Ozarks Environmental and Water Resources Institute (OEWRI)
Missouri State University (MSU)

Bacteria Source Tracking Assessment of Sequiota Spring, Springfield Missouri (June 2019-July 2020)

FINAL REPORT

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SCOPE AND OBJECTIVES

Sequiota Spring is located in one of Springfield, Missouri's most popular public parks and has a long history of pollution due to domestic wastewater contamination within the recharge area (Bullard et al., 2001; WWE, 2001). High levels of *E. coli* have been found in several local streams as Pearson Creek, Wilson Creek, and 39 miles of the James River upstream of Lake Springfield are all on the 2020 Missouri Department of Natural Resources 303d list of impaired streams for *E. Coli* concentrations that exceed whole body contact standards (MDNR, 2020). Sequiota Spring flows into Galloway Creek, which is not specifically on the 303d list, but is a tributary to Springfield Lake. The presence of *E. coli* bacteria is an indicator of fecal contamination by warm-blooded animals, including humans, and identifying bacteria sources are important for focusing management efforts that could ultimately reduce *E. coli* concentrations and potential waterborne diseases in the stream and cave system (Davis et al., 2005; Gentry et al., 2006; MSS, 2019). The City of Springfield has been working to remediate aging sanitary sewer infrastructure in the recharge area to help improve water quality and reduce human health risk in local streams. Furthermore, local agencies have been proactive by conducting several recent studies to help isolate hotspots, determine specific sources of contamination, and test the effectiveness of remediation efforts (Mirza et al., 2018; Owen et al., 2017; Owen et al., 2018, Owen et al., 2019).

For this study, the City of Springfield contracted the Ozarks Environmental and Water Resources Institute (OEWRI) at Missouri State University to perform a bacteria source tracking study on water entering the lake at the Sequiota Spring cave entrance. The purpose of this project is to identify the variability of bacterial contamination of Sequiota Spring from three potential sources (i.e., human, waterfowl, and dog) and total bacteria abundance using real-time PCR of specific marker genes. The specific objectives of this study are: (i) collect three sets of triplicate water samples for bacteria source tracking analysis at the cave entrance, approximately 2 weeks apart, over approximately a 1-year period; (ii) analyze samples for identification total bacteria, human, goose, and dog markers; and (iii) quantify the temporal variability of bacteria concentrations and contamination sources over the study period. Results of this study will be compared to data collected using similar methods after completion of sanitary sewer remediation in the recharge area.

STUDY AREA

Sequiota Spring is located in southeast Springfield within the Galloway Creek watershed. Galloway Creek is a tributary of the James River and is within the Lake Springfield-James River Watershed (12-digit Hydrologic Unit Code (HUC) 110100020108) (Figure 1). The underlying

geology of the watershed is Burlington-Keokuk Limestone (Mississippian) within which a karst landscape has formed where sinkholes, losing streams, and springs are common (Thomson, 1986). There are a total of 10 mapped springs within the Galloway Creek watershed with Sequiota Spring being the largest with flow rates varying from 0.6-11.0 million gallons per day (Vineyard and Feder, 1982). The Sequiota Spring recharge area is approximately 4.8 mi^2 and drains significant portions of urbanized southeast Springfield. Dye tracing experiments have linked several ponds and losing sections of Galloway Creek to the spring (WWE, 2001) (Figure 2). The spring exits a cave and flows down a short channel section into a small lake before overflowing the spillway into Galloway Creek (Figure 3).

METHODS

Water Sampling

Three sets of samples were collected in June 2019, November/December 2019 and July 2020. Each period included a set of two sampling events roughly two weeks apart. Triplicate water samples were collected in 8 L sterilized polypropylene carboy containers during each sampling event to quantify bacteria source tracking variability. All samples were placed on ice after collection and transported to the Microbiology Laboratory at MSU within two hours of sampling. During the bacteria source tracking sampling, additional samples were collected for quantifying bacteria counts using the IDEXX method to compare with the source tracking results. These samples were collected in sterile 125 mL plastic bottles and processed in the OEWRI laboratory at MSU within two hours of sample collection for comparison. While numerous waterfowl reside on the lake year-round, the short channel section between the cave and lake flows continuously during low flow. Water samples were collected approximately 20 feet upstream of the cave entrance to avoid contamination from the lake downstream as much as possible.

Laboratory Methods

IDEXX Methods

Samples were analyzed for the presence of *E. Coli* using the IDEXX Colilert® and Quanti-Tray® method for detection and enumeration (OEWRI, 2013). Both field duplicates and field blanks were collected for quality control purposes. Dilution of 1-part sample to 1-part deionized water were used when concentrations exceeded the upper detection limit of 2,419.9 MPN/100 mL. The lower detection limit for this method is 1 MPN/100 mL with accuracy of + or – 20%. IDEXX MPN Generator 3.2 software was used for confirming MPN of sample results, as well as calculation of 95% confidence intervals.

DNA Extraction

Approximately 1-2 liters of water per sample were filtered through 0.22 μm Sterivex filters (Millipore Corporation, MA) using a peristaltic pump (Masterflex, Cole–Pamer Co, Vernon Hills, IL, USA) (Table 1). Filters were broken and membranes were aseptically cut into small pieces in 2 mL micro-centrifuge tubes, which were used for DNA extraction. Genomic DNA from 18 samples was extracted using the DNeasy PowerLyzer DNA extraction kit (Qiagen, Valencia, CA, USA). All extraction steps were followed according to the manufacturer's instructions, and DNA samples were stored at –20°C until analyzed.

