Fate and Transport of *E. coli* Through Appalachian Karst Systems

Diana Schmidt

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Jonathan Czuba, Chair

Leigh Anne Krometis

Madeline Schreiber

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Karst waters serve as important water sources in rural Appalachia and are well-connected to surface waters, making them susceptible to anthropogenic contamination, including by fecal indicator bacteria which represent a public health risk. This work designed and implemented a watershed-scale monitoring program for a 26 km$^2$ sinking stream system in southwest Virginia to determine the fate and transport of *E. coli* in the system. This hydrologically complex watershed is predominantly agricultural and includes multiple key surface water sinks that enter Smokehole Cave and emerge at Smokehole Spring. Field campaigns at surface sites and within Smokehole Cave included bacteriological sampling, hydrologic measurement, and dye tracing. Field data was synthesized to: 1) examine variations in *E. coli* concentrations in the watershed during varying flows/seasonal conditions; and 2) calculate *E. coli* growth/decay coefficients for the karst system during different flow/antecedent conditions. *E. coli* concentrations at Smokehole Spring consistently peaked days after peak hydrologic stage. Flow conditions and storm event response were the largest drivers of *E. coli* transport through the system. Dye trace results revealed that water from sinks can be stored or move slowly through the karst system, resurging during storm events. *E. coli* was calculated to decay within the karst system, with a half-life of about 5-120 days which is longer than the travel time of water through the cave of approximately 0.5-2 days. Findings indicate that *E. coli* transport in Appalachian karst systems is hydrologically driven, roadside spring water collection is not recommended, and bacterial treatment is encouraged if performed. Targeted land-management practices should be explored to decrease *E. coli* loadings in karst waters.
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Abstract (general audience)

Karst (cave) waters serve as important water sources in rural Appalachia and are well-connected to surface waters, making them susceptible to contamination from human or animal waste – a public health risk. A field monitoring program was conducted in an agriculturally impacted stream and cave system in southwest Virginia to determine how *E. coli*, a bacteria found in the waste of humans and other animals, moves through the system. There are several places where surface water sinks into the cave system, eventually entering Smokehole Cave and emerging at Smokehole Spring. Field data collection was performed at surface sites and within Smokehole Cave including sampling for *E. coli*, water flow measurements, and dye tracing. Field data was combined to 1) examine variations in *E. coli* concentrations during varying flows/seasonal conditions and 2) calculate *E. coli* growth/decay coefficients for the cave system during different flow and soil moisture conditions. It was found that *E. coli* concentrations at Smokehole Spring consistently peaked days after the water depth. Flow conditions and storm events were the largest drivers in *E. coli* movement through the system. Dye trace results revealed that water from sinks can be stored or moves slowly through the cave system and resurges during storm events. *E. coli* was found to decay within the cave system. Findings indicate that *E. coli* movement in Appalachian cave systems is driven by storm events, roadside spring water collection is not recommended, and bacteria treatment is recommended if performed. Cave-specific land-management practices are recommended to keep *E. coli* from entering cave waters.
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# Contents

**Introduction** .................................................................................................................. 1

**Methods** .......................................................................................................................... 5  
+ Site Description ................................................................................................................. 5  
+ Field Data Collection Summary ......................................................................................... 9  
+ Water Quality Data Collection ......................................................................................... 10  
+ Bacteria Laboratory Analysis ......................................................................................... 11  
+ Hydrologic Measurements .............................................................................................. 12  
+ Dye Tracing ..................................................................................................................... 12  
+ Data Analysis .................................................................................................................. 13

**Results** ............................................................................................................................. 18  
+ Seasonal and Water Quality Analysis ............................................................................. 18  
+ Hydrologic Event Analysis .............................................................................................. 21  
+ Dye Trace Results ........................................................................................................... 25  
+ Growth/Decay Coefficient Generation ............................................................................ 29

**Discussion** .......................................................................................................................... 32  
+ Observations of Clover Hollow Watershed and Smokehole Cave .................................. 32  
+ Relevance to Prior Literature ......................................................................................... 33  
+ Relevance to Public Health ............................................................................................. 37  
+ Limitations and Future Work ............................................................................................ 38

**Conclusion** ....................................................................................................................... 39

**References** .......................................................................................................................... 40
List of Figures

Figure 1 Conceptual diagram of environmental factors that promote and limit E. coli survival in surface waters and karst systems. ................................................................. 4
Figure 2. Study area map of the Clover Hollow watershed with designated land-use, sampling locations, and associated caves. ................................................................. 6
Figure 3. Map of Smokehole Cave (Personal Communication, Wil Orndorff, VA DCR). ......................... 7
Figure 4. Study area map of Clover Hollow watershed with underlying geology, known karst features and dye trace efforts (Hubbard, 2003) ......................................................... 9
Figure 5. Streamflow stage of Sinking Creek (USGS 0317154954) for water years 2021 through 2023 relative to collected environmental data. .............................................................. 10
Figure 6. E. coli concentrations at surface sites during low flow with EPA recreational limit (logarithmic scale). .......................................................................................................................... 19
Figure 7. Average seasonal E. coli concentrations at surface sites during low flow with EPA recreational limit (logarithmic scale). ................................................................. 19
Figure 8. E. coli concentrations at in-cave sites (May 1, 2022, July 22, 2022, and Nov. 8, 2022) ........... 20
Figure 9. E. coli concentrations at R (red x) and all surface sites (black point) with corresponding water quality parameters ................................................................. 21
Figure 10. E. coli concentrations at R and CHB over storm event and Sinking Creek stage (USGS 0317154954), Sep. 2022. .................................................................................. 22
Figure 11. E. coli concentrations at CHB over storm event with corresponding stage, Sep. – Oct. 2022 .. 23
Figure 12. E. coli concentrations at R over storm event with corresponding stage, Sep. – Oct. 2022 ...... 23
Figure 13. E. coli concentrations and stage at CHB, Mar. 2023 (E. coli concentration on log scale). ...... 24
Figure 14. E. coli concentrations and stage at R, Mar. 2023 (E. coli concentration on linear scale) ...... 25
Figure 15. Dye concentrations at SA, E. coli concentrations at R, and time since release during dye trace 1 (Nov. 11-19, 2022). ........................................................................................................ 26
Figure 16. Dye concentrations at SA, E. coli concentrations at R, and time since release during dye trace 2 (Feb. 6-18, 2023). ........................................................................................................ 27
Figure 17. Dye concentrations at SA, E. coli concentrations at R, and time since release during dye trace 3 (Mar. 23 - Apr. 5, 2023). ........................................................................................................ 28
Figure 18. Fluorescein dye concentration detected at SA and time since injection at J. ........................ 28
Figure 19. Rhodamine WT dye concentration detected at SA and time since injection at CHB. .......... 29
Figure 20. Fluorescein dye concentration peaks at SA with occurrence time of chosen C_{R@t}. .......... 30
List of Tables

Table 1. Table of calculated E. coli decay coefficients for varying flow/antecedent conditions affecting the Jones Creek pathway.

