Image caption: Dr. Matthew Covington observes a dye-trace study.

Comparative Microbial Community Dynamics in a Karst Aquifer System and Proximal Surface Stream in Northwest Arkansas

Matthew D. Covington[1*], Kristen E. Gibson[2], Josue Rodriguez[1]

[1]Department of Geosciences, University of Arkansas, [2]Department of Food Sciences, University of Arkansas
*Corresponding author

Abstract: Northern Arkansas is underlain largely by carbonate bedrock, with relatively well-developed karst flow systems. Much of this region is rapidly urbanizing, leading to a variety of potential threats to groundwater including increased and redirected runoff and the potential introduction of contaminants into the subsurface via septic systems, effluent wastewater discharge, and agricultural runoff. Here, Blowing Springs Cave (BSC) and Little Sugar Creek (LSC) were selected to serve as a model for how non-point source pollution may move through the subsurface and subsequently impact springs as well as receiving streams via contaminated water and resuspension of contaminated sediments. The objectives of the study were to: 1) explore structure, diversity, and temporal variability of microbial communities in BSC and LSC; 2) differentiate allochthonous bacteria from land surface runoff with bacteria in the sediments and water of the karst aquifer; 3) determine impact of sediment movement from karst springs to LSC through comparison of microbial communities; and 4) delineate the recharge area of BSC and constrain potential sources of *E. coli*. Water and sediment samples were collected routinely once per month for 9 months and during 2 rain events in a 3-day time series (1, 2, 4 d). During the study period, 92 water samples and 89 sediment samples were collected. Analysis of water samples for *E. coli* showed significantly higher median levels in LSC (120 MPN/100mL) when compared to BSC (56 MPN/100mL). Moreover, there was a strong correlation between discharge and levels of *E. coli* at BSC (Spearman's R=0.79, $p<<0.05$); however, this same relationship was not observed in LSC. It is evident that there are significant differences in the microorganisms present in water and sediment samples regardless of event type and sampling location. Last, dye tracing indicated a connection between Blowing Spring and a sinkhole located approximately 1 km to the NE. The average flow velocity of the tracer between the injection point and spring was approximately 40 m/day. The results of the study suggest that sources of *E. coli*, and microbial diversity in general, are different between the karst system and surface stream, even though LSC is under the influence of BSC.

Key Points:

- *Escherichia coli* concentrations were significantly higher in Little Sugar Creek (median=120 MPN/100 mL) than in Blowing Spring Cave (median=56 MPN/100 mL).
- *E. coli* concentrations at Blowing Spring Cave were strongly correlated with discharge (Spearman's R=0.79, $p<<0.05$), whereas concentrations at Little Sugar Creek showed no statistically significant correlation with discharge.
- There was significant dissimilarity in microbial composition among water and sediment samples regardless of location or event type.

Introduction

Northern Arkansas is underlain largely by carbonate bedrock, with relatively well-developed karst flow systems. Much of this region is rapidly urbanizing, leading to a variety of potential threats to groundwater including increased and redirected runoff and the potential introduction of contaminants into the subsurface via septic systems, effluent wastewater discharge, and agricultural runoff (Heinz et al., 2009; Katz et al., 2010). Impacts to groundwater can harm fragile karst ecosystems, but also pose direct threats to the public utilizing groundwater (Johnson et al., 2011). The karst systems within the Ozark Plateaus contain numerous linkages to surface water, with water often repeatedly entering and leaving the subsurface through karst sinking streams and springs. A large percentage of the population of Northern Arkansas utilizes decentralized wastewater treatment systems located within karst terrain. Consequently, threats to groundwater quality are also threats to surface water quality, which is used widely in the region for both drinking water and recreation.

The sites selected for the present study—Blowing Springs Cave (BSC) and downstream receiving surface water, Little Sugar Creek (LSC)—do not currently reside in an ANRC 319 Nonpoint Source Pollution Program priority watershed nor is the LSC or its tributaries listed on the ADEQ 303(d) list; however, there are several reasons for selecting these study sites. The Elk River Watershed (ERW), in which LSC resides, was identified in 1998 as impaired by the Missouri Department of Natural Resources due to excess nutrients primarily related to livestock and population growth. The ERW is bound in the east and west by the White River and Illinois River basins, respectively. Finally, Sugar Creek in Missouri has been listed on the 303(d) list for impairment related to low dissolved oxygen levels since 2006, though the source has yet to be identified.

