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Hypothesis-Driven Approach for the Identification of Fecal Pollution Sources in Water Resources

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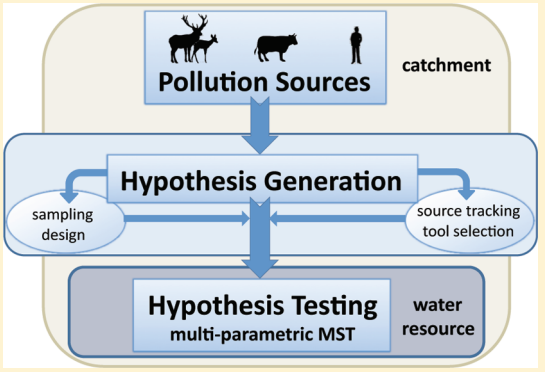
Supporting Information

ABSTRACT: Water resource management must strive to link catchment information with water quality monitoring. The present study attempted this for the field of microbial fecal source tracking (MST). A fecal pollution source profile based on catchment data (e.g., prevalence of fecal sources) was used to formulate a hypothesis about the dominant sources of pollution in an Austrian mountainous karst spring catchment. This allowed a statistical definition of methodical requirements necessary for an informed choice of MST methods. The hypothesis was tested in a 17-month investigation of spring water quality. The study followed a nested sampling design in order to cover the hydrological and pollution dynamics of the spring and to assess effects such as differential persistence between parameters. Genetic markers for the potential fecal sources as well as microbiological, hydrological, and chemo−physical parameters were measured. The hypothesis that ruminant animals were the dominant sources of fecal pollution in the catchment was clearly confirmed. It was also shown that the concentration of ruminant markers in feces was equally distributed in different ruminant source groups. The developed approach provides a tool for careful decision-making in MST study design and might be applied on various types of catchments and pollution situations.



INTRODUCTION

It is estimated that 884 million people worldwide lack access to safe drinking water.[1] In this context fecal pollution of water resources is one of the most serious risks. Regulatory limits for fecal impact on waters are still based on the cultivation of fecal indicator bacteria (FIB) such as *E. coli*.[2] Though constantly debated in terms of their reliability as indicators of actual fecal influence[3,4] FIB have proven to be good and representative parameters in many areas, e.g., for the important sources of fecal pollution in the Austrian alpine environment.[5,6] However, without further characterization, they do not allow source identification, which is crucial for remediation of the cause, verification of remediatory measures, and characterization of the hazards potentially caused by fecal pollution.[7] Microbial source tracking (MST) methods are proposed to solve this problem of source identification.[8]

Recently methods for the molecular detection of source-specific genetic markers have become available and have shown great promise in the search for reliable and affordable MST tools.[7] During the last years markers for fecal *Bacteroidetes* have been very popular targets and have been shown to possess a certain degree of host-specificity.[9,10] Like other genetic markers they are currently usually detected by quantitative real-time PCR (qPCR) (e.g., refs 11−15). Few application studies using this approach have set MST data in a contextual framework of microbiological, hydrological, and general water quality parameters.[6,16,17] Without such a reference MST data interpretation and actual source identification is currently extremely difficult.[18] In addition methods were rarely validated under the field conditions in the intended study areas in order to assess whether a specific source tracking problem is likely to be solvable

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Table 1. Calculation of Conditional Detection Probability of True Positive MST Results for Various Pollution Scenarios [a]

contribution of specific source to total pollution[b]	background pollution[c]	probability of correct detection of specific source P(H/T)[d]			
		specificity 99%	specificity 95%	specificity 90%	specificity 50%
0.999	0.001	1.000	1.000	1.000	0.999
0.990	0.010	1.000	0.999	0.999	0.995
0.900	0.100	0.999	0.994	0.989	0.947
0.500	0.500	0.990	0.950	0.909	0.667*
0.100	0.900	0.917	0.679*	0.526*	0.182*
0.010	0.990	0.503*	0.161*	0.092*	0.020*
0.001	0.999	0.091*	0.019*	0.010*	0.002*

[a] Approach based on Kildare et al.[14] using Bayes' theorem, modified for the purpose of this study. Sensitivity of the used assay is set to 100% (P (T/H) = 1);[14] for details see Experimental Section. [b] Given proportion of total fecal pollution contributed by a target source (P(H)).[14] [c] Corresponds to P(H').[14] [d] Probability of the event that there is a specific source of contamination (H) in an analyzed water sample given the event the test signals positive (T) with a source-specific assay targeting the specific source[14] with an experimentally determined level of specificity (P(T/H') = 1 − specificity). * Indicates conditional probability for correct, true positive detection <90%.

using the applied MST method.[7] This information together with background knowledge on the catchment under investigation is critical for the choice of appropriate MST methods and study design.

