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Presumptive Sources of Fecal Contamination in Four Tributaries to the New River Gorge National River, West Virginia, 2004

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Contents

Figures

Tables

Conversion Factors, Abbreviations, and Datums

Inch/Pound to SI

Multiply	By	To obtain
	Length	
foot (ft)	0.3048	Meter (m)
mile (mi)	1.609	Kilometer (km)
	Area	
square mile (mi^2)	259.0	Hectare (ha)
square mile (mi^2)	2.590	square kilometer (km^2)
	Volume	
ounce, fluid (fl. Oz)	0.02957	liter (L)
pint (pt)	0.4732	liter (L)
quart (qt)	0.9464	liter (L)
gallon (gal)	3.785	liter (L)
	Flow rate	
cubic foot per second (ft^3/s)	0.02832	cubic meter per second (m^3/s)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:
°F=(1.8× °C)+32
Temperature in degrees Fahrenheit (°F) may be converted to degrees Celsius (°C) as follows:
°C=(°F-32)/1.8
Specific conductance is given in microsiemens per centimeter at 25 degrees Celsius (µS/cm at 25 °C).
Concentrations of chemical constituents in water are given either in milligrams per liter (mg/L) or micrograms per liter (µg/L).
Vertical coordinate information is referenced to the National Geodetic Vertical Datum of 1929 (NGVD 29).
Horizontal coordinate information is referenced to the North American Datum of 1983 (NAD 83).
Altitude, as used in this report, refers to distance above the vertical datum.

Presumptive Sources of Fecal Contamination in Four Tributaries to the New River Gorge National River, West Virginia, 2004

By Melvin V. Mathes[1], Tara L. O'Brien[2], Kriston M. Strickler[3], Joshua J. Hardy[3], William B. Schill[1], Jerzy Lukasik[4], Troy M. Scott[4], David E. Bailey[2], and Terry L. Fenger[2]

Abstract

Several methods were used to determine the sources of fecal contamination in water samples collected during September and October 2004 from four tributaries to the New River Gorge National River -- Arbuckle Creek, Dunloup Creek, Keeney Creek, and Wolf Creek. All four tributaries historically have had elevated levels of fecal coliform bacteria. The source-tracking methods used yielded various results, possibly because one or more methods failed. Sourcing methods used in this study included the detection of several human-specific and animal-specific biological or molecular markers, and library-dependent pulsed-field gel electrophoresis analysis that attempted to associate *Escherichia coli* bacteria obtained from water samples with animal sources by matching DNA-fragment banding patterns. Evaluation of the results of quality-control analysis indicated that pulsed-field gel electrophoresis analysis was unable to identify known-source bacteria isolates. Increasing the size of the known-source library did not improve the results for quality-control samples. A number of emerging methods, using markers in *Enterococcus*, human urine, *Bacteroidetes*, and host mitochondrial DNA, demonstrated some potential in associating fecal contamination with human or animal sources in a limited analysis of quality-control samples. All four of the human-specific markers were detected in water samples from Keeney Creek, a watershed with no centralized municipal wastewater-treatment facilities, thus indicating human sources of fecal contamination. The human-specific *Bacteroidetes* and host mitochondrial DNA markers were detected in water samples from Dunloup Creek, Wolf Creek, and to a lesser degree Arbuckle Creek. Results of analysis for wastewater compounds indicate that the September 27 sample from Arbuckle Creek contained numerous human tracer compounds likely from sewage. Dog, horse, chicken, and pig host mitochondrial DNA were detected in some of the water samples with the exception of the October 5 sample from Dunloup Creek. Cow, white-tailed deer, and Canada goose DNA were not detected in any of the samples collected from the four tributaries, despite the presence of these animals in the watersheds. Future studies with more rigorous quality-control analyses are needed to investigate the potential applicability and use of these emerging methods. Because many of the detections for the various methods could vary over time and with flow conditions, repeated sampling during both base flow and storm events would be necessary to more definitively determine the sources of fecal contamination for each watershed.

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[2] Marshall University
[3] West Virginia Department of Agriculture
[4] Biological Consulting Services of North Florida, Inc.

Introduction

More than 200,000 visitors annually raft the whitewater rapids of the New River within the confines of the New River Gorge National River in West Virginia (Jesse Purvis, National Park Service, Glen Jean, W.Va., oral commun., 1999). Visitors also enjoy swimming, kayaking, canoeing, and fishing in the river. Many of these visitors come into direct contact with the waters of the New River during these activities.

Concentrations of fecal coliform bacteria have been monitored by the National Park Service and the West Virginia Department of Environmental Protection since 1985 at several mainstem sites and tributaries to the New River. During these monitoring activities, fairly high concentrations of fecal coliform bacteria—with some concentrations in excess of 100,000 colonies per 100 milliliters (mL) of water—were documented in many tributary inflows to the New River (Purvis and others, 2002; National Park Service, 1996). The standard set by the State of West Virginia for waters suitable for primary contact recreation is a concentration of fecal coliform bacteria colonies not to exceed 200 colonies per 100 mL of sample, based on a geometric mean of five samples per month. Alternatively, fecal coliform bacteria concentrations should not exceed 400 colonies per 100 mL of sample in more than 10 percent of the samples collected within one month (West Virginia Water Resources Board, 1998). The presence of high concentrations of fecal coliform bacteria indicates the possible presence of associated pathogens including bacteria such as *Shigella* and *Salmonella*, protozoans such as *Cryptosporidium* and *Giardia lamblia*, and more than 100 viruses associated with human feces (Britton and Greeson, 1988). All of these pathogens can be transmitted to humans through ingestion of contaminated water, and some can be transmitted by contact with contaminated water (Benenson, 1995). The presence of *Giardia lamblia* in Dunloup Creek, the tributary to the New River that flows past park headquarters for New River Gorge National River, has been reported previously (Messinger, 2002).

Suspected sources of the fecal bacteria found in tributaries to the New River Gorge National River include, but are not limited, to sewage from straight pipes from communities with no sewage treatment facilities; partially treated sewage from communities with insufficient sewage treatment, especially during storms with excessive amounts of surface runoff; leaking sewer lines; faulty septic systems; and runoff from farmlands, pasturelands, and forested areas. Identification of the sources of the high bacteria concentrations found in these tributaries could aid the National Park Service in findings ways to improve the water quality of the tributaries to the New River Gorge area in order to protect park visitors from waterborne diseases.

Bacterial contamination sources commonly are nonpoint sources that are difficult to track and identify. Management of nonpoint sources is one way to control and reduce contaminant loads to streams. Microbial source tracking and other source tracking methods (hereafter referred to as sourcing) are tools that can be used to help identify nonpoint sources of bacterial contamination (Stoeckel, 2005). Sourcing methods were selected for a study by the U.S. Geological Survey, in cooperation with the National Park Service, to attempt to determine, for the time of sample collection, sources of contamination for four tributaries –Arbuckle Creek, Dunloup Creek, Keeney Creek, and Wolf Creek.

In a previous study, Stoeckel and others (2004) compared the ability of selected microbial source tracking methods to associate a challenge set (blinded with respect to animal source) of *Escherichia coli* (*E. coli*) bacteria isolates to their correct animal sources. Sourcing methods compared included pulsed-field gel electrophoresis (PFGE) of macrorestriction digests using a *Not*I-based protocol, ribotyping using the restriction enzyme *Hin*dIII, ribotyping using the restriction enzymes *Eco*RI and *Pvu*II, repetitive-element DNA polymerase chain reaction (PCR) with REP primers, repetitive-element DNA PCR with BOX primers, carbon utilization profiling

using Biolog GN-2 microplates, and antibiotic resistance analysis (ARA). PFGE was the only method able to correctly classify 100 percent of a set of blinded replicate *E. coli* isolates (isolates pulled from the known-source library) into the correct species-level categories (animal sources) that included human (*Homo sapiens*), dog (*Canis familiaris*), white-tailed deer (*Odocoileus virginianus*), Canada goose (*Branta canadensis*), cow (*Bos taurus*), swine (*Sus scrofa*), horse (*Equus caballus*), and chicken (*Gallus gallus*). For this reason and because of the existence of large local source libraries of additional PFGE bacteria isolates, PFGE was selected for inclusion as a sourcing tool for this study. Several emerging sourcing methods were included in the study. These methods included a human-host associated molecular marker found in *Enterococcus faecium* (Scott and others, 2005); a urine-carried virus marker associated with humans (McQuaig and others, 2006); a general fecal *Bacteroidetes* marker (Dick and Field, 2004); a human-associated *Bacteroidetes* marker (Seurinck and others, 2005); species-specific host mitochondrial DNA markers independently derived but similar to those from Martellini and others (2005) for humans and several animals including dog, cow, chicken, pig, horse, white-tailed deer, and Canada goose; and analysis for wastewater compounds such as caffeine (Zaugg and others, 2002).

Purpose and Scope

This report identifies and describes the inferred sources of fecal contamination at the time of sample collection, September to October 2004, in four tributaries to the New River Gorge National River in West Virginia, as determined by the use of several microbial and related sourcing methods. To accomplish this task, 396 fecal samples from humans, Canada geese, white-tailed deer, dogs, cows, horses, chickens, coyotes, and turkeys were collected, and two sets of water samples were collected from each of four tributary streams - - Arbuckle Creek, Dunloup Creek, Keeney Creek, and Wolf Creek. This report also (1) describes the effect of increased source-library size on the ability of library-dependent methods such as PFGE to associate fecal bacteria with the animal source and (2) describes the potential for several emerging library-independent sourcing methods to differentiate between human and nonhuman fecal contamination sources.