Table 2. Calculated E. coli decay coefficients for varying flow/antecedent conditions affecting the Jones Creek pathway during non-dye traced events.

Table 3. T_{50} and T_{90} for E. coli travelling from Jones Creek for each dye trace.
Introduction

Karst topography is formed by the dissolution of soluble rocks, such as limestone, which creates caves, sinkholes, sinking streams, and springs. Resultant karst environments are unique ecosystems that host a diverse array of species, many of which are unique to individual systems or caves (Niemiller et al., 2015; Bonacci et al., 2008). Karst systems are also home to significant water resources that have long been recognized as critical to downstream anthropogenic and ecosystem needs (Parise et al., 2014). Karst aquifers provide water to hundreds of millions of people, making up 40% and 25% of groundwater utilized for drinking water in the United States and worldwide, respectively (Ghasemizadeh et al., 2012). Wells and springs in karst areas are also an important source of domestic water supply in rural Appalachian communities (Krometis et al., 2019; Pieper et al., 2016). Surface waters used for ecosystem and human services, such as recreation, agriculture, and municipal supplies, are also fed by resurfacing karst waters. However, due to variable travel times, direct input from surface water sources, and absence of ultraviolet (UV) light for degradation, karst systems have the potential to be highly susceptible to bacteria contamination (Green et al., 2006). For instance, a Camplyobacter and E. coli drinking water outbreak in Walkerton, Ontario in 2000, which led to seven deaths and 2,300 reported illnesses, was linked to heavy rainfall event and subsequent surface water contamination of the epikarst system that provided community source water (Worthington et al., 2001, 2002, 2012). Though the impacts of increased anthropogenic contamination of surface waters entering karst systems is assumed to adversely impact karst and surface ecosystems as well as human uses, these impacts are poorly quantified, limiting effective management strategies.
Fecal indicator bacteria (FIB), like *Escherichia coli* (*E. coli*), are currently the leading contaminant responsible for stream and river water quality impairments in Virginia and West Virginia (EPA, 2021a). Given the difficulty and expense associated with direct pathogen monitoring, many water quality efforts rely on measures of FIB as a surrogate of infectious risk (Paruch et al., 2012). FIB enter surface water from a variety of sources including runoff from agricultural fields and feedlots, sewage treatment effluent, septic tank leakages, polluted stormwater runoff, and stormwater runoff carrying animal feces (USGS-MWSC, 2017). At present, culturable *E. coli* is recommended by the Environmental Protection Agency (EPA) as an indicator of fecal contamination in freshwater (USGS-MWSC, 2017). Total coliforms, a broader bacterial family that includes native soil bacteria as well as commensal intestinal species, are recommended by the EPA as indicators of general bacterial contamination of water supplies (Leclerc et al., 2001; EPA, 2023). The Virginia threshold for impairment of recreational waters is 235 counts/100mL of *E. coli* (Virginia Water Control Board, 2016). The national maximum contaminant level goal for *E. coli* and total coliforms in public drinking water systems is 0 mg/L with no more than 5% of samples testing positive for a given month (EPA, 2023). Roadside springs are not regulated and not subject to drinking water standards (Krometis, 2019).

The predominant models and frameworks used in watershed modeling and public health decision making are informed by the study of FIB fate and transport in surface waters. Comparatively little is known about the fate and transport of FIB in karst waters. Fecal contamination of surface and groundwaters can occur from urban and agriculture sources (Buckerfield et al., 2020). Watershed agricultural land use is highly correlated with *E. coli* contamination in surface waters, particularly following livestock grazing and manure application.
Accordingly, high *E. coli* concentrations downstream from springs and cave streams in watersheds with a high percentage of agricultural land use are common (Boyer et al., 2007; Coxon et al., 2011). Leaking or poorly maintained septic systems in rural communities can also increase FIB loadings to groundwater that flow through karst springs (Knierim et al., 2015 & Laroche et al., 2010).

Although FIB such as *E. coli* are facultative anaerobes native to the intestines of warm-blooded animals, FIB persistence and even regrowth has been observed in aquatic habitats (van Elsas et al., 2010; Jang et al., 2017). The various environmental factors that promote and limit bacterial survival are complex and poorly understood. Differences in in karst systems compared to surface waters may limit or further environmental survivability (Figure 1). For example, specific conductivity within karst waters is often higher than that in surface waters due to the higher concentration of dissolved ions from the dissolution of soluble rocks (Hartmann et al., 2014). Higher salinity can assist in osmotic regulation of *E. coli* and promote survival (DeVilbiss et al., 2021). It is unclear if higher specific conductivity in karst systems, due to dissolved calcium carbonate, will have a similar effect of *E. coli* survival. However, higher flow dependent residence times through karst systems are also correlated with a reduction in *E. coli* concentrations, which may be due to dissolution processes, as longer residence times can increase the pH of karst waters and decrease survivability (Buckerfield et al., 2020). As UV radiation inhibits *E. coli* growth and can permanently damage nucleic acids, preventing replication (Vermeulen, 2008), once in the karst system, water is not exposed to UV light, enhancing the potential for survival. Conversely, karst systems in the Virginias retain a temperature of ~12°C (55°F) year-round, whereas the optimal temperature for *E. coli* growth is
37°C (98.6°F) (NCKRI, 2021; Albrecht, 2021). Due to seasonal temperature fluctuations, the survivability of *E. coli* in karst systems due to temperature may increase or decrease relative to surface waters.

**Figure 1** Conceptual diagram of environmental factors that promote and limit *E. coli* survival in surface waters and karst systems.

