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Identifying Hot-Spots of Fecal Contamination in the Royal Spring Karstshed

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IDENTIFYING HOT-SPOTS OF FECAL CONTAMINATION IN THE ROAYL SPRING KARSTSHED

THESIS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Civil Engineering in the College of Engineering at the University of Kentucky

By

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ABSTRACT OF THESIS

IDENTIFYING HOT-SPOTS OF FECAL CONTAMINATION IN THE ROYAL SPRINGS KARSTSHED

The City of Georgetown, Kentucky relies on a vast karst spring network as a drinking water source. This karst feature has several inputs from sinkholes and streams in the Cane Run Watershed: a watershed associated with a variety of land uses in the recharge area. The recharge area encompasses the area from North Lexington to Georgetown and is composed of urban, suburban, agricultural and industrial usage. A serious water quality issue exists with respect to the impact of fecal contamination within the spring recharge area. Identification of fecal contamination is quantified by microbial indicators adapted from surface water applications: fecal load (E. coli), fecal source (two human-host specific *Bacteroides* DNA markers) and fecal age (AC/TC ratio). These three criteria are used in a categorical Microbial Source Tracking (MST) model to assign a Sanitary Category Value (SCV) between 0 and 3 for each sample location. Low SCVs (<1.5) are associated with relatively clean water, while high SCVs (>1.5) are associated with high values of fecal load, low fecal age and detectable concentration of human-specific markers. SCV measured during dry weather conditions are indicative of potentially leaking human sewers.

Due to retention and conservation of fecal load (*E. coli*) and age (AC/TC) microbial indicators in the karstic environment, ambiguous SCV model results cannot pinpoint, with statistical confidence, fecal sources in a karstic environment. Human-host specific genetic markers (HF183 and HuBac) were also detected at all sample sites above limits of detection, indicating steady inflow of fecal material during all sample events. By adding a flow multiplier and expressing HF183 and HuBac values as a load, it was strongly indicated that a human fecal source was entering the groundwater conduit and impacting Royal Spring independent from other upstream fecal sources. Interpretation of these trends, while strongly indicated, cannot be supported with statistical evidence.

KEYWORDS: Microbial Source Tracking, Royal Spring karstshed, *Bacteroides*, *E. coli*, AC/TC

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1.0 Introduction

1.1 Research Background

The City of Georgetown, KY relies on a vast karst spring network as a drinking water source. This karst feature has several inputs from sinkholes and streams in the Cane Run watershed: a watershed associated with a variety of land uses in the recharge area. The recharge area encompasses the area from North Lexington to Georgetown and is composed of urban, suburban, agricultural and industrial usage. As discussed in Sections 2.6 – 2.9, historical water quality research highlights the impact of fecal contamination within the spring recharge area.

1.2 Problem Statement

Two previous studies conducted Dr. Gail Brion, University of Kentucky, show that the sole source of drinking water for Georgetown, Royal Springs, is under human fecal influence. This thesis will determine if the source of this fecal load is within the recharge area of Royal Springs.

1.3 Research Objective

The objective of this thesis is to identify hot-spots of fecal contamination within the Royal Spring Karstshed. This study will also define the impact of these sources on the water quality received at the Georgetown Water Treatment Plant (WTP). This research follows multiple preceding studies completed in the Cane Run watershed with improved microbiological genetic tools, allowing classification of fecal sources indicated in water quality samples. Previously inaccessible for sampling, a well directly tapping the primary underground karst conduit of the spring recharge area provided was also available during this study. Access to this feature provided water quality samples fully representative of the fecal load entering the Royal Spring karst system. Combining these two analytical improvements, this thesis builds on previous studies, screening the watershed to identify potential sources of untreated human and non-human sewage. Such sources often contain elevated levels of waterborne pathogens, impairing water quality of the receiving waters.

2.0 Literature Review

2.1 Royal Spring Karstshed Description

The Royal Spring karstshed is located in central Kentucky, north of Lexington, Kentucky. The karstshed shares a recharge basin with the Cane Run watershed; a HUC-14 watershed covering some 29,160 acres of pasture, cultivated crops, and developed urban environments in Fayette and Scott County (Figure A.1, Appendix A). Royal Spring is characterized by a massive groundwater conduit that pipes groundwater from Lexington to Georgetown, Kentucky.

As shown in Figure A.2 (Appendix A), The Royal Spring Recharge Basin is characterized with numerous karst features. Surface water is transferred to the groundwater conduit through numerous swallets and sinkholes, effectively transferring Cane Run to a massive karst environment. The central aquifer, as approximated from dye trace vectors published by the Kentucky Geological Survey (KGS), supplies Royal Spring with water infiltrating through sinkholes, swallets, and disappearing streams. Figure A.2 also introduces the location of sample sites (discussed in Section 3.2) relative to these karst hydrologic features. Data collected by Jim Currens, Kentucky Geological Survey (unpublished at the time of this study), describes the nature of the karst conduit:

- Average water temperature: 21˚C
- Average dissolved oxygen: 80% Saturation
- Average Turbidity: 11 NTU

Currens' data supports the idea that the Royal Spring conduit is a "pipe": the groundwater is very similar to surface water introduced to the aquifer. The water temperature, while cooler than surface water, is still warmed by stream water disappearing into swallets and sinkholes. Groundwater within conduit is not turbid, but only during dry weather; turbidity increases to much higher levels (100 NTU) after stormwater flushes particles into the karst conduit. Dissolved oxygen is also maintained at high levels due to turbulent inflow of stream water into the karst system. These data highlight the observation that parameters within the karst conduit reflect the water quality of surface waters contributing to the karstic environment.

The Karstshed is predominantly characterized by agricultural land use, as interpreted from Anderson Level II Landuse Categories shown in Figure A.3 (Anderson, 1972). Table 2.1 shows the

land use classification throughout the Karstshed. Very little of the Karstshed remains undeveloped; the urban environment in Lexington is divided from Georgetown by heavily used cultivated cropland and pasture.

Table 2.1: Land Use Classification in the Royal Spring Karstshed

	Land Use	Area (Acres)	Total Area (Acres)
Developed Urban (37.9%)	Developed - Open Space	1976.01	5972.68
	Developed - Low Intensity	2169.31	
	Developed - Medium Intensity	1297.31	
	Developed - High Intensity	530.05	
Developed Agricultural (58.5%)	Pasture/Hay	7678.12	9204.94
	Cultivated Crops	1526.82	
Undeveloped (3.6%)	Open Water	48.48	569.55
	Natural Barren	17.79	
	Deciduous Forest	295.41	
	Evergreen Forest	26.62	
	Mixed Forest	1.88	
	Scrub/Shrub	96.79	
	Grasslands - Herbaceous	79.25	
	Emergent Herbaceous Wetlands	3.34	
	TOTAL	15747.17	

The Royal Spring karstshed offers the following challenges for the identification of human sewage impact with microbial indicators (discussed in Section 2.2):

1. The Urban-Agricultural mix of land use: Since many fecal indicator organisms (such as *E. coli* and Fecal Coliforms) are present in both the feces of human and livestock (cattle, horses, sheep, etc.), the presence of a fecal indicator cannot confirm human sewage as a contributing source.
2. The enigmatic behavior of groundwater infiltration and discharge: As shown in Figure A.2, numerous swallets contribute surface water to the Royal Spring conduit. The recharge area of the conduit is much bigger than the Cane Run watershed. Therefore, a fecal source, as indicated by the presence of microbial organisms, may originate from a recharge area outside of Cane Run. This creates difficulty when "pinpointing" a possible source.

3. Application of surface water fecal indicators in a karstic environment: Although the Royal Spring conduit is strongly linked to surface water infiltrating the karstic environment, there are dissimilar factors affecting microbial growth and decay. The conduit is cooler and dark. The conduit has a lower dissolved oxygen concentration than surface water. Therefore, enteric bacteria are expected to survive longer in the conduit where the cool, dark, and more anoxic environment is beneficial. This adds to ambiguity when interpreting microbial indicator data: can we attribute detection of feces within the conduit to a possible source when indicator bacteria are surviving in the karstic environment?

2.2 Fecal Indicators

The wide variety of water-borne pathogens types, complexity of testing methods, and expense of pathogen monitoring make the detection of pathogens for water quality assessment a difficult, expensive, and time-consuming process. As a result, direct pathogen monitoring relies on the occurrence and concentration of "fecal indicators": fecal organisms whose presence in a water sample likely correspond to the presence of pathogens. These indicators, usually bacteria such as *E. coli*, fecal coliform, or enterococcus, are much easier to detect and less expensive to monitor than the pathogens themselves.

An ideal fecal indicator organism is one exclusively found the intestines of warm-blooded animals (not found naturally in water), unable to grow or reproduce in the environment outside of the intestine, and recovered in a density or concentration reflective of the degree of fecal pollution (Maier et al., 2000). Discussion of fecal indicators used in this study follows.

E. coli:

E. coli are pathogenic bacteria that naturally live in the digestive systems of humans and other warm-blooded animals (EPA, 2012). Human contact with these bacteria produces short-term health effects (diarrhea, cramps, nausea, etc.) when water containing the microbes is consumed or comes into contact with the eyes, mouth and skin. Since *E. coli* colonies are shed in large number in human feces, do not reproduce outside of the digestive system, and are easily quantified with laboratory analysis*, E. coli* is a common fecal indicator for water quality analysis (Madigan et. al, 2003).

Due to the commonality of *E. coli* analyses, multiple water quality studies used *E. coli* as a fecal indicator in karstic environments. A 1995 study in Bourbon County, Kentucky, sampled springs, swallets, and disappearing streams within an entirely agricultural environment (Howell et. al, 1995). Conclusions from this study linked proximity of domestic animals to streams and swallets to high exceedances of Kentucky water quality standards for *E. coli*. The authors followed this study with a 1998 experiment discussing the infiltration of fecal pollution through the soil profile to contaminate the groundwater (McMurry et. al, 1998). Soil amended with chicken feces was proven to pollute shallow groundwater with fecal bacteria conduits in exceedance of water quality standards. These studies applied *E. coli* as an indicator of agricultural fecal pollution, but literature also supports the use of *E. coli* for detecting human fecal pollution in karst groundwater. A 2001 study in the karstic environment of Sarasota Bay, Florida, found failing onsite sewage disposal systems using *E. coli* as a microbial indicator of fecal pollution (Lipp et. al, 2001). Indicator data collected at sample sites were ranked by relative risk (percent exceedance of water quality standards), grouped into clusters, and sorted to identify sample sites indicative of sewage pollution. In addition, significant difference between sample sites was detected by analyzing geometric means of *E. coli* data. Success with *E. coli* as a fecal indicator has resulted in detection of both agricultural and human fecal pollution in karstic environments.

Despite the aforementioned success of indicating fecal contamination with *E. coli*, considerable criticism of relying on *E. coli* alone as a microbial indicator is found. An important criterion for use of an indicator organisms is the inability of the organism to reproduce or survive outside of the host. Numerous studies find that *E. coli* is capable of surviving and reproducing outside of the host (McFeters and Stuart, 1972; Anderson et. al, 2005). Especially in elevated water temperature, such as subtropical and temperate estuaries, *E. coli* may persist and even grow in the environment for weeks (Desmarais et. al, 2002). Therefore, the concentration of *E. coli* recovered from water quality analysis does not indicate fecal contamination due to the ability of the microorganism to grown in the environment (Shanks et. al, 2006).

Considering this criticism, analysis of *E. coli* data alone will not indicate human fecal pollution in the Royal Springs Karstshed. In addition, the fact that *E. coli* are also present in the intestines of all warm-blooded animals, indication of human fecal pollution requires the analysis of multiple indicators.

<u>AC/TC</u>:

To indicate the age of sewage in a water quality sample, the AC/TC ratio relies on the concentration of introduced fecal bacteria relative to the concentration of indeginous bacteria native to the aqueous environment (Brion and Lingireddy, 1999). Total Coliform bacteria (TC) are a commonly used bacterial indicator (of which *E. coli* is a subset) found in the intestinal tract of warm-blooded animals and their feces (WHO, 2004). TC bacteria are all facultative anaerobic, non-sporeforming, gram-negative rod shaped bacteria that ferment lactose within 24 hours at 35˚ Celsius (APHA, 1998). Atypical colonies (AC) are detected growing on the same media as TC bacteria, but are bacteria unable to ferment lactose in 24 hours. AC concentrations in surface water have been shown to be relatively stable in comparison to TC, suggesting that a large portion of AC colonies are indigenous to nutrient-enriched waters (Brion and Mao, 2000). Therefore, the normalized change of TC counts relative to these indigenous AC counts provies a useful environmental tool to indicate the age of fecal pollution in a waterway (Nieman and Brion, 2003; Booth and Brion, 2004). Therefore, low AC/TC ratios (<10) indicate fresh fecal material while high AC/TC ratios (>10) indicate aged fecal material.

Fresh fecal inputs, defined by a lower AC/TC ratio, indicate the presence of a point-source of contamination (Brion et. al, 2000). In addition, fresh stormwater runoff carries more fecal-associated TC than indigenous AC, therefore indicating fresh fecal input during storm events in urban watershed. Research of AC/TC levels in the Kentucky River has resulted in successful delineation of fecal sources in surface water; the AC/TC ratio was proven to predict the presence of enteric viruses within the Kentucky River (Black, 2007; Black et al., 2007). These studies support the use of AC/TC as an indication of human fecal material.

Research on the utility of the AC/TC ratio does not recommend its use for indicating fecal age in groundwater. A 2006 study at a spring in Woodford County, Kentucky found that AC/TC ratios significantly decreased (indicating decrease in fecal age) during storm events (Reed, 2006). An observation presented by this study suggests that introduced fecal TC bacterial populations grow relative to AC populations due to an influx of bacteria in stormwater runoff. Pairing this observation with levels of fecal pollution detected in the urban environment of Woodford County, the study was able to indicate fecal pollution in a karst feature with the AC/TC ratio. However, a study by Ward criticized the applicability of AC/TC to assess water quality (Ward, 2008). In a bench-scale experiment, Ward sought to evaluate the behavior of the AC/TC ratio under karst-like conditions relative to surface water condition. Using raw sewage samples

diluted in water obtained from the Blue Hole Spring (Woodford County, KY), Ward found that "conditions within a karst aquifer should preserve the AC/TC ratio at lower levels longer than in surface waters after an input of fresh fecal material" (Ward, 2008). Ward observed that exposure to sunlight and higher temperatures decreased the survival of introduced fecal bacteria (TC) without inhibiting the growth of indigenous bacteria (AC). In the cool, dark conditions of a karst aquifer, the indigenous bacteria (AC) do not grow as they do in surface streams, whereas the survival of fecal bacteria (TC) is not affected. As such, the AC/TC ratio is preserved and changes very little while underground. Therefore, Ward's conclusions suggest that the AC/TC ratio cannot reliably indicate the impact of human fecal pollution in a karst environment because it remains stable.

HuBac:

Bacteroides bacteria, like *E. coli*, are indicator organisms present in the intestines of all warm-blooded mammals. *Bacteroides* are described as obligate anaerobic, gram-negative rod shaped bacterial colonies. Contrasted to *E. coli*, *Bacteroides* are unable to persist or reproduce in oxygenated environments and are shed in larger numbers in human feces, therefore satisfying more criteria as an indicator organism (Eckburg et al., 2005; Finegold et al., 1983). Most importantly, since *Bacteriodes* are involved in nutrient digestion in the host intestine, they are found to express different genes in different host species due to difference in food sources (Hooper et al., 2005). Therefore, examination of the genes expressed in *Bacteroides* is linked to the host, effectively indicating the source of fecal indicator. Although these bacteria are strict anaerobes, and actually do not persist long outside of their host, the genetic material can be found in the environment long after the bacteria has been inactivated. This genetic material can be examined for host specific genetic markers. It is important to remember that the presence of the genetic markers for the bacteria is not directly linked to the presence of actual living bacteria, and that short pieces of genetic material spread farther and persist longer in aqueous environments than longer pieces (Ficetola et al., 2008).

Host-specific DNA markers for enteric *Bacteroides* have been developed for several host species that are likely to contribute to fecal pollution in recreational and drinking source waters. Layton developed three *Bacteroides* fecal markers which utilize the TaqMan probe technology with quantitative real time PCR (qPCR) analysis of the 16s rRNA marker (Layton, 2006).One of these *Bacteroides* markers was present in fecal samples taken from human-hosts: the human-

associated fecal marker HuBac). HuBac markers are used in this study to indicate the fecal source of pollution detected in water quality samples.

Little evidence is present in the literature to support the application of *Bacteroides* markers in a karstic environment. One study demonstrated that karst aquifers in the Midwest were vulnerable to both human and non-human fecal contamination (Zang, 2012). Most water samples indicated both human and animal sources of fecal pollution. In addition human-sewage source as determined by *Bacteroides*-based qPCR was linked to known failing on-site wastewater treatment systems in rural areas. This study only sought to measure and rank the severity of fecal pollution in a karst environment, but did not explore the behavior of the *Bacteroides* genetic marker; it did not answer questions about how the concentration of *Bacteroides* genetic markers may persist, deposit, or change in the karstic environment.

Numerous studies have explored the persistence of *Bacteroides* 16s rRNA markers in a variety of environmental conditions. One study utilized qPCR and found the *Bacteroides* genetic marker was detectable for 24 days at 4°C and 12°C in surface water samples seeded with cultured *Bacteroides* colonies (Seurnick et al., 2005). Another similar study found persistence of genetic markers for 8 days at 24°C (Kreader, 1998). Studies exploring the effect of sunlight on *Bacteroides* genetic marker persistence found no differences in prepared samples exposed to sunlight and those kept in darkness (Walters, 2007). These studies suggest that the HuBac marker could survive for up to two weeks in the cool, dark environment of a karst conduit. However, these studies did not shed light on if the markers would be expected to behave like the bacteria with respects to sedimentation, or if they would be expected to behave like random fragments of DNA and remain in suspension.

Bell (2007) presented an in-depth study of the survivability of the HuBac marker relative to one primary environmental condition: removal of the *Bacteroides* genetic marker by biologic removal, such as “grazing” by protozoa. Unlike previous studies, Bell found that initial concentration and source of fecal matter did not affect the removal rate of HuBac markers. Therefore, significant environmental parameters of HuBac marker survivability are those that encourage removal by protozoa: disaggregation of initial fecal input and temperature. This study again found that cool temperatures saw higher survivability of *Bacteroides* genetic markers than warmer water temperatures. At 5°C, the marker was above detectable limits for 15 days. Contrasted to a water temperature of 25°C, the marker was detectable for 3 days. Bell’s

observation indicated longer survivability of HuBac in cool environments. In addition, Bell found that the disaggregation, described as the breaking up of feces before contact with receiving waters, resulted in the disappearance of the *Bacteroides* marker after two days. Conditions that minimize disaggregation (direct deposit of fecal matter in streams) may result in marker persistence of up to one week. Therefore, Bell concluded that feces input into receiving waters during storm runoff events will not survive as long as those directly input into the stream. These implications are applicable to *Bacteroides* genetic marker analysis in a karstic environment: the HuBac marker may survive in the cool groundwater conduit for two weeks. Also, HuBac markers should not survive as long in the conduit after rain events.