Description of Study Area

The study area consists of several tributary watersheds (fig. 1) that flow to the New River Gorge National River. Four of these tributaries, Arbuckle Creek, Dunloup Creek, Keeney Creek, and Wolf Creek, were chosen in consultation with National Park Service scientists from among watersheds that had been monitored for fecal bacteria. These tributaries were chosen on the basis of accessibility, location near high-usage areas of the river, the availability of several years of water-quality and microbiological data, and documented repeated or periodic fecal contamination. The New River Gorge, into which these four tributaries flow, constricts the river channel of the New River, causing it to be narrower, deeper, and steeper in the gorge than at other points along the river. This produces the world-class whitewater rafting for which this reach of the gorge is noted. The gorge walls rise to a maximum of 1,000 feet (ft) above the river channel, and the channel narrows from 1,000 ft upstream from the gorge to a minimum of 200 ft inside the gorge (Wilson and Purvis, 2003). The land in the study area and within the four selected watersheds is predominantly forest covered (90 percent). About 5 percent of the area consists of agricultural pastures, and only about 2 percent of the area consists of developed lands (Purvis and others, 2002).

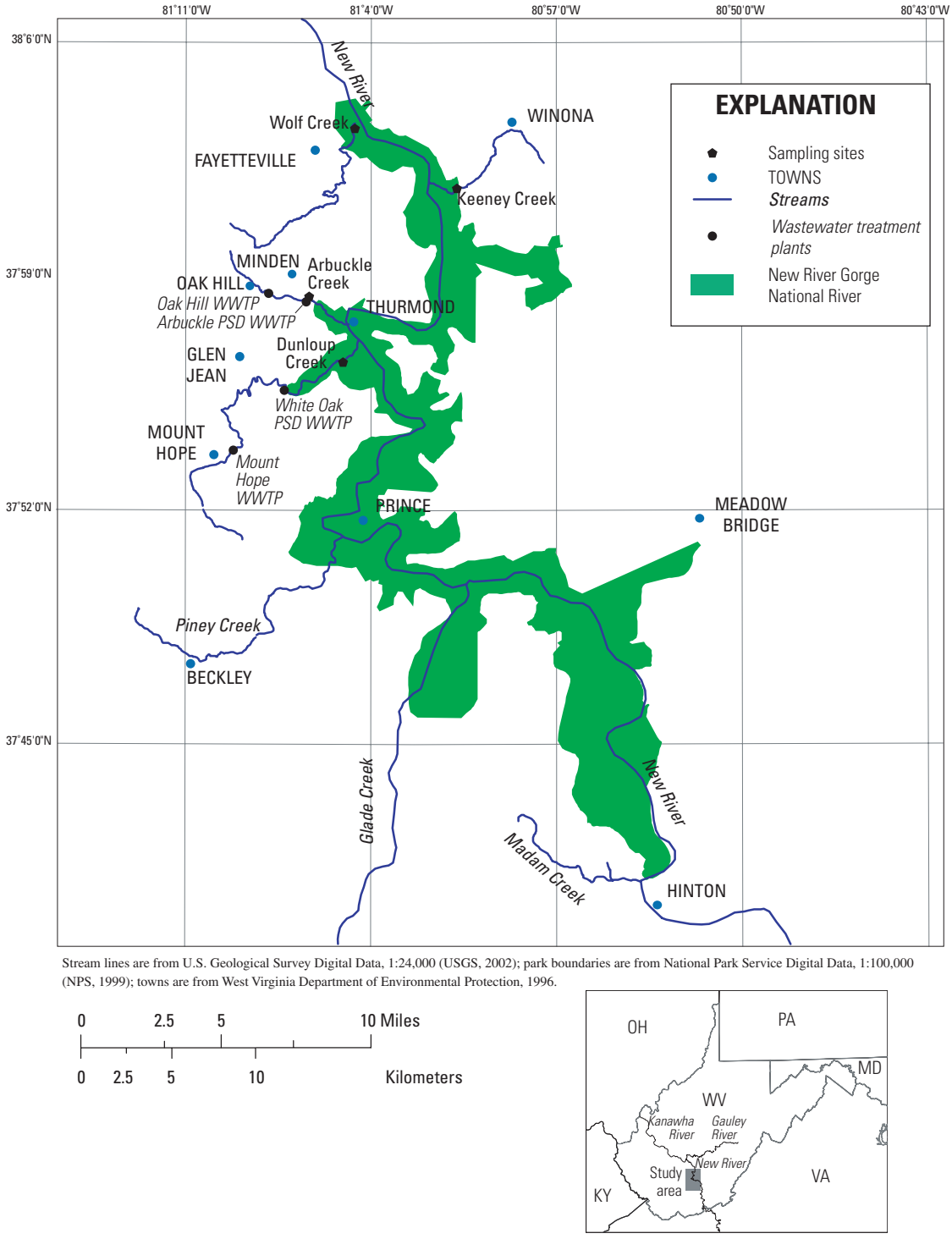

Figure 1. Location of the New River Gorge National River, West Virginia, with park area, towns, tributaries, and sampling sites.

The New River and its tributaries in the study area lie entirely within the Appalachian Plateaus Physiographic Province (Fenneman and Johnson, 1946). Rock units in the study area consist primarily of interbedded sandstone, shale, clay, and coal layers of Pennsylvanian age that

are flat lying or gently folded. The Kanawha and New River Formations are the major geologic units that outcrop in the four selected tributary watersheds (Purvis and others, 2002). Most of the sedimentary rock beds in the area are fractured in a manner consistent with stress-relief fracturing as described in Wyrick and Borchers (1981). Topography and stress-relief fracturing limit ground-water recharge of rivers and streams to the immediate area adjacent to the stream or river extending from the ridge on one valley side to the opposite ridge; therefore, each river gorge and tributary valley acts as a distinct recharge unit. Thus the tributary inflows to the New River are the major pathways for input of fecal contamination to the New River in the gorge.

Arbuckle Creek is a small tributary that drains 8.7 square miles (mi^2) and flows southeasterly through the communities of Oak Hill and Minden and into the western side of the New River at the town of Thurmond, 25.1 miles (mi) (Mathes and others, 1982) upstream from the confluence of the New and Gauley Rivers. Concentrations of fecal coliform bacteria in 12 water samples collected over a wide range of streamflow in Arbuckle Creek by the National Park Service from 1998 to 2000 ranged from 30 to 250,000 colonies per 100 mL of streamwater with a median concentration of 500 colonies per 100 mL (Wilson and Purvis, 2003). The Oak Hill and Arbuckle Public Service District wastewater-treatment plants discharge untreated or partially treated wastewater into Arbuckle Creek, and lift stations in the watershed overflow even during dry periods, according to Wilson and Purvis (2003).

Dunloup Creek drains 48.5 mi^2 and flows northeasterly through the communities of Mount Hope and Glen Jean and into the western side of the New River just upstream from the town of Thurmond, and 25.8 mi (Mathes and others, 1982) upstream from the confluence of the New and Gauley Rivers. Concentrations of fecal coliform bacteria in 13 water samples collected over a wide range of streamflow in Dunloup Creek by the National Park Service from 1998 to 2000 ranged from 13 to 1,600 colonies of bacteria per 100 mL of streamwater with a median concentration of 170 colonies per 100 mL (Wilson and Purvis, 2003). The Mount Hope sewage treatment plant lift station and the White Oak Public Service District wastewater-treatment plant are subject to overflows during storms and may discharge partially treated wastewater to Dunloup Creek, according to Wilson and Purvis (2003).

Keeney Creek drains 8.9 mi^2 and rises southeast of the community of Winona and flows to the west through the community of Winona and into the eastern side of the New River 16.0 mi (Mathes and others, 1982) upstream from the confluence of the New and Gauley Rivers. Concentrations of fecal coliform bacteria in 11 water samples collected over a wide range of streamflow in Keeney Creek by the National Park Service from 1998 to 2000 ranged from 430 to 9,100 bacteria colonies per 100 mL of streamwater with a median concentration of 3,000 colonies per 100 mL (Wilson and Purvis, 2003). The communities in the watershed do not have any centralized municipal wastewater-treatment facilities, according to Wilson and Purvis (2003).

Wolf Creek drains 17.4 mi^2 and flows northerly into the western side of the New River just upstream from the New River Gorge Bridge and 12.0 mi (Mathes and others, 1982) upstream from the confluence of the New and Gauley Rivers. Concentrations of fecal coliform bacteria in 11 water samples collected over a wide range of streamflow in Wolf Creek by the National Park Service from 1998 to 2000 ranged from 1 to 2,600 colonies per 100 mL of streamwater with a median concentration of 28 colonies per 100 mL, according to Wilson and Purvis (2003). Wilson and Purvis (2003) list houses without sewer or adequate septic systems, a malfunctioning sewer collection system, pastureland, and an overloaded sewer system lift station on a minor tributary as potential sources of bacteria for Wolf Creek.

Sample Collection

Fecal samples from animal and human sources were collected from the New River Gorge and nearby locations and were used to build a regional library of known-source *E. coli* bacteria isolates for use in the analysis by PFGE. Water samples for PFGE analysis were collected from the four selected tributaries to the New River Gorge National River for the enumeration of fecal coliform bacteria. Water samples also were examined for the presence of genetic markers associated with fecal sources and for the presence of a suite of wastewater compounds to aid in the determination of sources of fecal contamination. Quality-control samples for the library-dependent method PFGE were generated by cultivating *E. coli* bacteria from fecal samples. Quality-control samples for library-independent methods were generated by spiking sterile phosphate buffered saline (PBS) water with trace amounts of feces. Quality-control samples were submitted blind to the analytical laboratories.