For the present study, we established an integrative approach for MST study design and conduction. Information about potential fecal pollution sources in the study area was integrated into a pollution source profile, which formed the basis for the formulation of a hypothesis about the dominant pollution source. It was instrumental for assessing the ability to test the hypothesis in MST study design. The approach was applied on the catchment of limestone karst aquifer spring 8 (LKAS 8) to determine the dominant source of fecal pollution using qPCR-based MST marker detection and a nested sampling design adapted to the hydrological and pollution dynamics. In addition the abundance distribution of suspendable ruminant-specific marker in various sources was determined.

EXPERIMENTAL SECTION

Study Area. The studied karst catchment area in the Northern Calcareous Alps in Austria has an estimated area of approximately 11 km^2 at an average altitude of 1341 m above sea level. Vegetation cover is composed of summer pastures on calcareous alpine swards (41% of the catchment area) and open krummholz and forests (59%). There are no permanent settlements in the area, only temporary mountain hotels and cabins open during summer months. The outlet of investigated limestone karst aquifer spring 8 (LKAS8) is at 522 m above sea level. The catchment area is mainly built up from Triassic limestones and dolomites. On the plateau Paleocene sediments are found in closely bounded areas. Discharge shows high variations with a $discharge_{max}/discharge_{min}$ ratio of <14 based on daily mean discharges (2003−2007). The mean water residence time was estimated to be 1.2 years based on oxygen-18 calculations.[19] The discharge response after precipitation as observed during two event sampling campaigns was 2−3 hours. The spring's mean discharge between 2003 and 2007 was 589 L s^{-1}.

Hydrological and Chemo−Physical Data. In-field online sensors directly installed at the spring outlet of LKAS8 recovered all hydrological and chemo−physical data. Conductivity and water pressure were registered with the data collecting system GEALOG-S from Logotronic (Vienna, Austria). Probes used were WTW-Tetracon 325 (WTW, Weilheim, Germany) for measuring conductivity and PDCR 1830 (Druck, London, UK) for water pressure. Recorded gauge heights were converted with a discharge stage relation. All sensors were controlled with single measurements with an interval of 1−4 weeks, using instruments which were part of a certified quality management system. Turbidity and SAC_{254} were measured with a spectro:: lyser (s::can Measuring Systems, Vienna, Austria).

Conditional Probability Analysis of MST Target Detection and Pollution Scenarios. We used the approach described by Kildare et al.[14] based on Bayes' theorem to estimate the probability for the correct detection of a specific source of pollution under different pollution scenarios (Table 1). To evaluate possible pollution scenarios we assumed different levels of contribution from a targeted specific source to total fecal pollution in an area (P(H)) and assessed the effect of various assumed levels of assay source-specificity (i.e., 99%, 95%, 90%, 50%) on the ability to correctly detect this specific source by the respective assay (P(H/T)). The contributing nontarget fecal pollution levels (P(H′)) were calculated by 1 − P(H). Assay sensitivity (P(T/H)) was assumed to be 1. The applied approach differs from the original[13] in that the probabilities of P(H) and P(H′) were directly derived from the respective pollution scenarios.

Pollution Source Profiling (PSP). The assessment of the potential quantitative contribution of the fecal source groups in the catchment area of LKAS 8 was based on data about the catchment found in local official records and provided by officials, and forestry and water works professionals as well as expert knowledge and literature. The necessary calculations are extensively described in the Results section and in Table 2. The calculations were based on average values and point estimates.

