly to the lab (located approximately 5 min from sampling site).

A series of microcosms subjected to varying environmental conditions were used to evaluate *Bacteroides* genetic marker persistence. For each variable, triplicate microcosms were made consisting of 100 mL of stream water spiked with fresh fecal slurry contained in a 200 mL Erlenmeyer flask (referred to as experimental microcosms), and one microcosm was made consisting of 100 mL of filtered stream water spiked with the same fresh fecal slurry (referred to as filtered control microcosms). In addition, for each experiment, one 100 mL microcosm of raw unfiltered stream water without spiking with fecal matter was sampled daily to determine background concentration of *Bacteroides* in the stream water (referred to as background control microcosms). All fecal slurries were made by diluting a 10,000 mg (wet weight)/L slurry of fresh fecal matter mixed in stream water previously filtered through a 0.22-µm filter. Microcosms were covered with foil to reduce evaporation of water. The typical microcosm consisted of stream water spiked to 100 mg (wet weight)/L of moderately disaggregated fecal slurry and kept for 1 wk in the dark at 25°C in a PsycroTherm controlled environmental shaker (New Brunswick Scientific, Edison, NJ). A starting concentration of 100 mg/L was chosen because it represents the mid-range of fecal concentration previously measured in local streams (Layton et al., 2006). Samples (1 mL) were collected from the center of the microcosm then frozen in a –80°C freezer until qPCR analysis (described below). The effects of initial fecal aggregate size, initial fecal concentration and temperature on fecal concentration decay rates, as measured by the qPCR analysis of 16S rRNA *Bacteroides* gene, were evaluated independently, as described below.

Fecal Aggregate Size

The effects of initial fecal aggregate size on the persistence of the *Bacteroides* 16S rRNA genes were evaluated using microcosms prepared with three separate fecal slurries: coarse (hand-separated), medium (vortexed for 10 s), and fine (blended for 30 s on "low blend" in a Hamilton Beach 10 speed blender). Each slurry was diluted into one filtered and three unfiltered stream water samples of 100 mL to a concentration of 100 mg (wet weight)/L, incubated concurrently at 25°C for 1 wk, and sampled six times.

An aggregate size analysis was performed on three 100-mL samples of each size class to ensure consistency in methodology and provide a measure of the aggregate size distribution within each level. Each slurry was filtered through a column of U.S. standard sieves for the following size openings: no.10 (2 mm), no. 35 (500 µm), no. 60 (250 µm), no. 80 (180 µm), no.100 (150 µm), and no.120 (125 µm). Each sieve was backwashed with deionized water and the resulting liquid containing organic particles of the corresponding size class was collected on preweighed 22 µm glass fiber filters by vacuum filtration. The glass fiber filters were dried at 104°C for 6 h and weighed to determine the weight of the organic particles for each size class. For each particle size classification, the data is reported as the percent of the total mass.

Fecal Concentration

The effects of initial fecal concentration on the persistence of the *Bacteroides* were evaluated using microcosms with 10, 100, and 1000 mg (wet weight)//L fecal contamination. The experimental, filtered control and background control microcosms were held concurrently at 25°C for 1 wk and sampled daily.

Temperature

The effects of incubation temperature on the persistence of *Bacteroides* genes were evaluated using experimental, filtered control, and background control microcosms held at 5, 10, 15, and 35°C containing fecal slurries at concentrations of 100 mg (wet weight)/L. Since only one incubator was used during the course of these experiments the temperature microcosm tests were performed sequentially. The duration of each experiment was for 1 wk for the 15 and 35°C tests and 2 wk for the 5 and 10°C tests. For analysis of temperature effects, the data obtained from the fecal size and concentration microcosm experiments, which were performed at 25°C, were compared to the data obtained from the other temperatures.

Detection and Quantification of Bacteroides

Real-time PCR analysis with a nonhost-specific *Bacteroides* assay (AllBac) was used for detection and quantification of the target gene in water samples by direct PCR amplification (without DNA extraction) from the microcosm experiment subsamples as previously described (Layton et al., 2006). The PCR reaction mix for each PCR sample consisted of 12.5 µL PCR mix (QIAGEN, Valencia, CA or Stratagene, LaJolla, CA), 5 pmol of the forward primer AllBac 296f and reverse primer AllBac 412r, 15 pmol of AllBac 375r *Taqman* probe, 8

μL of sterile water and 2.5 μL of sample or standard. The PCR protocol consisted of 50°C for 2 min, 95°C for 10 min, and 45 cycles of alternating 95°C for 30 s and 60°C for 45 s. The PCR amplification and fluorescent probe detection were performed using the Chromo4 Real-Time PCR Detection system (BioRad, Hercules, CA). The threshold cycle for fluorescence detection was adjusted manually to coincide with the number of cycles to detection for the 10^7 and 10^5 plasmid standards.