Real-time PCR for specific marker genes

Bacteroidetes specific to human, waterfowl, and dog fecal bacteria were determined by using group-specific primers (Table 2). These assays were carried out using the same master mix concentrations as described previously (Mirza et al., 2017). Briefly, the TaqMan universal PCR master mix from Thermo Fisher Scientific was used for all TaqMan assays (human and dog markers). Each 20 μL PCR reaction contained 1X PCR master mix, 100 nm of each of the primers and probe, and 2 μL of template DNA. The DNA probes will be modified with 6-carboxyfluorescein (FAM) as a reporter fluorophore on the 5' end and N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) as a quencher on the 3' end (Table 2). For waterfowl and general bacterial testing iTaq Fast SYBR green supermix with ROX (Bio–Rad, Inc., Hercules, CA), 100 nM primers, and 2 μL of template DNA. PCR conditions were as follows: 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56-58°C (depending on marker gene) for 1 min, and extension at 72°C for 30 s. PCR grade water was used as a negative control. The specificity of the qPCR products for SYBR green supermix was confirmed by melting curve analysis. In both types of qPCR (Taq-man and SYBR green), a standard curve was generated from serial dilutions (10^0 to 10^{-9}) of the plasmid DNA of the specific marker gene. The qPCR efficiency (*E*) was calculated according to the equation:

$$E = 10^{[-1/\text{slope}]}.$$

The absolute quantification of the targeted gene was performed by interpolating CT values of unknown samples from the standard curve, the latter was prepared with serial dilutions of the known quantity of plasmid DNA inserted with the targeted gene (16S rRNA, human, waterfowl, and dog-specific marker genes). Details on the standards preparation procedure and quantification have been previously reported (Mirza et al., 2017).

QA/QC

The PCR primer combination used in this study has been previously well tested and optimized for the specific amplification of bacterial marker genes from human (Bernhard and Field, 2000; Seurinck et al., 2005; Harwood and Stoeckel, 2011), waterfowl (Green et al., 2011), and dog

(Kildare et al., 2007) fecal materials. The positive standard DNA material (plasmid with the inserts of specific marker genes) was used as reference material for our unknown water samples. The negative samples (sterile water) did not show any amplification. The regression line of the standard curve generated through serial dilutions of specific marker genes showed a coefficient of determination of 0.997 to 0.999 and a PCR amplification efficiency of 94 to 100%. The specificity of the SyBR Green PCR amplicon was confirmed by the melting curve analysis, which indicated the presence of a single peak for each of the marker genes. Each 96-well qPCR reaction plate included a standard (9 dilutions of plasmid insert with the specific gene) and six controls (three no template and three *Escherichia coli* DNA).

RESULTS AND DISCUSSION

Hydrology

During the sample collection period, 10-day rainfall totals were variable, however groundwater elevations and temperature were relatively consistent. For the June 2019 and November/December 2019 sample sets, groundwater elevations were within 0.1 ft, ranging from 1,228.83-1,228.93 ft from near groundwater wells operated by the City of Springfield (Figure 4, Table 3). At the time this report was written, groundwater elevation for the July 2020 samples were not available. Also, over the same sample periods, water temperature varied just under 4 °F over that time, ranging from 60.4-64.0 °F. The coldest water temperature was recorded during the June 13, 2019 sampling and the warmest during the November 18, 2019 sampling. However, 10-day rainfall totals were highly variable during the summer 2019 and winter 2019 sampling periods ranging from 0.1-3.5 inches measured at Sequiota Elementary School. The consistency of the water levels during sampling suggests that rainfall can move through the shallow groundwater system fairly quickly. Finally, seven-day average sanitary sewer flow rates from a nearby station were also variable, ranging from 1.08 MGD on July 16, 2020 to 2.78 MGD on June 28, 2019.

IDEXX Results

IDEXX *E. Coli* concentrations at Sequiota Spring exceed water quality standards and have an inverse relationship with 10-day rainfall totals suggesting storm water dilution of local sources. The average *E. Coli* concentration from all six samples was 1,185 MPN/100 mL ranging from 488 to 2,827 MPN/100 mL (Figure 5). The geometric mean of the warm weather samples collected in June 2019 and July 2020 was 1,092 MPN/100 mL. This is five times higher than the whole-body contact concentration of 206 counts per 100 mL for Class B streams (MSS, 2019). Site replicate samples collected 9-17 days apart show that in-season variability can range from 50-90% at this site with an average of 74% (Figure 6). In June 2019, the counts decreased from 921

MPN/100 mL on June 13, 2019 to 488 MPN/100 mL on June 28, 2019, while 10-day rainfall totals increase from 1.1" to 3.5" over that time. In contrast, during July 2020, counts increased from 1,120 MPN/100 mL on July 7, 2020 to 2,827 MPN/100 mL on July 16, 2020 while 10-day rainfall totals decreased from 0.4" to 0.0" over that period. In fact, the *E. Coli* concentration from the July 16, 2020 sample were the highest of the entire study. This may suggest a persistent local source that gets concentrated over time, like sewer exfiltration, is being diluted by storm water as new runoff flushes out the karst system. The same pattern can be seen when comparing *E. Coli* counts to average 7-day sanitary sewer flow rates. In general, like rainfall, *E. Coli* concentrations also decrease with increased sanitary sewer flows in seasonal sampling sets suggesting infiltration into the sewer system that is not consistent with a direct source of sewer water as a major factor. Field duplicate analysis shows sample site variability is less than 50% (n=4), with an average of about 26% (Figure 6). Finally, all field blanks collected for this project (n=4) were less than 1 MPN/100 mL.

