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ORIGINAL RESEARCH

Differentiating Sources of Fecal Contamination to Wilderness Waters Using Droplet Digital PCR and Fecal Indicator Bacteria Methods

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Introduction—Human activity in wilderness areas has the potential to affect aquatic ecosystems, including through the introduction of microorganisms associated with fecal contamination. We examined fecal microorganism contamination in water sources (lake outlets, snowmelt streams) in the popular Absaroka Beartooth Wilderness in the United States. Although the region is remote, increasing human visitation has the potential to negatively affect water quality, with particular concern about human-derived microorganism fecal contaminants.

Methods—We used standard fecal indicator bacterial assays that quantified total coliform bacteria and *Escherichia coli* concentrations, together with more specific polymerase chain reaction-based microbial assays that identified possible human sources of fecal microorganisms in these waters.

Results—Total coliforms were detected at all lake outlets (21 of 21 sites), and *E coli* was detected at 11 of 21 sites. Droplet digital polymerase chain reaction assays revealed the presence of human feces-derived microorganisms, albeit at abundances below the limit of detection (<10 gene copies per milliliter of water) at all but 1 of the sampling sites.

Conclusions—Our results suggest low prevalence of water-borne pathogens (specifically *E coli* and human-derived *Bacteroides*) in this popular wilderness area. However, widespread detection of total coliforms, *Bacteroides*, and *E coli* highlight the importance of purifying water sources in wilderness areas before consumption. Specific sources of total coliforms and *E coli* in these waters remain unknown but could derive from wild or domesticated animals that inhabit or visit the Absaroka Beartooth Wilderness. Hence, although contamination by human fecal microorganisms appears minimal, human visitation could indirectly influence fecal contamination through domesticated animals.

Keywords: microbial source tracking, water quality, Absaroka Beartooth Wilderness, visitor impacts, environmental management, wilderness character

Introduction

Designated wilderness areas in the United States are unique in that there are restrictions on permanent facilities to manage visitor impacts and human waste.[1] However, human visitation to these areas continues to increase,[2] creating challenges for managers seeking to minimize impacts of human activities on wilderness natural resources.[2-4] In particular, fecal contamination of wilderness water sources presents a growing problem, requiring managers to identify areas of potential contamination to protect public health.[5] Outbreaks of illnesses associated with fecal bacteria (eg, diarrhea) have been documented in congested recreational sites in wilderness areas,[6,7] with possible sources of fecal contaminants including livestock, dogs, wild animals, and humans.

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Fecal indicator bacteria (FIB) assays are often used to identify the presence of total coliform bacteria, fecal coliforms, and *Escherichia coli* in aquatic ecosystems.[8] Coliform bacteria can be introduced from various sources, and although the occurrence of *E coli* is frequently attributed to fecal contamination, many strains of *E coli* are not pathogenic and are not associated with feces.[8] Moreover, standard FIB tests do not discriminate among the potential sources (eg, wildlife or human) of these microorganisms. Therefore, increasingly, DNA and polymerase chain reaction (PCR)-based methods are used to aid microbial source tracking (MST), linking microbial pathogens to specific host sources.[9-12]

Bacteria belonging to the phylum Bacteroidetes are often used as targets in MST assays because of source specificity to mammalian guts and feces.[12-15] Members of *Bacteroides* can comprise upwards of 26 to 36% of the gut microbiota,[16,17] and several *Bacteroides* genetic markers have been developed and successfully applied in PCR assays targeting humans, cattle, pigs, and horses.[9,10,12,13,18-20]

Our study focused on potential fecal contamination of waters in the Absaroka Beartooth Wilderness (ABW) in south central Montana. The ABW is a mountainous wilderness area northeast of Yellowstone National Park. The region ranges in elevation from approximately 1.6 km to over 4.1 km and contains a network of subalpine forests and alpine tundra. With over 1100 km of trails, the area receives high recreational use, including backpacking and stock packing, but no livestock grazing. Most human visitation is concentrated around lakes and at water sources near popular summits. Backcountry campsites are required to be >60 m from lakeshores, and popular campsite locations have been cataloged in a geospatial database maintained by the US Forest Service for over 20 y. We sought to examine the occurrence of total coliform and *E coli* in ABW water sources adjacent to backcountry camping areas and use MST methods to quantify members of *Bacteroides* derived from human feces.