HF183:

Develpoed by Benhard and Field (2000), the HF183 *Bacteroides* 16S rRNA genetic marker indicates the presence of human-sources fecal material. Similar to the HuBac marker, this human-specific genetic marker is analyzed by a real time qPCR assay (Seurinck, 2005). Contrasted to the HuBac marker, HF183 is much more host-specific. In a host-specificity study, the HF183 marker was detected in only 13 nontarget host groups (Ahmed et al., 2012). This corresponds to a false positive rate of 6%. The Hubac marker was found in a study of fecal positive and negative controls to yield a 32% false positive rate (Layton et al., 2006). While both the HuBac and HF183 markers yielded a 100% true positive rate, the HuBac marker is presented in this study as "human-associated", indicating that the likelihood of isolating the marker from animals other than humans is quite high. Therefore, the HF183 marker is presented as "human-specific" due to the documented low probability of detecting the marker in non-human fecal sources.

HF183 is supported in multiple articles as a preferential microbial indicator. The host-specificity of the marker allows for consistent detection of human fecal pollution in various watersheds. Shanks et al. (2006) conducted an in-depth study with the HF183 marker of the Tillamook Bay, Oregon. A total of 2,912 samples were collected from 30 sites and analyzed for HF183 genetic markers from human hosts. By comparing percentage of events where the HF183 was present or absent at a sample site, this study successfully identified a point source of human fecal pollution from a wastewater treatment plant. Similar studies also support analyzing HF183 as present/absent: the larger frequency of detection of HF183 markers at a sample site relative to other sample sites indicates a significant human fecal source impacting that site (Seurinck et al.,

2006; Chong et al., 2012). As a human-specific marker, HF183 can successfully indicate the impact of human sewage sources in a surface watershed.

Relating these surface water applications to indicator use in a karstic environment begs the same of HF183 to Persistence studies examined the persistence of HF183 as a function of multiple environmental variables (Seurinck et al., 2005). Comparable to HuBac, these studies found disappearance of the genetic marker is primarily a result of predation. Therefore, factors favorable to biologic removal cause removal of the HF183 marker. Temperature is this factor. Seurinck et al. (2005) observed detectable levels of the HF183 genetic marker for 8 days at 28˚C. This study also observed the presence of HF183 at detectable levels for 24 days at 12˚C. Based on these data, the HF183 genetic marker is more susceptible to temperature than HF183. However, similar to conclusions drawn from literature review of the HuBac marker, it is expected that the cool, dark environment of the karst conduit will likely conserve the HF183 marker. This potential conservation can cause ambiguous results: both HF183 and HuBac will be detected in the karst conduit for longer periods after fecal pollution of the groundwater.

2.3 Sanitary Category Value (SCV) Model

First described in Brion's 2011 document "A Plan for Identifying Hot-Spots and Affirming Remediation Impacts on Surface Water Quality: Phase 1", the Sanitary Category Value (SCV) model is a categorical model based on observations of multiple fecal indicators found in surface water samples. The SCV offers a simple summation of values from 0 to 3 for observed indicators of fecal load (*E. coli*), fecal age (AC/TC) and fecal source (host-specific *Bacteroides* qPCR markers) (Brion, 2011). Fecal load and age indicator classes are assigned a value of 0 to 1.0 with small values (<0.5) representative of low fecal loads and high fecal ages. The SCV value for fecal source is modeled with a proportion of human-specific qPCR markers (HuBac) in a sample to the maximum qPCR signal found in sewage for the same marker. The qPCR values are log-transformed before ratios are taken so that small ratios (<0.5) represent a small proportion of human-specific signal. The SCV model is calculated per the following formula.

$$\mathrm{SCV} = \text{Categorical } E.coli + \text{Categorical AC/TC} + \frac{\log_{10}\mathrm{HuBac}}{\log_{10}\mathrm{HuBacSewageMax}}$$

The midpoint SCV (0.5) represents a threshold value for each indicator class. Any sample that meets or exceeds midpoint values for all three input classes has a summary SCV of 1.5 or higher,

indicating high fecal loading, low fecal age and a high proportion of human-specific signal. Therefore, this SCV of 1.5 is referred to as the "Tipping Point": where the categorical weight of the fecal indicators tips the SCV over the threshold of concern.

The midpoint (0.5) categorical value for *E. coli* is set at the threshold for the level of concern according to the KY Division of Water Quality Standards (WQS) for both *E. coli* and Fecal Coliform bacteria (Brion, 2011). Where WQS were not available for *E. coli*, Brion substituted WQS enforced for Fecal Coliforms. Since *E. coli* form a subgroup of Fecal Coliforms, the proportion of *E. coli* to Fecal Coliforms in freshwater has been reported to range from 0.5 to 0.95, with common proportions of 0.63 found in surface water and higher proportions found in raw sewage (EPA BIT, 2001). By assuming that the proportion of *E. coli* to Fecal Coliform measured in surface water was equal to 1.0, Brion used KYDOW WQS, shown in Table 2.2, as the categorical values outlined below (Brion, 2011):

Table 2.2: KY Primary and Secondary Contact Water Quality Standards. Taken from 401 KAR 10:031 "Surface Water Quality Standards

	Primary Contact Recreation WQS		**Secondary Contact Recreation WQS**	
	Instantaneous (CFU/100mL)	Geometric Mean (CFU/100mL)	Instantaneous (CFU/100mL)	Geometric Mean (CFU/100mL)
Fecal Coliform	400	200	2000	1000
E. coli	240	130	None	None

- Samples below the instantaneous *E. coli* KY WQS for Primary Contact (i.e. < 240 CFU/100mL) are assigned a SCV of 0. This shows that a body of water can fully support Primary Contact recreational activities (i.e. swimming) without undue risk of gastroenteritis (EPA, 2012).
- The midpoint (0.5) SCV for *E. coli* is set at the KY Fecal Coliform WQS for Secondary Contact. Water samples with *E. coli v*alues greater than the geometric WQS, but less than the instantaneous WQS (i.e. 1000 < value < 2000 CFU/100 mL) are assigned a SCV of 0.5
- The maximum SCV (1.0) for *E. coli* was set to screen water samples for combined (CSO) and sanitary sewer overflows (SSO) during storm events (Brion, 2011). The National Pollution Discharge Elimination System (NPDES) mandates that water samples screened for fecal indicators must be diluted at a 10-fold dilution to accurately detect SSOs and

CSOs (NPDES, 1992). The standard method to measure *E. coli*, IDEXX/Quantitray 2000 (discussed in Section 4.3) has a maximum reportable range of 2,400 CFU/100 mL. Therefore, the SCV classification reflects standard dilution and analytical limits by assigning the top SCV (1.0) to any water sample containing 10 times the maximum reportable limit (i.e. >24,000 MPN/100 mL).

- Categories are divided equally between these numbers for other *E. coli* SCVs (Brion, 2011).

An overview of *E. coli* and other SCV categories is displayed in Table 2.3. To determine SCV classifications for the fecal age indicator (AC/TC), Brion relied on previous research experience (Brion, 2011). A summation of AC/TC SCV categories follows:

Table 2.3: SCV Classifications of Fecal Load, Age, and Source. Taken from "A Plan for Identifying Hot-Spots and Affirming Remediation Impacts on Surface Water Quality: Phase I" (Brion, 2011).

Fecal Load Category		**Fecal Age Category**		**Human Fecal Source Category**
***E. coli* (MPN/100mLs)**	**Value**	**AC/TC**	**Value**	**HuBac qPCR**
<235	0.00	>20	0.00	Directly calculated ratio of log_{10}HuBac of sample: Log_{10}HuBacMax (inlet sewage)
>235, <576	0.17	<20, >15	0.25	
>576, <1,000	0.33			
>1,000, <2,000	0.50	<15, >10	0.50	
>2,000, <10,000	0.67			
>10,000, <24,000	0.83			
>24,000	1.00	<10	1.00	

- The level of concern for the AC/TC classification was based on the detection of fresh fecal inputs from cattle and other warm-blooded mammals. Numerous studies show that AC/TC values above 20 are associated with aged fecal materials (Brion and Mao 2000, Nieman and Brion 2003, Booth and Brion 2004). Therefore, AC/TC values greater than the threshold (i.e. >20) of concern are assigned a SCV of 0.
- Prior studies have shown that surface waters with AC/TC below 10 are associated with significant, raw sewage inputs into local creeks (Booth and Brion, 2004). This AC/TC also marked the appearance of detectable human enteric viruses in the Kentucky River (Brion and Lingreddy, 2003). Therefore, the SCV indicating highest level of concern (1.0) is assigned to water samples with AC/TC values below this threshold (i.e. <10) (Brion, 2011).

The SCV category for fecal source is a direct calculation of log-transformed *Bacteroides* qPCR HuBac marker values. A unitless value for a water sample is calculated by taking the $\log_{10}$ transformed value for HuBac divided by the $\log_{10}$ transformed value for the maximum amount of HuBac detected in sewage influent during Brion's 2011 study in the Wolf Run Watershed. This maximum sewage HuBac values was 4,750,000 DNA copies/µL of extract from a 100 mL raw sewage sample taken from the Town Branch Wastewater Treatment Plant (Brion, 2011). This proportion is referred to as HuBac/HuBacMax and shows the relative strength of human-sourced fecal signal found in a water sample:

- The midpoint SCV (0.5) corresponds to 2,178 DNA copies/µL of extract is 0.05% of the maximum HuBac signal found in human sewage (Brion, 2011). This is 200 times larger than the lower level of detection established for HuBac, as discussed in Section 4.4.

Average SCVs calculated from raw sewage samples taken at Town Branch Wastewater Treatment Plant during the 2011 Wolf Run study had a value near 3.0 (Brion, 2011). An overflowing sanitary sewer manhole was also sampled during the 2011 Wolf Run study, resulting in a SCV of 2.88., indicating a large load of fresh, human-sourced fecal material.

2.4 "A Plan for Identifying Hot-Spots and Affirming Remediation Impacts on Surface Water Quality": Sanitary Category Value Application in the Wolf Run Watershed

The Wolf Run watershed is a highly developed basin located in the predominantly urban of Central Lexington. In the Kentucky Division of Water's (KYDOW) "2010 Integrated Report to Congress: 303d List of Surface Waters", the entire Wolf Run watershed is listed as an impaired body (KYDOW, 2010). This report indicates that conditions in Wolf Run did not support the Primary Contact Recreation designated use due to "fecal coliform, nutrient/eutrophication biological indicators, specific conductance" due to the influence of "unspecified urban storm water and urban runoff/storm sewers". As such, this watershed provided an ideal test bed for the identification, location, and ranking of human sewage sources with the Sanitary Category Value (SCV) Model.

As discussed in Dr. Gail Brion's "A Plan for Identifying Hot-Spots and Affirming Remediation Impacts on Surface Water Quality", members of the volunteer watershed group Friends of Wolf

Run collected surface water samples at 20 sites within the Wolf Run watershed from April 6th through August 5th, 2010 (Brion, 2011). Under direction by Friends of Wolf Run volunteers and LFUCG officials, sample sites were selected to reflect areas were the designated use of the watershed, primary contact recreation, were likely to occur, such as city parks and golf courses. One of these sites was located at a known sewer overflow location to provide comparison of the water quality indicators between wet and dry weather conditions. Samples of raw human sewage were also taken at the inlet to Town Branch Wastewater Treatment Plant, which is located within the Wolf Run watershed, to provide a baseline of fecal indicators for the SCV model.

Samples collected were analyzed at the University of Kentucky's Environmental Research Training Laboratories (ERTL) for *E. coli*, AC/TC, and quantitative PCR markers (Brion, 2011). Six dry-weather sample events screened for indication of leaking sanitary infrastructure while four wet-weather sample events provided evidence of SSOs. Brion's conclusions from the study are summarized below:

1. Statistical analysis of *E. coli* load values showed that inlet sewage was significantly different from all other sample sites under summary, dry, and wet conditions (Brion, 2011). In addition, no statistically significant differences were found when comparing between the *E. coli* loads measured at sample sites within the watershed, even under similar weather conditions. Due to the large confidence intervals associated with the MPN assay method, and the variance found in concentrations on different days at the same sites, Brion concluded that "it is important not to rely upon levels of *E. coli* alone when trying to define differences between areas within a watershed." (Brion, 2011).
2. By modifying analysis of fecal load (*E. coli*) with trends observe in fecal age (AC/TC) values, Brion detected the impact of fresh sewage at a sample site. Suspicions of a leaking sanitary sewer, as indicated by low AC/TC values and high *E. coli*, were confirmed when Volunteers from Friends of Wolf Run documented a broken sewer pipe upstream of the sample site in question. Fecal age increased (indicated by increasing AC/TC values) at the sample site during rainfall, a trend explained as aged fecal materials entered the stream from overland scour. Even inlet sewage has a slight rise in AC/TC values during rain events. The successive decrease of AC/TC values from wet to dry events at the suspected sample site indicated fresh fecal material entering the

stream due to leaking sanitary infrastructure. Therefore, analysis of AC/TC alone can detect a leaking sanitary sewer in an urban watershed. However, similar to *E. coli*, wide variability around average AC/TC values at sample sites caused difficulty to detect statistical significance when comparing sites against each other. As discovered during interpretation of fecal load (*E. coli*) levels, Brion concluded that "the fecal age indicator should not be used alone".

3. During analysis of qPCR data (HuBac and HF183), Brion found that the HuBac marker was detected in larger concentrations than HF183 marker. Even inlet sewage had significantly less average HF183 marker signal than average HuBac signal. HuBac was also detected above limits of detection (LOD) more frequently than HF183: "Values for HuBac were below the LOD 28.5% of the time whereas values for qHF183 were below the LOD 74.5% of the time". Brion found significance in the percentage of sampling events that the HF183 marker was detected at a sampling site. At the same site where a documented leaking sewer connection was indicated by fecal age and load, the HF183 marker was detected above the LOD in all dry weather samples. During wet weather, the qHF183 signal was diluted to non-detectable levels 33% of the time. Therefore, Brion found that, while the human-associated HuBac marker was recovered more frequently and in larger numbers, detection of the more human-specific HF183 marker was meaningful. Brion concluded that "the importance of developing and proving multiple indicator systems" outweighs the search for a single, "silver bullet" indicator for monitoring water quality.
4. Application of the SCV in Wolf Run resulted in definitive success: all sample sites were significantly different than sewage during dry conditions except for the site where a leaking sanitary sewer connection was documented. Brion provided preliminary feedback based on depressed AC/TC values and elevated *E. coli* loadings to the Friends of Wolf Run, leading to the discovery of the aforementioned leaking connection. The SCV model had indicated a condition that was confirmed. This confirmed that application of the SCV, especially during dry conditions, can be used to "pinpoint hot-spots of human sewage leaking into the environment" (Brion, 2011).

2.5 "A Plan for Identifying Hot-Spots in West Hickman Watershed": Confirming Sanitary Category Value as an Analytical Tool

The West Hickman watershed is another basin located in the urban environment of Central Lexington. Relative to the Wolf Run watershed, the West Hickman is less impacted by human sewage. As reported in the "2010 Integrated Report to Congress: 303d List of Surface Waters", only one stream segment of the West Hickman is listed for partial support of the Primary Contact Recreation use designated to the watershed (KYDOW, 2010). However, like the Wolf Run watershed, the cause of water quality impairment was due to pathogens from sewage.

Brion organized the same approach and modeling system to water quality analysis in the West Hickman watershed: under dry weather conditions, SCVs were calculated to indicate leaking sanitary sewers. . Samples were collected by the employees of Third Rock Consulting from eighteen sample locations on four dry weather events from August through October, 2011 (Brion, 2012). These samples were again analyzed for viable *E. coli* bacteria, AC/TC, two host-specific *Bacteroides* markers (HuBac and HF183). Conclusions drawn by Brion reflected the KYDOW's 303(d) classification: West Hickman did not have sites as severely impacted by human sewage as were found in Wolf Run. However, sample sites did show the continuous presence of human sewage. As shown in the Wolf Run watershed, the SCV model approach identified and ranked sites in the West Hickman watershed. Conclusions drawn by Brion are summarized below (Brion, 2012):

1. These results of *E. coli* analysis indicate that not all streams within the watershed are impaired for primary contact recreational use under dry weather conditions. Two sites had geometric means below the geometric mean regulatory limit. However, detection of human-sourced HuBac and HF183 signals at these sites indicated that the sample sites were still impacted by human sewage. As concluded in Section 2.3, *E. coli* analysis alone cannot indicate the absence of human sewage.
2. AC/TC Results identified a number of sites impacted by fresh fecal sources. One site with the smallest average AC/TC (indicating freshest fecal age) confirmed the detection of large *E. coli* loads. However, trends based on AC/TC analysis alone did not result in any site identified significantly different than any other site.
3. Similar to the Wolf Run study, HuBac was recovered above limits of detection during dry weather at all sampling locations in West Hickman. Conclusions based on HuBac markers alone indicated that human sewage was detected, at some level, all the time during the sampling period. However, the more conservative human marker, qHF183,

was only detected at three sites during the study. These three sites also measured the largest *E. coli* loads and freshest AC/TC values relative to other sites, indicating the impact of fresh, human-sourced sewage. Again, the specificity of the HF183 marker supported conclusions not possible considering only specific fecal indicators.

4. While no sites were significantly similar to sewage during the dry sampling events, the highest average SCV values were found at the aforementioned sampling sites impacted by large loads of fresh, human-sourced sewage relative to other sampling sites. As such, Brion concluded that, even in a relatively "clean" watershed, the SCV is applicable in a predominantly urban environment.

A study prepared by Farrell and Evans at Third Rock Consultants was published parallel to Brion's 2012 study. This study, titled "West Hickman Microbial Source Tracking: Dry Weather Assessment of Pathogen Sources for Sanitary Sewer Priority Areas" chose to interpret the same data set of *E. coli* fecal indicators by expressing Total Maximum Daily Loads (TMDLs) at each sample site (Ferrell and Evans, 2012). Using flow data collected during sample events, *E. coli* yields were calculated and averaged at sample sites. Allowable yields of *E. coli* were calculated by multiplying the KY WQS for *E. coli* at that same flow. Sites where the calculated yield exceeded the allowable yield were classified as areas significantly impacted by fecal material. This application of fecal loading analysis is the current EPA recommended method for determining locations of fecal impact in surface watersheds (EPA, 2007; KYDOW, 2009). The conclusions of this study were identical to Brion's: the same three sample sites were identified as hot-spots of fecal impact in the West Hickman watershed. Therefore, the SCV model was again proven capable of identifying sites and ranking the relative impact of human sewage in the West Hickman watershed.

2.6 Report to the City of Georgetown: Water Quality Analysis 2005

During the period of March through May, 2005, the City of Georgetown contracted Dr. Gail Brion at the University of Kentucky to conduct a study within the Royal Spring Karstshed (Brion, 2005). This study identified sources of sewage contamination impacting Royal Spring and Georgetown's drinking water supply. Eight sample sites were tested weekly for a total of 11 sample events, capturing both wet and dry weather conditions. Three fecal indicators were selected to screen samples for human sewage: fecal load (*E. coli*); fecal age (AC/TC); and fecal source (F+ coliphage). Coliphage were used as fecal source identifiers prior to the development of qPCR markers for *Bacteroides*, but the application of fecal load, source, and age indicators was similar to later studies.