qPCR Results

Total Bacteria

Total bacteria counts were very high at Sequiota Spring and do not correlate necessarily with the results of the IDEXX method. High abundance of total bacteria cells were detected in all 18 samples ranged from $6.82x10^7$ to $3.29x10^8$ cells/L (Figure 7). In-season and site variability were relatively low between samples. Results do indicate a seasonal trend. In general, total bacteria concentrations were higher in the warm months and lower in the cooler months. The highest totals were in the July 2020 samples, which was also the period with the lowest 10-day rainfall totals. However, total bacteria results do not necessarily correlate well with the IDEXX results. For example, the IDEXX results for November 18, 2019 and July 7, 2020 are similar with both counts near 1,200 MPN/100 mL. In contrast, total bacteria results show the November 18, 2019 counts less than $1.0X10^8$ cells/L and July 7, 2020 counts greater than $1.0X10^8$ cells/L. This suggests the total bacteria analysis may not be necessary when trying to isolate specific potential sources in the future.

Human Fecal Bacteria

Human specific marker genes were detected in five out of the six samples, had the highest abundance of the three markers analyzed for the project, and suggests human fecal contamination within the spring system is still a significant health concern. Detected comparable numbers of human marker genes were relatively high across five of the six sampling dates ranging from $3.17x10^3$ to $1.11x10^5$ cells/L (Figure 7). This is the highest count of the three genetic markers analyzed. However, in one of six sampling events on December 5, 2019, human fecal indicator bacteria were not detected. This appears to be an outlier in this

dataset as IDEXX results and total bacteria counts were similar to the other sampling dates. It may also suggest there are water flow paths that are not connected to surface or sewer areas in the recharge area. In contrast, the last sampling event on July 16, 2020 had highest number of human fecal indicator bacteria ($1.11x10^5$ cells/L). This does correlate generally with the IDEXX and total bacteria results. Human fecal contamination of the Sequiota Spring has been well known for many years (Bullard et al. 2001, WWE 2001). Results of this study suggest human bacteria sources are still present in the Sequiota Spring recharge area. Bacterial contamination originated from human fecal material can pose a serious health risk due to the presence of human pathogens such as *Salmonella*, *Campylobacter*, *Legionella*, pathogenic strains of *E.coli*, *Vibrio vulnificus* etc. As the fecal material from infected individuals can carry high loading of bacterial pathogens, such as up to 10^{10} *Salmonella* cells and 10^9 *Shigella* cells per gram of human feces.

Waterfowl and Dog Fecal Bacteria

The relative abundance of markers from dog fecal indicator bacteria were 5-54 times lower, and waterfowl sources were 90-370 times lower than the abundance of the human marker genes. For five out of the six sampling dates, total abundance of waterfowl fecal indicator bacteria was less than $1X10^2$ cells/L, and on July 16, 2020, it was less than $1X10^3$ cells/L (Figure 7). The total abundance of dog fecal indicator bacteria was less than $1X10^3$ cells/L during five out of the six sampling periods, and on July 16, 2020 it was less than $1X10^4$ cells/L. These patterns generally follow the same trend as the IDEXX, total bacteria, and human specific marker results stated above with respect to 10-day rainfall totals. These results also suggest that fecal contamination from pet waste is higher source of contamination to the spring than from waterfowl. However, these results also show that human fecal contamination is a far greater concern in the system than from either waterfowl or pet waste.

CONCLUSIONS

There are five main conclusions from this project:

1. Groundwater elevations and temperatures were similar for each sample period, however 10-day rainfall totals were variable. Groundwater elevations varied 0.1 ft and temperature varied less than 4°F over both the summer and early fall sampling periods. At the time this report was written, groundwater elevations for the summer 2020 samples were not available. However, 10-day rainfall totals ranged from 0.1-3.5 inches suggesting rainfall moves through the shallow groundwater system fairly quickly. Similarly, 7-day average sewer flow rates were also highly variable.

2. Results of the IDEXX *E. Coli* analysis shows concentrations at Sequiota Spring exceed water quality standards and that variability of concentrations have an inverse relationship with 10-day rainfall totals suggesting storm water dilution of local sources. The geometric mean of the warm weather samples was 1,092 MPN/100 mL which is five times higher than Missouri water quality standards. Finally, sample duplicate analysis shows site variability is less than 50% while in-season variability was greater than 50%. Generally, *E. coli* concentrations decrease with increased 10-day rainfall totals and 7-day average sewer flow rates within the individual season. This may suggest a persistent local source, like sewer exfiltration, is being diluted by storm water that gets concentrated over time without new runoff to flush out the karst system.