Meanwhile, BSC is the site of several past and ongoing scientific studies. Specifically, Knierim et al. (2015) provided over six years of data on the presence of the *Escherichia coli* (*E. coli*) at the BSC discharge point as well as nitrate and chloride levels from 1992 to 2013. From 2007 to 2013, *E. coli* concentrations at BSC ranged from <1 to 2,420 most probable number (MPN) or colony forming units (CFU) per 100 mL. Median *E. coli* concentrations at base flow periods and during storm events were reported at 41 and 649 MPN or CFU per 100 mL, respectively, and storm event *E. coli* was significantly greater than base-flow concentrations. Based on the data, Knierim et al. (2015) hypothesized that septic tank effluents were a major contributor to chloride, nitrate, and *E. coli* levels in BSC. This hypothesis was largely based on the estimated recharge area for the spring, which was within a residential area that was known to have septic tanks present. Therefore, we selected the sites in the present study to serve as a possible model for how septic tank effluents may move through the subsurface and subsequently impact springs as well as receiving streams via contaminated water as well as resuspension of contaminated sediments.

The objectives of this study were to: 1) explore structure, diversity, and temporal variability of microbial communities in BSC and LSC; 2) differentiate allochthonous bacteria from land surface runoff with bacteria in the sediments and water of the karst aquifer; 3) determine impact of sediment movement from karst springs to LSC through comparison of microbial communities; and 4) delineate the recharge area of BS and constrain potential sources of *E. coli.*

Methods

Sample Collection

Routine sampling was conducted in BSC and LSC once per month from March to November of 2016. Samples were collected from three sites along the main stream of BSC and from LSC at four sites, one rural and three within the town of Bella Vista (Figure 1). Water samples consisted of 500 mL grab samples. Sediment samples (10 cm depth) were collected using a core sampler or scoop and placed in sterile Whirl-Pak® bags. Two storm events were also sampled at higher temporal resolution, with a threshold precipitation of 0.5 in in a 24-h period to trigger a storm sampling series. Storm sampling was conducted during the receding limb with samples taken approximately 1, 2, and 4 days following peak flow.

Dye Tracing

A dye tracing test was conducted to better constrain the recharge area of BSC. The hypothesized recharge area for BSC (Knierim et al. 2015) was searched for potential injection sites, and a single prominent sinkhole was identified within the basin. Fluorescein dye was chosen for the tracing experiment to minimize adsorption onto sediment within the sinkhole. Before introduction of dye into the sinkhole, approximately 50 gal of BSC water were dumped into the sinkhole. This was followed by 55 g of fluorescein dye dissolved in 500 mL of water, and then an additional 450 gal of spring water. Dye was detected using activated charcoal packets, which were deployed in the field to cumulatively absorb dye. Dye was extracted from the charcoal packets in the lab using an alcohol-potassium hydroxide eluent. Elutant was analyzed on a Shimadzu RF-5301 Spectrofluorophotometer. Before injection of dye, charcoal packets were placed in the field to determine any background fluorescence. Charcoal packets were placed in BSC, LSC, and all other nearby springs that were identified. To better determine the timing of the dye pulse, a GGUN-FL24 field fluorometer was deployed in the cave stream.

E. coli Analysis

For detection and enumeration of *E. coli* in water samples, Standard Method 9223B IDEXX Quanti-tray® 2000