As shown in Table 2.4, Highland Spring and IBM were identified as hotspots for fecal contamination (Brion, 2005). The greatest loads of fecal material were input into the Karstshed at Highland Spring, as shown by the relative magnitude of *E. coli* geometric means detected over the sampling period. This fecal source was also fresh, as indicated by low average AC/TC values (<5). While IBM also was a significant source of fecal load, as indicated by the *E. coli* geometric mean, the average fecal age was greater (AC/TC >20) than the fecal input at Highlands. Contrasting these fecal age and load values measured at Highland Spring and IBM to those measured at Royal Spring (Georgetown WTP in Table 2.4) indicates that very little fresh fecal material was detected at Royal Spring. The fecal load was very small and very aged, well beyond concern thresholds outlined in Section 2.3. Since fecal age and load indicators measured at Royal Spring were negligible, that sewage in the conduit had aged and diluted without any additional fecal input to the almost undetectable values at Royal Spring (Brion, 2005)

Table 2.4: Fecal Load and Age Indicators. Taken from "Report to the City of Georgetown: Water Quality Analysis 2005" (Brion, 2005).

Site	Geometric mean E. coli (cfu/100mL)	Average AC/TC Ratio
Highland Springs	454	3.5
IBM	243	24
Georgetown WTP	30	186

Since this 2005 study predated the availability of *Bacteroides* qPCR markers for water quality analyses, Brion relied on the presence of F+ specific RNA coliphage to indicate fecal source. Since F+ coliphage are viruses that infect coliform bacteria, their presence has been linked to the

presence of human sewage for microbial source tracking (Smith, 2006). As shown in Table 2.5, Brion used two methods to indicate human-sourced sewage with F+ coliphage: frequency of isolation of F+ coliphage and average F+ coliphage concentration.

Table 2.5: Fecal Source Indicators. Taken from "Report to the City of Georgetown: Water Quality Analysis 2005" (Brion, 2005).

Site	Average Concentration of F+ coliphage pfu/100mL (min-max)	Frequency of Isolation of F+ coliphage
Highland Springs	4 (1-10)	11 of 11 days
IBM	4 (0-16)	9 of 11 days
Georgetown WTP	32 (1-226)	11 of 11 days

Unlike fecal age and fecal source indicators, Brion found a consistent source of human fecal material indicators at Royal Spring. Average F+ coliphage values were an order of magnitude greater at Royal Spring (Georgetown WTP in Table 2.5) than values detected at Highland Spring and IBM, indicating human sewage was impacting the Karstshed downstream of Highland and IBM. This sewage source was also very consistent, as indicated by the 100% isolation of phage at Royal Spring during the sample period. As shown in Table 2.6, average F+ coliphage values increased by an order of magnitude during wet events, indicating that the fecal source impacting Royal spring was wet weather related (Brion, 2005).

Table 2.6 Wet versus Dry Weather Fecal Load, Age and Source Indicators. Taken from "Report to the City of Georgetown: Water Quality Analysis 2005" (Brion, 2005).

Highland Spring	Wet			Dry		
	E. coli	Phage	AC/TC	E. coli	Phage	AC/TC
Mean	219	3	**3.39**	845	4.5	**3.63**
Mininum	78	1	0.54	111	2	0.97
Maximum	488	5	9.42	2419	10	6.98

IBM	Wet			Dry		
	E. coli	Phage	AC/TC	E. coli	Phage	AC/TC
Mean	58	3.4	**7.07**	794	4	**38.02**
Mininum	1	2	0.87	12	0	3.06
Maximum	238	9	23.62	15553	16	152.4

G-town treatment plant	Wet			Dry		
	E. coli	Phage	AC/TC	E. coli	Phage	AC/TC
Mean	82	47.2	**149.41**	31	19.17	**215.91**
Mininum	15	1	1.6	8	1	9
Maximum	157	226	540.23	111	108	703

Based on data displayed in Table 2.6, Brion concluded the following:

1. There is a hotspot of human sewage input into the Royal Spring Karstshed at the Highland Spring. Since fecal load, age, and source indicators changed very little between wet to dry events, it was concluded that a consistent sewage source was impacting the Karstshed, such as a leaking sanitary sewer or septic system. Brion concluded that the aging infrastructure in the Highlands subdivision input human sewage into the Royal Spring Karstshed (Brion, 2005).
2. IBM also provided a hotspot of human sewage input into the Karstshed. Relative to Highland Spring, this source was indicated by smaller fecal load and older fecal age values, but comparable fecal source values. As shown in Table 2.6, fecal age measured at IBM decreased by an order of magnitude during wet weather events, indicating a fresh input of sewage during rain events. Brion concluded that the source at IBM was likely a leaking sewer that overflowed during rain events (Brion, 2005).
3. While the constant F+ coliphage signal at Royal Spring (G-town treatment plant in Table 2.6) was, in part, due to sewage inputs from Highland Spring and IBM, the order of magnitude increase in average value and consistency of signal indicates a large fecal source between these sites and Royal Spring. Brion concluded that a "large, undiscovered source of human sewage" was input into the spring system at Royal Spring

and “further study is required to identify this source so that a remediation plan can be developed” (Brion, 2005)

2.7 Report to the City of Georgetown: Water Quality Analysis 2006

In a follow-up study Spanning May through June, 2006, Brion collected and analyzed water quality samples from the same sample sites in the Royal Springs Karstshed. Again, three sample sites correspond to sample sites analyzed in this thesis: IBM, Highland Spring, and Royal Spring. Six samples were taken, during both wet and dry weather condition, and analyzed for the same fecal indicators as before: fecal load (*E. coli*); fecal age (AC/TC); and fecal source (F+ coliphage). In addition, three more human source indicators were analyzed from water samples: two Fecal Sterols (Coprostanol, Epicoprostanol) and Caffeine.

Fecal sterols are formed from the digestion of cholesterol in the guts of warm blooded animals and birds (Brostrom, 2005). Since Coprostanol and Epicoprostanol are shed in higher numbers from humans than in animals and are detected in streams contaminated with sewage, Brion sought to further pinpoint the source of F+ Coliphages (Brostrom, 2005). Caffeine has also been applied in Microbial Source Tracking to identify human sewage sources, but with limited success (Blanch, 2006). Averages of these indicators found in Cane Run are shown in Table 2.7.

Table 2.7 Average Fecal Indicators in Royal Spring Karstshed. Taken from “Report to the City of Georgetown: Water Quality Analysis 2006” (Brion, 2006)

Site	Phage (PFU/100mL)	Caffeine (ppt)	E. coli (MPN/100mL)	Total Coliforms (CFU/100mL)	Atypical Coliforms (CFU/100mL)	AC/TC	Coprostanol (ppt)	Epicoprostanol (ppt)
Highland	1.5	39.0	579.4	2150	16500	7.67	71.1	<3.3
IBM	1.5	28.0	547.5	2250	66500	29.65	10.0	4.7
G-town Water Plant	2.5	26.7	238.2	305	2700	8.85	3.8	<3.3

Conclusions drawn from this study support those from Brion’s 2005 report:

1. Hot-spots of fecal impact were detected at IBM and Highland Spring. Low AC/TC values and high *E. coli* loads detected at Highland Spring indicate the impact of fresh fecal material. Large *E. coli* loads at IBM are indicative of aged fecal material (high AC/TC). Again, *E. coli* values were diluted from Highland and IBM to Royal Spring.
2. F+ Coliphage increased at Royal Spring (G-town Water Plant in Table 2.7) relative to upstream sites (IBM and Highland Spring). In addition, the decrease of average AC/TC

values (relative to upstream sites) indicates a fresh, human-sourced fecal input between Royal Spring and upstream sites (Brion, 2006).

3. Coprostanol values were higher at Highland and IBM than Royal Spring. Epicoprostanol and Caffeine analyses did not show any significant results (Brion, 2006).

2.8 Cane Run Watershed Project

As described in the document "Cane Run and Royal Spring watershed-Based Plan", the University of Kentucky Biosystems and Department of Agricultural Engineering (BAE) collected monitoring data to document water quality in the Cane Run watershed and Royal Spring Karstshed (BAE, 2012). A sampling network combined biweekly grab samples with automated storm samples for sediment and bacterial data. Water samples collected in 2008, 2009 and 2010 at 14 sampling sites provided insight into sewage sources impacting Cane Run and Royal Spring, using *E. coli* as a fecal load indicator.

Of these 14 sampling sites, two are equivalent to sites selected for this thesis: IBM and Highland Spring. As shown in Table 2.8, samples collected at IBM (CR03) and Highland Spring (CR04) show a significant sewage load detected in the Royal Spring Karstshed. Table 2.8 also presents the amount of time *E. coli* values in water samples exceeded Kentucky's Water Quality Standards (WQS) for Primary and Secondary Contact. As stated in 401 KAR 10:031, geometric means of *E. coli* taken during a thirty day period shall not exceed 240 CFU/100mL for primary contact or 676 CFU/100mL for secondary contact.

Table 2.8: *E. coli* Geometric Means with Numbers of Sample Exceeding KY WQS. Taken from "Cane Run Watershed Based Plan" (BAE, 2012).

Site	*E. coli* Geometric Mean (cfu/100mL)	No. Samples *E. coli* >240 MPN/100mL	No. Samples *E. coli* >676 MPN/100mL
CR03	3076	21 (88%)	18 (75%)
CR04	7003	44 (100%)	44 (100%)

As shown in Table 2.8, Highlands (CR03) and IBM (CR04) were both influenced by large fecal loads, indicated by high geometric mean *E. coli* values. Samples at Highlands were above the KY DOW WQS for both Primary Contact and Secondary Contact 100% of the time of study. The study also found that "concentrations measured at the Highlands were strongly linked to those measured at a downstream site", indicating that a sewage source at Highlands was influencing the Karstshed (BAE, 2012). The Cane Run watershed Based Plan concluded the following:

1. Highland Spring and IBM were hotspots of fecal contamination in the Royal Spring Karstshed. *E. coli* values at Highland Spring and IBM were related to 48-hour prior rainfall, indicating that the sewage source is likely "linked to failing sewer lines and other sewer infrastructure" (BAE, 2012).
2. Smoke testing conducted in the Highlands Subdivision by LFUCG during the sample period concluded that there were many cross-connections between sanitary sewers and storm overflows. This confirmed that leaking sanitary sewers detected by *E. coli* values at Highland Springs.
3. Inspection of failing septic systems, leaking sanitary sewers, and other sanitary sewer infrastructure should be focused on sites draining to IBM and Highland Spring. Replacement of this infrastructure will reduce fecal loads detected in the Royal Spring Karstshed (BAE, 2012).

2.9 Development of Fecal Coliform TMDL for 303(d) Listed Stream in the Kentucky River Basin: Cane Run in Fayette County, Kentucky

Cane Run was first placed on the Kentucky Division of Water's (KY DOW) 303(d) list of impaired water in 1998 (KY DOW, 1998). By 2010, this 303(d) list had expanded to include the entire 17.4 miles of Cane Run, all tributaries to Cane Run, and Royal Spring itself (KY DOW, 2010). This updated list determined that Cane Run and Royal Spring could not support the designated water use of Primary Contact and Secondary Contact due to Fecal Coliform and Sewage Biological Indicators.

To meet the KY DOW's mandate to "safeguard from pollution the uncontaminated waters of the Commonwealth; to prevent the creation of any new pollution of the waters of the Commonwealth; and to abate any existing pollution", a Total Maximum Daily Load of human sewage was developed in 2010 by the Kentucky Water Resources Research Institute (KWWRI) (KRS 224.71). This TMDL process established a fecal pollutant load allowable in the Cane Run watershed while maintaining the designated watershed use.

From May 2002 to September 2002, the KWWRI sampled Cane Run and tributaries of the watershed (Ormsbee et al., 2010). Samples were analyzed for fecal loading with fecal coliform bacteria as an indicator. Both wet and dry weather samples were collected to better screen for both point and nonpoint fecal sources. Of the eight sites selected for this 2002 study, one site

(C0) corresponds to a sample site selected for this thesis: IBM. As shown in Figure 2.1, fecal loads, indicated by geometric means of fecal coliform colonies, were significantly higher at IBM than all other sampling sites (Ormsbee et. al, 2010).

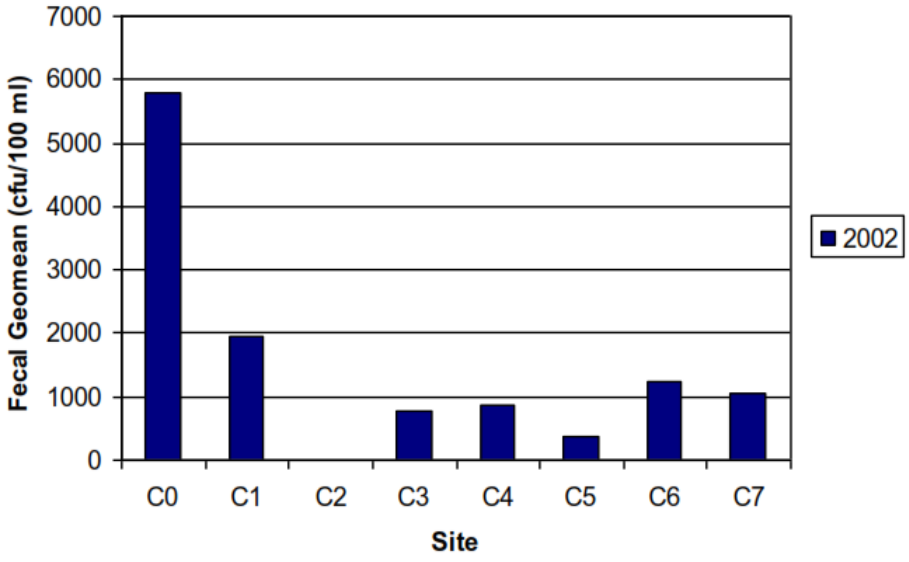

Figure 2.1: Fecal Coliform Geometric Means from KWWRI 2002 Sampling. Taken from "Development of Fecal Coliform TMDL for 303(d) Listed Stream in the Kentucky River Basin: Cane Run in Fayette County, Kentucky" (Ormsbee et. al, 2010).

As shown in Figure 2.1, fecal coliform data measured at IBM (C0 in Figure 2.1) exceeded the 30-day geometric limit (200 CFU/100mL) set by Kentucky's Surface Water Standards (401 KAR 5:031) for primary contact recreation. In addition to the data collected by the KWRRI, data obtained from the Georgetown Municipal Water Company and Lexington Fayette Urban County Government was analyzed. The authors concluded that "more than 90% of the time, pathogen values in Cane Run, and its tributaries, exceeded limits set for primary contact recreation" (Ormsbee et. al, 2010). The TMDL document concluded that fecal loading at IBM was likely due to failing On-Site Wastewater Treatment systems (septic tanks), leaking sewers or other illegal storm water cross-connections with sanitary sewers. In addition, fecal loading at IBM was directly related to numerous Sanitary Sewer Overflows, as documented by the increase of fecal coliform loading during wet-weather events (Ormsbee et. al, 2010).

3.0 Research Objectives and Approach

3.1 Hypothesis

An unidentified human sewage source is impacting the Georgetown Drinking Water supply at Royal Spring. This source is local to Royal Spring and independent of other fecal inputs to the Karstshed.

3.2 Research Approach

Four sample sites were selected for water sample collection. These sites are shown in Figure A.2 (Appendix A) and described in Table 3.1. A photo log documents these sampling sites in Appendix B. Six sample events were collected (3 wet weather, 3 dry weather) from these four sampling locations, May 2012 through July 2012. Dry weather samples were screened for indication of leaking sanitary sewers while wet samples indicated the impact of storm-related sanitary sewer overflows (SSO).

Table 3.1: Sample Site Descriptions

Site ID	Site Name	Site Description	Site Access	Lat / Long
RS	Royal Spring	Royal Spring is located on West Main Street in downtown Georgetown, KY. The spring has supplied drinking water to Georgetown since 1889 and is the primary drinking water source to 8,000 customers of Georgetown Municipal Water and Sewer Service (Georgetown Municipal Water, 2008)	Turn onto Royal Spring St. from West Main (US 460). Park at the WTP and access the spring from the maintenance walkway	38.208660° / -84.562108°
KYHP	KY Horse Park	The Kentucky Geological Survey (KGS) maintains a research station in the KY Horse Park. This station directly monitors the groundwater conduit that "pipes" water from Lexington to Georgetown, KY. A groundwater monitoring well provides access to water samples from the conduit.	Enter the KY Horse Park from Iron Works Pike (KY 1973). Take Cigar Lane to Walt Robinson Road. Access to the KGS monitoring Station is through a horse pasture.	38.164732° / -84.531542°
HS	Highlands Spring	The Highland Spring is an undeveloped artesian spring located near an aging subdivision (Highlands) in Lexington, KY. Groundwater from the spring feeds a small tributary to Cane Run which then disappears into the conduit after crossing Citation Blvd. Highlands marks the transition from urban to agricultural land use within the Karstshed	Park at unmarked farm access from Citation Blvd. Walk across corn field and enter wooded area. Samples are taken from abandoned concrete spring box.	38.091413° / -84.503089°
IBM	IBM Swallet	The IBM Swallet transfers surface water from Cane Run to the conduit. As discussed in Sections 2.4 – 2.7, leaking sewers impact Cane Run directly upstream of the Swallet	Enter IBM property on Nadino Blvd from Newton Pike. The swallet is close to the road (approximately 30 yards).	38.078106° / -84.490453°

3.3 Objectives

1) Detect, classify, and pinpoint sewage sources in the Royal Spring karstshed. Contrast these sources to those highlighted by previous studies in the karstshed.
2) Apply and analyze multiple fecal indicators in water quality samples taken from Karstic environment. Explore the applicability of these indicators for analysis of groundwater.
3) Analyze and explore applicability of load analyses in the karstshed.

4.0 Methods and Materials

4.1 Sample Collection

Surface and groundwater samples were collected in sterile 100mL polypropylene bottles and stored on ice before delivery to the Environmental Research Training Laboratory (ERTL) at the University of Kentucky in Lexington, KY. Samples were collected, transported and analyzed within the 6-hour window specified in the EPA document "SOP: Surface Water Collection" (US EPA, 2003).

4.2 AC/TC Analysis

AC/TC analysis followed EPA standardized methods (SM 9222B) for bacterial enumeration:

1. 100 mL of raw water samples were analyzed. One dilution, 1:100, was prepared with phosphate buffered saline (PBS).
2. Three volumes of water samples were filtered through a membrane: 0.1 and 1 mL from the 1:100 diluted sample; and 10mL from the undiluted sample. Bacterial colonies present in the sample remained on the filter membrane.
3. The filters were aseptically transferred from the filtration funnel to petri dishes containing pads saturated with M-Endo growth media. Each sample dilution and volume was filtered in duplicate. Funnels were sterilized between analyses with a UV disinfection booth.
4. Plates were inverted and incubated at 35°C±0.5°C for 24±2 hours.
5. After incubation, single colonies were counted on the plates. The reportable range for a plate count falls between 20 and 80 colonies. Two or more colonies touching were counted as one colony. All bacteria which produced a red colony were considered members of the Atypical Coliform group (AC).
6. Bacteria producing metallic, golden "beetle-wing" sheen were considered members of the Total Coliform group (TC).

As described in SM 9222B, AC and TC enumerations were calculated as colony forming units (CFU) per 100mL by the following:

$$\frac{\text{CFU}}{100\text{mL}} = \frac{\text{Total \# of Colonies} * 100}{\text{Total Volume Plated}}$$

Considering that analyses were performed with duplicate plates for each volume filtered, this calculation allows for an average CFU/100mL between duplicates. This calculation is also beneficial when plate counts were reported less than the countable range (< 20 colonies); CFU/100mL can be calculated across multiple dilutions. For example, AC data collected at KYHP on 6/7/2012 was calculated:

0.1mL: 2,0

1mL: 7,8

$$\frac{\text{CFU}}{100\text{mL}} = \frac{\text{Total \# of Colonies} * 100}{\text{Total Volume Plated}} = \frac{(2+0+7+8)*100}{(0.1+0.1+1+1)} = 773\frac{\text{CFU}}{100\text{mL}}$$

The AC/TC ratio was reported as a unit less value.