For each qPCR run, a set of standards was generated from either plasmid TnHu1–4 or TnBo1–5, which contain a partial *Bacteroides* 16S rRNA gene, by generating 10-fold serial dilutions from a starting concentration of 1.0×10^8 plasmid copies/μL (Layton et al., 2006). The qPCR amplification, cloning, extraction, and sequencing of these partial *Bacteriodes* 16S rRNA genes has been previously described (Layton et al., 2006). The initial plasmid concentration in the plasmid extract was determined by measuring the DNA concentration in ng/μL using a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences) followed by conversion to copy number/μL based on the total number of nucleotides on the plasmid (4607 bp), assuming an average mw of 660 per base pair and using Avogadro's number to convert from moles to molecules. The standard curve was generated in triplicate by adding 2.5 μL of each plasmid dilution into three wells on the 96-well plate from the 1×10^7 copies/μL dilution to the 10 copies/μL dilution resulting in plasmid standards ranging from 2.5×10^7 to 2.5×10^1 copies/qPCR reaction. Each qPCR run also contained triplicate blank reactions consisting of master mix and sterile water. Each microcosm subsample was analyzed in triplicate by adding 2.5 μL of the subsample into three wells containing the master mix. In addition, to determine whether the subsample inhibited the qPCR reaction, a qPCR reaction was generated for each subsample containing 2.5 μL of that sample along with 2.5 μL of 10^5 plasmid standard dilution. At the end of the qPCR run, the target recovery efficiency (RE) was calculated for each sample as follows:

RE = (measured copies in sample spiked with 2.5×10^5 plasmid copies – measured copies in unspiked water sample)/$(2.5 \times 10^5) \times 100$.

The REs for a single well spiked sample were considered acceptable if they were >30%. Subsamples with qPCR inhibition were diluted 10-fold and the assays were performed again with the diluted subsamples. In this study the subsamples from two of the experimental treatments (microcosm with a starting concentration of 1000 mg/L and the microcosm incubated 35°C) required 10-fold dilution to overcome qPCR inhibition. *Bacteroides* 16S rRNA genes in the 2.5-μL samples were calculated by comparison to the master standard curve and adjusted to copies/mL by dividing by 2.5 to yield copies/μL and multiplying by 1000 to yield copies/mL. For this procedure, the theoretical assay limit of quantification assuming one gene copy per PCR reaction would be 400 *Bacteroides* 16S rRNA genes/mL. However, the actual limit of detection for each experimental microcosm was higher because unfiltered stream water containing background levels of the *Bacteroides* 16S rRNA genes was used to construct the microcosm. Thus, for each experiment a microcosm consisting of stream water without the addition of the fecal slurry was analyzed and used to determine the background concentrations of *Bacteroides* 16S rRNA genes/mL.

Data and Statistical Analyses

For statistical analysis all *Bacteroides* 16S rRNA genes/mL were log-transformed to allow comparison of data spanning three orders of magnitude. Each experimental variable contained a set of three microcosms: (i) a microcosm consisting of unfiltered stream water plus the added fecal slurry (experimental), (ii) a microcosm consisting of filtered stream water plus the added fecal slurry (filtered control), and (iii) unfiltered stream water without added fecal slurry (background control). For each set of experimental variables and on each sample day, paired *t* tests (two-tailed assuming unequal variance) between *Bacteroides* 16S rRNA genes/mL in the experimental microcosm and the filtered control were used to determine the days for which the experimental microcosm was significantly different from the filtered control (indicating decay). Paired *t* tests between *Bacteroides* 16S rRNA genes/mL in the experimental microcosm and the background controls were used to identify the days for which the experimental microcosm was no longer significantly different from the background control using Microsoft Office Excel 2007.

Decay rates (k) for the measured log concentrations of *Bacteroides* 16S rRNA genes in each microcosm treatment were determined in SigmaPlot 2004, version 9. For each experimental treatment, decay rate calculations were based on all individual gene concentration measurements from Day 0 to the time point after the *Bacteroides* 16S rRNA gene concentration in the experimental microcosm was not significantly different from the *Bacteroides* 16S rRNA gene concentration in the stream water control. Time values were converted to hours from days to fit first-order exponential functions to the data as described for other microbiological water quality indicators (Kay et al., 2008, Jamieson et al., 2005). The following first-order rate equation was used:

$$C(t) = C_0 \times e^{-kt}$$

where C is the log concentration of *Bacteroides* 16S rRNA genes/mL at time t, C_0 is the initial log concentration of *Bacteroides* 16S rRNA genes/mL, and k is the decay rate associated with the decay of the log *Bacteroides* 16S rRNA genes/mL. Analysis of variance, parameter estimates, and fit were determined using all values. Multivariate regression analysis was performed between variables to determine which variables (temperature, measured initial concentration, and calculated initial concentration) had the largest effect on k using JMP 7.0.2 software package (SAS Institute, Cary, NC). Based on this analysis the k values vs. temperature was plotted and modeled to both a linear function ($f = y_0 + a \times x$) and a peak function ($f = a \times \exp\{-0.5 \times [(x - x_0)/b]^2\}$) in SigmaPlot, where x = temperature and f(x) = k.

