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ABSTRACT

Fecal contamination in Midwestern karst regions was evaluated by a combination of *Bacteroidales*-based microbial source tracking (MST), traditional bacterial indicators, and environmental variables. Water samples from springs and wells were collected from Illinois, Wisconsin, Kentucky, and Missouri. A quantitative polymerase chain reaction (PCR) with seven primer sets targeting different members of *Bacteroidales* was used to discriminate human and livestock fecal pollution. The results suggested that most samples were contaminated by a mixture of human and animal waste sources, with only a few samples showing pollution solely by humans or animals. Spring water tended to be more contaminated than well water, and fecal biomarkers were detected in higher numbers in urban spring systems than rural spring systems. Correlation between traditional bacterial indicators and fecal contamination determined by *Bacteroidales*-based MST was weak, with the exception of *E. coli*, chloride, and dissolved oxygen (DO), which correlated well with the level of *Bacteroidales* fecal biomarkers. The *Bacteroidales*-based MST method used in this study did not give definitive results, but nevertheless we believe it could be a powerful tool used in conjunction with other measures, including traditional bacterial indicators and a variety of environmental variables. It appears that there currently is no single "silver bullet" for MST in karst aquifers, and future studies should continue to combine chemical-MST and microbe-MST techniques.

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ABBREVIATIONS

AdV	adenoviruses
APHA	American Public Health Association
ARA	antibiotic resistance analysis
BMPs	best management practices
CA	cluster analysis
Cl^-	chloride
CTAB	cetyl trimethyl ammonium bromide
DGGE	denaturing gradient gel electrophoresis
DKN	dissolved Kjeldahl nitrogen
dNTP	deoxyribonucleotide triphosphate
DO	dissolved oxygen
DOC	dissolved organic carbon
E	amplification efficiency
gDNA	genomic DNA
HOPE	hierarchical oligonucleotide primer extension
IDPH	Illinois Department of Public Health
ISTC	Illinois Sustainability Technology Center
ISWS	Illinois State Water Survey
KDW	Kentucky Department of Water
MDOC	Missouri Department of Conservation
MST	microbial source tracking
NO_3-N	nitrate-nitrogen
ORP	oxidation-reduction potential
P_n	copy number of the product
P_0	starting number of the template
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
PPCPs	pharmaceuticals and personal care products
PSL	ISWS Public Service Laboratory
PyV	polyomaviruses
Q-PCR	quantitative PCR
rRNA	ribosomal RNA
SpC	specific conductance
TC	total coliform
TDS	total dissolved solids
TMDLs	total maximum daily loads
TN	total N
tRFs	terminal restriction fragments
T-RFLP	terminal restriction fragment length polymorphism
WGNHS	Wisconsin Geological and Natural History Survey
WNDR	Wisconsin Department of Natural Resources
WHO	World Health Organization

1. INTRODUCTION

1.1 Traditional microbial indicators of fecal pollution

Indicators that signify the presence of fecal pollution in water bodies and forecast the associated risk for human health have been in use for many years. These indicators are abundant in human and animal feces that can be easily differentiated from other microorganisms, typically using cultivation methods. An ideal indicator should have the following characteristics: (1) specific to fecal origin; (2) no replication or regrowth outside of the host; (3) similar survival characteristics with pathogens in natural environments; (4) strongly associated with the presence of pathogens; and (5) simple to test and easy to recover from the environment (Scott et al., 2002).

Commonly used indicators in water include total coliform (TC), *Escherichia coli* (*E. coli*), enterococci, *Clostridium perfringens* and their spores, and coliphages. Enumeration of TC, *E. coli,* and/or enterococci is recommended to indicate fecal pollution in freshwater and marine water. Although coliforms were originally thought to be primarily of fecal origin, they are in actuality widely distributed in the environment, making the representativeness of their presence problematic (Leclerc et al., 2001). *E. coli* is considered to be a better indicator of fecal contamination because it is more restricted to feces of human and warm-blooded animals (Edberg et al., 2000). Enterococci are often used as bacterial indicators for recreational surface waters due to their salt-resistant characteristics (APHA, 1998). However, they are approximately 100- to 1000-fold less numerous in aquatic environments than *E. coli*, making them a less effective indicator for low levels of fecal contamination (Edberg et al., 2000). *C. perfringens* is an anaerobic bacterium that turns into spores under unfavorable conditions in the environment. Its long survival time in the spore state makes it useful for indicating groundwater pollution (Edberg et al., 2000). However, detection of *C. perfringens* and its spores requires that testing be performed under strict anaerobic conditions, which increases complexity and expense. Coliphages are viruses that infect members of the coliform group, and thus are not specific only to *E. coli*. Coliphages are a useful indicator for the presence of enteric viruses in water because of their similarity to human viruses (WHO, 2006). However, coliphages are found in less than 50% of human feces samples (Edberg et al., 2000), and cultivating coliphages is time-consuming and technically difficult. As a result, the use of coliphages to indicate fecal contamination has not been widely used.

Despite their limitations, TC, *E. coli,* and enterococci remain the most widely used microbial indicators of fecal contamination, and they are easy and inexpensive to measure. However, it is difficult to differentiate sources of fecal pollution using these traditional indicators. Coliforms and *E. coli* are present in both human and animal feces, and they can survive outside hosts for long periods and even replicate in the environment. The correlation between the presence of indicators and human health risks is also typically not strong (Fiksdal et al., 1985; Weiskel et al., 1996; Solo-Gabriele et al., 2000; Griffin et al., 2001, 2003; Scott et al., 2002; Noble et al., 2006; Shanks et al., 2008). Knowing only the level of contamination without determining fecal sources provides little information for land use management and remediation. Therefore, effective microbial source tracking (MST) methods are needed to discriminate sources of fecal pollution.

1.2 Microbial source tracking (MST)

1.2.1 Concept of MST

The goal of MST is to trace the origin of fecal pollution by using chemical, microbiological, genotypic, and/or phenotypic methods (Scott et al., 2002). Its major assumption is that there are host-specific characteristics that are stable spatially and temporally. MST methods can be divided into two broad categories based on target types: chemical-based and microbe-based. Recently, researchers have promoted using a combination of methods from each category rather than relying on a single method (Noble et al., 2006; Peeler et al., 2006; Walters et al., 2007; Katz and Griffin, 2008; Reischer et al., 2008; Ritchey and Coyne, 2009; Sauer et al., 2011; Furtula et al., 2012). In addition to identifying and/or quantifying sources of contamination in the area of interest, MST can support the development and implementation of total maximum daily loads (TMDLs) and best management practices (BMPs), thus lowering health risks associated with fecal pollution (Scott et al., 2002; Simpson et al., 2002; Santo Domingo et al., 2007).

MST has been applied in various environments, such as freshwater in general (Layton et al., 2006; Okabe et al., 2007; Walters et al., 2007; Ritchey and Coyne, 2009; Savichtcheva and Okabe, 2009; Furtula et al., 2012), rural freshwater (Hagedorn et al., 1999; Noble et al., 2006; Peeler et al., 2006), urban marine water (Peeler et al., 2006), urban stormwater systems (Sauer et al., 2011), and groundwater from karst regions (Wicks et al., 2004; Katz and Griffin, 2008; Johnson et al., 2011; Reischer et al., 2011).

1.2.2 Chemical-based MST

Chemical-based fecal MST targets chemicals that are specific to human wastewater to indicate pollution by anthropogenic activities. A variety of human-specific chemicals has been proposed from compounds identified in wastewater effluent, including caffeine, stanols/sterols (metabolic by-products of cholesterols [coprostanol]), pharmaceuticals (carbamazepine and diphenhydramine), and fluorescent whitening agents (Glassmeyer et al., 2005). For examples, Furtula et al. (2012) used chemical (sterols) and microbial MST methods as well as nitrogen concentrations to explore the potential contamination sources of surface water in the Nathan Creek watershed, British Columbia, Canada. Sterol-based MST was shown to be effective in discriminating between human and non-human pollution. Another study (Peeler et al., 2006) utilized caffeine, nutrients, and indicator bacteria to track human and non-human sources of pollution in rural freshwater and urban marine systems. In the rural area, caffeine and nitrogen did not correlate well with traditional fecal indicators. In the urban system, caffeine was linked to traditional fecal indicators and human activity. Chemical-based MST can be done rapidly and avoids the problem of microbial regrowth and decay in the environment. However, analytical costs for some of these chemical compounds can be prohibitive. The concentrations of these compounds are also commonly very low in natural water systems. Information on the persistence of these chemicals under ambient environmental conditions and their relationship with traditional microbial indicators is also lacking. Lastly, these methods cannot always distinguish among different animal sources (Hagedorn and Weisberg, 2009).

1.2.3 Microbe-based MST

Microbe-based MST can be either library-independent or library-dependent. A library is a database of microbial characteristics from known sources of contamination. These source libraries are produced by collecting fecal samples from various sources and isolating distinctive fecal bacteria from them. Source libraries can be used to identify unknown-source fecal bacterial isolates by comparison with those in the library.

Library-independent techniques determine sources of pollution based on known host-specific characteristics of microorganisms without referring to a library. These techniques need the establishment of a library of bacterial isolates to determine fecal pollution sources. Generally, certain characteristics of targeting bacteria isolated from various feces (e.g., human and animal) are recorded to generate a library. A database of known sources is extracted from the library using statistical analysis (discriminant or clustering analysis). Then bacterial isolates from unknown sources are compared with the database of known sources to determine possible sources of pollution (Hagedorn et al., 1999).

Library-dependent methods can be divided into two approaches based on phenotypes and genotypes. The most commonly used phenotypic method is antibiotic resistance analysis (ARA). ARA relies on the detection of antibiotic resistance patterns of fecal streptococci, enterococci, or *E. coli* (Hagedorn et al., 1999; Wiggins et al., 1999; Simpson et al., 2002; Ritchey and Coyne, 2009). Many field studies have demonstrated that ARA is a simple and reliable approach to discriminate sources of fecal contamination. Hagedorn et al. (1999) established a large database of ARA for fecal streptococci with greater than 80% accuracy in source identification. The database was applied to a rural watershed in Virginia where cattle were identified as the dominant source of pollution. Based on these results, access of cattle to riparian areas was limited and water quality was observed to improve. Another study (Ritchey and Coyne, 2009) examined the resistance patterns to *E. coli* and fecal streptococci in small watersheds in Kentucky in order to discriminate human from non-human sources of pollution. Using their database, a correct classification of greater than 60% was achieved. The study concluded that ARA detection in combination with information on the watershed and its land use can help guide remediation of impaired water environments.

Although ARA is easy to use from technical aspects and can discriminate multiple fecal sources, its accuracy (e.g., correct classification) is still low (generally more than 50%) and dependent on the size and representativeness of the reference database (Hagedorn et al., 1999; Wiggins et al., 1999; Harwood et al., 2000; Graves et al., 2002; Ritchey and Coyne, 2009). Furthermore, it is difficult to apply this method to complex systems with multiple sources of pollution. Another concern is that horizontal gene transfer via plasmids could carry antibiotic resistance genes from target organisms to other non-fecal contamination-related organisms. This will change the antibiotic resistance profile of known isolates in the database (Simpson et al., 2002).

Genotypic-based library-dependent methods, including ribotyping, pulse field gel electrophoresis (PFGE), and repetitive element polymerase chain reaction (PCR), have principles similar to DNA fingerprinting. Bacteria from known sources are first isolated, cultured, and identified. Ribotyping identifies pieces of genomic DNA (gDNA) cut with restriction enzymes by oligonucleotide probes targeting ribosomal RNA (rRNA) genes, which are essential for protein synthesis in all living organisms. The process is highly

reproducible but labor-intensive. PFGE utilizes gel electrophoretic analysis to diagnose gDNA fingerprints after treatment with rare-cutting restriction enzymes. Correlation between PFGE profiles and the isolate source is weak due to the high variation in gDNA among closely related bacterial isolates at the strain level. Repetitive element PCR uses PCR targeting interspersed repetitive DNA sequences in prokaryotic genomes followed by gel electrophoresis (Scott et al., 2002; Simpson et al., 2002). This method is easier to perform than ribotyping and PFGE. Application of repetitive element PCR for MST has seldom been performed. In comparison with library-dependent phenotypic methods, library-independent genotypic methods are more expensive and sometimes more labor-intensive and time-consuming. Their use in field studies is still limited compared with ARA. Due to the disadvantages of library-dependent methods and the advances in molecular techniques, library-independent methods based on molecular markers are gaining in popularity. These methods include host-specific PCR/quantitative PCR (Q-PCR), gene-specific PCR, phage typing, and microbial community analysis (denaturing gradient gel electrophoresis [DGGE] and terminal restriction fragment length polymorphism [T-RFLP]) (Simpson et al., 2002).

Among all the possible host-specific bacteria, most analyses use *Bacteroidales* as the target. *Bacteroidales* is an order in the phylum *Bacteroidetes*. *Bacteroidetes* species are obligate anaerobes, which account for about half of all bacterial populations in the human intestine and occur in much larger numbers than TC or *E. coli* (Ley et al., 2006). The dominant members of the genus *Bacteroides* include *B. vulgatus* (31% of all *Bacteroidetes* and 1.4–15% of all bacteria), *B. thetaiotaomicron* (12% of all *Bacteroidetes* and 0.2–6.2% of all bacteria), *B. fragilis* (5% of all *Bacteroidetes* and 2.5–13.3% of all bacteria), *B. distasonis* (0.8% of all *Bacteroidetes* and 0.4% of all bacteria), and *B. caccae* (0.2–1.1% of all bacteria) (Suau et al., 1999; Eckburg et al., 2005; Ley et al., 2006; Hong et al., 2008). Because of their prevalence in feces, *Bacteroides* were considered a good candidate target for MST. Early studies focused on assessing the use of *B. fragilis* as an indicator for human fecal contamination. Cultivation methods were used to examine their prevalence in various animal feces and ambient environments and their degradation rate in the aerobic environment (Allsop and Stickler, 1984; 1985; Fiksdal et al., 1985). These studies demonstrated that *Bacteroides* are host specific and have limited survival in the environment. With its high abundance, it has the ability to discriminate human fecal contamination from other fecal sources.

Methods such as Q-PCR and T-RFLP targeting the 16S rRNA genes of *Bacteroides* were developed and applied to circumvent the difficulty of cultivating anaerobic *Bacterioides* species. T-RFLP is a community analysis technique that can identify all possible major microbial populations in one electropherogram (see Liu et al. [1997] for a detailed explanation on the fundamentals of the technique). Using primer sets targeting the order *Bacteroidales*, specific terminal restriction fragments (tRFs) representing different fecal origins in an electropherogram are selected. Fecal sources of unknown samples can be easily identified with the selected tRFs. This method is a semi-quantitative technique that can provide relative abundance information for different contamination sources (Liu et al., 1997; Bernhard and Field, 2000; Field et al., 2003; Savichtcheva and Okabe, 2009). T-RFLP is reported to be high throughput and reproducible, with analytical times of a few hours. Multiplex reactions are not needed since representative tRF peaks associated with different hosts can be determined in one

reaction. Several representative fragments are reported, including 117-119 bp human-specific marker and 222-227 bp cow-specific marker (Bernhard and Field, 2000; Savichtcheva and Okabe, 2009). However, T-RFLP can produce many false-positive results, possibly because each tRF can represent more than one target. At the same time, this feature might be beneficial in cases where false negatives are unacceptable (Savichtcheva and Okabe, 2009). In addition, analysis of T-RFLP needs sequencing instruments, which are expensive.

Artificial amplification of DNA pieces, or amplicons, can be quantified efficiently using Q-PCR, in what is referred to as "real-time." Assuming a constant amplification efficiency (E), a normal PCR reaction is:

$$P_n = P_0 \times (E+1)^n \qquad (1)$$

where *n* is the number of thermocycles, P_n is the copy number of the product and P_0 is the starting number of the template. To predict P_0, we need to know E:

$$P_0 = {}^{P_n}/_{(E+1)^n} \qquad (2)$$

Equation (2) can be converted using a logarithm transformation:

$$Log\ (P_0) = -n \times Log\ (E+1) + Log\ (P_n) \qquad (3)$$

If $Log\ (P_n)$ is constant, $Log\ (P_0)$ and *n* have a linear relationship with $Log\ (E+1)$ as the slope and $Log\ (P_n)$ as the y-intercept. If multiple values of *n* and $Log\ (P_0)$ are measured, then the slope $Log\ (E+1)$ can be calculated using linear regression. To satisfy all the requirements, a series of DNAs with known concentrations are amplified in individual tubes and compared at a point where the same amounts of DNA products were obtained. A series of *n* corresponding to the point are recorded and $Log\ (E+1)$ and $Log\ (P_n)$ can be calculated. For unknown DNA samples, *n* corresponding to the same point chosen with DNA templates of known quantity is recorded. Knowing all the parameters on the right side of equation (3), $Log\ (P_0)$ can be calculated (Higuchi et al., 1993; Raeymaekers, 2000; Rutledge and Cote, 2003).

A breakthrough for Q-PCR is the ability to quantify the accumulation of amplicons using fluorophores such as SYBR®. The point at which each reaction contains identical amounts of DNA products is determined arbitrarily where the fluorescent signal intensity increases above the background. That fluorescent intensity is defined as the threshold, and the corresponding value of *n* is called the threshold cycle (Ct). The regression line between Ct and $Log\ (P_0)$ is the standard curve. To minimize non-target amplification, a melting curve can be carried out after the normal amplification, which reduces the occurrence of false-positives (Higuchi et al., 1993; Raeymaekers, 2000; Rutledge and Cote, 2003; Okabe et al., 2007; Hong et al., 2009).

Q-PCR specifically targeting *Bacteroidales* is widely used for tracking fecal contamination from most human, cow, and pig feces in different environments (Layton et al., 2006; Noble et al., 2006; Okabe et al., 2007; Shanks et al., 2007; Sauer et al., 2011). Other possible contamination sources include dog, cat, elk, deer, and gull (Field et al., 2003; Dick and Field, 2004; Dick et al., 2005). The targeted DNA biomarkers of *Bacteroidales* include 16S rRNA genes, functional genes, and genes encoding hypothetical proteins. However, it can be difficult to design specific primers to target *Bacteroidetes* species from different sources due to the close homology (shared ancestry) of the DNA biomarkers among *Bacteroides* species. Dick et al. (2005) analyzed 16S rRNA gene sequences from the feces of eight different hosts: human, bovine, pig, horse,

dog, cat, gull, and elk. They observed that tight and high-similarity clusters were formed only for *Bacteroides* species detected in cow, pig, and horse feces. 16S rRNA gene sequences of *Bacteroides* species from human, gull, dog, and cat fecal samples were clustered together, making it difficult to design specific primers for Q-PCR to distinguish *Bacteroides* species from different hosts. A study tested ten different Q-PCR assays for human-associated *Bacteroidales*, and only three out of ten primer sets satisfied all six criteria used. Clearly, it is important to validate the primer sets before applying them in MST (Shanks et al., 2010).

Q-PCR detection is not only quantitative but also sensitive and specific. It was theoretically calculated that Q-PCR could detect as few as three copies of targeted DNA per reaction (Bustin et al., 2009), in comparison to the range of detection adopted in most MST studies, which is approximately $25 – 1\times10^6$ copies of targeted gene per reaction (Layton et al., 2006; Okabe et al., 2007; Shanks et al., 2008; 2010). To connect copies with cell numbers, the copy number of 16S rRNA genes in one cell would need to be assumed. In general, each genome of *Bacteroides* contains four to six copies of 16S rRNA genes with an average of 5.57 copies (Klappenback et al., 2001).

Generally, Q-PCR for *Bacteroidales*-based MST methods can correctly identify fecal sources with an accuracy of greater than 80% when samples from known sources are tested, which is much higher than the specificity of ARA and T-RFLP (Layton et al., 2006; Shanks et al., 2010; Reischer et al., 2011). The Q-PCR step using a 96-well reaction plate can be finished in three hours. Compared to T-RFLP, there is no need to use a DNA sequencing machine. Because of the advantages and successes in field studies, Q-PCR has become a more reliable method than methods like T-RFLP in MST studies.

1.3 Karst environments

Karst refers to terrain that has been shaped by the dissolution of rocks, usually carbonates (limestone and dolomite), and is characterized by features such as crevices, large conduits, sinkholes, caves, large springs, and sinking streams (Figure 1) (Ford and Williams, 2007; Kelly et al., 2009; Pronk et al., 2009). Karst aquifers are important sources of drinking water, supporting roughly a quarter of the world's population (Ford and Williams, 2007). Karst systems, however, are known to be vulnerable to human and animal waste contamination. For example, in the sinkhole plain of southwest Illinois, almost all of the springs and many wells sampled by Panno et al. (1996) and Kelly et al. (2009) were contaminated with fecal bacteria. The presence of fractures, conduits, and sinkholes results in the rapid movement of recharge from land surface into and through the subsurface. This rapid movement of water lessens the amount of time available for physical and biogeochemical reactions (e.g., sorption, ion exchange, degradation) to decrease the concentrations of surface-derived contaminants in the subsurface.

Transport of microbial contaminants in karst is facilitated by high groundwater flow velocities, particularly following heavy rainfalls or snowmelts. High loadings of contaminants, including nutrients, pesticides, and fecal materials are flushed into the groundwater system (Pasquarell and Boyer, 1995; Ryan and Meiman, 1996; Currens, 2002; Panno and Kelly, 2004; Kelly et al., 2009; Laroche et al., 2010). Sources of human waste include on-site disposal systems and community treated wastewater effluents. Contamination from animals can occur either from discharge from waste lagoons and pits at livestock facilities or from leaching of manure (Breed and Norton, 1937; Mahler et al.,

2000; John and Rose, 2005; Reed et al., 2011). Human and animal wastes carry not only high concentrations of nutrients, but also elevated levels of microorganisms and possibly pathogens. A close link between impaired water quality and karst topography has been reported in France (Beaudeau et al., 2010). In addition, disease outbreaks have been traced back to contaminated drinking water originating from karst systems (D'Antonio et al., 1985; Stevanovic, 1988; USEPA, 2006; Beaudeau et al., 2008).