4.3 *E. coli* Analysis

Standardized methods (SM 9222B) also describe the procedure for enumerating *E. coli*. Analysis was performed with the IDEXX Quanti-Tray/2000 as described in the document "SOP for *E. coli* and Total Coliform Quantification using the IDEXX Quanti-Tray/2000 system" (US EPA, 2003).

1. Surface water samples were stored on wet ice up to 6 hours
2. 100 mL of sample at two dilutions were prepared with PBS solution: no dilution and 1:10
3. One pre-measured packet of Coliert reagent was poured into a 100 mL sample. The mixture was shaken, poured into a Quanti-Tray/2000, and sealed with a Quanti-Tray sealer.
4. These sealed Quanti-Tray/2000 were incubated at 35°C±0.5°C for 24±2 hours
5. A color change from clear to yellow indicates the presence of Total Coliform bacteria. Fluorescence under a UV light of these same yellow wells indicates the presence of *E. coli*. Counting the number of small and large yellow and fluorescent wells determines a statistical estimate of the most probable number (MPN) of bacteria per 100 mL sample. These MPNs were generated from the "MPN Generator" software provided by IDEXX and reported as MPN/100mL.

The detection limit of the IDEXX Quanti-Tray/2000 is 2,419.6 MPN/100mL (IDEXX, 2012). Since analysis of water samples were performed at 1:10 dilution, the reporting range of this analysis is <1 to 24,196 MPN/100mL.

4.4 qPCR Analysis for Human-linked Markers

Bacteroides qPCR analyses follow the method described by Alice Layton in the document "Development of *Bacteroides* 16S rRNA Gene TaqMan-Based Real-Time PCR Assays for Estimation of Total, Human, and Bovine Fecal Pollution in Water" (Layton, 2006). Analyses of AllBac and HuBac genetic markers were performed by ERTL Lab Manager Trish Coakley as follows:

1. 250 mL of undiluted sample were filtered through a filter membrane. When samples were too turbid, clogging the filter before the entire 250mL volume was filtered, a smaller volume was selected.
2. The filter was rolled, placed into sterile 15mL centrifuge tubes, and stored at -20°C until extraction. Duplicates of one sample site per sampling event were prepared.
3. qPCR extractions were completed by ERTL Lab Manager Trish Coakley. DNA extractions were completed by a method described in the 2010 US EPA document "Method B: *Bacteroides* in Water by TaqMan(R) Quantitative Polymerase Chain Reaction (qPCR) Assay":

   > "The method uses AE buffer with 0.2 µg/mL Salmon testes DNA (Sketa 22) as an internal standard to determine the presence of PCR inhibition in the sample matrix. Each membrane filter was placed into a Mobio 5mL PowerWater® Bead Tube to which 1 mL of the Sketa spiked buffer solution was added. Bead tubes were vortexed for 10 minutes using a multi-tube vortex adapter. Tubes were centrifuged at 3,500 Xg for 5 minutes and 0.5 mL supernatant was recovered from each and transferred to a 1.5mL microcentrifuge tube. The microcentrifuge tubes were then centrifuged at 13,000 Xg for 1 minute and the supernatant was transferred to another 1.5mL microcentrifuge tube. Extracts were stored at -20°C until DNA analysis by qPCR." (US EPA 2010)

4. AllBac and HuBac *Bacteroides* genetic markers were analyzed by qPCR using primers and probes developed by Alice Layton at the University of Tennessee. This process is described by ERTL Lab Manager Trish Coakley:

   > "Real-time PCR was performed using a BioRad iCycler IQ™. Each 20 µL PCR reaction consisted of 10 µL TaqMan Environmental Mastermix (Life Technologies™), 10 pmol forward primer (Allbac, Hubac, HF183 or SKETA), 10 pmol of the corresponding reverse primer, 5 pmol of the corresponding FAM fluorescently-labeled molecular probe, and 2 uL of the filtered water DNA extract. PCR protocols consisted of a 50°C hold for 2 minutes and a 10- minute

activation at 95°C, followed by 50 cycles of 95°C denaturation for 30 seconds and 60°C annealing for 45 seconds" (Coakley, 2011)

AllBac, HuBac and HF183 quantities were reported as DNA copies per µL of extract for a 250 mL sample filtrate. The lower quantifiable reportable levels of detection (LOD) for AllBac and HuBac were established as 100 DNA copies per µL of filter extract (Brion, 2011). These limits are based on Brion's past analytical experience with the HuBac marker: analytical error (expressed as standard deviation) decreased in the real-time q PCR analysis at a HuBac concentration of 100 DNA copies per µL. Brion (2011) also recommended, based on analytical experience, the LOD for the human-specific HF183 genetic marker. This was LOD of 1 copy per µL of filter extract and a quantification level of 10 or greater copies per µL of filter extract. This classification allowed for low levels of HF183 (>1 but <10) to indicate the presence of human sewage, but not quantify the fecal signal. Data infilling for qPCR values less than LOD is summarized in Table 4.1. These differences in detection levels were due to the requirement of microbial source tracking models to use repeatable values in model calculations. As found in previous studies of the SCV model, repeatable values were determined as those greater than the 100 DNA copies per µL of extract (Brion 2011).

Table 4.1: Data Infilling for qPCR Values

AllBac	**HuBac**	**HF183**
<100 = BDL	<100 = BDL	<1 = BDL; <10 = BQL
IF BDL: AllBac = 50 copies/ µL	IF BDL & AllBac BDL: HuBac = 5 copies/ µL	IF BDL: HF183 = 1 copy/ µL
	IF BDL & AllBac > BDL: HuBac = 50 copies/ µL	IF BQL: HF183 = 5 copies/ µL

After data infilling, AllBac, HuBac and HF183 values were converted to quantifiable units by the adjusting for an initial 250 mL sample volume:

$$\text{qPCR}\left(\frac{\text{DNA Copies}}{\text{mL of Original Sample}}\right) = \text{qPCR}\left(\frac{\text{DNA Copies}}{\mu\text{L of Original Sample}}\right) * \frac{1000\ \mu\text{L/mL}}{250\ \text{mL of Original}}$$

In summation, qPCR values were converted to quantifiable units with a multiplication factor of 4.

4.5 Sanitary Category Value (SCV) Calculation:

As discussed in Section 2.2, Sanitary Category Value (SCV) is the sum of categorical classifications of *E. coli*, AC/TC, and HuBac:

$$\text{SCV} = \text{Categorical } E.coli + \text{Categorical AC/TC} + \frac{\log_{10}\text{HuBac}}{\log_{10}\text{HuBacSewageMax}}$$

Note that the HuBac SCV is a directly calculated as a ratio of log-transformed HuBac values measured at sampling sites to the largest log-transformed HuBac value determine of inlet human sewage (4×10^6 DNA Copies/ 100 µL of Extract) during Brion's (2011) development of the SCV model. Recalling discussion in Section 2.3, this HuBac normalization was utilized to provide relative rank of HuBac values to the largest HuBac value expected in human sewage.

Using the classification scheme presented in Table 4.1, a category value for each fecal indicator was calculated and summed to give a SCV for each sampling event. Average SCVs were calculated across sampling events for each site.

Table 4.2: SCV Classifications of Fecal Load, Age, and Source. Taken From "A Plan for Identifying Hot-Spots and Affirming Remediation Impacts on Surface Water Quality: Phase 1" (Brion, 2011).

Fecal Load Category		**Fecal Age Category**		**Human Fecal Source Category**
***E. coli* (MPN/100mLs)**	**Value**	**AC/TC**	**Value**	**HuBac qPCR**
<235	0.00	>20	0.00	Directly calculated ratio of $\log_{10}$HuBac of sample: Log_{10}HuBacMax (inlet sewage)
>235, <576	0.17	<20, >15	0.25	
>576, <1,000	0.33			
>1,000, <2,000	0.50	<15, >10	0.50	
>2,000, <10,000	0.67			
>10,000, <24,000	0.83			
>24,000	1.00	<10	1.00	

SCVs for each site are included in Appendix C.3. An example calculation for fecal indicators measured at the KY Horse Park on 5/14/2012 is seen as:

E. coli = 19863 MPN/100 mL
AC/TC = 4.03
HuBac = 218.01 Copies of DNA/µL of Extract from a 250 mL sample

$$\text{SCV} = 0.83 + 1.00 + \frac{\log_{10}(218.01)}{\log_{10}(4.75\times10^6)} = 0.83 + 1.00 + 0.35 = 2.18$$

Note that the categorical values for human-linked signal (HuBac/HuBacMax) were not corrected for the volume of sample filtered (Discussed in Section 5.7). Although the HuBacMax value

originated from a 100mL sample, the filter clogged before the entire volume was sampled, negating the need for correction.

4.6 Quality Control

Analytical quality control measures were similar to those described in the document "A Plan for Identifying Hot-Spots and Affirming Remediation Impacts on Surface Water Quality: Quality Assurance Project Plan" (Brion, 2010). Quality controls enacted while analyzing AC/TC were:

1. Positive controls for M-Endo media quality were analyzed for each sampling event. This was accomplished by analyzing duplicates of raw influent sewage samples collected during the study. These samples were collected during sample events at the West Hickman Wastewater Treatment Plant in Lexington, Kentucky. If these analyses produced no observable sheening (TC) colonies, then the m-Endo growth media was considered expired.
2. A negative control for media quality was performed at the beginning and end of filtration for each sampling event. A volume of PBS buffer was filtered, plated, and incubated to ensure both PBS and growth media quality.
3. Each water sample was filtered with a minimum of three dilutions and two replicate plates per dilution analyzed.

Quality controls used during *E. coli* analysis are:

1. Positive controls for Coliert growth media were analyzed during each sampling event. An IDEXX Quanti-Tray/2000 was analyzed with a dilution of raw influent sewage sample collected at the West Hickman Wastewater Treatment Plant.
2. A negative control for growth media and PBS buffer quality were performed for each sampling event. A Quanti-Tray/2000 was analyzed with 100 mL of PBS and Coliert growth media.
3. Duplicates of one sampling site were performed for each sampling event.

Quality control guidelines are recommended by the EPA for qPCR analysis (EPA 2010). These include:

1. qPCR positive control for each PCR run. These were collected from a duplicate sample extraction of sewage samples collected from the West Hickman Wastewater Treatment Plant during the sample period.
2. qPCR negative control for each PCR run. These were collected from sample blanks.
3. qPCR method blank and negative control for each sample run.

4.7 Statistical Analysis of Data

All statistical analyses of data collected during this study were performed with the SigmaPlot 12 software. One-Way Repeat Measures ANOVA (Holms-Sidak with significance = 0.05) tested for significant difference between sampling sites under wet and dry weather conditions. Data, raw and transformed, were checked with the statistical software for normality and equal variance during these procedures. ANOVAs were also applied to compare fecal indicators detected at sample sites to indicators characteristic of human sewage. These sewage values originated from samples taken from the Town Branch Wastewater Treatment during Brion's 2011 study (described in Section 2.2).

Paired t-Tests (95% Confidence, one-tailed) illuminated differences between sample sites during specific sample events. These tests were used to detect significant difference between changes in individual fecal indicators during both wet and dry sampling events.

4.8 Hydrologic Analysis of Precipitation and Flow Data

As described by the National Pollutant Discharge Elimination System (NPDES) in the "NPDES Storm Water Sampling Guidance Document", sampling days were classified as "wet" when the cumulative precipitation in the previous 48 hours equaled or exceeded 0.5 inches of rainfall (NPDES, 1992). Daily precipitation data was obtained from the University of Kentucky College of Agriculture Weather Center (UKAGWC, included in Appendix D), resulting in the classification summarized in Table 4.3.

Table 4.3: Rainfall Classification. Rainfall Data taken from the University of Kentucky College of Agriculture "UK Ag Weather Center" (UKAGWC, 2012).

Sample Date	Cumulative 24 Hour Precipitation (in)	Cumulative 48 Hour Precipitation (in)	Classification
5/8/2012	0.03	0.06	Dry
5/14/2012	0.37	0.60	Wet
5/29/2012	0.00	0.00	Dry
6/7/2012	0.00	0.00	Dry
7/16/2012	0.08	0.70	Wet
7/20/2012	0.95	1.38	Wet

The US Geological Survey operates and maintains a monitoring station at Royal Spring (USGS 03288110). Access to average daily discharge data was granted at the National Weather Information System (NWIS) Web Interface. These data are displayed in Appendix E.

The Kentucky Geological Survey (KGS) maintain a groundwater monitoring station at the KY Horse Park that directly taps into the groundwater conduit, as described in Section 3.2. A stage-discharge relationship, developed by Jim Currens at KGS, estimates discharge in the conduit based on observed water depth in the aquifer. Since this stage-discharge data was unpublished at the time of this study, access to data was supplied by Jim Currens through personal communication (Currens, 2012).

No flow stations monitored Highland Spring during the sampling period; therefore observed discharge data were not available for analysis. While stream discharge at ungagged surface sites may be estimated by area-averaged estimates, this method is not recommended for groundwater discharge at upwelling springs (Ries, 2006). As such, Highland Spring was omitted from load analysis.

Gaging stations monitored discharge at IBM was operated by the University of Kentucky Department of Biosystems and Agricultural Engineering from 2008 through 2011. This data provided a historical basis for estimating the expected discharge at IBM during the sampling period. Discharge data was measured daily at 15 minute intervals during the 2008 thorough 2011 monitoring period (BAE, 2012). Average daily discharges were calculated from these interval measurements and then averaged across the four-year sampling period. A summary of flow data determined at Royal Spring, KY Horse Park, and IBM is displayed in Table 4.4.

Table 4.4: Summary of Flow Data

				IBM Historical	
48-Hour Precipitation Classification	Date	Royal Spring Average Daily Discharge (CFS)	KYHP Average Daily Discharge (CFS)	Average Daily Discharge (CFS)	STDEV
Dry	**5/8/2012**	37.00	33.53	8.27	9.47
Wet	**5/14/2012**	36.00	38.11	2.27	2.23
Dry	**5/29/2012**	1.20	30.11	0.00	0.00
Dry	**6/7/2012**	0.61	29.93	0.00	0.00
Wet	**7/16/2012**	1.80	30.09	0.11	0.19
Wet	**7/20/2012**	5.00	31.07	3.12	5.31

As shown in Table 4.4, considerable error is associated when estimating stream discharges at IBM from historical data. Large variances around discharge means propagate to load calculations, as discussed in Section 5.7. Note also the historical zero discharge values estimated for two dates (5/29 and 6/7). These days were very dry with little discharge observed during the sampling event; Cane Run was observed during dry weather as a series of stagnant pools. However, surface water flow, while estimated as zero, may not be equivalent to flow entering the Karst aquifer through the swallet even during dry-weather events. This adds uncertainty to the use of zero-discharge values.

Error is also likely present when estimating discharge at the KY Horse Park since the flow was often greater than discharge measured at Royal Spring. Counterintuitive to expected results, KY Horse Park discharge was often greater than discharge measured at Royal Spring. One would expect that Royal Spring, downstream of the KY Horse Park, would observe larger discharges due to a larger discharge area. However, during the very dry period from 5/29 to 7/16/2012, agricultural demand on the conduit utilized groundwater from the Royal Spring conduit for irrigation. Farming operations downstream of the KY Horse Park pump water from the conduit for irrigation, creating a groundwater depression that causes backflow from Royal Spring This trend was observed in a previous study, "Determining Groundwater Travel Times in the Royal Spring Karst Basin of Kentucky", where Paylor and Currens observed an disappearance of dye tracers at Royal Spring during very dry events (Paylor and Currens, 2004).

5.0 Results and Discussion

5.1 *E. coli* Results

As shown in Table 5.1, the Royal Spring Karstshed is under significant fecal influence considering the magnitude of *E. coli* loading measured during the sampling period. Compared to the KYDOW Water Quality Standard for Primary Contact Recreation, all sites sampled exceed the criteria for *E.* coli at least once. This criterion is an instantaneous *E. coli* load of 240 MPN/100mL in any one sample (401 KAR 10:31).

Table 5.1: *E. coli* Results

***E. coli* (mpn/100mL)**	Dry	Wet	Dry	Dry	Wet	Wet	% of Days > 240 MPN/100mL
	5/8/2012	**5/14/2012**	**5/29/2012**	**6/7/2012**	**7/16/2012**	**7/20/2012**	
RS	613.10	19863.00	32.70	160.70	160.70	1986.30	50.00%
KYHP	24196.00	19863.00	151.50	129.60	19863.00	488.40	66.67%
HS	886.40	866.40	193.50	686.70	816.40	435.20	83.33%
IBM	6131.00	2613.00	1553.10	3448.00	2419.60	2755.00	100.00%

The sample sites at IBM and Highland Spring (HS) were significantly impacted by fecal loading, displaying the largest number of days exceeding the *E. coli* water quality standard (WQS). Samples taken from Highland Spring met the WQS on only one event while samples at IBM exceeded the WQS 100% of the time. Samples at the Kentucky Horse Park (KYHP) met the WQS on dry days, indicating that the fecal impact at KYHP was wet-weather related. Royal Springs (RS) was the least impacted by fecal loading, since samples exceeded the WQS 50% of the time. However, the KY WQS for *E. coli* also requires analysis of fecal load data with a Geometric Mean, mandating that "Escherichia coli content shall not exceed 130 colonies per 100 ml respectively as a geometric mean based on not less than five (5) samples taken during a thirty (30) day period" (401 KAR 10:31). Geometric Means for dry, wet and all sampling events were calculated and are displayed in Table 5.2. No sample sites were in compliance with KY WQS for *E. coli*. The *E. coli* Geometric mean characteristic of a sewage samples is also summarized in Table 5.2. This data was taken from the 2011 study of the Wolf Run watershed discussed in Section 2.3.

Table 5.2: Average *E. coli* Loads

	All Weather	Wet	Dry
RS	522.84	1850.84	147.69
KYHP	2122.94	5775.93	780.29
HS	575.30	675.21	490.18
IBM	2881.05	2592.20	3202.08
Sewage	834555.80	-	-

Table 5.2 presents an interesting observation: the Geometric mean *E. coli* concentrations at all sample sites were higher during wet weather than those measured during dry weather. This was true for all sample sites except for IBM. Larger *E. coli* loads resulting from rain events are indicative of sanitary sewer overflows (SSOs) or non-point source loading from overland flow, but do not implicitly indicate a leaking sewer. The large dry weather *E. coli* mean detected at IBM and Highland Spring reflect conclusions from previous studies (Section 2.4 – 2.7): Cane Run appears to still be under the influence of leaking sanitary sewers, as indicated by the elevated fecal load detected at the IBM and Highland Spring (HS) sample sites. As shown in Table 5.2, fecal loading (as indicated by *E. coli* geometric means) at IBM and HS varied little between dry and wet-weather events, indicating a consistent input of fecal material. Paired with the very little difference in load values between sample events observed in Table 5.1, it is likely that a leaking sewer was impacting IBM and Highland Spring during the sampling period.