Results

In this study multiple qPCR runs were required to collect data from the 10 independent microcosm experiments containing at least five time points per microcosm. To minimize variability between qPCR runs a master standard curve was generated from 31

standard curves obtained during the course of these studies. The data from the combined standard curves yielded a linear fit with an r^2 of 0.96 and the equation y = –0.31x + 11.49, where y = log plasmid copies per reaction and x = Ct (critical threshold) (Fig. 1). From this master standard curve, the *Bacteroides* 16S rRNA gene copies in each PCR reaction were calculated and converted to copies/mL. For each experiment a microcosm consisting of stream water without the addition of the fecal slurry was also analyzed and used to determine the background concentrations of *Bacteroides* 16S rRNA genes/mL. In the Day 0 stream water samples (n = 6) the average *Bacteroides* 16S rRNA genes/ml was 4.5 (± 3.1) × 10^4. The background concentrations of *Bacteroides* 16S rRNA genes/mL in stream water were also fivefold higher than the average blank values used to monitor cross contamination of qPCR mix and primers and probe. To monitor decay of *Bacteroides* 16S rRNA genes/mL, the microcosm with the lowest amount of fecal slurry added (10 mg/L) was chosen to have an initial concentration approximately 10-fold higher than the stream water concentration.

For each day of analysis in each experiment three microcosms types were evaluated: unfiltered stream water containing the added fecal slurry (experimental), filtered stream water containing the same fecal slurry (filtered control), and unfiltered stream water without added fecal slurry (background control). In the initial data analysis, two questions were addressed in this experimental design using paired t tests. First, for each day was there a statistical difference between the *Bacteroides* 16S rRNA genes/mL in the unfiltered stream water fecal slurry microcosm and the filtered stream water fecal slurry microcosm? Second, was there a statistical difference between the *Bacteroides* 16S rRNA genes/mL in the unfiltered stream water fecal slurry microcosm and the stream water without the fecal slurry addition? Experimental data that was not significantly different from the background control was considered to be at or below the AllBac limit of quantification for that sample date. In all experiments, at all time points, there was a statistically significant difference between the *Bacteroides* 16S rRNA genes/mL in the filtered controls and the background controls.

Effects of Initial Fecal Aggregate Size

Fecal slurries used to generate microcosms testing aggregate size were mechanically disaggregated to produce fine, medium, or coarse fecal aggregates each with a starting concentration of 100 mg/L of feces. A visible difference in the number of aggregates retained on the 2 mm and 120 µm sieves for the three disaggregation treatments was seen (Fig. 2A). For the coarse aggregate treatment, approximately 95% of the fecal particles were larger than 0.5 mm. For the fine aggregate treatment, approximately 95% of the fecal particles were smaller than 0.2 mm and in the medium sized aggregate treatment, particle sizes varied between these two extremes (Fig. 2B). Within 2 d, *Bacteroides* 16S rRNA genes/mL in the unfiltered stream water microcosms declined to the concentrations present in the stream water control microcosm for all three particle size treatments (coarse, medium, and fine) (Fig. 3). In contrast, the *Bacteroides* 16S rRNA gene concentrations in the filtered stream water microcosm remained significantly above the background concentrations present in the stream water control microcosm for the duration of the 7 d test for all three treatments

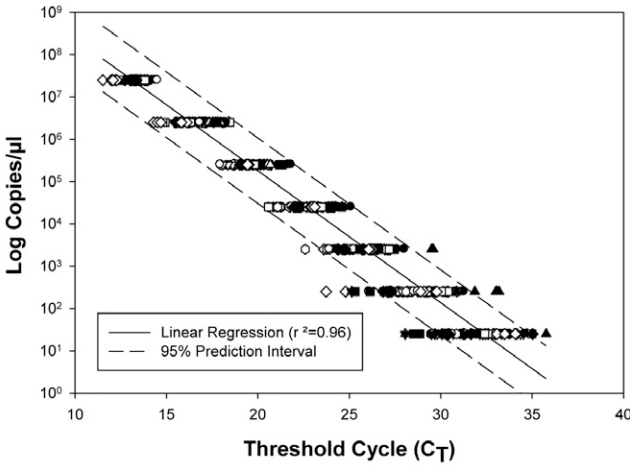

Fig. 1. Master standard curve generated from 31 individual standard curves resulting in a linear equation y = –0.31x + 11.49, where y = log (*Bacteroides* 16S rRNA genes) and x = C_T.

(Fig. 3). The first-order decay rate constants (k) for the three experimental aggregate size microcosms in unfiltered stream water were determined by fitting the log-transformed *Bacteroides* 16S rRNA genes/mL vs. time. The derived decay constants ranged from 0.0289 ± 0.0040 to 0.0336 ± 0.0042 and the goodness of fit to the curves ranged from r^2 of 0.73 to 0.81 (Table 1). In addition, the calculated starting concentrations in the first-order model (C_0), ranging from 6.38 to 6.65 log *Bacteroides* 16S rRNA genes/mL (Table 1) were similar to the measured starting concentrations of 6.29 to 6.61 log *Bacteroides* 16S rRNA genes/mL. There also appeared to be a decay trend in the *Bacteroides* 16S rRNA genes/mL in the filtered microcosm containing the fine fecal slurry (Fig. 3), but the trend was weak or absent for the medium and coarse particle microcosms. Due to this variability, the decay rate was not calculated for the microcosms using filtered stream water.