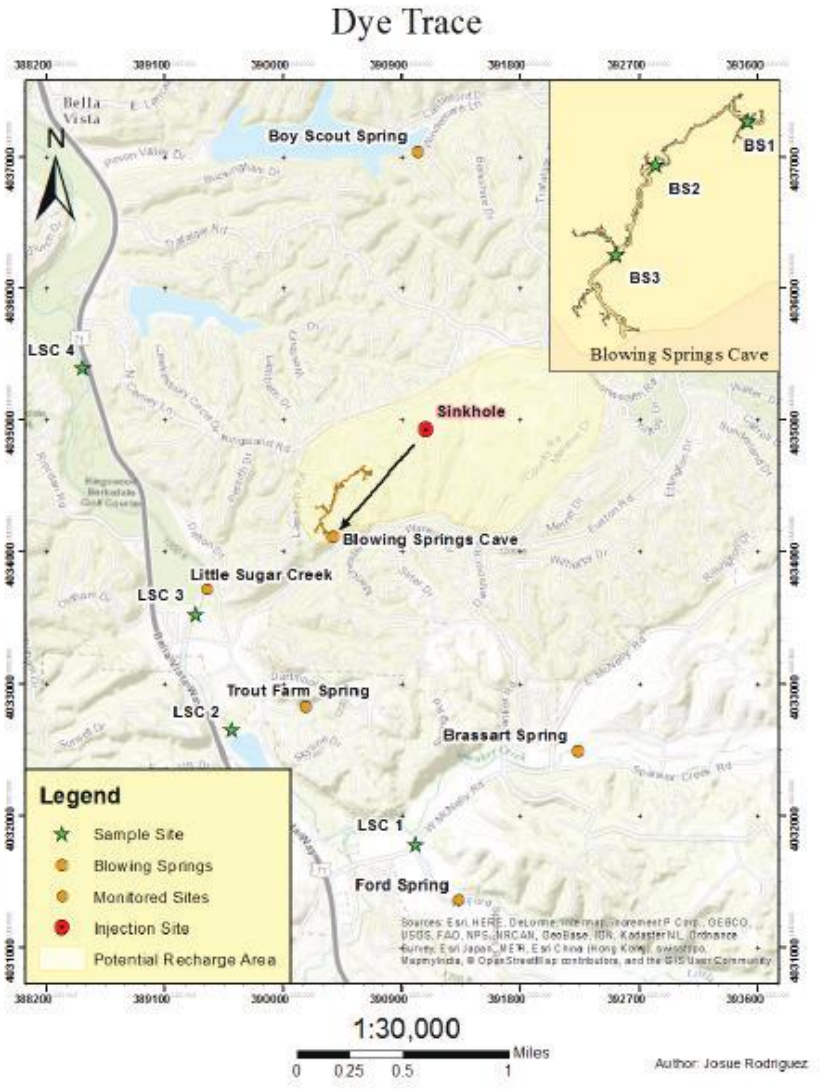

Figure 1. Locations of the sampling points, dye injection, and charcoal packet deployment. A positive trace was detected from the sinkhole site to Blowing Spring Cave (indicated by arrow), but not at the other monitored sites.

system with Colilert™ reagent was used to determine the most probable number (MPN) in each sample. A negative control containing 100 ml of 0.1% peptone was analyzed by Colilert™ for each batch of samples.

DNA Extraction – Water and Sediments

For each sampling event, 200 mL of water from BSC and LSC was filtered through a 0.2-µm, 47-mm Supor-200 filter membrane to capture total bacterial cells. Filter membranes were placed at −80°C in 500 µl of guanidine isothiocyanate buffer. The total genomic DNA (gDNA) was extracted from prepared filters using the Fast DNA Spin Kit for Soil (MP Biomedicals). Genomic DNA was extracted from sediment samples as described by Gomes et al. (2007). Total gDNA was quantified using a NanoDrop UV spectrophotometer.

16S rRNA Metagenomic Analysis

Extracted gDNA from water and sediment samples was used as template DNA for amplification of 16S ribosomal RNA (rRNA) gene by polymerase chain reaction (PCR) as described by Kozich (2013). The PCR analysis was completed through the service center at the University of Arkansas under the direction of Program Associate Dr. Si Hong Park. Briefly, forward and reverse primers targeting the 16S rRNA gene including the partial adapter overhang sequence, PCR master mix, and templated DNA were combined in a single PCR reaction well for each sample. The resulting PCR amplicons were verified by gel electrophoresis. 16S rRNA metagenomics for determination of bacterial community structures in water and sediment samples collected from the karst aquifer system (BSC) and receiving surface stream (LSC) over a 9-month period was completed at the University of Arkansas. The high quality sequence reads have been assembled. For data analysis, bioinformatics procedures using QIIME for operational taxonomic unit (OTU) assignment was applied as described by Kozich et al. (2013). Data are currently being analyzed to answer research questions.