Contrasting fecal loads measured on wet versus dry days at the Kentucky Horse Park (KYHP) shows a significant wet-weather trend detected at KYHP. Geometric mean *E. coli* values increased by nearly a factor of 10 in magnitude during wet-weather events. In addition, *E. coli* values were greater than those at upstream sites (HS and IBM) during wet-weather sampling events (as shown in Table 5.1), indicating a fecal source was directly impacting KYHP. Given this wet-weather relationship and the predominantly agricultural land use surrounding KYHP (as shown in Figure 4.3), this fecal source likely originates from nonpoint pastureland runoff during storm events.

Table 5.2 shows large differences between the groundwater conduit at KYHP and Royal Spring: the geometric mean *E. coli* value at KYHP was a factor of magnitude greater than that at Royal Spring. Fecal load also decreased from KYHP to RS during wet and dry-weather events. This indicates that the fecal input responsible for large *E. coli* loads measured at KYHP is diluted (or perhaps retained in the karstshed) before the groundwater emerges at Royal Spring. This

observation reflects conclusions from Brion's 2005 (Section 2.5) and 2006 (Section 2.6) studies: fecal loads present at IBM and HS were diluted to much smaller values measured at Royal Spring during the dry weather. These observations do not support the hypothesis of this study: *E. coli* loads do not indicate a fecal source impacting Royal Springs after the KY Horse Park. However, these results are not supported by statistical significance and there could be other reasons for the decreasing trend seen, such as retention of E. coli within the karstshed.

Average *E. coli* concentrations measured during wet, dry, and all weather were not significantly different between sampling sites. For example, the average *E. coli* concentration at the KY Horse Park (2122.94 MPN/100mL) measured during the study was not significantly different (Holms-Sidak; P = 1) than that measured at Royal Spring (522.84 MPN/100mL). Although the average *E. coli* concentration at KYHP was nearly 4 times the magnitude of the average concentration at RS, uncertainty inherent in the Quanti-Tray/2000 analysis introduces large variance in measured *E. coli* concentrations between sample events. Even events classified with similar weather conditions varied greatly during the sample period, adding to the variance around mean values. *E. coli* concentrations measured (Table 5.2) during wet-weather events at KYHP show an example of this variance: concentrations decreased a by a factor of 10 from 5/14 to 7/20 (19863 t0 1986.3 MPN/.100mL), even though both events were classified as wet sampling days. Log-transforming the *E. coli* concentrations before statistical analysis yielded the same results: no significant difference existed between sampling events. Comparing wet, dry and all-weather averages with *E. coli* concentrations indicative of raw human sewage showed that sewage fecal load was significantly different than those loads measured at all sampling sites during the Royal Spring sampling events.

Retention and propagation of bacterial cells within the karstic environment could explain the ambiguity surrounding statistical interpretation of *E. coli* data. An alternate hypothesis applicable to this fecal loading data suggests it was likely that *E. coli* survived in the karstic environment, causing statistical similarity of data collected at different sample sites. As discussed in Section 2.2, *E. coli* are criticized in the literature as a fecal indicator due to the ability of the microorganisms to persist and propagate in warm surface water environments. First mentioned in Section 2.1, water quality data collected at the KY Horse Park monitoring station suggest that the groundwater conduit was relatively warm (average water temperature of 21˚C) during the summer sampling events. Since *E. coli* are proven to propagate in surface

water ecosystems at 15 ˚C, the water temperature in the conduit was favorable for the microorganisms' survival (Medema et al., 1997). In addition, no sunlight is present in the groundwater aquifer, further removing a key determinant of *E. coli* removal (Medema et al., 1997). Therefore, this retention hypothesis can be explained in the following discrete steps:

1. Fecal loading, as indicated by *E. coli* concentration, of the Royal Spring karstshed occurred during wet-weather events: stormwater washed point and non-point fecal sources into the recharge area of the groundwater conduit. This is evident in the 7-fold increase of average *E. coli* loading measured at the KYHP monitoring station (Table 5.2).
2. As the rainfall ceased and groundwater flow decreased during dry-weather, fecal sources identified by previous studies of the Royal Spring karstshed continue to input *E. coli* into the underground aquifer. These sources include leaking sanitary sewers impacting the IBM (Sections 2.6 – 2.9) and Highland Spring (Sections 2.6, 2.8 and 2.8) sample sites. As indicated by larger average dry weather *E. coli* loads than wet-weather loads, the fecal sources impacting IBM were the primary source contributing fecal material to the karstshed.
3. *E. coli* survived in the groundwater conduit and may have settled with sediment in chambers where flow was slow. Warm water temperatures and lack of sunlight could have allowed *E. coli* to reproduce in the karstic environment, but most certainly would have enhanced their survival relative to surface water. This deposition, survival, and potential growth may have caused detection of fecal load above WQS at the KYHP and Royal Spring sample sites (Table 5.1 and 5.2), downstream of dry weather fecal inputs.
4. Rain, following these extended dry events, could wash *E. coli* retained in the conduit to downstream sample sites. Compounding of fecal inputs with resuspension caused large loads detected in the groundwater conduit at KYHP and Royal Spring. This alternate hypothesis would identify the fecal sources contributing to *E. coli* levels detected at IBM responsible for the majority of human sewage impacting the Royal Spring karstshed.

While trends can be interpreted from fecal loading data, the large variability of *E. coli* concentrations between sample events measured in Table 5.1 and the inherent uncertainty of the Quanti-Tray/2000 analysis yields a resounding conclusion: *E. coli* loads alone cannot characterize the presence of human sewage in the Royal Spring Karstshed. This is contiguous with conclusions drawn in previous studies discussed in Section 2.4 and 2.5: Interpretations of

fecal load indicators must be supported and modified by observations drawn from fecal age and source indicators.

5.2 AC/TC Results

Table 5.3 displays the AC/TC values measured during the sampling events. Recalling from Section 2.2 that low (<5) AC/TC values indicate fresh fecal material whereas high (>20) AC/TC values indicate aged fecal material, an interpretation of data in Table 5.3 indicates a discernible trend: fresh fecal material was detected at all sites in the Royal Spring Karstshed. Both Wet and Dry sampling events were characterized by smaller AC/TC values at the KY Horse Park relative to upstream sites (Highland Spring and IBM). This indicates an input of fresh fecal material at KYHP. AC/TC values then increased from the KHYP downstream to Royal Spring, indicating that no more fresh fecal material was input to the conduit. This trend was observable on all wet and dry sampling days, except during the 6/7/2012 sampling event.

Table 5.3: AC/TC Results

AC/TC	Dry	Wet	Dry	Dry	Wet	Wet
	5/8/2012	**5/14/2012**	**5/29/2012**	**6/7/2012**	**7/16/2012**	**7/20/2012**
RS	17.88	10.19	4.42	1.97	10.18	23.09
KYHP	12.08	4.03	1.52	2.81	6.73	6.09
HS	23.57	5.21	1.40	2.75	6.00	9.17
IBM	27.59	6.15	536.25	1.30	11.06	8.25

Average AC/TC values for wet, dry and all-weather events are displayed in Table 5.4. Ranking the sample sites based on lowest average AC/TC places KY Horse Park as the source of freshest fecal input in the Royal Spring Karstshed. Average AC/TCs at the KY Horse Park were always lower than upstream sites (Highland Spring and IBM) for all weather conditions. Again, no fresh fecal input between the KY Horse Park and Royal Spring were detected, as indicated by the increase of average AC/TC values during wet, dry, and all weather events. *E. coli* results (Section 5.1) show dilution of fecal loading between the KY Horse Park. AC/TC analysis supports this conclusion especially during wet events where dilution with aged fecal materials, low in E. coli, but higher in AC would cause the rise in AC/TC seen in Table 5.4. These findings do not support the hypothesis, but significant statistical evidence is difficult to draw from the AC/TC data as well.

Table 5.4: AC/TC Statistical Comparison

	All Weather AC/TC		Wet AC/TC		Dry AC/TC	
	MEAN	**STDEV**	**MEAN**	**STDEV**	**MEAN**	**STDEV**
RS	11.29	7.99	14.48	7.45	8.09	8.56
KYHP	5.54	3.75	5.62	1.41	5.47	5.76
HS	8.02	8.08	6.79	2.09	9.24	12.43
IBM	98.43	214.67	8.49	2.46	188.38	301.55
Sewage	2.40	1.39	-	-	-	-

As shown in Table 5.4, variability measured in the AC/TC is considerable. This variability obfuscated any statistical significance (Holms-Sidak, P = 0.05) detected between sampling sites and sampling events. Therefore, it is difficult to determine if any one site was significantly different than another site or sewage. AC/TC values characteristic of sewage, taken from the 2011 Wolf Run study, have low variance and provide a very consistent AC/TC value for comparison. Comparison of Royal Spring AC/TC values shows that no sampling sites produced AC/TC values significantly similar to sewage.

This lack of significant difference can be linked to difficulty of interpreting the age of fecal material in a karst groundwater environment from past studies. As discussed in Section 2.2, Ward observed that cool, dark environment of a karst aquifer preserves the AC/TC ratio at lower levels longer than in surface water after an input of fresh fecal material (Ward, 2008). Ward's conclusion is supported by AC/TC levels detected at KYHP: consistently low ratios, even between differing weather events, were detected. This indicates conservation of the AC/TC ratio between sampling events, but measurement error with large variances, creating statistical ambiguity.

The abundance of AC/TC values below the level of concern (<5) indicate that fresh fecal material was input into the Royal Spring karstshed, especially from urban areas at the Highland Spring and IBM. However, considering the apparent conservation of the low AC/TC signal in the karst conduit, the lack of statistical significance, and the variability of the AC/TC indicator, the AC/TC ratio alone cannot indicate or pinpoint the presence of human fecal sources within the Royal Spring Karstshed. Therefore, we must look to other indicators to provide clarity to these preliminary observations of relatively fresh and high fecal loads.

5.3 HuBac qPCR Results:

If we accept the highly quantifiable presence of HuBac as presumably linked to human sewage where it is found in large quantities, analyses of the HuBac qPCR data displayed in Table 5.5 suggests interpretations comparable to conclusions drawn from Brion's 2005 (Section 2.6) and 2006 (Section 2.7) studies of the Royal Spring karstshed: primarily that a human-sourced fecal material was influencing Royal Spring downstream of the KY Horse Park. A human fecal source independently impacting Royal Spring was present in the karstshed, as indicated a by an increase of average concentration of HuBac markers relative to the upstream sample sites at the KY Horse Park. This increase was observed during all, wet, and dry-weather conditions, but is a more observable trend under dry conditions.

Table 5.5: HuBac qPCR Results

	HuBac qPCR Averages (DNA Copies/µL of Extract)					
	All Weather		Wet		Dry	
	HuBac	**HuBac:HuBacMax**	**HuBac**	**HuBac:HuBacMax**	**HuBac**	**HuBac:HuBacMax**
RS	208.85	0.32	277.50	0.34	140.19	0.29
KYHP	160.07	0.32	223.21	0.35	96.93	0.29
HS	142.33	0.32	150.01	0.31	134.64	0.32
IBM	946.41	0.40	563.73	0.38	1329.08	0.42
Sewage	2.80E+06	0.89	-	-	-	-

As shown in Table 5.5, leaking sewers are impacting Cane Run as indicated by the very high HuBac values detected at the IBM sample site: human-sourced sewage entered the Karstshed in large concentrations during dry weather relative to concentrations detected during wet weather conditions. This sewage was diluted in the karstshed, as indicated by the steady decrease of average HuBac values under dry conditions from IBM to the downstream sample site at the KY Horse Park. This dry-weather HuBac signal diluted without any further inputs to the levels detected at the KY Horse Park. Considering this trend, it is concluded that leaking sanitary sewers upstream of, and impacting the sample site at IBM, introduced human sewage to the Royal Spring Karstshed during dry weather events. To a lesser extent, human sewage was also input into the Royal Spring Karstshed from Highland Spring. However the average HuBac values detected at the Highland Spring sample site at were a factor of 10 in magnitude smaller than those measured at IBM during dry weather, and inline with values found at the non-IBM sites in the karstshed.

Statistical evidence does not substantiate these observations to a high level of certainty. During dry, wet, and all weather events, any log-transformed, average HuBac values from a single

sampling site were not significantly different (Holms-Sidak, p = 0.05) than any other site. In addition, all average HuBac values were significantly different from sewage HuBac signal determined from the Wolf Run sewage data. Statistical analysis was also performed on the average ratio of HuBac values to the maximum HuBac Sewage value (as used in the SCV model, labeled HuBac:HuBacMax in Table 5.5); again no statistical significance was found.

To determine how HuBac values changed during sample events within the Karstshed, paired t-tests compared Royal Spring to all other sampling sites (i.e. RS vs. KYHP, HS, and IBM). Both log-transformed HuBac values and ratios (HuBac:HuBacMax) were compared. These tests showed that HuBac values measured as Royal Spring were not significantly different than those at any other site, save IBM. Such statistical evidence suggested a conclusion contrary to the hypothesis: sewage impacting the sample site at IBM provided a human signal significantly greater than that measured at all other sampling sites, indicating that a fecal source impacting the Cane Run watershed area contributing to the sample site at IBM was responsible for the human signal detected in the karstshed.

An alternate hypothesis similar to the one made for *E. coli* prior relating retention of the HuBac signal in the Karstic environment could explain the similarity between sample sites. As explained in detail in Section 5.1, human-associated fecal material (as indicated by the HuBac genetic marker) would enter the karstshed during dry weather from sewage sources impacting the sample site at IBM. This trend is interpreted from large HuBac values measured at IBM in Tables 5.4 and 5.5. During the dry weather flow, the genetic signal may be retained in the conduit if the genetic material was associated with particles or still contained within the anaerobic bacteria. The genetic signal would be expected to be conserved underground and accumulate where ever particulates dropped out of the flow streams in the conduit. Rain events, following these extended dry periods, could re-entrain and wash the sedimented HuBac signal downstream, resulting in large values detected at the KY Horse Park and Royal Spring. This hypothesis, congruent with Section 5.1, would identify the fecal sources impacting IBM as the primary contribution of human-associated fecal material in the Royal Spring karstshed.

As concluded from HuBac persistence studies, discussed in Section 2.2, this hypothesis is unlikely; the water temperature of the groundwater conduit is too warm for extended conservation of the HuBac signal. Predators should be active in the karstshed and would consume the signal, especially if it were immobilized onto particulates. However, the bulk of the

dry weather genetic signal should be in the water, spreading out and being carried along more like a chemical dye than a bacteria. Under dry weather conditions, when the concentration of the HuBac marker is highest at IBM, there is little sediment entrained in the water, so the potential for adsorption to soil particles is low due to the low frequency of interception. The genetic material, cut into short pieces by naturally occurring DNAses in the environment, would tend to remain entrained in the water and would be expected to persist at least 1 week based, if not a month (Ficetola et al, 2008). Since the average temperature (21°C) is favorable for biologic removal of the *Bacteroides* genetic material, it is more likely that the trends suggested by Table 5.5 support the hypothesis of this study: that a human-associated fecal source is impacting Royal Spring downstream of the KY Horse Park causing an increase, albeit nonsignificant, in HuBac signal at Royal Springs in spite of dilution. However, ambiguity of the HuBac data cannot support this interpretation with statistical significance.

5.4 SCV Model Results:

SCV values calculated in the Royal Spring Karstshed are compared in Table 5.6 to SCVs calculated from raw sewage samples collected during a study of the Wolf Run watershed in Lexington, KY (Brion, 2010). These sewage SCVs value varied little with an average SCV approximately equal to 3, providing a consistent SCV data set for statistical comparison. As shown in Table 5.6 and Figure 5.1, average SCVs calculated at the KY Horse Park indicate a significant fecal source impacting the Royal Spring Conduit. However, application of this model may well lead to more confusion than clarification due to the conservation of the AC/TC and HuBac signal in the karstshed, and the potential for retention and growth of *E. coli*.

Table 5.6: SCV Model Results

	All Weather SCV		Wet SCV		Dry SCV	
	MEAN	**STDEV**	**MEAN**	**STDEV**	**MEAN**	**STDEV**
RS	1.14	0.34	1.12	0.50	1.15	0.17
KYHP	1.71	0.42	1.96	0.38	1.46	0.31
HS	1.40	0.38	1.59	0.09	1.20	0.50
IBM	1.51	0.57	1.77	0.28	1.26	0.74
Sewage	2.85	0.26	-	-	-	-

Statistical analyses compared SCV data calculated at sample sites against other sample sites and sewage SCVs. No sample site was significantly different (ANOVA) than any other sampling site. In addition, all sample site SCVs were significantly different than sewage SCVs. As illustrated in

Figure 5.1, the variance of SCV at a sample site between sampling events introduced error, disallowing the detection of any significant differences.

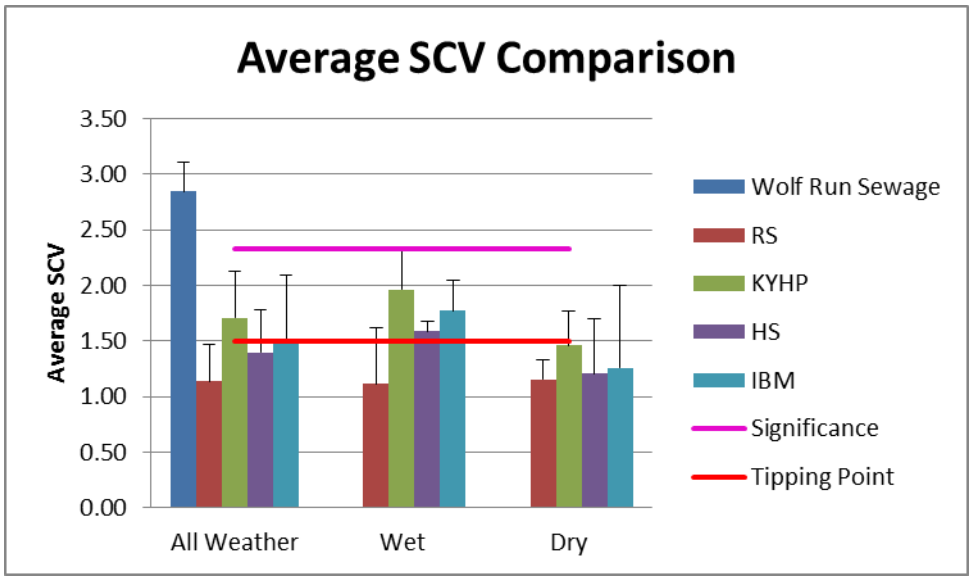

Figure 5.1: Average SCVs for All, Wet and Dry-Weather Events

Figure 5.1 illustrates the nature of SCV values measured during the sampling events. Average SCVs for each sampling site are plotted with one standard deviation of error, showing the variance in SCV between sampling events. As described in Section 2.2, the SCV indicates level of concern at a "Tipping Point" (SCV = 1.5) where all three indicators may be above the categorical threshold for concern (0.5 for each indicator or 0.75 for any one indicator). This "Tipping Point" is depicted as a line in Figure 5.1. Often, while a site's average SCV was below the model's concern threshold, error propagated from variance between sampling events pushed the SCV beyond the "Tipping Point". For example: while average SCVs calculated for dry-weather events at all sampling sites were below the threshold limit, the error depicted in Figure 5.1 exceeds the threshold limit. This disallows confidence in the observation that, during dry weather, sewage indicated by the SCV at all sampling sites was not above the level of concern. With respect to this concern threshold, average SCVs calculated KYHP during all, wet, and dry-weather events exceeded the "Tipping Point", therefore indicating a sewage source exceeding threshold values for individual fecal indicators impacting the groundwater conduit and detected at the KY Horse Park sampling site.