Effects of Concentration

Fecal slurry microcosms consisted of the medium fecal aggregate size and were diluted to starting concentrations of 10, 100, and 1000 mg/L. In the unfiltered stream water microcosms, the starting fecal concentration had little influence on the *Bacteroides* 16S rRNA gene concentrations, although the time needed for the *Bacteroides* 16S rRNA gene concentrations in the 1000 mg/L experimental microcosm to decay to the background concentrations was longer than the time needed in the 10 and 100 mg/L treatments to decay to the background concentrations (Fig. 4).

As in the fecal size aggregate microcosms, the *Bacteroides* 16S rRNA gene concentrations in the filtered stream water microcosm remained significantly above the background concentrations present in the stream water control microcosm for the duration of the 7 d test for all three treatments (Fig. 4). The decay rate for the *Bacteroides* 16S rRNA genes in the 1000 mg/L experimental microcosm (0.0236 ± 0.0018) was similar to that of the 100 mg/L experimental microcosm (0.0222 ± 0.0034) (Table 1). The calculated initial concentrations (C_0),

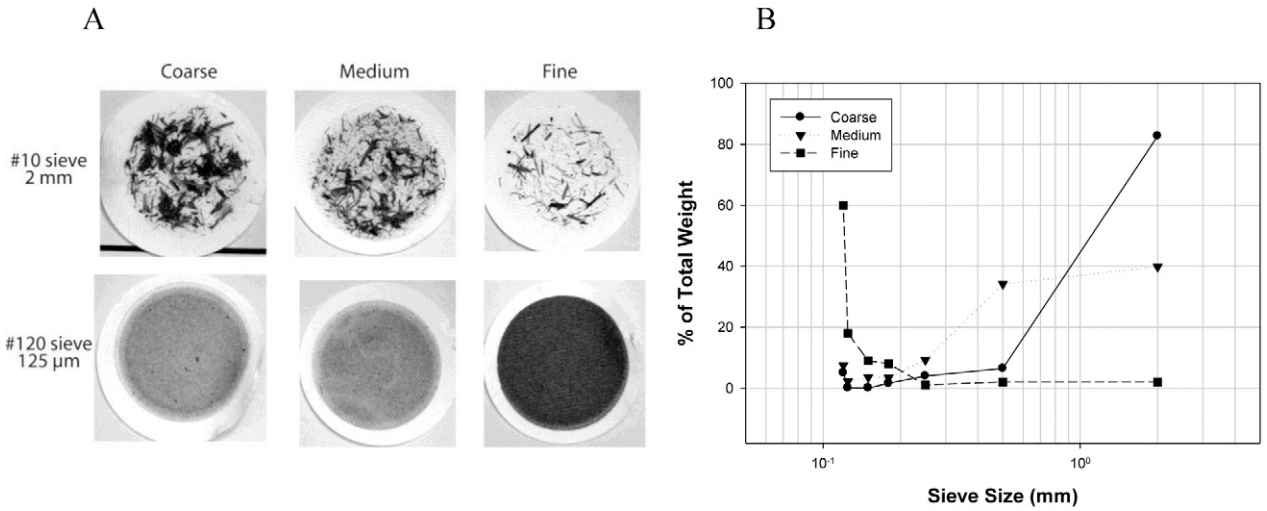

Fig. 2. Comparison of fecal particle size and the persistence of *Bacteroides* 16S rRNA genes from three separate slurries: coarse, medium, and fine. (A) visual comparison of the finest and coarsest particle size intervals evaluated in the particle size analysis, (B) size interval distribution for three aggregate size intervals (coarse, medium, and fine) as reflected in percent of total weight for each filtrate.

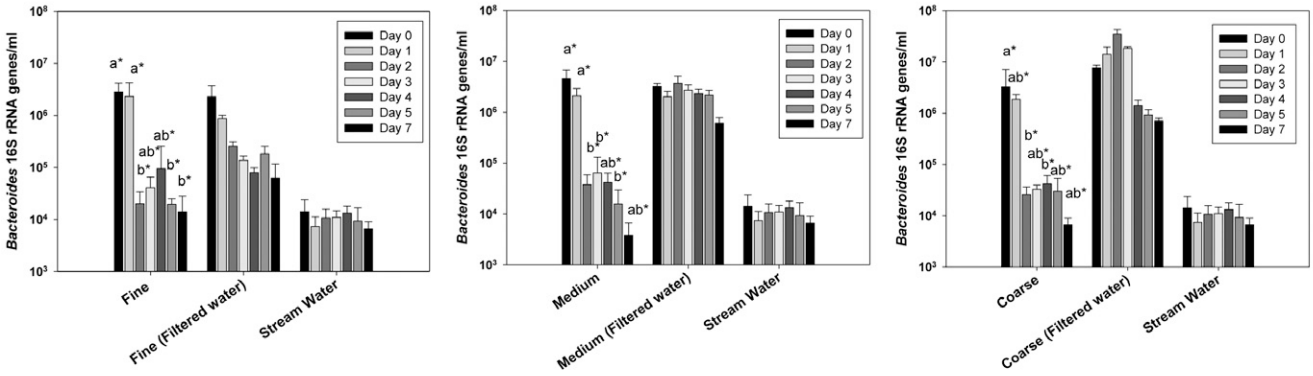

Fig. 3. Comparison of *Bacteroides* 16S rRNA genes/mL for fine, medium, and coarse fecal slurries in unfiltered water (experimental), in filtered water (filtered control) and stream water without fecal slurry (background control). All microcosms were incubated at 25°C. Error bars in the plots represent the standard deviations between triplicate microcosms for the microcosms containing fecal slurries and the standard deviations in triplicate PCR reactions for the filtered stream water control and the stream water only control. a* = samples from unfiltered stream water microcosms with significantly different *Bacteroides* 16S rRNA genes/ml concentrations than the corresponding stream water background controls. b* = samples from unfiltered stream water microcosms with significantly different *Bacteroides* 16S rRNA genes/mL concentrations than the corresponding filtered stream water control.