Fecal Samples

A total of 396 fecal samples were collected from November 2004 to July 2005 and were transported to Marshall University (Huntington, W.Va.) for cultivation of *E. coli* bacteria isolates that were compiled into a library of 579 known-source *E. coli*. Fecal samples were collected either by use of sterile toothpicks and stored in sterile plastic vials, or by use of Starswabs II bacteriology culture collection and transport system. Samples collected in sterile vials were chilled upon collection and during transport to the University laboratory. Fecal samples collected using Starswab II were stored at room temperature after collection and during transport. White-tailed deer, Canada goose, dog, cow, horse, cat, coyote, and wild turkey fecal samples were collected from field locations in the four tributary watersheds, in the New River Gorge, and in locations close to the tributaries and New River. Human-origin samples were solicited from volunteers in these same geographical areas and from New River Gorge National River employees, most of whom lived in or near the study area. Fecal samples were streaked onto eosin methylene blue (EMB) media at the Marshall University laboratory to isolate *E. coli*. Attempts were made to isolate three *E. coli* isolates from each fecal sample that exhibited *E. coli* growth. The number of fecal samples collected and the number of isolates cultured and added to the known-source library for each animal category are listed in table 1.

Water Samples

Water samples were collected twice in 2004 from Arbuckle Creek (September 27 and October 6), Dunloup Creek (September 22 and October 5), Keeney Creek (September 23 and 30), and Wolf Creek (September 29 and October 12). Water samples were collected for each site visit as follows: one 2-liter (L) sterile bottle for analysis for *Bacteroidetes*, for host DNA, and for other tests at the USGS Leetown Science Center (Leetown, W. Va.); two 1-L sterile bottles for analysis for human host-specific *Enterococcus* marker and human-urine marker by Biological Consulting Services of North Florida, Inc.(Gainesville, Fla.); one 1-L bottle for determination of wastewater compounds by the USGS National Water-Quality Laboratory (Denver, Colo.), and one or more 250-mL sterile bottles for membrane filtration of fecal coliform and fecal streptococcus bacteria onsite. All water samples were chilled and shipped by overnight mail. Measurements of

Table 1. Description of the 579-known-source *Escherichia coli* library with animal sources of fecal samples collected from the vicinity of the New River Gorge National River, West Virginia, 2004.

Animal source of fecal samples	Number of fecal samplescollected	Number of fecal samples with *E. coli* growth	Number of fecal samples from which usable data were collected	Number of *E. coli* isolates added to source library
Canada goose	179	59	57	160
White-tailed deer	70	43	43	126
Dog	51	37	36	103
Human	41	39	36	103
Cat	17	13	13	34
Cow	13	13	8	23
Horse	20	7	7	21
Coyote	3	3	3	9
Wild turkey	2	0	0	0
Totals	396	214	203	579

streamflow, water temperature, pH, specific conductance, and dissolved oxygen were made at each site (table 2). Water samples were collected by depth-width integrated methods into sterile bottles according to USGS protocols outlined in the National Field Manual (Wilde and others, 1999).

Water samples were analyzed in the field for fecal-indicator bacteria using a membrane filtration technique followed by cultivation on fecal coliform media (mFC) and fecal streptococcus media (KF) (Myers and Wilde, 1997) for fecal coliform and fecal streptococci analysis (table 2), respectively. Several different volumes of sample were filtered and plated at each site. For quality-control purposes, an equipment blank and a procedure blank were prepared by filtering sterile buffered water before and after each set of water samples. No colonies were observed to grow on any of the quality-control blanks. After incubation, the plates containing fecal streptococcus colonies were counted and destroyed. The plates containing fecal coliforms were counted, and the plates were chilled and shipped by overnight mail to the West Virginia State Agriculture Laboratory (Moorefield, W. Va.) for culturing and confirmation of *E. coli* bacteria isolates for analysis by PFGE.

Total coliforms, enterococci, and *E. coli* were assayed by standard filtration methodology for all water and quality-control samples received by the USGS Leetown Science Center laboratory. Total coliforms and *E. coli* were enumerated from growth on m-ColiBlue24, and enterococci were enumerated from growth on m-*Enterococcus* agar. Digital photographs of plates were made, and counts (table 2) were made using image analysis techniques.

The four sampled streams were generally well mixed and easily wadeable. Stream mixing was verified by water-quality cross-sectional measurements of water temperature, pH, specific conductance, and dissolved oxygen where possible. Values for these water-quality indicators varied only slightly across the width of the stream channel for Dunloup Creek, Wolf Creek, and Arbuckle Creek. Depths were too shallow in Keeney Creek to obtain water-quality cross-sectional data during both sampling visits, but conditions at this site indicated good mixing.

Quality-Control Samples

Quality-control samples were generated to challenge analyses by PFGE, for the human host-specific *Enterococcus* marker, for the human-urine marker, and for host DNA markers. A stock solution of sterile PBS water was prepared by adding one phosphate buffered saline tablet to each

Table 2. Water-quality characteristics of, and bacteria counts in, eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004

[ft^3/s, cubic feet per second; mm, millimeters; mg/L, milligrams per liter; µS/cm, microsiemens per centimeter; °C, degrees Celsius; mL, milliliters; E, estimated]

Sampling site	Sampling date	Flow (ft^3/s)	Air pressure, field (mm of mercury)	Dissolved oxygen (mg/L)	Dissolved oxygen (percent of saturation)	pH, field (standard units)	Specific conductance, field (µS/cm @25C)
Arbuckle Creek	09-27-04	1.4	719	10.1	113	8.0	375
Arbuckle Creek	10-06-04	2.5	728	10.4	105	7.8	368
Dunloup Creek	09-22-04	23	740	9.5	96	8.3	485
Dunloup Creek	10-05-04	22	739	9.7	97	8.3	498
Keeney Creek	09-23-04	1.3	724	9.4	97	7.6	155
Keeney Creek	09-30-04	5.2	720	9.3	97	7.5	122
Wolf Creek	09-29-04	15	736	9.2	97	8.0	274
Wolf Creek	10-12-04	3.0	731	10.3	103	8.6	631

Sampling site	Sampling date	Water temperature (°C)	Fecal coliforms, field (colonies per 100mL)	Fecal streptococcus, field (colonies per 100 mL)	Total coliform (colonies per 100 mL)	*E. coli* (colonies per 100 mL)	*Enterococcus* (colonies per 100 mL)
Arbuckle Creek	09-27-04	17.7	160	290	2,200	24	30
Arbuckle Creek	10-06-04	13.8	150	39	2,400	90	74
Dunloup Creek	09-22-04	14.5	87	36	1,000	14	15
Dunloup Creek	10-05-04	13.7	E17	E7	2,900	41	35
Keeney Creek	09-23-04	14.2	E44	E74	11,000	53	98
Keeney Creek	09-30-04	14.6	280	160	3,900	92	88
Wolf Creek	09-29-04	16.4	3,800	1,900	1,600	44	390
Wolf Creek	10-12-04	13.3	E3	E5	1,100	3	5

200 mL of distilled water and autoclaving the resultant solution at 121 degrees Celsius (°C) for 15 minutes. Small unmeasured quantities of fresh feces from known sources (1-3 individuals per source) were obtained by use of sterile toothpicks and were added to sterile bottles of this stock solution to challenge these sourcing methods. A 1-L sample containing all human feces (designated sample Unknown A) and a 1-L sample containing no human feces (designated sample Unknown B and containing white-tailed deer and Canada goose feces) were shipped for analysis for the human host-specific *Enterococcus* marker and the human-urine marker, and two 2-L samples (Unknowns A and B, same composition as in 1-L bottles) were shipped for analysis for host DNA. Because no bacterial growth resulted from the 2-L sample of Unknown B, a replacement sample (designated Unknown C and containing white-tailed deer and Canada goose feces) was requested by and sent to the lab performing the host DNA analyses. Four additional samples were generated by adding white-tailed deer feces to one bottle of PBS stock solution, Canada goose feces to a second bottle, human feces to a third bottle, and dog feces to a fourth bottle. Fecal coliform bacteria were obtained from these samples by the membrane filtration method (Myers and Wilde, 1997). Selected plates of the fecal coliform bacteria were chilled and shipped by overnight mail to the West Virginia Agriculture Laboratory from which 37 *E. coli* bacteria isolates were cultured, confirmed, and analyzed by PFGE to produce DNA-fragment banding patterns. The DNA-fragment banding patterns for these 37 blinded quality-control isolates

were sent to the Marshall University Laboratory for computer matching with the DNA-fragment banding patterns for the 579 known-source isolates to determine the animal sources of the isolates from the blinded quality-control samples.

Laboratory Methods and Procedures

Several methods were used to determine the source of fecal contamination in New River Gorge National River tributaries. These sourcing methods included analysis by pulsed-field gel electrophoresis (PFGE) and analyses for a human host-specific *Enterococcus* marker, for a human-urine marker, for human-specific and general *Bacteroidetes* markers, for several host DNA markers, and for wastewater compounds. PFGE is a library-dependent method that attempts to associate fecal bacteria from water samples with animal or human sources by matching DNA-fragment banding patterns generated from bacteria in the water samples to DNA-fragment banding patterns generated from fecal bacteria obtained from feces of known animal source. Thus this method depends on the existence of a library of known-source bacteria isolates with DNA-fragment banding patterns. The other methods with the exception of wastewater compounds are library-independent methods that attempt to detect the presence or absence of biological or molecular markers that are specific to human or animal source. Most of these biological markers actually contain genetic sequences of bacteria or viruses that are thought to be specifically associated with humans or animals. An experimental method also was tested. This experimental method is based on directly detecting human or animal DNA that is carried into receiving waters by cells shed from the gut of the source, rather than depending on the indirect association of microbes with hosts. Specific amplification (an increase in the number of copies of a specific DNA fragment) of source-specific sequences by quantitative polymerase chain reaction (qPCR) was used to detect source DNA and to measure its relative concentration.