This study designed and implemented a watershed scale monitoring effort to determine the potential for karst systems to serve as conduits for *E. coli*. In order to better characterize FIB fate and transport in karst systems to inform effective management, this study aims to: 1) quantify *E. coli* concentration change within a sinking stream/karst system through a field-scale monitoring program collecting samples at known sinks, in the cave, and at the cave outlet (the rise) during different seasons and flow conditions; and 2) combine these results with hydrologic measures of travel time to calculate generalizable *E. coli* decay/growth constants for karst systems.
Methods

Site Description

The study focused on monitoring within the Clover Hollow watershed near Newport in Giles County, Virginia (Figure 2). Land use within this roughly 26 km$^2$ watershed is just over 30% agricultural – predominantly beef cattle, row crop, and silage production – (in the valley) and approximately 65% forested (on steep hillslopes) (USGS, 2021). The hydrology is notably complex: there are several identified locations where surface water sinks into the karst system, eventually entering Smokehole Cave (Figure 3). The water then emerges from the cave at Smokehole Spring (R), and sometimes Tawney’s Spring (T), before draining to Sinking Creek. Clover Hollow Creek drains those areas not entering the karst system and is a tributary to Sinking Creek roughly 1 km upstream of Smokehole Spring. Smokehole Spring was selected as a focus as it is perennial, whereas Tawney’s Spring primarily flows following high rain or flow events. It is fed by an underground stream that is suspected to split from the Smokehole in-cave stream within Smokehole Cave between sample sites D1 and D3, before flowing through Tawney’s Cave and emerging at Tawney’s Spring (Personal Communication, Wil Orndorff, VA DCR). It is estimated that one-third of the total flow is diverted to Tawney’s Spring during these high flow events. There is also anecdotal evidence of home water collection at Smokehole Spring, which suggests it is both of community value and consistently hydrologically active.
Figure 2. Study area map of the Clover Hollow watershed with designated land-use, sampling locations, and associated caves.

Site identification and classification are as follows. Sinks: Clover Hollow Creek Sink (CHA), Stay High Cave Sink (SHC), Bull Creek Headwaters (BC), Jones Creek Sink (J). Rises: Smokehole Spring (R), Tawney’s Spring (T). Surface outlet (non-karst): Clover Hollow Creek Mainstem (CHB).

Tawney’s Spring was not regularly sampled.
Figure 3. Map of Smokehole Cave (Personal Communication, Wil Orndorff, VA DCR).
Site identification and classifications are as follows: Smokehole Cave Mainstem (SA), Smokehole Cave Tributaries (SB/C), Smokehole Cave Rise (R), Drips (D1-6), Standing Pools (P1-2).

Geologically, Clover Hollow watershed is underlain with soluble rocks common in karst areas, 37% limestone and 7% dolostone, predominantly in the valley (USGS, 2005) (Figure 4). Karst features (including sinkholes, stream sinks, and caves) have been observed throughout the watershed, many of which have been confirmed as hydrologically connected to Smokehole and Tawney’s Springs through prior dye tracing efforts (Schwartz et al., 2003; Saunders, 1981) (Figure 4). These known features and their surrounding land use were therefore used to inform sample site selection. The Clover Hollow karst system was selected for this study because of the well-defined and known connection between several surface water sinks and Smokehole Cave/Spring as well as surrounding agricultural land cover likely to contribute high FIB loadings (Boyer et al., 2007).
Figure 4. Study area map of Clover Hollow watershed with underlying geology, known karst features and dye trace efforts (Hubbard, 2003)

Field Data Collection Summary

Environmental data was collected between December 16, 2021, and April 5, 2023 (Figure 5). Grab samples were taken during low-flows and in series during storm events for the duration of the study. Water quality parameters were measured in conjunction with grab samples between Dec. 2021 and Nov. 2022. In-cave grab samples were taken during low flow conditions on May 1, 2022, July 22, 2022, and November 8, 2022. Pressure transducers were deployed in the field between Sept. 2022 and April 2022. Three dye traces were performed in
conjunction with grab samples between Nov. 10 – 19, 2022, Feb. 6 – 18, 2023, and Mar. 23 – Apr. 5. An automated sampler was deployed at CHB and R between March 25-28, 2023.

![Figure 5. Streamflow stage of Sinking Creek (USGS 0317154954) for water years 2021 through 2023 relative to collected environmental data.](image)

**Water Quality Data Collection**

A total of 350 water quality samples were collected from 6 surface sites in the Clover Hollow watershed from December 16, 2021, through March 31, 2023, during low flow conditions and across five storm events. Surface sampling sites were a combination of sinks (CHA, J, SHC, and BC), a karst spring outlet (R), and a surface outlet (CHB) (Figure 2). In-cave locations were manually sampled three times during low flow conditions in accordance with best practices for field safety. In addition to sampling in-cave streams, in-cave drip water samples were taken to differentiate between surface water inputs via the sinks and inputs entering as seepage through the epikarst. Standing pools were also sampled to determine bacteria concentrations in pools that were abandoned after the recession of high flows.
At each site two 100 mL water samples (as replicates) were collected for bacteria laboratory analysis. After field collection, samples were immediately placed into a cooler to limit bacterial growth and were analyzed immediately upon return. Water quality parameters were measured in-situ with a YSI ProDSS (digital sampling system) handheld multiparameter meter (YSI Inc. / Xylem Inc., Yellow Springs, Ohio): water temperature, dissolved oxygen, specific conductivity, conductivity, pH, and turbidity.

During hydrologic events, grab samples were taken in series before, during, and after the storm event and its associated hydrograph to determine their relationship to changes in *E. coli* concentrations. To isolate the peak *E. coli* concentrations during a few hydrologic events, a Model 6712 ISCO Automated Sampler (Teledyne ISCO, Lincoln, NE) was placed at both R and CHB.

**Bacteria Laboratory Analysis**

Concentrations of *E. coli* and total coliforms were determined from each individual sample via Standard Method 9223 with Quanti-Tray 2000s and Colilert (www.idexx.com, Wetsbrook, MN) following serial dilution as needed to maintain levels within the detection limit. Following 18-24hr incubation, trays were examined for macroscopic endpoints: yellow wells as total coliform positive and *E. coli*-positive wells that fluoresce under UV light. These recordings were entered into the IDEXX MPN Generator 1.4.4 to determine the Most Probable Number (MPN) of total coliform (yellow) and *E. coli* (luminous) in each sample (www.idexx.com, Wetsbrook, MN).
Hydrologic Measurements

To compare the *E. coli* concentration relationships with stage in karst and surface environments, HOBO Water Level Data Loggers/pressure transducers (Onset HOBO, Cape Cod, MA) were placed at two points: R and CHB (Figure 2). A pressure transducer was placed in-stream at R and CHB and a third was attached to a tree to record atmospheric pressure for barometric conversion of in-stream pressure to sensor depth. Similarly, two pressure transducers were placed in Smokehole Cave, one in-stream at SA and the other recording air pressure in the cave (Figure 3). Additionally, a USGS stream gage (0317154954, Sinking Creek Along Route 604 Near Newport, VA) continuously collected stage every 5 minutes in Sinking Creek just upstream of Smokehole Spring (USGS, 2023).

In addition to stage measurements, streamflow discharge was measured at R, CHB, and J. Velocity and stage measurements were made at equal increments across a specified cross-section at 60% of the measured depth using a Marsh-McBirney Flo-Mate 2000 electromagnetic velocity meter (Rantz, 1982).