Water Sampling and Sample Processing. Water samples were taken from LKAS8 between June 2007 and October 2008. Sampling was organized in three tiers (Figure 1): (i) basic monitoring (MONIT, $n = 23$) every three weeks, (ii) high-frequency monitoring (HFM, $n = 70$) with sampling twice a week during summer months (June to September 2007 and May to September 2008), and the investigation of a hydrological event (EVENT, $n = 27$) with strongly elevated discharge in August 2007 which was sampled up to several times a day. Water samples of usually 4.2 L were collected and processed (filtration on 0.2-μm polycarbonate filters and DNA extraction using bead-beating and phenol/chloroform) as described previously.[6] Enumeration of *E. coli*,

Table 2. Catchment Pollution Source Profiling[a]

		produced average fecal mass				est. environmentally available fecal material				average produced and available *E. coli* cells		
source		average abundance [units d^{-1}][b]	population-based defecation percentage[c]	average individual fecal amount [kg wet weight d^{-1}]	total fecal amount per source group [kg wet weight d^{-1}]	est. percentage of out-door and in-door defecation	est. rate of environmental availability	total environmental fecal amount [kg wet weight d^{-1}]	percentage of total fecal amount [%]	average *E. coli* concentration per source group [CFU g^{-1}][d]	environmentally available *E. coli* [CFU d^{-1}]	percentage of total *E. coli* [%]
human	tourist	405[e]	30%[i]	0.15[k]	18	100% (i.-d.)	1%[n]	0.18	0.00%	9.8×10^7	1.78×10^{10}	0.02%
	hiker + alpinist	203[f]	50%[i]	0.15[k]	15	10% (o.-d.)	100%	1.52	0.03%	9.8×10^7	1.48×10^{11}	0.15%
						90% (i.-d.)	1%[n]	0.14	0.00%	9.8×10^7	1.34×10^{10}	0.01%
wildlife	red deer	250[g]	100%	1.13[m]	283	100% (o.-d.)	100%	283	4.70%	3.3×10^7	9.35×10^{12}	9.50%
	chamois	450[g]	100%	1.13[m]	509	100% (o.-d.)	100%	509	8.45%	6.6×10^7	3.36×10^{13}	34.20%
	roe deer	240[g]	100%	1.13[m]	271	100% (o.-d.)	100%	271	4.50%	3.3×10^{7p}	8.98×10^{12}	9.10%
livestock	cattle	210[h]	100%	23.6[k]	4956	100% (o.-d.)	100%	4956	82.31%	9.3×10^6	4.63×10^{13}	47.00%

[a] Abbreviations: est., estimated; CFU, colony forming units; i.-d., in-door defecation; o.-d., out-door defecation. [b] Mean daily abundance during the time span of June until September in both 2007 and 2008. [c] Percentage of individuals defecating in the investigated catchment area. [d] According to ref 5. [e] Data obtained from operators of cog-railway terminating at 1800 m above sea level in the catchment area. [f] Assumption that two-thirds of total visitors are tourists and one-third are hikers and alpinists. [g] Data provided by gamekeepers responsible for the catchment area. [h] Data from official pasture management records; considering alpine pastures with relevance for catchment protection areas. The composition of the herds (cows and calves) was taken into consideration: 300 (total number of animals) × 0.7 (conversion factor) = 210 (equivalent number of adult animals). [i] Estimated value. [k] According to ref 39. [m] Due to lack of references, values are related to average fecal amount excreted by sheep.[39] [n] Percentage taking into account estimated leakage of raw sewage from sewers or septic tanks or disinfection efficiencies of sewage treatment with chlorinated lime before disposal in the environment. [p] Average *E. coli* concentration of roe deer was set equal to the value of red deer, due to insufficient data of roe deer feces.

enterococci, presumptive *Clostridium perfringens* (each with a detection limit of 1 CFU L^{-1}) and heterotrophic plate count at 22 °C was performed as described in the respective ISO standard methods.[20−23] Numbers of aerobic spore-forming bacteria were determined by pasteurization of the water sample at 60 °C for 15 min, membrane filtration, and incubation on yeast extract agar at 22 °C for 7 days.

qPCR Procedures. Human- (BacH) and ruminant (BacR)-specific qPCR assays were performed as described previously.[12,13] These assays have been developed and evaluated in the Eastern Austrian region where they have shown high levels of source-sensitivity (100% for BacR, 95% for BacH) and source-specificity (100% for BacR and 99.7% for BacH).[12,13] All sample DNAs were measured in duplicate in at least two 4-fold DNA dilution steps and the results were compared in order to rule out PCR inhibition. Controls included no-template controls, as well as filtration and DNA extraction blanks. Marker concentration results were expressed as marker equivalents (ME) per liter taking into account the filtration volume. A 4.2-L filtration volume, the use of 2.5-μL of undiluted DNA extract in qPCR, and the minimal theoretically detectable marker concentration per reaction defines the threshold of detection that is shown in Figures 2 and S1.[12,13]