Methods

STUDY AREA AND SAMPLING SITES

We selected a total of 23 sampling locations within the ABW (21 remote alpine lake outlets and 2 snowmelt streams) using geospatial information on campsite condition and popular recreational sites. Sampling sites were identified using ArcMap (version 10.5.1), overlaying campsite point locations with a wilderness-wide opportunity class layer representing 3 zones of recreational use: 1) pristine, 2) primitive, and 3) transition zones. Pristine zones were those with negligible anthropogenic influence, primitive zones were those with measurable anthropogenic influence, and transition zones were those where human impacts were moderate to substantial. All of our sampling was done within the transition zone, between 4.5 and 24 km from main trailheads (Figure 1).

WATER SAMPLING, FIB DETECTION, AND DNA EXTRACTION

Water samples from 21 different alpine lake outlets and 2 snowmelt streams were collected in triplicate over 13 d in July and August 2018 (Table 1). Water samples were placed into sterilized 250-mL polypropylene bottles and transported to the laboratory in the dark and on ice. In the laboratory, samples were stored at 4°C until processing (within 48 h of sample collection). Two 100-mL aliquots of sample water from each of the triplicate 250-mL sample bottles were vacuum filtered onto separate 47-mm diameter, 0.45-µm pore size (mixed cellulous ester) gridded, pre-sterilized filters (Millipore Sigma**,** Burlington, MA). One of these filters was used for total coliforms and *E coli* analyses, and the other filter was used for subsequent extraction of DNA. No filter was processed for DNA from Sylvan Lake, and only filters for subsequent extraction of DNA were collected from the 2 snowmelt streams.

Filters for FIB analyses were placed in M-ColiBlue24 broth petri dishes and incubated at 35°C for 24 h (Millipore Sigma). Colony-forming units (CFUs) were quantified by counting and recording the number of red (non-*E coli* coliforms) and blue (*E coli* coliforms) plate-forming colonies. Total coliforms were calculated as the sum of red and blue colonies.

DNA was extracted from filters using the MasterPure DNA Purification kit (Lucigen Corporation, Middleton, WI). Triplicate blank filters (ie, no sample filtered onto them) were processed alongside samples. Filters were transferred from 15-mL centrifuge tubes to 2-mL microcentrifuge tubes containing 600 µL of a cell lysis solution and 100 µL of 0.1-mm and 100 µL of 0.5-mm glass beads. The tubes containing filters were frozen at -80°C, thawed, and placed into a mechanical bead beater for 2 min, followed by the addition of proteinase-K (50 $\mu g \cdot \mu L^{-1}$ final concentration). Samples were incubated at 65°C for 15 min and placed on ice for 3 to 5 min, and DNA was extracted following the MasterPure DNA Purification kit protocols. DNA was resuspended in 100 µL of nuclease-free water and stored at -80°C.

DROPLET DIGITAL PCR FOR DETECTION OF UNIVERSAL AND HUMAN-SPECIFIC BACTEROIDES GENE MARKERS

We used 2 different droplet digital PCR (ddPCR) assays: one targeting bacteria belonging to the genus *Bacteroides*