Error is also evident as average SCVs calculated for sampling sites are compared to SCVs calculated from Wolf Run sewage data. As shown in Figure 5.1 (as a line labeled "Significance"),

SCVs significantly different from sewage fall outside of 2 standard deviations from the mean sewage SCV. The average SCV calculated at KYHP for wet-weather events was significantly different than sewage; the SCV falls below the “Significance” threshold. However, variation in the SCV between sampling events at KYHP introduces uncertainty and error pushes the average SCV above the “Significance” threshold. In conclusion, the average wet-weather SCV calculated at KYHP were significantly different than sewage, but were not to a high degree of statistical confidence. This affirms the observation of a significant fecal source at the KY Horse Park sampling site. However, it does not appear that sewage inputs impacting the sample sites at Highland Spring and IBM are entirely responsible for this SCV: SCVs calculated at HS and IBM were consistently lower than SCVs calculated at KYHP.

Without context of the individual indicators that comprise the model, conclusions drawn from SCV analysis do not add support to the hypothesis of this study: there would not appear to be a significant, independent sewage source after the KY Horse Park impacting Royal Spring based on face-value examination of the SCV. Average SCVs at Royal Spring were always less than the concern threshold and significantly different than sewage SCVs. Addition of error shows that the average dry-weather SCV is significantly less than the concern threshold. In fact, average SCVs calculated indicate a much more significant potential sewage source at any of the other sampling site relative to Royal Spring. As discussed, the most prominent potential sewage source, as indicated only by SCV values, was detected impacting the KYHP sample site. However, Figure 5.2 illustrates the SCV as a compilation of interpretations, leading to dissimiliar observations.

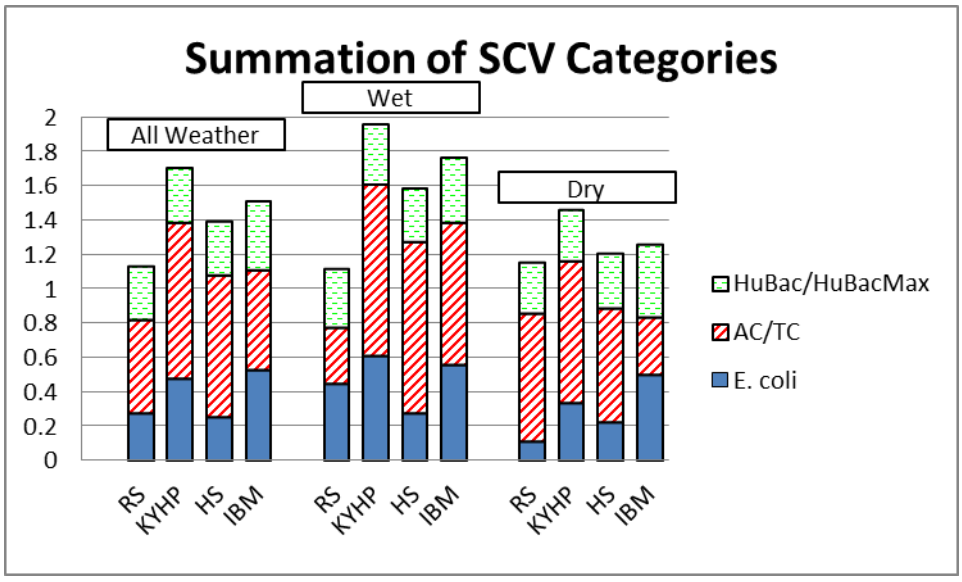

Figure 5.2: Summation of Average Individual SCV Categories

Figure 5.2 illustrates an observation drawn from data in Section5.2 critical to the interpretation of SCV data: the AC/TC ratio is conserved in a Karst environment and changes little. Therefore, the value of the AC/TC as an indicator in Karst systems is nominal. All that is gained from analysis of the AC/TC values is that fecal inputs, human or other animal, are relatively fresh. Categorical AC/TC values at the Karstic sites show very little variance relative to the total SCV at sampling sites between wet and dry events, indicating that the categorical AC/TC reflects conservation of raw AC/TC values. This observed conservation artificially "inflates" the SCV calculated at each site since, as shown in Figure 5.2, the AC/TC category accounted for a relative majority of the total SCV.

The following observations consider the compilation of these microbial indicators illustrated in Figure 5.2. These interpretations are strongly indicated by trends observed from microbial indicator data, but, as discussed previously, cannot be supported with statistical significance.

- Average SCVs at the KY Horse Park for wet-weather events were greater than average dry SCVs. This increase was driven by the *E. coli* category of the SCV model: *E. coli* concentrations increased by a large magnitude from dry to wet events (Table 5.2). Only marginal decrease in fecal age (increases of AC/TC in Table 5.4) and increase of human signal (increase of HuBac:HuBacMax values in Table 5.5) were detected by the SCV. This suggests that the SCV detected a wet-weather linked, non-human sewage source impacting the KYHP sampling site.

- Average SCVs calculated at IBM increased from dry weather to wet weather events. Unlike KYHP, the SCV saw a decrease of average *E. coli* paired with a considerable increase of fecal age, as indicated by decrease of average AC/TC categorical signal. In addition, HuBac:HuBacMax also decreased from dry to wet events. All of these indicate a leaking sanitary sewer: dry-weather load (*E. coli*) and source (HuBac:HuBacMax) values were diluted during wet weather event as the leaking sewage source was combined with clean precipitation. The fecal age (AC/TC) decreased as fresh sewage was washed into the karstshed. Therefore, leaking sanitary sewers were impacting the sample site at IBM, as indicated by the SCV model.
- During all, wet, and dry-weather events, average SCVs calculated at Highland Spring were less than those at another urban site (IBM). Highland Spring saw a marginal increase of fecal source (*E. coli)*, a marginal decrease of fecal age (AC/TC) and no change in fecal source (HuBac:HuBacMax) from dry to wet weather events. This indicates that a constant source of sewage is influencing Highland Spring, perhaps originating from leaking sanitary infrastructure in an urban environment.
- Average SCVs at Royal Spring showed an increase of *E. coli* concentration, increase in AC/TC value (decrease in categorical signal) and increase in HuBac:HuBacMax during wet weather. This indicates that fecal materials influencing RS are wet-weather related. More importantly, Figure 5.2 illustrates that the human source category (HuBac/HuBacMax) accounted for an increase in the SCV sum during wet weather, leading to the conclusion that sewage originating from human fecal material is being input into the karst aquifer between the conduit at the KY Horse Park and the upwelling at Royal Spring.

In summation, interpretations of individual components of the SCV model support the hypothesis of this study: a human sewage source was directly impacting Royal Spring. The origination of the human sewage signal for Cane Run is seen at IBM and Highland Spring with dilution to the KY Horse Park. However, the magnitude of HuBac increases slightly at Royal Spring relative to the KY Horse Park during rain events when clean water dilution should have decreased HuBac signal. This observation, visualized in Table 5.7, supports the hypothesis. However, lack of statistical significance cannot support these interpretations with an appropriate level of confidence. Due to lack of significant difference between sample sites

experienced applying the SCV model, as a whole, did not indicate this observation, application of the SCV failed to support the hypothesis.

Table 5.7: Change of Fecal Load, Age, and Source Indicators from KY Horse Park to Royal Spring. Note that trends presented are not supported by statistical significance.

	Fecal Load (*E. coli*)	**Fecal Age (AC/TC)**	**Fecal Source (HuBac)**	**SCV Categorical Fecal Source (HuBac/HuBacMax)**
Dry Weather Change from KYHP to RS	Decrease	Increase	Increase	No Change
Wet Weather Change from KYHP to RS	Decrease	Increase	Increase	No Change

5.5 HF183 qPCR Results:

With the difficulties experienced with bacterial and non-specific genetic markers, it was thought that another approach my lend credence to the suspicions of prior studies and the results from the HuBac markers if another, more specific marker was investigated. Human-specific qPCR markers (HF183) supported observations drawn from human-associated (HuBac) markers: that a human fecal source was detected at Royal Spring. As shown in Table 5.8, increases in average HF183 markers at Royal Spring relative to an upstream sample site (KY Horse Park) indicated a human source more local to Royal Spring. However, observed average HF183 values during dry weather events at Royal Spring were not greater than values at the KY Horse Park, indicating that the source of human fecal material may be wet weather related. Such sources could include a SSO, illegal cross-connections between storm drains and sanitary sewers, or a faulty septic tank.

Table 5.8: HF183 qPCR Results

	All Weather HF183 (DNA Copies/µL of Extract)		Wet HF183 (DNA Copies/µL of Extract)		Dry HF183 (DNA Copies/µL of Extract)	
	MEAN	**STDEV**	**MEAN**	**STDEV**	**MEAN**	**STDEV**
RS	45.63	52.57	65.16	73.54	26.10	18.91
KYHP	31.07	20.30	17.78	16.84	44.37	14.72
HS	69.17	55.63	84.98	70.10	53.36	45.52
IBM	224.46	371.79	328.96	524.90	119.95	193.11
Sewage	2.50E+05	4.47E+05	-	-	-	-

A strong human-specific sewage signal was detected at IBM. Comparable to trends detected in HuBac qPCR analysis, human-sewage entered the karstshed and was diluted during dry weather flow. This was indicated by the steady decrease of average HF183 values from IBM to the KY Horse Park and then to Royal Spring. This dry-weather trend indicates leaking sanitary sewers impacting the sample site at IBM. Human fecal signal was also detected at Highland Spring, but not at the magnitude of signal detected at IBM. HF183 signal input at Highland Spring was also diluted during wet and dry weather to levels measured at the Kentucky Horse Park.

No statistical significance was detected to support these observations: ANOVAs and paired t-tests compared the average human-specific HF183 signal at RS versus all other sites. No site was significantly different than another. HF183 values associated with human sewage were also significantly different than values at each sampling site. Natural-log transformed HF183 values were also analyzed, but resulted in the same conclusion.

An alternate hypothesis could explain trends observed in HF183 values detected in the Royal Spring karstshed: HF183 was retained and conserved in the groundwater environment. Comparing HF183 values measured in Royal Spring to HF183 data from Brion's 2011 Wolf Run Study (Section 2.3) unearths an interpretation supportive of this alternate hypothesis. Using the same level of detection scheme for HF183 (LOD < 1), HF183 was below detectable limits in the Wolf Run watershed 75% of the time (Brion, 2011). HF183 was never below detectable limits in the Royal Spring Karstshed. The relative recovery of human-specific signal in Royal Spring indicates a trend: HF183 was not decaying at the same rate within the Karst environment relative to surface water. Human-specific signal detected at any one site was likely conserved in

the Karst environment, resulting in the similarities between HF183 values amongst all sites. However, fate and persistence studies of the HF183 genetic marker cannot support this alternate hypothesis. As taken from the literature reviewed in Section 2.2, water temperature in the conduit was too warm for conservation of the HF183 signal if it was sorbed to particles and deposited like bacteria within the karstshed. Average water temperatures measured at the KYHP monitoring station were beneficial for removal *Bacteroides* genetic material by protozoan grazing, which is highest in sedimented beds of particulates. Therefore, similarities between sampling sites were more likely caused by constant input of human fecal material. However, these similarities complicate any statistical significance drawn solely on HF183 concentrations. Given the lack of statistical significance and large variance in HF183 values between sampling events, HF183 observations alone cannot completely indicate a human sewage source in the Royal Spring Karstshed

5.6 Statistical Significance of HF183 values: Royal Spring Versus All Other Sites:

To better define the meaning of the human sewage signal detected at Royal Spring, a non-parametric test compared HF183 values detected at Royal Spring versus values measured all other sampling sites. These tests determined the significance of sampling events where HF183 values at Royal Spring are greater than those measured at other sample sites. Considering that the probability that HF183 measured at Royal Spring was greater than the any other during a sample event was 0.5, comparison with a binomial distribution was utilized. Table 5.9 shows the results of this analysis. Note that "# of Events" in Table 5.8 corresponds to "Number of Successes" when utilizing the binomial distribution. For example: the probability that, during wet weather events, HF183 at Royal Spring was greater than KY Horse Park is equivalent to the cumulative binomial probability of observing, at most, 2 successes from 3 observations. Therefore, Royal Spring HF183 values were greater than those detected at KY Horse Park 88% of the time.

Table 5.9: Binomial Comparison of HF183; RS vs. Upstream Sites

	Binomial (Probability of Success = 0.5) Comparison of HF183 Concentrations at RS vs. Upstream Sites					
	All Weather		**Wet**		**Dry**	
	# of Events	Probability	# of Events	Probability	# of Events	Probability
>KYHP	3	0.66	2	0.88	1	0.50
>HS	2	0.34	1	0.50	1	0.50
>IBM	1	0.11	1	0.50	0	0.13

Trends similar to those detected from HuBac and HF183 analysis existed in this test: it was probable that HF183 values detected at Royal Spring exceeded those at the KY Horse Park, indicating a human-specific human sewage source was influencing Royal Spring. Note that the observed probability was greater during wet weather events versus dry weather events (88% wet versus 50% dry), also indicating that this sewage input is wet-weather related, such as an SSO or leaking septic system. During dry weather, it appears that the HF183 signal was conserved, and not diluted, in the Karst Conduit between the KY Horse Park and Royal Spring.

HF183 values measured at Royal Spring were seldom greater than those measured at IBM. Especially during dry-weather events; RS HF183 values were never greater than IBM values. This trend is repeated from previous analyses: sewage sources impacting the sample site at IBM was responsible for the majority of human sewage signal during dry events. Note that the probability that RS was greater than IBM increased during wet-weather events, further solidifying the observation that RS is influenced by a wet-weather human-specific sewage source. However, the difference in flow is significant between Royal Spring and IBM (as discussed in Section 4.8). Since IBM has a much smaller flow during dry weather, this decrease in HF182 signal could be due to dilution.

The human-specific sewage signal observed at Highland Spring is difficult to interpret: Royal Spring HF183 values were greater than Highland Spring values 50% of the time during both wet and dry-weather events. This lack of observable trend is potentially due to the conservation of HF183 signal within the Karst environment. To confirm this inference, Binomial tests compare HF183 values at the KY Horse Park to upstream sites in Table 5.10.

Table 5.10: Binomial Comparison of HF183; KYHP vs. Upstream Sites

	Binomial (Probability of Success = 0.5) Comparison of HF183 Concentrations at KYHP vs. Upstream Sites					
	All Weather		**Wet**		**Dry**	
	# of Events	Probability	# of Events	Probability	# of Events	Probability
> HS	3	0.66	1	0.50	2	0.38
>IBM	2	0.34	1	0.50	2	0.88

As shown in Table 5.10, HF183 signal at the KY Horse Park was also greater than signal at Highland Spring 50% of the time during dry events. In addition, the human sewage signal at the KY Horse Park was rarely greater than signal at IBM during dry events. This indicates that the IBM signal was conserved in the Groundwater Conduit during dry days and accounted for the HF183 signal detected at the KY Horse Park.

This analysis is mired by the same obstacle encountered during other analyses: HF183 had a high variance between sampling events (as shown in Table 5.7). As with analyses centered on comparing average values (ANOVA, t-test), little significance can be attributed to the probabilities calculated with the binomial probability. For example: the probability that HF183 values at RS exceeded those at KYHP during wet events (88%), while greater, was not significantly greater than the probability of exceedance during dry events (50%). Although a trend supports the hypothesis, it cannot do so with statistical certainty.

5.7 HF183 and HuBac Loading:

Since the SCV model uses ratios of fecal indicators that do not change with dilution from clean water sources, it was speculated that a better method of investigating the hypothesis should involve a fecal indicator modified by the flow at each site. Fecal source indicators (HF183 and HuBac) provided a signal not impacted by dilution in the groundwater conduit. By accounting for flow at sample sites, fecal source signal can be expressed as a load. This avoids problems utilizing fecal indicators that change due to retention in karst (*E. coli*) and those that do not change when decay is slowed by underground conditions (AC/TC).

Using flow data presented in Section 4.8, HuBac and HF183 loads were calculated from quantifiable units by the following:

$$\text{Load}\left(\frac{\text{DNA Copies}}{\text{Day}}\right) = \frac{\text{DNA Copies}}{\text{mL of Original}} * \text{Flow}\ \frac{\text{ft}^3}{\text{s}} * \frac{28{,}316.85\ \text{mL}}{\text{ft}^3} * \frac{86{,}400\ \text{s}}{\text{day}}$$

HF183 loads calculated at sample sites for all, wet, and dry-weather events are displayed in Table 5.11. Note that, as discussed in Section 4.8, there were no means to estimate flow originating from Highland Springs. Therefore, HuBac and HF183 loads cannot be calculated at Highland Spring.

Table 5.11: HF183 Loading

	HF183 Loads (Copies DNA/Day)					
	All Weather		**Wet**		**Dry**	
	Average	Stdev	Average	Stdev	Average	Stdev
RS	1.16E+13	2.06E+13	1.80E+13	2.93E+13	5.09E+12	8.60E+12
KYHP	9.83E+12	6.29E+12	6.21E+12	6.59E+12	1.35E+13	4.05E+12
IBM	1.33E+12	1.62E+12	1.53E+12	1.64E+12	1.12E+12	1.94E+12

Data displayed in Table 5.11 provides a trend supportive to the hypothesis of this document: human-specific loads measured at Royal Spring (RS) exceeded loads measured at any other site in the Karstshed. During all-weather and wet-weather events, Royal Spring HF183 loads were greater than loads measured at an upstream site (KYHP). This indicates that the magnitude of human sewage input at RS was clearly greater than human sewage input into the Karstshed at KYHP. Therefore, suspicions of a sewage source local to Royal Spring are confirmed. During wet-weather events, this source is located between the KY Horse Park and Royal Spring

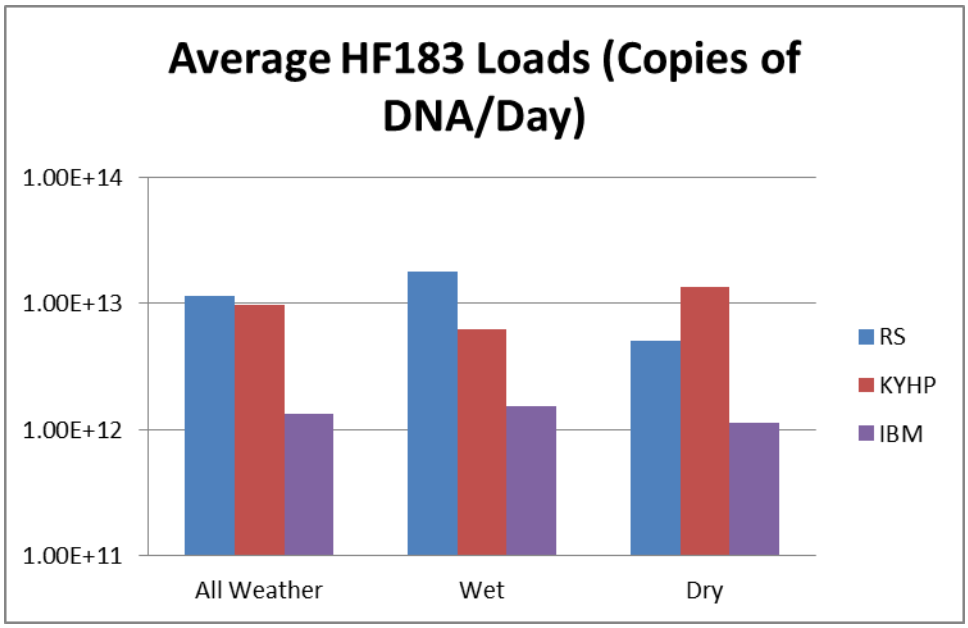

Figure 5.3: Average HF183 Loads

Figure 5.3 presents perspective to calculated HF183 loading: while the sewage source at IBM had larger average HF183 concentrations than Royal Spring (Table 5.7), the magnitude of flows measured at RS exceed those at IBM (Table 4.), resulting in larger loads at Royal Spring. Again, a wet-weather trend is observed at Royal Spring: HF183 loads were a log-step greater during wet-weather events than dry-weather events. This confirms conclusions drawn in previous analyses: the human-sewage source influencing Royal Spring is wet-weather related.

