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I am submitting herewith a thesis written by Ryan Michael Ragsdale entitled “Bacterial Source Tracking in an Eastern Tennessee Stream using *Bacteroides* Host Associated Real-Time Polymerase Chain Reaction Assays.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental and Soil Science.

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**Bacterial Source Tracking in an Eastern Tennessee Stream using *Bacteroides* Host
Associated Real-Time Polymerase Chain Reaction Assays**

A Thesis
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Ryan Michael Ragsdale
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ABSTRACT

A bacterial source tracking study using *Bacteroides* host associated real-time PCR assays was performed to determine the sources of fecal contamination in Pond Creek (HUC 06010201013). Pond Creek, located in the Ridge and Valley physiographic region in Eastern Tennessee, is a 303(d) listed stream that fails to meet water quality standards for pathogens. Water samples and discharge were measured monthly at eight locations from November 2005 to November 2006. Grab samples were analyzed for several chemical parameters and for microbial fecal indicator organisms, namely *Bacteroides* spp., *Escherichia coli*, and *Enterococcus*. The objectives of the study were to quantify total, human, and bovine associated *Bacteroides*. Additionally, we investigated spatial and temporal variation of fecal indicator organisms and created load duration curves for each sampling site.

The results showed that *Escherichia coli* concentrations regularly exceeded water quality standards. *Bacteroides* host associated real-time PCR assays indicated that cattle were the dominant source of fecal pollution (99 percent of total *Bacteroides*). Although human-associated *Bacteroides* were detected, their concentrations remained relatively low across the watershed. Load data show that fecal contamination from bovine sources occurs at elevated levels throughout the watershed; no statistical differences between sites were observed for bovine associated *Bacteroides* (BoBAC) and *Bacteroides* spp. belonging to the *Bacteroides* genus (AllBAC) loads. Additionally, bovine-associated *Bacteroides* concentrations were very highly correlated ($r^2 = .903$) with the total *Bacteroides* concentrations. Load Duration Curves (LDCs) also indicated extensive bovine fecal pollution. Load data separated into human and bovine LDCs showed that

Escherichia coli loads from bovine sources were mostly flow dependent whereas human associated *Escherichia coli* loads were generally flow independent.

Temporal variations followed seasonal weather patterns; mean loads of all fecal indicators, except *Enterococcus*, were greatest during the months of highest precipitation and lowest in the drier months. No temporal patterns were established for concentrations of fecal indicator organisms. This suggests that runoff transported the majority of fecal inputs to Pond Creek. Best management practices (BMPs) such as improving pastures, nutrient management, proper manure storage, controlling livestock stocking densities, vegetative filter strips, and riparian fencing with careful riparian grazing, should be implemented to reduce fecal inputs from cattle and help Pond Creek meet TMDL guidelines.

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CHAPTER I

INTRODUCTION

To comply with Federal Regulations, the State of Tennessee must implement measures to reduce pollutant loads in contaminated streams on the 303(d) list of impaired streams. One of Tennessee's impaired streams is Pond Creek (HUC 06010201013), located in portions of Loudon, McMinn, and Monroe counties in Eastern Tennessee. Pond Creek and its tributaries (Mud Creek and Greasy Branch) are listed on Tennessee's 2006 303(d) list of impaired waterways for pathogens, specifically for *Escherichia coli* (TDEC, 2006b). Pasture grazing, livestock in the stream, and animal feeding operations are believed to be the primary sources of the pathogens (TDEC, 2006b). The State of Tennessee must create total maximum daily load (TMDL) guidelines in an effort to reduce pathogen inputs to Pond Creek. To facilitate pathogen TMDL development, it is necessary to know all the sources of fecal contamination.

Recent advances in microbial source tracking (MST) have made it possible to identify the origin of fecal contamination by using *Bacteroides spp.* (Bernhard and Field, 2000a; Bernhard and Field, 2000b; Bernhard et al. 2003; Dick and Field, 2004; Dick et al. 2005; Kreader, 1998; Seurinck et al. 2005; Layton et al., 2006; Reischer et al., 2007). Additionally, the use of real-time polymerase chain reaction (qPCR) assays allows quantification of fecal indicator organisms, not just their presence or absence in a water sample (Layton et al., 2006; Reischer et al., 2007). Microbial source tracking using *Bacteroides* host associated real-time PCR assays allows estimation of the total fecal

contamination in addition to the amount of bovine- and human- associated fecal contamination in water samples.

The primary goal of this research was to quantify total, human, and bovine associated *Bacteroides* in Pond Creek and to understand spatial and temporal patterns of fecal indicator organisms (*Bacteroides spp.* and *Escherichia coli*). *Escherichia coli* loads were plotted on load duration curves to show how their concentrations vary with streamflow. Spatial variations in *Bacteroides* between sample sites were examined to help locate pathogen hotspots. Overall, this information will help guide the design and implementation of cost effective best management practices. More specifically, data gathered by this project will help partnering agencies such as the Tennessee Department of Environment and Conservation (TDEC) and the Tennessee Valley Authority (TVA) develop implementation plans to meet total maximum daily load (TMDL) guidelines for Pond Creek. Objectives of this study were to:

- 1) Quantify total, human, and bovine associated *Bacteroides* in Pond Creek.
- 2) Understand spatial and temporal patterns of *Bacteroides* and *Escherichia coli*.
- 3) Develop Load Duration Curves for all sample sites.
- 4) Identify areas to target remediation efforts.

CHAPTER II

LITERATURE REVIEW

Point vs. Non-point Regulations

The first attempts to regulate water pollution in the United States began in 1948. In 1972 the United States Congress revised the regulations and passed the Federal Water Pollution Control Act (Copeland, 2001). After amendments in 1977, the legislation became known as the Clean Water Act (CWA) (USEPA, 2002b). The CWA serves as the foundation for regulations aimed at protecting water quality in the United States (Copeland, 2001). The purpose of the CWA is to restore or maintain the chemical, physical and biological integrity of the nation's waters so they can support the protection and propagation of fish, shellfish, and wildlife and recreation in and on the water (USEPA, 2002b).

The CWA charged the United States Environmental Protection Agency (EPA) with the task of implementing pollution control programs by setting standards for pollutants in surface waters (USEPA, 2002b). Additionally, the CWA made it illegal to discharge pollutants from a point source into navigable waterways without a permit (USEPA, 2002b). This permitting program is described in section 402 of the CWA, and is known as the National Pollutant Discharge Elimination System (NPDES)(USEPA, 2002b). The NPDES program sets limit on the quantities and types of pollutants that can be discharged from discrete and identifiable sources into waterways of the United States (USEPA, 2002b). Moreover, it requires monitoring and reporting of discharges to the EPA (USEPA, 2002b).

Section 502 of the CWA defines a point source as “any discernible, confined and discrete conveyance, including but not limited to any pipe, ditch, channel, tunnel, conduit, well, discrete fissure, container, rolling stock, concentrated animal feeding operation, or vessel or other floating craft, from which pollutants are or may be discharged” (USEPA, 2002b). This term does not include agricultural storm water discharges and return flows from irrigated agriculture (Houck, 1999). A non-point source (NPS) is anything that does not fit in the definition of a point source, and is generally considered to be runoff from land, drainage, seepage, or atmospheric deposition (USEPA, 1999). Non-point source pollution results from pollutants being carried by rain or snowmelt over and/or through the ground and deposited into streams, lakes, estuaries, oceans, or groundwater (USEPA, 1999).

The CWA has been effective at controlling surface water pollution from point sources (USEPA, 1996). However, non-point sources continue to pollute surface waters, and they are difficult to regulate (USEPA, 1996). Non-point source pollution was addressed in 1987 when Congress amended the CWA to include section 319 (USEPA, 2002b). However, the EPA has no authority to regulate NPS pollution (USEPA, 1996). Section 319 delegates NPS regulation to individual states (USEPA, 1996). The EPA funds grants to help states and management agencies implement programs and practices aimed at reducing NPS pollution (USEPA, 1996).

Approximately 40 percent of waters nationwide are affected by NPS pollution (TDEC, 2006d). In Tennessee, 15,060 stream kilometers or 15 percent of assessed waters are impaired or threatened (TDEC, 2006c). The causes of impairment are summarized in Figure 1. Pathogens, sediment, and habitat alteration are the leading causes of impairment

in Tennessee (TDEC, 2006c). Nationwide, the most common NPS pollutants are sediment and nutrients (USEPA, 2000). Depending on the watershed pesticides, pathogens, salts, oil grease, toxic chemicals, or heavy metals can also be pollutants (USEPA, 1996). These pollutants are carried from agricultural land, animal feeding operations, construction sites, urban areas, and disturbed lands (USEPA, 1996). Non-point source pollution can cause environmental and human health problems in addition to aquatic habitat destruction, fish kills, and beach closures (USEPA, 1996).