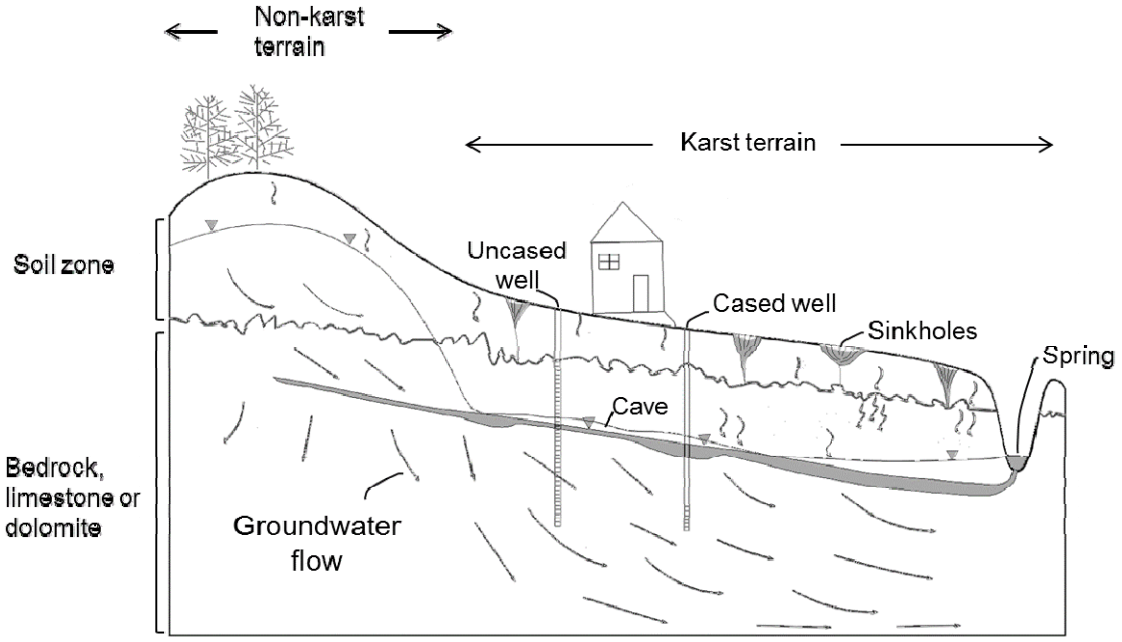

Figure 1. Karst topography (from Kelly et al., 2009)

Springs and wells are the two main sources of drinking water in karst regions. Spring discharge represents an amalgam of soil water and shallow and deep groundwater draining into a central conduit system. All sources of contaminated water (point and non-point) are mixed with water from uncontaminated areas such as forests. Conversely, wells are usually designed to intersect crevices and conduits, and the groundwater contributing to a particular well typically comes from a much smaller area than springs. Thus spring water samples are more likely to exhibit contamination than well water samples, but mixing tends to prevent extremely high contaminant concentrations. Consequently, point sources of contamination can go unrecognized in spring water samples. Conversely, a contaminated well would be diluted or flushed out at a somewhat slower rate than a contaminated spring.

Karst regions are common in the Midwestern United States (Figure 2). In Illinois, these regions are primarily in the northwest and southwest, but also include the northeast, west, and south. Every state bordering Illinois has significant karst regions, including Indiana, Iowa, Kentucky, Missouri, and Wisconsin. The karstified carbonate rocks (limestones and dolomites) are Paleozoic in age, ranging from Cambrian to Mississippian (USGS, 2012). The Midwest has the highest density of caves in the country, including Mammoth Cave in Kentucky, the largest cave in the world.

1.4 Studies on tracking contaminations in karst region

Direct monitoring of inorganic and organic compounds, bacterial indicators, and viruses associated with contamination can give clues to the state of the water system, and is often the first step in recognizing the presence of non-point source pollution. In most cases, elevated levels of chloride (Cl^-), nitrate-nitrogen (NO_3-N), and traditional fecal indicators are observed in the karst area being studied. However, the correlation between the presence of enteric bacteria and elevated levels of Cl^- and/or NO_3-N is not always statistically significant (Neill et al., 2004; Katz and Griffin, 2008; Kelly et al., 2009). In addition to routine monitoring of traditional indicator bacteria, enteric viruses have been monitored in some studies (Leguyader et al., 1993; 1994; Noble and Furman, 2001; Griffin et al., 2003; Noble et al., 2006; Katz and Griffin, 2008; Johnson et al., 2011; Pedersen et al., 2011).

Once contamination is detected, the next step is to identify the possible pollution sources. Due to the relatively high concentrations of pesticides and pharmaceuticals in vulnerable karst regions, chemical-based MST may be suitable in addition to the widely-used microbe-based MST. Detections of previously-banned and currently-used pesticides and their degradation products have been used (Katz and Griffin, 2008; Fox et al., 2010). Another study (Wicks et al., 2004) investigated concentrations of natural estrogen in the

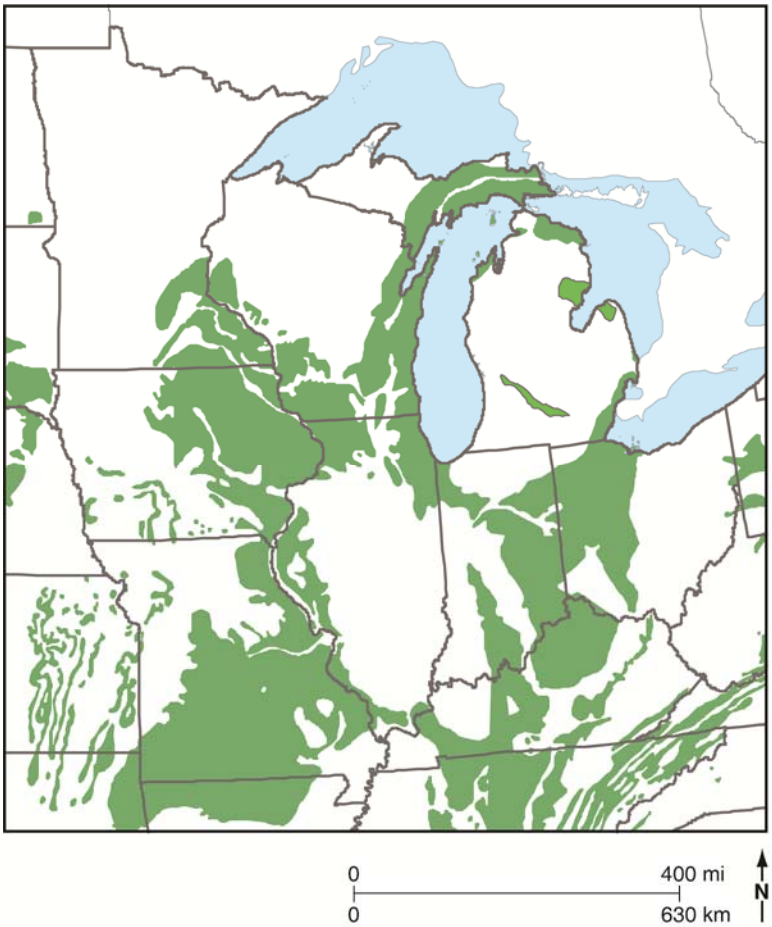

Figure 2. Karst regions (green) in the Midwestern U.S. (USGS, 2012)

Missouri Ozark Plateau Aquifer. The concentrations of estrogen changed seasonally and were at levels that could lead to adverse health effects in fish more than 60% of the time. The sources of estrogen were identified as sewage-treated wastewater and agricultural runoff.

Although many studies have been conducted to trace sources of pollution from karst regions in Austria (Reischer et al., 2011), Switzerland (Pronk et al., 2009), Florida (Katz and Griffin, 2008), Tennessee (Johnson et al., 2011), Arkansas (Peterson et al., 2000), Kentucky (Reed et al., 2011), Missouri (Wicks et al., 2004; Fox et al., 2010), and Wisconsin (Pedersen et al., 2011), direct quantification of human and animal fecal pollution is lacking except for one study (Reischer et al., 2011). The comparison of different studies from various regions is difficult because of different methods used in these studies, thus a systematic investigation of karst regions covering a large area is needed to reflect the spatial differences.

1.5 Objective of the study

The objective of this study was to distinguish between human and livestock sources of fecal contamination in karst aquifers in four Midwestern states, Illinois, Wisconsin, Kentucky, and Missouri. Q-PCR was used for MST, along with traditional bacterial indicators and environmental parameters to assist in interpretation.

Seven *Bacteroidales*-based primer sets were used in the Q-PCR analysis, one for overall contamination, four for human, and one each for swine and bovine. Traditional bacterial indicators and water quality data were determined to explore their relationship with fecal contamination determined by *Bacteroidales*-based MST. Ordination analysis was used to extrapolate the correlation between Q-PCR results and these environmental variables. Where available, land use data were also used to inform the MST process.

This was the first study to examine and compare fecal contamination in Midwestern karst aquifers using a multi-metric approach. Results of the study could provide useful information for BMP implementation and better land use management. Also, identifying environmental parameters associated with fecal contamination are important for future monitoring activities.

2. MATERIALS AND METHODS

2.1 Study sites

Our initial plan was to sample contaminated wells in karst areas, but early in the project it became clear that this would be difficult for several reasons: (1) contamination is often intermittent, thus it can be difficult to time the sampling of a well when it is contaminated; (2) contaminated wells were often sanitized before we could sample them; and (3) wells that exhibit persistent contamination are often abandoned. Almost all of the contaminated wells that we sampled in the Sinkhole Plain 5 to 15 years ago (Panno et al., 1996; 2001; Hackley et al., 2007; Kelly et al., 2009) had been abandoned by the time this project started. Because of these limitations, we expanded our sampling to include springs in karst areas, which tend to be persistently contaminated, and wells in non-karst areas. A total of 25 wells in Illinois and Wisconsin and 23 springs in Illinois, Kentucky, and Missouri were sampled during this study (Figure 3). Most of the sampled wells were domestic wells, but five non-community public wells in Illinois and three public supply

wells in Sturgeon Bay, WI, were also sampled. A sample was also collected of runoff from a field in Wisconsin in which manure had recently been applied.

2.1.1 Illinois

Wells sampled in Illinois are shown in Figure 4. Six domestic wells in Illinois were selected from wells in the Sinkhole Plain (Monroe and Randolph Counties) that exhibited contamination when sampled in the 1990s (Wells IL-1 – IL-6). This area is well known for having over ten thousand large (typically 30 to >100 meters [m] in diameter) cover-collapse sinkholes, large karst springs, and numerous branchwork caves with actively flowing streams (Panno and Kelly, 2004). The bedrock is primarily calcite-rich Mississippian-Age limestone. The upland area (about two-thirds of Monroe County) is, for the most part, covered with Illinois Episode glacial till and/or residuum that are overlain by a layer of loess (windblown silt). Glacial drift and other materials that overlie the limestone bedrock in most of the upland areas of Monroe County are relatively thin (typically less than 15 m) and bedrock exposures are common (Panno et al., 1997). About 65% of the land use in this area is row-crop agriculture, with some livestock including

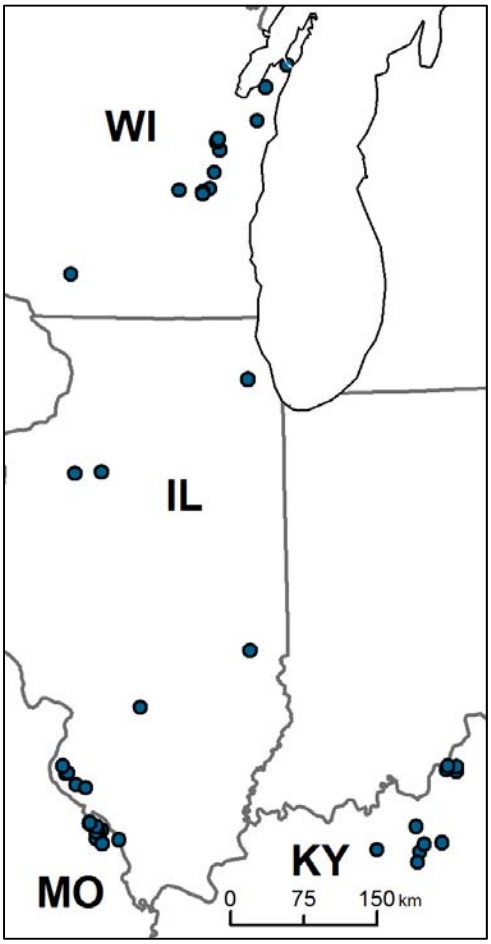

Figure 3. Locations of sampling sites. Wells were sampled in Illinois and Wisconsin, springs were sampled in Kentucky and Missouri.

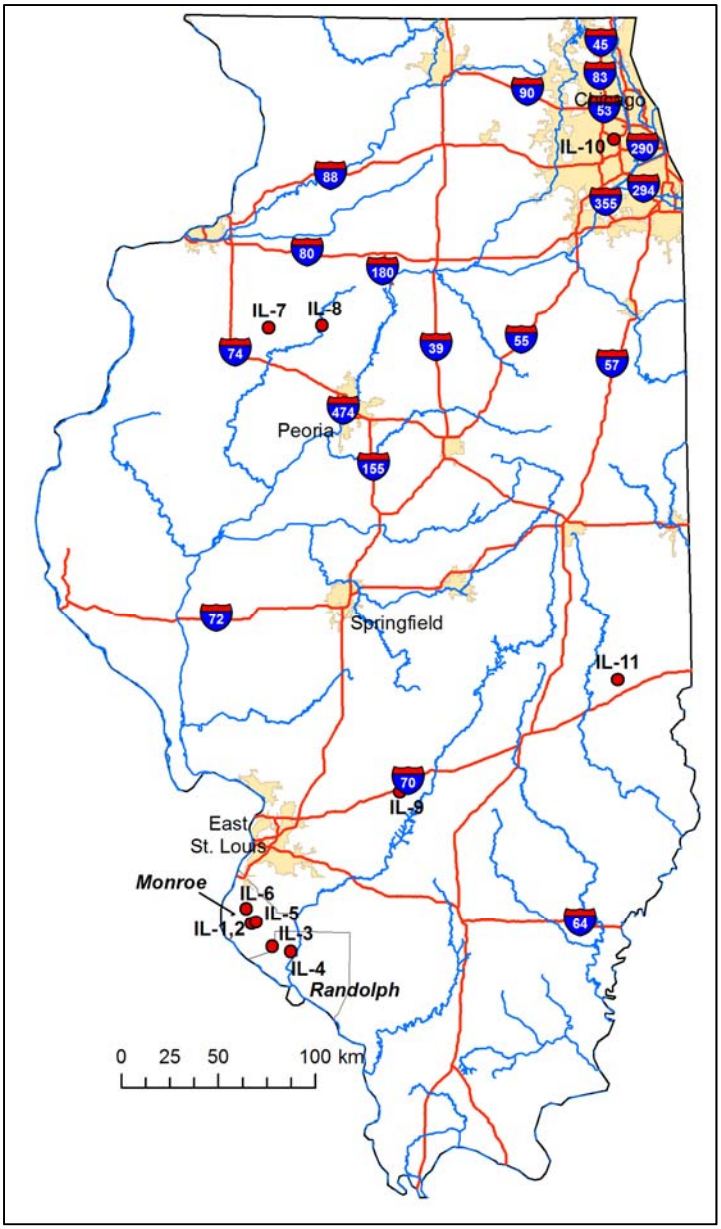

Figure 4. Locations of wells sampled in Illinois

hogs and cattle. Hogs are generally confined indoors while cattle are allowed to graze in pastures most of the year. Hog manure is applied as fertilizer for crop lands, but is a minor part of the fertilizer applied in this region. Livestock manure and on-site wastewater treatment systems all contribute to the local groundwater contamination as suggested from previous studies (Kelly et al., 2009). Wells IL-1 and IL-2 were located on a hog farm.

Five non-community public wells outside of the Sinkhole Plain were recommended to us for sampling by the Illinois Department of Public Health (IDPH) because of a positive bacteria sample. These included three campgrounds and two restaurants (Wells IL-7 – IL-11).

2.1.2 Wisconsin

Locations of wells sampled in Wisconsin are shown in Figure 5. Sampling was focused in five counties: Calumet, Door, Fond du Lac, Kewaunee, and Manitowoc. Three of the wells in Wisconsin were public supply wells in Sturgeon Bay (Door County), which have had a history of positive coliform detections. Sturgeon Bay well #10 was sampled five times, and well #6 twice.

The domestic wells in Wisconsin had persistent contamination issues and were recommended by the Wisconsin Department of Natural Resources (WDNR) or the Wisconsin Geological and Natural History Survey (WGNHS). Two of the domestic wells were located in residential areas (WI-1 and WI-3), and the rest were in rural areas. Five wells were sampled four times each (WI-3, WI-4, WI-5, WI-6, and WI-7), and one was sampled twice (WI-2). At two of the wells (WI-3 and WI-9), contamination had previously caused sickness in the household. MST was done for WI-9, which identified human bacteria and other household waste indicators (M. Gotkowitz, WGNHS, personal communication).

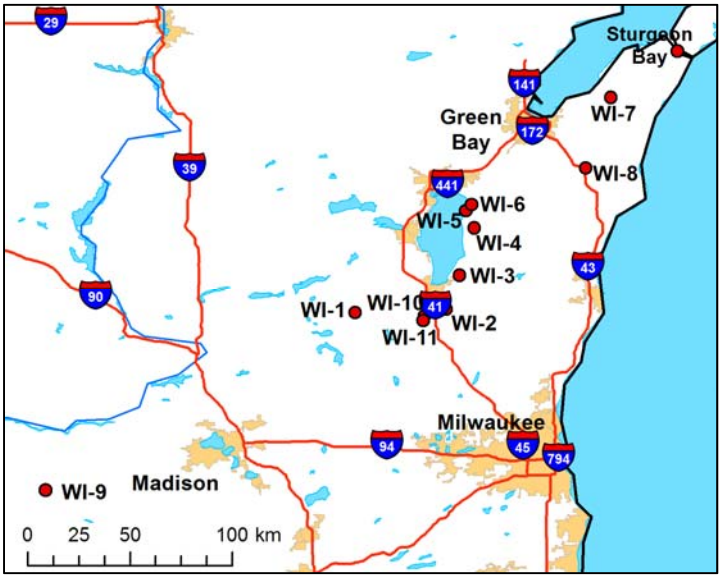

Figure 5. Locations of wells sampled in Wisconsin

Karst features exist in a large portion of Wisconsin (Figure 2), primarily in fractured and creviced dolomitic limestones of Silurian age. These carbonate units are not extensively weathered, and sinkholes and caves are rare. Vertical development of karst features is limited by the underlying Maquoketa Shale (Johnson and Stieglitz, 1990). The carbonates are fractured and groundwater flows through the fractures anisotropically (Northeast Wisconsin Karst Task Force, 2007). Contaminated well water has been a concern in this region for decades.

Wisconsin is a major dairy state, and in 2012 there were approximately 383,000 head of cattle in the five-county region study area (USDA, 2012). By comparison, there were approximately 281,000 people in that region in 2011 (U.S. Census Bureau, 2012). County-level hog data are not available.

2.1.3 Kentucky

Springs in Kentucky had been previously sampled on several occasions by the Kentucky Division of Water (KDW) and were always contaminated. Springs sampled were in two general localities, in Louisville, an urban setting, and the Green River basin, a rural region in western Kentucky (Figures 6 and 7).

The springs in Louisville are in the Beargrass Creek watershed, which is located in west-central Jefferson County, and covers an area of 157 square kilometers) km^2 (Blair and Ray, in press). This bedrock includes limestone, dolostone, and shale of Ordovician, Silurian, Devonian and Mississippian ages. Limestones within the Beargrass Creek watershed are very soluble and have well developed karst drainage. The predominant land cover is Urban/Residential (73%), which includes significant impervious cover (Blair and Ray, in press).

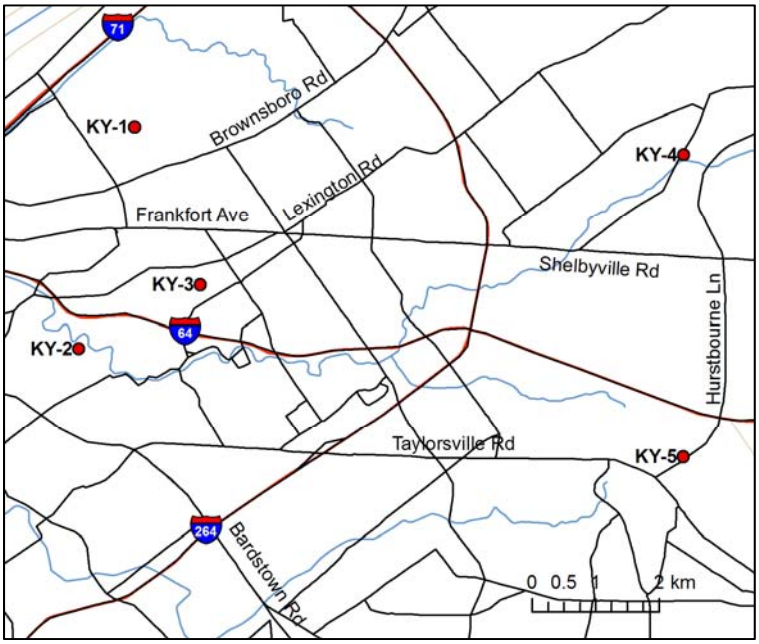

Figure 6. Locations of springs sampled in Louisville, KY

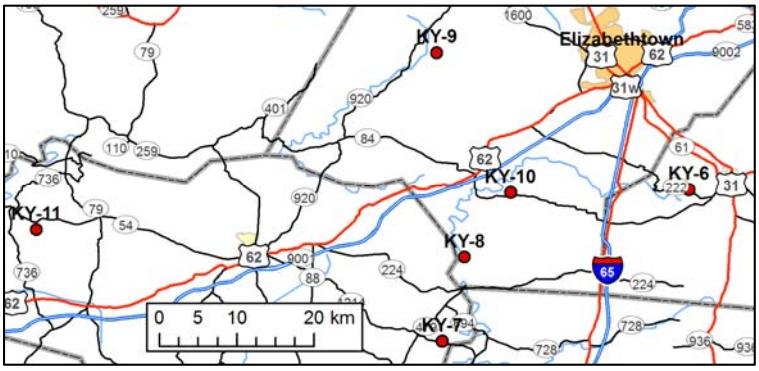

Figure 7. Locations of springs sampled in the Green River Basin of western Kentucky

The springs sampled in the Green River basin are in the Mississippian Plateau. A complete description of the geology and hydrogeology of the region is found in Blair et al. (2012). Briefly, the region has Mississippian-age carbonate rocks, primarily limestone, with well-developed karst drainage. There are a large number of sinkholes, caves, and influent streams; Mammoth Cave National Park is just south of the study area. Groundwater flow is primarily through solutionally enlarged conduits, and springs have relatively large discharges, with base flow discharges measured up to 24 cubic feet per second (cfs) (59 million Liters/day). The primary land uses in the basin are forest (49%) and agriculture (row crop or pasture; 47%), but with considerable variability for individual springsheds (Table 1). The region is rural, and urban/residential land cover is low. The USDA (2011) reports that in 2011 there were approximately 134,500 head of cattle in the four counties in which the sampled springs are located (Grayson, Hardin, Hart, and Larue); we could not find county-level numbers for hogs or other livestock, although it appears their numbers are much less than for cattle. The human population in the four-county study area was approximately 166,000 in 2011 (U.S. Census Bureau, 2012). Groundwater is the major source of drinking water in the basin, and Blair et al. (2012) report that it is sensitive to surface-derived contamination.

Table 1. Land Use Percentages within Springsheds Sampled in Kentucky (From Blair et al., 2012)

ID	Spring Name	Major Land Use Categories (% of springshed)			Total basin area (mi^2)
		Urban/Residential	Agriculture	Forest	
KY-6	Nolynn	6.1	61.5	32.4	56.4
KY-7	Mill	2.5	18.5	79.1	7.1
KY-8	Goodman	3.0	53.2	43.8	14.7
KY-9	Head of Rough	3.3	63.2	33.5	17.7
KY-10	Skees Karst Window	5.3	75.5	19.2	27.5
KY-11	Mahurin	3.9	31.8	64.3	25.3

2.1.4 Missouri

Springs sampled in Missouri were located in Perry County and were recommended by the Missouri Department of Conservation (MDOC), who had sampled them on previous occasions and found them to be always contaminated (Figure 8). Perry County is on the northeastern edge of the Salem Plateau within the Ozark Plateau. Rocks in the county are primarily Ordovician carbonates (Unklesbay and Vineyard, 1992). The eastern part of the county (east of I-55) is characterized by an extensive sinkhole plain that overlies the highest density of caves in Missouri. Sinkholes in this area range in size from several m to several km in diameter and can reach up to 30 m in depth (Vandike, 1985). Streams in the area support a variety of species, including some rare species listed in Missouri Species of Concern (Fox et al., 2010). Descriptions of some of the springs we sampled are found in Vandike (1985).