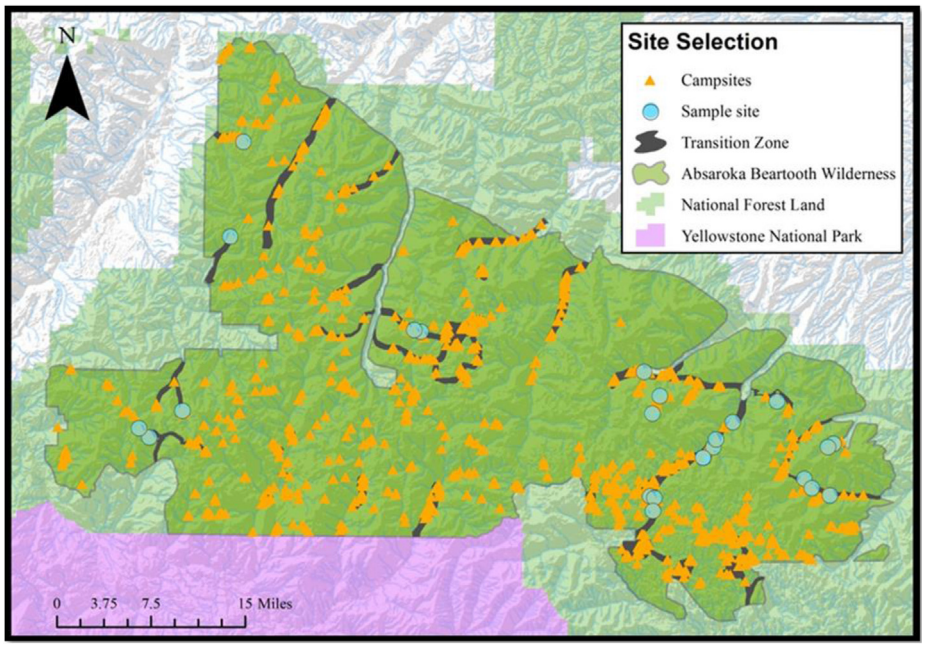

Figure 1. Sampling sites in the Absaroka Beartooth Wilderness. Backcountry campsites are indicated with orange triangles, and sampling sites for this study are indicated with blue circles.

and another specific to members of *Bacteroides* known to be associated with human feces. Both assays relied on previously published MST PCR methods.[9,18] Two different pairs of PCR primers were used for each assay (Table 2): The AllBac primer pair was designed to amplify 16S rRNA genes of members of *Bacteroides*, inclusive of those previously recovered from mammalian feces,[13,18] and the BacH primer pair targets 16S rRNA genes from the HF183 cluster of human feces-associated *Bacteroides*.[9,10,14,21,22] ddPCR assays were applied to sites where *E coli* was detected using the FIB assays (excluding Sylvan Lake). We also used both ddPCR assays on samples collected from 3 lake outlets (Diamond, Rainbow, September Morn) where no *E coli* was detected but total coliform abundances were relatively high. Finally, both ddPCR assays were used on the samples collected from the 2 snowmelt streams.

Triplicate filter extracts from the lake outlets and snowmelt streams were analyzed by ddPCR using a QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA). Each ddPCR reaction (20 µL total) contained EvaGreen Supermix (Bio-Rad Laboratories), nuclease-free water, DNA extract, and 0.18 µM (final concentration) of each primer. Triplicate controls (with no added DNA) were included as negative controls. Filter blank DNA extracts were also analyzed in triplicate to estimate the detection limits associated with both ddPCR assays (see description in Statistics subsection). PCR reaction mixes were combined with droplet generation oil specific for EvaGreen, and droplets were generated using the droplet generator (Bio-Rad Laboratories). Droplets (40 µL total, including PCR reaction mix and oil) were transferred by multichannel pipettor into 0.2-mL 96-well PCR plates. Plates were heat sealed and placed in a Bio-Rad C1000 thermal cycler.

Amplification conditions were as follows: 95°C for 6 min, followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 45 s.[13] A QX200 Droplet Reader (Bio-Rad) was used to quantify droplet specific fluorescence. The fluorescence amplitude threshold was manually adjusted to distinguish positive droplets from those that demonstrated no amplification (negative droplets). We excluded reactions with <10,000 accepted droplets from subsequent analyses (n=3). Gene abundances (copies per milliliter of sample water) were quantified for each sample using the manufacturer's software (QuantaSoft, Bio-Rad Laboratories).