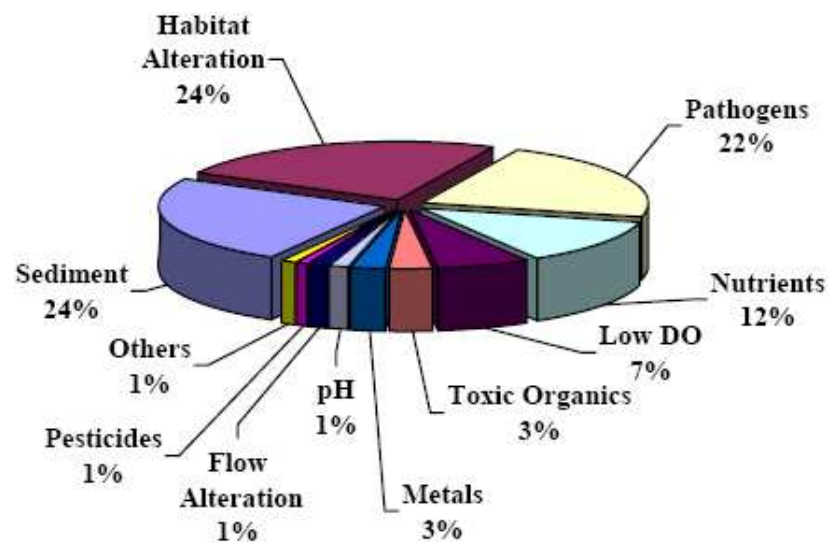


Figure 1. Leading causes of water quality impairments in Tennessee in 2006. Courtesy of TDEC. (TDEC, 2006c).

Urban NPS pollution results from storm water runoff, septic tank failures, and construction activities (USEPA, 1996). The extent of urban NPS pollution is a function of land use, and the amount of impervious surfaces, number of vehicles, intensity of industrial activities, and other factors such as slope, soil type, and climate (Ventura and Kim, 1993). Schueler (1995) reports that as little as 10 percent impervious cover has been linked to stream impacts which increase in severity as impervious cover increases.

Agriculture is generally considered to be the largest source of NPS pollution in the United States (Tim and Jolly, 1994; USEPA, 1996). The main pollutants coming from agricultural lands include sediments, nutrients, pesticides, and fecal coliforms (Farrell-Poe et al. 1997). The amount of NPS pollution generated by a site depends on physical features like vegetation, soil type and slope (Tim and Jolly, 1994).

Management practices can greatly increase the amount of pollution coming from agricultural lands (Tim and Jolly, 1994). Allowing cattle free access to streams causes stream bank destabilization and loss of the riparian buffer zone, which increases the amount of nutrients and pathogens entering waterways from pasture (Agouridis et al, 2005). Overgrazing pastures exposes soil and also makes it easier for nutrients and pathogens to move overland to waterbodies (Krivak, 1978).

Animal feeding operations (AFO) are another source of NPS pollution. These are areas where livestock are kept and raised in confined situations. Pollution from AFOs increases as the number of animals increases (USEPA, 1999). In fact, beef cattle operations with more than 1,000 confined animals are considered a point source of pollution, and are called Concentrated Animal Feeding Operations (TDEC, 2006c). They are required to get a NPDES permit (USEPA, 2002b).

One of the biggest problems with livestock operations, especially the larger ones, results from the manure generated by the livestock (Mancl and Veehuizen, 2006, Agouridis, 2005). Manure and wastewater can contribute pollutants such as nitrogen, phosphorus, pathogens, organic matter, sediments, heavy metals, hormones, antibiotics, and ammonia. In 1989 pollution of waterways by animal manure caused 16 fish kills (over 20,000 fish) and has been implicated in 19 other investigations in Ohio alone (Mancl and Veehuizen, 2006). Animal manure is also a source of pathogens in waterways (USEPA, 1996). Many studies show strong correlation between the presence of cattle with pollution of adjacent waterways or increases in fecal indicator organisms from background levels after cattle are introduced (Milne, 1976; Stephenson and Street, 1978; Gary et al., 1983; Howell et al., 1995; Line, 2003).

Water Quality Standards

Section 305(b) of the CWA requires an assessment of water quality in every state. Many states use a rotating basin approach to assess water quality, which means streams are assessed at least once during a three to five year period. The Tennessee Department of Environment and Conservation (TDEC) published the reports titled 2006 305(b) Report Status of Water Quality in Tennessee and Year 2006 303(d) List in accordance with the CWA and the Tennessee Water Quality Control Act. Tennessee water quality standards have three sections. The first defines the designated uses of the state's waterways. The second section establishes the numeric pollution limits for each stream use class. The third section is designed to protect existing water uses and prevent future abuses (TDEC, 2006b).

All waterbodies are classified for multiple uses, which may or may not have the same water quality standards. When multiple standards are applied to the same waterway, the most stringent standard must be met. Figure 2 presents the stream use classifications in Tennessee. All streams, rivers, lakes, and reservoirs are classified for at least two public uses: recreation and protection of fish and aquatic life, which also complies with the federal CWA. Designated uses do not necessarily indicate that a waterway is currently used for that activity. Rather, it sets a goal to preserve the ability for that waterway to support a certain use in the future (TDEC, 2006b).

After assessment, waterways are classified in one of three ways: 1) Fully supporting its designated use, 2) Partially supporting designated uses, and 3) Not supporting designated uses. A waterbody not supporting its designated uses is said to be “water quality limited,” and placed on the 303(d) list (TDEC, 2006b). The 303(d) list is a compilation of all the streams and lakes that are water quality limited for specific pollutants or are expected to exceed water quality standards within the next two years. Once a waterbody is placed on the 303(d) list, it is targeted for water quality improvement programs and establishment of total maximum daily load (TMDL) guidelines. Moreover, additional loadings of the pollutant into the waterwbody cannot be permitted by the State (TDEC, 2006a).

Bacteriological water quality standards for Pond Creek must meet limits established for recreational use (TDEC, 2006b).

- | |
|---|
| <ol style="list-style-type: none">1. Fish and Aquatic Life2. Recreation3. Irrigation4. Livestock Watering and Wildlife5. Drinking Water Supply6. Navigation7. Industrial Water Supply |
|---|

Figure 2. Tennessee's Stream Use Classifications.

According to the *State of Tennessee Water Quality Standards, Chapter 1200-4-3, General Water Quality Criteria, October, 2007*, the concentration of the *Escherichia coli* group shall not exceed 941 colony forming units (CFU) 100mL⁻¹ for monthly grab samples or 126 CFU 100ml⁻¹ for the geometric mean of at least five samples in a 30 day period.

Pathogens and Fecal Indicator Organisms

The USEPA describes a pathogen as a microbe that causes disease in humans, animals, plants, or other microorganisms (USEPA, 2006). Pathogens can be classified as bacteria, protozoans, viruses, and certain types of fungi or parasitic worms (USEPA, 2006). Important bacterial pathogens include *E. coli* O157:H7, *Salmonella*, *Shigella*, *Campylobacter jejuni* and *Vibrio cholerae* (Maier et al., 2000). In May 2000 an outbreak of *E. coli* O157:H7 occurred in Walkerton, Ontario in Canada killing 7 people and sickening more than 2,300. The cause of the outbreak was due to cattle manure being land applied and entering the groundwater near the town's well (O'Connor, 2002). *Campylobacter jejuni* is the leading cause of bacterial diarrheal illness in the United States, more than *Shigella* and *Salmonella* spp. combined (CDC, 2005).

Protozoans of concern include *Cryptosporidium*, *Giardia lamblia*, and *Entamoeba histolytica*. *Cryptosporidium* is transmitted by ingestion of oocysts excreted in human or animal feces (Maier et al., 2000). In 1993 *Cryptosporidium parvum* caused the largest documented waterborne disease outbreak in United States history. Over 403,000 people suffered from gastroenteritis, and more than 100 people died. The outbreak was blamed on a failed filtration process at the Milwaukee Wisconsin treatment plant (Corso et al., 2003).

Compared to bacteria and protozoans, fewer waterborne disease outbreaks in the United States are attributed to viruses. (Figure 3) (American Chemistry Council, 2003). Infectious viruses found in water systems include Enterovirus, Rotavirus, Hepatitis A, and Reovirus (Bosch, 1998).

From 1991 to 2000, there were 155 outbreaks and 431,846 cases of illness in public and individual U.S. water systems (American Chemistry Council, 2003). Table 1 is a compilation of waterborne disease outbreak data from the Center for Disease Control from 1991 to 2000. It lists reported outbreaks, their causes, the number of cases of associated illness reported, and the types of water systems affected. The actual number of cases may be higher due to underreporting. Additionally, many acute gastrointestinal disease events have unknown etiology (Ford, 1999).

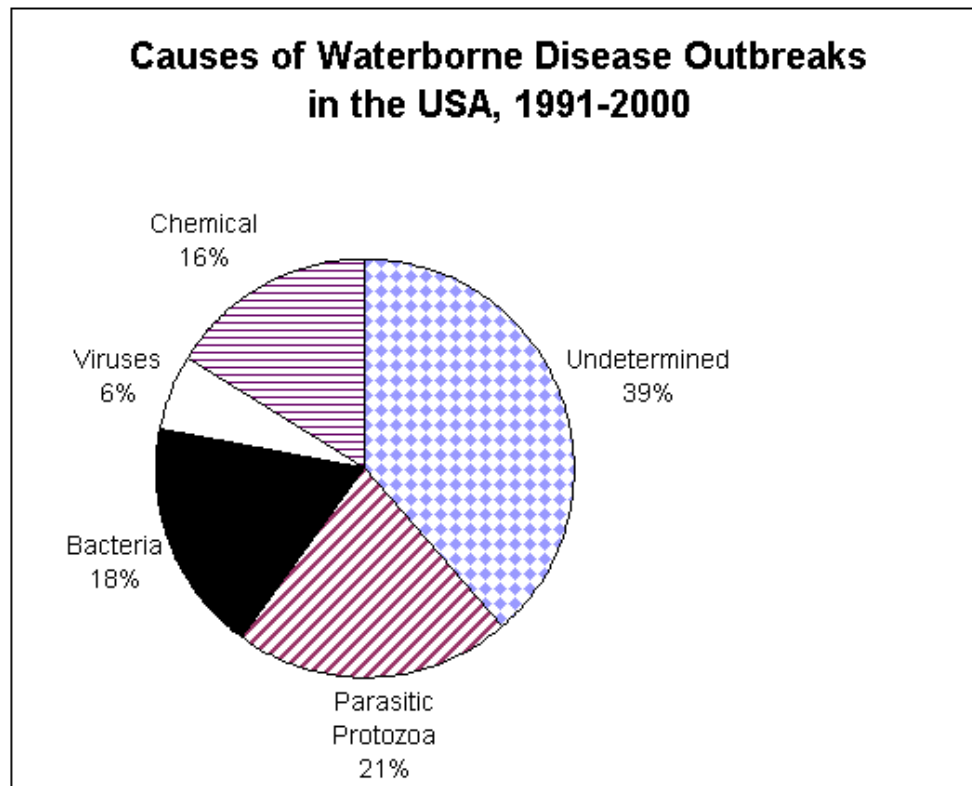


Figure 3: Causes of Waterborne Disease Outbreaks in the USA, 1991 – 2000.
(American Chemistry Council, 2003).