The land use in the karst watershed of Perry County is almost equally divided between agriculture (47%) and silviculture (45%). Land uses in some of the springsheds sampled are reported in Table 2. In 2007, there were approximately 36,000 head of cattle and 7000 hogs in Perry County (USDA, 2007). The human population in 2011 was approximately 19,000 (U.S. Census Bureau, 2012).

Groundwater is the sole source of drinking for almost all public water supplies in Perry County (Perryville purchases ~65% of its drinking water from a surface water source) (Missouri Department of Natural Resources, 2012).

Table 2. Land Use Percentages in Sub-watersheds in the Karst Region of Perry County, MO (from L. Taylor [MDOC], personal communication, 2012)

ID	Spring Name	Major Land Use Categories (%)				Total basin area (mi^2)
		Urban	Cropland	Grassland	Forest/wetland	
MO-1,6	Mystery Cave	0	57	30	10	7.0
MO-5	Thunder Hole	1	42	42	14	2.1
MO-9	Keyhole	1	65	25	8	1.3
MO-11	Ball Mill	0	41	41	16	0.66

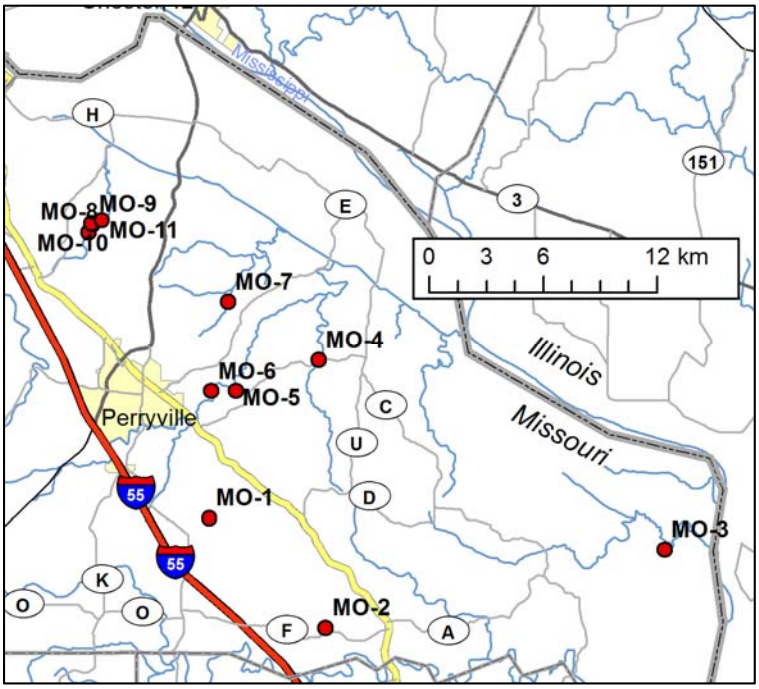

Figure 8. Locations of springs sampled in Perry County, Missouri

2.2 Sample collection and analysis

A total of 68 samples were collected during this study (not including duplicates), 46 from wells and 22 from springs. We collected 61 samples, five were collected by the water operator at Sturgeon Bay, WI, one was collected by the WGNHS, and one by WDNR.

Aseptic techniques were used during sample collection. Wells were purged until field parameters (temperature, pH, DO, SpC, and ORP) stabilized (at least 15 minutes). The field parameters were measured using a multi-sonde (Hydrolab® MS-5, Hach) that was calibrated at the beginning of a sample trip. Prior to collecting unfiltered samples for bacterial analysis, the spigot was flame sterilized. Samples were collected in autoclaved bottles, and latex gloves were worn during sample collection. Filtered samples for

chemistry were then collected in appropriate containers and preserved with acid to pH < 2 as appropriate. An in-line 0.45-μm filter capsule was used. Samples were stored in ice-filled coolers for transport back to the analytical laboratories. Bacteria samples from springs were collected by dipping containers as close to the spring discharge point as possible. A peristaltic pump with an in-line 0.45-micrometer (μm) filter capsule was used to collect filtered samples for chemical analysis.

Bacterial indicators (TC, *E. coli*, enterococci) were determined using the IDEXX method (Neill, 2004) at either the Illinois Department of Agriculture (IDOA) Animal Disease Laboratory in Centralia, IL, or at the Wisconsin State Laboratory of Hygiene (WSLH) in Madison, WI. Samples were either delivered to these laboratories the day they were collected or shipped overnight in insulated shipping containers with ice packs. Inorganic chemistry and dissolved organic carbon were analyzed at the Illinois State Water Survey (ISWS) Public Service Laboratory (PSL) in Champaign, IL. Inorganic parameters included major ions, metals, nutrients (nitrogen and phosphorous species), and alkalinity.

Samples collected from wells in Wisconsin in June 2010 were analyzed for selected pharmaceuticals and personal care products (PPCPs). Unfiltered samples were collected in 1 L silanized amber glass bottles. Extraction and analysis were done at the Illinois Sustainable Technology Center (ISTC) in Champaign, IL.

For molecular-based MST analysis, water samples were processed according to the procedure shown in Figure 9. Water samples were filtered through 0.22 μm filters to concentrate bacterial cells. The concentrated bacterial biomass was used for molecular analysis.

2.3 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from biomass of water samples using a modified protocol described by Schmidt et al. (1991). In brief, filters with biomass were cut into pieces and put into 2 mL centrifuge tubes. Then, 600 microliters (μL) of extraction buffer (0.1 M Tris-HCl, 0.1 M EDTA, 0.75 M Sucrose) was added to 2 grams of the filter/biomass with 0.3 gram glass beads, and homogenized using beadbeater-8 (Biospec Product, OK) for 90 seconds. The mixture was combined with 6 μL of lysozyme (100 mg/mL), briefly vortexed, and incubated at 37°C for 30 min. To fully lyse the cells, 3 μL of 20 mg/mL proteinase and 60 μL of 10% SDS were added to the tube, and incubated at 37 °C for another 2 hours. Cetyl trimethyl ammonium bromide (CTAB) (60 μL of 10 % CTAB with 84 μL of 5M NaCl) was used to remove polysaccharides. The supernatants were purified twice with phenol-chloroform extraction followed by isopropanol precipitation and ethanol wash. After DNA extraction, the purity of gDNA was evaluated by measuring the absorbance at wavelengths of 230, 260, and 280 nm with a UV-spectrophotometer. Ratios of 2.0 – 2.2 for OD260/OD230 and 1.7 – 2.0 for OD260/OD280 suggested that good quality of gDNA was extracted. If otherwise, the DNA extraction process was repeated. The gDNA was also analyzed through gel electrophoresis using 1% agarose gel.

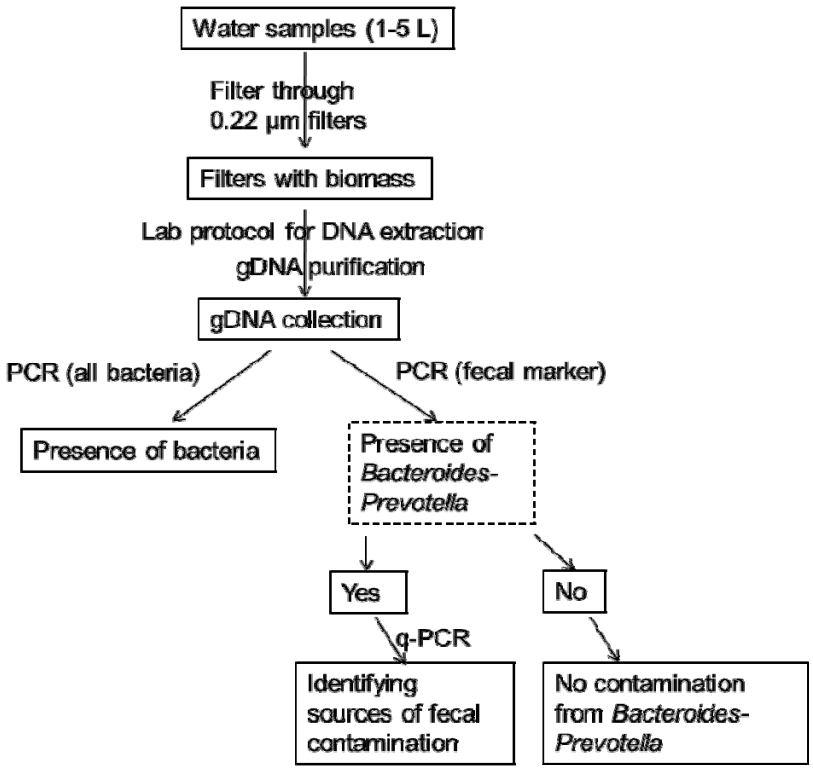

Figure 9. Experimental procedure for molecular-based MST analysis

2.4 PCR and gel electrophoresis analysis

All the gDNA were purified before PCR to ensure successful amplification using a Wizard® Genomic DNA Purification Kit (Promega). Genomic DNA from individual water samples was PCR-amplified separately using a domain *Bacteria*-specific primer set, 11F (5'-GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGY TAC CTT GTT ACG ACT T-3'), and a *Bacteroides-Prevotella*-specific primer set, Bac32F (5'-AAC GCT AGC TAC AGG CTT-3') and 1492R (5'-GGY TAC CTT GTT ACG ACT T-3') (Hong et al., 2009). Each PCR mixture (25 µL in volume) contained 20 to 30 nanograms of gDNA in 1X DNA polymerase buffer, 25 nanomoles of one set of forward and reverse primer, 100 millimoles of deoxyribonucleotide triphosphate (dNTP), and 0.5 U of Ex-Taq DNA polymerase. The reaction mixture was subjected to 30 to 35 cycles of thermal amplification, consisting of denaturation (95^{o}C for 30 seconds), annealing (55^{o}C for 45 seconds), and extension (72^{o}C for 60 seconds) to generate amplicons. PCR products were visualized with gel electrophoresis.

2.5 Q-PCR

Q-PCR was used to identify the sources of fecal pollution. A total of seven primer sets were tested for each sample (Table 3). PCR amplicons from four bacterial reference strains (*B. fragilis* BCRC10619, *B. uniformis* JCM5828, *B. caccae* JCM9498, and *B. vulgatus* BCRC12903), one uncultivated pig-specific *Bacteroidales* clone, and one uncultivated cow-specific *Bacteroidales* clone were used as reference 16S rRNA genes. The PCR condition was 96°C for 5 min., followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 45 seconds, and

after the cycles a further extension at 72°C for 10 min. PCR was performed with a C1000 Thermal Cycler (Bio-Rad). The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The Q-PCR experiments were performed as described previously (Hong et al., 2009). Standard curves were established in duplicates with 400 nM of forward and reverse primers, 1×iQ SybrGreen mastermix (Bio-Rad, CA), and different concentrations of the six reference DNA (0.0001 – 100 pg per 25 µL of reaction mixture). Forty cycles of thermal program (denaturation at 96°C for 10 seconds, annealing at 60°C [57°C for cow-specific primer set] for 30 seconds, extension at 72°C for 30 seconds) were performed with CFX96 Real-Time System (Bio-Rad). A melting curve was carried out following the normal Q-PCR program to check the specificity of annealing. Ct values were plotted against the logarithmic concentration of the DNA template to generate standard curves using the software by CFX96 Real-Time System (Bio-Rad). Quantification of bacterial targets from water samples was performed in duplicate with 10 ng of gDNA. The molecular weight of each reference strains was calculated to be 9.2×10^5 g/mole. The mass per copy of reference strains was estimated to be 1.53×10^{-18} g of DNA.

Table 3. Primers Used in Q-PCR for Determining Sources of Fecal Contamination (Layton et al., 2006; Hong et al., 2009)

Target	Primer	Primer sequence (5'-3')
Bacteroides-Prevotella	Bac32F	AAC GCT AGC TAC AGG CTT
	Bac303R	CCA ATG TGG GGG ACC TT
Pig-specific uncultivated *Bacteroidales*	Bac32F	AAC GCT AGC TAC AGG CTT
	P163R	TCA TAC GGT ATT AAT CCG C
Cow-specific uncultivated *Bacteroidales*	C367F	GGA AGA CTG AAC CAG CCA AGT A
	C467R	GCT TAT TCA TAC GGT ACA TAC AAG
B. fragilis	927F	GGG CCC GCA CAA GCG G
	Bfrg1024R	TCA CAG CGG TGA TTG CTC A
B. uniformis	927F	GGG CCC GCA CAA GCG G
	Bufm1018R	CTG CCT TGC GGC TGA CA
B. caccae	927F	GGG CCC GCA CAA GCG G
	Bcc1066R	CGT ATG GGT TTC CCC ATA A
B. vulgatus	927F	GGG CCC GCA CAA GCG G
	Bvg1016R	ATG CCT TGC GGC TTA CGG C

2.6 Statistical analysis

Cluster analysis (CA) was performed using selected important environmental parameters to evaluate differences among sampling sites. CA was done with Primer 6 (http://www.primer-e.com/primer.htm). Gradient analysis was created with the software Canoco (http://www.pri.wur.nl/uk/products/canoco/). These analyses were used to identify important environmental factors, and facilitate the interpretation of findings from bacterial indicators and fecal markers (Singh et al., 2005; Terrado et al., 2011; Furtula et al., 2012). These parameters included pH, SpC, ORP, B, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Si, Sr, Zn, alkalinity, F^-, Cl^-, NO_3-N, SO_4^{2-}, *o*-PO_4-P, DOC, dissolved Kjeldahl nitrogen (DKN), NH_3-N, and total dissolved solids (TDS).

3. RESULTS

3.1 Bacterial Indicators and Chemistry-Wells

Of the 45 well samples tested for bacterial indicators (one sample was lost), 23 were positive for TC, nine positive for *E. coli*, and eight positive for enterococci (Table 4 and Appendix). Of the five domestic wells in Wisconsin that were sampled four times, each well tested positive for TC on three occasions; three tested positive once and two positive twice for *E. coli*; and three positive once and two positive twice for enterococci. One well (WI-6) tested positive twice for both *E. coli* and enterococci.

For the Wisconsin wells sampled four times, the samples with positive results for *E. coli* and/or enterococci had slightly different water chemistry compared with negative results. Positive samples tended to have higher concentrations of NO_3-N, DOC, potassium (K), ortho-phosphate (*o*-PO_4-P), copper (Cu), and zinc (Zn) and lower SpC (TDS) values (Figure 10). None of the differences were significant based on t-test or rank sum tests (tested at 95% level), but this is probably mainly due to the small sample size (N = 10 and 19, respectively). These differences in chemistry are consistent with contamination due to rapid infiltration events, such as snow melt or heavy or long-term rainfalls. The input of low TDS precipitation water into the aquifer causes dilution, resulting in lower concentrations for most dissolved parameters. However, these rapid infiltration events tend to entrain soil and, in livestock areas, manure, which is reflected in the relatively elevated NO_3-N, DOC, and *o*-PO_4-P concentrations. Complete water quality results are reported in the Appendix.

Table 4. Testing Results for Bacterial Indicators

	All samples	Well samples	Spring samples
Positive for total coliform	45	23	22
Positive for *E. coli*	30	9	21
Positive for enterococci	29	8	21
≥2419 CFU for total coliform	14	1	13
≥2419 CFU for *E. coli*	3	0	3
≥2419 CFU for enterococci	1	1	0
Total	67	45	22

Of special note is the sample collected from WI-6 in February 2011, during a snow melt event. The water was a light brown color and foamy as it discharged from the spigot, indicating serious contamination. The sample had very high levels of DKN, NH_3-N, *o*-PO_4-P, and DOC. The bacterial concentrations were the highest of any samples, with *E. coli* being 31 cfu/milliliters and TC and enterococci greater than their upper detection limits (2,419 cfu/mL). Less than 48 hours after we sampled, another researcher collected a sample at WI-6 and reported that the water was no longer colored or foamy

and TC, *E. coli*, and enterococci were not detected (S. Sibley, personal communication; Pedersen et al., 2011). This illustrates the rapid movement of water through karst aquifers, and that contamination patterns can be similar to slugs of contamination in surface streams.

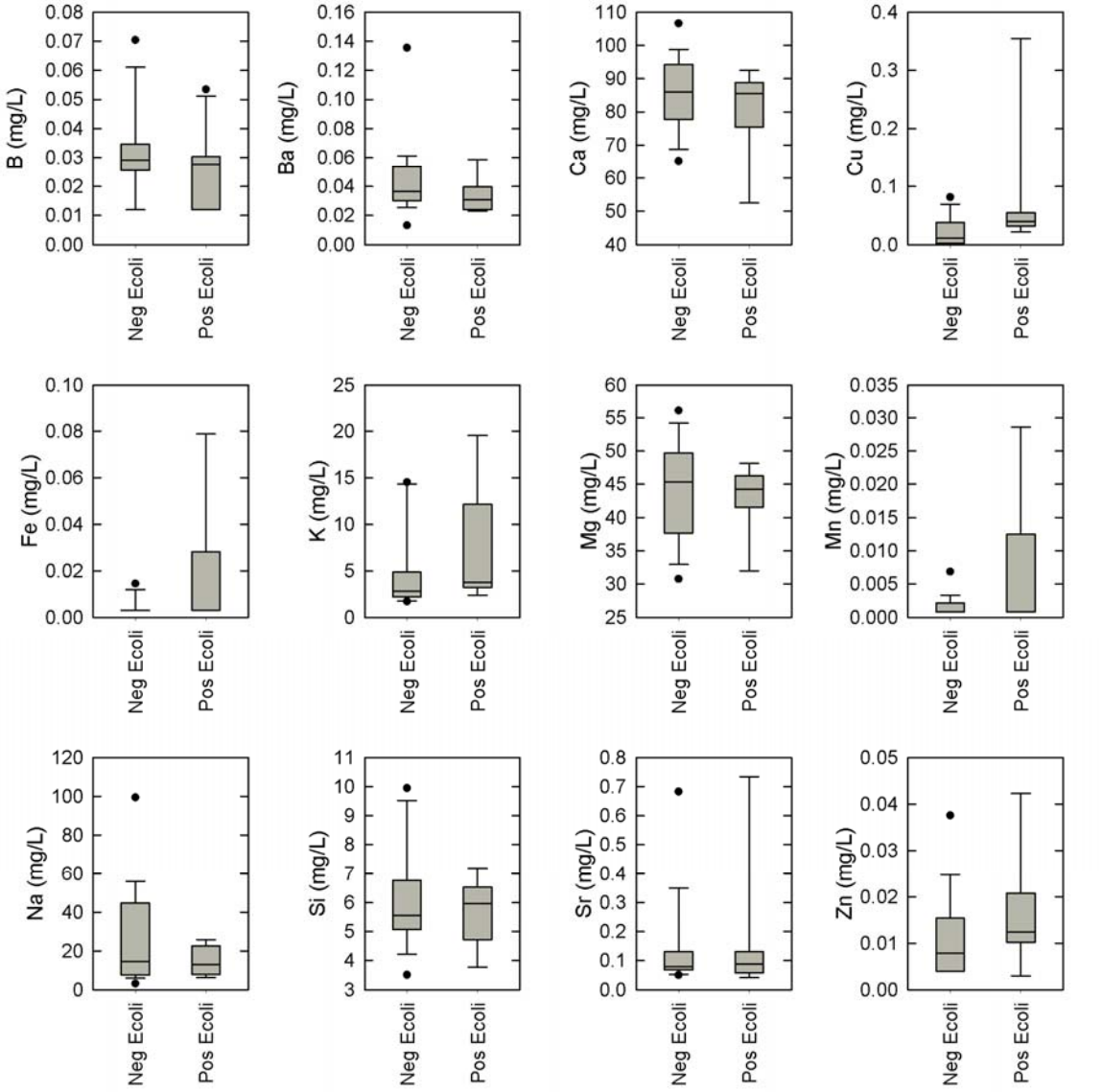

Figure 10. Box and whisker diagrams for well samples from Wisconsin showing concentrations of various parameters as a function of whether *E. coli* was detected. Line in middle of box represents median value, top and bottom of box 75th and 25th percentiles, respectively, top and bottom whiskers 90th and 10th percentile respectively, and black circles are outliers. Values below detection where bottom of box is missing (Fe, Mn, DKN). N = 19 for negative *E. coli*, N = 10 for positive *E. coli*.

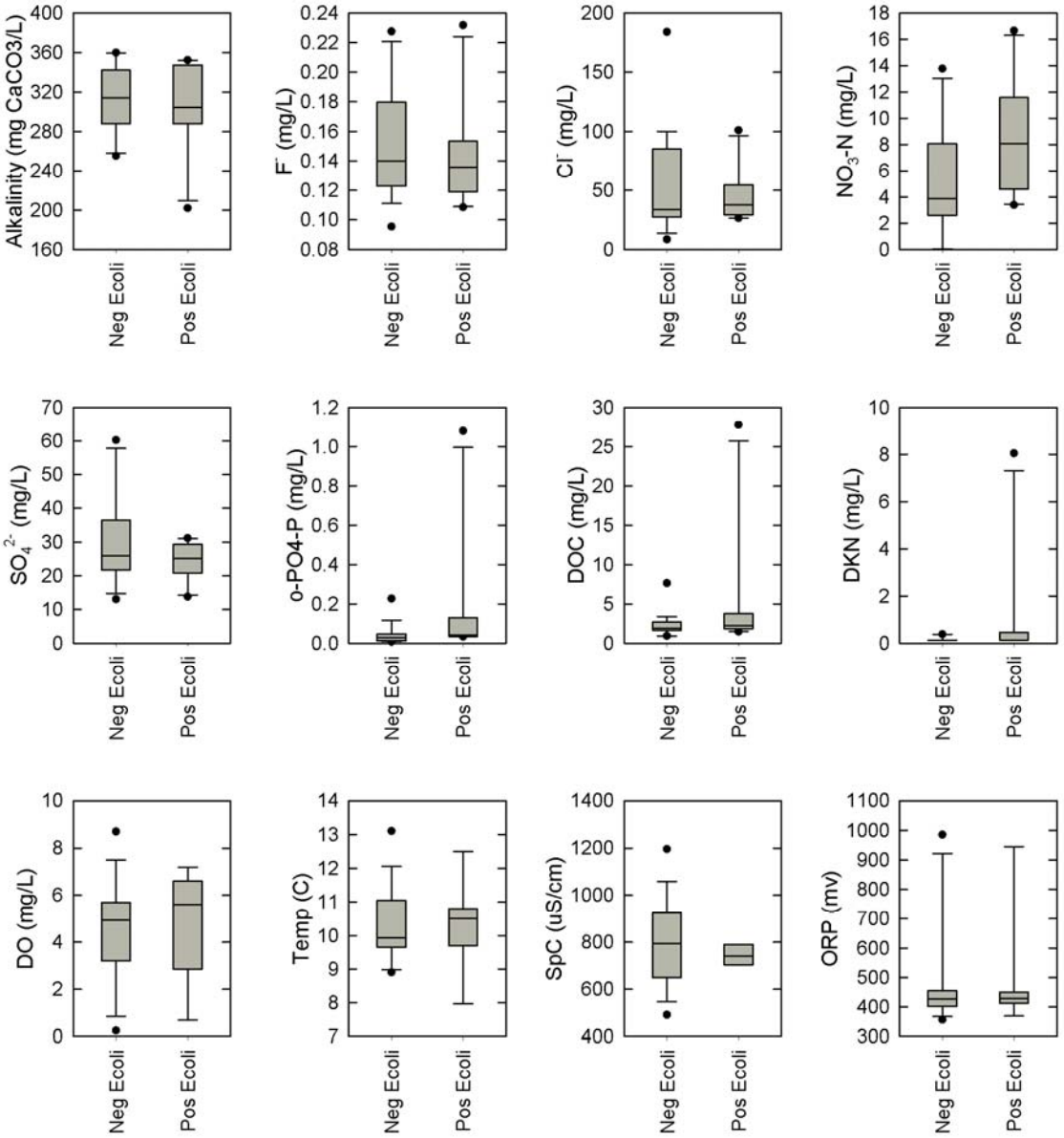

Figure 10. Continued

Of the four domestic wells that were sampled once or twice in Wisconsin, only WI-8 had a positive enterococci result (none had positive *E. coli*). This well had unusual chemistry compared to the other well samples, including very high TDS, calcium (Ca), and sulfate (SO_4^{2-}) concentrations. Other parameters that were somewhat elevated included iron (Fe), magnesium (Mg), strontium (Sr), and fluoride (F^-). Dissolved oxygen was not detected, indicating anoxic conditions, which was unusual compared to the other wells we sampled in Wisconsin. The well was located near an interstate highway, and it was suggested that road salt runoff might have been entering the well's capture zone. However, Cl^- and Na concentrations, while relatively elevated, were not exceptionally

high. The very high levels of Ca (530 mg/L) and SO_4^{2-} (1392 mg/L) suggest gypsum dissolution, although we are not aware if there are natural gypsum deposits in these formations. There is a possibility that contamination was caused by improper disposal of drywall (gypsum board), but that was beyond the scope of this project.