3. Total bacteria counts were very high at Sequiota Spring and do not correlate necessarily with the results of the IDEXX method. There was a relatively high abundance of total bacteria cells detected in all 18 samples collected ranged from $6.82x10^7$ to $3.29x10^8$ cells/L with relatively low in-season and site variability. Sample counts were higher in the warm months and lower in the cooler months. However, total bacteria results do not necessarily correlate well with the IDEXX results suggesting the total bacteria analysis may not be necessary when trying to isolate specific potential sources in the future.

4. Human specific marker genes were detected in five out of the six samples, had the highest abundance of the three markers analyzed for the project, and suggests human fecal contamination within the spring system is still a significant health concern. Detected comparable numbers of human marker genes had the highest counts of the three sources analyzed for this project ranging from $3.17x10^3$ to $1.11x10^5$ cells/L. It's well known that Sequiota Spring has been contaminated by domestic wastewater for a long time. Results of this study suggest it is a source of higher concern than pet waste and waterfowl. Bacterial contamination originated from human fecal material can pose a serious health risk due to the presence of human pathogens and pathogenic strains of *E.coli*.

5. The relative abundance of markers from dog fecal indicator bacteria were 5-54 times lower, and waterfowl sources were 90-370 times lower than the abundance of the human marker genes. Waterfowl and dog sources were detected at the spring but are at far lower levels than human sources. Results suggest that fecal contamination from pet waste is higher source of contamination to the spring than from waterfowl. However, these results also show that human fecal contamination is a far greater concern in the system than from either waterfowl or pet waste.

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TABLES

Table 1. Water sampling dates, time, and volume of water sample filtered for DNA extraction

Sample ID	Date	Time	Volume of water filtered
S1	6/14/2019	14:15	1.75 L per replicate sample
S2	6/28/2019	14:00	2 L per replicate sample
S3	11/18/2019	14:00	1.5 L per replicate sample
S4	12/5/2019	13:00	1.5 L per replicate sample
S5	7/7/2020	12:30	1 to 1.25 L per replicate sample
S6	7/16/2020	11:00	1.5 L per replicate sample

Table 2. PCR Primers for the microbial source tracking of different fecal sources.

Primer	Sequence (5' to 3)	Marker	Reference
F Primer HF183	ATCATGAGTTCACATGTCCG	Human	Bernhard and Field, 2000
R Primer SSHBacR	TACCCCGCCTACTATCTAATG		Seurinck et al., 2005
SSHBac-PRB:	(FAM)-TTAAAGGTATTTTCCGGTAGACGATGG-(TAMRA)		Harwood, 2011
GFDF	TCGGCTGAGCACTCTAGGG	Waterfowl	Green et al., 2011
GFDR	GCGTCTCTTTGTACATCCCA		
BacCan-545f1	GGAGCGCAGACGGGTTTT	Dog	Kildare et al., 2007
BacUni-690r2	AATCGGAGTTCCTCGTGATATCTA		
BacUni-656p	FAM-TGGTGTAGCGGTGAAA-TAMRA		
UniBac 515F	GTGC CAGCMGCC GCGG	All bacteria	Lane 1991
UniBac 907R	CCGT CAATTCMTTTRAGTTT		

Table 3. Groundwater level, 10-day rainfall totals, and average sanitary sewer flow rates at the time of sample collection

Sample	Date	Time	Water Level (ft)	Water Temp (°F)	10-Day Rainfall (in)	7-Day Avg. Sewer Flow Rate (MGD)
S1	6/13/2019	14:15	1,228.83	60.1	1.1	1.25
S2	6/28/2019	14:00	1,228.91	63.0	3.5	2.78*
S3	11/18/2019	14:00	1,228.87	64.0	0.1	1.63
S4	12/5/2019	13:00	1,228.93	61.0	0.9	2.26*
S5	7/7/2020	12:30	NA	NA	0.4	1.24
S6	7/16/2020	11:30	NA	NA	0.0	1.08