Table 1. Causes of Waterborne Outbreaks, by Type of Water System, 1991-2000. Courtesy of American Chemistry Council, 2003.

Etiological Agent	Community Water Systems ³		Noncommunity Water Systems ³		Individual Water Systems ⁴		All Systems	
	Outbreaks	Cases	Outbreaks	Cases	Outbreaks	Cases	Outbreaks	Cases
<i>Giardia</i>	11	2,073	5	167	6	16	22	2,256
<i>Cryptosporidium</i> *	7	407,642	2	578	2	39	11	408,259
<i>Campylobacter</i>	1	172	3	66	1	102	5	340
<i>Salmonella</i> , nontyphoid	2	749	0	0	1	84	3	833
<i>E. coli</i>	3	208	3	39	3	12	9	259
<i>E. coli</i> O157:H7/C <i>jeuni</i>	0	0	1	781	0	0	1	781
<i>Shigella</i>	1	83	5	484	2	38	8	605
<i>Plesiomonas shigelloides</i>	0	0	1	60	0	0	1	60
Non-O1 <i>V. cholerae</i>	1	11	0	0	0	0	1	11
Hepatitis A virus	0	0	1	46	1	10	2	56
Norwalk-like viruses	1	594	4	1806	0	0	3	2400
Small, round-structured virus	1	148	1	70	0	0	2	218
Chemical	18	522	0	0	7	9	25	531
Undetermined	11	10,162	38	4,837	11	238	60	15,237
Total	57	422,364	64	8,934	34	548	155	431,846

¹Data in Table 1 are compiled from CDC Morbidity and Mortality Weekly Report Surveillance Summaries for 1991-1992, 1993-1994, 1995-1996, 1997-1998 and 1999-2000. Figures include adjustments to numbers of outbreaks and illness cases originally reported, based on more recent CDC data.

²Community water systems are those that serve communities of an average of at least 25 year-round residents and have at least 15 service connections.

³Non-community water systems are those that serve an average of at least 25 residents and have at least 15 service connections and are used at least 60 days per year.

⁴Individual water systems are those serving less than 25 residents and have less than 15 service connections.

*There were 403,000 cases of illness reported in Milwaukee in 1993.

Despite the need to know if pathogens are in the drinking water supply or surface waters, most regulatory agencies test for fecal indicator organisms (FIO) instead of pathogens (Maier et al., 2000). It is too difficult and expensive to test for specific pathogens on a routine basis. The inherent assumption is that if FIO are present in a water sample, enteric pathogens may also be present. There are several different species used, but a good indicator possesses the following characteristics: 1) environmental survival and transport is similar to pathogen behavior, 2) strong correlation with pathogen presence in water samples, 3) rapidly detected, 4) easily enumerated, and 5) nonpathogenic (Scott et al., 2002). Total coliforms, fecal coliforms, *Escherichia coli*, and *Enterococcus* spp. have all been used as indicator organisms (Scott et al., 2002; Maier et al., 2000).

The coliform group consists of all the aerobic and facultatively aerobic, gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35°C (Brock and Madigan, 1991). Absence of this group of organisms in drinking water samples ensures the prevention of bacterial waterborne disease outbreaks (Maier et al., 2000). However, members of the coliform group have been observed to regrow in natural surface and drinking water distribution systems (Gleeson and Gray, 1997). It should be noted that coliforms are naturally present in the environment, so their presence in a water sample does not guarantee that pathogens are present (USEPA, 2002a).

Fecal coliforms (FC) are generally members of the *Escherichia* and *Klebsiella* genera and are defined by their ability to ferment lactose with the production of acid and gas at 44.5°C within 24 hours. They live in the intestines of humans and warm-blooded

animals (Maier et al., 2000). Therefore, their presence in surface water indicates fecal contamination (Scott et al. 2001; Maier et al., 2000). *Escherichia coli* is the principal fecal coliform (Maier et al., 2000). The EPA now recommends that *Escherichia coli* be used as the principle indicator organism in freshwaters, instead of FC. Research has shown *Escherichia coli* densities are strongly correlated with swimming-associated gastroenteritis (USEPA, 2002a). Toranzos (1991) suggests that both coliforms and fecal coliforms occur naturally and regrow in surface water with elevated temperatures and high levels of organic matter. Additionally, *Escherichia coli* and other fecal coliforms can attach to sediment and survive (Stephenson and Rychert, 1982; Howell et al., 1996). Howell et al. (1996) reported lower mortality rates for fecal coliforms in the clay sized sediments than for coarser sediments. There is a potential for *Escherichia coli* concentrations to increase in streams when bottom sediments are resuspended during high flows (Stephenson and Rychert, 1982).

Fecal streptococci belong to the genera *Enterococcus* and *Streptococcus* (Gleeson and Gray, 1997). The genus *Enterococcus* is made up of all the streptococci that are of fecal origin and grow in 6.5 percent sodium chloride and high pH (9.6) and temperature (45°C) (Maier et al, 2000). Enterococci are especially reliable as indicators of health risk in marine environments (Cabelli et al., 1982). In the genus *Streptococcus* there are only two species considered to be true fecal streptococci, *S. bovis* and *S. equines* (Maier et al., 2000). The fecal coliform to fecal streptococci (FC-FS) ratio was formerly used to indicate the relative contribution of human to animal sources of fecal contamination in water samples (Scott et al., 2002). A ratio of 4 or more indicated human fecal pollution, where as a ratio below 0.7 indicated contamination by animal sources (Geldreich and

Kenner, 1969). However, this method is no longer accepted for use in source tracking (Pourcher et al., 1991).

Total Maximum Daily Loads (TMDLs)

In accordance with the CWA, waterbodies on the 303(d) list must undergo TMDL assessment (TDEC, 2006b). A TMDL is a calculation of the maximum amount of a pollutant that a waterbody can receive and still meet water quality standards (USEPA, 2002c). A TMDL is a tool to develop controls for reducing pollution from both point and non-point sources (USEPA, 2002c). A TMDL is explained mathematically in the following equation:

$$\text{TMDL} = \sum \text{LA} + \sum \text{WLA} + \text{MOS}$$

Where $\sum \text{LA}$ = Load Allocation
 $\sum \text{WLA}$ = Waste load Allocation
MOS = Margin of Safety

The load allocation refers to existing and future non-point sources and natural background levels. Waste load allocation corresponds to existing and future point sources. The margin of safety (MOS) attempts to account for the uncertainty of many estimates that are sometimes required in TMDL development. Two approaches for MOS are available, implicit and explicit. The former uses assumptions from a conservative model while the later reserves part of the total TMDL as the MOS and uses the rest for

allocations. Tennessee uses the explicit approach by employing a 10 percent MOS (TDEC, 2006e).

After states develop a TMDL for a watershed, it must be submitted to the EPA just as states must submit a listing of impaired waterways. If the EPA does not approve the TMDL, the EPA is responsible for creating an appropriate TMDL (USEPA, 2002c). Although this law has been on the books since 1972, it was not enforced initially (Brader, 2006). Lawsuits by citizen groups in the 1990s forced the EPA to enforce surface water regulations concerning TMDLs (Brader, 2006; Leclair, 1997). Total maximum daily load plans are simply guidelines; they do not include implementation plans that suggest methods to actually reduce pollutant loads (TDEC, 2006e). Best management practice implementation plans are critical to restore water quality and meet TMDL guidelines (Agouridis et al., 2005).

Microbial Source Tracking

Historically, bacteriological water quality testing was limited to testing for the presence or absence of fecal indicator organisms (Scott et al., 2002). It was assumed that if fecal indicator organisms were present, there is a chance, but no guarantee, that enteric pathogens were also present. Total maximum daily loads are developed to reduce fecal inputs, but the effectiveness of TMDLs is limited if the source of the pathogens is unknown (Scott et al., 2002).

Microbial source tracking (MST) allows the sources of fecal contamination to be determined (USEPA, 2005). Microbial source tracking is a promising tool for TMDL development and watershed management, but its strengths and weaknesses must be

considered before using MST (Scott et al., 2002). Additionally, with current technologies, there is no single method that can be used for all situations involving fecally contaminated water systems (USEPA, 2005).

