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Semi-quantitative evaluation of fecal contamination potential by human and ruminant sources using multiple lines of evidence

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ABSTRACT

Protocols for microbial source tracking of fecal contamination generally are able to identify when a source of contamination is present, but thus far have been unable to evaluate what portion of fecal-indicator bacteria (FIB) came from various sources. A mathematical approach to estimate relative amounts of FIB, such as *Escherichia coli*, from various sources based on the concentration and distribution of microbial source tracking markers in feces was developed. The approach was tested using dilute fecal suspensions, then applied as part of an analytical suite to a contaminated headwater stream in the Rocky Mountains (Upper Fountain Creek, Colorado). In one single-source fecal suspension, a source that was not present could not be excluded because of incomplete marker specificity; however, human and ruminant sources were detected whenever they were present. In the mixed-feces suspension (pet and human), the minority contributor (human) was detected at a concentration low enough to preclude human contamination as the dominant source of *E. coli* to the sample. Without the semi-quantitative approach described, simple detects of human-associated marker in stream samples would have provided inaccurate evidence that human contamination was a major source of *E. coli* to the stream. In samples from Upper Fountain Creek the pattern of *E. coli*, general and host-associated microbial source tracking markers, nutrients, and wastewater-associated chemical detections—augmented with local observations and land-use patterns—indicated that, contrary to expectations, birds rather than humans or ruminants were the predominant source of fecal contamination to Upper Fountain Creek. This new approach to *E. coli* allocation, validated by a controlled study and tested by application in a relatively simple setting, represents a widely applicable step forward in the field of microbial source tracking of fecal contamination.

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1. Introduction

1.1. Background

Fountain Creek is a high-gradient stream on the Front Range of the Rocky Mountains in Colorado. The headwaters of Fountain Creek drain Pikes Peak, a major landmark and destination for tourism. In addition, Fountain Creek is a drinking water source for the City of Colorado Springs, Colorado, and is used for irrigation, recreation, and other purposes between Colorado Springs and the confluence with the Arkansas River at Pueblo, Colorado. In 2008, Fountain Creek was placed on the Colorado

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303(d) list of impaired streams because of *Escherichia coli* (*E. coli*) contamination (CDPHE, 2008). The level of impairment is evident in historical data collected by the U.S. Geological Survey (USGS; available at http://waterdata.usgs.gov/nwis). Fecal coliform densities measured by USGS on 241 dates between 1980 and 2004 at Fountain Creek near Colorado Springs, Colorado (USGS site ID 07103700, site 12 in Fig. 1) ranged from 2 to 150,000 colony-forming units per 100 millilters (CFU/100 mL) with a median value of 200 CFU/100 mL. Similarly, *E. coli* densities measured by USGS on 95 dates between 2000 and 2005 ranged from 2 to 130,000 CFU/100 mL with a median value of 140 most probably number per 100 mL (MPN/100 mL).

Fountain Creek is representative of a large number of contaminated streams that require remedial action by U.S. law and analagous regulations in other countries. With regards to Fountain Creek, Colorado historically has used a Class 1E (existing recreational usage) fecal coliform 30-day geometric mean standard of 200 per 100 mL or an *E. coli* geometric mean standard of 126 per 100 mL (CDPHE, 2005). By comparison to this and similar standards, over 15% of evaluated stream miles in the U.S. are not suitable for their designated use because of excessive fecal-indicator bacteria (FIB), making "pathogens" the largest listed cause of stream and river impairment (USEPA, 2010). This level of water-quality degradation remains despite the fact that nearly 9000 total maximum daily load (TMDL) assessments for pathogens have been done since 2002 (USEPA, 2010). Better and more effective tools clearly are called for to make more effective use of these efforts.

The USEPA Protocol for Developing Pathogen TMDLs (USEPA, 2001) provides general guidance for development of management plans for waters impaired by fecal contamination, but no specific guidance on partitioning the relative contribution of contamination from, for instance, human sources as opposed to domestic animals, birds, or wildlife. Professionals in the field of microbial source tracking (MST) recognized this challenge to water management and proposed that MST tools might be used to partition sources of fecal contamination in support of TMDL development (USEPA, 2005). Existing MST tools have limitations, however. Although several reports over the past decade purport to partition fecal contamination loads according to source by use of MST tools (Booth et al., 2003; Samadpour et al., 2002; Shanks et al., 2006; Vogel et al., 2007), none has succeeded in controlled tests using artificial "aqueous test samples" of fecal contamination (Griffith et al., 2003; Vogel et al., 2007). Thus, no prior application of MST has the proven ability to measure the amount of fecal contamination coming from a particular source, as needed to effectively generate a TMDL plan.

For this reason MST is characterized as an emerging field of research, typically with the intent to inform management decisions in recreational, fisheries, or drinking source waters (Reischer et al., 2008; Boehm et al., 2003; Gentry et al., 2007). Various researchers use different approaches to apply MST for sanitary water-quality management—a typical design uses a tiered strategy in which non-problem areas or areas with obvious sources of fecal contamination are identified and excluded during a sanitary survey and coarse-level monitoring (Boehm et al., 2003; Noble et al., 2006). Elimination of both non-problem areas and areas with obvious fecal contamination sources allows efficient use of resources in later, more-intensive tiers of research.

Field application of MST, particularly for watershed management systems such as the TMDL program in the U.S., is hindered by several factors. First, host-associated markers do not have absolute host specificity (Stoeckel and Harwood, 2007) and chemical tracers such as pharmaceuticals and caffeine can have non-fecal sources. This adds Type 1 (false positive) error to presence–absence evaluations of fecal sources. The second issue, which also can lead to Type 1 error, is that both MST markers (Bower et al., 2005) and chemical tracers (Haack et al., 2009) can be detected in water that meets FIB-based water-quality standards. Although it remains to be proven that this error is relevant to true public-health risk, at this time regulations meant to protect human health are driven by FIB concentration. This opens the possibility that human-origin fecal contamination (contributing FIB at levels lower than the relevant standard) can be detected and wrongly implicated as the major source of impairment in samples that are, in fact, grossly contaminated by a different source. These issues underscore the importance of quantification, which hitherto has not been possible, in MST studies.

1.2. *Theoretical approach*

Early protocols for detection of host-associated markers for MST (reviewed in Stoeckel and Harwood, 2007) provided data in presence or absence format. More recent protocols (such as Reischer et al., 2007; Savichtcheva et al., 2007; Seurinck et al., 2005; Shanks et al., 2008, 2009) allow measurement of MST markers by use of quantitative polymerase chain reaction (qPCR). Furthermore, limited surveys of MST markers in human (Savichtcheva et al., 2007; Shanks et al., 2009) and ruminant (Shanks et al., 2008) fecal material indicate that the concentration ranges of both FIB and MST markers, while broad, have limits. Given knowledge of concentration distributions for FIB and MST markers in feces, we propose that it is mathematically possible to estimate the upper limit of host-origin FIB in a sample based upon the measured concentration of host-associated MST markers. In cases where the calculated maximum level of FIB from a source is less than the measured FIB in water, then specific sources may be excluded as likely sources of fecal contamination.

This report describes development and application of a semi-quantitative approach to fecal source tracking in validation samples and in a relatively simple example study area. A small portion of Fountain Creek, Colorado, was selected for initial evaluation of the approach because accuracy in larger, more complex, study areas would be more difficult to evaluate. Streams such as the one described in this study, on the other hand, drain areas of manageable size, potential sources of fecal contamination are relatively few and well characterized, and flow is unidirectional. This exemplar study area serves to demonstrate this semi-quantitative approach to MST, which is broadly applicable to water bodies in many geographic regions.

1.3. *Specific objectives*

The long-term objective for development of quantitative MST data analysis is to inform management decisions that are

designed to reduce FIB concentrations and, thus, to meet locally applicable standards. In this study, our objective was to make innovative use of information including MST marker concentration measurements in water and feces, along with a suite of traditional environmental measurements, to evaluate potential contributions of FIB from various sources. Knowledge of sources can allow evaluation of public-health risk based on contamination levels from various sources (human, ruminants) as recommended for risk-based assessments by the World Health Organization (2003). A further objective of monitoring activities in this research was to provide detailed information about where and when fecal contamination entered Fountain Creek, specifically. These objectives represent a crucial first step in evaluating this approach, and thus to validate its application in similar study areas as well as more complex study areas.

2. Materials and methods

2.1. Study design

The research described in this report was designed in three steps: 1) collection and analysis of fecal samples from various sources 2) evaluation of quality-control fecal slurries and 3) application to a relatively simple watershed. The watershed study was a thorough investigation accomplished by collection of data related to FIB density, hydrology, chemical constituents, and MST marker concentration. The first tier of data collection was a sanitary survey, which included traditional measures such as monitoring FIB densities under different hydrologic conditions at 16 sites, collection of land-use and National Pollutant Discharge Elimination System (NPDES) discharge information (USEPA, 2009), and a visual sanitary survey. During the visual survey, scientists walked the watershed looking for leaky pipes and other apparent sources of fecal contamination and tested these sources for *E. coli*. On the basis of sanitary-survey results, 5 sites were selected for intensive monitoring in the second tier of the study.

2.1.1. Known-source fecal sample collection

Known-source reference feces were collected from human sources (both septic systems and consolidated sewer flow), pets (cats and dogs), ruminants (range-fed beef cattle, elk, big horn sheep), horses, and birds (duck) (Table 2). Reference feces were composited by species; each sample composite represented 8–10 individuals of the species. Several species (human, dog, cat, cattle, horse) were sampled on two occasions (August and October 2008) and the remainder were sampled only in August. Human-source septic dips were collected from seven residences and human sewage flow was collected by dip sampling from main collector sewerlines. non-human feces samples were collected by taking equal-sized portions (approximately 2 g wet wt) from fresh feces and compositing them, by species, in a sterile container. Combined reference feces from each species were manually homogenized in plastic bags and subsamples of the homogenized samples were removed for subsequent analysis. Percent dry weight was measured by evaporating a subsample of each fecal reference sample at 105 °C to constant weight.