STATISTICS

The method detection limit (MDL) of the ddPCR assays was defined as the lowest number of genes that could be

Table 1. Elevation, distance to trailhead, number of campsites, groundcover, and types of use for all sites surveyed in this study

Sampling site	*Date sampled (2018)*	*Elevation (m)*	*Distance to trailhead (km)*	*No. of established campsites*[a]	*Ground cover*[b]	*Use type*[c]
Bald Knob	Jul 22	2871	12	11	Rock	Foot, stock
Diamond	Aug 12	2982	13	1	Alpine tundra	Foot, stock
Elbow	Jul 17	2664	11.2	5	Open forest	Foot, stock
Elk	Jul 29	2071	5	16	Open forest	Foot, stock
Fish	Jul 23	2732	8	2	Forest, meadow	Foot, stock
Horseshoe	Aug 14	2922	11.2	8	Alpine tundra	Foot
Keyser Brown	Aug 4	2650	11.2	9	Open forest	Foot, stock
Knox	Jul 23	2584	5.6	9	Open forest	Foot, stock
Lake at Falls	Jul 29	2499	14.5	5	Open forest	Foot, stock
Lake Gertrude	Aug 3	2924	7	4	Forest, meadow	Foot, stock
Lost	Aug 4	2583	8	18	Open forest	Foot, stock
Mystic	Jul 18	2337	5	44	Open forest	Foot, stock
Ouzel	Jul 22	2870	11.6	7	Rock	Foot, stock
Pine Creek	Jul 11	2801	8	14	Forest, meadow	Foot
Rainbow	Jul 29	2376	13.6	23	Open forest	Foot, stock
Rimrock	Jul 29	2317	9.6	5	Rock	Foot
Russell	Jul 22	2667	9.6	9	Open forest	Foot, stock
September Morn	Aug 4	3005	11.2	13	Open forest	Foot, stock
Sylvan	Jul 28	2799	9.6	3	Forest, meadow	Foot, stock
Thompson	Jul 12	2447	9.6	16	Open forest	Foot, stock
Timberline	Aug 3	2985	8	14	Forest, meadow	Foot, stock
Snowmelt stream 1	Aug 22	3547	11.2	5	Alpine tundra	Foot
Snowmelt stream 2	Aug 22	3642	16	3	Alpine tundra	Foot

[a]The US Forest Service does not designate campsites in this area, but it monitors well-established but illegal camping sites.
[b]Ground cover designations defined by US Forest Service monitoring.
[c]Use-type: Foot refers to human foot traffic; foot and stock refers to both human and stock animal traffic.

distinguished based on replicate (n=4) amplification of the filter blank DNA extracts. We estimated the MDL for each assay as

$$MDL = Mean_{blank} + 3 \times SD_{blank}$$

We defined the target gene as "present" if 1 or more of the triplicate ddPCR reactions amplified above the detection limits after 40 cycles. We quantified gene abundances only for those samples for which all triplicate ddPCR reactions amplified above the detection limits. Data are presented as mean±SD with range.

Least-squares linear regression analyses of square root transformed count data were used to examine relationships between CFUs of coliform and *E coli* and between CFUs and site-specific characteristics (eg, elevation, distance from trailhead). Given the large number of 0 values (for the FIB assays) or values below the MDL (for ddPCR assays), data were square root transformed as

$$y_t = (y + 0.5)^{1/2}$$

where y_t is the transformed variable, y is the measured property (eg, CFUs, gene abundances), and 0.5 is a constant. For those sites with gene abundances below the MDL, we assumed y=0 before transforming; for those sites where total coliform CFUs were above the upper threshold for accurate quantification (>200 CFUs), we assumed y=200 before transformation.

Results

TOTAL COLIFORM AND *E COLI* OCCURRENCES

Total coliforms were found in all 21 of the lake outlets sampled, and *E coli* was found in approximately half (11 of 21) of the sampled sites. Total coliform CFUs ranged from 27 to >200 CFUs per 100 mL of water, whereas *E coli* ranged from undetectable to 23 CFUs per 100 mL of lake outlet water (Figure 2, Table 3). Total coliform levels of >100 CFUs per 100 mL of lake water were found in 14 of the 21 (67%) lake outlet sites sampled (Table 3). Total coliform and *E coli* CFUs were correlated across the various sampling sites (least-squares linear regression, R^2=0.26, P=0.018); however, neither total coliform nor *E coli* CFUs were correlated with site-specific properties such as elevation, distance from trailhead, or number of established campsites (least-squares linear regressions, P>0.05).