ranging from 5.90 to 7.65 (Table 1), were similar to the measured initial concentrations which ranged from 5.72 to 7.46 log *Bacteroides* 16S rRNA genes/mL.

Effects of Temperature

Microcosms containing 100 mg/L of medium aggregate size fecal slurries were incubated for 7 to 15 d at 5, 10, and 15°C in addition to the microcosms incubated at 25°C described above. *Bacteroides* 16S rRNA gene concentrations in unfiltered stream water microcosms incubated at lower temperatures decayed more slowly than those incubated at higher temperatures. The time required for the *Bacteroides* 16S rRNA gene concentrations in the experimental microcosm to become significantly different from the filtered control microcosm ranged from 1 d for the microcosms incubated at 35°C to 11 d for the microcosms incubated at 5°C (Fig. 5). In addition, the time needed for the *Bacteroides* 16S rRNA gene concentrations to reach the background control concentrations ranged from 4 to 13 d. The differences in decay of the *Bacteroides* 16S rRNA gene concentrations were also reflected in the calculated k values which ranged from 0.0071 ± 0.0006 for the 5°C microcosms to 0.0291 ± 0.0061 for the 35°C microcosms (Table 1). The calculated initial concentrations (C_0), ranging from 6.45 to 7.57 (Table 1), were also similar to the measured initial concentrations which ranged from 6.15 to 7.57 log *Bacteroides* 16S rRNA genes/mL. The *Bacteroides* 16S rRNA gene concentrations in the filtered control microcosm treatments remained significantly higher than the background control microcosms throughout the entire experiment (Fig. 5).

Comparison of Decay Rates between Unfiltered Stream Water Microcosms

Visual comparison of bar graphs for each treatment variable in Fig. 3 to 5 suggest that incubation temperature had the largest influence on the *Bacteroides* 16S rRNA genes decay rate (k) in the experimental microcosms (Table 1). To determine whether initial fecal concentration also had an influence on the decay rate, a multivariate analysis was performed using the following parameters: k, temperature (°C), measured C_0 and calculated C_0 from the first order rate equation. Initial fecal aggregate size was not included in this analysis because it was only measured in three experimental microcosms. In this analysis, the measured C_0 and calculated C_0 were highly correlated (r^2 = 0.97), but both were poorly correlated with k (Table 2). This analysis also confirmed that temperature had the strongest relationship with k (r^2 = 0.85). The relationship between the *Bacteroides* 16S rRNA genes decay rate (k) and temperature was further evaluated by graphing k for all experimental microcosms (Fig. 6). In this analysis, the decay rate for the *Bacteroides* 16S rRNA genes vs. temperature best fit a peak Gaussian equation with all three parameters being significant ($P < 0.005$). An alternative linear equation is also shown, however, the r^2 for this equation (0.73) was less than the r^2 for the peak function equation and the y-intercept (Y_0) in this equation was not statistically significant ($P = 0.2132$).

Table 1. Summary of *Bacteroides* decay kinetics determined for microcosm experiments using unfiltered stream water shown in Fig. 3 to 5, where *C(t)* = log *Bacteroides* 16S rRNA genes/mL at a particular time, *t* = hours of incubation and C_0 and k are solved.

Treatment	r^2†	Calculated C_0‡	k§
Particle size: fine 25°C	0.73	6.42 (± 0.16)	0.0336 (± 0.0042)
Particle size: medium 25°C	0.81	6.65 (± 0.13)	0.0316 (± 0.0034)
Particle size: coarse 25°C	0.74	6.38 (± 0.15)	0.0289 (± 0.0040)
Concentration: 10 mg 25°C	0.83	5.90 (± 0.13)	0.0325 (± 0.0030)
Concentration: 100 mg 25°C	0.64	6.25 (± 0.21)	0.0222 (± 0.0034)
Concentration: 1000 mg 25°C	0.88	7.65 (± 0.12)	0.0236 (± 0.0018)
Temperature: 5°C	0.70	7.57 (± 0.10)	0.007 (± 0.0006)
	0.82¶	7.33(0.1270)#	0.011 (0.0017)††
Temperature: 10°C	0.73	6.78 (± 0.10)	0.013 (± 0.0012)
Temperature: 15°C	0.88	6.96 (± 0.08)	0.0207 (± 0.0015)
Temperature: 35°C	0.71	6.45 (± 0.23)	0.0291 (± 0.0061)

† r^2 = goodness of fit to a linear equation, (y = y0+a × x), where x = time (h) and y = log *Bacteroides* 16S rRNA genes/mL.