2.1.2. Preparation and treatment of QC blind samples

One set of known-source reference feces was used to create positive-control QC blind samples. The slurries created for *E. coli* analysis were used as starting material, and dilutions were made in phosphate-buffered saline. Because these samples were created in the analytical laboratory, a technician not associated with the project was assigned to create QC blind samples to enhance independence: 1) all human, 2) all ruminant, 3) a single source neither human nor ruminant, and 4) a combination of human and a second source. The order, composition, and strength of each sample were unknown to those involved in the project and remained unknown until after the data were interpreted. Samples were processed in the same way as all other samples for FIB and MST markers.

2.1.3. Sample and field data collection

Stream-water samples were collected by the hand-dip method according to standard methodology (USGS, 2006). The hand-dip method was used instead of depth-width integrated sampling because the stream is high gradient and expected to be well mixed. Streamflow was measured at each sampling site by use of Price AA or Pygmy current meters (Rickly Hydrological Company, Columbus, Ohio), depending on depth and velocity (Rantz et al., 1982). In addition to collecting water samples, field properties (pH, specific conductance, and temperature) were measured using a Beckman ϕ240 m (Beckman Coulter, Fullerton, Calif., for pH) and an Orion model 128 m (Thermo Fisher Scientific, Pittsburgh, Pa., for temperature and specific conductance). Samples were preserved, as appropriate, by filtration, addition of acid, and (or) chilling until analysis (Wilde et al., 2004). Sixteen sites were sampled seasonally during the sanitary survey. For the year-long investigation, 5 sites were sampled monthly between October 2007 and May 2008 and semi-monthly between June and September 2008.

2.2. Study area description

The study area is described in detail to enable full evaluation of experimental results. The upper Fountain Creek watershed extends approximately from Woodland Park, Colorado, to the confluence of Monument Creek with Fountain Creek at Colorado Springs, Colorado (Fig. 1). A continuously monitored USGS streamgage, Fountain Creek near Colorado Springs (station 07103700; sampling site 12 in Fig. 1), is located downstream from Manitou Springs, approximately 3 miles upstream from the confluence with Monument Creek. The drainage area at the streamgage is 103 sq mi (Bossong, 2001), and from 1958 to 2008 the mean daily streamflow was 16.0 cubic feet per second (CFS). The overall drainage of the study area is 118 sq mi. Elevation in the main channel drops from approximately 9250 feet at Woodland Park to approximately 5950 feet at the confluence of Monument Creek (PPACG, 2003).

Streamflow in the Fountain Creek watershed has been described as seasonally variable with three distinct flow regimes: base flow, snowmelt, and summer flow (Stogner, 2000). Generally uniform base flow begins in late September or early October and extends until the following April. Snowmelt occurs from about mid-April to about mid-June with a peak in early to mid-May. Typically, more variable,

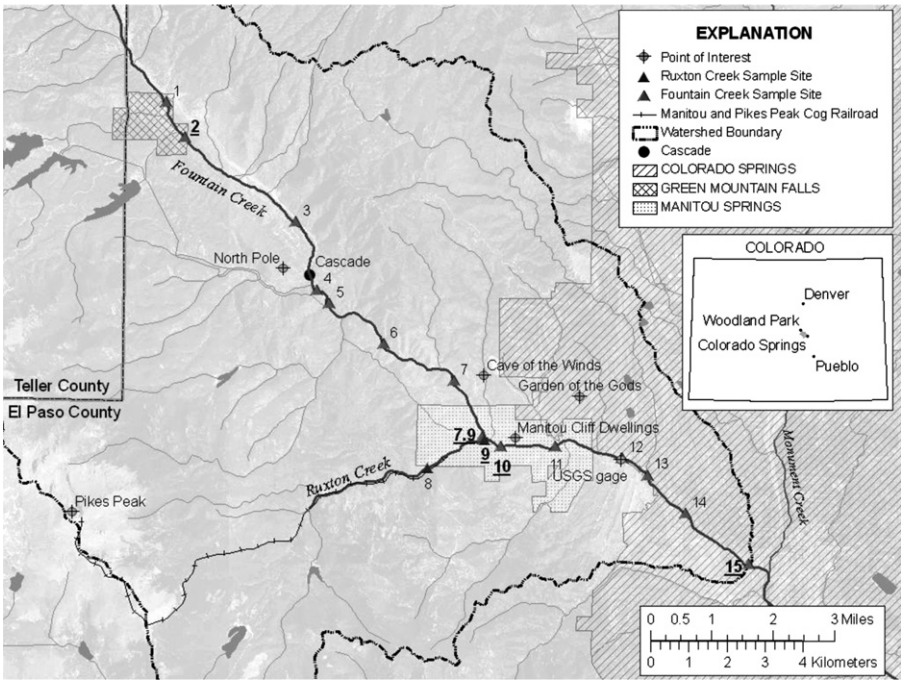

Fig. 1 – Map showing locations of sampling sites in the Fountain Creek and Ruxton Creek watersheds, Colorado, 2007–2008. Various municipalities are shown as shaded areas. Sample sites are indicated with numerals (1–15). Numerals that are bold underlined indicate intensive-monitoring sites at which a full suite of analytes was evaluated.

rainfall-driven summer streamflow follows the snowmelt period beginning about mid-June and extending through September (Stogner, 2000).

Land use in upper Fountain Creek is primarily undeveloped except for areas associated with Manitou Springs and Colorado Springs (Table 1). Residential uses increase from about 2% in the headwaters to approaching 8% as Fountain Creek enters Colorado Springs. Land areas designated for agricultural and industrial uses are relatively minor (less than 3% agricultural, less than 0.5% industrial). Land designated for commercial use increases from less than 1% in the headwaters to approaching 5% at Colorado Springs. The land area designated as streets and easements is between 1% and 2% throughout the watershed. Thus, this is characterized as a generally rural, undeveloped watershed with major use as undeveloped woodland.

Based on sanitary-survey results at 15 sites distributed throughout the study area (Fig. 1), 5 sites were sampled during the one-year intensive study period. These represented drainage of the area upstream from Manitou Springs (site 2, which frequently meets state standards for recreational water quality), drainage within Manitou Springs (sites 7.9, 9, and 10, which bracket the area where Fountain Creek frequently changes from meeting to exceeding recreational water-quality standards), and drainage between Manitou Springs and Colorado Springs (site 15, which frequently exceeds state standards for recreational water quality). Upstream from and surrounding Manitou Springs, most of the study area is undeveloped (Table 1). Tourism results in dramatic population fluctuations seasonally and, seasonal increases in weekend populations. There were no NPDES-permitted discharges to Fountain Creek and tributary streams upstream from Manitou Springs (Rich Muzzy, PPACG, written commun., October 2007, USEPA, 2009).

Within Manitou Springs are a significant tributary stream (Ruxton Creek) and the Pikes Peak Cog Railroad. Manitou Springs is heavily affected by seasonal tourism. Between Manitou Springs and Colorado Springs are a substantial number of NPDES discharge points located on tributary streams and the main stem, but few of the discharge-point descriptions (USEPA, 2009) indicate that they would carry fecal contamination. The main stem of Fountain Creek becomes a straightened urban/industrial reach beginning upstream from the USGS streamgage (site 12) and extending to the confluence with Monument Creek. The City of Colorado Springs intermittently diverts flow from Fountain Creek for use as drinking source water at a location downstream from the USGS streamgage.

Likely controllable sources of fecal contamination, identified by local water managers, include horses, grazing cattle, wildlife, humans, and domestic pets. Horse access to Fountain Creek and its tributaries is distributed throughout the watershed, especially in the headwater areas and around the Garden of the Gods parkland in the lower reach. Some reaches in the headwaters, near Green Mountain Falls, run through horse paddocks. A small number of grazing beef cattle have access to Fountain Creek and its tributaries in the upper reaches. Pet and human access to the waterways generally is coincident, with many recreational trails running alongside waterways. Water could be contaminated by human feces directly (hikers in recreational areas, vagrants and the homeless population in peri-urban areas, and partial-immersion

Table 1 – Study site ID (as in Fig. 1), site description and USGS NWISWeb identifier, stream kilometer relative to the confluence of Monument Creek, drainage area contributing to the reach, and percent of area classified according to various land uses.

Site ID	Site name and USGS NWISWeb identifier	Stream kilometer	Reach area (km^2)	Land use					
				Agriculture	Commercial	Industrial	Residential	Streets and easements	Undefined/undeveloped
Fountain Creek sites									
1	At Green Mountain Falls (site 07099990)	22.4	42.99	NA[a]	NA	NA	NA	NA	NA
2	Below Crystal Creek (site 385550105003401)	21.2	90.38	0.9%	0.8%	<0.1%	2.3%	0.9%	95%
3	Below Wellington Gulch (site 385438104583401)	17.5	109.92	2.5%	0.9%	<0.1%	5.0%	1.0%	91%
4	Below Cascade Creek (site 385340104581001)	15.6	136.38	2.1%	0.8%	<0.1%	4.7%	1.9%	90%
5	Above French Creek (site 385329104575801)	15.0	137.54	2.0%	0.8%	<0.1%	4.9%	1.9%	90%
6	Below French Creek (site 385254104565901)	12.9	168.10	1.7%	2.4%	<0.1%	4.4%	1.6%	90%
7	Above Cavern Gulch (site 385223104554201)	10.5	176.27	1.6%	3.4%	<0.1%	4.3%	1.5%	89%
7.9	Above Ruxton Creek (site 385137104551001)	8.9	180.25	1.6%	3.8%	<0.1%	4.5%	1.4%	89%
10	Below Ruxton Creek (site 385126104545101)	8.5	235.07	1.3%	3.5%	0.1%	4.4%	1.1%	90%
11	At Schryver park (site 385127104535201)	6.9	237.49	1.3%	3.8%	0.2%	4.7%	1.2%	89%
12	Near Colorado Springs (site 07103700)	5.0	266.77	1.2%	3.6%	0.2%	8.0%	1.1%	87%
13	Below Camp Creek (site 385102104521101)	4.2	295.56	1.4%	4.6%	0.2%	7.7%	1.1%	85%
14	Above 21st Street (site 385029104513001)	2.7	300.24	1.4%	4.6%	0.3%	8.1%	1.1%	84%
15	Below 8th Street (site 07103707)	0.5	305.62	1.4%	4.7%	0.4%	8.8%	1.1%	85%
Ruxton Creek sites									
8	1 mi above mouth (site 385111104560801)	9.9	42.80	0.1%	0.2%	<0.1%	0.2%	<0.1%	99%
9	At mouth (site 385134104550901)	8.9	45.70	0.1%	0.6%	<0.1%	2.1%	<0.1%	97%

a NA, not available, data were not available for land use in this reach.

bathing; recreational boating is not practical on these streams because of shallow depth), leachate from outdated on-site wastewater systems, leaky underground wastewater pipes, or accidental wastewater discharges directly to the streams. There are several campgrounds in the area, including those near 8th Street, 36th Street, and Garden of the Gods (Colorado Springs), near Schryver Park (Manitou Springs), and near Pikes Peak Highway.