Sampling and Analysis of Ruminant Fecal Samples. Samples were collected on July 31 and August 1, 27, and 28, 2008 in the catchment area of LKAS8 and an adjacent catchment. Fecal material was collected using either sterile fecal sampling tubes or plastic sampling bags. Sixty-one fresh and single fecal samples from ruminant sources, i.e., cattle, red deer, chamois, and roe deer, from well described habitats were collected. Homogenized feces (141 ± 37 mg) were suspended in 45 mL of sterile-filtrated spring water in 50-mL centrifugation tubes (Sterilin, Aberbargoed, UK) on a vortex machine for 10 s each. The tubes were incubated at 4 °C and after gentle shaking on a vortex machine and sedimentation of plant residues (10 s for each step) the suspensions were diluted 100 fold (v/v). One mL of this dilution was immediately filtered through polycarbonate filters, DNA was extracted, and the concentration of BacR marker was determined as described above for water samples.

Sampling and DNA extraction of soil samples and data processing and statistical analysis are covered in the Supporting Information.

RESULTS

Fecal Pollution Source Profiling and Hypothesis Formulation. To assess the relative contribution of potential sources of fecal pollution in the catchment of the limestone karst aquifer spring 8 (LKAS 8) a fecal pollution source profile was established. The sources with potential significance in this alpine area are human sources (sewage from mountain huts and restaurants), cattle kept on pastures during summer months, and game (deer, roe deer, and chamois). Other potential sources such as birds or ground-dwelling mammals were considered to be negligible and were therefore disregarded. The resulting assessment is elaborated in Table 2. Data on abundances of sources were obtained from public information on tourism in the area, official records on livestock numbers, and estimates of game numbers by local authorities. After estimation of the percentage of daily defecation by the subpopulation in the catchment area (i.e., tourists spend only a part of the day in the catchment), the total amount of wet feces per source group and day was calculated. After estimating the environmental availability (i.e., a large part of human feces is collected in sanitary facilities and disposed outside the catchment) the amount of fecal material potentially available in the environment was calculated and converted to the total number of standard FIB *E. coli* introduced per day (Table 2). *E. coli* was chosen because it is one of the main parameters for the monitoring of microbial quality of drinking water in Austria and worldwide.

The pollution source profile estimated that on average more than 99.8% of *E. coli* from the investigated sources in the catchment can be expected to be shed by ruminant animal sources (9.9×10^{13} CFU d^{-1}), roughly half of which is