Three of the public-supply wells in Sturgeon Bay, WI, were sampled in April 2010 and had no bacterial detections. Subsequently, two of these wells (once for #6 and four times for #10) had positive results for TC, and samples were shipped overnight to us for chemical analysis. There was almost no difference in chemistry among the samples for either of these wells.

Of the 11 Illinois well samples, four were referred to us as being positive for *E. coli*. Unfortunately, two of the wells (IL-9 and IL-11) were chlorinated without our knowledge before we were able to collect samples.

The well at IL-10, located in an urban area (Elmhurst), was obviously impacted by road salt runoff (Cl^- = 154 mg/L, Na = 97.0 mg/L). Two wells (IL-1, IL-2) were located on a hog farm. These wells had very high NO_3-N (33.3 and 17.3 mg/L) and Cl^- (56.7 and 52.5 mg/L) concentrations, suggesting contamination from hog manure, but surprisingly had no detectable *E. coli* or enterococci. IL-1 did have a TC concentration of 214 cfu/100 milliliters. Three other wells had NO_3-N concentrations greater than probable background (2 – 3 mg/L): IL-5, IL-7, and IL-11.

Typical background concentrations of NO_3-N in groundwater have been reported to be between 2.0 and 3.0 mg/L (Madison and Brunett, 1985; Panno et al., 2006b), with concentrations greater than background indicating contamination. Five wells in Illinois had NO_3-N concentrations > 3.0 mg/L, the two wells at the hog farm (IL-1 and IL-2), IL-5, IL-7, and IL-11. Seven of the 11 private wells in Wisconsin and two of the three Sturgeon Bay wells had at least one sample greater than 3.0 mg/L, with a total of 26 samples exceeding that value. Of the five wells sampled four times, four had a range of more than 3 mg/L in the NO_3-N concentration, although the season in which the highest concentrations were measured was not consistent (Figure 11).

3.2 Bacterial Indicators and Chemistry-Springs

Of the 22 springs sampled, all but one (Jesse's Spring, KY) tested positive for the three bacterial indicators (Table 4 and Appendix). Two springs (Mockingbird Spring, KY; Mystery Entrance, MO) had *E. coli* concentrations greater than the upper detection limit (2,419 cfu/100 mL), and three others had concentrations > 1,000 cfu/100 mL (Huber Branch, MO; Blue Spring, MO; CSO 206 spring, KY). The highest levels of enterococci (> 100 cfu/100 mL) were measured in two Missouri springs (Thunder Hole and Ball Mill). Almost all of the Missouri springs had total coliform levels at or greater than the upper detection limit (2,419 cfu/100 mL), and three of the five urban Kentucky springs had TC above the upper detection limit.

Besides the differences in land uses between the three spring regions sampled (KY urban, KY rural, MO rural), there are differences in geology, soil cover, and climate, which limits the ability to compare the water quality among these different regions. Nevertheless, a few pertinent observations can be made. The water quality of springs in urban Kentucky (Louisville) had significantly higher TDS values, mainly the result of elevated Cl^-, Na, and sulfate (SO_4^{2-}) concentrations, than the rural springs. The Cl^- and Na likely come from road salt runoff (Louisville averages about 15 inches of snow annually).

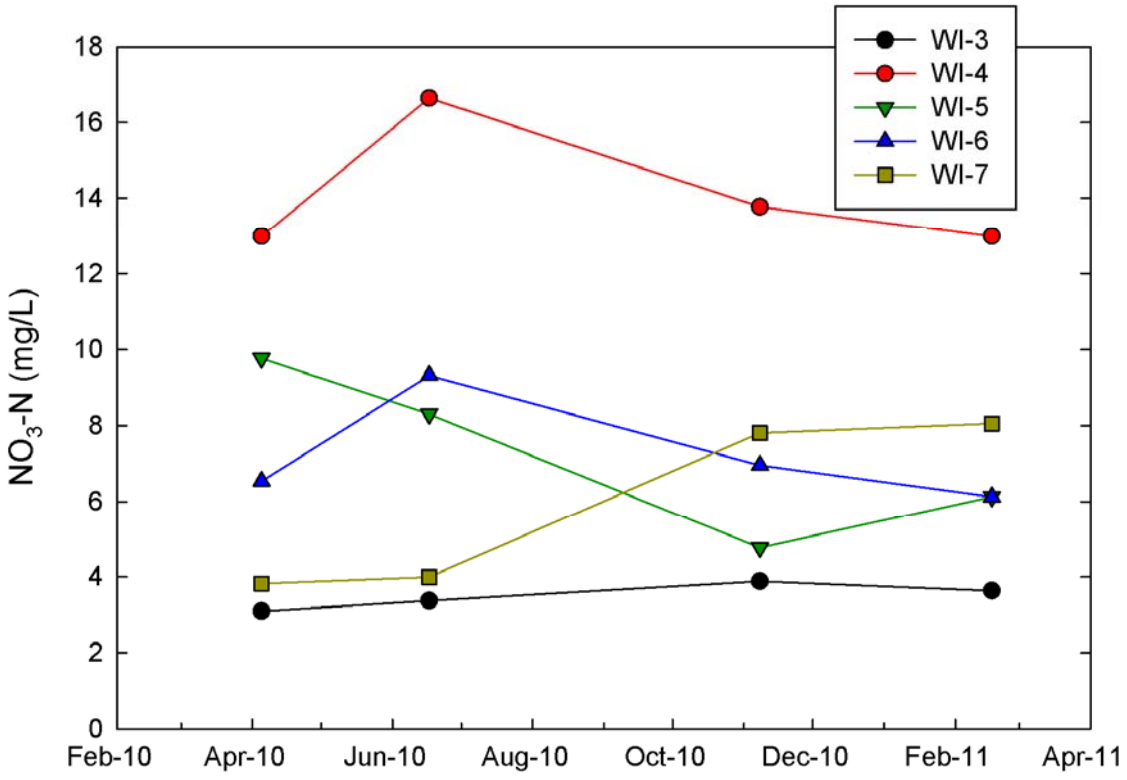

Figure 11. NO_3-N concentrations in Wisconsin wells that were sampled four times

Sulfate is often elevated in developed areas, where soil layers and sediments are disrupted, potentially exposing sulfide minerals (e.g., pyrite) to atmospheric oxygen, leading to the oxidation of the sulfide phases and producing SO_4^{2-}. Three of the Kentucky urban springs also had relatively elevated levels of boron (B).

Nitrate-N concentrations were above background (2–3 mg/L) in about half of the springs, in both rural and urban settings. The Missouri springs tended to have higher levels of reduced forms of N (DKN and NH_3-N) and DOC. Two of the Missouri springs, Mystery Entrance and Mystery Resurgence, had water quality problems. These included elevated levels of DKN, NH_3-N, *o*-PO_4-P, DOC, K, Fe, and manganese (Mn). A sewage/manure smell was noted at Mystery Entrance, and the elevated levels of reduced N species and *o*-PO_4-P suggest fecal contamination. These two springs are in the same springshed, the entrance and exit of a cave, thus their similar water quality is not surprising.

3.3. PPCPs

The 16 PPCPs analyzed by ISTC were below their detection limits in the Wisconsin well samples collected in June 2010. The ISTC lab technician estimated concentrations for several of the compounds. These are reported in Table 5.

Table 5. PPCP results for samples 22 – 26 collected from Wisconsin wells collected June 17, 2010. Results in ng/L. Estimated concentrations are reported for several samples (in parentheses).

	22	23	24	25	26
Compound					
Naproxen	< 5.2	< 5.2	< 5.2	< 5.2	< 5.2
Ibuprofen	< 10	< 10	< 10	< 10	< 10
Gemfibrozil	< 10	< 10	< 10	< 10	< 10
Triclosan	< 44	< 44	< 44	< 44	< 44
Trichlorocarb	< 5.3	< 5.3	< 5.3	< 5.3	< 5.3
Acetaminophen	< 33 (26)	< 33 (27)	< 33 (30)	(39)	< 33 (20)
Caffeine	< 23	< 23	< 23	< 23	< 23
Trimethoprim	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0
Sulfamethazine	< 19	< 19	< 19	< 19	< 19
Ciprofloxacin	< 450	< 450	< 450	< 450	< 450
Sulfamethoxazole	< 10 (0.9)	< 10 (1)	< 10 (0.7)	< 10 (1)	< 10
Diphenhydramine	< 2.8	< 2.8	< 2.8	< 2.8	< 2.8
Erythromycin	< 65 (15)	< 65 (8)	< 65 (4)	< 65 (6)	< 65
Carbamazepine	< 7.6 (0.07)	< 7.6 (0.05)	< 7.6 (0.04)	< 7.6	< 7.6
Norfluoxetine	< 10	< 10	< 10	< 10	< 10
Fluoxetine	< 10	< 10	< 10	< 10	< 10

3.4 Statistical Evaluation of Environmental parameters

All samples were clustered based on selected environmental parameters (Figure 12). Most samples formed one single cluster (in the center of Figure 12A), indicating a high similarity among these samples. Four samples from Illinois were grouped together as a result of elevated boron concentrations, which typically originate from detergents containing sodium perborate (Widory et al., 2005; Katz and Griffin, 2008). Two Wisconsin samples (cluster "3") had high level of the metals Cu, Fe, and Mn. Two Missouri samples (cluster "2") had elevated levels of nutrients (*o*-PO_4-P, DOC, DKN, and NH_3-N). Samples S19, S22, S34, and S47 each formed distinct clusters. S19 (IL-8) had elevated concentrations of SpC, Ba, Sn, SO_4^{2-} and TDS. S34 (WI-8) had elevated concentrations of B, Ca, Fe, Mg, Mn, Ni, alkalinity, SO_4^{2-} and TDS. S22 (WI-3: June 2010) had elevated concentrations of Na and Cl^-. S47 (runoff at WI-11) had high concentrations of B, Cu, Fe, K, Mn, *o*-PO_4-P, DKN, and NH_3-N. The center cluster "1" in Figure 12A could be further divided into five subgroups and four singletons (Figure 12B). The grouping pattern of those samples mostly coincided with the classification of geological regions, except for three samples at the intersection of the Illinois wells and Louisville springs (KY_ Louisville).

3.5 PCR screening

To determine the source of fecal contamination, two PCR screening steps were used (Figure 13). First, all samples were tested for a primer set targeting all bacteria. Among the 73 samples, 62 tested positive for the presence of bacterial cells. From those

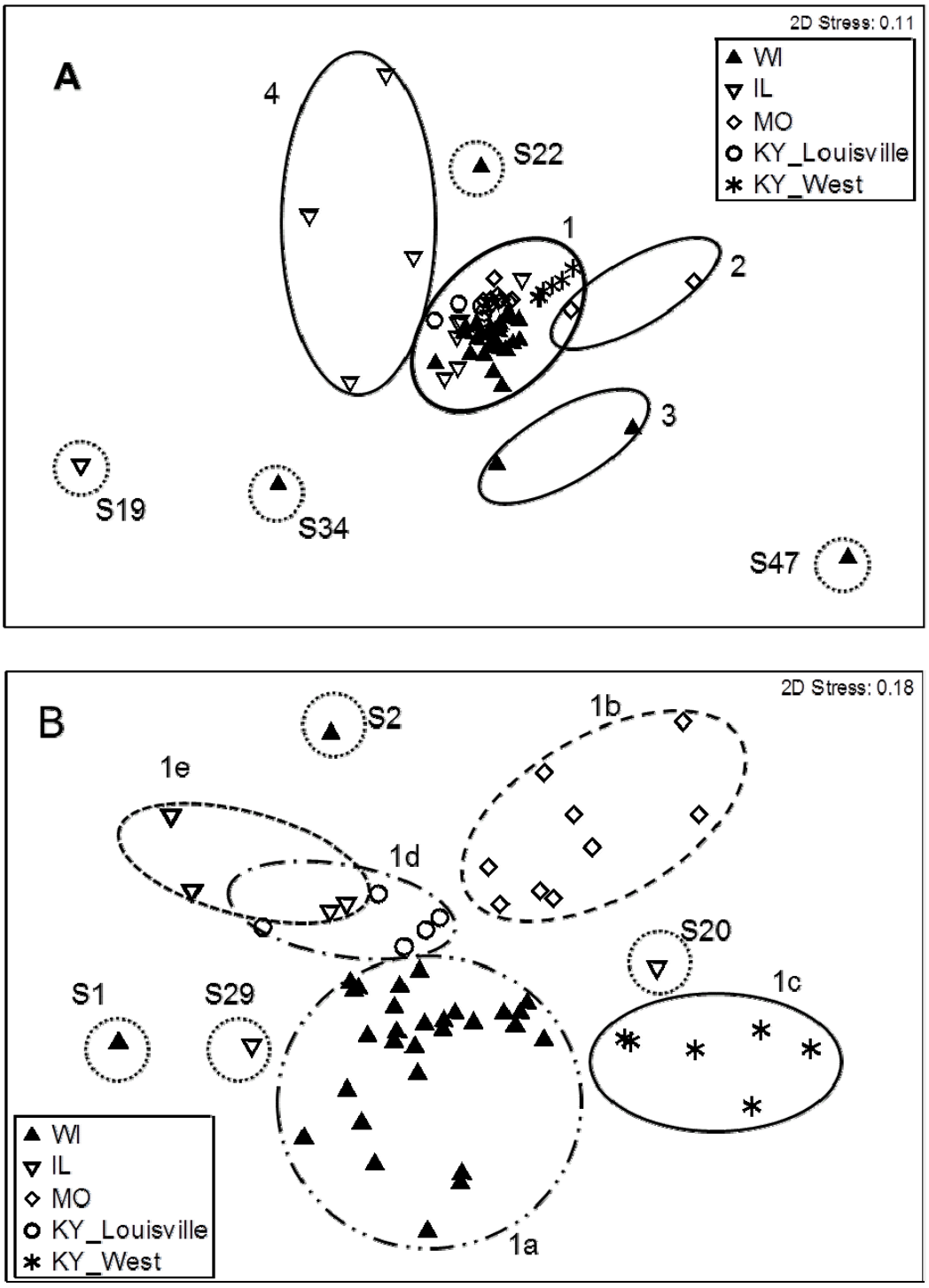

Figure 12. Multidimensional scaling (MDS) analysis plot based on D1 Euclidean distance with log (X+1) transformation of environmental parameters. Environmental parameters used in the analysis included pH, SpC, ORP, B, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Si, Sr, Zn, alkalinity, F^-, Cl^-, NO_3-N, SO_4^{2-}, *o*-PO_4-P, DOC, DKN, NH_3-N, and TDS. All the samples except five negative controls were included in Panel A. The centered cluster "1" (60 samples) was further clustered into subgroups in Panel B.

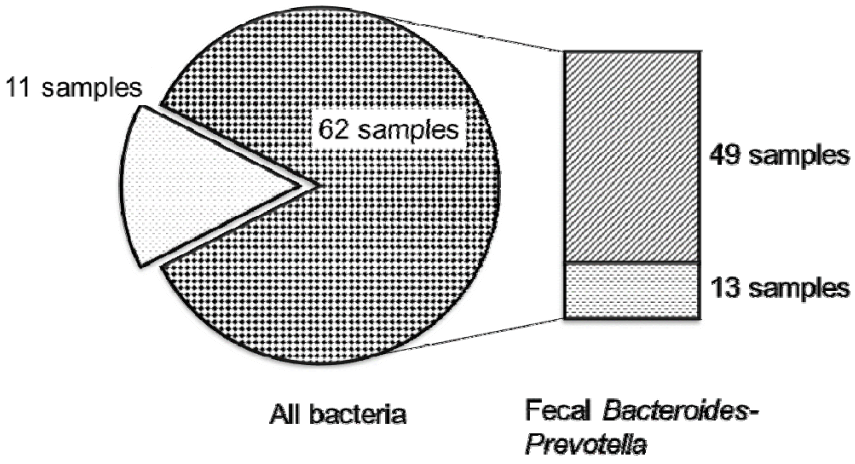

Figure 13. Water sample screening process. All the samples were first tested against primer sets targeting all bacteria. Samples that were positive were further analyzed using primer sets targeting fecal *Bacteroides-Prevotella*.

bacteria-positive samples, 49 tested positive with the primer set specific for fecal *Bacteroides-Prevotella*, and were furthered analyzed using Q-PCR-based MST.

3.6 Standard curves for Q-PCR

Standard curves were determined using the six reference 16S rRNA genes obtained (Table 6). The correlation coefficient (R^2) was always greater than 0.99. The amplification efficiency (E) was 52% for the primer set targeting *B. uniformis* and between 60 and 75% for the other six primer sets. These efficiencies were not as high as in other studies, where efficiencies between 78 and 102% were reported. The target DNA concentration was between 653 and 6.53×10^6 copies, which is comparable to other studies, which reported values between 10 and 2.5×10^6 copies (Layton et al., 2006; Okabe et al., 2007; Shanks et al., 2008; 2010). The standard curve was repeated each time water samples were tested, thus statistics for those curves varied slightly.

3.7 Using Q-PCR technique to identify sources of fecal contamination

Seven Q-PCR were conducted with one primer set targeting the fecal *Bacteroides-Prevotella* group and the remaining six primer sets targeting different host-specific contamination (four for human, and one each for swine and bovine). Among the 49 samples that tested positive for Q-PCR targeting fecal *Bacteroides-Prevotella*, six samples had concentrations below the detection limit and were excluded from testing with the remaining primer sets. Primer sets targeting *B. fragilis*, *B. caccae*, *B. uniformis*, and *B. vulgatus* were used to indicate human fecal contamination, and primer sets targeting certain *Prevotella* groups were used to indicate fecal contamination from animals (swine and bovine). ANOVA analysis ($p < 0.05$) confirmed the observation that tests with *B. fragilis*, *B. vulgatus,* and the bovine primer sets gave significantly higher concentrations (median > 10^2 copies/ng-gDNA) than all the other three primer sets (Figure 14). One sample that was negative for a fecal marker in the PCR screening

process was randomly chosen to check with primer sets targeting *Bacteroides-Prevotella* by Q-PCR. No target could be detected or the target was below the detection limit.

Table 6. Q-PCR Standard Curve Equations and Performance Characteristics

Assay	Standard curve	Amplification efficiency (E)	Range of quantification (ROQ) (copies) for target DNA	R^2	%CV across ROQ
Bacteroides-Prevotella	y=8.28-4.33x	70.25%	6-6.53×10^6	0.998	2.27
B. fragilis	y=6.84-4.83x	61.05%	65-6.53×10^6	0.996	4.19
B. caccae	y=6.39-4.30x	70.88%	6-6.53×10^6	0.998	2.48
B. uniformis	y=10.17-5.45x	52.56%	653-6.53×10^6	0.994	0.87
B. vulgatus	y=7.33-4.13x	74.70%	6-6.53×10^6	0.998	2.60
Swine	y=8.19-3.96x	78.96%	65-6.53×10^6	0.999	1.61
Bovine	y=9.77-4.47x	67.46%	65-6.53×10^6	0.999	2.55

"Amplification efficiency" is equal to ($10^{-1/slope}$) - 1.
"Range of quantification for target DNA" is the range of quantifications of reference DNA for each standard curve.
"%CV across ROQ" is the mean percent coefficient of variations measured for quantifications of standards.

The levels of fecal biomarkers between spring and well samples were separated and compared (Figure 15). In general, spring samples contained higher levels of biomarkers than well samples. Concentrations of biomarkers for *B. vulgatus*, swine, and bovine were significantly different ($p < 0.05$) between the two water types. Average concentrations of fecal markers within each sampling area are shown in Table 7. The biomarkers were highest in samples collected from the Louisville springs, and were likely associated with the urban spring systems. Spring samples tended to have higher levels of bovine markers than well samples, but well samples had higher concentrations of biomarkers for human pollution.

Based on Q-PCR results, we could discriminate sources of fecal pollution for each water sample (Table 8). Human fecal pollution was determined based on the presence of at least two or three detectable primer sets. When two primer sets were used, almost all spring samples were contaminated with human and bovine feces. Contamination by swine feces occurred to a lesser extent, but was still observed for more than half of the spring samples. Only two of the 11 well samples from Illinois contained more than two detectable biomarkers for human pollution, compared with 18 of 25 samples (72%) from Wisconsin. When three positive biomarkers were used, samples from Wisconsin, rural Kentucky, and Missouri were affected the most, suggesting that the level of contamination for a few samples were near or below the detection limit. Although the presence of all four human biomarkers would be ideal, it could not be applied in this study because one of the human primer sets did not work well.

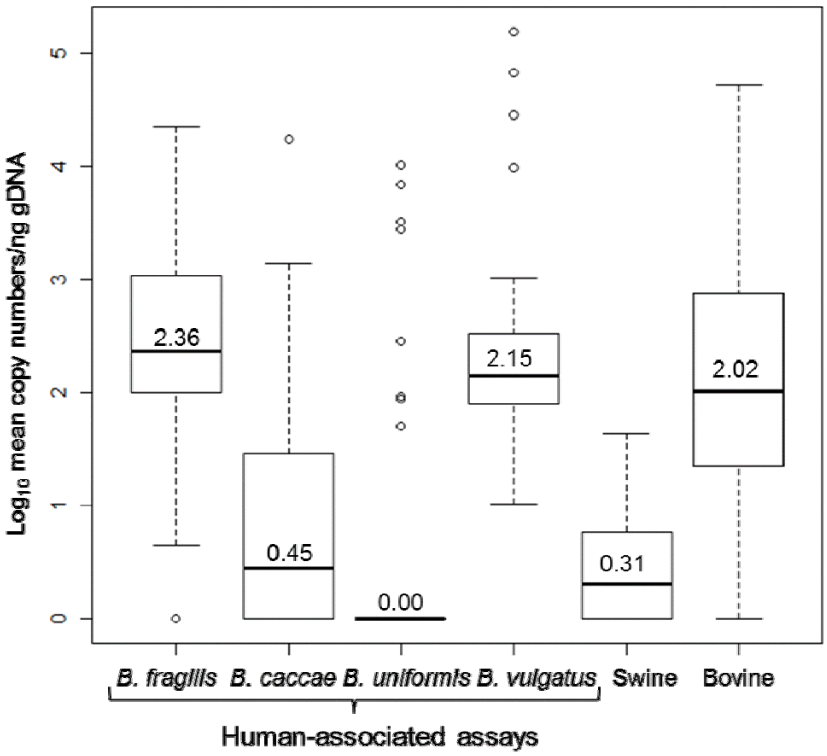

Figure 14. Q-PCR results for determining fecal contamination. Estimated gene copy numbers are shown in the unit, copies/(ng-gDNA) (log10 scale). The middle line within the box represents the median value and the boundaries of the box indicate the 25th and 75th percentile values. Whiskers above and below the box are the 10th and 90th percentile values. A "◦" marks outliner measurements. The total number of samples included is 43.