Pulsed-Field Gel Electrophoresis Method (PFGE)

PFGE of macrorestriction digests using a *Not*I-based protocol was applied to the 579 isolates in the known-source library by researchers at Marshall University and on the 37 isolates from the quality-control samples and the 373 isolates from water samples from the four tributaries to the New River Gorge National River by researchers at the West Virginia Agriculture Laboratory. This PFGE protocol generates DNA-fragment banding patterns that result from whole-genome digestion and that are based on variability anywhere in the genome (Stoeckel and others, 2004). PFGE generally is considered to be an extremely sensitive method of detecting genetic differences between strains (Myoda and others, 2003; Tenover and others, 1997). The protocol for PFGE applied in this study uses the same restriction enzyme as that used by Simmons and others (2000). DNA extraction and digestion by PFGE were performed by streaking *E. coli* bacteria isolates from the 579-isolate known-source library, the 37 quality-control isolates, and the 373 water-sample isolates for confluent growth by a method similar to that reported in Stoeckel and others (2004). The DNA-fragment banding patterns generated for bacteria cultured from water samples were matched to DNA-fragment banding patterns generated from bacteria isolates cultured from known sources of feces in an attempt to associate bacteria in water samples with their correct animal or human source.

Human Host-Specific *Enterococcus* Marker Method

Scott and others (2005) proposed the use of a molecular marker in *Enterococcus faecium* to identify human sources of contamination in the environment. The human fecal contamination

marker that they used targets a putative *Enterococcus* virulence factor, the enterococcal surface protein (*esp*). A validation study conducted by Scott and others (2005) detected this gene in 97 percent of sewage and septic samples analyzed, but did not detect the gene in samples collected from livestock waste lagoons, birds, or from other nonhuman fecal samples.

Water samples were analyzed using this method by isolating enterococci from the samples by membrane filtration and by performing PCR on composite DNA samples from the cultivated enterococci. Filters were incubated for 18 to 24 hours (hr) on mE agar (Difco) supplemented with indoxyl substrate (Sigma, Inc.). The filters are next lifted, suspended in azide dextrose broth (Difco), vortexed vigorously, and incubated for 3 hr at 41°C to wash bacteria from the filters and enrich the bacteria. DNA extraction is performed on the resulting culture by use of a QIAamp DNA extraction kit (Qaigen, Inc.). Primers specific to the *esp* gene in *Enterococcus faecium* were developed after DNA sequence alignment of the two available variants of the gene (GenBank Accession Numbers AF444000 and AF443999). PCR was performed in a 50-microliter (µL) reaction mixture containing 1X PCR buffer, 1.5 millimolar (mM) magnesium chloride, 200 micromolar (µM) of each of the four deoxyribonucleotides, 0.3 µM of each primer, 2.5 units (U) of HotStarTaq DNA polymerase (Qiagen), and 5µL of template DNA. Amplification was performed initially at 95°C for 15 minutes to activate Taq polymerase, followed by 35 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. PCR products were separated on a 1.5 percent agarose gel stained with GelStar nucleic acid stain (BioWhittaker) and were viewed under ultraviolet light. The resultant PCR product is 680 base pairs in length. Positive and negative controls were run with each set of reactions. The *Enterococcus faeciu*m strain C68, which contains the *esp* gene, was used as the positive control, and sterile water was used as a negative control. All DNA extracts were spiked with positive-control DNA to assess whether samples contained PCR inhibitors. Samples in which the positive control failed to amplify were reprocessed under more stringent conditions designed to remove PCR inhibitors and were reanalyzed by PCR.

Human-Urine Marker Method

Researchers in Florida (McQuaig and others, 2006) used a method to concentrate and extract DNA of human polyomaviruses from water samples. Polyomaviruses have a demonstrated prevalence in the worldwide human population (Del Valle and others, 2004). Serological studies have shown that 60 to 90 percent of human adults harbor antibodies against polyomaviruses (Hirsch and Steiger, 2003). These adults can shed as many as one million viral particles of polyomaviruses in 1 mL of urine (Bofill-Mas and others, 2000). Individuals with suppressed immune systems and individuals with healthy immune systems can shed these viruses (Bofill-Mas and others, 2000; Behzad-Behbahani and others, 2004). The human-urine marker uses primers specific to the conserved t-antigen of the JC virus and BK virus, both of which are polyomaviruses, to detect the polyomaviruses in a nested PCR. The method can detect as little as one µL of raw sewage (which contains urine in addition to fecal material and other constituents) added to 100 mL of water (McQuaig and others, 2006).

Bacteroidetes Method

Water samples collected from the four tributaries were analyzed at the USGS Leetown Science Center to detect potential fecal contamination and its nature. To accomplish this, the methods developed by Dick and Field (2004) and Seurinck and others (2005) were employed to detect both general and human-specific *Bacteroidetes* markers. SYBR Green detection was used to

detect the general *Bacteroidetes* marker rather than the 5-prime nuclease (TaqMan) detection method originally described.

Water samples (1,000 mL) were filtered (47 mm, 0.45 micron cellulose acetate) through sterile filtration devices to collect and concentrate bacteria and particulate-bound DNA. Sterilization and decontamination of filtration setups was accomplished by soaking the apparatus (without filters) in 10 percent household bleach for 30 minutes, rinsing thoroughly with sterile distilled water, and autoclaving for 20 minutes. Just before use and after installation of the membrane filter, the device was irradiated with ultraviolet light to further ensure against laboratory contamination. For quality control, 100 mL of distilled water was passed through the filter apparatus to detect any possible laboratory contamination from filter units and extraction/purification reagents. Filters were frozen for later processing. DNA from bacteria and particulates retained by filtration were obtained from the frozen filters using a Water Kit (MoBio, Carlsbad, Calif.) and following the manufacturer's instructions. The filtered water was processed to concentrate and purify dissolved DNA (see "Host DNA Markers Method").

All purified and concentrated DNA samples were tested for possible matrix interferences by performing PCR on samples composed of 50 percent purified sample DNA and 50 percent known, unrelated DNA, then verifying that amplification kinetics were identical to those of the controls containing 50 percent known, unrelated DNA and 50 percent distilled water.

Purified DNA was tested for the presence of general and human-specific *Bacteroidetes* markers using a SYBR Green Jumpstart for Quantitive PCR kit (Sigma, Chicago, Ill.). Reactions (25 μL) were conducted in a Corbett RotorGene 3000 thermocycler (Corbett Life Sciences, Sydney, Australia) using thermocycling conditions as originally described. Data analysis was performed using the instrument-associated software. Because no absolute standards were available, quality-control sample Unknown A (human fecal sample) was used as a surrogate after being determined to be a human or primarily human fecal sample by host DNA analysis. Decimal dilutions (dilutions of one part substance to be diluted added to nine parts water) of Unknown A were used to generate standard curves and determine relative concentrations of general and human-specific *Bacteroidetes* markers in the water samples collected from the four tributaries.

Host DNA Markers Method

The eight water samples collected from the four tributaries to the New River Gorge National River were tested for the presence of mitochondrial cytochrome b sequences in both filter-bound and dissolved fractions. DNA was measured in both fractions separately (rather than as a composite sample) because it is generally thought that sediment-bound DNA is protected somewhat from degradation and may reflect more historic DNA contributions to the environment than the dissolved fraction, whereas DNA in the dissolved fraction is more subject to degradation than filter-bound DNA and, therefore, should have a shorter half-life and be more reflective of recent DNA contributions to the environment. DNA in the dissolved fraction was obtained from the filtrate of the samples and was concentrated and purified according to the method of Matsui and others (2004) with minor modifications. Specifically, 0.25 molar ethylenediaminetetraacetic acid (EDTA) was used to redissolve precipitated DNA complexes and to chelate calcium and magnesium rather than the TE buffer (a combination of Tris and EDTA) used in the original procedure. Recovered DNA was purified further by extraction with a Water DNA Kit (Epicentre, Madison, Wis.) following the manufacturer's instructions and further concentrated by ultrafiltration using an Amicon Ultra-4 30,000 MW spin concentration device (Millipore, Billerica, Mass.).

PCR was used to detect the host DNA sequences, specifically the presence of mitochondrial cytochrome b sequences from human, dog, cow, chicken, horse, white-tailed deer, Canada goose

and pig using recently developed methods. (Primer sequences and cycling parameters are not discussed here). These methods are similar in approach, but developed independently, from that of Martellini and others (2005). Differences that should increase the sensitivity of detection are that targeted sequences were smaller in this study (approximately 100-200 base pairs) as opposed to those published by Martellini (400-934 base pairs). Targeting smaller sequences should yield increased sensitivity in detecting the DNA that is expected to be degraded in the environment. Martellini and others (2005) analyzed for DNA that was retained by the filters or sedimented by centrifugation, whereas in this study, techniques were adapted for concentrating and purifying dissolved DNA as described above. Moreover, this study used real-time PCR that is inherently more sensitive than the gel electrophoresis used in the previous study.

Reactions (25 μL) were conducted using a Corbett RotorGene instrument using SYBR Green detection as described in the section "*Bacteroidetes* Method." Absolute standards for mitochondrial sequences were not available, so human placental DNA (Sigma, Chicago, Ill.) was used as a surrogate. Decimal dilutions of the human DNA were used to generate a standard curve that was, in turn, used to estimate the amounts of human DNA in dissolved and filter-bound fractions.

Wastewater Compounds Procedure

Zaugg and others (2002) developed techniques for the analytical determination of more than 60 wastewater compounds in natural waters by solid-phase extraction and capillary-column gas chromatography and mass spectrometry. These techniques were developed in response to concerns regarding the effect of endocrine-disrupting chemicals in wastewater on aquatic organisms, but the method may be useful in evaluating the effect of combined sanitary and storm-sewer overflow on the water quality of urban streams. The method focuses on the determination of compounds that are indicators of wastewater or are potential endocrine disruptors. Unfiltered water samples were collected in 1-L amber glass bottles for each of the four tributaries and were chilled and shipped by overnight mail to the USGS National Water Quality Laboratory (Denver, Colo.) for these analytical determinations.