2.3. *Sample analysis*

2.3.1. *Fecal-indicator bacteria*

E. coli density was measured in each sample by use of Colilert reagents and Quantitray 2000 most-probable number (MPN) trays (Idexx, Westbrook, Maine). Stream-water samples were processed within 6 h of collection. Dilutions (1:10 or 1:100) were made as necessary using 90 or 99 mL of sterile distilled water buffered with the Colilert reagent.

E. coli density in reference fecal material was measured by first diluting a measured mass (approximately 1 g wet weight) in 100 mL of phosphate-buffered water (USEPA, 2000). Dilutions up to the final dilution were made in buffered water; the final dilution was made with sterile distilled water buffered with the Colilert reagents. Samples were incubated at 35 °C in Quantitray 2000 trays for 24–28 h, and a manufacturer-provided MPN table (Idexx, Westbrook, Maine) was used to generate *E. coli* density estimates based on the proportion of positive reactions in each tray.

2.3.2. *Microbial source tracking analysis*

Water samples were concentrated for MST analysis, prior to DNA extraction, by use of 47-mm diameter, 0.45-μm pore size acetate plus filters (GE Osmonics). Reusable polycarbonate filter funnels (Nalge Nunc International, Rochester, N.Y.) were cleaned and autoclaved, residual DNA was removed by use of DNA-Zap reagent (Applied Biosystems/Ambion, Austin, Tex.), and filters were loaded with 100 mL of sample (straight or diluted, as processed to obtain *E. coli* measurements within the range of detection). Vacuum was applied to dryness and loaded filters were removed, folded, and sliced with a non-oiled razor blade into 2-mm strips directly into lysis buffer (MoBio Power Soil, Carlsbad, Calif.). For reference feces samples, approximately 0.1 g of wet composited fecal sample was weighed and added directly to lysis buffer.

Once in lysis buffer, samples were held frozen at −80 °C for up to 6 months before extraction. Extraction was completed by lysing the sample in a BeadBeater (BioSpec Products, Bartlesville, OK) for 30 s on high setting followed by extraction with the DNA EZ kit (GeneRite, North Brunswick, N.J.) for all subsequent steps. Extracted and purified DNA was eluted from the column by use of two aliquots of 100 μL elution solution that were combined for analysis. Samples were stored at −20 °C following extraction. Losses of DNA during the processes of concentration, holding, extraction, and subsequent storage were measured and corrected for by use of an exogenous spike and recovery control plasmid containing the dsRed2 sequence (Stoeckel et al., 2009).

Purified, extracted DNA was analyzed for various microbial source tracking markers by use of previously published protocols. In brief, the AllBac marker (Layton et al., 2006) was used to estimate total Bacteroidales; qHF183 (Seurinck et al., 2005) and BacHum (Kildare et al., 2007) were used to measure human-associated Bacteroidales; and BoBac (Layton et al., 2006) was used to measure ruminant-associated Bacteroidales in each sample. All qPCR analyses were done using 5 μL of DNA extract and 20 μL of master mix in an Applied Biosystems 7500 (Foster City, Calif.) thermal cycler. Applied Biosystems 2x master mix (universal TaqMan or Sybr Green formulation, as applicable) with uracil-N-glycosylase (UNG, to degrade qPCR product and avoid cross contamination) was used for each analysis.

Quality-control samples were incorporated into the analysis at various stages (results summarized in Section 3.1). A processing blank (phosphate-buffered water processed in the same manner as a water sample) was run with water samples each day. An extraction blank (sterile distilled water processed in the same way as a DNA-containing sample) was done with each batch of DNA extracts. No-template controls and plasmid-based standard curves were included in all qPCR runs.

Each quantitative PCR analysis was done in duplicate. Matrix inhibition was tested by addition of approximately 1000 copies per reaction volume of the qHF183 or BoBac standard-curve plasmid (respectively for Sybr and TaqMan-based protocols) to the master mix (Stoeckel et al., 2009). Samples in which matrix inhibition was detected (threshold cycle of the sample more than 1 cycle higher than the threshold cycle of the spiked no-template control) were diluted 1:5, 1:10, 1:20, or 1:100. The lowest sample dilution at which matrix inhibition was no longer detected was used to generate qPCR data for that sample.

2.3.3. *Chemical and nutrient analyses*

Water-quality data were collected and processed for wastewater compounds and nutrients using standard USGS techniques and procedures (USGS, 1977, 2006; Sylvester et al., 1990; Horowitz et al., 1994). All water-quality samples collected during this study were chilled and shipped by overnight express courier and analyzed by the USGS National Water-Quality Laboratory (NWQL), Lakewood, Colorado. Water-quality data reported in this report, along with associated quality-control information, are available through the USGS National Water Information System (NWISWeb, available at http://waterdata.usgs.gov/nwis; NWISWeb identifiers are given in Table 1).

2.4. *Calculations and statistical treatment of data*

2.4.1. *Calculation of MST marker concentration*

Samples were analyzed by qPCR over the course of a year. A composite standard curve was used to relate observed threshold cycle to marker concentration in each sample. For all runs, acceptance criteria for standard-curve coefficient of determination was 0.99 and amplification efficiency was between 0.8 and 1.1. If acceptance criteria were not met, the run was rejected and the analysis was redone. Each composite standard curve included results from 7 to 12 individual runs. In order to calculate a concentration per 100 mL of water sample from observed marker concentration per qPCR reaction, factors were applied to adjust for known volumetric

losses in the DNA extraction process and volumes conversions according to the following equation:

$$\text{Observed Concentration(copies/100 mL)} = \text{Concentration(copies/reaction)} * (\text{Volume extract/Volume reaction}) * (100/\text{Volume filtered (mL)})$$

The recovery efficiency of the exogenous DNA control was calculated as the ratio of marker recovered to marker added (Stoeckel et al., 2009). The calculated marker concentration for each sample was then adjusted to the spike and recovery control according to the following equation:

$$\text{Adjusted Concentration} = (\text{Observed Concentration/Recovery Efficiency})$$

2.4.2. *Calculation of upper threshold levels*

The upper credible limit for *E. coli* from a particular source was estimated using the range of MST marker concentrations and *E. coli* densities among the collection of reference materials. The 95% confidence interval was calculated for log-transformed *E. coli* density and log-transformed concentration of each MST marker in fecal reference materials. These values were used to estimate a maximum number of *E. coli* represented by one copy of each MST marker, for each source, according to the following equation.

$$\text{Ratio}_{\text{max,source}}\ (\text{MPN/copy}) = \frac{\text{Upper limit } E.coli\ \ (\text{MPN/g dry weight})}{\text{Lower limit marker (copy/g dry weight)}}$$

The value of $\text{Ratio}_{\text{max, source}}$ was then multiplied against the concentration of MST marker detected in the water sample to estimate the maximum concentration of *E. coli* in the water sample that could have come from that source ($\text{Est}_{\text{max,source}}$). For evaluation of human sources other sources which were found to carry more than one marker, the lower value of $\text{Est}_{\text{max,source}}$ between the markers present in that source was taken as a conservative estimate of the maximum number of *E. coli* that could have originated from the source, as indicated by measured MST marker concentration.

2.4.3. *Statistical regressions and means comparisons*

Relations among variables were investigated by the traditional method of linear regression of untransformed or transformed data, as appropriate. The coefficient of determination (R^2) was used to evaluate strength of the various relations. Microsoft Excel software was used to create these plots and generate regression equations. Means were evaluated by use of the *t*-test function in Microsoft Excel. The probability of equal means (p value) was used to evaluate differences between means.

3. Results

3.1. *Quality control*

Quality-control blanks and replicates were done for each analysis. For *E. coli* analysis, no false-positive results were obtained in 24 blank samples analyzed. The average relative percent difference for 29 duplicate sample analyses was 16% (range 0–59%). For MST marker analysis, the 95th percentile value of 80–130 blank analyses was used to set detection limits that represented 45, 4, 10, and 8 copies/reaction for AllBac, qHF183, BacHum, and BoBac, respectively. The average relative percent differences among 6 duplicate measurements (base 10 values calculated from log(concentration) data) ranged from 8% to 70% for the four MST markers evaluated (excluding samples in which the target was not detected).

3.2. *MST marker distribution in reference feces and upper thresholds of* E. coli *per source*

Reference feces samples were collected and analyzed for *E. coli* and MST marker concentration as described. Sample data for 5 human-source sewage samples, 4 ruminant-source fecal composite samples, 4 fecal composites from domestic pets, and 3 samples from other sources are compiled in Table 2. In general, the markers showed complete sensitivity—each marker was detected in each sample from the expected hosts. Specificity, however, was in general poor. One or both of the human-associated markers was detected, albeit at low concentration, in 9 of 13 non-human samples. Similarly, the ruminant-associated marker, BoBac, was detected in 10 of 13 non-ruminant samples.

Despite the lack of specificity observed for the host-associated markers in reference feces, each marker showed strong differential distribution. For example, although the human-associated marker qHF183 was detected in 9 of 13 non-human feces samples, the detected concentrations were at least two orders of magnitude higher in human sewage than they were in non-human feces. This differential distribution was combined with the observed *E. coli* densities in the reference feces to calculate an upper threshold of *E. coli* that could be expected to originate with the represented source categories.