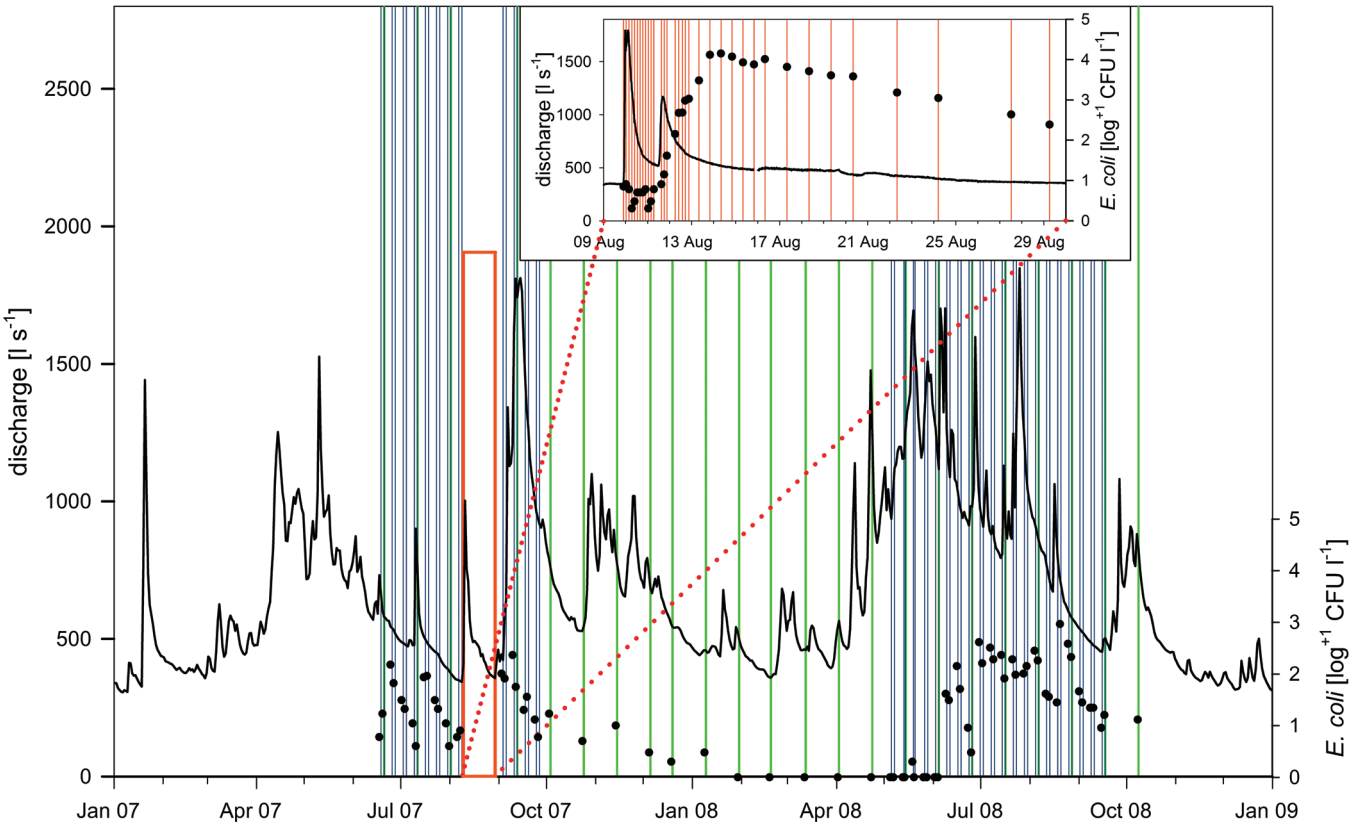

Figure 1. Hydrological situation, nested sampling scheme, and fecal pollution levels in LKAS8. Daily mean discharge is shown for the years 2007 and 2008; vertical lines are sampling dates (green lines, basic monitoring (MONIT); blue lines, high frequency monitoring (HFM); red lines in zoomed-in box, flood event monitoring 2007 (EVENT); discharge levels in the zoomed-in box are values measured every 15 min; FIB *E. coli* levels in colony forming units (CFU) per liter for all samples (black dots) after adding 1 to a measured value and $\log_{10}$ transformation.

contributed by livestock and half by wildlife, respectively. The hypothesis for the subsequent investigation of spring water quality in LKAS 8 was therefore that the main source of fecal influence in the catchment of LKAS8 is ruminant animals. Consequently we expected (i) elevated levels of ruminant-specific MST signals when compared to other sources, (ii) a correlation between FIB parameters and ruminant-specific MST signal levels, and (iii) the ability to explain variations in FIB *E. coli* levels by ruminant-specific quantitative MST signal variations.

MST Study Design. The study design for the investigation of LKAS8 was based on the information gathered in the pollution source profiling as well as on the hydrological and pollution dynamics of the spring. The pollution source profile defined the requirements for the MST methods to be applied in the study. As demonstrated by the calculation scenarios of the conditional probabilities for correct source identification (Table 1), dominant potential sources of pollution—e.g. ruminant sources in this study—can be detected with high confidence even with assays that have less than perfect source-specificity (e.g., 50−90%). On the other hand sources with lower proportional contribution to fecal pollution (<50%) will require assay specificity levels higher than 90% to provide appropriate confidence in the results. Sources contributing less than 10% need specificity levels larger than 99% and will be very hard to detect reliably with an acceptable confidence level based on the Bayes′ theorem calculation. Therefore the BacR assay should allow the detection of the expected high levels of ruminant pollution and the prediction of *E. coli* levels in spring water. In contrast even the high source-specificity of the BacH assay might not allow us to distinguish true positive from potentially false positive results at the low levels of human fecal pollution (<1%) expected in the catchment.

The applied nested sampling design (Figure 1) with the tiers basic monitoring (MONIT), high-frequency monitoring (HFM), and event monitoring (EVENT) covered most of the pollution dynamics in the spring during the study period. In relation to the hypothesis this nested sampling allowed investigation of water with different mean residence times in the aquifer in the different tiers and therefore assessment of the effect of differential persistence of parameters (e.g., cultivation-based FIB and molecular ruminant-specific MST markers) on the results.