Results

Both monthly and rain event water samples were collected at BSC (n=42) and LSC (n=56) (Tables 1 and 2). *E. coli* MPN/100 mL ranged from 0.9 to 921 at BSC and 4 to >2419.6 at LSC. *E coli*. concentrations were compared against discharge at both sites (Figure 2). Similar to Knierim et al. (2015), the highest *E. coli* concentrations at BSC in the present study were seen during and following high flow events. The correlation between discharge and *E coli*. was strong at BSC as quantified using Spearman's rank correlation coefficient (Rs=0.79, p<<0.05). In contrast, LSC showed no statistically significant correlation between discharge and *E coli.* concentrations (R_s=-0.1, p=0.33). Though *E. coli* concentrations generally increase at BSC during high discharge events, the relationship between discharge and *E. coli* displays some hysteresis, with peak concentrations occurring after peak discharge and during the time of flow recession (Figure 3). *E. coli* concentrations were statistically higher in LSC than in BSC as indicated by a nonparametric Mann-Whitney U test (p<0.005). The median *E. coli* concentration at BSC was 56 MPN/100 mL, whereas the median at LSC was 120 MPN/100 mL. While

Table 1. *E. coli* concentrations (MPN/100 mL) and stream discharge at the Blowing Spring Cave sites.

	E. coli			Q_{bs} (cms)
Date	BSC1	BSC2	BSC3	
3/7/2016	1	0.9	0.9	0.038
4/4/2016	10.9	12.2	23.3	0.04
5/2/2016	435.2	285.1	290.9	0.097
5/25/2016	63.7	63.7	63.7	0.055
5/26/2016	165	165	165	0.093
5/27/2016	866.4	920.8	648.8	0.062
6/6/2016	143	165.8	117.8	0.041
7/11/2016	224.7	209.8	325.5	0.052
8/8/2016	161.6	88.2	88	0.052
9/8/2016	4.1	4.1	4.1	0.032
10/5/2016	48.7	48.7	48.7	0.015
10/6/2016	34.1	44.8	35.5	------
10/7/2016	18.3	18.9	24.3	------
11/10/2016	2	9.7	4.1	0.029

Table 2. *E. coli* concentrations (MPN/100 mL) and stream discharge at the Little Sugar Creek sites.

	E. coli				
Date	LSC1	LSC2	LSC3	LSC3	Q_{lsc} (cms)
3/7/2016	22.7	45.3	15.4	22.7	2.41
4/4/2016	22.8	116.2	4.1	12.2	4.08
5/2/2016	137.6	86	100.8	93.2	7.4
5/25/2016	920.8	2419.6	2419.6	2419.6	3.73
5/26/2016	78.9	2419.6	816.4	770.1	7
5/27/2016	275.5	1413.6	344.8	365.4	5.34
6/6/2016	61.3	23.5	73.8	124.6	4.79
7/11/2016	36.4	461.1	113.7	41.4	7.84
8/8/2016	30.5	58.3	75.4	13	4.34
9/8/2016	1413.6	106.1	125.9	31.5	1.06
10/5/2016	160.7	2419.6	816.4	488.4	1.74
10/6/2016	95.9	980.4	410.6	248.1	1.94
10/7/2016	114.5	920.8	579.4	547.5	2.07
11/10/2016	52.8	298.7	218.7	83.9	1.54

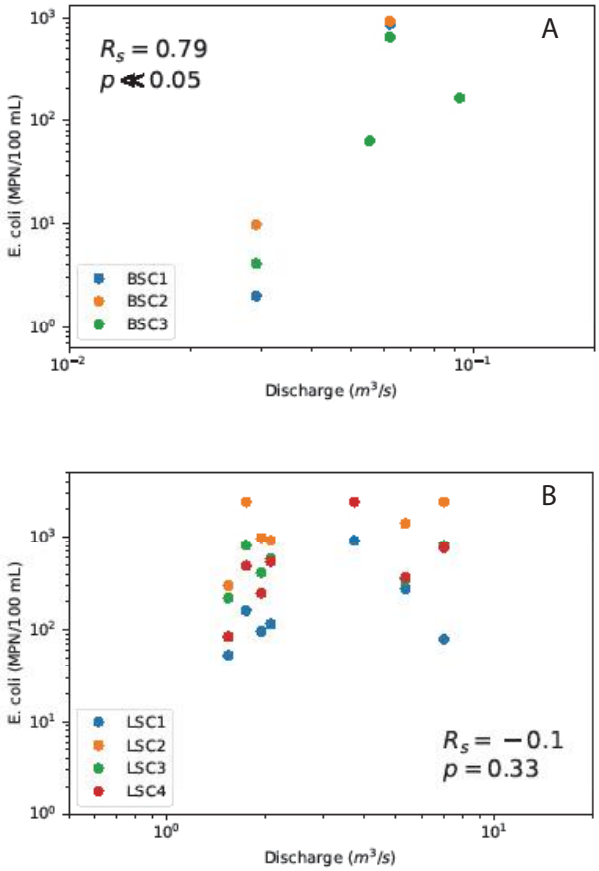

Figure 2. Discharge versus *E. coli* concentrations in Blowing Spring Cave (a) and Little Sugar Creek (b) during the study period. BSC1 is the site that is furthest downstream within the cave, and BSC3 is furthest upstream. LSC1 is the site that is furthest upstream, and LSC4 is furthest downstream. Spearman rank correlation coefficients (Rs) indicate that there is a strong positive correlation between *E coli.* and discharge at BSC, but there is no statistically significant correlation at LSC.