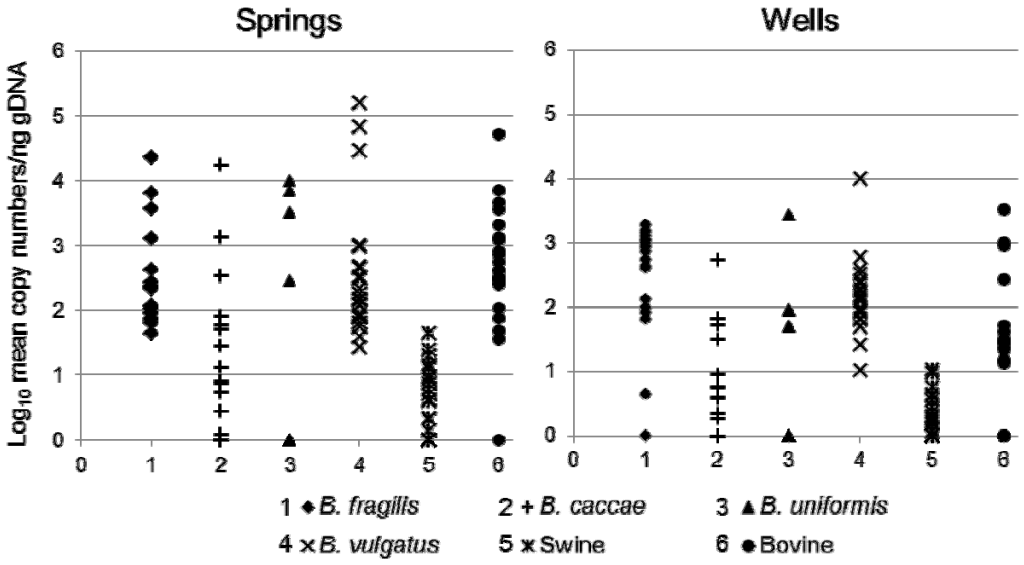

Figure 15. Concentrations of fecal biomarkers from springs (22 samples) and wells (21 samples)

Table 7. Average Concentration of Each Biomarker in the Unit of Copies/ng gDNA Among Water Samples (combining results from PCR screening process and Q-PCR process)

Sample Location	Water Source	Number of Samples	Human				Animal	
			B. fragilis	*B. caccae*	*B. uniformis*	*B. vulgatus*	Swine	Bovine
Illinois	Wells	10	87.0	54.0	284.1	989.8	0.4	332.0
Wisconsin	Wells	25	594.5	9.9	7.3	128.0	2.0	99.3
KY_Louisville	Springs	5	6821.8	3760.3	4125.2	50672.1	17.2	12288.6
KY_West	Springs	6	107.7	14.6	0.0	142.7	6.1	701.0
Missouri	Springs	11	143.9	40.3	0.0	258.8	3.8	981.7

Five negative controls and 11 samples that did not have enough gDNA are not included.

Table 8. Number of Samples that were Contaminated by Human and Animal Wastes

Sample Location	Water Source	Number of Samples	Human		Animal	
			+ in ≥ 2 primer sets	+ in ≥ 3 primer sets	Swine	Bovine
Illinois	Wells	10	2	2	2	1
Wisconsin	Wells	25	18	13	13	14
KY_Louisville	Springs	5	5	4	4	5
KY_West	Springs	6	6	2	4	5
Missouri	Springs	11	11	7	6	11

Five negative controls and 11 samples that did not have enough gDNA are not included.

Contamination by both human and animal fecal pollution was the dominant type of pollution among all samples (25 out of 57) (Table 9). Only three samples showed pollution solely by humans, whereas 15 samples were contaminated solely by animals. Forty-three samples were contaminated by at least one source, with all the spring samples contaminated by human or animal sources. Six well samples from Wisconsin were contaminated only by animals. Table 9 indicates a correlation between the presence of contamination and TC. However, when data from each sample were examined individually, there was a discrepancy between the two methods (Figure 16). Five samples could not be detected with *Bacteroidales*-MST, but showed positive for traditional indicators. Another nine samples contained detectable *Bacteroidales*-MST biomarkers, but were negative for traditional indicators.

3.8 Comparison between land-use data and MST

Using land-use data provided by various state agencies and observations in the field, we made a preliminary estimate of the potential sources of fecal contamination for each sample (Table 10). The urban springs in Louisville were assumed to have only human (sewage) sources. Sites IL-1 and IL-2 (located on the same property) were the

Table 9. Number of Samples Contaminated within Each Sampling Area Compared to Number of Samples that were Positive for Traditional Indicators

Sample Location	Number of Samples	Human only	Animal only	Both Human and Animal	Contaminated by at least one source	Traditional indicators		
						Total coliform	*E. coli*	Enterococci
Illinois	10	0	0	2	2	2	0	0
Wisconsin	25	2	6	11	19	17	8	8
KY_Louisville	5	0	1	4	5	4	4	4
KY_West	6	1	4	1	6	6	6	6
Missouri	11	0	4	7	11	11	11	11

Five negative controls and 11 samples that did not have enough gDNA are not included.

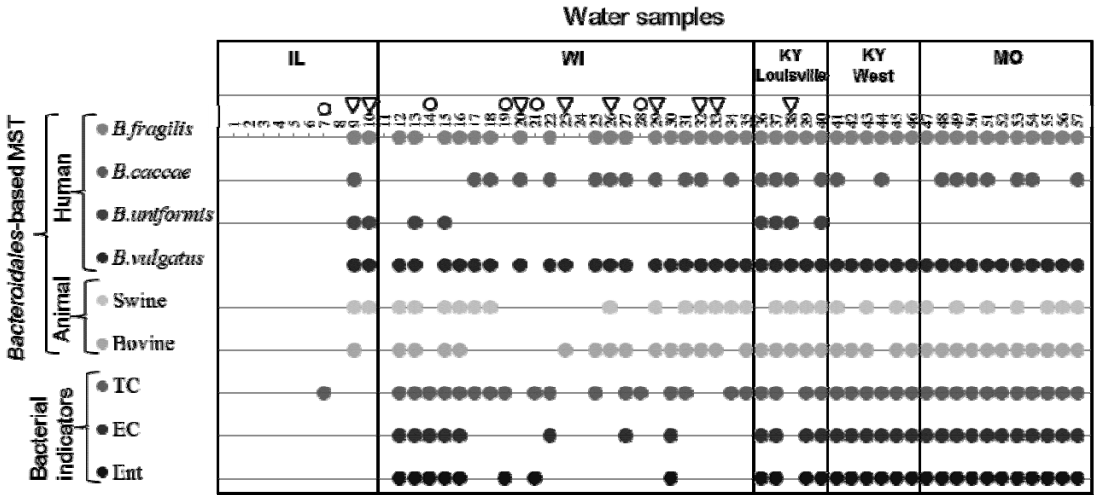

Figure 16. Comparison of contamination identified by *Bacteroidales*-based MST and traditional bacterial indicators. Open circles indicate samples that were positive for traditional bacterial indicators but could not be detected with *Bacteroidales*-MST. Inverted triangles indicate samples that contained detectable *Bacteroidales*-MST biomarkers, but were negative for traditional indicators.

only ones where swine were observed. At site WI-11 (well) and WI-11X (runoff onto the WI-11 property), liquid cow manure had been applied to a field up-gradient and was observed to be contaminating the property during a snowmelt. For most of the sites, however, no definitive sources were observed (although cow manure was strongly suspected at WI-5, 6, and 7). These were mainly rural wells and springs, and had the possibility of both human and livestock (primarily bovine) contamination.

Because we used four bacterial strains for determining human contamination in our MST method, we used two criteria to evaluate whether human contamination was present. The base case was if at least two strains had a concentration > 10^2 copies/ng gDNA, and the more rigorous criterion if at least three strains had concentrations > 10^2

copies/ng gDNA. In general, there was agreement between land use data and our MST results with respect to potential sources (Table 10). However, there were some discrepancies that are difficult to explain. The oddest discrepancies are for the Louisville springs (sites KY-1 – KY-5). We expected only human sources of contamination, but bovine sources were detected for all five springs and swine sources for three of the springs. With no obvious livestock sources in these springsheds, we are unable to explain these results.

3.9 Comparison between MST and Adenovirus and Polyomavirus Study

Pedersen et al. (2011) sampled several of the wells we sampled in Wisconsin as part of a concurrent (but unrelated) study investigating the use of human and bovine adenoviruses (AdV) and polyomaviruses (PyV) as fecal contamination indicators. They sampled WI-3, WI-5, WI-6, and WI-7 three or four times each. Two of their sampling events occurred the day after our last two sampling events (8 November, 2010, and 17 February, 2011).

Pedersen et al. (2011) were able to detect human and bovine AdV and PyV in some of their samples. However, only for site WI-5 were they able to definitively determine the source, which they concluded was animal. They suspected the source to be bovine, but could not rule out wildlife. For the other sites, they reported the putative sources to be "unknown" (WI-3 and WI-7) or "ambiguous" (WI-4).

3.10 Chloride and nitrogen concentrations and bacterial contamination

Infiltration of fecal materials into groundwater is often associated with elevated levels of N and Cl^-. Panno et al. (2006a; 2006b) used N and Cl^- concentrations to identify possible sources of contamination (e.g., synthetic fertilizer, road salt, human/animal waste) in various regions. In a study of the karst region in southwest Illinois, Panno et al. (2006b) estimated a threshold concentration of total N (TN) of approximately 2.5 mg/L; concentrations above this threshold indicated anthropogenic contamination. Panno et al. (2006a) likewise determined a threshold concentration for Cl^- of 15 mg/L for shallow groundwater in the northern two-thirds of Illinois. Panno et al. (2006a) concluded that samples with elevated TN concentrations but low Cl^- levels were generally associated with contamination by synthetic fertilizers, while elevated Cl^- levels with low TN levels usually indicated road salt runoff. Concentrations of TN greater than 15 mg/L indicated significant contamination by human/animal waste sources.

Most of the well sample data plot in the "Mixed Sources" domain (Figure 17A). The sample from site IL-10 (sample S27) in the Chicago region is obviously affected by road salt, and several others with elevated levels of both Cl^- and TN (e.g., WI-1) are also likely influenced by road salt runoff. Most of these well samples are from Wisconsin, which receives more snow and thus road salt than in the areas we sampled in Illinois (with the exception of IL-10). The sample from site IL-4 (sample S14) is somewhat puzzling, as it is located in a rural part of southwestern Illinois, an area with relatively little snowfall or road salt application. The elevated Cl^- is most likely from water softening salts passing through the septic system.

Several sites had TN levels suggesting significant animal or human waste sources, including IL-1, IL-2, WI-4 (sample S23), and WI-11X. Sites IL-1 and IL-2 are located at

Table 10. Suspected source of contamination of samples based on land use information. Bolded sources indicate strong suspicion. Where *Human* is italicized, indicates human source not detected using more rigorous criteria (see text).

State/location	Site No.	Sample No.	Suspected source(s)	MST source(s)
Illinois	IL-1	S-11	**Swine**	*
Illinois	IL-2	S-12	**Swine**	ND
Illinois	IL-3	S-13	Human, Bovine	ND
Illinois	IL-4	S-14	Human, Bovine	ND
Illinois	IL-5	S-15	Human, Bovine	ND
Illinois	IL-6	S-16	Human, Bovine	ND
Illinois	IL-7	S-18	Human	ND
Illinois	IL-8	S-19	Human	ND
Illinois	IL-9	S-20	Human	ND
Illinois	IL-10	S-27	Human	Bovine, Human, Swine
Illinois	IL-11	S-29	Human	Human
KY_Louisville	KY-1	S-48	**Human**	Human, Bovine
KY_Louisville	KY-2	S-49	**Human**	Human, Bovine, Swine
KY_Louisville	KY-3	S-50	**Human**	Bovine, Human, Swine
KY_Louisville	KY-4	S-51	**Human**	Human, Bovine
KY_Louisville	KY-5	S-52	**Human**	Human, Bovine, Swine
KY_West	KY-6	S-53	Bovine, Human	Bovine, *Human*, Swine
KY_West	KY-7	S-54	Bovine, Human	Bovine, Human
KY_West	KY-8	S-55	Bovine, Human	Bovine, Human
KY_West	KY-9	S-56	Bovine, Human	*Human*
KY_West	KY-10	S-57	Bovine, Human	*Human*, Bovine
KY_West	KY-11	S-58	Bovine, Human	Bovine, *Human*
Missouri	MO-1	S-63	Bovine, Human	Human, Bovine, Swine
Missouri	MO-2	S-64	Bovine, Human	*Human*, Bovine
Missouri	MO-3	S-65	Bovine, Human	*Human*
Missouri	MO-4	S-66	Bovine, Human	Bovine, Human
Missouri	MO-5	S-67	Bovine, Human	Bovine, Human
Missouri	MO-6	S-68	Bovine, Human	Human
Missouri	MO-7	S-69	Bovine, Human	Human, Bovine
Missouri	MO-8	S-70	Bovine, Human	Human, Bovine
Missouri	MO-9	S-71	Bovine, Human	Human, Bovine
Missouri	MO-10	S-72	Bovine, Human	Human
Missouri	MO-11	S-73	Bovine, Human	Human, Bovine
Wisconsin	WI-2	S-2,21	Bovine, Human	ND
Wisconsin	WI-3	S-22,30,40	Human, Bovine	Human, Bovine
Wisconsin	WI-4	S-23,31,41	Bovine, Human	Human, Swine
Wisconsin	WI-5	S-24,32,43	Bovine, Human	Human
Wisconsin	WI-6	S-25,33,44	Bovine, Human	*Human*, Bovine
Wisconsin	WI-7	S-26,35,45	Bovine, Human	Human
Wisconsin	WI-8	S-34	Bovine, Human	ND
Wisconsin	WI-9	S-37	Bovine, Human	ND
Wisconsin	WI-10	S-39	Bovine, Human	*Human*
Wisconsin	WI-11	S-47	**Bovine**	*Human*, Bovine
Wisconsin	WI-11X	S-46	**Bovine**	Human, Bovine
Wisconsin	Sturgeon Bay	S-38,42,61,62,75	Human, Bovine	*Human*, Bovine

* not enough gDNA for analysis ND: no source detected

a hog farm, WI-4 was located close to an area where cow manure was applied to agricultural fields, and WI-11X was a surface runoff sample collected down-gradient of a field on which cow manure had been applied. It's likely that other sites where elevated TN concentrations were measured also had human and/or animal waste inputs.

The spring data were quite distinct from the well data, reflecting both the geographic differences (Kentucky and Missouri vs. Wisconsin and Illinois) and the hydrogeological nature of springs, with significantly larger capture zones than wells (Figure 17B). Except in the Louisville springs, Cl^- concentrations were generally lower than in wells, and TN concentrations covered a narrower range of values (1.26–8.71 mg/L vs. below detection – 33.4 mg/L). About 24% of the well samples had TN concentrations > 10 mg/L.

The main difference in water quality for the springs was between urban vs. rural land use. The Louisville springs had much greater Cl^- concentrations compared to the rural springs, suggesting a road salt source in Louisville. The rural samples in both Kentucky and Missouri tended to fall along a line with a positive slope. This would suggest that springs with relatively elevated Cl^- and TN concentrations would be more likely to have animal/human waste sources. There was, however, no positive relationship between TN (or Cl^-) and *E. coli* concentrations (Figure 18). This lack of correlation was not unexpected, being commonly observed in other studies.

Chloride and TN results showed only partial correlation with the *Bacteroidales*-MST method (Figure 19). Our MST results were negative (i.e., no contamination source detected) for almost all well samples that had background TN concentrations. Four of the samples that had TN > 2.5 mg/L but negative MST results were collected in the first round of sampling when we did not collect a sufficient sample for analysis. (IL-1, IL-2, IL-5, IL-7). Except for the Louisville springs, most of the spring samples had Cl^- concentrations < 15 mg/L. It is likely that the threshold Cl^- concentrations in these basins is much lower than determined by Panno et al. (2006a) in Illinois. The rural Kentucky springs generally had very large discharges, suggesting the potential for considerable dilution. Even so, it was not surprising to find that all the springs sampled in this study were microbially contaminated, using either traditional or advanced MST methods. Even when a springshed is mainly uncontaminated, a small amount of fecal contamination may be detected at the spring outlet.

In the late 1990s, springs and wells were sampled in the Sinkhole Plain region of southwestern Illinois (Panno et al., 2001; Hackley et al., 2007). The water quality data were similar to the samples collected in this study. *E. coli* were detected in fewer wells than in our study, and Cl^- levels were lower than the Wisconsin well samples, which were likely more heavily impacted by road salt runoff (Figure 20A). The water quality of the springs sampled in Illinois was very similar to the Missouri and rural Kentucky springs sampled in this study (Figure 20B). This suggests similar hydrological processes and land use activities in these separate karst basins.

3.11 Correlation between environmental variables and fecal contamination

Ordination analysis was performed to find putative correlations between environmental variables and fecal contamination. Three environmental parameters, DO, Cl^-, and *E. coli*, were correlated with levels of *Bacteroidales* fecal biomarkers. In Figure 21, the two axes explain 30.9% of all variation among samples. The DO of almost all of

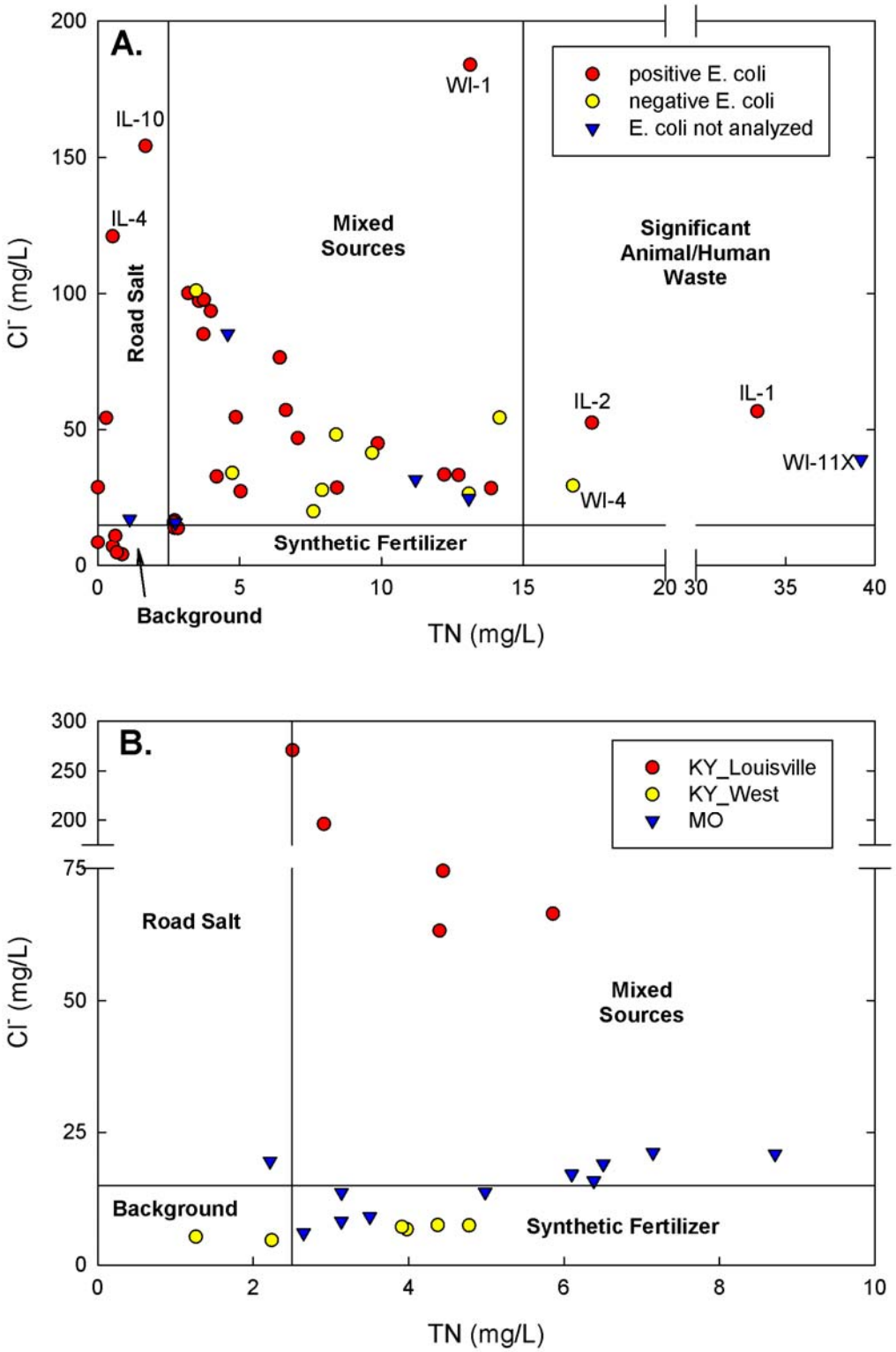

Figure 17. Cl^- vs. TN concentrations showing contamination source domains for (A) wells and (B) springs sampled in this study. Wells are differentiated based on the presence or absence of *E. coli*. Labeled wells are discussed in the text.