Detection of Contaminants, by Analytical Method

PFGE analysis of *E. coli* bacteria isolates cultured from water samples and fecal samples collected in the New River Gorge area, the detection of molecular and biological markers, and wastewater compounds were used to analyze potential sources of fecal contamination in Arbuckle Creek, Dunloup Creek, Keeney Creek, and Wolf Creek tributaries to the New River Gorge National River. Discussion and individual results of each sourcing method follow. Future studies with more rigorous quality-control analyses are needed to investigate the potential applicability and use of these emerging methods.

Field and Laboratory Determinations of Fecal Indicator Bacteria

Bacteria counts (table 2) revealed no particular trends or abnormalities and were generally what might be expected from surface-water samples; however, field and laboratory counts often differed, probably reflecting die-off or regrowth of bacteria during overnight transit. The Wolf Creek sample collected September 29, 2004, was delayed in shipment by the contracted carrier.

Pulsed-Field Gel Electrophoresis

Computers were used to match DNA-fragment banding patterns generated from *E coli* bacteria isolates cultured from water samples to DNA-fragment banding patterns generated from *E. coli* cultured from fecal samples of known animal or human source. A curve-based matching procedure was used by researchers at Marshall University to classify the 37 blinded quality-control isolates and the 373 watershed isolates of unknown source to source categories with the aid of BioNumerics image analysis software using the following parameters: 2.0 optimization, 5.0 percent tolerance, 2.5 percent end of pattern change, and unweighted pair-group method using arithmetic averages (UPGMA) clustering. In the previous microbial sourcing methods comparison study by Stoeckel and others (2004), the same research laboratory assigned patterns that did not match known database patterns with better than 85 percent similarity to a "not identified" category.

Using the above criteria, only 1 of the 37 quality-control isolates was matched against the 579-isolate known-source library to its correct animal source (a white-tailed deer). Similarities of the 37 pattern matches ranged from 66 to 92 percent with only 4 of 37 matches having similarities equal to or greater than the 85 percent identification threshold. Curve-based matching of the same 37 quality-control isolates to an expanded known-source library of 1,266 isolates (an additional 687 isolates from the New River area were collected independently by Marshall University researchers), and a known-source library of 8,043 isolates (all available known-sources isolates from West Virginia) again correctly associated only 1 of 37 isolates to its correct animal source (a white-tailed deer). Although increasing the size of the known-source library increased the average similarity of the matches from 78 percent for the 579-isolate known-source library to 84 percent for the 8,043-isolate known-source library, it did not change the number (one white-tailed deer) of correct animal-source associations.

The use of different selective-growth media in the various steps of culturing of *E coli* bacteria isolates in this study could partially explain the errors in attempted association of quality-control isolates to animal source. Not all *E. coli* isolates will grow on each of the available selective-growth media for *E. coli* (Don Stoeckel, U.S. Geological Survey, written commun., 2006). Thus, it is possible that the subset of *E. coli* quality-control isolates that were originally obtained from fecal coliform colonies grown on mFC media was different from the subset of *E. coli* isolates in the known-source library that were obtained from fecal samples of known source that were streaked onto EMB media for *E. coli* growth. It is also possible that the population of DNA-fragment banding patterns derived from *E. coli* in human and animal feces is too diverse and variable to allow accurate associations of watershed bacteria isolates to animal sources by use of PFGE analysis. Results of curve-based matching of the 373 watershed isolates to known-source libraries are shown (tables 3 and 4) in tabular form, but no interpretations were made from these data because of the failure of the PFGE method in this specific project to identify correctly no more than 1 of 37 quality-control isolates. Curve-based matching of the 373 watershed isolates to the 579-isolate known-source library generated 53 of 373 pattern matches (table 3) with a similarity of 85 percent or greater. Sources represented in this library include human, dog, white-tailed deer, Canada goose, cow, horse, cat, and coyote (table1). Curve-based matching of the 373 watershed isolates to an expanded library of all 8,043 available *E. coli* known-source isolates for West Virginia generated 167 of 373 pattern matches (table 4) with similarities equal to or greater than 85 percent. This expanded known-source library includes the 579 isolates in this project; 687 isolates collected independently in the New River area by Marshall University; 1,872 isolates collected in Berkeley County, West Virginia, to include the isolates collected by Stoeckel and others (2004); and 1,902 isolates collected in the Huntington, West Virginia, area. Available animal sources to be

matched in the expanded library included those for the 579-isolate library plus swine, chicken, and raccoon.

Human Host-Specific *Enterococcus* Marker

Two water samples were collected in sterile bottles from each of the four tributaries to the New River Gorge National River and were submitted for detection analysis for the human molecular marker in *Enterococcus faecium*. Two quality-control samples, each containing minute amounts of feces in a sterile PBS solution, also were submitted for detection. Quality-control sample Unknown A contained strictly human feces, and quality-control sample Unknown B contained Canada goose and white-tailed deer feces. The method correctly detected the human marker in sample Unknown A and did not detect the marker in sample Unknown B. The human marker was detected in one of the two water samples from Keeney Creek (September 23), but was not detected in any of the water samples from Arbuckle Creek, Dunloup Creek, or Wolf Creek. The number of enterococci colonies exceeded 200 and were too numerous to count in the enrichment samples for both quality-control samples and for all water samples collected from the four tributary streams, thus reducing the chance of false negative results for the marker in any of the analyzed samples.

Human-Urine Marker

Two water samples were collected in sterile bottles from each of the four tributaries to the New River and were submitted for detection analysis for the human-urine marker for the JC and BK polyomaviruses. Two negative quality-control samples, each containing minute amounts of feces in a sterile PBS solution, also were submitted for detection. No positive-control samples were submitted. Quality-control sample Unknown A contained human feces, but no human urine, and quality-control sample Unknown B contained Canada goose and white-tailed deer feces, but no human urine. The method correctly did not detect the human-urine marker in sample Unknown A and sample Unknown B. The human-urine marker was detected in both of the water samples from Keeney Creek, but was not detected in any of the water samples from Arbuckle Creek, Dunloup Creek, or Wolf Creek.

Bacteroidetes

PCR was used to detect bacterial sequences for *Bacteroidetes* markers. The two markers detected are a fecal *Bacteroidetes* marker for general contamination (Dick and Field, 2004) and a human-specific *Bacteroidetes* marker (Seurinck and others, 2005). Relative fluorescence of the SYBR Green I dye as obtained from real-time PCR analysis for each marker indicated the presence of the marker and was used to calculate the concentrations of these markers (figs. 2 and 3) relative to the surrogate standard (quality-control sample Unknown A). Decimal dilutions of quality-control sample Unknown A were used to generate standard curves by determination of the linear regression of the logarithm of standard concentration against Ct (threshold cycle). Ct is defined as the fractional cycle number at which relative fluorescence exceeds a set threshold that is in the exponential part of the sample growth curves and significantly above background noise levels. Equations describing the standard curves for total and human-specific *Bacteroidetes* concentrations were calculated to be Concentration = $10^{(-0.380*Ct + 6.540)}$, $R^2 = 0.99396$ and Concentration = $10^{(-0.312*Ct + 10.819)}$, $R^2 = 0.99025$, respectively. Surprisingly, the distribution patterns of these two markers were similar, although the concentrations were different. The authors observed that in the

Table 3. Summary of 373 *Escherichia coli* isolates from water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004, that were compared with the 579-isolate known-source library

[Data are presented according to sample, followed by totals across all samples. Results for two samples are not shown because no *E. coli* bacteria were isolated from these two samples. Patterns with similarities greater than or equal to 85 percent were accepted. **RESULTS IN THIS TABLE ARE EXPECTED TO BE INCORRECT, BASED ON QUALITY-CONTROL EVALUATION**]

Animal source	Arbuckle Creek sample 1 (9/27/04)	Arbuckle Creek sample 2 (10/6/04)	Dunloup Creek sample 1 (9/22/04)	Dunloup Creek sample 2 (10/5/04)	Keeney Creek sample 2 (9/30/04)	Wolf Creek sample 1 (9/29/04)	Total matches by source
Human	0	0	2	3	2	0	7
White-tailed deer	4	1	0	1	2	13	21
Canada goose	0	0	1	1	4	1	7
Dog	1	4	2	0	1	4	12
Cat	2	0	0	0	1	0	3
Coyote	1	0	0	0	0	0	1
Cow	0	1	0	0	0	0	1
Horse	0	0	0	1	0	0	1
Total matches	8	6	5	6	10	18	53
Matches not accepted	39	52	16	45	93	75	320
Total isolate patterns	47	58	21	51	103	93	373

environmental samples, the human-specific marker was measured to be 100 to 1,000 times the general marker. This appears to be an artifact of using quality-control sample Unknown A that was compounded from only three individuals as a surrogate standard. The environmental samples have (presumably) more contributors and thus are more likely to have a higher frequency of the marker. Therefore, the concentration of this marker would be expected to be elevated when compared with samples (including Unknown A) containing feces from low numbers of contributors. Both the general-contamination and the human-specific *Bacteroidetes* markers were detected in all eight water samples, but were comparatively elevated in the Dunloup Creek samples and in the second Keeney Creek sample (September 30). The concentration of the general-contamination marker was elevated (fig. 2) in the first Wolf Creek sample (September 29) in comparison with the second sample (October 12). The human-specific marker was most pronounced (fig. 3) in the Keeney Creek sample of September 30 and to a lesser degree in the Dunloup Creek sample of October 5.