Dye Tracing

Fluorescent dye tracing was used to determine the travel time of water from two sinks to R and to explore connections between in-cave tributary (SB/SC) specific sinks (Goldscheider et al., 2008). Two sinks that contributed the highest *E. coli* concentrations to the system, J and CHA as determined by the first sampling efforts, were selected as dye injection sites. Fluorescein and rhodamine WT were injected at J and CHA, respectively, to isolate travel time to R from each sink. A Turner Designs C3 in-situ data logging submersible fluorometer (Turner Designs, San Jose, CA) was calibrated to sense fluorescein, rhodamine WT, and 1,3,6,8-
Pyrenetetrasulfonic Acid-Tertrasodium Salt (PTSA) and placed in-cave at SA to capture dye concentrations. PTSA (another common tracer) was included in sensor calibration, but was not released during the study. The presence of PTSA in the environment would serve as a marker for potential anthropogenic contamination. Charcoal packets were placed in each in-cave tributary (SB/SC) as well as CHB to determine flow pathways in the karst system and surface. Charcoal packets were also placed at R prior to the first dye trace to detect any ambient dye present in the system. Three dye traces were performed and categorized by flow/antecedent conditions as follows: 1) Nov. 10 – 19, 2022: storm/dry, 2) Feb. 6 – 18, 2023: low/wet, and 3) Mar. 23 – Apr. 5: storm/wet. Each dye trace was performed in conjunction with surface-water quality data collection. The first peak of rhodamine WT and fluorescein of each dye trace were examined to compare shape and spread. Additionally, travel time (t) from each sink to SA was determined for each dye trace event as the time to peak dye concentration in the karst system from dye injection (t=0).

Data Analysis

*E. coli* concentrations and karst system travel times for a known pathway were assume to follow a classical first order decay model (Chick, 1908).

Equation 1

\[ \frac{dC}{dt} = kC \]

Where:

\[ C = E.\ coli\ concentration\ (MPN/100mL) \]

\[ t = time\ (hr) \]
The solution to the differential equation is below.

Equation 2:

\[
\frac{C_t}{C_o} = kt
\]

Where:

\( C_t \) = E. coli concentration at spring at time \( t \) (MPN/100mL)

\( C_o \) = E. coli concentration at the dye injection time (MPN/100mL)

\( t \) = travel time between dye injection and spring (hr)

\( k \) = growth/decay constant (positive for growth, negative for decay) (hr\(^{-1}\))

\( E. coli \) growth/decay coefficients were calculated for the \( E. coli \) carried by the water entering the karst system at sink J, which contributed the highest \( E. coli \) concentrations as determined by the first sampling efforts. Many inputs to the system are unknown and/or unquantified, therefore the coefficients are calculated under the assumption that no additional \( E. coli \) was added into the system from other surface nor karst inputs. Due to potential dilution from other inputs into the karst system, an estimate of the amount of flow J is responsible for at R was necessary for isolation of the sink. To account for this dilution, three versions of this estimate were formulated to account for the complex hydrology and unknown inputs. The three estimates were calculated using: 1) the drainage areas of sink J and of the outlet at CHB, 2) the calculated discharge at both the sink J and R, accounting for a portion of flow diverted to Tawney’s Spring and 3) the calculated discharge at both the sink J and R without the Tawney’s
diversion. The modified version of the solved differential equation including the estimate is below.

Equation 4:

\[
\frac{(C_{R@t} \times a_{1-3})}{C_{J@o}} = kt
\]

Where:

- \(C_{R@t}\) = *E. coli* concentration emerging from the rise at time \(t\) (MPN/100mL)
- \(C_{J@o}\) = *E. coli* concentration entering J at \(t = 0\) (MPN/100mL)
- \(a_{1-3}\) = Predicted ratio of *E. coli* concentrations entering the sink to total *E. coli* concentrations at the spring

The drainage area estimate \(a_1\) is the ratio of the drainage area of J \(A_J\) to the total area potentially draining to Smokehole Spring (R), assumed to be the area of Clover Hollow watershed before the surface outlet \(A_{CHB}\). The drainage areas of each point were found using the USGS Stream Stats delineating tool (USGS 2021). This estimate has limitations specific to karst. The drainage area of R was assumed equal to the drainage area of CHB due to widespread linkages between sinks in the Clover Hollow watershed to R (Figure 4). However, it is unknown if any non-traced sinks outside of the Clover Hollow watershed may contribute water, and therefore drainage area to R. This estimate also does not account for the portion of water that does not sink into the karst system, but instead leaves via the surface outlet at CHB. The method used to develop the drainage area estimate \(a_1\) is outlined below.
Equation 5:

\[ a_1 = \frac{A_J}{A_{CHB}} \]

Where:

\( a_1 \) = Ratio of sink (J) drainage area to total watershed drainage area

\( A_J \) = Sink (J) drainage area (mi\(^2\))

\( A_{CHB} \) = Watershed drainage area (mi\(^2\))

Due to the limitations of \( a_1 \), two additional estimates (\( a_{2-3} \)) were calculated using the ratio of in-field-informed discharge calculations of J and R. Due to the hydrology at J, in which a small stream flows directly into a sinkhole with no remaining surface flow, the uncertainty of non-sinking flow, as described in \( a_1 \), is eliminated. However, discharge was only calculated for one flow event. Therefore, it is unknown if/how the discharge ratio fluctuates. Additionally, the estimated ratio of flow that splits within Smokehole Cave and flows to Tawney’s Spring (T) is included in \( a_2 \). This estimate is formed from visual observation and could potentially introduce error. The methods used to develop the flow ratio estimates (\( a_{2-3} \)) are outlined below.

Equation 6:

\[ a_2 = \frac{Q_J}{Q_R} \times (1 - T) \]

Where:

\( a_2 \) = Ratio of sink (J) discharge to spring (R) discharge.

\( Q_J \) = Sink (J) discharge (m\(^3\)/s)

\( Q_R \) = Spring (R) discharge (m\(^3\)/s)
\( T = \) Estimated ratio of flow diverted to Tawney's Spring

Equation 7:

\[ a_3 = \frac{Q_J}{Q_R} \]

Where:

\( a_2 = \) Ratio of sink (J) discharge to spring (R) discharge

Travel time (t) from J to SA was determined for each dye trace event as the time to peak dye concentration in the karst system from dye injection at the target sink (t=0). For each dye trace event, the \( E.\ coli \) concentration at sink J at t=0, \( C_{J@0} \), and closest measured concentration to the travel time at spring R, \( C_{R@t} \), were used in conjunction with the estimates from Equations 5-7 to determine three coefficients (k) describing the decay or growth of \( E.\ coli \) in the karst system if no \( E.\ coli \) inputs from other sources were added to the system. For each dye trace, k-values were negative and therefore representative of decay. For each dye trace, the hydrologic (storm events or low flow) and antecedent (prolonged wet or dry period) conditions were evaluated, and the travel time calculated for each event were applied to non-dye-traced events that shared similar conditions and k-values were calculated for each. The median k-value estimate (\( k_1, k_2, \) or \( k_3 \)) was chosen to summarize the decay coefficients. The time required for the initial \( E.\ coli \) concentration (\( C_{J@0} \)) to decrease by 50% and 90% of its population, half-life (\( T_{50} \)) and \( T_{90} \), respectively, were calculated for each dye trace using the chosen k-value in the calculations below.