3.3. *Test of approach against known-composition fecal suspensions*

To test whether the proposed approach could overcome limitations imposed by incomplete specificity of these markers, a series of known-source suspensions (prepared by non-project personnel) was analyzed as blind samples. Results for *E. coli* density observed in the test sample, upper

Table 2 – Characteristics of reference feces collected in the study area. Measured concentrations of *E. coli* were normalized to water content (percent dry weight) of reference samples (most-probable number per gram dry weight, MPN/g dry). Measured concentrations of fecal source tracking markers were normalized to water content and adjusted to account for recovery efficiency (copies per gram dry weight, copy/g dry). Shaded areas indicate tests that should result in no detection. All samples were composites of at least 10 individuals except Big Horn Sheep and Bear, for which only one individual scat was sampled.

Source	Season	Percent dry weight	General		Human		Ruminant
			E. coli (MPN/g dry)	AllBac (copy/g dry)	qHF183 (copy/g dry)	BacHum (copy/g dry)	BoBac (copy/g dry)
Human-source fecal reference materials							
Septic	Warm	0.067%	2.5×10^{6}	1.9×10^{12}	3.5×10^{9}	1.2×10^{10}	1.3×10^{8}
Sewer 1	Cool	0.060%	9.1×10^{7}	3.5×10^{12}	6.8×10^{10}	2.4×10^{11}	6.3×10^{9}
Sewer 1	Warm	0.054%	1.6×10^{7}	8.2×10^{11}	3.2×10^{10}	7.0×10^{10}	1.1×10^{9}
Sewer 2	Cool	0.066%	2.0×10^{8}	8.8×10^{12}	2.0×10^{11}	7.3×10^{11}	1.1×10^{10}
Sewer 2	Warm	0.041%	6.6×10^{7}	1.1×10^{12}	3.0×10^{10}	7.6×10^{10}	1.1×10^{9}
Ruminant-source fecal reference materials							
Cow	Cool	16%	1.0×10^{7}	4.3×10^{12}	9.8×10^{6}	3.2×10^{7}	2.2×10^{11}
Cow	Warm	16%	3.9×10^{6}	3.9×10^{12}	6.9×10^{6}	$<4.9 \times 10^{6}$	1.2×10^{12}
Cow	Warm	15%	8.3×10^{6}	3.3×10^{12}	$<1.2 \times 10^{7}$	$<3.9 \times 10^{5}$	8.2×10^{11}
Big Horn Sheep ($n = 1$)	Cool	35%	6.0×10^{5}	2.6×10^{12}	E4.9×10^{6}	1.1×10^{7}	7.4×10^{10}
Elk	Warm	23%	1.0×10^{7}	9.6×10^{11}	2.5×10^{6}	$<2.6 \times 10^{6}$	1.9×10^{11}
Alternate sources in domestic contact with humans							
Cat	Cool	49%	5.4×10^{7}	1.8×10^{12}	1.1×10^{7}	5.8×10^{7}	7.9×10^{9}
Cat	Warm	49%	4.9×10^{5}	2.4×10^{11}	4.3×10^{7}	1.2×10^{8}	6.5×10^{9}
Dog	Cool	30%	1.3×10^{7}	7.8×10^{12}	7.1×10^{7}	6.4×10^{8}	8.1×10^{10}
Dog	Warm	33%	1.7×10^{7}	1.7×10^{12}	2.2×10^{6}	2.8×10^{7}	1.1×10^{11}
Alternate sources not in close contact with humans							
Horse	Warm	28%	2.1×10^{6}	6.0×10^{11}	$<2.0 \times 10^{6}$	$<5.4 \times 10^{5}$	$<4.9 \times 10^{4}$
Duck	Warm	28%	2.8×10^{7}	4.6×10^{7}	$<2.6 \times 10^{5}$	$<6.9 \times 10^{5}$	$<2.8 \times 10^{6}$
Pigeon	Warm	36%	3.4×10^{8}	8.0×10^{6}	$<3.7 \times 10^{5}$	$<6.5 \times 10^{5}$	$<7.5 \times 10^{5}$
Bear ($n = 1$)	Warm	21%	7.4×10^{7}	1.0×10^{9}	2.6×10^{6}	5.5×10^{7}	E1.0×10^{7}

E, estimated value, value reported is below the lowest standard in the standard curve. <, less than, value reported is calculated from the analytical detection limit.

threshold *E. coli* from each source calculated based on MST marker concentration, and the expected density of *E. coli* added to the test mixture (based on the mass of fecal material added) are compiled in Table 3. In all four cases, the source added was detected by the analysis and the calculated upper limit of *E. coli* from that source greatly exceeded the *E. coli* added. In some cases, false-positive indications of fecal contribution were derived (such as 67,000 MPN/100 mL calculated upper limit of *E. coli* from ruminants in QC blind 1, and calculated upper limit densities of *E. coli* from pets in QC blind 2, 3, and 4 that were higher than the observed densities of *E. coli* despite absence of these sources in the respective samples). Thus, the approach did not allow discrimination between pet contamination and ruminant or human

Table 3 – Results for four quality-control (QC) samples. Observed *E. coli* densities (MPN/100 mL) are presented for each sample in italics followed by calculated upper limit (potential) *E. coli* contribution per fecal contamination category (Human, Ruminant, Pet, in MPN/100 mL). Sources for which host-associated MST markers were measured (Human, Ruminant) are denoted in italic when the detection was above the limit of quantification. *E. coli* added to the QC blind test mixture from each source (sewage for human, cattle for ruminant, cat for pets, horse for other) are presented last.

		QC blind 1	QC blind 2	QC blind 3	QC blind 4
Observed	*E. coli*	>24,000	24,000	830	930
Calculated upper limit	Human	62,000	ND	ND	7900
	Ruminant	67,000	350,000	ND	ND
	Pets	1,300,000	200,000	4700	3500
Added to test mixture	Human	810	0	0	500
	Ruminant	0	42,000	0	0
	Pets	620,000	0	0	0
	Other	0	0	710	0

ND, not detected, analyte below limit of detection.

contamination because pets tended to shed qHF183, BacHum, and BoBac marker with their feces (Table 2) and no pet-associated MST marker was used. It was, however, possible to distinguish human from ruminant contamination. In no case was a false-negative result derived—when a fecal source was present, it always was detected.

3.4. *Application to the upper fountain creek test watershed*

3.4.1. *Sanitary survey*

Fifteen sites in the study area were sampled three or more times in 2007 (Fig. 1). One sample set was collected at high streamflow during snowmelt with no precipitation (May and early June), the second sample set was collected at low streamflow during the summer with no precipitation (July), and the third sample set was collected at medium streamflow during the winter with no precipitation (October). Based on early sanitary-survey results, a sixteenth site (designated 7.9) was added. The sanitary survey also included 5 partial sample sets, for which 5 of the 16 sites were sampled approximately monthly for 4 months. An additional late July "walking survey" was done of reaches where increases in fecal contamination previously were observed in early results. During the walking survey, samples were collected from all discharging pipes and tributaries in the segment, as well as from the main stem at locations between established sample locations.

Three pipe leaks were identified during and after the walking survey. One cracked pipe released a trickle of flow containing 200,000 MPN/100 mL *E. coli*. Mass balance comparison of this discharge and the associated in-streamflow indicated that the cracked pipe contributed some, but not most, of the *E. coli* observed in Fountain Creek at the time of sampling (data not shown). One leaking pipe was on private property and the third leaking pipe was identified below ground during street repairs, shortly after the sanitary survey—neither was sampled for *E. coli*. All three leaking pipes were repaired before the one-year study was initiated. None of eight discharging pipes or tributaries (with the exception of Ruxton Creek) that were sampled during the sanitary survey carried higher *E. coli* density relative to the receiving water. Each sample was analyzed for E. coli (Fig. 2).

3.4.2. *Year-long survey*

During the year-long survey of Fountain Creek, the median flow (24 CFS) was higher than the long-term median for the entire period of record extending to 1959 (16 CFS). The maximum flow during the study period was, however, substantially lower than the maximum over the period of record (92 CFS compared to 813 CFS). Though typical streamflow was similar to or higher than normal during this investigation, there were no intense precipitation events. None of the samples collected was substantially influenced by precipitation runoff, though some of the samples (early May) were influenced by snowmelt (Fig. 2).

3.4.2.1. E. coli *patterns*. For the purposes of this study, samples were divided into cool-weather months (cool season) and warm-weather months (warm season) based on data patterns, hydrology, and tourism. The warm season began

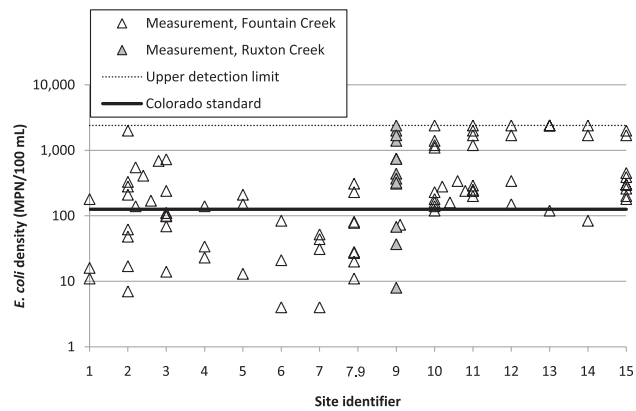

Fig. 2 – *E. coli* density measurements collected during the sanitary survey, May–October 2007. Site identifiers can be cross-referenced to Fig. 1 and Table 1; samples collected between established sites are plotted at corresponding locations in the plot. The upper detection limit for the method utilized (without dilution) is 2400 MPN/100 mL. The Colorado 30-day geometric mean standard of 126 MPN/100 mL is shown for reference.