E. coli concentrations were typically similar at all of the cave sites (Figure 4a), the LSC site located just downstream from Bella Vista Lake (LSC2) frequently had higher concentrations (Figure 4b), with a median value of 380 MPN/100 mL.

Figures 5a and 5b show the genus level metagenomic results for water and sediment samples from the different sampling sites in BSC and LSC during a routine sampling event on May 2, 2016. The most abundant bacterial genus in water samples was *Acinetobacter*--a gram negative bacteria commonly found in soil and water--followed by *Pseudomonas* and *Flavobacterium*, again both common to the soil and freshwater environments (Figure 5a). The family *Enterobacteriaceae* which includes *E. coli* is also represented at most water sampling locations though at lower percentages. With respect to sediment collected during the same routine sampling event, the microbial make up is quite different than paired water samples across all sampling sites (Figure 5b). The major bacterial families identified in sediment were *Bacillaceae* and *Enterobacteriaceae*, and one of the primary genera detected was *Clostridium*. The family *Bacillaceae* includes *Bacillus*, a microbe ubiquitous in nature. Meanwhile, *Clostridium* is also a soil microbe as well as an inhabitant of the intestinal tract of animals, including humans.

Samples were also analyzed by sample type for beta diversity which is the diversity of microbes between samples within a specific group. The weighted principal coordinate

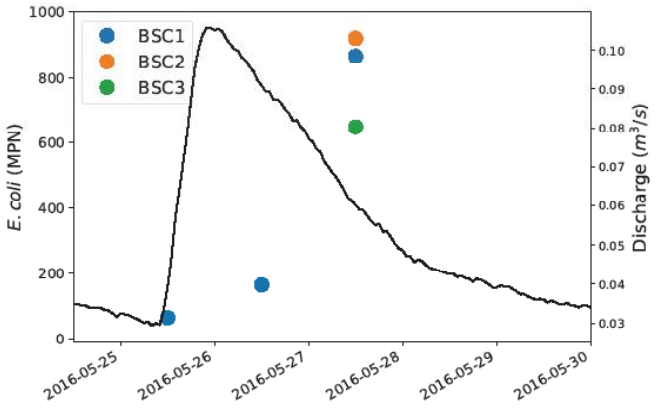

Figure 3. Hydrograph and *E. coli* concentrations at Blowing Spring Cave during a storm event. Peak *E. coli* concentrations occur after the time of peak discharge, during recession flow.