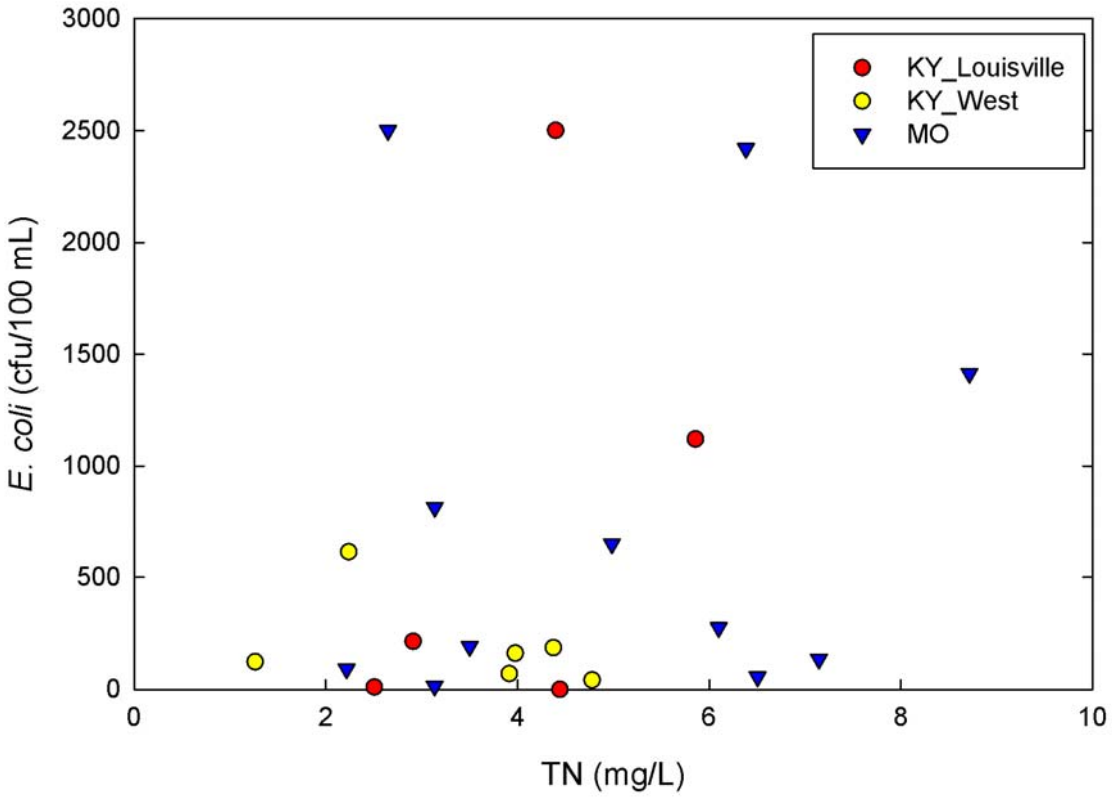

Figure 18. E. coli vs. TN concentrations for spring samples collected in this study

the samples was greater than 3 mg/L, suggesting that the samples were oxygenated. Spring and well samples had similar DO levels, with the exception of several well samples with values ≤ 1 mg/L (S-2, S-14, S-19, S-27, S-29, S-34, S-35, S-45). *E. coli* was an important factor associated with fecal contamination, but not total coliform. Well samples were affected by DO and Cl^- the most, and to a lesser extent by *E. coli*, whereas spring samples were influenced by all three parameters. Bacterial indicators were less frequently detected from well samples than from spring samples, and negative controls were distantly separated from well and spring samples. The distribution of samples from springs was more dispersed compared with well samples. Spring samples were correlated with high concentrations of DO, TN, and Cl^-. No strong regional distribution was detected.

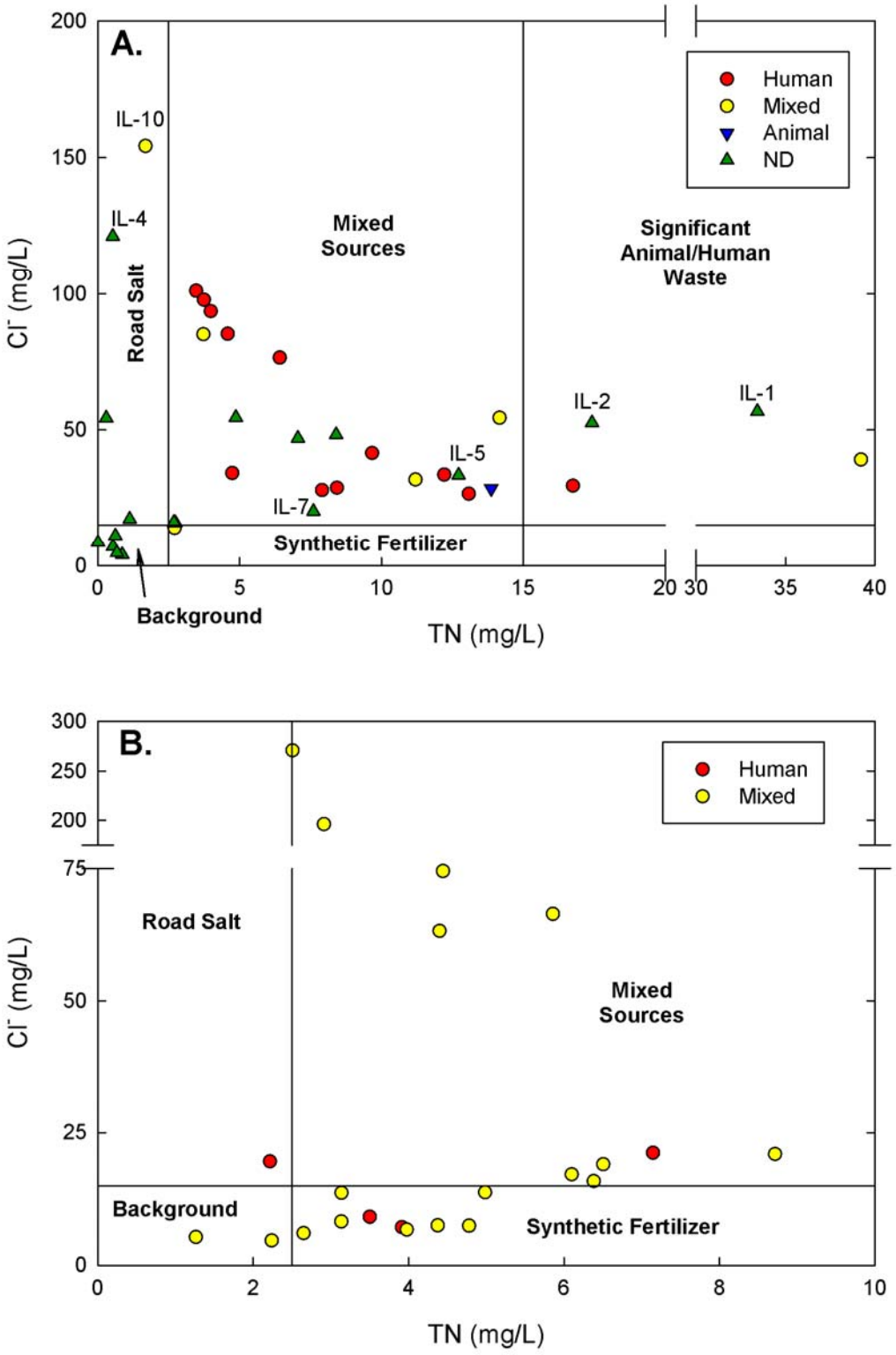

Figure 19. Cl^- vs. TN concentrations showing contamination source domains for (A) wells and (B) springs sampled in this study. Samples are differentiated based on identity of contamination source as determined by MST.

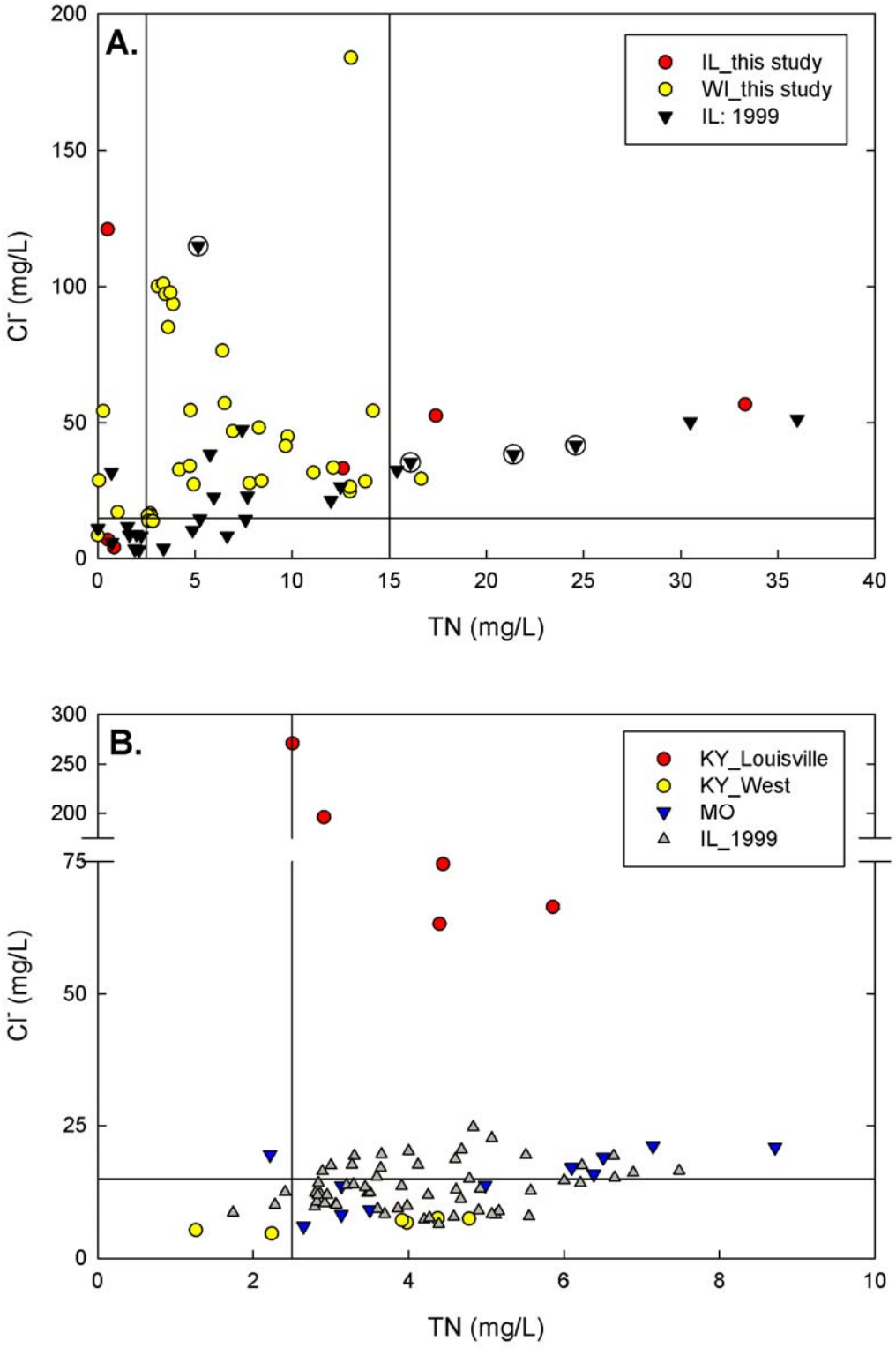

Figure 20. Cl^- vs. TN concentrations for samples collected in this study and in Illinois by Panno et al. (2001) and Hackley et al. (2007); (A) wells, and (B) springs. Only samples collected in karst regions are shown (IL-1 – IL-6). TN data for previous study are for NO_3-N only. Data points circled in (A) represent wells in which *E. coli* were detected.

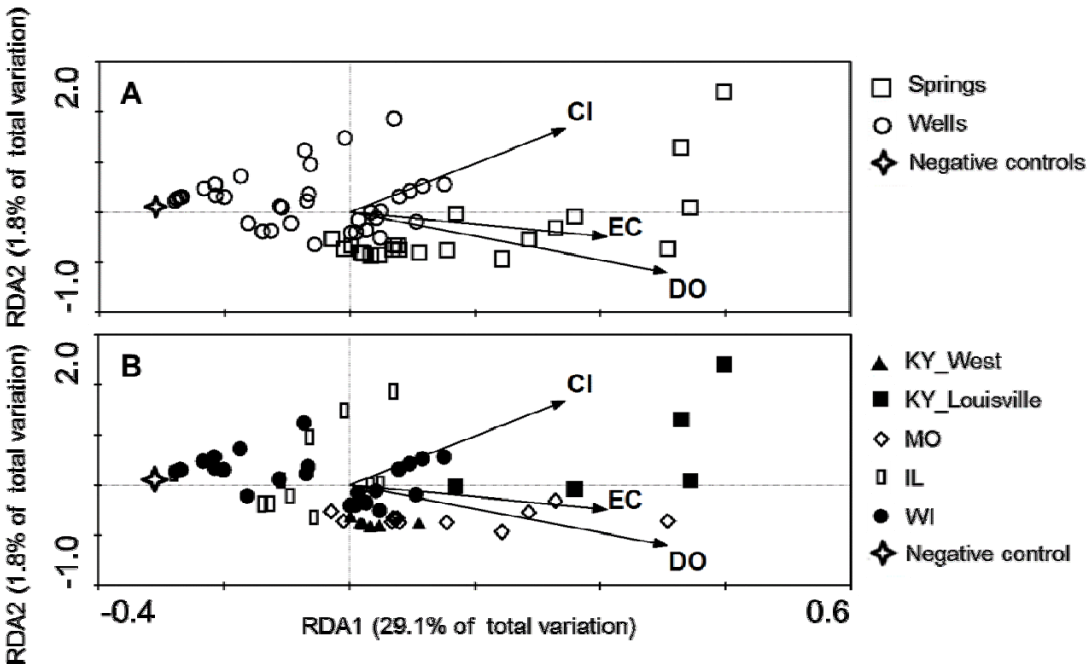

Figure 21. Relationship between environmental variables and *Bacteroidales* fecal biomarkers by linear redundancy analysis (RDA). Six individual biomarkers were included in addition to selected important environmental parameters. DO, *E. coli* (EC), and Cl^- were identified as the significant factors ($p < 0.05$). This diagram accounted for 31.4% of the total variation. Samples in Panel A were categorized by water types (springs and wells), whereas Panel B was grouped by sampling areas. Eleven samples that did not have enough gDNA were excluded. Each arrow represents an environmental variable and the direction of the arrow points to the steepest increase of values of the environmental variable. Distance between sample points indicates the dissimilarity of their fecal biomarker composition measured by Euclidean distance.

4. DISCUSSION

4.1 Sensitivity and specificity of Q-PCR-based MST

Useful MST information includes not just Q-PCR results but also the entire analytical process, including selection of sampling sites, sample collection and filtration, DNA extraction, removal of PCR inhibitors, establishment of reference DNA and standard curves, control of cross contamination, and estimation of gene copy numbers based on raw data. Although the Q-PCR technique is reliable and easy to apply to field studies, maintaining acceptable quality control during each step in the process can be difficult. Field et al. (2003) reported that in a comparison of methodologies among five participating laboratories, on average one laboratory could not obtain sufficient gDNA from the samples tested. Another study reported a spike recovery rate of as low as 63% for plasmids (Layton et al., 2006), indicating the likelihood of low DNA recovery. For the initial ten samples collected in this study (S-1 – S-10), only one L water was collected, which was insufficient for extracting sufficient good quality gDNA. Even when sufficient concentrations of gDNA were obtained, the presence of inhibitors in gDNA could lead to the failure of PCR and Q-PCR. To prevent inhibition, gDNA extracts were purified using a commercially available kit and then diluted to 10 ng/μL, which reduced the concentrations of potential inhibitors such as humic acids. Another potential approach is to use internal amplification controls to evaluate the effect of inhibitors in PCR-based reactions (Field et al., 2003; Shanks et al., 2010).

One problem with Q-PCR is the low amplification efficiency. The amplification efficiency for most primer sets used in our study was between 61 and 79%, lower than previously published values of 78 to 102% (Layton et al., 2006; Okabe et al., 2007; Shanks et al., 2008; 2010). We further examined the secondary structure of amplicons obtained from primer sets targeting *B. fragilis*, *B. uniformis*, and bovine, and observed the formation of complex secondary structures. This could also affect the amplification efficiency, give false-negative results, and underestimate the concentrations of *Bacteroidales* biomarkers in all samples.

Shanks et al. (2010) tested ten Q-PCR assays for human-associated *Bacteroidales*. Only three out of ten primer sets satisfied all the criteria, suggesting that vigorous validation on the designed primer sets is very important for obtaining convincing MST results. Our primer sets were not included in their study, but were previously tested using another molecular technique, where high levels of specificity and sensitivity were demonstrated (Hong et al., 2009).

A few groundwater samples tested in our study were highly contaminated with human and/or bovine feces. Shank et al. (2008) reported a mean of three copies per ng gDNA for bovine fecal *Bacteroidales* marker in ruminant feces. Our results showed a much higher copy number, ranging from 15 to 5.12×10^4 per ng gDNA. This difference might come from highly polluted groundwater, the sampling collection efficiency, and/or the DNA extraction process. In another study, where different primer sets targeting *Bacteroidales* for human fecal pollution were compared, concentrations of human fecal markers in 54 sewage samples were reported to be $10^2 – 10^5$ copies/ng gDNA (Shanks et al., 2008). Our tests got similar ranges of concentrations for human markers with primer sets targeting *B. fragilis* ($44 – 2.24\times10^4$ copies/ng gDNA), *B. caccae* ($1 – 5.37\times10^2$ copies/ng gDNA), *B. uniformis* ($51 – 1\times10^4$ copies/ng gDNA), and *B.vulgatus* (10 –

1.58×10^5 copies/ng gDNA). The high level of fecal contamination identified in our study is not surprising, as elevated levels of nutrients, bacterial indicators, natural estrogen, pesticides, and the presence of male-specific coliphages have been previously reported in Midwestern karst regions (Wicks et al., 2004; Kelly et al., 2009; Reed et al., 2011; Furtula et al., 2012).

Because different units were used to report the final concentrations of fecal biomarkers, the results obtained in this study were converted to copy number/100 mL water sample to enable further comparison. On average, the gDNA concentrations extracted from two L of water samples were 90 ng/μL with a final volume of 50 μL. Thus, a total of 4,500 ng gDNA could be recovered from two L, and the concentration was equal to 225 ng gDNA/100 mL. If Q-PCR identified 10^3 copies/ng gDNA, then the copy number would be 2.25×10^5 in 100 mL water sample. River water samples collected in a previous study had $2.5\times10^2 – 1.0\times10^4$ copies/100 mL of total *Bacteroides*, which was ten times lower than our estimated number (Savichtcheva and Okabe, 2009). Another study reported much higher concentrations of total and human-specific *Bacteroides* markers with averages of 9.8×10^8 and 4.8×10^7 copies/100 mL, respectively, for stormwater outfalls (Sauer et al., 2011). This result could be expected based on extremely high fecal indicator concentrations (4,900 – 3,410,000 cfu/100 mL water samples).

4.2 Multi-metric approaches: relationship among traditional indicators, environmental variables, and *Bacteroidales*-MST

The complexity and cost of MST are dependent on the composition and knowledge of the water system. If landscape, infrastructure, and human and animal populations are well understood, then the potential sources of contamination can be easily screened and confirmed with methods effectively targeting the specific sources. In this case, dominant fecal sources (contributing > 50%) can be detected with high confidence with assays having a specificity greater than 50%, while sources contributing 10–50% of the pollution would need methods with a specificity greater than 90%. Pathogens, if present, can be identified and isolated following a confirmation step. In more complex study areas, such as urban systems with significant amounts of aging infrastructure and numerous non-point sources of pollution, Q-PCR may serve as an easy and cost-effective method to gain an initial knowledge of the sources (Sauer et al., 2011). With the previous statistical analysis, only dominant sources (contributing more than 10% of pollution) might be effectively detected considering a specificity of Q-PCR at 80–100%. Combined with information of local land use and human and animal populations, more specific and focused MST methods can be applied to hotspot areas, which can lead to a better understanding of the sources of fecal contamination.

Although traditional bacterial indicators are easy, less expensive, and suitable for routine monitoring, their correlation or occurrence with fecal pollution and the presence of pathogens is weak (Leguyader et al., 1993; 1994; Noble et al., 2001; 2006; Peeler et al., 2006; Okabe et al., 2007; Katz and Griffin, 2008; Johnson et al., 2011; Sauer et al., 2011), except in a few studies (Dick and Field, 2004; Layton et al., 2006; Farnleitner et al., 2010). This weak relationship also occurred in our study. Five samples contained traditional indicators that could not be detected with *Bacteroidales*-MST, and another nine samples had detectable *Bacteroidales*-MST biomarkers without traditional indicators. At the same time, the presence of *Bacteroidales* markers correlates well with the presence

of pathogens (Noble et al., 2006; Walters et al., 2007). This discrepancy of indication function between traditional indicators and *Bacteroidales* markers may come from the different survival ability of the two groups. In addition, this ability can also be affected by factors such as temperature, sunlight, nutrient levels, availability of oxygen, and presence of predators (Roslev et al., 2004; Okabe et al., 2007).

In addition to traditional indicators, we considered the concentrations of Cl^- and TN to discriminate fecal source pollution. However, these methods did not correlate with *Bacteroidales*-MST. Because the various approaches (i.e., traditional indicators, *Bacteroidales*-MST, and nutrient levels) often do not agree, multi-metric or multi-tiered approaches have been proposed by a number of researchers (Noble et al., 2006; Peeler et al., 2006; Walters et al., 2007; Katz and Griffin, 2008; Reischer et al., 2008; Ritchey and Coyne, 2009; Sauer et al., 2011; Furtula et al., 2012). In these studies, two or more of the following methods were used to validate the sources of contamination: organic and inorganic compounds (N, P, Cl^-, organic wastewater compounds, pharmaceutical compounds), indicator bacteria, viruses, and MST (caffeine, sterols, ARA, *Bacteroidales*). The selection of a proper combination of techniques, which can verify results from different techniques as well as get different perspectives on the problem of interest, depends on availability of resources. For example, Furtula et al. (2012) adopted a multi-metric approach to investigate the effects of agricultural activities on surface water quality. Their toolbox included inorganic nitrogen measurements, sterol analysis, and *Bacteroidales*-based MST. Sterol ratios showed a combination of human and animal contamination for most sampling events. *Bacteroides*-MST methods showed animal contamination for fewer samples and no human contamination. To solve the discrepancy between the two methods, other tiers need to be considered.

The *Bacteroides*-MST methods used in our study gave ambiguous results when considered by themselves. One limitation is that we used four bacterial reference strains for human fecal contamination, but only one each for pigs and cows. We detected human sources in every sample with positive results. This may suggest widespread septic contamination in all sampled regions, or it may suggest our criteria for determining human contamination were not rigorous enough, especially with respect to bovine and swine sources.

An unexpected result of our MST analyses was the presence of bovine and swine contamination in urban springs in Louisville. Without any obvious sources of livestock in these springsheds, it is difficult to explain why we obtained those results.

The fact that most of the spring samples showed both human and animal sources of contamination was not unexpected. The land area contributing to springs is usually much larger than for wells and thus more likely to have multiple sources of contamination. Thus current MST methods may not be particularly useful for determining specific contamination sources in karst springs. Determining these sources would require a more detailed study than just collecting samples from the spring.

5. CONCLUSIONS

Our results demonstrated that karst aquifers in the Midwest were vulnerable to both human and non-human fecal contamination. Most water samples were contaminated by both human and animal wastes as determined by *Bacteroidales*-based Q-PCR. This result was in accordance with the observation on the presence of on-site wastewater treatment systems in rural areas and anthropogenic activities in urban areas. Samples from urban spring systems (Louisville, KY) had the highest level of fecal contamination, suggesting the vulnerability of karst aquifers due to human activities. Generally, spring systems were more vulnerable due to recharge from a much larger area. The overall contamination level from wells was much lower than from springs. Dilution was not enough to mitigate contamination in karst systems associated primarily with springs.

The Q-PCR method was effective at distinguishing fecal contamination sources and more sensitive than traditional bacterial indicators to evaluate the overall contamination level. The Q-PCR method used in this study involved even reactions (each primer set was tested separately) for each sample, which was not convenient for analyzing a large number of samples. To enhance productivity in the future, studies could apply multiplex Q-PCR or other multiplexing analysis such as hierarchical oligonucleotide primer extension reaction (HOPE) (Hong et al., 2009) to obtain both qualitative and quantitative information for fecal contamination.

The *Bacteroidales*-based MST method used in this study did not give definitive results, but nevertheless we believe it could be a powerful tool used in conjunction with other measures, including traditional bacterial indicators and a variety of environmental variables. *E. coli*, Cl^-, and DO were identified as the most important environmental parameters, which correlated well with the level of *Bacteroidales* fecal biomarkers.