after snowmelt and was defined as June 15–September 15. Geometric mean *E. coli* density was higher in the warm season than the cool season at all five sites (Fig. 3; *t*-test $p < 0.05$). The standard generally was met in samples collected during cool season at all sites (Fig. 3)—when evident, exceedances were at the mouth of Ruxton Creek (site 9) and Fountain Creek below Ruxton Creek and below 8th stream (sites 10 and 15) rather than at upstream sites (sites 2 and 7.9). In contrast, the standard was exceeded more frequently during the warm season – routinely at the sites upstream from Ruxton Creek (sites 2 and 7.9) and always at Ruxton Creek (site 9) and sites downstream from Ruxton Creek (sites 10 and 15). In both seasons, upward trends in *E. coli* density were noted in an upstream-to-downstream direction (linear regression of log *E. coli* with river mile, slope = −0.089, $R^2 = 0.25$ (cool season); slope = −0.104, $R^2 = 0.46$ (warm season), data not shown). The lower coefficient of determination in the cool-season regression appears

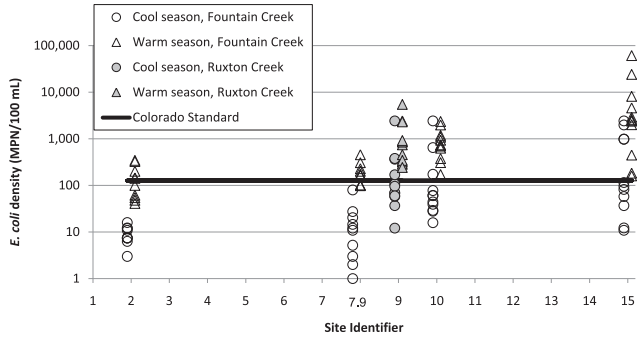

Fig. 3 – *E. coli* density measurements during the year-long intensive monitoring, August 2007–September 2008. Cool season is from September 16 to June 14; warm season is June 15–September 15. Warm season and cool-season values are associated with the same sites, but are offset from the true site identifier to enhance visual clarity.

largely to be caused by the presence of a step increase in *E. coli* density between Fountain Creek above Ruxton Creek (site 7.9) and Fountain Creek below Ruxton Creek at Manitou Springs (site 10) rather than the more consistent trend observed in the warm-season regression (Fig. 3).

3.4.2.2. Microbial source tracking marker patterns in stream water. The longitudinal and seasonal trends observed for the various MST markers generally were distinct from the trends observed for *E. coli* (Fig. 4). Like *E. coli*, AllBac had an upward trend and stepwise increases in concentration upstream to downstream, though site-to-site pairwise differences were significant only during warm season ($p < 0.05$). In contrast, the concentrations of human-associated markers (qHF183 and BacHum) showed significant stepwise increases only at sites 7.9 and 10, in Manitou Springs, and only during the cold season (Fig. 4). The ruminant-associated marker (BoBac) showed no concentration differences longitudinally in either season ($p < 0.05$ for all pairwise significant differences).

Curiously, host-associated markers of fecal contamination showed seasonal differences that were inverted relative to the trends observed for *E. coli* (Fig. 4B–D compared to Fig. 3). Unlike *E. coli*, for which densities always were higher in warm-season samples compared with cool-season samples, average human-associated marker concentrations were not significantly different between seasons at the Fountain Creek sites. Human-associated markers were higher in cool-season samples than in warm-season samples at Ruxton Creek (site 9; $p < 0.05$). The BoBac marker of ruminant contamination tended to be higher in cool-season samples than warm-season samples at all sample sites (Fig. 4D; $p < 0.05$).

Analysis of MST marker concentration against *E. coli* density by linear regression indicated that the AllBac marker of general fecal contamination was correlated with *E. coli* density in cool ($R^2 = 0.36$; $p = 0.0035$) and to some extent warm ($R^2 = 0.21$; $p = 0.17$) seasons (Fig. 5A). The qHF183 and BacHum human-associated markers were more strongly correlated with *E. coli* in cool ($R^2 = 0.84$; $p = 0.024$) season than warm ($R^2 < 0.1$; $p > 0.1$) season (Fig. 5B). Similarly, the BoBac ruminant-associated marker was more strongly correlated with *E. coli* density in cool ($R^2 = 0.33$; $p = 0.0011$) than warm ($R^2 < 0.1$; $p > 0.1$) season samples (Fig. 5C). Regression lines and equations are shown on the plots of Fig. 5A–C only for those relationships in which the coefficient of determination (R^2) was greater than 0.1.

MST samples from three cool-season dates were analyzed to obtain characteristic conditions when *E. coli* densities typically did not exceed the water-quality standard. Upper thresholds of *E. coli* calculated based on MST marker concentrations did not exclude any of the sources tested as a major or minor source of contamination to the samples (Table 4) except that ruminant and pet sources likely were not the major sources of 110 MPN/100 mL *E. coli* to Ruxton Creek (site 9) on February 20, 2008. In the two instances that *E. coli* density exceeded 126 MPN/100 mL (both collected on May 1, 2008), data did not exclude any of the sources tested as a potential primary contributor of *E. coli* to the samples. High upper thresholds for the bird category were caused by lack of a bird-associated marker (only the general marker was used to create

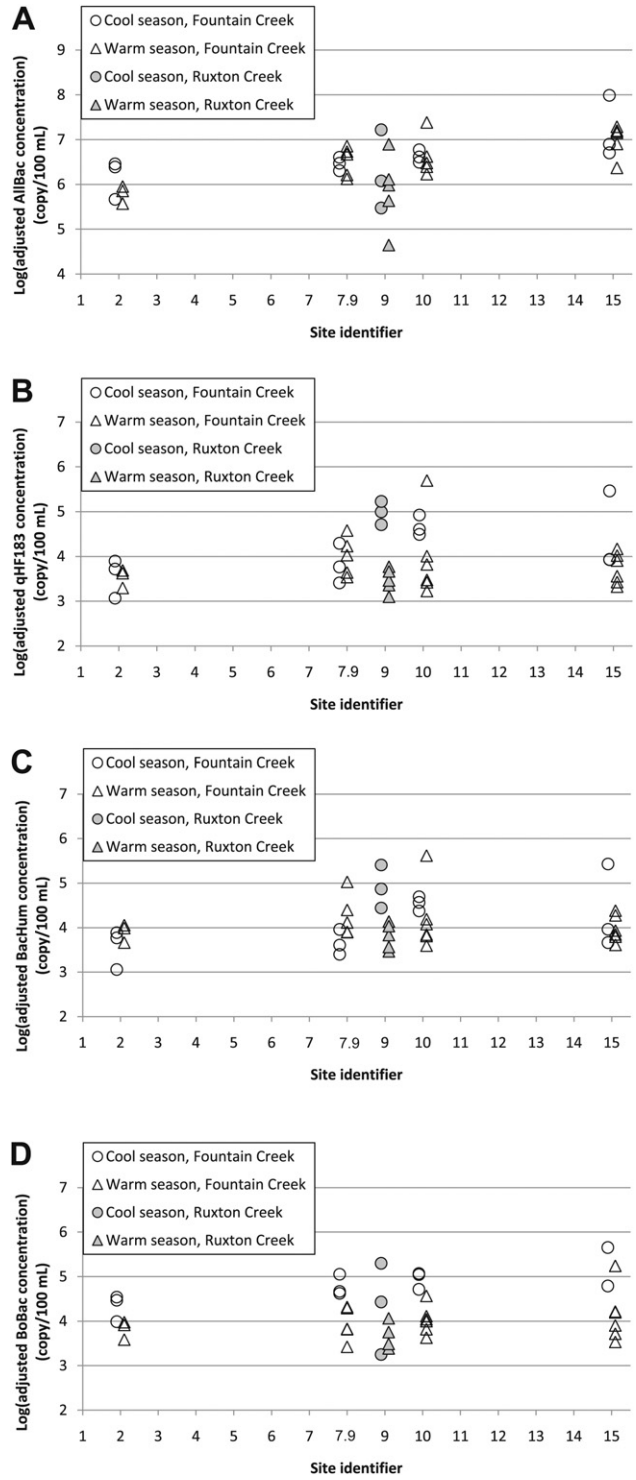

Fig. 4 – Microbial source tracking marker concentration measurements for (A) AllBac, (B) qHF183, (C) BacHum and (D) BoBac during the year-long intensive monitoring, August 2007–September 2008. Cool season is from September 16 to June 14; warm season is June 15–September 15. Warm season and cool-season values are associated with the same sites, but are offset from the true site identifier to enhance visual clarity.

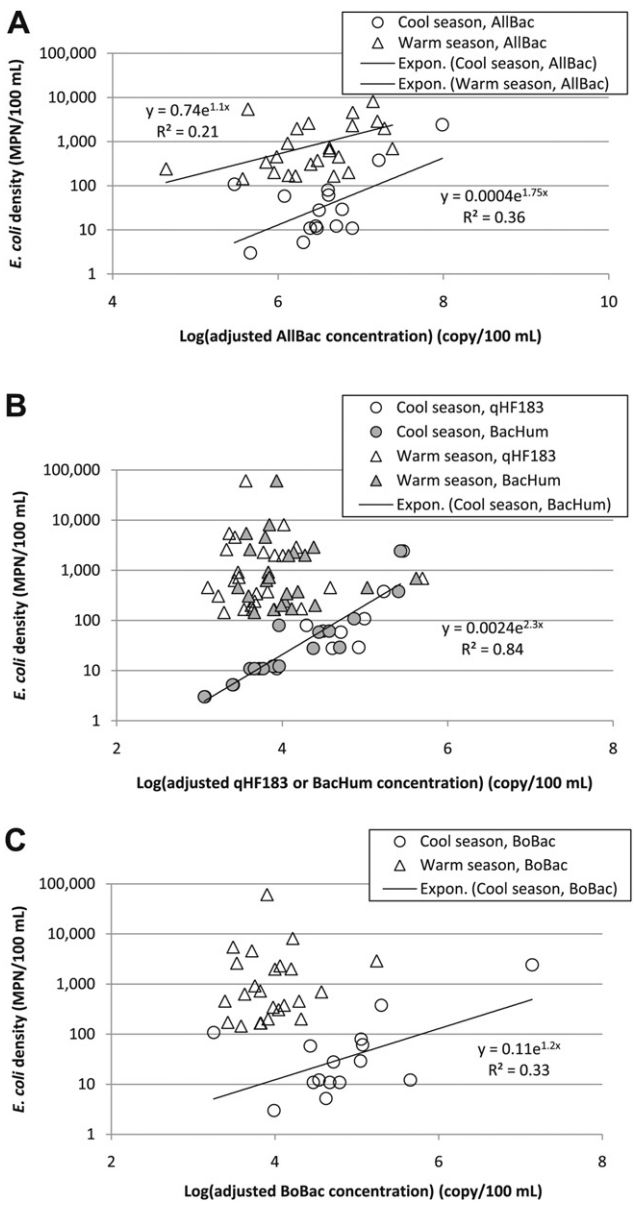

Fig. 5 – MST marker concentrations plotted against *E. coli* density measurements for (A) AllBac, (B) qHF183 and BacHum, and (C) BoBac during the year-long intensive monitoring, August 2007–September 2008. Cool season is from September 16 to June 14; warm season is June 15–September 15. The regressions for the two human-associated markers were nearly identical; therefore, both are plotted in (B) and only the regression for BacHum is shown on the chart.

this estimate) and scarcity of data (only two composite bird samples were analyzed).