Table 2. Polymerase chain reaction primers used for microbial source tracking analyses

Target	*Primer*	*Sequence*	*Amplicon size (bp)*	*Ref.*
Universal *Bacteroides*	AllBac 296f	5′- GAGAGGAAGGTCCCCCAC -3′	116	17
	AllBac 412r	5′- CGCTACTTGGCTGGTTCAG -3′		
Human *Bacteroides*	BacH_f	5′- CTTGGCCAGCCTTCTGAAAG -3′	93	18
	BacH_r	5′-CCCCATCGTCTACCGAAAATAC-3′		

UNIVERSAL AND HOST-SPECIFIC *BACTEROIDES* MST

The amplitudes of fluorescence (in relative fluorescence units [RFUs]) for droplets binned as positive for the AllBac and BacH assays were 1.7- and 1.6-fold greater, respectively, than fluorescence amplitudes of the negative droplets. The amplitude of fluorescence for positive droplets in the AllBac assay was 15,571±1195 (11,628−17,579) RFUs, whereas the fluorescence amplitude of negative droplets was 9247±773 (8044−11,026) RFUs. The fluorescence amplitude of the positive droplets for the BacH assay was somewhat lower at 9206±2849 (6270−19,885) RFUs, with negative droplets at 5799±1030 (4129−9971) RFUs. The number of accepted droplets for both assays was 15,043±1877 (10,404−18,558). The MDL for the BacH ddPCR assay was 18 copies per ddPCR reaction, equivalent to 9 copies per milliliter of lake water, whereas the MDL for the AllBac ddPCR assay was 42 copies per reaction, equivalent to approximately 21 copies per milliliter of lake water.

AllBac genes were present in all of the sites examined by ddPCR, with gene abundances quantifiable in 11 of 15 sites (Table 3). For those sites where gene abundances were quantifiable, AllBac gene abundances ranged from 5×10^3 to 131×10^3 genes per 100 mL of sampled water, with peak abundances observed in the same 2 lakes where *E coli* abundances were greatest (Lost and Keyser Brown lakes; Table 3). One of the snowmelt streams sampled near the base of Granite Peak also contained high abundances of the AllBac gene targets, with AllBac gene targets below the limit of detection in the other snowmelt stream sampled (Table 3).

Human-associated *Bacteroides* were found in very low abundances at all sites tested. BacH genes targets were present, but not quantifiable, at 52% (7 of 15 sites) of the sites examined. Only 1 of the sampled sites (Elk Lake) had quantifiable but relatively low BacH gene abundance (Table 3). Elk Lake was notable as the site closest (4.8 km) to the main trailhead into the ABW and hence likely receives the most visitation of any of the sites sampled. All sites that tested positive for the presence of BacH gene targets also tested positive for the presence of AllBac gene targets. Neither of the snowmelt streams sampled contained quantifiable abundances of BacH gene targets (Table 3).

Based on least-squares linear regressions of the square root transformed data, total coliform CFUs and the AllBac gene abundances were correlated (R^2=0.57, P=0.004); however, there were no significant relationships between the AllBac and BacH gene abundances or between AllBac abundances and any of the site-specific environmental properties (ie, elevation, distance from trailhead, number of established campsites; least-squares linear regressions; P>0.05).