A leaking sanitary sewer was indicated by HF183 loads at IBM, a trend consistent with analyses in previous Sections. Note that while HF183 loads at IBM were greater during wet weather than dry weather, loads did not increase by a very large value. Relative to Royal Spring, where a log-step increase of HF183 load was calculated, the wet weather loads at IBM are only marginally greater than dry weather loads. This is consistent with a leaking sanitary sewer: high sewage concentrations at low flows result in the same load of diluted sewage concentrations at high flows. Any conclusion drawn from IBM HF183 loading must be evaluated with an appreciation for the roughness of the estimation: loads were calculated from historical flows, not observed flows. Since dry weather flows were estimated from historical data as zero discharge, these dry-weather loads cannot provide a level of statistical confidence necessary to support the hypothesis.

Interpretation of human-specific loading at KYHP (KYHP) shows that a human-specific sewage source directly influenced the conduit at a greater load during dry weather than during wet weather. This load also exceeded loads calculated at an upstream site, IBM. Therefore, a human-sourced sewage influenced the karstshed between IBM and the KY Horse Park.

Table 5.12: HuBac Loading

	HuBac Loads (Copies DNA/Day)					
	All Weather		Wet		Dry	
	Average	Stdev	Average	Stdev	Average	Stdev
RS	3.84E+13	5.05159E+13	3.78E+13	1.48E+13	3.90E+13	6.68E+13
KYHP	5.11E+13	2.53004E+13	7.21E+13	7.97E+12	3.00E+13	1.43E+13
IBM	7.97E+12	1.08676E+13	7.28E+12	9.47E+12	8.66E+12	1.50E+13

Human-related HuBac loads are displayed in Table 5.12 and Figure 5.4. Contrasting HuBac and HF183 loads highlights the relative specificity of the HF183 and HuBac markers discussed in Section 2.2: HuBac loads calculated at each sampling site were greater than calculated HF183 loads, indicating that the HuBac signal is detected from a larger number of sources. This elevated signal does result in the same interpretation of the sewage load since Royal Spring HuBac loads were greater than IBM loads, indicating a human sewage source directly impacting Royal Spring after IBM. However, Royal Spring HuBac loads were smaller than loads calculated at the KY Horse Park, a reverse of wet-weather HF183 observation. This indicates that fecal sources supply HuBac at a greater magnitude than HF183 at the KY Horse Park.

This trend can be explained by the presence of HuBac marker in the feces of animals other than humans (Layton et. all, 2006): during wet-weather events, HuBac signal was detected from non-point sources (overland agricultural feces as indicated by *E. coli* analyses in Section 5.1) at KYHP in a greater amount than the signal detected from urban sources at RS, resulting in a larger HuBac load. This HuBac load decreases during dry-weather events since the fecal non-point sources did not input any sewage at KYHP, resulting in the greater HuBac load detected from a human source at RS. This conclusion is also confirmed by the consistent HuBac load calculated at IBM from a known human sewage source; HuBac loads at IBM did not increase by a noticeable magnitude since no non-point agricultural sewage source inputs a HuBac signal. This justifies the use of “human-associated” when presenting HuBac signals rather than the “human-specific”

characterization of HF183: human sewage loads at KYHP are indicated by the HuBac marker, but are also artificially inflated by the false positive signal from non-human sources.

The relative recovery of the HuBac signal is also apparent in Table 5.12 and Figure 5.4: HuBac loads had similar magnitudes at a sampling site regardless of the weather conditions. HF183 loads varied greatly between weather events, as shown in in Table 5.11. However, HuBac loads are only marginally greater at Royal Spring during dry weather events than wet weather events. This interpretation contradicts an observation deduced from HF183 loads, where a wet-weather sewage source resulted in the largest loads. However, the hypothesis is again proven by HuBac loads since a sewage source at Royal Spring is independent of that at IBM.

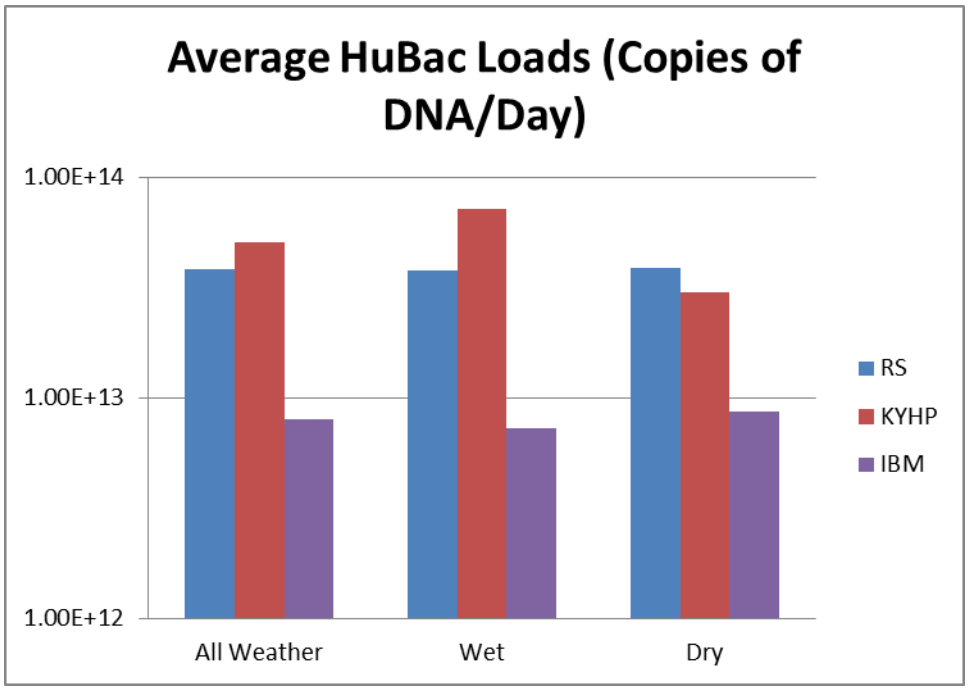

Figure 5.4: Human-associated (HuBac) Loading

The difference between the HF183 and HuBac markers in human specificity emphasizes the need to apply the appropriate fecal marker in Microbial Source Tracking. When attempting to pinpoint human sewage in an urban environment, HuBac will differentiate between human and non-human sources because of its great abundance in sewage. Discussion of Brion's 2011 Report in Section 2.3 supports this conclusion: HuBac successfully identified human sewage sources in the highly developed Wolf Run watershed (Brion, 2011). However, the presence of HuBac in non-human feces hinders application of the signal in a mixed urban and agricultural environment, such as the Royal Spring Karstshed where the sheer volume of animal fecal

material can increase the concentration of HuBac. A false-positive human signal was detected at the KY Horse Park due to the source of HuBac signal in significant volumes of non-point source fecal material. The HF183 marker was appropriate in the mixed environment of the Royal Spring Karstshed where there is not a dominance of human sewage in the water: detection of human sewage between the KY Horse Park and Royal Spring supports conclusions of Brion's two previous studies in the Karstshed (Brion, 2005 and 2006).

All interpretations drawn from HF183 and HuBac loading analyses are not supported by statistical significance. The large variance in loads between sampling events, as indicated by the standard deviation of HF183 and HuBac values in Tables 5.9 and 5.10, disallows the detection of any significance difference between average loads at sample sites (ANOVA) or loads during sample events (Paired t-Tests). Combined with the lack of observed flow data at IBM or any flow data, historical or observed, at Highland Spring, conclusions from loading alone cannot indicate sewage sources in the Royal Spring Karstshed. However, considering fecal source as an integral part of a comprehensive analysis is essential: HF183 and HuBac loads support and modify conclusions drawn from the analysis of multiple fecal indicators.

5.8 Discussion Summary:

Discussion of microbial indicators satisfies research objectives of this study. Interpretations of indicator data suggested sewage sources in the Royal Spring karstshed both known (IBM and HS sites) and as yet unknown (between the HP and RS). Multiple fecal indicators were analyzed, highlighting any observable caveats necessary when interpreting water quality samples taken from a karstic environment. As summarized in Table 5.13, conclusions were drawn concerning the applicability of these indicators for analysis of groundwater.

Table 5.13: Summary of Microbial Indicator Discussion

Indicator	**Indicator Applicable to Royal Spring's Environment?**	**Results supportive of the Hypothesis?**
Fecal Load: *E. coli*	No. Retention and propagation of *E. coli* in the karst causes ambiguity	No. No independent Sewage source impacting Royal Spring
Fecal Age: AC/TC	No. AC/TC ratio conserved in groundwater environment.	No. AC/TC ratio similar at all sample sites
Fecal Source: HuBac	Yes. Retention of genetic material unlikely.	Yes. Increase of HuBac values at Royal Spring relative to KYHP
Fecal Source: HF183	Yes. Retention of genetic material unlikely.	Yes. Increase of HF183 values at Royal Spring relative to KYHP
MST Model: Sanitary Category Value	No. SCV model skewed by AC/TC category.	No. KYHP pinpointed as primary site impacted by human sewage
HF183 and HuBac Loading	Yes. Flow multiplier considers dilutionial effects of fecal-source analysis.	Yes. Increase of human-sourced loads at Royal Spring from KYHP. Royal Spring Loads were always greater than IBM loads.

6.0 Conclusions

1. Fecal source indicators suggest a wet-weather, human-sewage source influencing Royal Spring after the Kentucky Horse Park. However, ambiguous results, caused by similar levels of human signal detected at all sample sites, cannot attest this trend with statistical confidence. Therefore, the hypothesis of this study, while supported, cannot be proven.
2. Use of microbial load and source indicators indicate human-sewage sources impacting the sample sites at IBM and Highland Spring. These were likely aging, leaking sanitary infrastructure inputting a steady amount of sewage into the karstshed. These results show strong correlation with previous studies of the Royal Spring karstshed and the Cane Run watershed.
3. There appears to be a wet-weather, non-human sewage source impacting the KY Horse Park. Probable retention of fecal load indicators within the karst provided a likely alternate hypothesis to explain this observable impact.
4. Use of the AC/TC ratio in karstic environments is not supported. Since the SCV model relies on fecal indicator ratios and genetic markers which were conserved underground, the SCV model alone cannot detect human sewage in a Karstic environment.
5. The HuBac signal was recovered in greater concentrations than the HF183 signal. Both signals originate from human sewage, but the HuBac signal was likely greater due to false-positives detected from other fecal material (such as livestock). As long as HF183 can be reliably detected, it should be the marker of choice, especially for determining signal loading.

7.0 Recommendations

1. The SCV model is enhanced and modified by analysis of the human-specific HF183 marker. HF183 loads supported conclusions drawn by the SCV. Parallel application of HF183 analysis with the SCV is necessary to detect human sewage. A new SCV model that incorporates more load components will be more applicable in a Karstic environment.
2. Ambiguity between sample sites may be relieved with a greater number of sample events. Experience gained during this study recommends that at least 10 sample events (5 dry, 5 wet) are utilized during microbial indicator studies.
3. Flow data should be collected at every sampling site during sampling events. Flow data obtained at sampling sites during sampling events will relieve reliance on historical flow data. This will eliminate the uncertainty in calculating loads with historical data. The USGS describes a feasible field method of obtaining flow data in “Measurment and Computation of Streamflow: Computation of Discharge” (Rantz et. al, 1982).
4. Access to the Groundwater Conduit between the Kentucky Horse Park and Royal Spring will provide even more insight to water quality issues. More sample sites are needed to accurately pinpoint the source of human sewage detected between the Kentucky Horse Park and Royal Spring.

Figure A.1: Royal Spring Karstshed Location

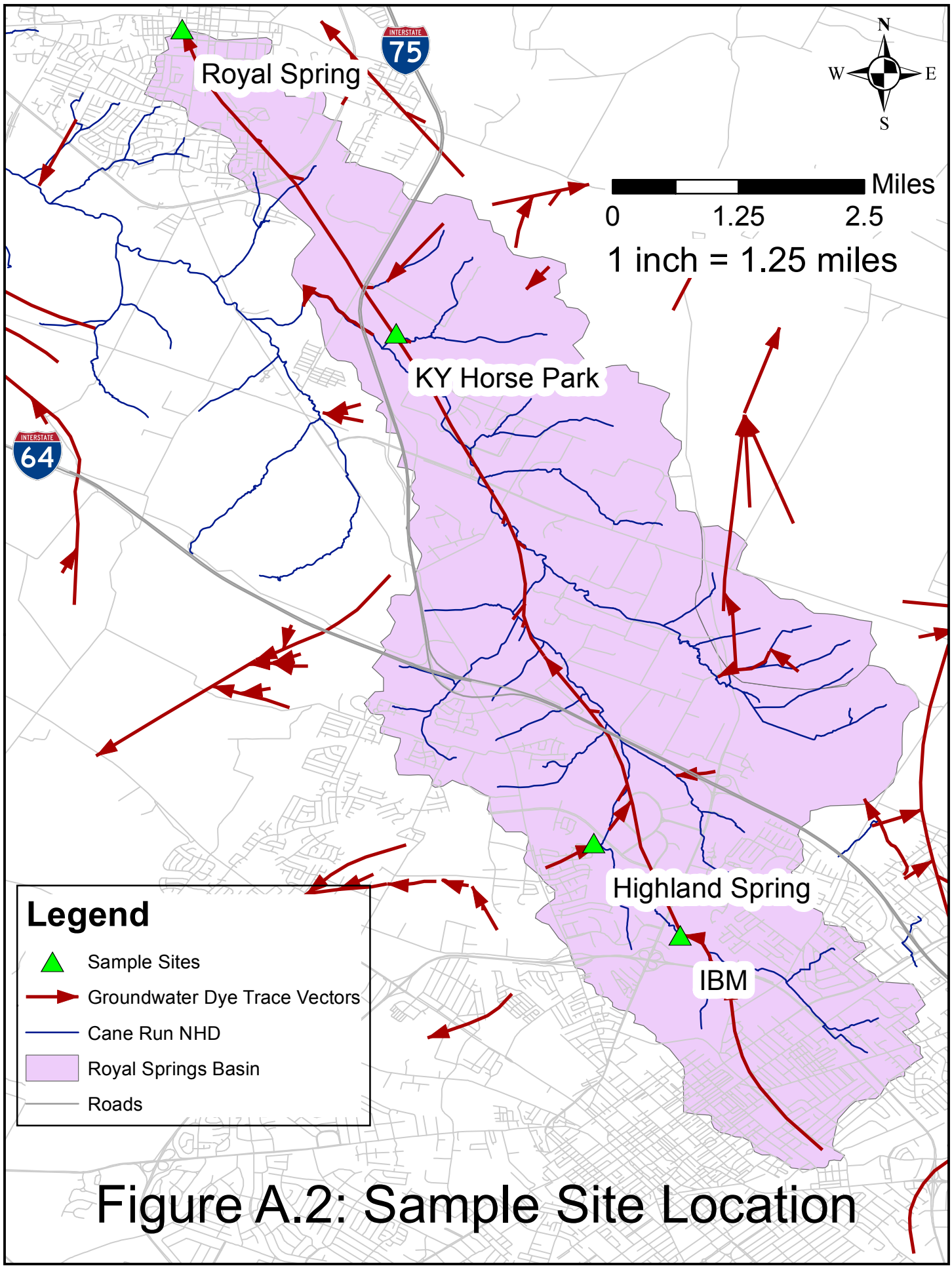

Figure A.2: Sample Site Location

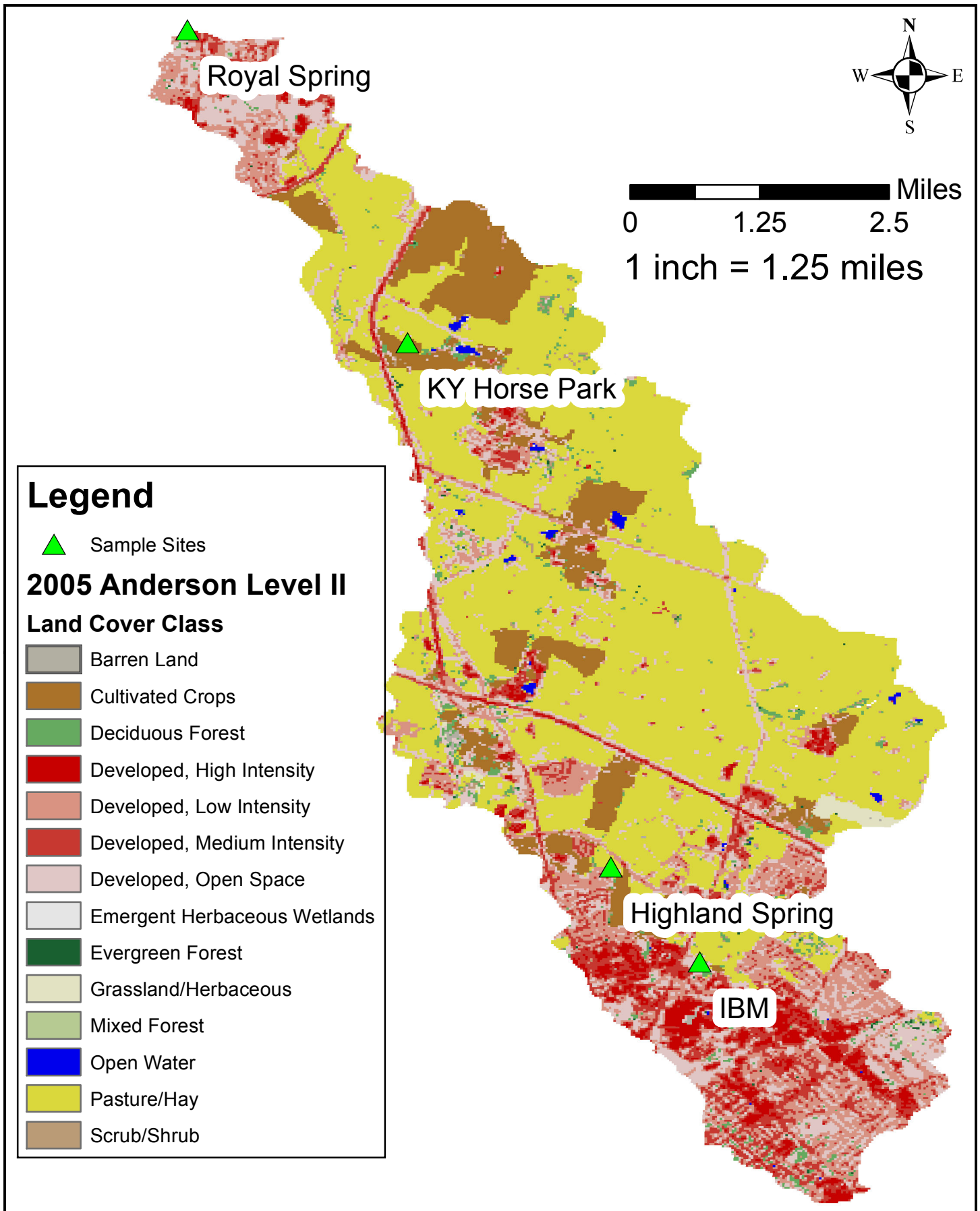

Figure A.3: Land Use Map

Royal Spring: Photo Courtesy James C. Currens, Kentucky Geological Survey

KY Horse Park: Kentucky Geological Survey Monitoring Station at Kentucky Horse Park

Highland Spring: Spring Box at Highland Spring

IBM: Swallet In Cane Run: Downstream View

IBM: Sewage Warning Signs in Lexmark Park, Upstream of IBM. Photo courtesy of Dr. Carmen Agouridis, University of Kentucky, Department of Biosystems and Agricultural Engineering.