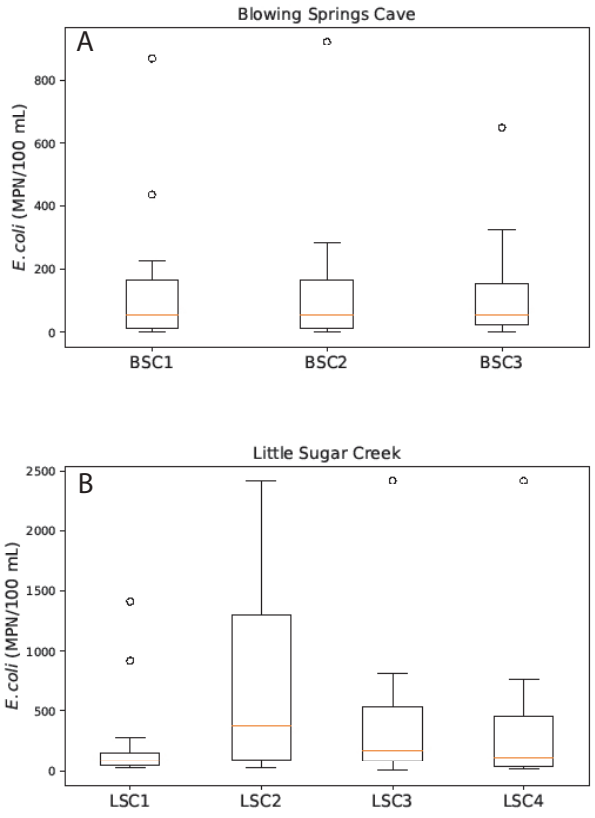

Figure 4. Boxplots of *E. coli* concentrations at: a) the three sites within Blowing Spring Cave from downstream (BSC1) to upstream (BSC3), and b) the four sites within Little Sugar Creek. Boxes indicate the median and quartile values and whiskers represent the range. Circles depict outliers, which are data points that lie outside of the box by more than 1.5 times the interquartile range. Note that the y-axis range on the Little Sugar Creek plot is much larger than on the Blowing Spring plot.

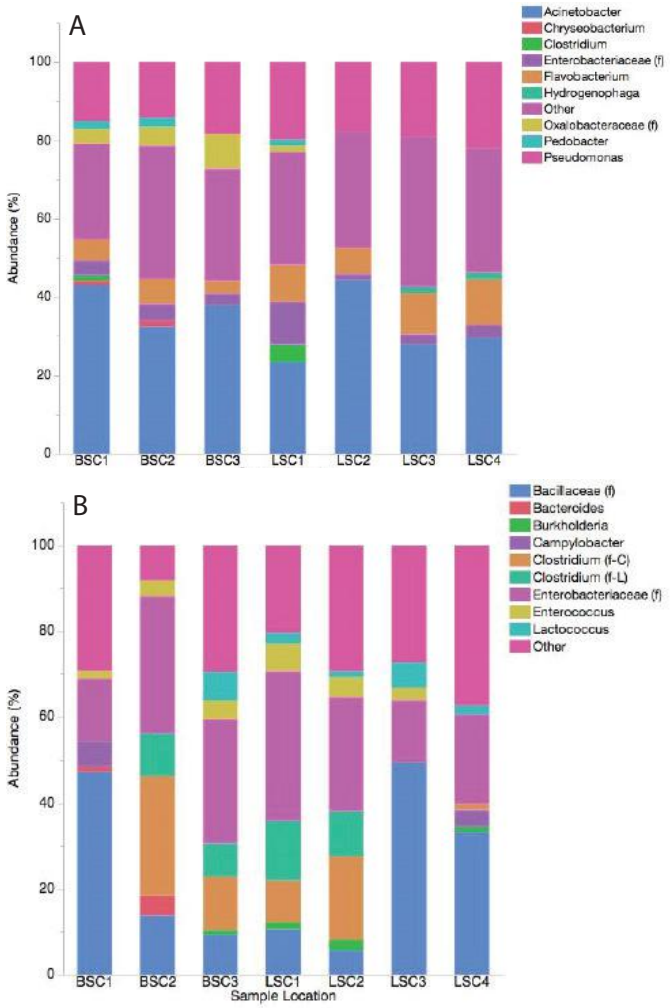

Figure 5. Relative abundance of major bacteria across the various sampling locations at the genus level in water (a) and sediment (b) collected on 5/2/2016. f in parenthesis indicates family, while f-C indicates family *Clostridiaceae* and f-L indicates family *Lachnospiraceae*--two families containing the genus *Clostridium*.

analysis (PCoA) UniFrac plot shown in Figure 6 illustrates the level of abundance of operational taxonomic units (OTUs) among sample types and their respective phylogenetic distances. In Figure 6, each data point representing an individual sample was aligned in parallel on the PC1 axis with 38.68%. An R value close to 1 was used to indicate that there was dissimilarity among sample type while an R value near 0 meant no separation. An R value from the weighted PCoA plot was 0.71 which implied a significant dissimilarity among water and sediment samples regardless of location or event type.

Fluorescein dye (55 g) was injected into the sinkhole site on February 27, 2017, during a relatively dry period. Following heavy rains, dye was detected at Blowing Spring within a charcoal packet that was deployed from March 13-27, 2017. Additionally, a fluorescein pulse was detected on the field fluorometer on March 25, 2017. This suggests a travel time of approximately 26 days over a straight-line distance of 1100 m, giving an average velocity of roughly 40 m/day. There were no positive detections at the other monitored