Other methods are available that could assist in MST, although these suffer their own limitations. For example, virus sampling in the Wisconsin study area by another research group (Pedersen et al., 2011) had similar ambiguities. Detection of organic chemicals, such as pharmaceutical compounds, pesticides, and their degradation products, could corroborate the identification of human fecal contamination. Previous studies have shown the presence of these chemicals in karst groundwater systems (Glassmeyer et al., 2005; Peeler et al., 2006; Furtula et al., 2012). It appears that there currently is no single "silver bullet" for MST in karst aquifers. Future studies should continue to combine chemical-MST and microbe-MST techniques.

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APPENDIX

Table A-1. Complete water quality data from wells sampled in Illinois. NA = not analyzed; ND = not detected.

Sample ID	Site ID	City	County	Date	Time	T C	pH	SpC µS/cm	ORP mv	DO mg/L
S11	IL-1	Burksville	Monroe	4/8/2010	10:30	13.6	6.88	943	389	5.57
S12	IL-2	Burksville	Monroe	4/8/2010	10:45	14.0	7.06	852	379	5.41
S13	IL-3	Ames	Monroe	4/8/2010	11:45	14.6	7.24	669	162	4.04
S14	IL-4	Evansville	Randolph	4/8/2010	12:45	15.0	8.07	986	-50	0.18
S15	IL-5	Burksville	Monroe	4/8/2010	13:45	14.8	7.00	805	307	3.03
S16	IL-6	Waterloo	Monroe	4/8/2010	14:15	14.5	8.37	650	121	4.21
S18	Cabin Fever Campground	Victoria	Knox	6/2/2010	10:15	12.1	6.97	929	338	4.20
S19	Allendale Conservation Club	Wyoming	Stark	6/2/2010	11:45	12.5	6.71	2263	167	0.10
S20	Bonanza Campground	Smithboro	Bond	5/25/2010	9:15	13.0	7.54	338	965	6.10
S27	Fred's Place	Elmhurst	DuPage	6/18/2010	10:40	14.4	7.09	1207	175	0.13
S29	Oilfield Restaurant	Oilfield	Clark	8/9/2010	10:15	21.3	7.21	846	911	1.00

Table A-1. Continued.

Sample ID	Site ID	alkalinity	Cl^-	SO_4^{2-}	Ca	K	Mg	Na	F^-	NO_3-N	TKN	NH_3-N	Total N
		mg/L $CaCO_3$	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
S11	IL-1	254	56.7	61.7	155	1.61	20.8	37.5	0.15	33.3	<0.26	<0.06	33.3
S12	IL-2	287	52.5	48.0	113	1.35	17.5	68.2	0.18	17.3	<0.26	0.08	17.4
S13	IL-3	371	3.95	24.3	81.7	4.07	31.6	31.6	0.68	<0.07	0.85	0.92	0.85
S14	IL-4	335	121	28.2	12.0	1.68	5.21	230	1.12	<0.07	0.51	0.53	0.51
S15	IL-5	330	33.4	36.2	98.9	0.903	22.7	69.6	0.26	12.6	<0.26	<0.06	12.6
S16	IL-6	335	6.93	13.0	3.60	2.60	3.86	170	10.0	<0.07	0.51	0.47	0.51
S18	Cabin Fever Camp.	373	20.0	154	126	0.653	54.4	25.6	0.29	7.34	0.27	<0.06	7.60
S19	Allendale Conserv.	755	11.0	859	292	7.11	206	58.7	0.22	<0.07	0.60	0.26	0.60
S20	Bonanza Camp.	167	4.63	19.8	43.2	0.880	14.8	13.7	0.23	0.58	<0.26	<0.06	0.58
S27	Fred's Place	359	154	76.7	93.3	7.97	54.9	97.0	0.52	<0.07	1.66	1.35	1.66
S29	Oilfield Restaurant	212	85.2	73.8	77.2	0.953	26.8	58.4	0.16	4.49	<0.26	<0.06	4.49

Table A-1. Continued.

Sample ID	Site ID	Fe	Mn	Si	Sr	Al	B	Ba	Cu	Li	Mo
		mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	µg/L	µg/L	mg/L	µg/L
S11	IL-1	<0.0059	<0.0015	13.6	0.346	<37	<0.023	97.9	2.31	<0.058	<22
S12	IL-2	<0.0059	<0.0015	11.1	0.212	<37	<0.023	91.5	4.71	<0.058	<22
S13	IL-3	0.537	0.0643	5.43	1.29	<37	0.145	226	<0.79	<0.058	<22
S14	IL-4	1.69	0.0266	4.53	0.421	<37	0.673	163	<0.79	0.086	<22
S15	IL-5	<0.0059	0.0035	9.16	0.190	<37	<0.023	102	11.4	<0.058	<22
S16	IL-6	0.0252	0.0032	4.07	0.572	<37	0.495	45.8	<0.79	0.236	55.9
S18	Cabin Fever Camp.	0.0094	0.0030	8.06	0.208	<37	0.101	66.6	3.12	<0.058	<22
S19	Allendale Conserv.	3.51	2.95	5.84	1.47	<37	0.806	23.0	1.20	<0.058	<22
S20	Bonanza Camp.	0.0167	<0.0015	8.86	0.130	43.1	0.024	28.5	4.72	<0.058	<22
S27	Fred's Place	0.662	0.0151	7.61	2.58	<37	0.464	92.3	6.05	<0.058	40.4
S29	Oilfield Restaurant	<0.0059	<0.0015	8.18	0.148	<37	0.040	22.9	44.7	<0.058	<22

Table A-1. Continued.

Sample ID	Site ID	Ni	P	Pb	S	Ti	Tl	V	Zn	Br^-	*o*-PO_4-P	DOC
		µg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L
S11	IL-1	<14	0.078	<41	22.8	<0.56	20.7	<47	41.2	NA	0.078	1.14
S12	IL-2	<14	<0.063	<41	17.4	<0.56	20.3	<47	18.1	NA	0.061	1.25
S13	IL-3	<14	<0.063	<41	8.73	<0.56	<17	<47	9.20	NA	0.017	0.94
S14	IL-4	<14	<0.063	<41	10.4	<0.56	<17	<47	<7.3	NA	0.009	0.54
S15	IL-5	<14	<0.063	<41	13.3	<0.56	<17	<47	15.3	NA	0.034	0.76
S16	IL-6	<14	<0.063	<41	4.77	<0.56	<17	<47	<7.3	NA	0.010	0.50
S18	Cabin Fever Camp.	26.6	<0.063	<41	53.6	<0.56	<17	<47	9.60	NA	0.009	1.53
S19	Allendale Conserv.	97.3	<0.063	<41	288	<0.56	<17	<47	10.7	NA	0.009	2.17
S20	Bonanza Camp.	<14	<0.063	<41	14.4	<0.56	<17	<47	13.4	NA	0.020	2.35
S27	Fred's Place	25.0	<0.063	<41	26.5	<0.56	<17	<47	23.4	NA	0.008	2.38
S29	Oilfield Restaurant	<14	<0.063	<41	24.8	<0.56	<17	<47	40.7	NA	0.009	1.00

Table A-1. Concluded.

Sample ID	Site ID	Calculated TDS	Hardness	TC	*E. coli*	Enterococci
		mg/L	mg/L	cfu/100 mL	cfu/100 mL	cfu/100 mL
S11	IL-1	619	472	214	0	0
S12	IL-2	530	354	0	0	0
S13	IL-3	365	334	0	0	0
S14	IL-4	567	51	0	0	0
S15	IL-5	489	340	0	0	0
S16	IL-6	368	25	0	0	0
S18	Cabin Fever Camp.	604	537	ND	positive	ND
S19	Allendale Conserv.	1812	1579	22	0	0
S20	Bonanza Camp.	190	169	ND	ND	ND
S27	Fred's Place	669	459	ND	ND	ND
S29	Oilfield Restaurant	455	303	NA	NA	NA

Table A-2. Complete water quality data from private wells sampled in Wisconsin. NA = not analyzed.

Sample ID	Site ID	City	County	Date	Time	T	pH	SpC	ORP	DO
						C		µS/cm	mv	mg/L
S1	WI-1	Alto	Fond du Lac	4/5/2010	11:45	13.1	6.89	1196	452	4.4
S2	WI-2	Eden	Fond du Lac	4/5/2010	13:00	9.9	7.01	685	371	<0.5
S21	WI-2	Eden	Fond du Lac	6/17/2010	12:15	11.7	7.08	568	426	3.2
S3	WI-3	St. Peter	Fond du Lac	4/5/2010	14:15	10.5	7.31	915	413	8.7
S22	WI-3	St. Peter	Fond du Lac	6/17/2010	13:05	12.5	6.96	970	430	5.4
S30	WI-3	St. Peter	Fond du Lac	11/8/2010	11:45	11.6	*	1024	417	5.0
S40	WI-3	St. Peter	Fond du Lac	2/17/2011	13:30	10.4	6.73	986	403	5.0
S4	WI-4	Chilton	Calumet	4/5/2010	15:15	9.6	7.07	641	392	6.8
S23	WI-4	Chilton	Calumet	6/17/2010	14:10	10.5	7.03	698	431	7.2
S31	WI-4	Chilton	Calumet	11/8/2010	12:35	9.7	*	773	436	6.3
S41	WI-4	Chilton	Calumet	2/17/2011	14:15	9.5	6.81	765	424	6.2
S5	WI-5	Stockbridge	Calumet	4/5/2010	16:15	10.1	7.15	688	378	7.2
S24	WI-5	Stockbridge	Calumet	6/17/2010	14:40	11.0	6.99	756	403	5.9
S32	WI-5	Stockbridge	Calumet	11/8/2010	13:05	10.5	*	866	428	2.8
S43	WI-5	Stockbridge	Calumet	2/17/2011	14:50	9.9	6.69	935	425	5.1
S6	WI-6	Menasha	Calumet	4/5/2010	17:15	9.0	6.88	748	399	4.9
S25	WI-6	Menasha	Calumet	6/17/2010	15:05	10.6	6.93	723	945	5.6
S33	WI-6	Menasha	Calumet	11/8/2010	13:30	11.8	*	899	985	3.2
S44	WI-6	Menasha	Calumet	2/17/2011	15:15	8.0	6.71	657	422	7.0
S7	WI-7	Luxemburg	Kewaunee	4/5/2010	18:30	9.7	7.05	615	906	4.4
S26	WI-7	Luxemburg	Kewaunee	6/17/2010	17:20	10.3	6.81	719	468	2.9
S35	WI-7	Luxemburg	Kewaunee	11/8/2010	16:15	9.9	*	799	369	0.7
S45	WI-7	Luxemburg	Kewaunee	2/17/2011	16:55	9.4	6.71	795	433	1.0
S34	WI-8	Maribel	Manitowoc	11/8/2010	15:00	11.0	*	2676	290	0.1
S37	WI-9	Mineral Point	Iowa	12/6/2010	NA	NA	NA	NA	NA	NA
S39	WI-10	Byron	Fond du Lac	2/17/2011	12:30	9.9	6.99	824	357	6.4
S46	WI-11	Brownsville	Dodge	2/24/2011	NA	NA	NA	NA	NA	NA
S47	WI-11 (runoff)	Brownsville	Dodge	2/24/2011	NA	NA	NA	NA	NA	NA

* pH probe not working

Table A-2. Continued.

Sample ID	Site ID	Date	alkalinity	Cl^-	SO_4^{2-}	Ca	K	Mg	Na	F^-	NO_3-N	TKN	NH_3-N	Total N
			mg/L $CaCO_3$	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
S1	WI-1	4/5/2010	325	184	57.8	107	12.6	56.1	99.4	0.10	13.0	<0.26	<0.06	13.0
S2	WI-2	4/5/2010	318	28.8	60.2	94.3	3.18	45.8	7.27	0.21	<0.07	<0.26	0.07	0.07
S21	WI-2	6/17/2010	301	8.48	28.4	77.7	1.70	37.0	3.18	0.22	<0.07	<0.26	<0.06	0.00
S3	WI-3	4/5/2010	360	100	34.0	98.8	1.74	54.2	56.0	0.12	3.09	<0.26	<0.06	3.09
S22	WI-3	6/17/2010	352	101	31.2	0.178	0.232	0.078	248	0.14	3.37	<0.26	<0.06	3.37
S30	WI-3	11/8/2010	359	93.5	36.2	92.5	2.03	47.1	51.1	0.14	3.88	<0.26	<0.03	3.88
S40	WI-3	2/17/2011	352	85.0	41.6	95.5	1.80	51.3	44.6	0.13	3.63	<0.26	<0.03	3.63
S4	WI-4	4/5/2010	282	24.8	24.4	84.4	2.76	45.5	6.07	0.12	13.0	<0.26	<0.06	13.0
S23	WI-4	6/17/2010	275	29.5	24.2	81.4	2.85	45.1	6.46	0.11	16.7	<0.26	<0.06	16.7
S31	WI-4	11/8/2010	296	28.5	25.9	82.2	2.68	45.4	5.97	0.11	13.8	<0.26	<0.03	13.8
S41	WI-4	2/17/2011	298	26.5	29.9	85.6	2.40	48.1	6.18	0.11	13.0	<0.26	<0.03	13.0
S5	WI-5	4/5/2010	288	44.9	25.6	82.1	3.84	44.1	21.8	0.12	9.77	<0.26	<0.06	9.77
S24	WI-5	6/17/2010	311	48.2	29.1	86.1	3.77	46.2	21.6	0.13	8.30	<0.26	<0.06	8.30
S32	WI-5	11/8/2010	338	54.6	28.6	85.8	4.15	46.3	25.8	0.12	4.77	<0.26	<0.03	4.77
S43	WI-5	2/17/2011	342	76.5	34.1	90.4	4.84	50.2	34.8	0.13	6.13	0.29	<0.03	6.42
S6	WI-6	4/5/2010	322	57.2	21.3	78.5	14.5	44.7	35.8	0.14	6.53	<0.26	<0.06	6.53
S25	WI-6	6/17/2010	296	41.5	21.4	70.1	19.0	40.1	21.1	0.15	9.32	0.36	<0.06	9.67
S33	WI-6	11/8/2010	340	46.9	36.4	86.0	14.3	47.7	19.7	0.13	6.96	<0.26	<0.03	6.96
S44	WI-6	2/17/2011	202	54.4	26.1	52.5	19.5	32.0	23.4	0.23	6.12	8.04	1.37	14.2
S7	WI-7	4/5/2010	291	32.8	22.9	81.8	2.81	39.1	9.27	0.12	3.82	0.38	<0.06	4.20
S26	WI-7	6/17/2010	348	34.1	13.8	92.5	3.62	43.6	12.4	0.15	3.99	0.75	<0.06	4.74
S35	WI-7	11/8/2010	347	27.9	19.2	91.6	3.77	43.0	8.95	0.16	7.81	<0.26	<0.03	7.81
S45	WI-7	2/17/2011	344	28.7	21.6	98.5	3.84	47.5	9.01	0.16	8.06	0.38	<0.03	8.44
S34	WI-8	11/8/2010	246	54.3	1392	530	4.05	81.3	23.7	1.16	<0.07	0.28	<0.03	0.28
S37	WI-9	12/6/2010	328	17.2	39.2	75.7	0.649	47.0	6.63	0.10	1.03	<0.26	<0.03	1.03
S39	WI-10	2/17/2011	314	33.5	38.7	90.4	5.58	49.7	14.4	0.12	12.1	<0.26	<0.03	12.1
S46	WI-11	2/24/2011	292	31.7	21.7	80.8	5.24	44.3	12.9	0.12	11.1	<0.26	<0.03	11.1
S47	WI-11 (runoff)	2/24/2011	NA	39.1	22.4	43.2	88.2	34.4	20.7	0.17	1.56	37.7	20.4	39.2

Table A-2. Continued.

Sample ID	Site ID	Date	Fe	Mn	Si	Sr	Al	B	Ba	Cu	Li	Mo
			mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	µg/L	µg/L	mg/L	µg/L
S1	WI-1	4/5/2010	<0.0059	<0.0015	7.92	0.350	<37	0.070	136	55.4	<0.058	<22
S2	WI-2	4/5/2010	<0.0059	0.0033	5.19	0.682	<37	0.025	38.1	<0.79	<0.058	39.4
S21	WI-2	6/17/2010	0.0060	0.0027	6.76	0.272	<37	<0.023	13.3	8.64	<0.058	<22
S3	WI-3	4/5/2010	<0.0059	<0.0015	9.52	0.0558	<37	0.029	33.0	15.2	<0.058	<22
S22	WI-3	6/17/2010	<0.0059	<0.0015	8.54	<0.00037	<37	0.027	<0.85	8.09	<0.058	<22
S30	WI-3	11/8/2010	<0.0059	<0.0015	9.51	0.0795	<37	0.041	53.6	11.0	<0.11	<22
S40	WI-3	2/17/2011	<0.0059	<0.0015	9.94	0.0727	<37	0.032	54.8	11.2	<0.11	<22
S4	WI-4	4/5/2010	<0.0059	<0.0015	6.24	0.146	<37	<0.023	25.9	7.02	<0.058	<22
S23	WI-4	6/17/2010	<0.0059	<0.0015	6.36	0.102	<37	<0.023	24.1	21.7	<0.058	<22
S31	WI-4	11/8/2010	<0.0059	<0.0015	6.48	0.130	<37	<0.023	25.4	11.9	<0.11	<22
S41	WI-4	2/17/2011	<0.0059	<0.0015	7.18	0.131	<37	<0.023	22.9	26.9	<0.11	<22
S5	WI-5	4/5/2010	0.0145	<0.0015	5.80	0.0524	<37	<0.023	29.8	38.3	<0.058	<22
S24	WI-5	6/17/2010	<0.0059	<0.0015	5.95	0.0611	<37	<0.023	30.8	55.7	<0.058	<22
S32	WI-5	11/8/2010	<0.0059	<0.0015	6.52	0.0712	<37	0.030	34.5	36.6	<0.11	<22
S43	WI-5	2/17/2011	<0.0059	<0.0015	6.48	0.0699	<37	0.026	36.4	43.8	<0.11	<22
S6	WI-6	4/5/2010	<0.0059	<0.0015	5.07	0.0502	<37	0.030	30.3	8.32	<0.058	<22
S25	WI-6	6/17/2010	0.0129	<0.0015	4.85	0.0525	<37	0.028	29.5	49.3	<0.058	<22
S33	WI-6	11/8/2010	<0.0059	<0.0015	5.08	0.0632	<37	0.035	33.8	35.6	<0.11	<22
S44	WI-6	2/17/2011	0.0436	0.0242	3.77	0.0406	38.5	0.030	23.7	53.2	<0.11	<22
S7	WI-7	4/5/2010	<0.0059	<0.0015	3.51	0.0673	<37	0.061	27.3	69.5	<0.058	71.7
S26	WI-7	6/17/2010	0.0080	<0.0015	4.58	0.0872	<37	0.053	33.6	40.2	<0.058	<22
S35	WI-7	11/8/2010	<0.0059	<0.0015	5.29	0.131	<37	0.030	58.6	36.2	<0.11	<22
S45	WI-7	2/17/2011	<0.0059	<0.0015	5.55	0.127	<37	0.027	60.7	81.4	<0.11	<22
S34	WI-8	11/8/2010	0.218	0.0018	5.93	8.21	<37	0.073	10.3	4.74	<0.11	<22
S37	WI-9	12/6/2010	0.0130	0.0018	4.70	0.0558	<37	<0.023	24.7	<0.79	<0.11	<22
S39	WI-10	2/17/2011	<0.0059	0.0029	6.40	0.261	<37	0.028	42.7	15.7	<0.11	<22
S46	WI-11	2/24/2011	0.0787	0.0286	6.53	0.734	<37	<0.023	44.9	355	<0.11	<22
S47	WI-11 (runoff)	2/24/2011	0.524	0.313	4.23	0.172	<37	0.080	62.1	111	<0.11	<22

Table A-2. Continued.

Sample ID	Site ID	Date	Ni	P	Pb	S	Ti	Tl	V	Zn	Br^-	*o*-PO_4-P	DOC
			µg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L
S1	WI-1	4/5/2010	21.6	<0.063	<41	21.3	<0.56	<17	<47	20.3	NA	0.049	3.39
S2	WI-2	4/5/2010	<14	<0.063	<41	22.2	<0.56	<17	<47	<7.3	NA	0.009	0.94
S21	WI-2	6/17/2010	18.7	<0.063	<41	9.57	<0.56	<17	<47	8.7	NA	0.008	0.92
S3	WI-3	4/5/2010	33.2	<0.063	<41	12.5	<0.56	<17	<47	8.0	NA	0.033	2.13
S22	WI-3	6/17/2010	<14	<0.063	<41	10.6	<0.56	<17	<47	11.5	NA	0.034	1.85
S30	WI-3	11/8/2010	29.7	0.177	<41	12.6	<0.56	<17	<47	16.2	NA	0.035	1.89
S40	WI-3	2/17/2011	<14	<0.073	<41	14.3	<0.56	<17	<47	7.9	NA	0.028	1.36
S4	WI-4	4/5/2010	16.9	<0.063	<41	9.21	<0.56	<17	<47	<7.3	NA	0.032	1.81
S23	WI-4	6/17/2010	22.8	<0.063	<41	8.13	<0.56	<17	<47	12.4	NA	0.033	2.12
S31	WI-4	11/8/2010	31.9	0.131	<41	8.76	<0.56	<17	<47	12.9	NA	0.031	1.84
S41	WI-4	2/17/2011	<14	<0.073	<41	10.2	<0.56	<17	<47	28.2	NA	0.035	1.47
S5	WI-5	4/5/2010	<14	<0.063	<41	9.54	<0.56	<17	<47	<7.3	NA	0.046	1.83
S24	WI-5	6/17/2010	26.5	<0.063	<41	9.96	<0.56	<17	<47	13.3	NA	0.047	1.80
S32	WI-5	11/8/2010	31.7	0.191	<41	9.91	<0.56	<17	<47	10.0	NA	0.047	2.28
S43	WI-5	2/17/2011	<14	<0.073	<41	11.6	<0.56	<17	<47	7.3	NA	0.047	1.90
S6	WI-6	4/5/2010	<14	0.278	<41	7.76	<0.56	<17	<47	15.4	NA	0.227	2.68
S25	WI-6	6/17/2010	26.7	0.184	<41	7.13	<0.56	<17	<47	12.7	NA	0.228	2.64
S33	WI-6	11/8/2010	37.1	0.299	<41	13.0	<0.56	<17	<47	24.8	NA	0.116	1.70
S44	WI-6	2/17/2011	<14	1.43	<41	9.54	1.29	<17	<47	42.3	NA	1.08	27.7
S7	WI-7	4/5/2010	<14	<0.063	<41	8.36	<0.56	<17	<47	<7.3	NA	0.082	7.63
S26	WI-7	6/17/2010	24.7	0.093	<41	4.82	<0.56	<17	<47	<7.3	NA	0.097	7.15
S35	WI-7	11/8/2010	30.2	0.178	<41	6.52	<0.56	<17	<47	10.5	NA	0.035	2.65
S45	WI-7	2/17/2011	<14	<0.073	<41	7.55	<0.56	<17	<47	37.5	NA	0.028	2.11
S34	WI-8	11/8/2010	48.8	0.169	<41	471	<0.56	<17	<47	17.8	NA	0.010	1.51
S37	WI-9	12/6/2010	46.0	0.096	<41	14.3	<0.56	<17	<47	47.3	NA	0.008	1.41
S39	WI-10	2/17/2011	<14	<0.073	<41	13.3	<0.56	<17	<47	14.4	NA	0.028	1.65
S46	WI-11	2/24/2011	<14	<0.073	<41	7.46	<0.56	<17	<47	12.4	NA	0.040	2.13
S47	WI-11 (runoff)	2/24/2011	<14	7.44	<41	12.7	6.77	<17	<47	43.9	NA	4.71	NA

Table A-2. Concluded.