Discussion

By leveraging culture-based FIB assays and culture-independent MST assays, we evaluated the spatial distribution and potential sources of fecal bacteria in selected ABW waters. Total coliforms were present in all of the lake outlets sampled, with positive occurrence of *E coli* at 52% of these sites. The highest *E coli* CFUs were found in lake outlets that are popular recreational water sources and accessible to stock animals and human foot traffic. Total coliform and *E coli* counts were positively related, suggesting that for these waters, the occurrence of elevated *E coli* covaries with abundances of total coliforms. Use of both FIB and PCR-based assays to examine fecal contamination of ABW waters revealed that in some cases, despite testing positive for the presence of *E coli*, human-derived *Bacteroides* were not present. These results point to other possible sources of fecal contaminants, including animals (wild or domesticated).

Our study highlights the utility of combining FIB and MST methods for discerning possible sources of feces-associated microorganism contaminants. For example, we did observe a weak but significant positive relationship between the FIB-derived total coliform CFUs and AllBac gene abundances, suggesting the presence of mammalian *Bacteroides* may be related to increased prevalence of total coliform bacteria. However, there were no clear patterns linking those lakes with high CFUs or *Bacteroides* gene abundances to

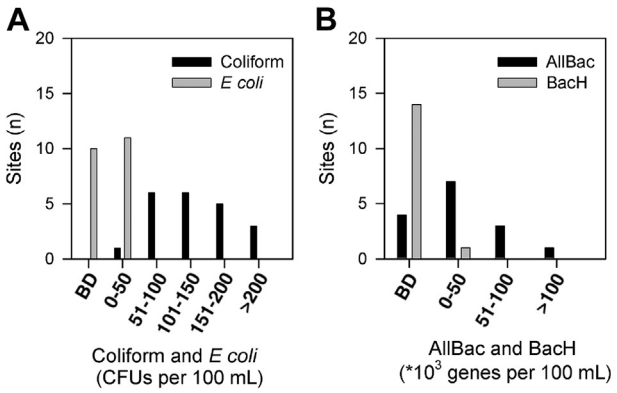

Figure 2. Histograms of colony-forming units (per 100 mL of water) for total coliforms and *E coli* (panel A) and ddPCR quantification of *Bacteroides* gene abundances for AllBac and BacH gene targets (panel B). BD = below limits of detection.

their surrounding environment or use types. Moreover, there were no apparent relationships between *Bacteroides* gene abundances or coliform CFUs and landscape types (eg, open forest, meadow, alpine tundra), elevation, or distance to trailhead. The extent to which variations in the local environment or watershed influenced the resulting microorganism distributions remains unclear; however, pack or domesticated animals may have contributed to the presence of coliform bacteria and *Bacteroides*.

Use of the AllBac ddPCR assay revealed widespread occurrence of members of *Bacteroides*. These microorganisms can be dominant members of ruminant, human, and waterfowl gut microbiomes[14,21,23]; thus, the occurrence of these microorganisms could indicate fecal contamination from various sources. We detected the presence of human feces-associated *Bacteroides* at 7 of the 15 of the sampling sites, but in all but 1 of these sites (Elk Lake) abundances were too low to quantify. Elk Lake was the lowest elevation site sampled, the closest to the trailhead (4.8 km), and it lies downstream of lakes that also tested positive for the presence of human-derived *Bacteroides* using the ddPCR assay. Thus, although most lakes in the ABW had very low human feces contamination, we did observe the presence of human-derived microorganisms in waters from highly trafficked areas.

We also sampled 2 snowmelt streams near Granite Peak, Montana's highest point and a popular summit within the ABW. The scarcity of water around Granite Peak creates congested basecamp areas that place disproportionate pressures on adjacent water resources. Prior studies on wilderness waters noted that both total coliform and *E coli* counts correlated with periods of peak human visitation.[24-26] Both of the snowmelt streams tested positive for the presence of enteric members of *Bacteroides*, and human feces-associated *Bacteroides* were found in 1 of these streams. We attribute these findings to fecal contamination from various sources, including wildlife and human fecal bacteria. The detection of human-derived fecal bacteria in 1 of the 2 snowmelt streams emphasizes that future studies should include sampling of running waters in this region.