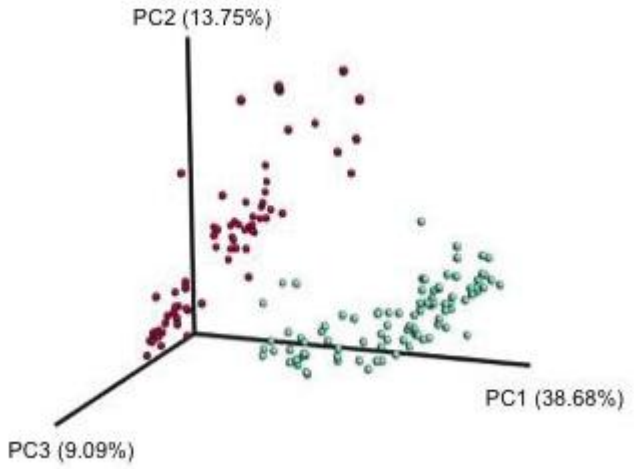

Figure 6. Beta diversity analysis among sample type, water (green) and sediment (red). Weighted principal coordinate analysis (PCoA) Unifrac plot of individual samples for each sample type.

sites. This trace confirms a positive connection between BSC and a portion of the recharge area hypothesized by Knierim et al. (2015) that lies within a residential area that contains some remaining septic tanks.

Conclusions, Recommendations, and Benefits

Even though Little Sugar Creek (LSC) receives contributions from numerous karst springs, such as Blowing Spring, the *E. coli* dynamics at the two sites are quite different, with concentrations at BSC displaying a strong positive correlation with discharge, and LSC showing no statistically significant correlation. *E. coli* concentrations at BSC peak during the recession period of storm events rather than during peak discharge. This could indicate that the contaminants are not mobilized from storage within the system but rather are delivered after recharging storm water has reached the spring. LSC frequently shows *E. coli* concentrations above the primary contact limit (410 CFU/100 mL) and sometimes above the secondary contact limit (2050 CFU/100 mL), indicating potential concerns for recreational users of the stream. The lack of correlation with discharge suggests that introduction of *E. coli* into the stream is not strongly linked with runoff, and that the sources are different than in BSC, where the contamination is hypothesized to result from septic tanks in the recharge area (Knierim et al. 2015). Concentrations just downstream of Bella Vista Lake (at LSC2) are particularly high, suggesting a source near that reach of the stream. Metagenomic analysis indicates that the microbial communities within the water and sediment are significantly different, and the cave and surface stream communities also display some differences. This study provides insight into the microbial communities of karst spring and surface waters within a mixed urban and agricultural setting, where much of the population relies on decentralized wastewater treatment. This combination of geology and land use is common throughout the Ozark Plateaus and more widely throughout the southern and eastern United States. Therefore, insight gained here is likely to apply widely across the region.

Acknowledgements

This material is based upon work supported by the United States Geological Survey under grant agreement No. G16AP00040 and administered by the Arkansas Water Resources Center. The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the opinions or policies of the U.S. Geological Survey.

References

Gomes, N.C.M., L.R. Borges, R. Paranhos, F.M. Pinto, E. Krögerrecklenfort, L.C.S. Mendonça-Hagler, and K. Smalla. 2015. Diversity of ndo genes in mangrove sediments exposed to different sources of polycyclic aromatic hydrocarbon pollution. Applied and Environmental Microbiology. 73(22):7392-7399.

Heinz, B., S. Birk, R. Liedl, T. Geyer, K.L. Straub, J. Andresen, K. Bester, and A. Kappler. 2009. Water quality deterioration at a karst spring (Gallusquelle, Germany) due to combined sewer overflow: evidence of bacterial and micro-pollutant contamination. Environmental Geology. 57:797-808.

Johnson, T.B., L.D. McKay, A.C. Layton, S.W. Jones, G.C. Johnson, J.L. Cashdollar, D.R. Dahling, et al. 2011. Viruses and bacteria in karst and fractured rock aquifers in East Tennessee, USA. Ground Water. 49(1):98-110.

Katz, B.G., D.W. Griffin, P.B. McMahon, H.S. Harden, E. Wade, R.W. Hicks, and J.P. Chanton. 2010. Fate of effluent-borne contaminants beneath septic tank drainfields overlying a karst aquifer. Journal of Environmental Quality. 39:1181-1195.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., & Schloss, P.D. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology. 79:5112–5120.

Knierim, K.J., P.D. Hays, and D. Bowman. 2015. Quantifying the variability in Escherichia coli (E. coli) throughout storm events at a karst spring in northwestern Arkansas, United States. Environmental Earth Sciences. 74:4607-4623.