* Missing data

FIGURES

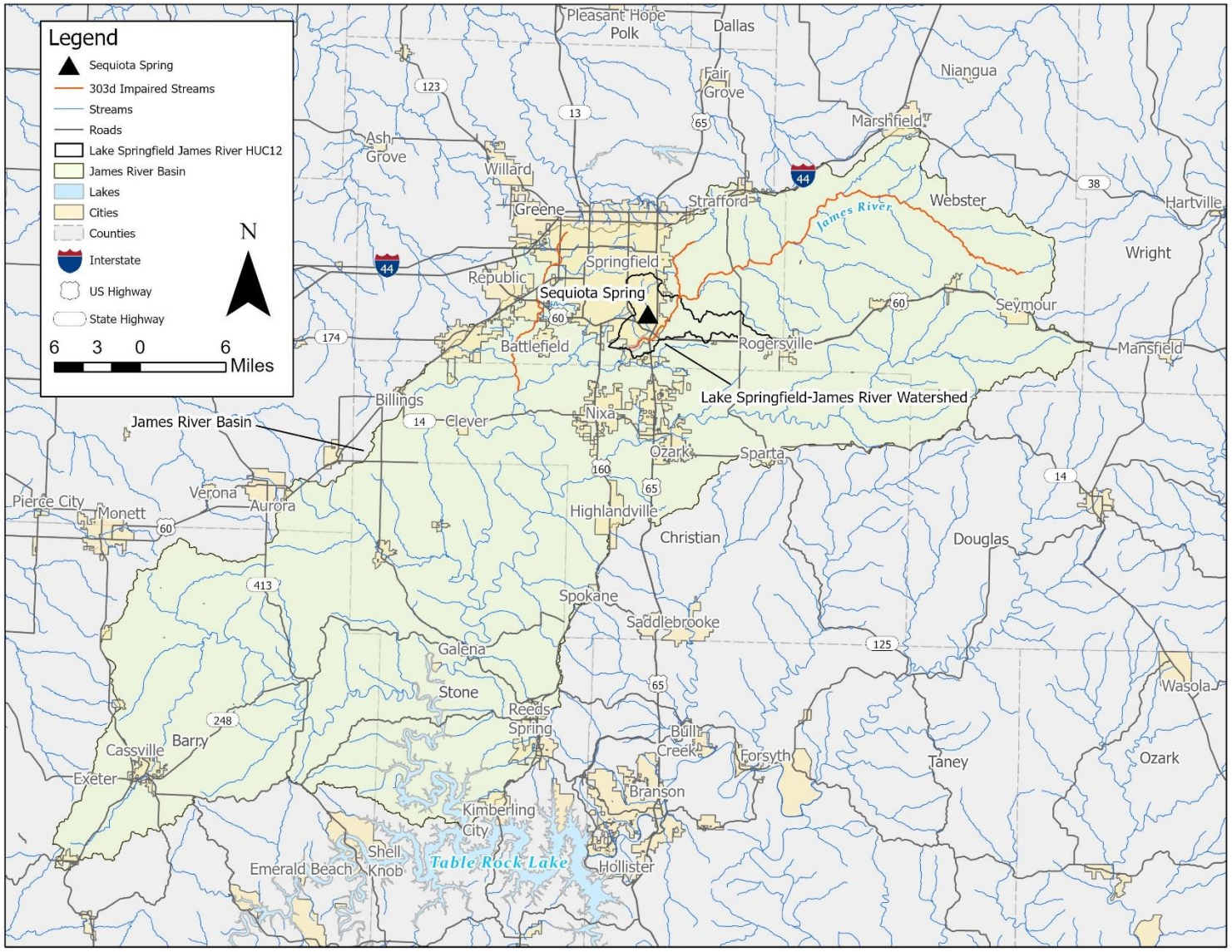

Figure 1. Location of Sequiota Spring within the James River Basin in Southeast Springfield.