Early attempts to classify fecal sources using microbial source identifiers were more general (human vs non-human) (Scott et al., 2002). The fecal coliform to fecal streptococci (FC-FS) ratio is an example (Maier et al. 2000). This method is no longer used, because it was observed that the coliforms and streptococci have different die-off rates (Geldreich and Kenner, 1969). However, that work paved the way for future MST studies. Present day MST methods can be classified into four different categories (Scott et al. 2002, Simpson et al. 2002). The basic categories and examples of each are presented in Figure 4.

Genotypic library-based methods distinguish bacteria based on identifying patterns in the genetic material of bacterial isolates and matching them with libraries from known sources (Scott et al, 2002). Phenotypic library based methods rely on growth patterns produced when bacterial isolates are subjected to antibiotics or grown on differing carbon sources (Griffith et al., 2003). Non-library based culture independent methods differentiate by identifying the presence of genetic markers unique to the fecal bacteria of the targeted host species (Scott et al., 2002).

Several studies have shown the usefulness of MST. Hagedorn et al. (1999) used antibiotic resistance analysis (ARA) of enterococci to determine cattle were responsible for the majority of fecal pollution in a Virginia watershed. Subsequent management efforts resulted in a 94 percent reduction in fecal coliforms (Hagedorn et al., 1999).

- | |
|---|
| <ol style="list-style-type: none">1) Genotypic library based<ul style="list-style-type: none">RibotypingPulsed-field gel electrophoresis (PFGE)Box PCR2) Phenotypic library based<ul style="list-style-type: none">Antibiotic resistance analysis (ARA)Carbon source Utilization3) Non-library based culture independent<ul style="list-style-type: none">Host specific Polymerase Chain Reactiont-RFLPtoxic gene biomarkers4) Direct measurement of human or bacterial viruses |
|---|

Figure 4. Four general categories and examples of microbial source tracking Methods.

Boehm et al. (2003) combined measurements of fecal indicator bacteria with detection of human-specific markers for *Bacteroides* and enterovirus to identify waters contaminated with human sewage.

There are disadvantages associated with all the methods as well. The library-based methods require the formation of an extensive database, which requires a lot of time and labor (Simpson, 2002). Culture based methods like ARA cannot produce rapid results (Scott et al, 2002); they often take several days to complete. This can be important during time sensitive situations, such as during beach closures. Scott et al. (2002) cites several other disadvantages. The first is that library-based methods may only work in the watershed in which they are developed. They go on to report that some methods are too complex for widespread acceptance or use by relatively untrained workers and that some methods are prohibitively expensive. Additionally, temporal or spatial characteristics of the indicator organism itself can cause inaccurate results. Perhaps the greatest concern regarding MST studies is the lack of comparative studies and blind testing performed on

the numerous methods (Griffith et al., 2003). Without this information it is difficult for regulatory agencies to decide which methods to devote their resources to. Currently, no single method can be completely rejected or universally recommended (Stoeckel and Harwood, 2007). Depending on the goal of the research, a combination of several methods usually works best (Scott et al, 2002; Griffith et al. 2003). Researchers must accurately define the problem they hope to address with their particular method, including weighing the tolerance for an incorrect answer (Griffith et al., 2003). Stoeckel and Harwood (2007) recommend common performance measures and validation strategies of all MST methods in order to improve expand the use and accuracy of MST.

A comparative study between 12 different methods conducted in 2002 provided interesting results. No MST method predicted the source material in blind samples perfectly, and every method performed poorly in identifying all sources of contamination. Some methods performed better than others, but host-specific PCR performed best at differentiating between human and non-human sources. Additionally, host-specific PCR had false positive rates for human source material at or near zero (Griffith et al., 2003). Host-specific PCR is also advantageous, because it does not require a culturing step and samples can be analyzed in just a few hours (Scott et al., 2002).

Bacteroides

Several host associated polymerase chain reaction (PCR) methods are using *Bacteroides spp.* as an indicator organism instead of traditional indicators such as *Escherichia coli* (Allsop and Stickler, 1985; Fiksdal et al., 1985; Kreader, 1998; Layton et al., 2006). The traditional fecal indicator organisms have been shown to grow and

survive in the environment (Toranzos, 1991), so their effectiveness as indicators has been questioned (Scott et al., 2002). *Bacteroides spp.*, however, are fecal anaerobes, and do not regrow in the environment (Allsop and Stickler, 1985; Fiksdal et al., 1985; Kreader, 1998). *Bacteroides spp.* also compose 10 percent of fecal mass (Layton et al., 2006). Additionally, due to differences in physiology (digestive tracts) between host animals, *Bacteroides spp.* have a high degree of host specificity (Dick et al., 2005). Others have reported that species with similar diets (humans and swine) may have closely related *Bacteroides* species present in their feces (Layton et al., 2006).

Microbial source tracking studies using *Bacteroides* target specific diagnostic sequences within the *Bacteroides* 16S rRNA gene present in feces from different animals (Bernhard and Field, 2000a; Bernhard and Field, 2000b; Bernhard et al. 2003; Dick and Field, 2004; Dick et al. 2005; Kreader, 1998; Seurinck et al. 2005). Assays are designed that are specific for the host animal. The genetic marker (16S rRNA sequence) is amplified by a PCR step (Scott et al, 2002). Other studies have already developed PCR primers for the 16S rRNA gene to differentiate between human-, bovine-, porcine-, and equine- associated *Bacteroides* 16s rRNA genes (Bernhard and Field, 2000a, Kreader, 1995, Layton et al., 2006).

Compared to traditional PCR, real-time PCR (qPCR) with fluorogenic probes is faster and allows genes to be simultaneously identified and quantified; therefore, real-time PCR is advantageous for measuring bacteria in food, water, and fecal and tissue samples (Balenger et al., 2003; Ibeke et al., 2002; Matsuki et al., 2004; Sails et al., 2003; Santo Domingo et al., 2003, Savill et al., 2001). Reischer et al. (2007) performed the first quantitative human-specific MST tool sensitive enough to allow quantitative source

tracking of human fecal impact in karstic spring water. Layton et al. (2006) reported that their AllBAC assay allows estimation of total fecal contamination and the BoBAC assay allows estimation of the amount and percentage of bovine-associated fecal contamination relative to the total fecal contamination. Their HuBAC assay also allows estimation of human-associated fecal contamination, but it has the potential for cross-amplification with dogs and pigs.

In summary, real-time PCR assays without DNA extraction can be used to quantify fecal concentrations and provide preliminary fecal source identification in watersheds (Layton et al., 2006, Reischer et al., 2007). The method is relatively inexpensive and can provide results within 6 hours of being analyzed (Layton et al., 2006).

Load Duration Curves

When used alone, concentrations of fecal indicator organisms provide little information about the origins of pathogens (Cleland, 2002a; Cleland 2002b). To interpret and present water quality data many researchers use load duration curves. Water resource planners have long employed stream flow data to assist with management decisions. Leopold (1994) suggests that a flow duration curve can be used to determine the frequency that a particular flow is equaled or exceeded. A flow duration curve plots stream flow on the y-axis, and the percent of time the flow is exceed on the x-axis (Leopold, 1994). If stream flow is measured while taking grab samples, it is possible to calculate “instantaneous loads.” This load can be plotted in a duration curve format known as a Load Duration Curve, which can help describe the characteristics of

impairment (Cleland, 2002a). Loads that are above the curve exceed allowable levels, while those below the curve indicate compliance. Pollutant loads that are greatest during high flows and decline as flows decrease suggest the pollutant is from a non-point source, and the pollutant is said to be flow dependent (Figure 5). Stormwater runoff is the source of these pollutants (USEPA, 2007). Exceedances that occur under low-flow conditions are attributed to sources directly delivered to the stream such as straight pipes, failing septic systems, and livestock with access to the stream (USEPA, 2007; McPherson, 2002). Flow independent pollutants (Figure 6) tend to be point sources, because their input is more constant (Cleland, 2002a; Cleland 2002b).

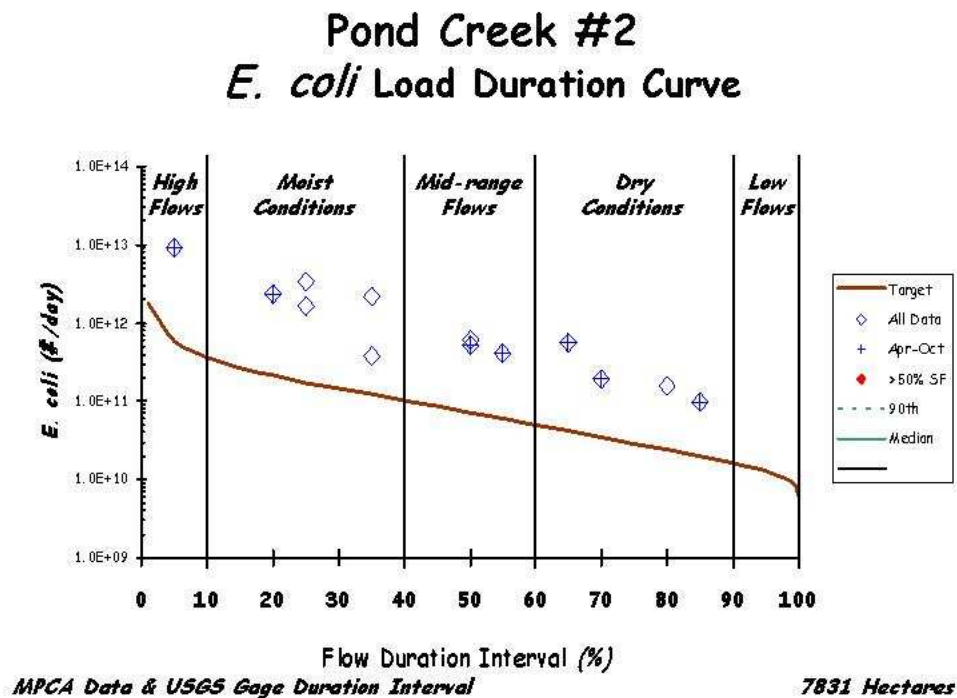


Figure 5. Load Duration Curve with flow dependent data points developed from Sample Site 2 based on a regional flow duration curve and 13 data points collected between November 2005 and November 2006 in Pond Creek, TN (HUC 06010201013).