Although use of culture-based FIB- and PCR-based MST methods revealed that several lakes in the ABW appear to be influenced by fecal microorganism contaminants, for most lakes we were unable to identify sources of these potential contaminants. The human-specific fecal indicator MST assay indicated that most of these lakes received little fecal input from humans. These results highlight the utility of combining more traditional FIB assays with emerging MST methods for information on both the occurrence and specific sources of microorganism contaminants to aquatic systems.

LIMITATIONS

A limitation in this study was the relatively high MDLs estimated for both ddPCR assays. Detection limits for both assays were somewhat higher than has been previously reported for quantitative PCR-based MST methods[27]; the detection limits for the ddPCR assay derive from any amplification in the blanks, together with the volume of water filtered to concentrate microorganism biomass. We processed relatively small volumes of water for subsequent extraction of DNA, constrained in part by the remote locations and the need to transport water back to the laboratory. We also did not include PCR inhibition controls as part of our ddPCR analyses; hence, we do not have information on the extent to which the presence of inhibitors may have resulted in underestimation of the target gene abundances. In addition, our sampling was restricted to a single summer season; hence, we do not have information on how seasonal- to episodic-scale changes in the local environment (eg, rainfall, snowfall) might affect the introduction of fecal contaminants to these waters.

Conclusions

Our results suggest relatively low prevalence of waterborne pathogens (specifically *E coli* and human-derived *Bacteroides*) in this popular wilderness area. The sources of total coliforms and *E coli* to these waters are unknown but could derive from wild or domesticated animals that inhabit or visit the ABW. Hence, increased human

Table 3. Occurrence of total coliform and *E coli* CFUs and detectable occurrences of AllBac and BacH gene markers in lake outlets and snowmelt streams sampled in the Absaroka Beartooth Wilderness

Sampling site (lake outlets and streams)	*Total coliform (CFUs per 100 mL)*	*E coli (CFUs per 100 mL)*	*AllBac gene abundances (× 10^3 copies per 100 mL)*	*AllBac present?*	*BacH gene abundances (× 10^3 copies per 100 mL)*	*BacH present?*
Bald Knob	89	0	NA	NA	NA	NA
Diamond	144	0	5.0±0.7	Yes	BD	No
Elbow	93	0	NA	NA	NA	NA
Elk	152	2	33±19	Yes	3.3±2.2	Yes
Fish	>200	0	NA	NA	NA	NA
Horseshoe	>200	1	15±19	Yes	BD	No
Keyser Brown	>200	5	96±56	Yes	BD	No
Knox	73	1	78±29	Yes	BD	No
Lake at Falls	142	1	29±2.2	Yes	BD	Yes
Lake Gertrude	174	1	20±11	Yes	BD	No
Lost	192	23	92±48	Yes	BD	Yes
Mystic	85	0	NA	NA	NA	NA
Ouzel	62	0	NA	NA	NA	NA
Pine Creek	27	0	NA	NA	NA	NA
Rainbow	82	0	BD	Yes	BD	Yes
Rimrock	124	1	BD	Yes	BD	Yes
Russell	127	1	14±4.3	Yes	BD	No
September Morn	162	0	15±3.2	Yes	BD	No
Sylvan	180	4	NA	NA	NA	NA
Thompson	129	0	NA	NA	NA	NA
Timberline	149	2	BD	Yes	BD	Yes
Stream 1	NA	NA	BD	Yes	BD	Yes
Stream 2	NA	NA	131±11	Yes	BD	No

Presence of target gene indicates detectable but not quantifiable genes (ie, at least 1 sample from the triplicate ddPCR reactions amplified; "No" indicates none of the triplicate polymerase chain reaction reactions amplified above the lower limit of detection). Mean±SD of triplicate analyses from each lake outlet or snowmelt stream. BD indicates gene abundances below detection (<900 gene copies per 100 mL of lake water for BacH and <2100 gene copies per 100 mL of lake water for AllBac). NA indicates sample not analyzed.

visitation to the ABW could have indirect impacts on water quality via fecal contamination attributable to domesticated animals. Furthermore, widespread occurrence of total coliforms, *Bacteroides*, and *E coli* highlights the importance of purifying water sources in wilderness areas before consumption.

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