‡ Calculated $C_0 = y_0$ parameter in the linear equation. The *P* value for all calculated y_0 was <0.005 for all treatments.

§ k = decay rate. The *P* value for k was <0.005 for all treatments.

¶ r^2 goodness of fit to a Sigmoidal, 3 Parameter equation, y = a/{1+ exp[–(x – x0)/b]}, where x = time (h) and y = log *Bacteroides* 16S rRNA genes/mL.

Calculated C_0 = a in the sigmoidal equation. The *P* value for all calculated a was <0.001.

†† k = –1/b a in the sigmoidal equation. The *P* value for all calculated b was <0.001.

Discussion

In this study we examined the persistence of the *Bacteroides* 16S rRNA genes in equine manure, which may be a significant source of pasture or barnyard runoff in some areas of the United States. In environmental waters decline of fecal bacteria is usually attributed to transport and dilution, inactivation (including cell death by starvation, chemical attack, or bacteriophage infection) and grazing (Artz and Killham, 2002; Barcina et al., 1997; Boehm et al., 2005, Menon et al., 2003). The role of inactivation vs. grazing in fecal bacterial decline can be differentiated by removal of protozoa by selective filtration or the use of protozoan inhibitors (Menon et al., 2003). In this study, comparison of the *Bacteroides* 16S rRNA gene concentrations in the unfiltered fecal-spiked stream water microcosm and the filtered fecal-spiked stream water microcosm (Fig. 3, 4, and 5) indicated that the decline in the *Bacteroides* population is primarily due to biological agents present in the stream water, such as protozoa, rather than inactivation of the *Bacteroides* by physical or chemical factors. This result is similar to those of Menon et al. (2003) which found that grazing by protozooplankton was responsible for >90% of the mortality rate of fecal bacteria in river and costal water. Because *Bacteroides* 16S rRNA genes did not decay to background levels in the filtered stream water microcosms, decay rate constants (k) were calculated

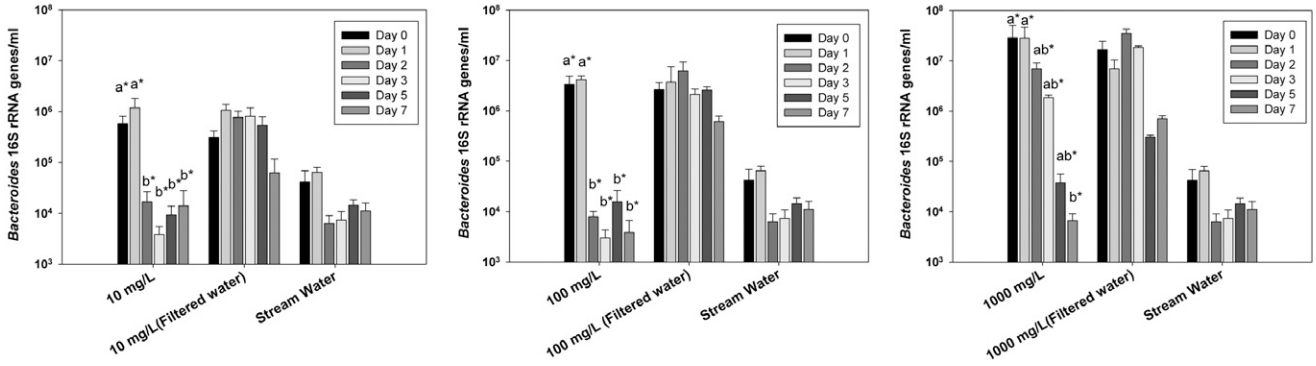

Fig. 4. Comparison of *Bacteroides* 16S rRNA genes/mL with initial starting fecal concentrations in microcosms of 10, 100, and 1000 mg/L in unfiltered water (experimental), filtered water (filtered control) and in stream water without fecal slurry (background control). All microcosms were incubated at 25°C. Error bars in the plots represent the standard deviations between triplicate microcosms for the unfiltered stream water microcosms containing fecal slurries and the standard deviations in triplicate PCR reactions for the filtered stream water microcosm containing fecal slurries and stream water only microcosm. a* = samples from unfiltered stream water microcosms with significantly different *Bacteroides* 16S rRNA genes/mL concentrations than the corresponding stream water background controls. b* = samples from unfiltered stream water microcosms with significantly different *Bacteroides* 16S rRNA genes/mL concentrations than the corresponding filtered stream water controls.