Pond Creek #2 *E. coli* attributable to Humans Load Duration Curve

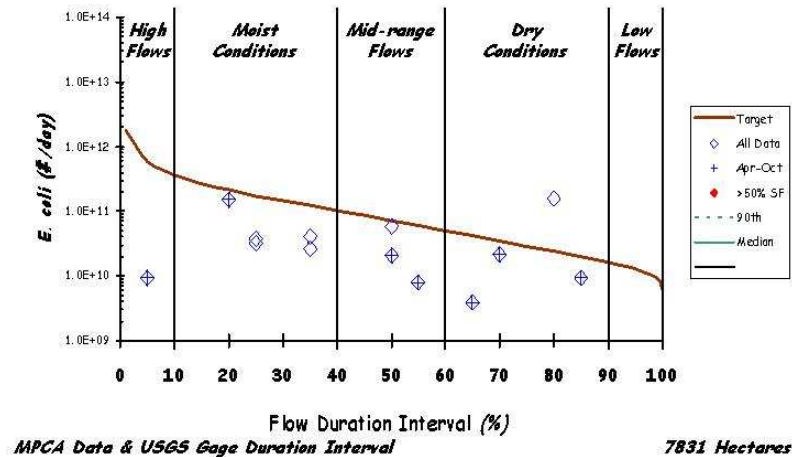


Figure 6. Load Duration Curve with flow independent data points developed from Sample Site 2 based on a regional flow duration curve and 13 data points collected between November 2005 and November 2006 in Pond Creek, TN (HUC 06010201013).

In summary, LDCs provide easy to read information about pollutant loads in streams (Cleland, 2002a; Cleland, 2002b). Specifically, they show if pollutant loads for sampling dates are above or below water quality standards. Examining the general trends among the data points can help explain the source of the pollutant (USEPA, 2007). Pollutant loads that are directly proportional to streamflow are coming from runoff, and are said to be flow dependent (USEPA, 2007; McPherson, 2002). Flow independent pollutants likely come from point sources (USEPA, 2007; McPherson, 2002).

CHAPTER III

MATERIALS AND METHODS

Study Area

The Pond Creek Watershed (HUC: TN06010201013) was chosen for the bacterial source tracking study in order to improve the existing watershed management plan. Pond Creek has been on the 303(d) list since at least 1998. Pond Creek and its tributaries (Mud Creek and Greasy Branch) are listed on Tennessee's 2006 303(d) list of impaired waterways for pathogens, specifically for *Escherichia coli* (TDEC, 2006b). The 303(d) list cites pasture grazing, livestock in the stream, and animal feeding operations as the primary source of the pathogens. Additionally, Pond Creek fails to support its designated use due to physical substrate habitat alterations and high levels of nitrates.

Pond Creek is a small watershed (approximately 10,000 hectares) in the Upper Tennessee Basin of Eastern Tennessee (Watts Bar Watershed). The Pond Creek watershed is located in the Ridge and Valley physiographic region, covering portions of Loudon, Monroe, and McMinn Counties (Figure 7 and 8). The watershed is predominantly agricultural consisting of beef cow-calf and dairy operations typical of the region.

Based on Tennessee Valley Authority (TVA) integrated pollutant source inventory (IPSI) data, Hagan and Walker (2006) estimated that there were 1,960 beef cattle, 960 calves and dry dairy cows, 1,575 mature, lactating dairy cows and 45 horses. Additionally, they report that there are 59 beef cattle and 12 dairy operations in the watershed; the majority of which are located adjacent to Pond Creek. The dairy

operations are all considered large operations. TVA's IPSI report defines large operations as those with more than 150 cows.

Water quality in Pond Creek has been monitored by the University of Tennessee since 2003 at eight different locations. In this study, the same eight sites were sampled monthly from November 2005 until November 2006 (Figure 9). General site descriptions and the top three landuse classes per subwatershed are presented in Table 2.

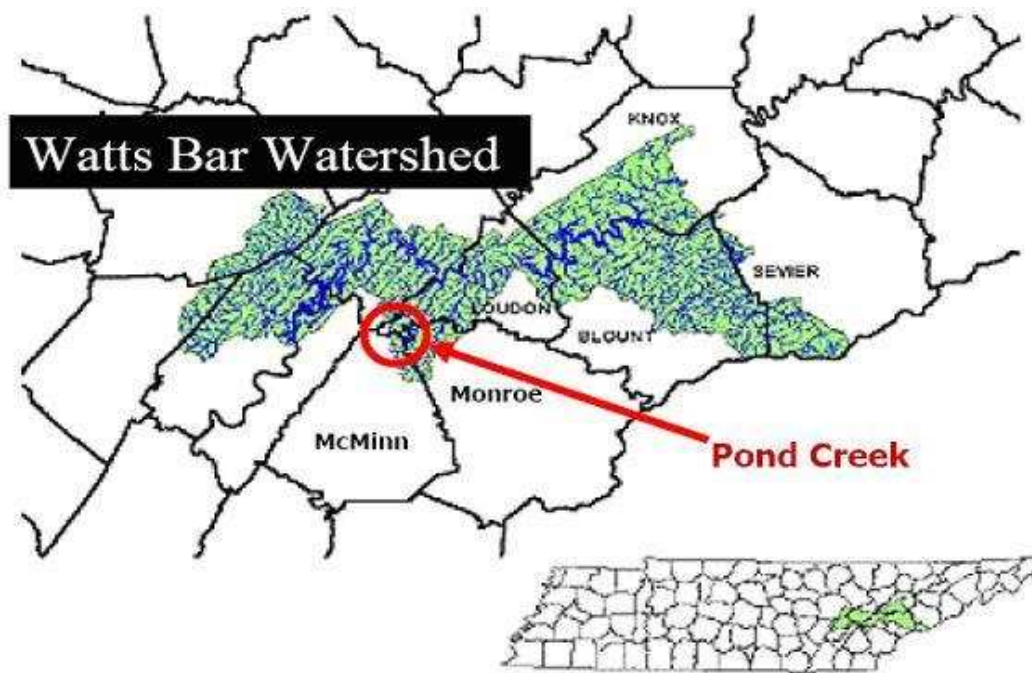


Figure 7. Location of Pond Creek Watershed (HUC 06010201013) in Tennessee. Pond Creek flows into the Tennessee River (Watts Bar Reservoir).

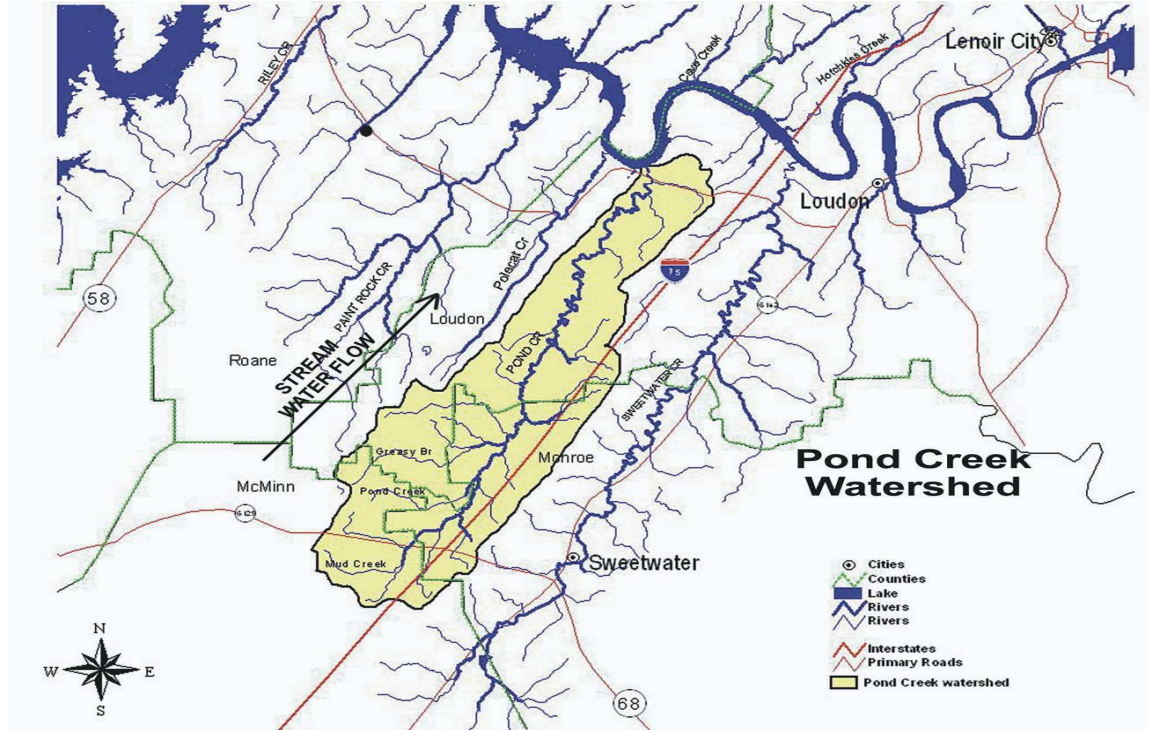


Figure 8. Map of the Pond Creek Watershed (HUC 06010201013) showing its proximity to Sweetwater, TN and Watts Bar Reservoir.