Equation 8.
\[ T_{50} = \frac{(0.5)}{k \times 24} \]

Where:

- \( k \) = decay coefficient (hr\(^{-1}\))
- \( T_{50} \) = half-life (d\(^{-1}\))

Equation 9.

\[ T_{90} = \frac{(0.9)}{k \times 24} \]

Where:

- \( k \) = decay coefficient (hr\(^{-1}\))
- \( T_{90} \) = time until initial \( E. \ coli \) concentration decreases by 90% (d\(^{-1}\))

Results

Seasonal and Water Quality Analysis

\( E. \ coli \) concentrations at all surface sites at low flow were highest during the spring and summer months (Figures 6 and 7). Concentrations at sink J varied year-round even during low flow conditions, ranging from 50 to 11,900 MPN/100mL (Figure 6).
**Figure 6.** *E. coli* concentrations at surface sites during low flow with EPA recreational limit (logarithmic scale).

**Figure 7.** Average seasonal *E. coli* concentrations at surface sites during low flow with EPA recreational limit (logarithmic scale).

In-cave concentrations were highest in summer (Figure 8). Additionally, sites SC and SA had the highest concentrations across represented seasons. This suggests that SC is the major
tributary supplying *E. coli* to the SA. Concentrations in all drip samples and pool samples were between >1.0 – 4.1 MPN/100mL of *E. coli*.

![E. coli Concentration (MPN/100mL)](image)

**Figure 8.** *E. coli* concentrations at in-cave sites (May 1, 2022, July 22, 2022, and Nov. 8, 2022).

*E. coli* concentrations were not visually correlated with any of the measured water quality parameters (Figure 9). The pH measured at R was consistently lower pH than the surface sinks, with a range of 6.96 to 7.69. Water temperature at R ranged between 10.5 to 12 °C and dissolved oxygen and specific conductivity ranged from 9.38 to 10.38 mg/L and 242 to 353 us/cm, respectively.
Figure 9. *E. coli* concentrations at R (red x) and all surface sites (black point) with corresponding water quality parameters

Hydrologic Event Analysis

*E. coli* concentrations at R and CHB fluctuated in response to storm events (Figure 10).

The highest recorded *E. coli* concentration at CHB (1,112 MPN/100mL) occurred at least 2 days before that of R (2,203 MPN/100mL).
**Figure 10.** *E. coli* concentrations at R and CHB over storm event and Sinking Creek stage (USGS 0317154954), Sep. 2022.

After the pressure transducers were deployed at R and CHB, time between relative stage and *E. coli* concentration peaks was estimated (Figure 11 and 12). Peak *E. coli* concentrations occurred closer to peak stage at CHB than at R. The highest recorded *E. coli* concentration at CHB (2,420 MPN/100mL) occurred 4 hrs after peak stage (range 24 before - 30 hrs after). The highest recorded *E. coli* concentration at R (1490 MPN/100mL) occurred 50 hrs after peak stage (range 24 - 144 hrs after). Ranges are due to gaps in grab sample collection times.
Figure 11. *E. coli* concentrations at CHB over storm event with corresponding stage, Sep. – Oct. 2022.

Figure 12. *E. coli* concentrations at R over storm event with corresponding stage, Sep. – Oct. 2022.

The *E. coli* peak concentration was further isolated for a storm event by combining prior grab sampling techniques and automated sampling at 1-hour intervals for three 24-hr periods. A small precipitation event occurred on March 25. The resulting stage peak at CHB was more defined than at R. The highest *E. coli* concentration at CHB (43,520 MPN/100mL) occurred
roughly 1hr before peak stage at CHB (Figure 13). The highest recorded *E. coli* concentration (260 MPN/100mL) and occurred approximately 13hr after the stage peak (Figure 14). The actual *E. coli* concentration peak at R could have occurred between 0-13hrs or 64-136hrs after stage peak.

Figure 13. *E. coli* concentrations and stage at CHB, Mar. 2023 (*E. coli* concentration on log scale).
**Figure 14.** *E. coli* concentrations and stage at R, Mar. 2023 (E. coli concentration on linear scale).

**Dye Trace Results**

Dye concentrations measured in-situ with the fluorometer at SA recorded that each traced sink (CHA and J) arrived at different times, with water from J arriving at SA before the water from CHA (Figure 15-17). Charcoal packet analysis revealed that no detectable fluorescein or rhodamine WT signatures were present at R prior to dye tracing. In-cave charcoal packet analysis revealed that both dyes entered Smokehole Cave through in-cave stream SC. No dye signatures were recorded at the CHB before nor during the first dye trace event (Nov. 10-19, 2022). Additionally, PTSA was observed in the environment during dye trace 2 and 3 (Figures 16-17). The fluorometer was not calibrated for PTSA during dye trace 1.

After the dye release on Nov. 11, 2022 the first precipitation occurred at 12 hrs. Subsequently, the fluorescein tracer injected at J rose to its peak 40.5 hours after dye release.
The rhodamine WT tracer rose to its peak 53 hours after dye release. The highest *E. coli* concentration at R (2,240 MPN/100mL) was recorded 68.5 hrs after dye release.

![Figure 15. Dye concentrations at SA, E. coli concentrations at R, and time since release during dye trace 1 (Nov. 11-19, 2022).](image)

On Feb. 6, 2022, dye was released during low flow conditions. Subsequently, the fluorescein tracer injected at J rose to its peak 18.5 hours after dye release. The rhodamine WT tracer rose to its peak 160 hrs after dye release during peak stage of a later storm event. Fluorescein dye was also found to resurge in a smaller second peak during this event at 162 hrs after dye release. In addition to injected dyes, PTSA was observed in the environment peaking 6 hours after peak stage of the storm event. The highest *E. coli* concentration at R (2,420 MPN/100mL) was recorded 242 hrs after dye release.
Figure 16. Dye concentrations at SA, E. coli concentrations at R, and time since release during dye trace 2 (Feb. 6-18, 2023).