Host DNA Markers

Results of the analysis of quality-control samples using the host DNA method are shown in table 5. Each of the quality-control samples (Unknowns A, B, and C) were examined for each of the nine markers specific to the following animal sources: human, Canada goose, white-tailed deer, cow, pig, chicken, horse, dog, and sheep. Average sensitivity for the three unknowns was 1.000, and average specificity was 0.911. That is, the test correctly identified the presence of host DNAs

Table 4. Summary of 373 *Escherichia coli* isolates from water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004, that were compared with the expanded 8,043-isolate known-source library

[Data are presented according to sample, followed by totals across all samples. Results for two samples are not shown because no *E. coli* bacteria were isolated from these two samples. Patterns with similarities greater than or equal to 85 percent were accepted. **RESULTS IN THIS TABLE ARE EXPECTED TO BE INCORRECT, BASED ONQUALITY-CONTROL EVALUATION.]**

Animal source	Arbuckle Creek sample 1 (9/27/04)	Arbuckle Creek sample 2 (10/6/04)	Dunloup Creek sample 1 (9/22/04)	Dunloup Creek sample 2 (10/5/04)	Keeney Creek sample 2 (9/30/04)	Wolf Creek sample 1 (9/29/04)	Total matches by source
Human	2	0	4	4	7	4	21
White-tailed deer	2	4	2	4	8	17	37
Canada goose	3	5	0	2	11	4	25
Dog	0	4	0	0	0	0	4
Cat	0	0	1	0	0	0	1
Coyote	0	0	0	2	0	0	2
Cow	2	4	0	2	3	7	18
Horse	2	0	0	0	0	0	2
Swine	4	13	2	3	8	1	31
Chicken	4	1	3	2	4	1	15
Raccoon	0	2	0	0	4	5	11
Total matches	19	33	12	19	45	39	167
Matches not accepted	28	25	9	32	58	54	206
Total isolate patterns	47	58	21	51	103	93	373

that were in the quality-control samples 100 percent of the time, but incorrectly identified as present host DNAs that were not in the quality-control samples 8.9 percent of the time. Quality-control sample Unknown A (containing human feces) was identified as human and cow. Quality-control sample Unknown B (containing white-tailed deer and Canada goose feces) was identified as Canada goose, white-tailed deer, and human. Because no bacterial growth was obtained from this sample, a replacement quality-control sample Unknown C (containing white-tailed deer and Canada goose) was requested and was correctly identified as white-tailed deer and Canada goose. Because quality-control samples were not prepared quantitatively, it is impossible to accurately determine the actual mixture composition of the unknown samples.

A standard curve was established using decimal dilutions of human placental DNA similar to those of the *Bacteroidetes* markers previously described; the equation describing this standard curve is Concentration = $10^{(-0.284*Ct + 7.332)}$, $R^2 = 0.99972$. Results of analyses of the eight water samples collected from the four tributaries to the New River indicate detections of human DNA in all eight samples and in relatively high amounts in several of the samples (figs. 4 and 5).

Relative fluorescence of the SYBR Green I dye as obtained from real-time PCR analysis for each marker indicates the presence of these markers and is used to calculate the concentration of these markers relative to the surrogate standard. In the second Keeney Creek sample (September 30), the relative concentration of human DNA was particularly elevated in both the dissolved and filter-bound fractions. In both Wolf Creek samples, the relative concentration of human DNA was

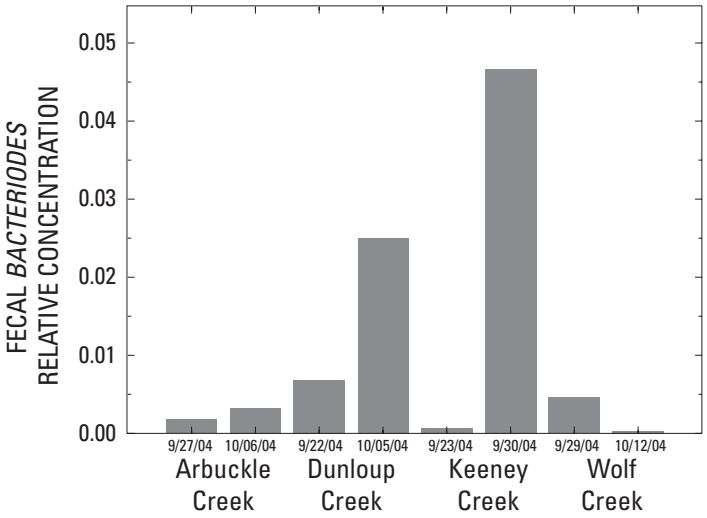

Figure 2. Relative concentrations of fecal *Bacteroidetes* in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004. (From Dick and Field, 2004) (Concentration is relative to quality-control sample Unknown A, where the concentration of this marker in a 1:1000 dilution of the standard equals 1000.)

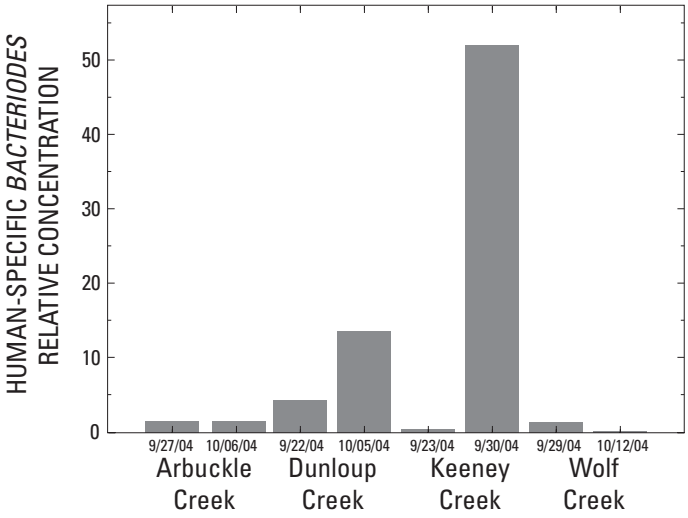

Figure 3. Relative concentrations of human-specific *Bacteroidetes* marker in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004. (From Seurinck and others, 2005) (Concentrations are relative to quality-control sample Unknown A, where the concentration of this marker in a 1:1000 dilution of the standard equals 1000.)

elevated in both fractions. The relative concentration of filter-bound DNA (fig. 4) was slightly higher in the Wolf Creek sample of September 29 than in the Keeney Creek sample of September 30, but the dissolved human DNA (fig. 5) was much higher in the Keeney Creek sample of September 30 than in any other sample. Human DNA was detected in elevated amounts for both filter-bound and dissolved DNA for the second Keeney Creek sample (September 30) and for both Wolf Creek samples, and for the filter-bound DNA for the second Dunloup Creek sample (October 5). Animal DNA for dog, chicken, horse, and pig only (table 6) was detected in smaller amounts than human DNA, but was not quantified. Animal DNA was detected in all eight water samples with the exception of the October 5 sample from Dunloup Creek. Dog DNA was detected in both samples from Arbuckle Creek and in the September 22 sample from Dunloup Creek. Chicken DNA was detected in both samples from Keeney Creek and in the October 12 sample from Wolf Creek. Horse DNA was detected in both samples from Wolf Creek, in the September 23 sample from Keeney Creek, and in the September 22 sample from Dunloup Creek. Pig DNA was detected only in the September 27 sample from Arbuckle Creek. White-tail deer, Canada goose, and cow DNA were not detected in any of the eight water samples from the four tributaries, despite the fact that deer and cows were observed in the study area, and geese were observed near the study area.

Table 5. Host mitochondrial DNA analysis of quality-control samples expressed as sensitivity and specificity

Quality-control Unknown	Sample composition	Species detected	Species not detected	Sensitivity	Specificity
Unknown A	Human	Human	Horse	1.000	0.875
		Cow	Dog		
			White-tailed deer		
			Pig		
			Chicken		
			Sheep		
			Canada goose		
Unknown B	White-tailed deer	White-tailed deer	Horse	1.000	0.857
	Canada goose	Canada goose	Dog		
		Human	Pig		
			Chicken		
			Sheep		
			Cow		
Unknown C	White-tailed deer	White-tailed deer	Horse	1.000	1.000
	Canada goose	Canada goose	Dog		
			Pig		
			Chicken		
			Sheep		
			Cow		
			Human		
Sample average				1.000	0.911

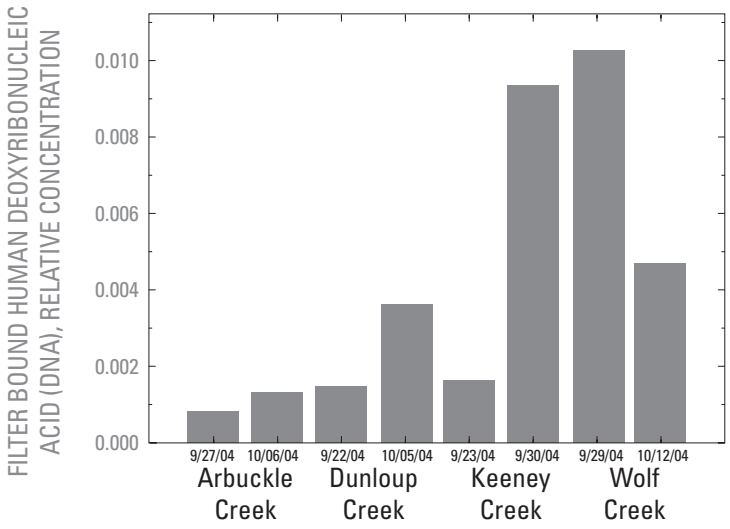

Figure 4. Relative concentrations of filter-bound human DNA in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004. (Concentrations are relative to a human placental DNA standard that had a concentration of 18.5 nanograms per microliter. A 1:1000 dilution of this standard is equal to 1000 on this scale.)