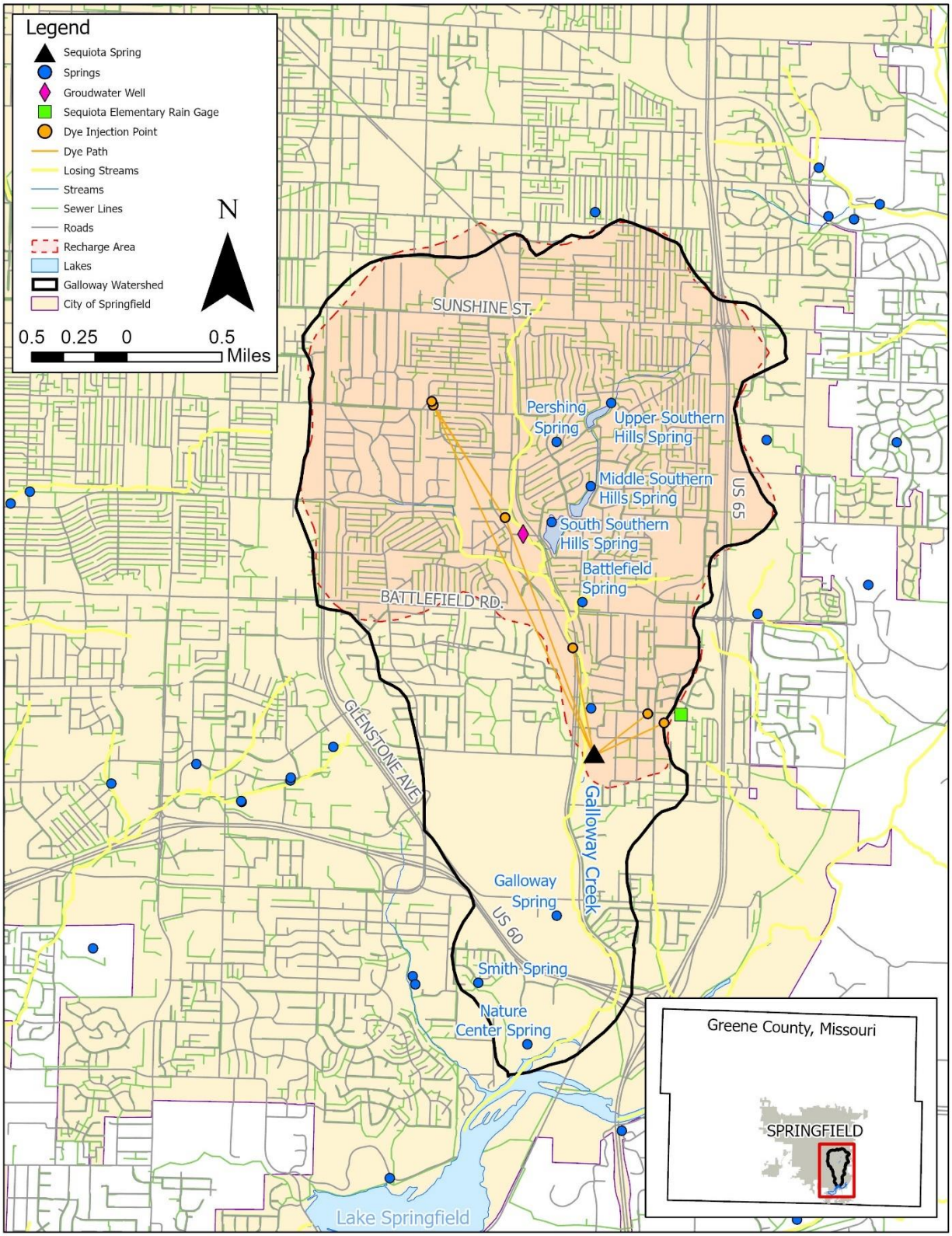

Figure 2. Location of Sequiota Spring and recharge area within the Galloway Creek watershed.

Figure 3. Site map of the cave entrance, spring branch, and lake in Sequiota Park.

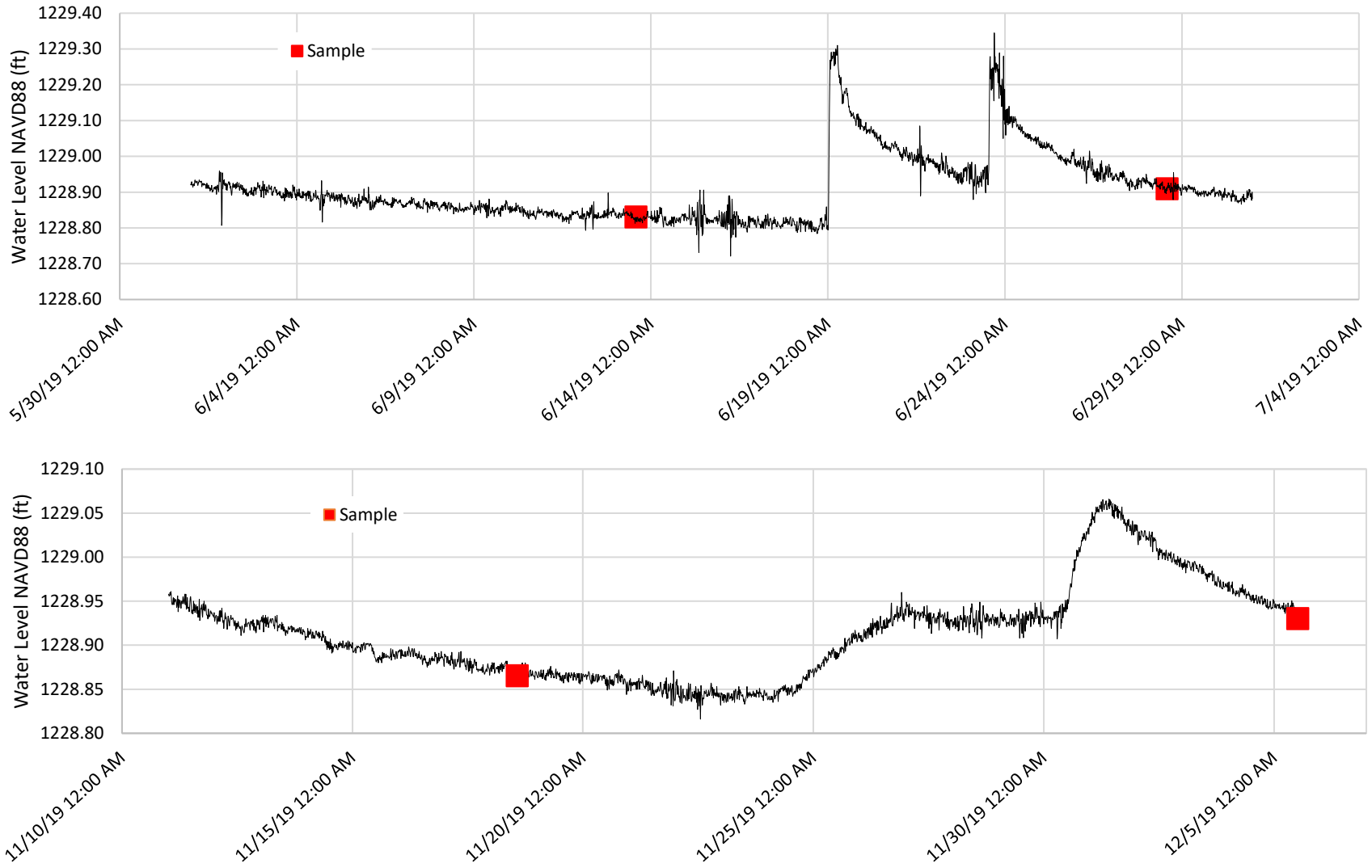

Figure 4. Groundwater elevation and sampling date for A) June 2019 and B) November 2019.