MST Marker Levels in Spring Water. Figure 2 shows that the levels of BacR were consistently higher than the levels of BacH in all data sets. The median BacR concentrations were 6.3×10^2 marker equivalents (ME) per liter during MONIT, 1.6×10^3 ME L^{-1} during HFM and 5.0×10^4 ME L^{-1} during the EVENT (Table S1). In contrast, the median concentration of the BacH maker was at the threshold of detection in MONIT and HFM and only slightly higher during the EVENT (Figure 2). It has been previously shown that the concentrations of the BacH marker in human fecal material are around 1 order of magnitude higher than BacR marker concentrations in ruminant feces.[12,13] For this reason, a corrected BacH parameter was calculated compensating for this discrepancy in abundance in fecal material

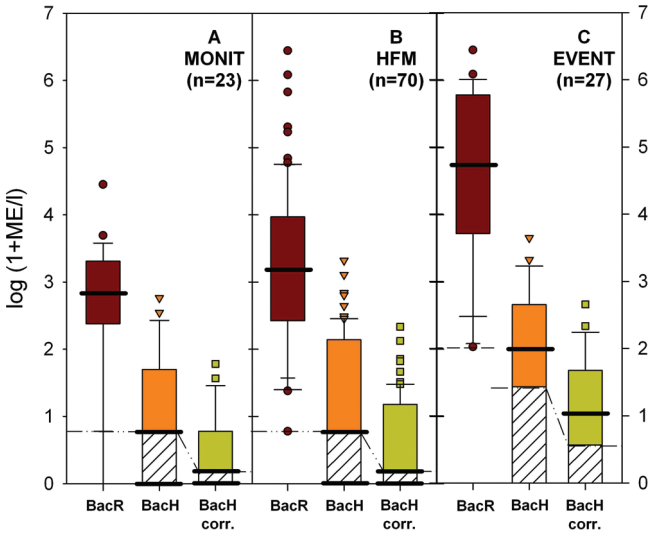

Figure 2. Levels of BacR and BacH MST markers in LKAS8 during basic monitoring (MONIT), high frequency monitoring (HFM), and flood event monitoring (EVENT). Box plots with whiskers indicating 10th and 90th percentiles, boxes indicating 25th and 75th percentiles, and lines within boxes showing the median. BacR, ruminant-specific marker (brown boxes and dot symbols); BacH, human-specific marker (orange boxes and triangle symbols); BacH corr., human-specific marker after correction for higher abundance in feces as compared to ruminant marker (green boxes and square symbols), ME, marker equivalent; n, number of samples; data is given after $\log^{+1}$ transformation; dash-dot-dot lines (undiluted samples) represent "threshold of detection" levels.

(Figure 2). In contrast to BacH, the BacR marker was consistently detectable in the event sample set and the large majority of samples in the other sample sets (88% in MONIT and 91% in HFM, Figure S1).

Relating MST to Other Measures of Water Quality. To put the MST results in a broader water quality context, the samples were further characterized using a broad set of parameters including microbiological, hydrological, and chemo−physical parameters (Table S1). Spring discharge and the chemo−physical parameters turbidity (Turb), spectral absorption coefficient at 254 nm (SAC_{254}), and conductivity (Cond) were measured online at the spring outlet. Median values and ranges for HFM were very similar to MONIT. In contrast, fecal indicator (FI) counts, i.e., *E. coli* and enterococci, and presumptive *Clostridium perfringens* as well as copiotrophic indicators (i.e., heterotrophic plate count at 22 °C and aerobic spore-formers) measured during HFM were higher than in MONIT. In general all parameters were strongly elevated during EVENT sampling when compared to the MONIT with the expected exception of conductivity (Table S1). Fecal pollution levels were highest during high discharge periods in summer (*E. coli* concentrations up to 1.6×10^4 CFU L^{-1}) many of which were covered by HFM but not by MONIT. In contrast, stronger discharge during the spring snowmelt period did not lead to elevated FI counts (Figure 1).

Multiple correlation analysis (Spearman rank correlation coefficient r; significance level <0.05, Bonferroni corrected) was used to investigate relationships among all investigated parameters (Table S2). In general correlation coefficients were higher in HFM and EVENT data sets than in the monitoring data. Correlation coefficients among microbiological parameters were higher than among other parameters. The BacR parameter showed significant correlation with the FIB parameters *E. coli* and enterococci in all data sets. In contrast the human-specific BacH marker showed low, nonsignificant correlations with these FIB parameters. To evaluate the predictability of *E. coli* by the BacR marker, regression analysis of these parameters was performed for the HFM and the EVENT data sets. Regression analysis between BacR and *E. coli* yielded coefficients of determination (R^2) of 0.85 and 0.86 for the HFM and the EVENT investigation, respectively (Figure S2).