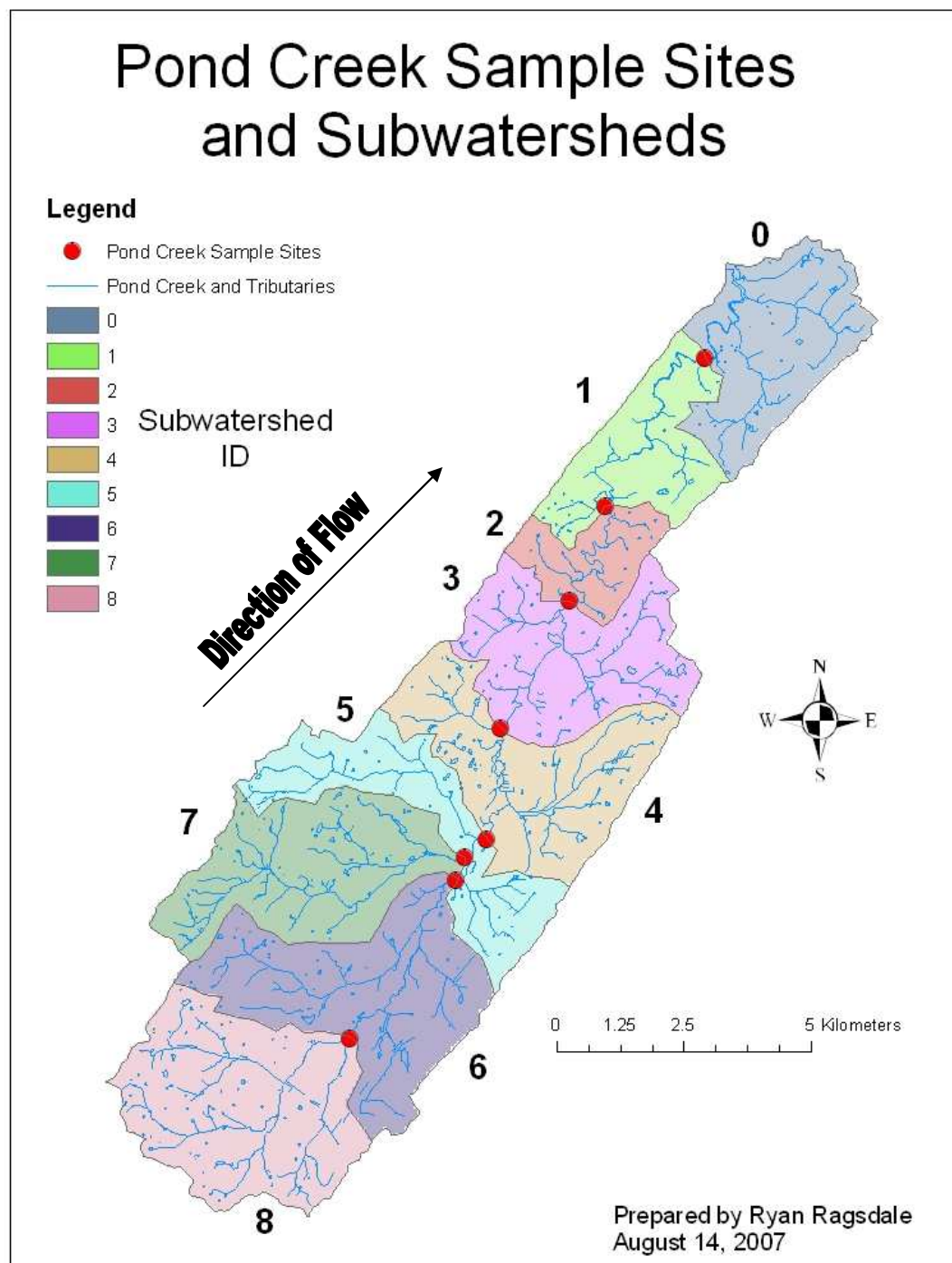


Figure 9. Location of sampling sites in Pond Creek, TN (HUC 06010201013) and subwatershed identification based on data acquired with a handheld GPS device and watershed delineation tools in ArcMap 9.2.

Table 2. General Characteristics of the Pond Creek Watershed (HUC 06010201013) including latitude and longitude of sampling sites, subwatershed drainage area, and the relative percent of each subwatershed the top three land use classes occupy.

Site	Lat (N), Long (W) Decimal Degrees	Drainage Area (Hectares)	Land Use 1	Land Use 2	Land Use 3
			Percent		
1	35.7309 N, -84.43635 W	743.4	FOREST LAND	FAIR PASTURE	HEAVILY OVERGRAZED
			49.5	17.1	15.6
2	35.70494 N, -84.4585 W	416.7	FOREST LAND	HEAVILY OVERGRAZED	FAIR PASTURE
			33.0	27.4	18.4
3	35.68826 N, -84.46643 W	1112.0	HEAVILY OVERGRAZED	GOOD PASTURE	FAIR PASTURE
			25.7	22.1	19.3
4	35.66618 N, -84.48199 W	1200.5	GOOD PASTURE	FOREST LAND	FAIR PASTURE
			22.8	19.9	14.9
5	35.64628 N, -84.48525 W	916.0	FOREST LAND	FAIR PASTURE	HEAVILY OVERGRAZED
			32.7	22.0	16.9
6	35.63905 N, -84.49201 W	1443.5	FAIR PASTURE	FOREST LAND	HEAVILY OVERGRAZED
			25.4	20.3	12.7
7	35.64326 N, -84.49015 W	1227.7	FAIR PASTURE	GOOD PASTURE	FOREST LAND
			32.2	20.4	17.4
8	35.61147 N, -84.51575	1516.2	GOOD PASTURE	FAIR PASTURE	FARMSTEAD
			38.4	17.4	9.2

Reference Streams

The State of Tennessee utilizes ecoregion reference streams as a baseline to compare physical, chemical, and biological quality of streams within an ecoregion. An ecoregion is a relatively homogenous area defined by similarity of climate, landform, soil, potential natural vegetation, hydrology, or other ecologically relevant variables (Denton, 2004). The reference stream is essentially a control stream for the ecoregion. It is a least impacted, but representative, waterbody within an ecoregion that can be monitored to establish a baseline for comparison with other streams in the ecoregion. Reference streams are not necessarily pristine or undisturbed by humans (Denton, 2004). To judge the relative water quality of a stream, it should be compared to the reference stream for that ecoregion. Pond Creek Watershed lies within the Ridge and Valley Ecoregion (67) and the Southern Limestone/Dolomite Valleys and Low Rolling Hills subregion (67f). Wolf Creek is the reference stream for this ecoregion. Wolf Creek flows into Watts Bar Reservoir in Roane County Tennessee. Bacteriological data for Wolf Creek, the reference stream, is presented in Table 3.

Table 3. Bacteriological Data for Ecoregion 67 reference stream (Wolf Creek) from 1996-1999 (TDEC, 2000). Pond Creek is in this Ecoregion.

Organism	Maximum	Minimum	Mean
	CFU / 100 mL		
Fecal Coliform	6700	0	452.4
<i>Escherichia coli</i>	2419	0	222
<i>Enterococcus</i>	1733	0	64.3

Land Use in the Watershed

Generally, Pond Creek is a rural watershed dominated by agriculture. Pasture to support the beef cattle and dairy operations is the dominant land use in the watershed. It makes up 55 percent of the total land area, mostly in the valley and toe slopes of the ridges. The ridge tops are mostly forested with mixed hardwoods and a few small pine plantations. Forested areas make up 29 percent of the land area. Cropland to produce corn for silage and soybeans covers 6.6 percent of the watershed. Residential areas are estimated to only make up 4 percent of the watershed (Hagan and Walker, 2006). Commercial and industrial areas are limited to areas near Interstate 75, and therefore, are a small component of land use. Wetlands and open water are also rare. General land use patterns are displayed in Figures 10 and 11.