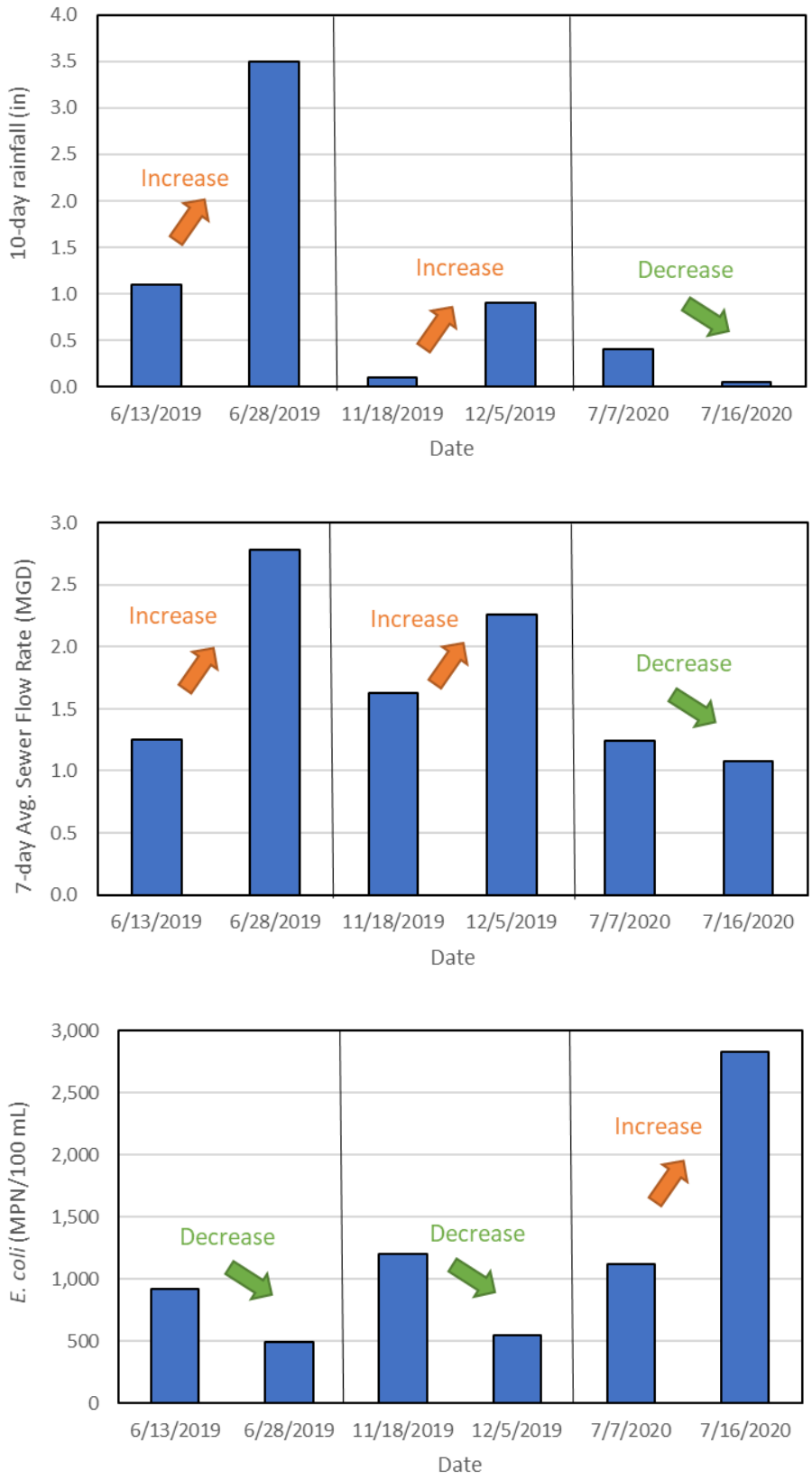

Figure 5. A) 10-day rainfall totals, B) 7-day average sewer flow rate, and C) IDEXX E.coli results.