Quantitative Distribution of Suspendable BacR Marker in Feces from Different Ruminant Sources. There are very little data on the prevalence and abundance of MST markers in different groups of animals, especially wildlife populations. To evaluate whether the BacR marker is shed at comparable concentrations by the four most important species of ruminants (chamois, deer, roe deer, and cattle) in the LKAS8 catchment, BacR marker concentrations were determined in 61 ruminant fecal samples collected in and close to the catchment area of LKAS 8. Samples were suspended in sterile filtered spring water and analyzed according to the procedure for spring water samples. BacR marker concentrations were remarkably similar and showed low variation among samples from all ruminant sources (Figure S3). The overall median concentration was 2.7×10^8 BacR ME g^{-1} wet feces. Elevated concentrations were found in roe deer samples, which in some cases were slightly desiccated. Altitude and vegetation type (krummholz, forest, pasture) at the sampling site did not have a discernible effect on marker abundance in feces (data not shown).

Detection Frequency of *Bacteroidetes* Markers in Soil of the Catchment. As stated above, the BacH marker was frequently detected at very low levels in LKAS8, not showing any apparent correlations to the hydrological or fecal pollution situation. A possible background level of *Bacteroidetes* markers motivated us to investigate pristine soil in the catchment as a source of the markers. Forty-eight soil samples originating from and close to the catchment area were investigated for BacR and BacH marker concentrations. Thirty-one percent of the samples were positive for the BacR marker and 50% of the samples were positive for the BacH marker. Mean concentrations were 2.1×10^4 BacR ME g^{-1} and 3.5×10^4 BacH ME per g soil, respectively.

DISCUSSION

Hypothesis-Driven MST. The outcome of the pollution source profiling approach was a valuable resource for evaluating the applicability of the available microbial source tracking methods and choosing an appropriate tool. Alternatively, sanitary surveys or fecal source apportionment[24−26] can also provide an estimate of the contribution to fecal pollution by potential sources. Ultimately any such estimation or model has to be put to the test by applying reliable source identification tools to the affected water resource itself. Previous investigations in Austrian karst catchments had shown that application of library-based MST approaches in this environment are very laborious and expensive.[27] Based on the pollution source profile an informed decision was made to apply the human-specific BacH and the ruminant-specific BacR qPCR assays targeting source-specific *Bacteroidetes* populations which had been developed and evaluated for Eastern Austria[12,13] and successfully applied in a similar catchment in the Eastern Calcareous Alps.[6] Following this strategy it was assured that the used MST methods covered all

relevant source groups in the catchment (humans, ruminant livestock, and wildlife)[5] and had the source-sensitivity and -specificity necessary for reviewing the hypothesis with appropriate confidence.[12,13]

The hypothesis that ruminant fecal sources are the main source of the FIB *E. coli* in the catchment of LKAS8 was corroborated on three main levels: first, the BacR marker was found in higher concentrations in spring water than the BacH marker; second, high and significant correlations were found between BacR and FIB in contrast to BacH; third, regression analysis between *E. coli* and BacR showed that during high-frequency monitoring and event monitoring total fecal pollution could be quantitatively related to ruminant fecal sources. Despite the unique catchment characteristics of LKAS8 these results are in accordance with a study done in the mountainous karst spring LKAS2,[6] 60 km distant from LKAS8. LKAS2 had a very large and less accessible alpine catchment (i.e., lower amount of potential human fecal sources) where ruminant animals were also the dominant fecal source group. The higher anthropogenic pressure made the LKAS8 catchment an ideal study area to develop and test the hypothesis-driven approach integrating information from the catchment, statistical considerations, and improved nested sampling design.

Verifying MST Results. Despite the promising results achieved in this and several other studies (e.g., refs 6,18,28) some fundamental restrictions and conditions apply to available marker-based MST methods and MST study design. The following section will elaborate on how this study tried to meet those challenges by (i) interpreting MST data in relation to fecal pollution in general, (ii) choosing appropriate markers and detection methods, and (iii) applying an integrated study design.