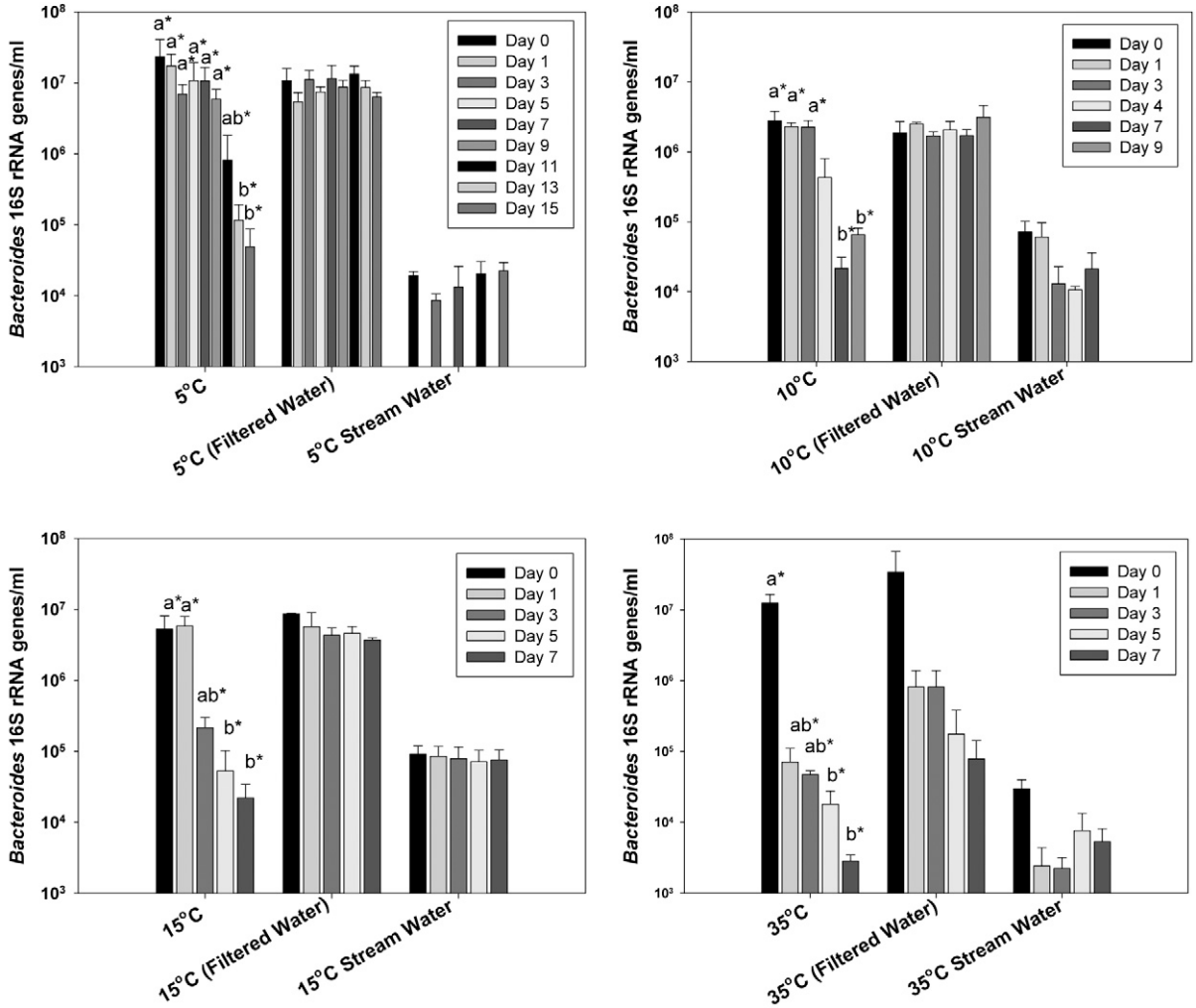

Fig. 5. *Bacteroides* 16S rRNA genes/mL in microcosms incubated with variable temperature ranging from 5 to 35°C. For each temperature three microcosms were used: (1) A fecal slurry of 100 mg/L in unfiltered stream water (experimental) (2) A fecal slurry of 100 mg/L in filtered stream water (filtered control), and (3) the unfiltered stream water without fecal slurry (background control). Error bars in the plots represent the standard deviations between triplicate microcosms for the microcosms containing fecal slurries and the standard deviations in triplicate PCR reactions for the stream water only microcosm. a* = samples from unfiltered stream water microcosms with significantly different *Bacteroides* 16S rRNA genes/mL concentrations than the corresponding stream water background controls. b* = samples from unfiltered stream water microcosms with significantly different *Bacteroides* 16S rRNA genes/mL concentrations than the corresponding filtered stream water controls.

for all unfiltered stream water microcosm treatments, but not for filtered stream water microcosm treatments.

Comparison of the decay rate constants between the unfiltered stream water microcosm treatments indicated that starting concentration and aggregate size had little effect on k and starting particle size had no apparent effect on k. Aggregate size was examined because bacteria present in manure runoff applied intentionally or unintentionally by pastured animals typically exist within an organic matrix, rather than as cell suspensions (Unc and Goss, 2004). We hypothesized that *Bacteroides* contained in finer manure aggregates may be more susceptible to decay than *Bacteroides* contained in larger organic aggregates due to protection from biological agents. Unexpectedly, the aggregate size experiment did not show reduction in decay rate with respect to increased size in unfiltered stream water. This indicates that large fecal aggregate size did not protect the *Bacteroides* 16S rRNA genes from biological decay.