On Mar. 23, 2023, dye was released 14 hrs prior to the start of a small precipitation event. The fluorescein tracer injected at J rose to its peak 23.5 hours after dye release. The rhodamine WT tracer rose to its first peak 106 hrs after dye release. Both fluorescein and rhodamine WT were found in a resurgence that began during peak stage of a later storm event and reached its peak 26 hrs after. This larger fluorescein peak occurred 219 hrs after dye release. In addition to injected dyes, PTSA was observed in the environment 3 hrs after peak stage of the storm event, peaking 30 hrs after peak stage. The highest E. coli concentration at R (260 MPN/100mL) was recorded 43 hrs after dye release.
Figure 17. Dye concentrations at SA, E. coli concentrations at R, and time since release during dye trace 3 (Mar. 23 - Apr. 5, 2023).

Travel time (t) for each dye trace event was determined by the largest dye concentration peak. The travel time from J to R for each dye trace event were 40.5, 18.5, and 23.5 hrs, respectively (Figure 18). Additionally, travel times from CHA to R for each dye trace event were 53, 161, and 219 hrs, respectively (Figure 19).

Figure 18. Fluorescein dye concentration detected at SA and time since injection at J.
Figure 19. Rhodamine WT dye concentration detected at SA and time since injection at CHB.

During all dye trace events, fluorescein was detected at SA sooner after injection than rhodamine WT and following the first-peak, both dyes were observed to resurge during later storm events.

Growth/Decay Coefficient Generation

Jones Creek sink (J) was chosen as the target sink to calculate *E. coli* growth/decay coefficients for the karst system due to its consistently high FIB loadings. The drainage areas of J and CHB (total watershed) were computed to be 1.9 mi² (4.9 km²) and 10.2 mi² (26.4 km²), respectively (USGS, 2021). The resultant corresponding drainage area ratio estimate ($a_1$) was roughly 0.2, meaning the area that sinks at J makes up about 20% of the total watershed area. The during high flow conditions, the flow at J and R were calculated to be 0.27 and 0.67 cms, respectively. The portion of flow diverted to Tawney’s Spring was estimated to be 1/3 of flow before reaching Smokehole Cave (Personal communication, Wil Orndorff). The corresponding flow ratio estimates ($a_2$ and $a_3$) were roughly 0.3 and 0.4, respectively, meaning the flow that sinks at J makes up 30-40% of discharge resurfacing at Smokehole Spring.
Concentrations of *E. coli* at sink J at time t=0 \((C_{j@0})\) were measured as 1285, 1322, and 8545 MPN/100mL, respectively. Due to variations in grab sample collection times, *E. coli* concentrations at R are not always known at the calculated travel time \((t)\). Concentrations of *E. coli* at R at time \(t\) \((C_{R@t})\) were chosen at the closest occurrence to the calculated travel times and were 685, 413, and 116 MPN/100mL, respectively. These chosen concentrations occurred 3.5, 18.5, and 1 hrs after the calculated travel time, respectively (Figure 20).

![Figure 20](image)

**Figure 20.** Fluorescein dye concentration peaks at SA with occurrence time of chosen \(C_{R@t}\).

Due to the *E. coli* concentrations decreasing through the system, the coefficients were negative, reflecting decay. The resulting coefficients using the drainage area and flow estimations are outlined below (Table 1).
Table 1. Table of calculated *E. coli* decay coefficients for varying flow/antecedent conditions affecting the Jones Creek pathway.

<table>
<thead>
<tr>
<th>Dye Trace</th>
<th>Date</th>
<th>Flow/Antecedent Conditions</th>
<th>$k_1$ (hr$^{-1}$)</th>
<th>$k_2$ (hr$^{-1}$)</th>
<th>$k_3$ (hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nov.10-19</td>
<td>storm, dry</td>
<td>-0.0026</td>
<td>-0.0039</td>
<td>-0.0053</td>
</tr>
<tr>
<td>2</td>
<td>Feb.6-8</td>
<td>low, wet</td>
<td>-0.0034</td>
<td>-0.0051</td>
<td>-0.0068</td>
</tr>
<tr>
<td>3</td>
<td>Mar.23-Apr.5</td>
<td>storm, wet</td>
<td>-0.00012</td>
<td>-0.00017</td>
<td>-0.00023</td>
</tr>
</tbody>
</table>

Calculated travel times from dye traces were applied to other sampling events with similar flow/antecedent conditions. Decay coefficients were calculated using $C_{J0}$ and $C_{Rt}$ from each non-dye-trace event where $t$ is the travel time calculated from the dye trace with similar flow/antecedent conditions (Table 2). The estimations ($a_{1-3}$) were used to calculate $k_{1-3}$, respectively. The $k_2$ decay coefficient was chosen, as the median of the k-values, to summarize the decay coefficients because the true partitioning of the flow contribution is unclear. The calculated $k_2$ coefficients of the dye traced and non-dye traced events have a range of $\approx 1.7 \times 10^{-4}$ to $\approx 5.1 \times 10^{-3}$ hr$^{-1}$ and a median of $\approx 4.3 \times 10^{-4}$ hr$^{-1}$ (Table 1 and 2).

Table 2. Calculated *E. coli* decay coefficients for varying flow/antecedent conditions affecting the Jones Creek pathway during non-dye traced events.

<table>
<thead>
<tr>
<th>Dates</th>
<th>Dry/Wet</th>
<th>Storm/Low</th>
<th>$k_1$ (hr$^{-1}$)</th>
<th>$k_2$ (hr$^{-1}$)</th>
<th>$k_3$ (hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/30-10/5</td>
<td>Dry</td>
<td>Storm</td>
<td>-0.00024</td>
<td>-0.00035</td>
<td>-0.00047</td>
</tr>
<tr>
<td>9/3-9/7</td>
<td>Dry</td>
<td>Storm</td>
<td>-0.00024</td>
<td>-0.00036</td>
<td>-0.00048</td>
</tr>
<tr>
<td>5/1/2022</td>
<td>Wet</td>
<td>Low</td>
<td>-0.00033</td>
<td>-0.00049</td>
<td>-0.00066</td>
</tr>
</tbody>
</table>

The half-life and time until 90% of the initial *E. coli* concentrations sinking at J were calculated for each dye trace (Table 3). The half-life of *E. coli* for each dye trace was calculated as 5, 4, and 120 days, respectively. The $T_{90}$ was calculated for each dye trace as 9, 7, and 216
days, respectively. The longest time to reach 50% and 90% *E. coli* decay occurred during the third dye trace (Mar. 23-Apr. 5). The shortest time to achieve 50% and 90% *E. coli* decay occurred during dye trace 2 (Feb. 6-8).