(i). "Quantitative" MST. qPCR is a quantitative method and its application for detection of genetic MST markers yields quantitative results. However the many unknown factors influencing this data (e.g., transport mechanisms) and the lack of a broad basic understanding of the ecology and fate of the microbial target cells (e.g., persistence) currently prohibit a direct quantification of fecal sources from qPCR-based MST data alone. In this investigation MST parameters were embedded in a multiparametric data set to relate MST to other measures of water quality and more specifically with total fecal pollution to get an impression of the role of a specific source group in the contamination of a water resource. As it is the case in this study, total fecal pollution will most often be determined using FIB.[5] In this study 85% (HFM) and 86% (EVENT) of the variations in *E. coli* data could be explained by the variations in BacR data. An alternative to the application of FIB would be genetic markers for total fecal pollution.[11,14,29] Unfortunately their reliability has been studied very little up to now.[30]

(ii). MST Method Performance Characteristics. In any application of qPCR-based MST marker detection basic methodical characteristics have to be investigated. These include the performance of enrichment and DNA extraction procedures as well as the method's source-specificity and -sensitivity in the study area.[7] The methods applied in this study were developed in the alpine karst environment and thoroughly validated in Eastern Austria.[6,12,13] Our assessment of the probabilities of correct MST detection shows that it is relatively easy to detect dominant sources of pollution (Table 1). However low contributions (<1%) are very hard to identify with high confidence in results because of the high probability of false-positive signals. Therefore it is impossible to be sure whether the observed low and intermittent occurrence of the BacH markers in the spring in our study is caused by human contamination or by false-positive signals from the dominating ruminant sources.

In addition MST markers should not be present in relevant concentrations in nonintestinal habitats that might influence the water resource under investigation. In the present study it was shown that soil in the catchment might contribute to a low background level of BacR or BacH MST marker detection. However the concentrations and coherence of BacR with the cultivation-based FIB parameters in spring water cannot be attributed to the low concentrations of this marker found in soils in the catchment. In contrast the contribution of soil could be another potential contributing factor for the low and intermittent occurrence of BacH in spring water. Theoretically the application of MST methods with higher specificity (e.g., host-specific mitochondrial markers[31]) on larger sample volumes might allow the confirmation of possible low human fecal influence on LKAS8. However any doubt cast on the results by the low BacH marker levels in no way affects the identification of ruminant animals as the dominant source of fecal pollution.

A subject that has been insufficiently studied up to now is the actual abundance distribution and prevalence of MST markers in source feces.[7] The finding that suspendable marker levels were very similar in ruminant livestock and wildlife populations makes it a reliable indicator for all ruminant fecal sources in this area.

(iii). Integrated Study Design. The nested sampling design developed for this study was based on extensive knowledge about the hydrological and fecal pollution dynamics of LKAS8. By using an integrated study design a holistic assessment of the system based on a broad set of parameters was possible. In this study the mean residence time of water, and consequently of fecal pollution introduced by infiltration, in the aquifer increased from event monitoring via high-frequency monitoring to basic monitoring tiers. This allowed the in situ assessment of possible effects of differential environmental persistence between and among MST and FIB parameters, respectively, in the system itself without resorting to selected microcosm experiments under laboratory conditions. Generally the environmental conditions in the LKAS8 aquifer, i.e., darkness, 5 °C, and ultraoligotrophic conditions, are favorable for the persistence of microbes when compared to other environments.[32,33] Our own investigations have shown relatively high persistence of *E. coli* in karst spring water under ambient spring conditions[34] comparable to the values found for genetic markers.[35,36] Remarkably the results of the source identification were in agreement in all sampling tiers despite the possible differential persistence between *E. coli* and BacR.

Implications for the Studied Catchment. The present study shows how information about a catchment and the corresponding water resource can be integrated in a hypothesis-driven study design. In combination with locally evaluated, state-of-the-art MST methods it was possible to obtain quantitative information on the dominant fecal sources. For the case of LKAS8 the results signify that the focus for remediation should be on ruminant animals in the area, and efforts in sanitation and sewage disposal are effectively contributing to the reduction of potential human fecal impact. Risk assessment efforts should be concentrating on the possible presence of zoonotic pathogens including bacterial (e.g., pathogenic *E. coli*, *Campylobacter*[37]) or parasitic (e.g., *Cryptosporidium*, *Giardia*[38]) pathogens. In this respect a future issue for site directed management will be the question of the relative importance of livestock (cattle) versus wildlife ruminants.

The development and application of MST methods to tackle this issue will be of high practical value as optimal management strategies as well as associated health risks for these two animal fecal source groups may differ significantly.[40] Finally, the low and uncertain BacH values should be further verified in order to clarify whether human fecal pollution at trace concentrations also need to be included at respective risk assessment scenarios.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary experimental details, Tables S1 and S2, and Figures S1, S2 and S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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