All MST samples from three dates and several MST samples from four other dates during warm season, when the *E. coli* standard typically was exceeded, were characterized (Table 5). In most of these cases, the human- and ruminant-associated markers were not detected. When markers were detected, the marker concentration was so low that the calculated upper limit of *E. coli* density from these sources usually was lower than the *E. coli* densities observed in the sample; in other words, the low MST marker concentration indicated that most of the *E. coli* present were not from human or ruminant sources. The major exception was on August 12, 2008, when the MST marker distribution indicated that human sources may have been responsible for high *E. coli* densities in Fountain Creek above and below Ruxton Creek (sites 7.9 and 10) but not below 8th street (site 15). Pets frequently were excluded as major sources of *E. coli* based on absence of cross-carried human- and ruminant-associated MST markers in the samples. Birds, however, could not be excluded as the source of fecal contamination to these samples, because no bird-associated marker was used and because of the low concentrations of MST markers detected in the bird reference samples. In other words, bird was the only source category tested that had high *E. coli* but low AllBac general MST marker concentration in their feces, similar to the pattern in samples collected during warm season. Other non-human, non-ruminant sources that were not sampled may have a similar pattern, but avian is the common factor among sources with high *E. coli* and low MST markers (Table 2 and unpublished data representing avian (chicken, Canada goose, duck, pigeon) and nonavian (raccoon, horse, bear, cat, dog) sources).

3.4.2.3. *Additional lines of evidence*. Evaluation of MST markers indicated that, in general, humans, ruminants, and (or) pets could have contributed *E. coli* at the levels observed during the cool season but not during the warm season (as defined in this study). To support this preliminary hypothesis, and to provide further support for the proposed approach, alternate lines of evidence (physical and chemical characteristics, nutrient concentrations, presence of wastewater organic chemicals) were evaluated.

Analysis of physical and chemical characteristics revealed little novel information. Temperature was significantly higher in warm-season samples at all sites (Table 6), and correlation between temperature and *E. coli* density was observed (global $R^2 = 0.35$). Although specific conductance may be expected to vary with water source (base flow dominated as opposed to snowmelt or rainfall dominated), the few seasonal differences in specific conductance ($p < 0.05$) did not follow any interpretable pattern. Specific conductance, in general, was lower in Ruxton Creek compared with Fountain Creek because of geology (Ruxton Creek drains a granitic watershed, while the headwaters of Fountain Creek are dominated by sandstone). The increase in specific conductance at the bottom of the watershed (site 15) may be caused by more urban influence, but specific conductance did not vary seasonally as did *E. coli* density. Observations of pH and streamflow did not vary with season. Streamflow in Fountain Creek increased in an upstream-to-downstream direction except for the reach between Manitou Springs and Colorado Springs (site 10 to site 15), likely because of withdrawals by Colorado Springs as a source for drinking water.

Increased *E. coli* density was expected to coincide with elevated nutrient concentrations, because fecal material carries high levels of each. In agreement with this expectation, total nitrogen was significantly higher in the warm

Table 4 – Results of routine field sampling during cool season (September 16–June 14) in Fountain Creek and Ruxton Creek. Ruxton Creek (site 9) data are presented between bracketing main stem sites but shaded to offset from main stem data. Observed *E. coli* densities (MPN/100 mL) are presented for each site in italics. Calculated upper limit (potential) *E. coli* contribution per fecal contamination category (Human, Ruminant, Pet, Bird, in MPN/100 mL) follow. Sources for which host-associated MST markers were measured (Human, Ruminant) are separated from other estimates and denoted in italic when the detection was above the limit of quantification.

		Site 2	Site 7.9	Site 9	Site 10	Site 15
Wednesday, February 20, 2008						
Observed	*E. coli*	*3*	*5*	*110*	*28*	*12*
MST marker	Human	38	84	350	790	310
	Ruminant	24	100	4	130	520
No MST marker	Pets	240	1100	86	1700	2700
	Bird	250,000	560,000	95,000	2,800,000	2,000,000
Wednesday, April 23, 2008						
Observed	*E. coli*	*12*	*11*	*58*	*29*	*11*
MST marker	Human	260	140	930	1700	160
	Ruminant	85	120	67	270	150
No MST marker	Pets	1500	1600	630	3100	3000
	Bird	1,700,000	900,000	1,400,000	5,900,000	1,000,000
Thursday, May 01, 2008						
Observed	*E. coli*	*11*	*80*	*380*	*61*	*2400*
MST marker	Human	200	310	8500	1200	9000
	Ruminant	72	280	490	290	10,000
No MST marker	Pets	1300	2100	8800	2200	51,000
	Bird	1,300,000	2,000,000	11,000,000	6,300,000	60,000,000

season compared to the cool season at three of four Fountain Creek sites (all except site 15; Table 7) but not at Ruxton Creek (site 9). When observed, increases in nitrogen were attributable to the nitrate-plus-nitrite component rather than organic nitrogen or ammonia nitrogen (Table 7). Unlike nitrogen, phosphorus tended to be enriched in warm season compared with cool season at downstream sites (sites 10 and 15) but not at upstream sites. A large portion of phosphorus enrichment was caused by orthophosphate concentration (Table 7). Although seasonal nutrient enrichment coincided with elevated *E. coli* density, nutrient concentrations were not correlated with *E. coli* concentrations in any combination of site or season (data not shown) except Fountain Creek at Manitou Springs (site 10).

Increased *E. coli* density also was expected to coincide with detection of wastewater-associated chemicals at sites where the *E. coli* originated with human sewage. Various wastewater organic chemicals were detected more frequently in the urbanized sections of Fountain Creek than in the undeveloped sections (Tables 8 and 9) although some chemicals (particularly industrial and asphalt-associated chemicals such as isophorone, camphor, and naphthalene) were detected throughout the watershed. Many chemicals, including human-waste-associated chemicals, were never detected in samples (Table 8).

4. Discussion

A new conceptual approach to analysis of microbial source tracking data was developed and data were collected from reference fecal material to test its applicability. The absolute distribution of markers in reference fecal material collected from the study area underscored the well-characterized risk of false-positive results when using presence–absence data to indicate fecal contamination sources (Shanks et al., 2010; Stoeckel and Harwood, 2007). Host-associated markers commonly were found in non-target reference materials; i.e., the lack of specificity was clear, particularly for the ruminant-associated marker BoBac. The markers were, however, more abundant in the respective associated hosts than they were in non-target hosts. The differential distribution of markers indicated that the proposed approach, using ratios of marker concentrations and *E. coli* densities to place an upper bound on the *E. coli* that could have come from a particular host, might provide useful information.

The proposed approach was tested against fecal suspensions prepared in a similar way to previous studies (Griffith et al., 2003; Wang et al., 2010). The analytical results identified both strengths and limitations of the proposed approach. In the positives column, there were no false-negative results, the minority contributor was correctly discriminated from the majority contributor in the mixed sample, and the practical calculation steps were quite simple. These findings are in contrast with previously described false-negative results and inability to discriminate minority from majority contributors (Griffith et al., 2003) and the need for complex calculation steps that may be beyond commonly accessible capabilities (Wang et al., 2010). Although the ability to quantify contributions from fecal contamination sources under constrained conditions was reported by the latter researchers (Wang et al., 2010)

Table 5 – Results of routine field sampling during warm season (June 15–September 15). Observed *E. coli* densities (MPN/100 mL) are presented for each site in italics. Calculated upper limit (potential) *E. coli* contribution per fecal contamination category (Human, Ruminant, Pet, Bird, in MPN/100 mL) follow. Sources for which host-associated MST markers were measured (Human, Ruminant) are separated from other estimates and denoted in italic when the detection was above the limit of quantification. Ruxton Creek (site 9) data are presented between bracketing main stem sites but shaded to offset from main stem data. Warm season calculated upper limit *E. coli* contributions in MPN/100 ml.