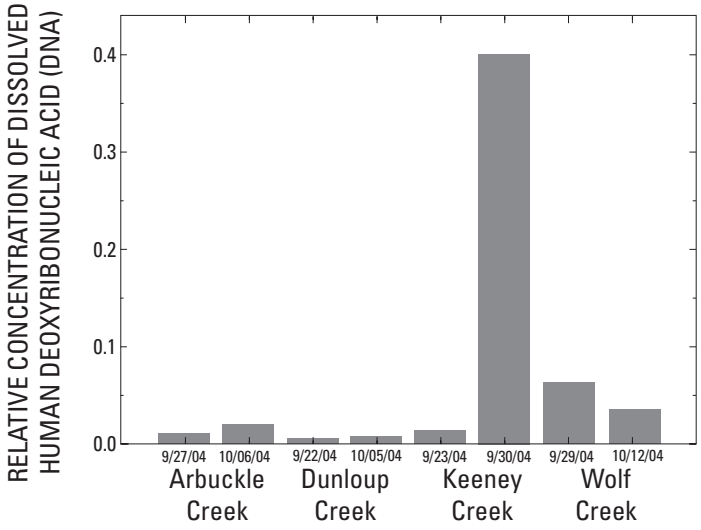

Figure 5. Relative concentrations of dissolved human DNA in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004. (Concentrations are relative to a human placental DNA standard that had a concentration of 18.5 nanograms per microliter. A 1:1000 dilution of this standard is equal to 1000 on this scale.)

Wastewater Compounds

The analyses for 62 wastewater compounds yielded detections (table 7) for 24 of the compounds (excluding surrogates); only phenol was detected at concentrations that exceeded the reporting limit. Although detections for 23 of the wastewater compounds were either not

Table 6. Host mitochondrial DNA markers for species other than human detected, but not quantified, in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004

[X, indicates that the detection was near, but higher than background values; ---, indicates no detection]

Stream	Date	Dog	Pig	Cow	White-tailed deer	Canada goose	Horse	Chicken
Arbuckle Creek	9-27-04	X	X	---	---	---	---	---
Arbuckle Creek	10-06-04	X	---	---	---	---	---	---
Dunloup Creek	9-22-04	X	---	---	---	---	X	
Dunloup Creek	10-05-04	---	---	---	---	---	---	---
Keeney Creek	9-23-04	---	---	---	---	---	X	X
Keeney Creek	9-30-04	---	---	---	---	---	---	X
Wolf Creek	9-29-04	---	---	---	---	---	X	---
Wolf Creek	10-12-04	---	---	---	---	---	X	X

quantified or were estimated detections that were less than the instrument reporting level, these detections, especially the estimated detections, are still considered to be significant (Stephen Zaugg, U.S. Geological Survey, written commun., 2006). Analysis of the water sample collected from Arbuckle Creek on September 27 yielded detections for 22 of 62 of the wastewater compounds. The compounds 1,4-dichlorobenzene, AHTN, cotinine, and triclosan that were detected in this sample are considered to be good indicators of wastewater, especially sewage. Thus, the Arbuckle Creek sample of September 27 likely was affected by human tracer compounds with sewage as the likely source of these compounds. These four compounds were not found in any of the other water samples except for detections of AHTN in the Arbuckle Creek sample of October 6 and the Keeney Creek sample of September 30. Caffeine indicates wastewater, but historically has been detected in about 50 percent of all field blanks. Phenol is ubiquitous in surface waters at the levels detected in this study (Stephen Zaugg, U.S. Geological Survey, written commun., 2006).

Presumptive Sources of Fecal Contamination, by Tributary

Descriptions of the findings regarding animal sources of fecal matter in the water samples from the four tributaries are summarized, by method, in Table 8 and by sample in Table 9. Field and laboratory determinations of bacteria concentrations in the eight water samples are listed in table 2. A discussion of the presumptive sources of contamination for each of the four tributaries to the New River follows. Any inferences of possible sources of fecal contamination are limited to the specific dates and times of sample collection. Because many of the detections for the various methods could vary over time and flow conditions, repeated sampling during both base flow and storm events would be necessary to more definitively determine the sources of fecal contamination for each tributary.

Arbuckle Creek

Arbuckle Creek was sampled September 27 and October 6, 2004; streamflows at the times of sample collection were 1.4 and 2.5 ft3/s, respectively (table 2). Concentrations of fecal coliform

Table 7. Wastewater compounds detected in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004

[µg/L, micrograms per liter; <, less than; E, estimated; M, presence verified, not quantified]

Sampling date	1,4-Dichloroben-zene (µg/L)	AHTN (µg/L)	Benzophenone (µg/L)	Caffeine (µg/L)	Camphor (µg/L)	Cholesterol (µg/L)
09-27-04	M	E.1	M	E.1	M	M
10-06-04	<.5	E.2	<.5	E.2	<.5	<2
09-22-04	<.5	<.5	<.5	M	<.5	<2
10-05-04	<.5	<.5	<.5	<.5	<.5	<2
09-23-04	<.5	<.5	<.5	<.5	<.5	<2
09-30-04	<.5	M	<.5	M	<.5	<2
09-29-04	<.5	<.5	<.5	M	<.5	M
10-12-04	<.5	<.5	<.5	<.5	<.5	<2

Sampling date	Cotinine (µg/L)	DEET (µg/L)	Fluoranthene (µg/L)	HHCB (µg/L)	Isophorone (µg/L)	Methyl salicylate (µg/L)
09-27-04	E.2	E.2	M	M	M	<.5
10-06-04	<1.00	<.5	<.5	<.5	<.5	<.5
09-22-04	<1.00	<.5	M	<.5	<.5	<.5
10-05-04	<1.00	<.5	<.5	<.5	<.5	<.5
09-23-04	<1.00	<.5	<.5	<.5	<.5	<.5
09-30-04	<1.00	<.5	M	<.5	<.5	M
09-29-04	<1.00	<.5	<.5	<.5	<.5	<.5
10-12-04	<1.00	<.5	<.5	<.5	<.5	<.5

Sampling date	p-Cresol (µg/L)	Phen-anthrene (µg/L)	Phenol (µg/L)	Prometon (µg/L)	Pyrene (µg/L)	Tetrachloro-ethene (µg/L)
09-27-04	M	M	1.1	E.1	M	<.5
10-06-04	<1	<.5	<.5	<.5	<.5	<.5
09-22-04	<1	<.5	<.5	<.5	M	M
10-05-04	<1	<.5	E1.0	<.5	<.5	<.5
09-23-04	<1	<.5	<.5	<.5	<.5	<.5
09-30-04	<1	<.5	2.9	<.5	M	<.5
09-29-04	<1	<.5	<.5	<.5	<.5	<.5
10-12-04	<1	<.5	0.8	<.5	<.5	<.5

Sampling date	Tributyl phosphate (µg/L)	Triclosan (µg/L)	Triethyl citrate (µg/L)	Tris (2-butoxy-ethyl) phosphate (µg/L)	FYROL CEF (µg/L)	FYROL PCF (µg/L)
09-27-04	E.1	M	E.1	E.2	E.2	E.1
10-06-04	<.5	<1	<.5	<.5	<.5	<.5
09-22-04	<.5	<1	<.5	<.5	<.5	<.5
10-05-04	<.5	<1	<.5	<.5	<.5	<.5
09-23-04	<.5	<1	<.5	<.5	<.5	<.5
09-30-04	<.5	<1	<.5	<.5	<.5	<.5
09-29-04	<.5	<1	<.5	<.5	<.5	<.5
10-12-04	<.5	<1	<.5	<.5	<.5	<.5

Table 8. Descriptions of findings, by method, for determining animal sources of fecal matter detected in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004

Method	Finding
Pulsed-field gel electrophoresis	Because PFGE correctly identified the source of only one of 37 quality-control bacteria isolates, no source inferences as to the source were based on the PFGE analysis.
Human host-specific *Enterococcus* marker	This marker, indicating a human source of contamination, was found only in one sample (September 23) for Keeney Creek. The marker was detected in one positive-control sample and was not detected in one negative-control sample.
Human-urine marker	This marker, indicating a human source of contamination, was found only in both Keeney Creek samples. The marker was not detected in either of two negative-control blind samples, but a positive-control blind sample was not submitted.
Wastewater compounds	At least one wastewater compound was found in all samples, except for the Keeney Creek sample of September 23. Detections were all low; the greatest number of detections occurred in the Arbuckle Creek sample of September 27. Detections in this sample indicate the presence of human tracer compounds likely derived from sewage. These analytes were not tested in quality-control samples.
Bacteroidetes human-specific marker	Quantitative detection of this marker, indicating human sources of contamination, was positive in all samples. In general, Keeney Creek contained the highest concentration of this marker, followed by Dunloup Creek. Detections in Arbuckle and Wolf Creeks were near the limit of detection. This marker was not tested against control samples.
Host-specific DNA	Detections of host-specific DNA were predominantly of human DNA for all four tributaries. Concentrations of filter-bound human DNA were slightly higher in the Wolf Creek sample of September 29 than in the Keeney Creek sample of September 30. Concentrations of dissolved human DNA were much higher in the Keeney Creek sample of September 30 than in any other sample. Trace amounts of dog, pig, horse, and chicken DNA were detected in some of the samples. The human marker was detected in one positive-control sample and in one of two negative-control samples (white-tailed deer and Canada goose). White-tailed deer and Canada goose DNA markers were detected in both tests against the positive-control sample. Markers for the other non-human hosts were not tested against positive-control samples.

and fecal streptococcus bacteria measured in the field were low to moderate and were 160 (Sept. 27) and 150 (Oct. 6) colonies of fecal coliform per 100 mL of sample and 290 (Sept. 27) and 39 (Oct. 6) colonies of fecal streptococcus per 100 mL of sample, respectively (table 2). Several wastewater compounds were detected at low concentrations for both samples (table 7). The detections of AHTN, cotinine, triclosan, and 1,4-dichlorobezene in the September 27 sample indicate that this sample contains human tracer compounds likely derived from sewage. Potential sources of these wastewater compounds are two wastewater-treatment plants that discharge sewage to Arbuckle Creek during high streamflow caused by storm runoff and lift stations that have been observed to overflow even during dry periods. Also straight pipe discharges, leaking sewer lines,

Table 9. Summary of human-specific markers present in eight water samples collected from four tributaries to the New River Gorge National River, West Virginia, 2004.