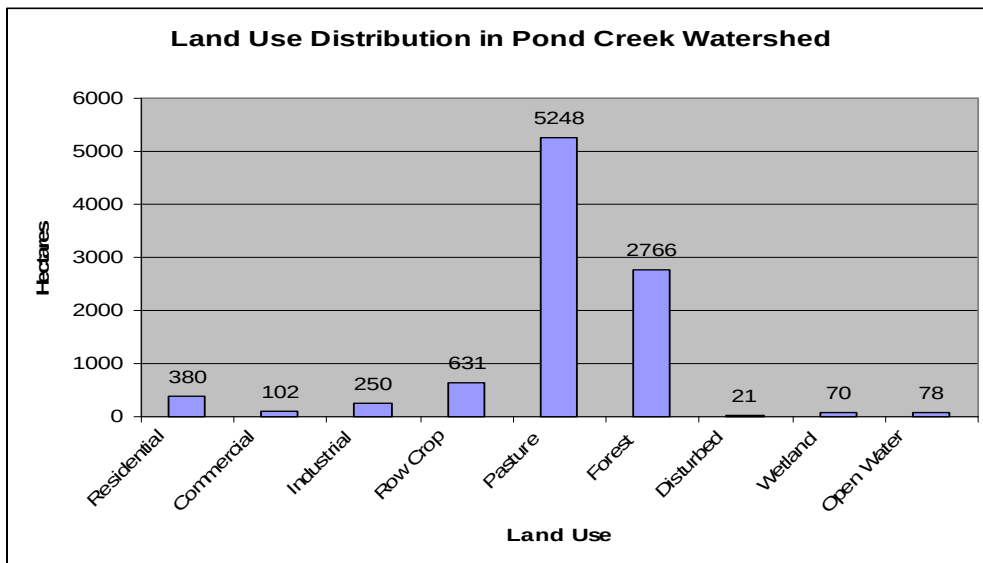


Figure 10. Distribution of Land Use Types in Hectares in Pond Creek, TN (HUC 06010201013) as calculated by the tabulate area function in ArcMap 9.2.

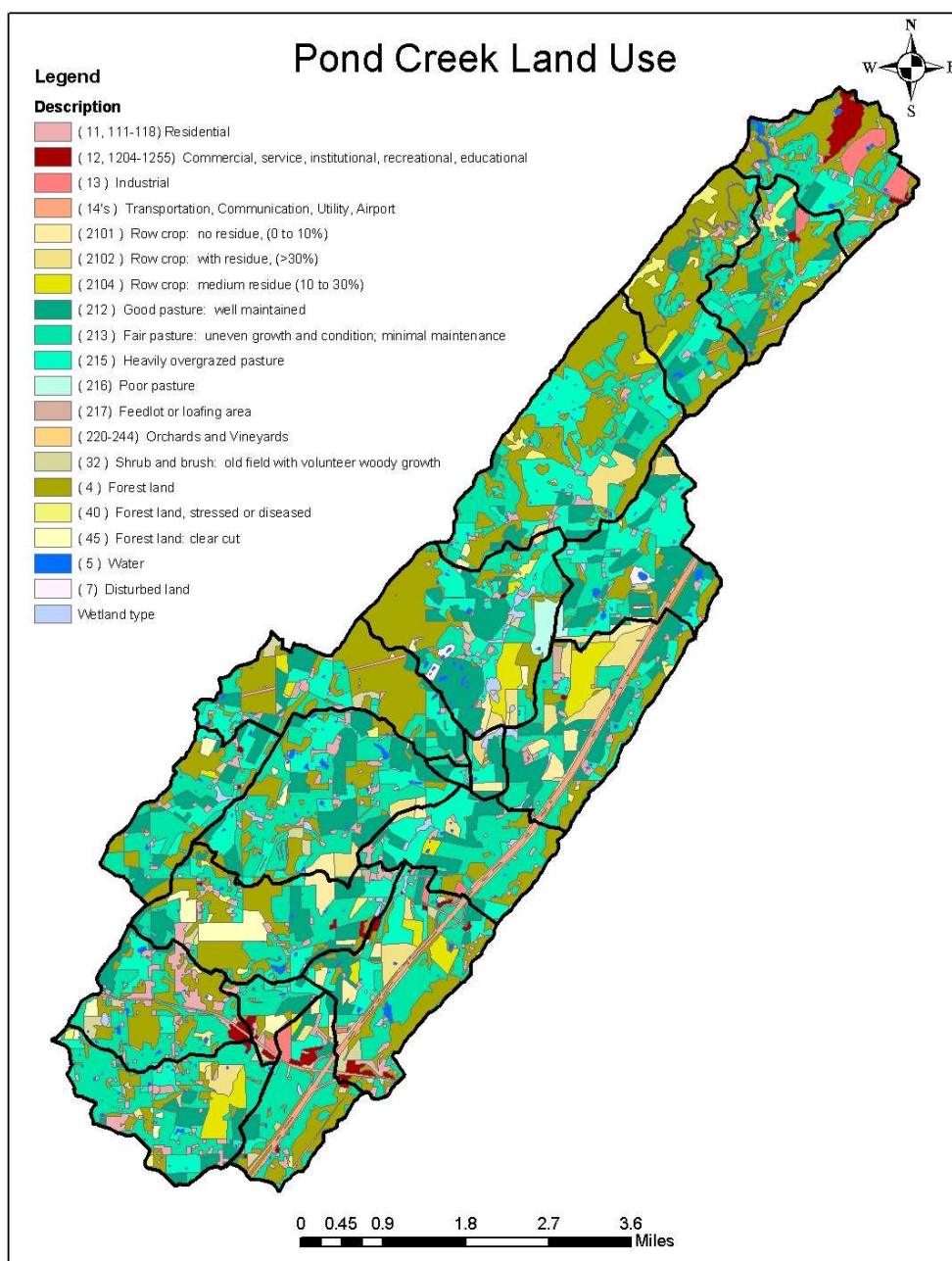


Figure 11. Land Use Map of Pond Creek (HUC 06010201013) generated by the Tennessee Valley Authority's Integrated Pollutant Source Inventory Geographic Database.

Sample Collection and Handling

From November 2005 through November 2006 monthly grab samples were collected at each site (Table 2 and Figure 9) from the mid channel of the stream. To collect the samples, the sampler waded to mid-channel and faced upstream. The sample bottle was then submerged with the opening towards the flow allowing water to enter and fill the bottle. The bottles were rinsed three times in the stream before submerging and filling the bottle for storage. Sample bottles were stored on ice in a cooler until they are brought to the Biosystems Engineering and Soil Science Water Quality Lab at the University of Tennessee, Knoxville within 5 hours of collection.

Two different bottles were used at each site for storing grab samples. A sterilized 250 mL Nalgene bottle was used to store samples for microbial analysis. A one-liter, acid-washed Nalgene bottle for each site stored water for all other procedures. Five-day biological oxygen demand (BOD₅) and microbial analysis began as soon as the samples reached the water quality lab. The bottles then remained under refrigeration until analysis began the following day. Water from Pond Creek was analyzed for: BOD₅, turbidity, total Solids (Dissolved and Suspended Solids), total organic carbon, ammonia, nitrate, total Kjeldahl nitrogen, ortho-phosphate, total phosphorus, chloride, sulfate, and fecal indicator organisms (total coliforms, *Enterococci*, *Escherichia coli*, *Bacteroides spp.*). To store samples for *Bacteroides* analysis, approximately 1 mL of creek water was pipetted using sterile pipette tips from the 250mL Nalgene bottle into sterile 1.5mL Eppendorf® tubes and frozen at -80°C until samples were analyzed.

During the sampling trips, discharge data was also collected. Discharge was calculated by the velocity area method using a Swoffer Model 3000 Flow Meter (Seattle,

WA). Velocity readings were measured at 6/10 depth of the stream on 1-meter intervals unless the last segment was less than 1 meter; irregular segment width was noted and measured accordingly.

Other field data collected on sampling trips were pH, temperature ($^{\circ}\text{C}$), dissolved oxygen (mg L^{-1}), and electrical conductivity (ms cm^{-1}). These parameters were collected by using a YSI Model 600QS (Yellow Springs, OH).

Microbiological Methods

Coliforms and *Escherichia coli*

Total coliforms and *Escherichia coli* were analyzed using Colilert kits from IDEXX Corporation (Westbrook, Maine). The kit uses Defined Substrate Technology® (DST®), allowing simultaneous detection of total coliforms and *Escherichia coli* (IDEXX, 2000). Basically, this method is based on the ability of enzymes produced by the targeted microbe to hydrolyze a specific substrate, which produces a color change (Katamay, 1990). Two nutrient-indicators, o-nitrophenyl- β -D-galactopyranoside (ONPG) and 4-methylumbelliferyl- β -D-glucuronide (MUG), are the major sources of carbon in Colilert and can be metabolized by the coliform enzyme β -galactosidase and the *E. coli* enzyme β -glucuronidase, respectively (IDEXX, 2000). Specifically, as coliforms grow in Colilert, they produce the enzyme β -galactosidase, which hydrolyzes and cleaves the specific substrate ONPG. o-nitrophenol is released in the process, which produces a yellow color (Figure 12).

Similarly, *Escherichia coli* produces the enzyme β -glucuronidase when it grows in Colilert (IDEXX, 2000). The enzyme hydrolyzes and cleaves the specific substrate MUG, resulting in the release of 4-methyl-umbelliferone (IDEXX, 2000). 4-methylumbelliferone fluoresces and can be detected under a blacklight (Figure 13). Most non-coliforms are unable to grow and interfere with the reaction, because they do not have these enzymes (IDEXX, 2000). The few non-coliforms that do have these enzymes are selectively suppressed by Colilert's specifically formulated matrix (IDEXX, 2000).

IDEXX claims this method is advantageous to other methods that grow both target and non-target organisms, because it reduces the number of false positives that are reported. Additionally, the use of salts, detergents, or other selective agents to suppress non-targets can inadvertently suppress target organisms, leading to false negatives (IDEXX, 2000).



Figure 12. Colilert ONPG reaction from IDEXX literature.