		Site 2	Site 7.9	Site 9	Site 10	Site 15
Tuesday, June 17, 2008						
Observed	*E. coli*	140	100	280	170	8200
MST marker	Human	–	–	–	–	ND
	Ruminant	–	–	–	–	ND
No MST marker	Pets	–	–	–	–	800
	Bird	–	–	–	–	890,000
Monday, June 30, 2008						
Observed	*E. coli*	55	99	460	310	2600
MST marker	Human	–	–	ND	ND	ND
	Ruminant	–	–	ND	27	ND
No MST marker	Pets	–	–	120	530	160
	Bird	–	–	130,000	590,000	180,000
Tuesday, July 15, 2008						
Observed	*E. coli*	140	170	5500	2000	4600
MST marker	Human	ND	ND	ND	ND	ND
	Ruminant	ND	ND	ND	ND	ND
No MST marker	Pets	190	130	150	480	250
	Bird	210,000	142,000	170,000	540,000	280,000
Tuesday, July 29, 2008						
Observed	*E. coli*	340	170	860	630	2000
MST marker	Human	ND	ND	–	ND	ND
	Ruminant	ND	ND	–	ND	ND
No MST marker	Pets	380	320	–	210	760
	Bird	510,000	360,000	–	230,000	850,000
Tuesday, August 12, 2008						
Observed	*E. coli*	<100	460	2300	740	61,000
MST marker	Human	330	3600	ND	14,000	290
	Ruminant	ND	ND	ND	91	20
No MST marker	Pets	400	950	560	1800	390
	Bird	440,000	1,100,000	620,000	2,000,000	430,000
Tuesday, August 26, 2008						
Observed	*E. coli*	62	170	860	730	>2400
MST marker	Human	–	260	ND	ND	810
	Ruminant	–	ND	ND	ND	430
No MST marker	Pets	–	320	270	320	8400
	Bird	–	350,000	300,000	350,000	5,300,000
Tuesday, September 09, 2008						
Observed	*E. coli*	41	200	240	380	160
MST marker	Human	–	ND	–	ND	–
	Ruminant	–	ND	–	ND	–
No MST marker	Pets	–	1000	–	620	–
	Bird	–	1,100,000	–	690,000	–

–, analysis not done.
ND, not detected, analyte below the limit of detection.

Table 6 – Physical and chemical characteristic averages, 95% confidence intervals about the means (CI), and numbers of observations (*n*) measured at study sites between May 2007 and September 2008. Data are divided into warm season (June 15–September 15), roughly corresponding to summer vacation season, and cool season (September 16–June 14), roughly corresponding to the off season. Ruxton Creek (site 9) data are presented between bracketing main stem sites but shaded to offset from main stem data.

Season	Fountain Creek below Crystal Creek (site 2)	Fountain Creek upstream from Ruxton Creek (site 7.9)	Ruxton Creek Mouth (site 9)	Fountain Creek below Ruxton Creek (site 10)	Fountain Creek below 8th Street (site 15)
	Average (CI, *n*)	Average (CI, *n*)	Average (CI, *n*)	Average (CI, *n*)	Average (CI, *n*)
Temperature (°C)					
Cool	5.0 (2.9–7.1, 12)	5.9 (3.7–8.2, 12)	6.0 (4.0–8.0, 14)	7.0 (4.4–9.6, 12)	10.3 (7.0–13.5, 12)
Warm	12.2 (11.4–13.0, 11)	13.9 (12.4–15.3, 10)	13.4 (12.7–14.1, 18)	14.3 (13.8–14.8, 16)	19.1 (18.3–19.9, 17)
Specific conductance (μS/cm)					
Cool	322 (303–341, 12)	321 (281–361, 12)	122 (97–146, 14)	317 (267–367, 12)	507 (401–613, 12)
Warm	321 (292–351, 11)	298 (233–363, 10)	176 (141–211, 19)	326 (272–380, 18)	829 (470–1190, 17)
pH (unitless)					
Cool	7.97 (7.72–8.22, 12)	7.30 (7.19–7.41, 12)	7.66 (7.43–7.88, 14)	7.48 (7.38–7.57, 12)	8.06 (7.90–8.21, 12)
Warm	8.06 (7.92–8.20, 10)	7.36 (7.21–7.52, 8)	7.63 (7.50–7.77, 16)	7.51 (7.41–7.62, 14)	8.14 (7.99–8.29, 15)
Streamflow (CFS)					
Cool	4.78 (3.77–5.80, 12)	8.94 (6.61–11.3, 12)	3.50 (1.83–5.17, 13)	14.3 (8.87–19.7, 12)	12.3 (6.05–18.6, 12)
Warm	4.72 (3.37–6.07, 9)	10.4 (5.38–15.4, 7)	2.21 (0.83–3.60, 14)	12.9 (7.84–18.0, 13)	14.4 (7.63–21.2, 14)

the samples in that study were not analyzed blind. The current research is the first report of correct semi-quantitative results from analysis of fecal suspensions that were analyzed blind.

In the negatives column, incomplete specificity of the host-associated markers resulted in common false-positive results, particularly for classes such as domestic pets that carried both human- and ruminant-associated markers (this report, Shanks et al., 2010; Stoeckel and Harwood, 2007). These false-positive results were pronounced for sources such as pets that were incidental carriers, and not purposefully targeted with a host-associated marker. Further development of markers with better specificity and inclusion of additional host-associated markers, such as the dog-associated marker (Kildare et al., 2007; Wang et al., 2010) and bird-associated markers that under evaluation (such as Lu et al., 2008; Weidhaas et al., 2010) may at least partially alleviate this problem. In sum, results for the fecal suspension samples indicated that the proposed approach could be used reliably to exclude sources of fecal contamination, though not to directly implicate sources of fecal contamination. It remained to be determined whether these limitations to the approach would render it ineffective in environmental application, or whether practical value would be obtained despite the limitations imposed by incomplete host specificity.

A thorough field study was done to provide a practical test of the approach. The sanitary survey allowed effective allocation of available resources, with a sample design that emphasized differences between a long cool season and a short warm season, and sites that bracketed a focus point in the watershed at which stream-water quality (as indicated by *E. coli* density) tended to breach the local water-quality standard of 126 MPN/100 mL. Furthermore, the sanitary survey uncovered unexpected sources of human wastewater to the stream that were repaired and (or) controlled before the year-long intensive monitoring began.

Despite these infrastructure repairs, data from the year of intensive monitoring (monthly during cool season and biweekly during warm season) showed the same trends indicated by the sanitary survey. Combined evaluation of *E. coli*, MST marker, nutrient, and wastewater organic chemical data clearly indicated that human sources of fecal contamination typically were not the major cause of *E. coli* standard exceedances in Fountain and Ruxton Creeks during the summer of 2008. Though *E. coli* densities increased to more than 100 times the recreational water standard, host-associated markers for human and ruminant fecal contamination stayed the same or decreased during warm season (Fig. 4), nutrient concentrations remained steady (Table 7), and human- and wastewater-associated organic chemicals rarely were detected (Tables 8 and 9). Though human sources of fecal contamination did not appear to cause chronic warm-season water-quality standard exceedances, instances of human-source contamination were apparent. On occasion, increased *E. coli* density coincided with increased MST markers.

Further evaluation of the integrated data set was done to test the validity of field study results and to emphasize the value of the proposed upper limit calculations. In one example, Fountain Creek below 8th street (site 15) had relatively high *E. coli* and MST markers on May 1, 2008 (Table 4), indicating that human, ruminant, or pet sources could have caused the observed *E. coli* contamination level. In support of this hypothesis, ammonium was measured at 0.56 ppm (compared with a 90th percentile value of 0.08 ppm among all samples; Table 7). Ammonium concentrations in wastewater are estimated to be 12–50 ppm (as N; Tchobanoglous and Burton, 1991) so this ammonium concentration would be expected in 1:100 diluted wastewater. In addition, 3 of 4 wastewater-associated chemicals and 5 of 6 human-associated chemicals detected in this study were detected in the May 1st sample (see Tables 4 and 5). In this instance, presence of human-origin fecal contamination was supported by *E. coli*, MST marker, nutrient, and wastewater organic chemical analytical data although presence of fecal contamination from other sources could not be excluded.

Table 7 – Nutrient concentration averages, 95% confidence intervals about the means (CI), and numbers of observations (*n*) measured at study sites between May 2007 and September 2008. Data are divided into warm season (June 15–September 15), roughly corresponding to summer vacation season, and cool season (September 16–June 14), roughly corresponding to the off season. Ruxton Creek (site 9) data are presented between bracketing main stem sites but shaded to offset from main stem data.

Season	Fountain Creek below Crystal Creek (site 2)	Fountain Creek upstream from Ruxton Creek (site 7.9)	Ruxton Creek Mouth (site 9)	Fountain Creek downstream from Ruxton Creek (site 10)	Fountain Creek below 8th Street (site 15)
	Average (CI, *n*)	Average (CI, *n*)	Average (CI, *n*)	Average (CI, *n*)	Average (CI, *n*)
Total nitrogen (parts per million as N)					
Cool	1.58 (1.47–1.69, 10)	1.05 (0.95–1.14, 10)	0.29 (0.19–0.40, 10)	0.95 (0.83–1.07, 10)	1.10 (0.75–1.46, 10)
Warm	1.76 (1.69–1.83, 8)	1.15 (1.12–1.17, 8)	0.29 (0.22–0.37, 10)	1.03 (1.01–1.06, 10)	1.09 (0.95–1.23, 10)
Ammonium (parts per million as N)					
Cool	0.018 (0.015–0.020, 10)	0.023 (0.015–0.031, 10)	0.031 (0.008–0.053, 10)	0.026 (0.007–0.044, 10)	0.075 (−0.031–0.182, 10)
Warm	0.021 (0.018–0.024, 8)	0.018 (0.015–0.021, 8)	<0.02 (no detect, 10)	0.018 (0.016–0.021, 10)	0.017 (0.013–0.020, 10)
Nitrate-plus-nitrite (parts per million as N)					
Cool	1.41 (1.30–1.51, 10)	0.905 (0.791–1.02, 10)	0.210 (0.170–0.250, 10)	0.784 (0.683–0.885, 10)	0.797 (0.737–0.857, 10)
Warm	1.64 (1.56–1.72, 8)	0.989 (0.941–1.04, 8)	0.199 (0.183–0.215, 10)	0.914 (0.881–0.947, 10)	0.796 (0.671–0.921, 10)
Total phosphorus (parts per million as P)					
Cool	0.037 (0.031–0.043, 10)	0.012 (0.010–0.015, 10)	0.017 (0.004–0.029, 10)	0.012 (0.010–0.015, 10)	0.016 (0.002–0.030, 10)
Warm	0.047 (0.036–0.058, 8)	0.038 (0.021–0.056, 8)	0.029 (0.004–0.053, 10)	0.039 (0.014–0.065, 10)	0.033 (0.023–0.043, 10)
Orthophosphate (parts per million as P)					
Cool	0.018 (0.017–0.020, 9)	0.005 (0.004–0.006, 9)	0.007 (0.002–0.013, 9)	0.005 (0.004–0.006, 9)	0.005 (0.004–0.006, 9)
Warm	0.024 (0.020–0.027, 8)	0.009 (0.006–0.012, 8)	0.009 (0.008–0.010, 10)	0.012 (0.007–0.017, 10)	0.013 (0.011–0.015, 10)

Table 8 – Wastewater organic chemicals detected fewer than three times in the study area. Chemicals are divided according to type and/or origin. Ruxton Creek (site 9) data are presented between bracketing main stem sites but shaded to offset from main stem data.