[Yes, detected; No, not detected; numbers following "Yes" indicate rank of detection level of samples with especially high levels of detection.]

Sampling site	Sampling date	*Enterococcus* marker	Urine marker	*Bacteroidetes* marker	Dissolved mitochondrial DNA	Filter-bound mitochondrial DNA
Dunloup Creek	9/22/2004	No	No	Yes -3	Yes	Yes
Dunloup Creek	10/5/2004	No	No	Yes -2	Yes	Yes -4
Arbuckle Creek	9/27/2004	No	No	Yes	Yes	Yes
Arbuckle Creek	10/6/2004	No	No	Yes	Yes	Yes
Keeney Creek	9/23/2004	Yes	Yes	Yes	Yes	Yes
Keeney Creek	9/30/2004	No	Yes	Yes -1	Yes -1	Yes -2
Wolf Creek	9/29/2004	No	No	Yes	Yes -2	Yes -1
Wolf Creek	10/12/2004	No	No	Yes	Yes -3	Yes -3

and faulty septic systems are possible sources. The human-specific *Enterococcus* marker and the human-urine marker were not detected in either Arbuckle Creek sample. The human-specific *Bacteroidetes* (fig. 3) marker and the human-specific mitochondrial DNA marker (figs. 4 and 5) were detected in both samples from Arbuckle Creek, but were not especially elevated in either sample. Dog DNA was detected in both Arbuckle Creek samples, and pig DNA was detected in the sample of September 27 (table 6).

Dunloup Creek

Dunloup Creek was sampled September 22 and October 15, 2004; streamflows at the times of sample collection were 23 and 22 ft^3/s (cubic feet per second), respectively (table 2). Concentrations of fecal coliform and fecal streptococcus measured in the field were relatively low at 87 and 17 (estimated) colonies of fecal coliform per 100 mL of sample, respectively, and 36 and 7 (estimated) colonies of fecal streptococcus per 100 mL of sample, respectively (table 2). The human-specific *Enterococcus* marker and the human-urine marker were not detected in either Dunloup Creek sample. The human-specific *Bacteroidetes* (fig.3) marker was detected in relatively elevated amounts in both water samples collected from Dunloup Creek. The human-specific marker for host mitochondrial DNA (figs. 4 and 5) also was detected in both water samples collected from Dunloup Creek Filter-bound mitochondrial DNA (fig. 4) was elevated in the second Dunloup Creek sample (October 5). As previously stated, two wastewater-treatment plants in the Dunloup Creek watershed could discharge partially treated sewage to Dunloup Creek during high streamflow caused by storm runoff. This was obviously not a factor in the base-flow samples collected from Dunloup Creek in this study. The fact that human-specific markers for *Bacteroidetes* and host mitochondrial DNA were detected at base flow seems to indicate that human waste was reaching Dunloup Creek during the base-flow conditions at the time of sampling. Possible sources of the human-specific markers indicating human contamination sources are human waste from straight pipe discharges to the stream, from leaking sewer lines, or from faulty septic systems. Dog and horse DNA (table 6) were detected in the September 22 sample.

Keeney Creek

Keeney Creek was sampled September 23 and 30, 2004; streamflows at the times of sample collection were 1.3 and 5.2 ft^3/s, respectively (table 2). Fecal coliform and fecal streptococcus

bacteria concentrations measured in the field were 44 (estimated) (Sept. 23) and 280 (Sept. 30) colonies of fecal coliform per 100 mL of sample and 74 (estimated) (Sept. 23) and 160 (Sept. 30) colonies of fecal streptococcus per 100 mL of sample, respectively (table 2). The human-specific *Enterococcus* marker was detected in the first but not the second sample, and the human-urine marker was detected in both samples (table 9). These two human-specific markers were not detected in samples from the other three tributaries. The human-specific *Bacteroidetes* marker (fig. 3) and the human-specific host mitochondrial DNA markers (figs. 4 and 5) were detected in both samples and were especially elevated in the second sample of September 30. Three of the four human-specific marker detections were for the second Keeney Creek sample which had higher fecal coliform bacteria concentrations than the first sample. Curiously, the human-specific marker for *Enterococcus* was detected in the first Keeney Creek sample that contained lower concentrations of fecal coliform bacteria. The presence of multiple human-specific markers in the Keeney Creek samples indicates a human source of fecal waste for these samples. The Keeney Creek watershed has no centralized municipal wastewater-treatment facilities. Thus, possible sources for the human-specific markers during base-flow conditions are straight pipe discharges and faulty septic systems. Chicken DNA was detected in both water samples, and horse DNA was detected in the September 23 sample (table 6).

Wolf Creek

Wolf Creek was sampled September 29 and October 12, 2004; streamflows at times of sample collection were 15 and 3.0 ft^3/s, respectively (table 2). The second sample was collected during base flow, but the first sample could have contained some surface runoff. Concentrations of fecal coliform and fecal streptococcus bacteria measured in the field were 3,800 (Sept. 29) and 3 (estimated) (Oct. 12) colonies of fecal coliform per 100 mL of sample and 1,900 (Sept. 29) and 5 (estimated) (Oct. 12) colonies of fecal streptococcus per 100 mL of sample, respectively (table 2). The first sample collected from Wolf Creek contained bacteria concentrations an order of magnitude higher than bacteria concentrations for the remaining seven samples. The human-specific *Enterococcus* and human-urine markers were not detected in either Wolf Creek sample. The human-specific *Bacteroidetes* marker (fig. 3) and the human-specific host mitochondrial DNA marker (figs. 4 and 5) were detected in both samples. The human-specific DNA marker was especially elevated in both samples. Possible sources of the human-specific markers are straight pipe discharges, leaking sewer lines, and faulty septic systems. The Wolf Creek watershed has a sewage distribution system that carries sewage to a sewage-treatment plant located in an adjacent watershed. Thus, municipal sewage outfalls or overflows are not possible sources of sewage in Wolf Creek. Horse DNA was detected in both samples, and chicken DNA was detected in the October 12 sample (table 6).

Summary and Conclusions

A study was conducted in the vicinity of the New River Gorge National River to determine the source of fecal contamination (sourcing) in four tributaries to the river. Inferences to possible sources of fecal contamination are limited to dates and times of sample collection from the tributaries. The sourcing methods used in this study to attempt to associate fecal contamination in water samples collected from the four tributaries to the New River with animal sources yielded various results.

Evaluation of results for quality-control samples indicated that PFGE could only correctly identify 1 of 37 known bacteria isolates. It could not identify any human, dog, or Canada goose quality-control bacteria isolates and correctly identified only one white-tailed deer quality-control

bacteria isolate. Increasing the size of the PFGE library of known-source isolates did not improve quality-control results. The use of different growth media in the various steps of culturing of *E coli* bacteria isolates in this study could partially explain the errors in attempted association of quality-control isolates to animal source. *E. coli* isolates for the water samples and quality-control samples were obtained from fecal coliform colonies grown on mFC media, and *E. coli* isolates were obtained from known sources of feces by streaking the fecal samples onto EMB media. It is also possible that the population of DNA-fragment banding patterns derived from *E. coli* in human and animal feces is too diverse and variable to allow accurate associations of bacteria isolates in water samples from the four tributaries with animal sources by use of PFGE analysis. Due to the poor results for the quality-control bacteria isolates, PFGE results for water samples were not used for sourcing.

Results of quality-control tests for emerging sourcing methods that use human-specific and animal-specific biological or molecular markers indicate that some of these methods may have potential for identifying the source of fecal waste in natural waters. A human-specific *Enterococcus* marker was correctly detected in a positive control sample and was not detected in a negative control sample. A human-specific urine marker was not detected in two negative control samples. A human-specific host mitochondrial DNA marker was correctly detected in a positive control sample, but was detected in one of two negative control samples. Canada goose-specific and white-tailed deer-specific host mitochondrial DNA markers were correctly detected in two positive control samples and not detected in one negative control sample. A cow-specific marker was detected in one of three negative control samples. Horse-, pig-, sheep-, dog-, and chicken-specific markers were not detected in any of three negative-control samples. Future studies will need rigorous quality-control components on the potential applicability and use of these emerging sourcing methods.

The highest relative concentrations of four human-specific markers for *Enterococcus* and human urine (both markers only detected in Keeney Creek), *Bacteroidetes*, and host mitochondrial DNA were detected in water samples from Keeney Creek, a watershed with no centralized municipal wastewater-treatment facilities, thus indicating human sources of fecal contamination. The human-specific *Bacteroidetes* and host mitochondrial DNA markers also were detected in water samples from Dunloup Creek, Wolf Creek, and to a lesser degree Arbuckle Creek. The results of analysis for wastewater compounds indicate that one sample from Arbuckle Creek contained numerous human tracer compounds, likely from sewage. Lesser amounts of dog, horse, chicken, and pig host mitochondrial DNA were detected in some of the water samples; no animal mitochondrial DNA was detected in one water sample from Dunloup Creek. Cow, white-tailed deer, and Canada goose DNA were not detected in any of the samples collected from the four tributaries, although these animals are present in the watersheds.

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