**Table 3.** $T_{50}$ and $T_{90}$ for *E. coli* travelling from Jones Creek for each dye trace.

<table>
<thead>
<tr>
<th>Dye Trace</th>
<th>Date</th>
<th>$T_{50}$ (d)</th>
<th>$T_{90}$ (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nov. 10-19</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Feb. 6-8</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Mar. 23-Apr. 5</td>
<td>120</td>
<td>216</td>
</tr>
</tbody>
</table>

**Discussion**

**Observations of Clover Hollow Watershed and Smokehole Cave**

Clover Hollow watershed and its karst system support hydrologically variable dependent *E. coli* transport between the surface and subsurface. Storm events are the main driver of *E. coli* at both the Smokehole Spring (R) karst outlet and mainstem Clover Hollow Creek (CHB) surface outlet of the watershed. Agricultural land-use in the valley is hypothesized to influence *E. coli* loadings at both R and CHB (Buckerfield, 2019). Sink J contributed the highest *E. coli* loadings to the karst system across seasons and storm events, which is hypothesized to be a result of its drainage of cattle grazing pasture (Figure 6). Additionally, the close relationship between peak stage and peak *E. coli* concentration at CHB is hypothesized to be in response to increased runoff from upstream pasture resulting in higher *E. coli* concentrations (Figures 10 and 12).

In addition to hypothesized agricultural influence on karst waters in Clover Hollow watershed, the presence of PTSA in the Clover Hollow karst system and its emergence during peak stage after storm events is an indicator of anthropogenic contamination from an unknown source being stored within the karst system.
*E. coli* concentrations within Smokehole cave drips and pools were lower than the in-cave stream and surface sites (Figure 7). All except two pool and drip samples tested below the minimum analytical *E. coli* detection limit (<1 MPN/100mL). The lack of *E. coli* in drip samples indicates that autogenic (flowing directly into the system via local epikarst) transport processes do not contribute considerable amounts of *E. coli* to the system during low-flow periods. Additionally, the lack of *E. coli* in standing pools fed by rising of the in-cave stream during high flows may indicate the decay of *E. coli* in long-term karst storage in between significant storm events.

**Relevance to Prior Literature**

Hydrologic event analysis showed that *E. coli* concentrations consistently peaked later after storm event stage at Smokehole Spring (R) than at the watershed surface outlet (CHB). Additionally, dye trace results indicated that after the initial dye peak, dye resurfaced during the peak stage of following storm events, resulting in maximum residence times within the karst system that may have exceeded our fluorometer deployment times. The combination of the lag in *E. coli* concentration peak and alignment of dye resurgence during peak stage indicate that water entering through the J and CHA sinks was being stored/moving slowly within the karst system and “flushed out” during storm events. This can be equated to the old water paradox that has been observed in small surface catchments, in which water is stored for months or weeks in subsurface pathways and remobilized quickly in response to storm events (Kirchner, 2003).

Prior studies have examined the interaction of water particle velocity and the celerity as drivers of water movement and age selection in karst systems (Zhang et al. 2020). Due to being
well-connected to surface waters, rapid infiltration of rainfall or runoff into karst storage favors young water (Harman et al., 2015; Zhang et al. 2020). While water particle velocity is the main driver for water age selection entering karst storage (favoring younger water), celerity has been the main controlling factor of water movement out of karst storage during storm events (remobilizing old water stored in small fractures and reservoirs) (Zhang et. al, 2020). Residence times in these storage reservoirs have been found to range from days to years (Zhang et. al, 2020). Additionally, our E. coli peak findings are consistent with prior studies on contaminant transport through karst systems, such as nutrients, which have found that maximum nitrate concentrations at springs experience a lag behind peak discharge (Yue et al, 2019).

The difference in dye breakthrough curve shape, magnitude, and travel time is hypothesized to be due to differences in flow distances and advective processes (Peely et al., 2021). Prior studies have found that breakthrough peak concentration decreases with increased conduit diameter and long distances, and that flattening can occur with low hydraulic conductivity, meaning that it is difficult for water to pass through fractures (Peely et al., 2021).

The fluorescein dye signature from J arrives at R quickly with a high, defined peak which could be indicative of its closer proximity to R and perhaps well-defined conduit (Figure 18). This is contrasted with the dampened rhodamine WT dye signature from CHA that arrives on the magnitude of hours or days after the fluorescein signature, which could be indicative of a longer flow distance to R and/or more tortuous flow pathways (Figure 19).

In addition to variable storm flows, E. coli concentrations were also visually correlated with seasonality. Low-flow E. coli concentrations at sinks and R were highest during the summer months (Figure 7). The in-cave low-flow samples also follow the same trend, with
concentrations in both tributaries peaking in July (Figure 8). This could be due to an increase in intensive agricultural practices in the Clover Hollow watershed that occur during the summer months, like spreading manure for fertilizer. Other studies have reported similar results in which changes in farming practices, often seasonal, increased *E. coli* concentrations in overland flow during rain events and at springs (Buckerfield et al., 2020).

Water quality parameters were found to have no visual correlation with *E. coli* concentration (Figure 9). However, several water quality parameters (pH, specific conductivity, temperature, and dissolved oxygen) were visually correlated with water emerging from Smokehole Spring (R). These correlations are likely due to hydrogeologic processes in karst systems that are known to impact water chemistry at springs (NCKRI, 2021, Stroj et al., 2020, Hartmann et al., 2014, Buckerfield et al., 2020). Consistent water temperature at R is likely due to the temperature inside the karst system staying relatively constant at about 12°C (55°F) year-round (NCKRI, 2021). Increased dissolved oxygen (DO) concentrations in springs are an indicator of excess air due to flow dynamics during transition from open to closed flow in fractures during high recharge conditions (Stroj et al., 2020). However, open channel flow through large conduits, such as that in Smokehole Cave, decrease DO (Stroj et al., 2020). The consistently saturated water at R may be due to a combination of these processes: closed flow in fractures during sinking and open channel flow in large conduits. Specific conductivity is higher in karst and spring waters due to the higher concentration of dissolved ions from the dissolution of soluble rocks (Hartmann et al., 2014). Additionally, variations in pH in karst systems depend on storage and residence time (Buckerfield et al. 2020). Storage in soils and epikarst result in a lower pH than storage within the rock matrix (Buckerfield et al., 2020). The observed lower pH
at R may be due to this storage differentiation. Turbidity was not visually correlated with R nor E. coli concentration magnitude. This is surprising because E. coli is commonly correlated with suspended and stored sediment and turbidity due to re-suspension of sediment and/or overland flow and accompanying erosion during storm events (Buckerfield et al., 2020).