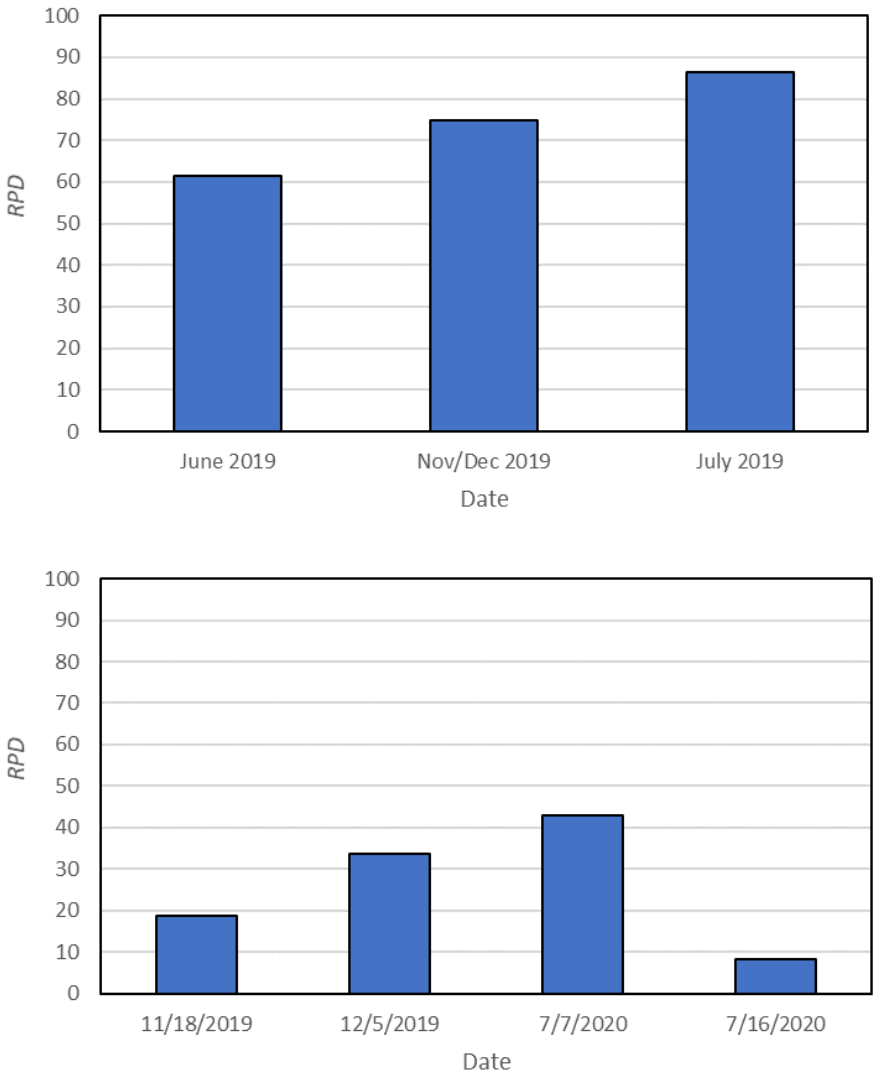

Figure 6. A) In-season variability by sample set and B) field duplicate by sample period for IDEXX *E.coli* results.

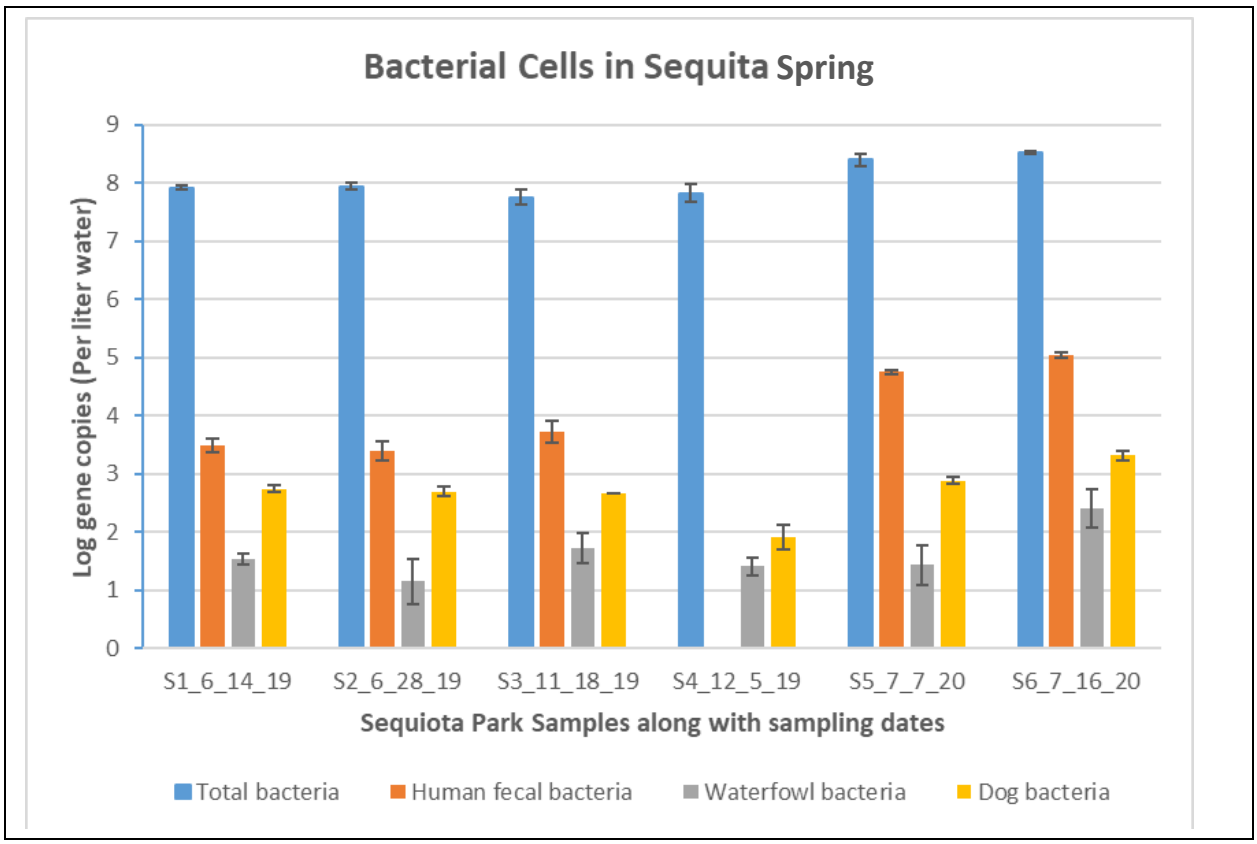

Figure 7. Real-time PCR-based quantification for four marker genes in water samples from the Sequiota cave. The gene copy numbers are given on a log scale. Error bars represent standard errors.