Sample ID	Site ID	Date	calculated TDS	Hardness	TC	*E. coli*	Enterococci
			mg/L	mg/L	cfu/100 mL	cfu/100 mL	cfu/100 mL
S1	WI-1	4/5/2010	742	497	<1.0	<1.0	<1.0
S2	WI-2	4/5/2010	401	424	<1.0	<1.0	<1.0
S21	WI-2	6/17/2010	312	346	Absent	Absent	<1.0
S3	WI-3	4/5/2010	545	470	2	<1.0	<1.0
S22	WI-3	6/17/2010	577	1	Present	Present	461
S30	WI-3	11/8/2010	526	425	17	<1.0	<1.0
S40	WI-3	2/17/2011	519	449	0	0	<1.0
S4	WI-4	4/5/2010	390	398	NA	NA	NA
S23	WI-4	6/17/2010	405	389	Present	Present	39
S31	WI-4	11/8/2010	404	392	4	<1.0	<1.0
S41	WI-4	2/17/2011	410	412	1	1	<1.0
S5	WI-5	4/5/2010	413	386	5	<1.0	<1.0
S5X	WI-5	4/6/2010	NA	NA	13	NA	NA
S24	WI-5	6/17/2010	431	405	Present	Present	35
S32	WI-5	11/8/2010	439	405	12	<1.0	1
S43	WI-5	2/17/2011	493	432	0	0	<1.0
S6	WI-6	4/5/2010	444	380	9	<1.0	<1.0
S25	WI-6	6/17/2010	405	340	Present	Present	299
S33	WI-6	11/8/2010	454	411	<1.0	<1.0	<1.0
S44	WI-6	2/17/2011	340	263	>2419.0	31	>2419.0
S7	WI-7	4/5/2010	352	365	<1.0	<1.0	<1.0
S26	WI-7	6/17/2010	393	410	Present	Present	1986
S35	WI-7	11/8/2010	405	405	110	1	<1.0
S45	WI-7	2/17/2011	419	441	5	0	<1.0
S34	WI-8	11/8/2010	2211	1656	68	<1.0	1
S37	WI-9	12/6/2010	357	382	NA	NA	NA
S39	WI-10	2/17/2011	447	430	15	0	<1.0
S46	WI-11	2/24/2011	396	384	NA	NA	NA
S47	WI-11 (runoff)	2/24/2011	NA	249	NA	NA	NA

Table A-3. Complete water quality data from public supply wells sampled in Sturgeon Bay, Wisconsin. NA = not analyzed.

Sample ID	Well	Date	Time	T	pH	SpC	ORP	DO
				C		µS/cm	mv	mg/L
S8	8	4/6/2010	8:30	10.3	7.07	560	463	<2.75
S9	10	4/6/2010	9:00	8.9	7.22	492	448	<3.7
S10	6	4/6/2010	9:30	9.6	7.10	812	457	4.98
S38	10	1/5/2011?		NA	NA	NA	NA	NA
S42	10	11/1/2010		NA	NA	NA	NA	NA
S61	6	4/4/2011		NA	NA	NA	NA	NA
S62	10	5/2/2011		NA	NA	NA	NA	NA
S75	10	6/1/2011		NA	NA	NA	NA	NA

Table A-3. Continued.

Sample ID	Well	Date	alkalinity	Cl^-	SO_4^{2-}	Ca	K	Mg	Na	F^-	NO_3-N	TKN	NH_3-N	Total N
			mg/L $CaCO_3$	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
S8	8	4/6/2010	267	27.4	22.2	74.0	3.05	37.7	10.8	0.18	4.94	<0.26	<0.06	4.94
S9	10	4/6/2010	258	16.7	15.9	68.8	2.40	34.3	8.59	0.17	2.37	0.34	<0.06	2.71
S10	6	4/6/2010	308	97.2	23.8	87.5	2.74	39.1	53.7	0.15	3.47	<0.26	<0.06	3.47
S38	10	1/5/2011?	270	15.8	15.2	68.8	2.22	32.1	7.08	0.19	2.46	0.28	<0.03	2.74
S42	10	11/1/2010	264	16.0	14.8	65.2	2.19	30.8	7.48	0.23	2.58	<0.26	<0.03	2.58
S61	6	4/4/2011	312	97.7	23.9	81.3	3.05	39.7	60.0	0.17	3.45	0.29	0.04	3.74
S62	10	5/2/2011	250	13.9	13.3	66.9	2.09	31.9	7.06	0.23	2.61	<0.26	<0.03	2.61
S75	10	6/1/2011	255	13.8	13.1	71.6	2.25	32.9	7.18	0.22	2.17	0.67	<0.03	2.83

Table A-3. Continued.

Sample ID	Well	Date	Fe	Mn	Si	Sr	Al	B	Ba	Cu	Li	Mo
			mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	µg/L	µg/L	mg/L	µg/L
S8	8	4/6/2010	<0.0059	0.0069	4.22	0.0724	<37	0.030	33.9	1.58	<0.058	<22
S9	10	4/6/2010	<0.0059	<0.0015	5.17	0.0969	<37	0.035	37.6	1.00	<0.058	<22
S10	6	4/6/2010	<0.0059	<0.0015	4.40	0.0736	<37	0.026	61.1	<0.79	<0.058	<22
S38	10	1/5/2011?	0.0069	<0.0015	5.06	0.0907	<37	0.031	35.1	1.79	<0.11	<22
S42	10	11/1/2010	<0.0059	0.0022	5.03	0.100	<37	0.031	35.5	2.28	<0.11	<22
S61	6	4/4/2011	0.043	0.0029	4.42	0.0843	<37	<0.023	61.9	2.32	<0.11	<22
S62	10	5/2/2011	<0.024	<0.0015	5.17	0.0924	<37	0.031	35.6	1.75	<0.11	<22
S75	10	6/1/2011	<0.024	<0.0015	5.12	0.0952	<37	0.028	39.7	2.29	<0.11	<22

Table A-3. Continued.

Sample ID	Well	Date	Ni	P	Pb	S	Ti	Tl	V	Zn	Br^-	*o*-PO_4-P	DOC
			µg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L
S8	8	4/6/2010	<14	<0.063	<41	NA	<0.56	<17	<47	<7.3	NA	0.010	1.87
S9	10	4/6/2010	<14	<0.063	<41	5.82	<0.56	<17	<47	<7.3	NA	0.011	2.26
S10	6	4/6/2010	19.4	<0.063	<41	9.03	<0.56	<17	<47	<7.3	NA	0.010	1.43
S38	10	1/5/2011?	<14	<0.073	<41	4.87	<0.56	<17	<47	<7.3	NA	0.011	3.76
S42	10	11/1/2010	26.3	0.159	<41	5.09	<0.56	<17	<47	<7.3	NA	0.010	2.96
S61	6	4/4/2011	<43	0.106	<41	7.70	<0.56	<17	<47	<9.7	NA	0.008	1.80
S62	10	5/2/2011	<43	0.139	<41	4.74	<0.56	<17	<47	<9.7	NA	0.009	1.82
S75	10	6/1/2011	<43	0.185	<41	4.69	<0.56	<17	<47	<9.7	NA	0.008	2.70

Table A-3. Concluded.

Sample ID	Well	Date	calculated TDS	Hardness	TC	*E. coli*	Enterococci
			mg/L	mg/L	cfu/100 mL	cfu/100 mL	cfu/100 mL
S8	8	4/6/2010	333	340	<1.0	<1.0	<1.0
S9	10	4/6/2010	289	313	<1.0	<1.0	<1.0
S10	6	4/6/2010	475	379	<1.0	<1.0	<1.0
S38	10	1/5/2011?	290	304	Present	Absent	Absent
S42	10	11/1/2010	283	289	Present	Absent	Absent
S61	6	4/4/2011	479	366	Present	Absent	Absent
S62	10	5/2/2011	275	298	Present	Absent	Absent
S75	10	6/1/2011	281	314	Present	Absent	Absent

Table A-4. Complete water quality data from springs sampled in Kentucky. NA = not analyzed.

Sample ID	Site ID	Location	City	County	Date	Time	T	pH	SpC	ORP	DO
							C		µS/cm	mv	mg/L
S48	KY-1	Mockingbird Spring	Louisville	Jefferson	3/16/2011	11:15	13.4	6.64	753	382	7.3
S49	KY-2	CSO 206 spring	Louisville	Jefferson	3/16/2011	11:45	13.4	7.36	739	354	8.0
S50	KY-3	Jesse's Spring	Louisville	Jefferson	3/16/2011	12:15	13.4	6.81	683	574	7.5
S51	KY-4	AB Sawyer Spring	Louisville	Jefferson	3/16/2011	14:20	13.0	6.72	982	378	8.2
S52	KY-5	Nunnlea Spring	Louisville	Jefferson	3/16/2011	15:10	15.0	6.56	1312	367	6.2
S53	KY-6	Nolynn Spring	Hodgenville	LaRue	3/16/2011	17:15	12.6	6.63	230	427	6.6
S54	KY-7	Mill Spring	Millerstown	Grayson	3/16/2011	18:20	10.9	6.91	235	395	7.4
S55	KY-8	Goodman Spring	Sonora	Hardin	3/16/2011	19:15	12.3	6.90	420	475	7.9
S56	KY-9	Head of Rough Spring	Radcliff	Hardin	3/17/2011	9:00	12.9	6.73	388	414	7.6
S57	KY-10	Skees karst window	Sonora	Hardin	3/17/2011	10:15	13.2	6.81	400	416	8.0
S58	KY-11	Mahurin Spring	Short Creek	Grayson	3/17/2011	11:30	10.9	6.79	279	266	7.7
S59	KY-11	Mahurin Spring (duplicate)	Short Creek	Grayson	3/17/2011	11:30	10.9	6.79	279	266	7.7

Table A-4. Continued.

Sample ID	Location	alkalinity	Cl^-	SO_4^{2-}	Ca	K	Mg	Na	F^-	NO_3-N	TKN	NH_3-N	Total N
		mg/L $CaCO_3$	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
S48	Mockingbird Spring	227	63.2	55.8	121	2.02	12.5	27.2	0.11	4.13	0.27	0.037	4.40
S49	CSO 206 spring	206	66.4	49.5	108	2.19	10.4	32.5	0.14	5.76	<0.26	<0.03	5.76
S50	Jesse's Spring	162	74.5	48.2	97.4	2.47	7.58	33.3	0.22	4.34	<0.26	0.052	4.40
S51	AB Sawyer Spring	136	196	29.9	64.6	2.00	27.3	88.0	<0.08	2.81	<0.26	<0.03	2.81
S52	Nunnlea Spring	176	271	45.2	107	2.09	25.6	116	0.09	2.24	0.27	<0.03	2.51
S53	Nolynn Spring	148	7.49	6.14	62.2	1.86	6.12	4.69	0.13	4.10	0.27	<0.03	4.37
S54	Mill Spring	95	5.32	11.5	41.6	1.62	3.90	2.87	0.10	1.16	<0.26	<0.03	1.16
S55	Goodman Spring	183	6.70	10.7	79.2	1.69	5.61	2.60	0.09	3.88	<0.26	<0.03	3.88
S56	Head of Rough Spring	168	7.15	7.82	71.6	1.74	7.36	3.05	0.11	3.82	<0.26	<0.03	3.82
S57	Skees karst window	172	7.42	5.13	73.8	1.69	5.86	2.86	0.13	4.68	<0.26	<0.03	4.68
S58	Mahurin Spring	110	4.65	16.9	47.8	1.51	5.43	2.76	<0.08	1.82	0.42	<0.03	2.24
S59	Mahurin Spring	110	4.69	17.1	47.6	1.62	5.46	2.94	0.08	1.77	<0.26	<0.03	1.77

Table A-4. Continued.

Sample ID	Location	alkalinity	Fe	Mn	Si	Sr	Al	B	Ba	Cu	Li	Mo
		mg/L $CaCO_3$	mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	µg/L	µg/L	mg/L	µg/L
S48	Mockingbird Spring	227	<0.024	<0.0015	4.54	0.157	<37	0.037	49.5	1.61	<0.11	<22
S49	CSO 206 spring	206	<0.024	0.0070	5.13	0.144	<37	0.051	39.9	0.99	<0.11	<22
S50	Jesse's Spring	162	<0.024	0.0045	4.45	0.130	<37	0.042	30.9	0.87	<0.11	<22
S51	AB Sawyer Spring	136	<0.024	0.0036	4.45	0.139	<37	<0.023	76.3	0.87	<0.11	<22
S52	Nunnlea Spring	176	<0.024	0.0046	3.99	0.140	<37	<0.023	86.1	1.06	<0.11	<22
S53	Nolynn Spring	148	0.040	0.0030	4.11	0.161	<37	<0.023	33.0	0.88	<0.11	<22
S54	Mill Spring	95	0.045	0.0037	3.37	0.133	53.3	<0.023	26.3	<0.79	<0.11	<22
S55	Goodman Spring	183	0.031	0.0107	3.58	0.189	<37	<0.023	31.4	<0.79	<0.11	<22
S56	Head of Rough Spring	168	0.033	0.0041	4.40	0.120	<37	<0.023	30.5	<0.79	<0.11	<22
S57	Skees karst window	172	<0.024	0.164	4.09	0.135	<37	<0.023	35.1	<0.79	<0.11	<22
S58	Mahurin Spring	110	0.054	0.0212	3.49	0.164	<37	<0.023	30.1	<0.79	<0.11	<22
S59	Mahurin Spring	110	0.042	0.0210	3.51	0.166	<37	<0.023	30.3	<0.79	<0.11	<22

Table A-4. Continued.

Sample ID	Location	Ni	P	Pb	S	Ti	Tl	V	Zn	Br^-	*o*-PO_4-P	DOC
		µg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L
S48	Mockingbird Spring	<43	0.198	<41	20.0	<0.56	18.0	<47	<9.7	NA	0.074	1.30
S49	CSO 206 spring	<43	0.267	<41	17.3	<0.56	<17	<47	<9.7	NA	0.131	1.35
S50	Jesse's Spring	<43	0.278	<41	16.9	<0.56	20.3	<47	<9.7	NA	0.118	0.90
S51	AB Sawyer Spring	<43	0.155	<41	10.6	<0.56	<17	<47	<9.7	NA	0.018	1.27
S52	Nunnlea Spring	<43	0.147	<41	15.7	<0.56	<17	<47	<9.7	NA	0.042	0.73
S53	Nolynn Spring	<43	0.130	<41	2.37	<0.56	<17	<47	<9.7	NA	0.030	1.25
S54	Mill Spring	<43	0.161	<41	4.17	1.01	<17	<47	<9.7	NA	0.024	1.97
S55	Goodman Spring	<43	0.147	<41	3.85	<0.56	<17	<47	<9.7	NA	0.028	1.27
S56	Head of Rough Spring	<43	0.151	<41	2.81	<0.56	<17	<47	<9.7	NA	0.037	1.25
S57	Skees karst window	<43	0.175	<41	1.90	<0.56	<17	<47	<9.7	NA	0.048	0.88
S58	Mahurin Spring	<43	<0.073	<41	5.97	<0.56	<17	<47	<9.7	NA	0.017	2.09
S59	Mahurin Spring	<43	0.107	<41	5.97	<0.56	<17	<47	<9.7	NA	0.018	1.97

Table A-4. Concluded.

Sample ID	Location	calculated TDS	Hardness	TC	*E. coli*	Enterococci
		mg/L	mg/L	cfu/100 mL	cfu/100 mL	cfu/100 mL
S48	Mockingbird Spring	416	352	>2419	>2419	435
S49	CSO 206 spring	401	312	>2419	1119	517
S50	Jesse's Spring	367	274	0	0	0
S51	AB Sawyer Spring	491	273	>2419	214	102
S52	Nunnlea Spring	666	372	291	10	54
S53	Nolynn Spring	183	180	816	186	199
S54	Mill Spring	122	120	387	122	72
S55	Goodman Spring	217	221	365	161	61
S56	Head of Rough Spring	203	209	285	70	105
S57	Skees karst window	206	208	185	41	68
S58	Mahurin Spring	145	142	770	613	88
S59	Mahurin Spring	145	141	NA	NA	NA

Table A-5. Complete water quality data from springs sampled in Missouri.

Sample ID	Site ID	Location	City	Date	Time	T	pH	SpC	ORP	DO
						C		µS/cm	mv	mg/L
S63	MO-1	Mystery Entrance	Longtown	5/18/2011	8:50	15.8	6.78	223	181	5.4
S64	MO-2	Apple Creek Shrine	Apple Creek	5/18/2011	9:35	14.3	6.82	491	366	5.3
S65	MO-3	Scholl Creek	Altenberg	5/18/2011	10:35	14.0	7.42	674	354	8.2
S66	MO-4	Dry Fork	Crosstown	5/18/2011	11:15	14.7	7.62	555	336	9.6
S67	MO-5	Thunder Hole	Perryville	5/18/2011	11:55	13.9	7.27	498	358	8.1
S68	MO-6	Mystery Resurgence	Perryville	5/18/2011	12:45	14.9	6.90	342	387	5.7
S69	MO-7	Huber Branch	Perryville	5/18/2011	13:35	18.4	8.06	530	340	9.3
S70	MO-8	Blue Spring	Lithium	5/18/2011	14:00	14.9	7.32	504	361	7.7
S71	MO-9	Keyhole Spring	Lithium	5/18/2011	14:25	13.8	7.37	508	365	8.1
S72	MO-10	Briar Spring	Lithium	5/18/2011	14:40	14.6	7.32	582	356	7.7
S73	MO-11	Ball Mill Spring	Lithium	5/18/2011	15:25	13.9	7.20	605	369	7.5

Table A-5. Continued.

Sample ID	Location	alkalinity	Cl^-	SO_4^{2-}	Ca	K	Mg	Na	F^-	NO_3-N	TKN	NH_3-N	Total N
		mg/L $CaCO_3$	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
S63	Mystery Entrance	95	6.05	3.33	20.6	11.1	8.35	2.77	0.108	<0.07	2.63	1.28	2.63
S64	Apple Creek Shrine	218	8.22	17.4	83.0	1.95	7.79	8.34	0.157	2.52	0.62	0.17	3.14
S65	Scholl Creek	299	19.5	29.7	95.2	2.71	20.2	23.2	0.238	1.62	0.60	0.06	2.22
S66	Dry Fork	247	13.6	20.1	95.9	1.67	8.68	17.0	0.222	2.53	0.61	<0.03	3.14
S67	Thunder Hole	208	13.7	15.8	73.4	3.98	14.0	9.91	0.248	4.23	0.75	<0.03	4.98
S68	Mystery Resurgence	141	9.09	10.4	38.0	7.64	15.4	6.08	0.213	1.93	1.57	0.65	3.50
S69	Huber Branch	215	15.8	19.6	85.2	1.50	7.66	16.7	0.202	5.58	0.81	<0.03	6.38
S70	Blue Spring	183	20.9	19.3	52.6	2.10	24.5	14.3	0.293	8.23	0.48	<0.03	8.71
S71	Keyhole Spring	197	19.0	17.9	53.6	2.37	26.4	13.3	0.310	5.68	0.82	<0.03	6.51
S72	Briar Spring	229	21.2	25.2	64.3	1.36	30.1	15.3	0.269	6.71	0.43	<0.03	7.14
S73	Ball Mill Spring	252	17.1	21.5	77.1	2.04	27.3	14.2	0.272	5.84	0.26	<0.03	6.10

Table A-5. Continued.

Sample ID	Location	Fe	Mn	Si	Sr	Al	B	Ba	Cu	Li	Mo
		mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	µg/L	µg/L	mg/L	µg/L
S63	Mystery Entrance	0.845	0.828	4.11	0.0572	229.8	<0.023	62.3	1.37	<0.11	<22
S64	Apple Creek Shrine	0.050	0.0313	7.46	0.225	<37	<0.023	90.4	1.13	<0.11	<22
S65	Scholl Creek	<0.024	0.0175	10.4	0.233	<37	<0.023	106.6	<0.79	<0.11	<22
S66	Dry Fork	<0.024	0.0342	7.87	0.218	<37	<0.023	90.0	0.91	<0.11	<22
S67	Thunder Hole	<0.024	0.0082	8.16	0.340	<37	<0.023	91.0	1.12	<0.11	<22
S68	Mystery Resurgence	0.195	0.280	5.66	0.132	97.8	<0.023	94.4	1.15	<0.11	<22
S69	Huber Branch	<0.024	0.0215	10.7	0.266	<37	<0.023	78.1	0.87	<0.11	<22
S70	Blue Spring	<0.024	0.0176	9.59	0.160	<37	<0.023	96.5	1.13	<0.11	<22
S71	Keyhole Spring	0.102	0.0820	9.23	0.160	<37	<0.023	106.1	0.91	<0.11	<22
S72	Briar Spring	<0.024	0.151	11.3	0.157	<37	<0.023	110.7	<0.79	<0.11	<22
S73	Ball Mill Spring	<0.024	0.0162	10.9	0.212	<37	<0.023	105.4	1.03	<0.11	<22

Table A-5. Continued.

Sample ID	Location	Ni	P	Pb	S	Ti	Tl	V	Zn	Br^-	*o*-PO_4-P	DOC
		µg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L
S63	Mystery Entrance	<43	1.48	<41	1.42	9.65	<17	<47	<9.7	<0.08	1.17	12.9
S64	Apple Creek Shrine	<43	0.188	<41	5.80	<0.56	<17	<47	<9.7	<0.08	0.103	2.97
S65	Scholl Creek	<43	0.175	<41	10.4	<0.56	19.5	<47	<9.7	<0.08	0.109	2.57
S66	Dry Fork	<43	0.182	<41	7.24	<0.56	<17	<47	<9.7	<0.08	0.076	2.18
S67	Thunder Hole	<43	0.310	43.8	5.49	<0.56	<17	81.5	<9.7	<0.08	0.168	4.56
S68	Mystery Resurgence	<43	0.705	<41	3.80	3.84	<17	<47	<9.7	<0.08	0.465	8.21
S69	Huber Branch	<43	0.155	<41	6.82	<0.56	<17	<47	<9.7	<0.08	0.095	2.37
S70	Blue Spring	<43	0.207	<41	6.65	<0.56	<17	<47	<9.7	<0.08	0.110	2.27
S71	Keyhole Spring	<43	0.226	<41	6.32	<0.56	<17	<47	<9.7	<0.08	0.104	3.37
S72	Briar Spring	<43	0.201	<41	8.84	<0.56	<17	<47	<9.7	<0.08	0.086	2.45
S73	Ball Mill Spring	<43	0.230	<41	7.58	<0.56	<17	<47	<9.7	<0.08	0.137	2.20

Table A-5. Concluded.

Sample ID	Location	calculated TDS	Hardness	TC	*E. coli*	Enterococci
		mg/L	mg/L	cfu/100 mL	cfu/100 mL	cfu/100 mL
S63	Mystery Entrance	103	86	>2419	>2419	120
S64	Apple Creek Shrine	253	239	2419	15	1
S65	Scholl Creek	355	321	1,300	91	30
S66	Dry Fork	297	275	>2419	816	38
S67	Thunder Hole	260	241	>2419	649	1300
S68	Mystery Resurgence	170	158	2419	192	80
S69	Huber Branch	287	244	2419	2419	4
S70	Blue Spring	270	232	>2419	1413	34
S71	Keyhole Spring	264	242	>2419	56	12
S72	Briar Spring	311	284	>2419	135	72
S73	Ball Mill Spring	320	305	>2419	276	1203