According to our decay coefficient (k) calculations, E. coli experiences decay in the karst system during low flows, storm events, and wet/dry antecedent conditions. The k_2 decay coefficient is summarized here as the median estimate and represents the dilution from other sinks and flow lost to Tawney’s Spring. The calculated k_2 coefficients of the dye traced and non-dye traced events have a range of $-1.7 \times 10^{-4}$ to $-5.1 \times 10^{-3}$ hr$^{-1}$ and a median of $-4.3 \times 10^{-4}$ hr$^{-1}$ (Table 1 and 2). Studies of surface waters have calculated k-values ranging from $-8 \times 10^{-3}$ to $-34 \times 10^{-3}$ hr$^{-1}$ in large rivers and $-5.8 \times 10^{-3}$ to $-6.4 \times 10^{-2}$ hr$^{-1}$ in mountainous, tropical headwater wetlands (Menon et al. 2003, Nakhle et al., 2021). Another study calculated average k-values of $-2.1 \times 10^{-2}$ hr$^{-1}$ in groundwaters and $-3.0 \times 10^{-2}$ hr$^{-1}$ in rivers (Blaustein et al., 2013). The calculated decay coefficients (k_2) for the Clover Hollow karst system from J were considerably smaller than those calculated for surface and groundwaters in other studies. Therefore, E. coli decays slower within the Clover Hollow karst system than in surface waters and groundwaters of prior studies.

The observed travel times from J to R for each dye trace event were 40.5, 18.5, and 23.5 hrs, respectively (Figure 18). The T_{50} of E. coli for each dye trace was 5, 4, and 120 days, respectively. The T_{90} was calculated for each dye trace as 9, 7, and 216 days, respectively. The calculated T_{50} and T_{90} of E. coli travelling from J are not likely to be reached before emergence at R, meaning E. coli likely moves through the system faster than it can decay. However, for water that remains stored within the system and resurges during later storm events, the T_{50}
and $T_{90}$ may be attainable. Longer deployment time of the fluorometer and/or regular rotation of charcoal traps at R would be necessary to determine the residence time of water from J in the system.

Relevance to Public Health

Like many other roadside springs in rural Appalachia, Smokehole Spring is utilized for domestic water collection by the surrounding community. This study has found that \textit{E. coli} concentrations at Smokehole Spring fluctuate over storm events, with concentrations ranging from the 10s (MPN/100mL) before storm events, peaking in the 1000s multiple days after peak stage, and returning to the 10s up to a week post-storm. This dramatic increase in concentration multiple days after storm events emphasizes the importance of disinfecting spring water for collected for domestic use to minimize exposure to fecal pathogens. To minimize health risk, these findings suggest that water should not be collected from Smokehole Spring for domestic purposes. Any water that is collected from Smokehole Spring should be treated using bacteria disinfection techniques. Additionally, one grab sample of spring water for bacteriological testing purposes may not be indicative of the full public health risk. Instead, multiple samples taken over a storm event may provide further insight into the health risk associated with water collection at that spring in different conditions.

The effective transport of \textit{E. coli} through karst systems during storm events found in this study also raises public health concerns for other karst water uses including agriculture, private/community well systems, and downstream recreation. \textit{E. coli} concentrations at Smokhole Spring during storm events consistently exceeded the EPA recreational standard in place for freshwater, which could be a public health concern for those recreating downstream.
in Sinking Creek and the New River. Resurfaced karst waters can also be used for irrigation or for cattle watering. Fecal contamination of irrigation waters can lead to crop contamination, which is a health risk if consumed (Solomon et al., 2002). Similarly, fecal contamination can also have adverse health effects on cattle. Fecal pathogens that originate from cows, such as \textit{C. parvum} (\textit{Cryptosporidium parvum}) can cause sickness and/or death of cattle and be spread through drinking fecal contaminated water (Olson et al., 1999). Due to the many uses of karst waters and their effective transport of FIB, it is important to implement karst-specific land management practices to decrease FIB loading of karst systems, such as cattle exclusion from known sinking streams and sinkholes.

Limitations and Future Work

Limitations of this study include the “built-upon” nature of the field data collection (Figure 5). Much of the data collection methods were added as the project progressed, making it difficult to compare data between storms, seasons, and dye traces (lack of pressure transducers at the outlets, no sample dilution to expand detection limits, no automated sampler, etc.).

When calculating the decay coefficients, \( C_t, C_0, \) and \( t \) were not known a priori. Therefore, concentrations at \( R \) were not collected at the precise travel times, resulting in some uncertainty in calculating the \( k \)-values. Further work should expand on continued dye tracing with increased sampling frequency, ideally using an automated sampler, to isolate the concentrations at \( R \) at the travel time. It was also assumed that no additional \textit{E. coli} was added to the system from other sources during \( k \)-value calculation. While this assumption is acceptable given the relatively low concentrations of \textit{E. coli} at the other regularly tested sinks in
this study, it does not take other unknown or untested sinks into account and may result in some error in k-value calculation. The flow percentage estimations \((a_{2.3})\) were calculated using flow estimations for only one storm-flow condition, which could vary under differing conditions. Additionally, the \(a_2\) estimate was calculated using a visual observation of flow difference between Smokehole and Tawney’s Springs which could be a source of error in calculating \(k_2\).

Future work expanding on this study could include expanding sampling efforts to include Tawney’s Spring to better quantify flow percentages at each spring and subsequent \(E. coli\) concentrations. Additionally, a dye trace during low flow and wet antecedent conditions could be conducted to determine subsequent travel times and \(E. coli\) decay coefficients. This could also be combined with flow measurements at CHA to calculate associated k-values.

**Conclusion**

\(E. coli\) fate and transport in Appalachian karst systems, as with other overland anthropogenic contaminants, is hydrologically driven. \(E. coli\) concentrations at Smokehole Spring consistently peaked days after peak stage and peak surface \(E. coli\) concentrations. Dye trace results revealed that water from sinks can be stored or move slowly through the karst system and resurge during storm events. \(E. coli\) was found to decay within the karst system, with a half-life of about 5-120 days which is longer than the travel time of water through the cave of approximately 0.5-2 days, meaning that \(E. coli\) travels through the karst system faster than it can significantly decay. The decay of \(E. coli\) in the Clover Hollow karst system was also slower than that calculated in both surface and groundwater systems in other studies. Karst
waters provide critical ecosystem services in rural Appalachia and are well-connected to surface waters, making them susceptible to fecal contamination. Collection of water from Smokehole Spring is not recommended and preemptive bacterial treatment is recommended for any water collected from other roadside springs. Feature-targeted land-management practices should be investigated to decrease E. coli loading in karst waters.

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https://doi.org/10.1016/j.watres.2012.10.027


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