We expect that the decay properties for equine *Bacteroides* 16S rRNA genes observed in this study will be similar to the decay properties of *Bacteroides* 16S rRNA genes in other fecal material, based on the research by Okabe and Shimazu (2007), which recently demonstrated that there is little difference in fecal *Bacteroides* 16S rRNA gene decay in human, cattle (*Bos taurus*), and pigs (*Sus scrofa*) as measured using host-specific and general assays. In this study, decay rates were calculated using a first order equation which is similar to the methodology used by others for examining *E. coli* inactivation in stream sediments, in sewage contaminated waters and in shellfish microcosms (Easton et al., 2005; Jamieson et al., 2004; Jamieson et al., 2005; Kay et al., 2008). Although the literature also suggests that nonlinear equations sometimes provide a better fit to model enteric bacterial decay in complex matrices (Gonzalez, 1995), the use of the first-order decay curve on log-transformed concentration data in this study provided a method

that could be used across all experimental treatment microcosms. In addition, the first order k values obtained in each fitted equation were significant ($P < 0.005$) (Table 1) and the calculated C_0 were highly correlated with the measured C_0 (Table 2).

The decay rates measured in this study varied with temperature and have first-order decay constants which are in the ranges reported for *E. coli* (0.03226 h^{-1} [Kay et al., 2008], 0.503 d^{-1} [= 0.0221 h^{-1}]) (Easton et al., 2005) and for *Bacteroides* [0.06–0.71 d^{-1} × (= 0.0025–0.0295 h^{-1})] (Okabe and Shimazu, 2007). In this study the comparison of decay rates at five temperatures (5, 10, 15, 25, and 35°C) suggests that the temperature effects on k can be best described by a Gaussian peak equation with a maximum at 30°C. Given that most environmental waters are below 30°C, a linear equation may also adequately describe the relationship between k and temperature. Additional data from different animal fecal sources as well as from experiments performed at different temperatures would be useful to further determine the comparative effectiveness of each model. These data also support the hypothesis that biological degradation by protozoa plays a major role in controlling decline of the *Bacteroides* 16S rRNA genes in stream water. At 5 and 10°C grazing microbes, such as protozoa, are much less active, so the persistence of *Bacteroides* 16S rRNA genes at these temperatures may be attributable to decreased grazing (Rose and Caron, 2007; Sherr et al., 1988).

More research is needed to determine whether other abiotic factors commonly associated with poor water quality, including turbidity and chemical pollutants, influence stream biota and indirectly affect *Bacteroides*16S rRNA gene decay. The temperature effect on *Bacteroides*16S rRNA gene decay presented here and by others (Okabe and Shimazu, 2007) suggests that the persistence of the *Bacteroides*16S rRNA gene could be affected by seasonality and geographic location. For instance, during the warmer seasons the *Bacteroides* 16S rRNA genes may only be detectable for 1 to 2 d after it is introduced to the environment, whereas in cooler seasons the detection period may be increased to over 2 wk. In addition more research is needed in directly comparing *Bacteroides* 16S rRNA gene decay curves to other pathogen indicator die-off curves with respect to temperature, because at warm temperatures (30°C) *Bacteroides* may be removed very rapidly, but *E. coli* may regrow (reviewed by Nwachuku and Gerba, 2008). The ability to predict the decay of *Bacteroides* with respect to temperature may be applicable to microbial source tracking as well as developing or implementing remediation plans for watersheds.

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Table 2. Correlations between incubation temperatures measured and calculated initial concentrations (C_0) and rates (k) derived from unfiltered and fecal-spiked stream water microcosms using a multivariate analysis.

	Temperature, °C	Measured C_0	Calculated C_0	Calculated k
Temperature, °C	1.0000	–0.5827	–0.5315	0.8545
Measured C_0	–0.5827	1.0000	0.9717	–0.6751
Calculated C_0	–0.5315	0.9717	1.0000	–0.6445
Calculated k	0.8545	–0.6751	–0.6445	1.0000

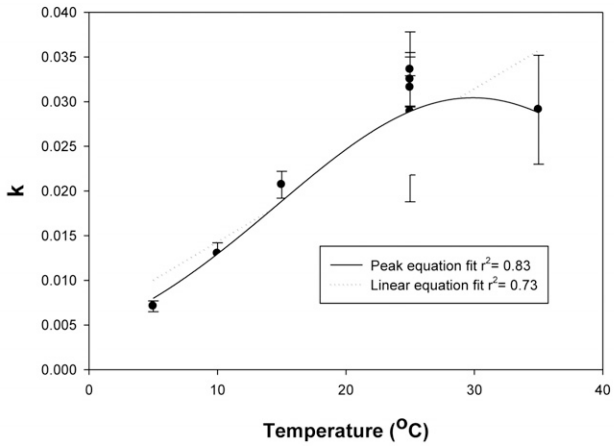

Fig. 6. Relationship between decay rate (k) of the log concentration *Bacteroides* 16S rRNA genes/mL with temperature in microcosms in unfiltered stream water using a Peak, Gaussian, 3 Parameter equation ($y = a \times \exp\{-0.5 \times [(x - x_0)/b]^2\}$) and a linear equation ($y = y_0 + a \times x$). The error bars represent the standard deviation of k for all experimental microcosms. For the peak Gaussian equation: Parameter a = 0.0304 ± 0.0032 (P = <0.001), parameter b = 15.2113 ± 3.0242 (P = 0.0015) and X_0 = 29.8817 ± 3.4623 (P < 0.001). For the linear equation Y_0 = 0.0057 ± 0.0032 (P = 0.2132) and a = 0.0009 ± 0.0002 (P = 0.0016).

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