Site	Date Collected	Total Coliform		E. Coli		MPN/100 mL	
		+ Large Wells	+ Small Wells	+ Large Wells	+ Small Wells	Total Coliform	E. coli
RS	5/8/2012	49	46	49	30	19863	613.1
RS-DUP	5/8/2012	49	47	49	34	24196	770.1
KYHP	5/8/2012	49	48	49	48	24196	24196
HS	5/8/2012	49	47	49	36	24196	886.4
IBM	5/8/2012	49	48	49	30	24196	6131
Blank	5/8/2012	0	0	0	0	1	1
		1:10 Dilution					

Site	Date Collected	Total Coliform		E. Coli		MPN/100 mL	
		+ Large Wells	+ Small Wells	+ Large Wells	+ Small Wells	Total Coliform	E. coli
RS	5/14/2012	49	48	49	46	24196	19863
KYHP	5/14/2012	49	48	49	46	24196	19863
KYHP-DUP	5/14/2012	49	48	49	42	24196	12297
HS	5/14/2012	49	36	49	36	8664	866.4
IBM	5/14/2012	49	48	49	15	24196	2613
Blank	5/14/2012	0	0	0	0	1	1
		1:10 Dilution					

Site	Date Collected	Total Coliform		E. Coli		MPN/100 mL	
		+ Large Wells	+ Small Wells	+ Large Wells	+ Small Wells	Total Coliform	E. coli
RS	5/29/2012	49	20	23	2	3448	32.7
KYHP	5/29/2012	49	43	46	11	14136	151.5
HS	5/29/2012	49	44	48	12	1553.1	193.5
HS-DUP	5/29/2012	49	47	46	21	19863	210.5
IBM	5/29/2012	49	48	49	44	24196	1553.1
Blank	5/29/2012	0	0	0	0	1	1
		1:10 Dilution					

Site	Date Collected	Total Coliform		E. Coli		MPN/100 mL	
		+ Large Wells	+ Small Wells	+ Large Wells	+ Small Wells	Total Coliform	E. coli
RS	6/7/2012	49	31	47	10	6488	160.7
RS-DUP	6/7/2012	49	30	48	19	6131	260.3
KYHP	6/7/2012	49	27	44	11	5172	129.6
HS	6/7/2012	49	14	49	32	2481	686.7
IBM	6/7/2012	49	48	49	20	24196	3448
Blank	6/7/2012	0	0	0	0	1	1
		1:10 Dilution					

Site	Date Collected	Total Coliform		E. Coli		MPN/100 mL	
		+ Large Wells	+ Small Wells	+ Large Wells	+ Small Wells	Total Coliform	E. coli
RS	7/16/2012	49	47	47	10	24196	160.7
KYHP	7/16/2012	49	48	49	46	24196	19863
HS	7/16/2012	48	20	49	35	3448	816.4
HS-DUP	7/16/2012	49	27	49	30	5172	613.1
IBM	7/16/2012	49	45	49	47	17329	2419.6
Blank	7/16/2012	0	0	0	0	1	1
		1:10 Dilution					

Site	Date Collected	Total Coliform		E. Coli		MPN/100 mL	
		+ Large Wells	+ Small Wells	+ Large Wells	+ Small Wells	Total Coliform	E. coli
RS	7/20/2012	49	48	49	46	24196	1986.3
KYHP	7/20/2012	49	30	49	26	6131	488.4
HS	7/20/2012	49	30	49	24	6131	435.2
IBM	7/20/2012	49	48	49	16	24196	2755
IBM-DUP	7/20/2012	49	48	49	13	24196	2359
Blank	7/20/2012	0	0	0	0	1	1
		1:10 Dilution					

Site	Date Collected	AC						CFU/100mL	TC						CFU/100mL	AC/TC
		0.1 mL		1 mL		10 mL			0.1 mL		1 mL		10 mL			
RS	5/8/2012	12	14	TNTC	TNTC	TNTC	TNTC	13000	1	1	6	8	TNTC	TNTC	727	17.88
KYHP	5/8/2012	28	65	TNTC	TNTC	TNTC	TNTC	46500	2	0	50	27	TNTC	TNTC	3850	12.08
HS	5/8/2012	4	16	72	78	TNTC	TNTC	7500	1	1	2	3	TNTC	TNTC	318	23.57
IBM	5/8/2012	79	81	TNTC	TNTC	TNTC	TNTC	80000	3	5	33	25	TNTC	TNTC	2900	27.59
Blank	5/8/2012	0	0	0	0	1	1	10					1	1	10	1.00

< values, but sign removed for calculations

Site	Date Collected	AC						CFU/100mL	TC						CFU/100mL	AC/TC
		0.1 mL		1 mL		10 mL			0.1 mL		1 mL		10 mL			
RS	5/14/2012	35	23	185	83	TNTC	TNTC	14818	2	2	12	16	TNTC	TNTC	1455	10.19
KYHP	5/14/2012	30	29	61	115	TNTC	TNTC	10682	3	8	29	24	TNTC	TNTC	2650	4.03
HS	5/14/2012	0	0	23	76	TNTC	TNTC	4950	0	0	2	17	TNTC	TNTC	950	5.21
IBM	5/14/2012	20	12	TNTC	TNTC	TNTC	TNTC	16000	0	0	12	40	TNTC	TNTC	2600	6.15
Blank	5/14/2012	0	0	0	0	1	1	10					1	1	10	1.00

< values, but sign removed for calculations

Site	Date Collected	AC						CFU/100mL	TC						CFU/100mL	AC/TC
		0.1 mL		1 mL		10 mL			0.1 mL		1 mL		10 mL			
RS	5/29/2012	0	0	12	7	TNTC	TNTC	950	0	0	0	0	22	21	215	4.42
KYHP	5/29/2012	2	0	21	20	TNTC	TNTC	2050	2	0	12	15	TNTC	TNTC	1350	1.52
HS	5/29/2012	0	0	5	2	TNTC	TNTC	350	0	0	3	2	TNTC	TNTC	250	1.40
IBM	5/29/2012	17	22	TNTC	TNTC	TNTC	TNTC	19500	0	0	0	4	0	4	36	536.25
Blank	5/29/2012	0	0	0	0	1	1	10					1	1	10	1.00

< values, but sign removed for calculations

Site	Date Collected	AC						CFU/100mL	TC						CFU/100mL	AC/TC
		0.1 mL		1 mL		10 mL			0.1 mL		1 mL		10 mL			
RS	6/7/2012	0	0	7	5	65	132	985	0	0	3	2	37	63	500	1.97
KYHP	6/7/2012	0	2	7	8	TNTC	TNTC	773	0	0	1	1	20	35	275	2.81
HS	6/7/2012	0	0	3	8	TNTC	TNTC	550	0	0	2	2	TNTC	TNTC	200	2.75
IBM	6/7/2012	32	37	TNTC	TNTC	TNTC	TNTC	34500	23	30	TNTC	TNTC	TNTC	TNTC	26500	1.30
Blank	6/7/2012	0	0	0	0	1	1	10					1	1	10	1.00

< values, but sign removed for calculations

Site	Date Collected	AC						CFU/100mL	TC						CFU/100mL	AC/TC
		0.1 mL		1 mL		10 mL			0.1 mL		1 mL		10 mL			
RS	7/16/2012	3	7	85	88	TNTC	TNTC	8650	0	1	11	6	TNTC	TNTC	850	10.18
KYHP	7/16/2012	18	23	167	210	TNTC	TNTC	18850	2	1	27	29	TNTC	TNTC	2800	6.73
HS	7/16/2012	3	3	24	30	TNTC	TNTC	2700	0	1	4	5	TNTC	TNTC	450	6.00
IBM	7/16/2012	12	11	89	110	TNTC	TNTC	9950	0	1	11	7	TNTC	TNTC	900	11.06
Blank	7/16/2012	0	0	0	0	1	1	10					1	1	10	1.00

< values, but sign removed for calculations

Site	Date Collected	AC						CFU/100mL	TC						CFU/100mL	AC/TC
		0.1 mL		1 mL		10 mL			0.1 mL		1 mL		10 mL			
RS	7/20/2012	16	15	123	131	TNTC	TNTC	12700	0	0	7	4	TNTC	TNTC	550	23.09
KYHP	7/20/2012	3	0	39	31	TNTC	TNTC	3500	0	0	3	1	53	62	575	6.09
HS	7/20/2012	2	1	10	15	TNTC	TNTC	1250	1	0	0	2	TNTC	TNTC	136	9.17
IBM	7/20/2012	14	15	130	134	TNTC	TNTC	13200	1	2	17	15	TNTC	TNTC	1600	8.25
Blank	7/20/2012	0	0	0	0	1	1	10					1	1	10	1.00

< values, but sign removed for calculations

HuBac-Human Specific

DNA copies/uL ext	Dry 5/8/2012	Moist 5/14/2012	Dry 5/29/2012	Desert 6/7/2012	Rain 7/16/2012	Wet 7/20/2012
RS	320.56	244.85	39.77	52.74	94.35	537.66
KYHP	131.02	218.01	109.78	74.66	231.62	220
HS	161.29	14.84	122.54	120.1	237.47	162.56
IBM	3007.71	1281.08	115.82	863.72	121.4	288.705

Underlined=duplicate average

BDL = less than 100

AllBac

DNA copies/uL ext	Dry 5/8/2012	Moist 5/14/2012	Dry 5/29/2012	Desert 6/7/2012	Rain 7/16/2012	Wet 7/20/2012
RS	2788.8	2854.14	438.18	559.65	700.19	2937.6
KYHP	2586.28	2814.97	871.41	744.64	2372.15	359.12
HS	2269.67	158.3	979.6	960.94	1615.325	752.51
IBM	30197.12	13765.92	5739.78	6473.79	3327.06	4755.525

Underlined=duplicate average

BDL = less than 100

qHF183-Human Specific

DNA copies/uL ext	Dry 5/8/2012	Moist 5/14/2012	Dry 5/29/2012	Desert 6/7/2012	Rain 7/16/2012	Wet 7/20/2012
RS	41.50	147.14	6.41	31.80	5.94	43.35
KYHP	35.61	36.86	61.36	36.14	11.47	5.33
HS	13.16	114.23	44.12	102.79	2.84	135.72
IBM	342.90	935.02	2.79	11.96	19.28	32.59

BDL < 1

1 < BQL < 10

Strong Signal > 100

Underlined = Duplicate Average

SCV SITE

	Dry 5/8/2012	Moist 5/14/2012	Dry 5/29/2012	Desert 6/7/2012	Rain 7/16/2012	Wet 7/20/2012
RS	0.96	1.69	1.25	1.25	0.75	0.91
KYHP	1.82	2.18	1.31	1.25	2.18	1.52
HS	0.66	1.58	1.31	1.64	1.69	1.50
IBM	0.85	1.80	0.81	2.11	1.48	2.04

DATE	PRECIP	DATE	PRECIP	DATE	PRECIP
5/1/2012	0.03	6/12/2012		7/29/2012	
5/2/2012		6/13/2012		7/30/2012	
5/3/2012		6/14/2012		7/31/2012	
5/4/2012		6/15/2012		8/1/2012	
5/5/2012	0.66	6/16/2012		8/2/2012	
5/6/2012		6/17/2012	0.52	8/3/2012	0.53
5/7/2012	0.17	6/18/2012		8/4/2012	0.46
5/8/2012	0.46	6/19/2012		8/5/2012	0.01
5/9/2012		6/20/2012			
5/10/2012		6/21/2012			
5/11/2012		6/22/2012			
5/12/2012		6/23/2012			
5/13/2012	1.29	6/24/2012			
5/14/2012	0.27	6/25/2012			
5/15/2012		6/26/2012			
5/16/2012		6/27/2012			
5/17/2012		6/28/2012			
5/18/2012		6/29/2012			
5/19/2012		6/30/2012			
5/20/2012		7/1/2012			
5/21/2012		7/2/2012	0.03		
5/22/2012		7/3/2012			
5/23/2012		7/4/2012			
5/24/2012		7/5/2012			
5/25/2012		7/6/2012			
5/26/2012		7/7/2012			
5/27/2012		7/8/2012	0.05		
5/28/2012		7/9/2012	0.02		
5/29/2012	0.01	7/10/2012			
5/30/2012		7/11/2012			
5/31/2012		7/12/2012	2.41		
6/1/2012	1.1	7/13/2012	0.4		
6/2/2012		7/14/2012	0.7		
6/3/2012	0.02	7/15/2012	0.08		
6/4/2012	0.03	7/16/2012			
6/5/2012		7/17/2012			
6/6/2012		7/18/2012	1.38		
6/7/2012		7/19/2012	0.95		
6/8/2012		7/20/2012	0.07		
6/9/2012		7/21/2012			
6/10/2012		7/22/2012			
6/11/2012	0.68	7/23/2012			

Date	Max (CFS)	Min (CFS)	Mean (CFS)	Date	Max (CFS)	Min (CFS)	Mean (CFS)	Date	Max (CFS)	Min (CFS)	Mean (CFS)
5/1/2012	6.9[P]	1.1[P]	2.8[P]	6/9/2012	1.8[P]	0.01[P]	0.24[P]	7/25/2012	0.00[P]	0.00[P]	0.00[P]
5/2/2012	6.2[P]	0.79[P]	2.2[P]	6/10/2012	3.5[P]	0.00[P]	0.48[P]	7/26/2012	0.00[P]	0.00[P]	0.00[P]
5/3/2012	5.9[P]	0.55[P]	2.0[P]	6/11/2012	7.7[P]	0.00[P]	3.1[P]	7/27/2012	37[P]	0.00[P]	16[P]
5/4/2012	5.4[P]	0.43[P]	1.5[P]	6/12/2012	7.9[P]	1.2[P]	3.3[P]	7/28/2012	31[P]	10[P]	18[P]
5/5/2012	21[P]	0.45[P]	11[P]	6/13/2012	6.4[P]	0.57[P]	2.2[P]	7/29/2012	14[P]	6.1[P]	9.4[P]
5/6/2012	12[P]	4.1[P]	7.0[P]	6/14/2012	5.3[P]	0.27[P]	1.2[P]	7/30/2012	9.0[P]	3.5[P]	5.5[P]
5/7/2012	9.2[P]	3.1[P]	5.5[P]	6/15/2012	4.7[P]	0.03[P]	0.81[P]	7/31/2012	6.4[P]	1.5[P]	3.0[P]
5/8/2012	52[P]	6.4[P]	37[P]	6/16/2012	3.3[P]	0.00[P]	0.41[P]	8/1/2012	4.3[P]	0.83[P]	1.4[P]
5/9/2012	25[P]	9.4[P]	15[P]	6/17/2012	7.7[P]	0.00[P]	1.8[P]				
5/10/2012	15[P]	5.9[P]	9.0[P]	6/18/2012	11[P]	2.6[P]	5.8[P]				
5/11/2012	11[P]	3.7[P]	6.1[P]	6/19/2012	7.7[P]	1.1[P]	3.1[P]				
5/12/2012	9.0[P]	2.6[P]	4.5[P]	6/20/2012	6.1[P]	0.20[P]	1.9[P]				
5/13/2012	52[P]	2.5[P]	26[P]	6/21/2012	4.7[P]	0.02[P]	0.75[P]				
5/14/2012	50[P]	25[P]	36[P]	6/22/2012	2.8[P]	0.00[P]	0.33[P]				
5/15/2012	30[P]	12[P]	19[P]	6/23/2012	0.40[P]	0.00[P]	0.04[P]				
5/16/2012	18[P]	7.9[P]	14[P]	6/24/2012	0.75[P]	0.00[P]	0.08[P]				
5/17/2012	21[P]	11[P]	15[P]	6/25/2012	0.14[P]	0.00[P]	0.02[P]				
5/18/2012	17[P]	8.3[P]	11[P]	6/26/2012	0.02[P]	0.00[P]	0.00[P]				
5/19/2012	14[P]	6.1[P]	8.3[P]	6/27/2012	0.00[P]	0.00[P]	0.00[P]				
5/20/2012	11[P]	4.4[P]	6.5[P]	6/28/2012	0.00[P]	0.00[P]	0.00[P]				
5/21/2012	9.7[P]	3.1[P]	5.2[P]	6/29/2012	[P]	[P]	[P]				
5/22/2012	8.5[P]	2.7[P]	4.2[P]	6/30/2012	[P]	[P]	[P]				
5/23/2012	7.9[P]	2.1[P]	3.4[P]	7/1/2012	[P]	[P]	[P]				
5/24/2012	7.3[P]	1.8[P]	2.8[P]	7/2/2012	0.00[P]	0.00[P]	0.00[P]				
5/25/2012	6.9[P]	1.4[P]	2.5[P]	7/3/2012	0.00[P]	0.00[P]	0.00[P]				
5/26/2012	6.6[P]	0.87[P]	2.1[P]	7/4/2012	0.00[P]	0.00[P]	0.00[P]				
5/27/2012	5.9[P]	0.47[P]	1.9[P]	7/5/2012	0.00[P]	0.00[P]	0.00[P]				
5/28/2012	5.4[P]	0.20[P]	1.5[P]	7/6/2012	0.00[P]	0.00[P]	0.00[P]				
5/29/2012	5.3[P]	0.14[P]	1.2[P]	7/7/2012	0.00[P]	0.00[P]	0.00[P]				
5/30/2012	5.1[P]	0.11[P]	1.0[P]	7/8/2012	0.00[P]	0.00[P]	0.00[P]				
5/31/2012	3.7[P]	0.06[P]	0.60[P]	7/9/2012	0.00[P]	0.00[P]	0.00[P]				
6/1/2012	12[P]	0.09[P]	7.1[P]	7/10/2012	0.00[P]	0.00[P]	0.00[P]				
6/2/2012	9.4[P]	2.6[P]	5.1[P]	7/11/2012	0.00[P]	0.00[P]	0.00[P]				
6/3/2012	7.7[P]	1.5[P]	3.9[P]	7/12/2012	0.00[P]	0.00[P]	0.00[P]				
6/4/2012	6.6[P]	0.75[P]	2.3[P]	7/13/2012	0.00[P]	0.00[P]	0.00[P]				
6/5/2012	5.9[P]	0.43[P]	2.0[P]	7/14/2012	0.00[P]	0.00[P]	0.00[P]				
6/6/2012	5.3[P]	0.17[P]	1.3[P]	7/15/2012	6.9[P]	0.00[P]	2.8[P]				
6/7/2012	3.9[P]	0.04[P]	0.61[P]	7/16/2012	5.9[P]	0.12[P]	1.8[P]				
6/8/2012	2.8[P]	0.02[P]	0.39[P]	7/17/2012	4.7[P]	0.00[P]	1.0[P]				
6/9/2012	1.8[P]	0.01[P]	0.24[P]	7/18/2012	3.0[P]	0.00[P]	0.21[P]				

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