	Site 2	Site 7.9	Site 9 (Ruxton Creek)	Site 10	Site 15	Never detected
Wastewater chemicals	None	None	Triethyl citrate Hexahydrohexamethyl cyclopentabenzopyran	None	Triethyl citrate	Benzophenone Triclosan Acetyl hexamethyl tetrahydro naphthalene
Human associated	None	None	DEET Triphenyl phosphate	DEET Menthol	DEET Cotinine Triphenyl phosphate Skatole[a]	3-tert-Butyl-4-hydroxyanisole D-Limonene 5-Methyl-1H-benzotriazole
Sterols and stanols	None	None	None	None	None	Beta-sitosterol Cholesterol Beta-stigmastanol 3-Beta-coprostanol
Pesticides/Herbicides	Metolachlor (M)	None	None	Bromacil Metolachlor	Prometon Tributyl phosphate	Chlorpyrifos Diazinon 1,4 Dichlorobenzene Metalaxyl
Synthesis, solvents, plasticizers	None	None	Phenol	Phenol	Tris(2-butoxyethyl) phosphate Phenol Acetophenone Isopropylbenzene	4-tert-Octylphenol diethoxylate 4-tert-Octtylphenol monoethoxylate 4-Nonylphenol diethoxylate 4-Nonylphenol 4-n-Octylphenol 4-tert-Octylphenol Indole Tribromomethane Isoquinoline Isoborneol Bisphenol A 4-Cumylphenol
Asphalt	None	1-Methylnaphthalene 2-Methylnaphthalene	None	1-Methylnaphthalene 2-Methylnaphthalene	1-Methylnaphthalene 2-Methylnaphthalene Skatole[a]	Anthracene Benzo[a]pyrene 2,6-Dimethylnaphthalene

a This chemical classified in more than one category.

Table 9 – Wastewater organic chemicals quantified and/or detected three or more times. Chemicals are divided according to type and/or origin. Values represent the number of samples in which the chemical was quantified and/or detected. Ruxton Creek (site 9) data and overall sums are shaded to offset from main stem data.

		Site 2	Site 7.9	Site 9	Site 10	Site 15	Overall
	Total	13	12	13	13	13	64
Wastewater chemicals	*Tris(2-cholorethyl) phosphate*						
	Detected	0	0	1	0	3	4
	Quantified	0	0	0	0	2	2
	Tris(dicholoroisopropyl) phosphate						
	Detected	0	1	1	0	3	5
	Quantified	0	0	0	0	2	2
Human associated	*Methyl salicylate*						
	Detected	1	1	2	1	4	9
	Quantified	0	0	1	0	1	2
	Caffeine						
	Detected	0	3	5	5	9	22
	Quantified	0	1	2	1	4	8
Synthesis, solvents, plasticizers	*Isophorone*						
	Detected	1	2	3	4	4	14
	Quantified	0	1	0	0	1	2
	Tetrachloroethene						
	Detected	1	0	0	0	11	12
	Quantified	0	0	0	0	2	2
	Phenanthrene						
	Detected	0	0	1	3	4	8
	Quantified	0	0	0	0	1	1
	Carbazole						
	Detected	0	0	0	0	3	3
	Quantified	0	0	0	0	2	2
	9,10-Anthraquinone						
	Detected	0	1	0	0	3	4
	Quantified	0	0	0	0	2	2
	Camphor						
	Detected	3	4	4	3	3	17
	Quantified	0	1	1	1	1	4
Asphalt	*Fluoranthene*						
	Detected	0	0	1	1	8	10
	Quantified	0	0	0	0	2	2
	Naphthalene						
	Detected	1	1	0	2	1	5
	Quantified	0	0	0	0	0	0
	Pyrene						
	Detected	0	0	1	0	6	7
	Quantified	0	0	0	0	1	1
	p-Cresol						
	Detected	0	1	2	2	4	9
	Quantified	0	0	0	0	0	0

On the same date, Ruxton Creek (site 9) had high *E. coli* and human-associated MST markers (Table 4), indicating that human or pet (but less likely ruminant) sources may have caused the observed fecal contamination. Nutrient concentrations measured in this sample were high (ammonium at 0.13 ppm, total nitrogen and total phosphorus higher than any other date during the study at this site). In addition, 3 of 4 wastewater-associated chemicals and 5 of 6 human-associated chemicals detected in this study were detected in the sample. The upper threshold limit approach was again supported by these additional lines of evidence in the Ruxton Creek sample.

In other cases, the inverse happened – host-associated MST markers did not increase when *E. coli* densities increased, indicating a source that was not detectable by the MST markers applied. On these dates, nutrients were present at baseline levels and wastewater- and human-associated chemicals were rarely detected. As an example, Fountain Creek below 8th street (site 15) had relatively high *E. coli* densities in four samples between June 17 and July 29, 2008 (Table 5), without an associated increase in ruminant or human-associated MST markers. In all four cases, nutrient concentrations were similar to those in other samples. Wastewater compounds were analyzed for 3 of the 4 samples – two human-associated chemicals (caffeine and skatole) and two wastewater chemicals (chloroethyl phosphate and chloroisopropyl phosphate) were detected, albeit

at relatively low concentrations (below the median of all observed and estimated concentrations). Similarly, Ruxton Creek had unusually high *E. coli* density on July 15 (Table 5) without increased host-associated MST marker, nutrient, or human- or wastewater-associated organic chemical concentrations.

In this report, a novel approach that numerically relates *E. coli* density and MST marker concentration in reference feces was used to estimate the upper in-stream threshold of *E. coli* that one could reasonably expect from a source. In-depth evaluation of the integrated data set was done as a final evaluation of the upper threshold limit approach to exclude potential sources of FIB to stream water. Overall, though human and ruminant sources could explain observed levels of FIB primarily during the cool season an unexpected source was indicated during the warm season, when *E. coli* densities are chronically elevated. Similar observations of seasonality to fecal contamination sources have been made in other studies of small and medium-sized streams by use of other semi-quantitative MST methods (Vogel et al., 2007; Wijesinghe et al., 2009).

By a process of elimination, the unexpected source of warm-season fecal contamination to the Fountain Creek watershed could be detected. Bird (two samples of composted fecal material, Table 2) was not excluded as the source of *E. coli* to the stream, based on absence of host-associated marker and relatively low observed concentrations of general marker relative to *E. coli*. Prior studies also have shown that bird feces has a low ratio of MST marker per *E. coli* (Haack et al., 2003, Stoeckel unpublished data) though no prior study indicated that birds may be a primary source of fecal contamination against land-use patterns that initially pointed to other sources. In this study, no substantial roosting by migratory birds was observed in the watershed and the only birds observed in substantial density were pigeons, associated with bridges and overpasses at Manitou Springs. Limited available information (Al-Harbi, 2003; Haack et al., 2003) indicates that the density of *E. coli* in pigeon feces in the summer time is between 10^6 and 10^{10} MPN/g wet weight. In this study, *E. coli* in pigeon feces was measured to be 3.4×10^8 MPN/g dry wt so a representative range was taken to be 10^8 to 10^9 MPN/g dry wt. During the warm season, typical streamflow in Manitou Springs was 13 CFS. If a pigeon defecates at a rate of 25–50 g wet wt fecal material per day, at 30–40% dry weight (typical of bird fecal material; Table 2) from a roost directly over water (in other words, 100% transfer efficiency) then the combined defecation of between 16 and 420 pigeons, distributed over 24 hours, would generate a sustained in-stream *E. coli* density increase of 1000 MPN/100 mL. It is, thereby, feasible that pigeons contribute significantly to warm season increases in FIB density in the study area.

5. Conclusions

1. Host-associated markers were not specific to host
   a. Concentrations of human-associated marker were highest in human-source waste, but frequently were present in ruminant and pet feces as well as other sources.
   b. Concentration of ruminant-associated marker was highest in ruminant-source waste, but consistently was present in pets and human sources. The ruminant-associated marker was present at low level in duck and bear feces, but not detected in horse feces.
   c. The general marker (AllBac) was differentially distributed among hosts, with the lowest concentrations in a bird feces sample.
2. Use of calculated *E. coli* upper threshold limits, the maximum concentration of *E. coli* expected from a source based on the pattern of MST marker concentrations, was validated through the use of blind-analyzed positive-control suspensions of reference feces and supported by multiple lines of evidence collected in this study.
3. In the study area, *E. coli* densities were lower during cool season (September 16–June 14) than warm season (June 15–September 15). Only minor differences were observed in physical and chemical characteristics of the stream, except for a seasonal difference in temperature.
4. Levels of *E. coli* detected during cool season, which rarely exceeded the criterion of 126 MPN/100 mL, consistently could be explained by upper threshold limit *E. coli* contributions based on measured concentrations of human- and ruminant-associated MST markers. Because of incomplete specificity, pets and birds could not be excluded as alternate contamination sources during this season.
5. Levels of *E. coli* detected during warm season, which routinely exceeded the criterion of 126 MPN/100 mL at all but the uppermost site on Fountain Creek, did not fall within the human or ruminant-origin upper threshold limits. Because of observed cross-carriage of human- and ruminant-associated markers, pets also were excluded as sources of observed FIB in most samples during warm season.
6. On-site observations and calculations using literature-derived data indicated that birds, particularly pigeons, could not be excluded as sources of chronic FIB contamination. This source would not have been identified except through application of the novel upper threshold of *E. coli* contamination calculation, and would have been less credible without the supporting lines of evidence collected.

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