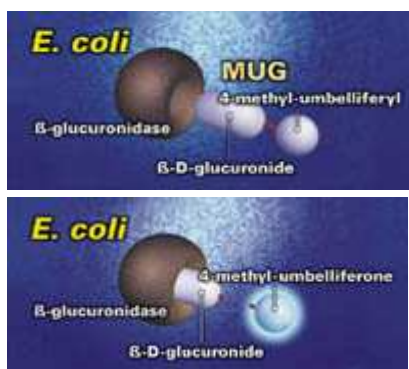


Figure 13. Colilert MUG reaction from IDEXX literature.

To prepare the samples for analysis in the Colilert Kit, the original sample bottle (sterile, 250 mL Nalgene) containing the grab sample was agitated by hand for 30 seconds. Then, 100mL of sample water was poured into a sterile, labeled bottle supplied by IDEXX. One package of Colilert reagent was then added to the 100mL sample. After which the bottle was capped and agitated by hand until the Colilert reagent had dissolved. The sample was poured into a Quanti-Tray® 2000 and sealed with a Quanti-Tray® Sealer. After being sealed, the trays were incubated at 35°C for 24 ± 4 hours. The following day the Quanti-Trays were compared to the reference color comparator provided with the media. Total coliforms were detected if the wells had a yellow color greater or equal to the comparator wells. After determining total coliform levels, the Quanti-Tray was placed in a UV-light box to look for fluorescence. Wells that fluoresced under UV light were positive for *Escherichia coli*. Most probable number (MPN) counts of total coliform and *Escherichia coli* per 100 mL were determined by counting the number of positive large and small wells and looking at the appropriate place on the table.

The IDEXX Quanti-Tray 2000 is a semi-automated quantification method based on the Standard Methods Most Probable Number (MPN) model. The Quanti-Tray 2000 is considered an autodilution, because the large wells hold ten times the sample/reagent mixture than the small wells. The Quanti-Tray® Sealer automatically distributes the proper amount of sample/reagent mixture into separate wells. After incubation, the number of positive wells is converted to an MPN using a table provided by IDEXX. The Quanti-Tray 2000 displays counts from one to 2,419 CFU 100 mL⁻¹. Samples were not diluted, so 2,419 CFU 100 mL⁻¹ was the maximum value recorded.

Enterococcus

Enterolert, a kit from IDEXX, was used to measure *Enterococcus*. It uses the same Defined Substrate Technology® as Colilert. With this method, the enzyme β -glucuronidase is produced by *Enterococcus*, which metabolizes MUG and subsequently releases 4-methyl-umbelliferone. 4-methyl-umbelliferone produces a blue fluorescent color visible under UV light (Figure 14). Samples analyzed for *Enterococcus* were prepared the same way samples were prepared for the Coli-ert Kit Method. The only difference was that the samples were incubated at 41°C instead of 35°C.

Real-time PCR assays

Probe and primer sequences, gene targets, and amplicon size for the all real-time PCR assays used in this study are summarized in Table 4. Alignments of partial *Bacteroides* 16S rRNA genes acquired from fecal source libraries and sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>) were used to develop the following

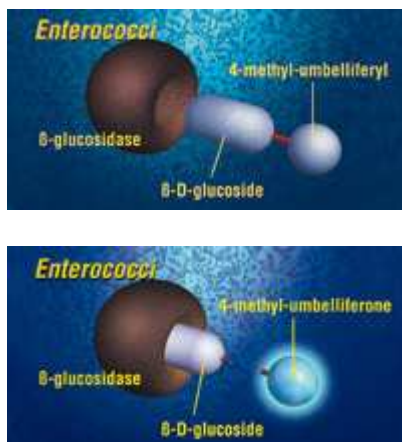


Figure 14. Enterolert MUG reaction from IDEXX literature.

Table 4. Real-time PCR assays used to detect *Bacteroides* 16S rRNA genes. The primers and probes used for each assay, and the PCR protocols for each assay.

Assay	Primer/probe name and sequence (5'-3')*	Size (bp) of product	Annealing temp (°C)
AllBAC (all <i>Bacteroides</i>)	AllBAC296f, 5'-GAGAGGAAGGTCCCCCAG-3' AllBAC412r, 5'-CGCTACTTGGCTGGTTCAG-3' AllBAC375Bhqf, 5'-(FAM)CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)-3'	106	60
BoBAC (bovine cluster of <i>Bacteroides</i>)	BoBAC367f, 5'-GAAG(G/A)CTGAACCAGCCAAGTA-3' BoBAC467r, 5'-GCTTATTCATACGGTACATACAAG-3' BoBAC402Bhqf, 5'-(FAM)TGAAGGATGAAGGTTCTATGGATTGTAACTT(BHQ-1)-3'	100	57
HuBAC (human cluster of <i>Bacteroides</i>)	HuBAC566f, 5'-GGGTTTAAAGGGAGCGTAGG-3' HuBAC692r, 5'-CTACACCACGAATTCGCGCT-3' HuBAC594Bhqf, 5'-(FAM)TAAGTCAGTTGTGAAAGTTTGC GGCTC(BHQ-1)-3'	116	60

*Numbers within the primer/probe name indicate the nucleotide position within the *Bacteroides* 16srRNA gene.

three assays: 1) *Bacteroides* genus (AllBAC), human-associated (HuBAC), and bovine-associated (BoBAC). The DNA sequence regions chosen for host specificity; either they were conserved in all *Bacteroides* species or conserved only in *Bacteroides* species from bovine or human fecal samples. The primers and probes were selected based on the guidelines provided by Applied Biosystems (Foster City, CA) for these DNA sequence regions. The oligonucleotide properties calculator (www.basic.northwestern.edu/biotools/oligocalc.html) was used to determine oligonucleotide melting temperature and self-complementarity. Oligonucleotide specificity for all *Bacteroides* 16S rRNA genes or for human-associated and bovine associated *Bacteroides* 16S rRNA genes was verified using the BLAST program at the National Center for Biotechnology Information (Altschul et al. 1990) and the probe match program of the Ribosomal Database Project (Cole et al. 2005). Oligonucleotide primers and 6-carboxyfluorescein (FAM)-BHQ probes were obtained from Biosearch Technologies.

Application of real-time PCR assays to creek water samples

All real-time PCR assays were performed using real-time PCR mix (QIAGEN, Valencia, CA) with 15pmol of the primer and 5 pmol of the probe. PCR amplification protocols consisted of consisted of 50°C for 2 minutes, followed by 95°C for 10 minutes and up to 50 cycles of 95°C for 30 seconds and 57°C (BoBAC assay) or 60°C (AllBAC and HuBAC assays) for 45 seconds. PCR amplification and detection of the fluorescent signal was performed using the DNA Engine Opticon continuous fluorescence detection system (MJ Research, Waltham, MA). The threshold cycle (Ct) value for all measurements was determined as the cycle at which fluorescence reached 5 standard deviations above the background, averaged over 5 cycles collected within the first 15 cycles of PCR amplification. In addition, PCR inhibition was tested by adding 2.5×10^5 copies of the plasmid standard into a fourth well for each sample. For all PCR runs, standards, negative controls (no DNA), and samples were run in triplicate. Fecal concentrations were calculated from standard curves based on the log transformation of known concentrations versus the threshold cycle. Because the concentrations are not determined directly, it should be noted that a label of equivalents of kg feces day⁻¹ is more appropriate than simply kg feces day⁻¹. Direct PCR without DNA extraction (Fode-Vaughan et al., 2001) was performed on 2.5-μl creek water samples in 25-μl PCRs containing QuantiTect master mix and primers and probes (see Table 4). Sterile Tris buffer (10mM) served as a negative control. Each assay plate contained a fecal dilution standard curve in addition to the test samples. All dilutions for standard curves were run in triplicate. For the AllBAC and HuBAC assays, human fecal samples ranging in concentration from 5,000 mg/liter to 0.32 mg/liter were used as the standard for

calculating the concentration of total feces and human-associated feces in each sample. For the BoBAC assay, a bovine fecal sample ranging in concentration from 10,000 mg/liter to 1.0mg/liter was used as the standard for calculating the concentration of bovine-associated feces in each sample. For each assay, the fecal concentration was determined using triplicate 2.5- μ l creek water samples.

Chemical Methods

All procedures were performed in accordance with Standard Methods for the Examination of Water and Wastewater, 21st Edition (Greenberg et al., 2005). Special care was taken to test for ammonia and nitrate first, because concentrations of these parameters can change rapidly with time.

The Nessler Method (Method 8038) was used to test for Ammonia. A HACH 2500 spectrophotometer (Loveland, CO) measured absorbance at 425 nm wavelengths to compute the concentration of ammonia. The procedure was adapted from Greenberg et al. (2005). The estimated detection limit is 0.06 mg NH₃-N L⁻¹. Each 25mL sample was mixed with a mineral stabilizer, polyvinyl alcohol-dispersing agent, and Nessler reagent. Blanks, used for zeroing, were created by adding the reagents to 25mL of deionized water. “Zeroing” creates a baseline for ammonia measurements, and was performed before testing samples for ammonia. The mineral stabilizer was zinc sulfate. It reacts and complexes with the calcium to inhibit precipitation of the residual calcium ions. The polyvinyl alcohol-dispersing agent helped produce the color in the reaction of the Nessler reagent and ammonium ions. As the intensity of the yellow color increased, the concentration of ammonium also increased (HACH, 1995).

Ion chromatography (IC) was used to test for chloride, sulfate, and nitrate in accordance with SM4500C from Standard Methods for the Examination of Water and Wastewater 21st Edition (Greenberg et al., 2005). Creek water was first filtered through a 0.45 μm pore diameter filter. This prevented large particles from damaging the IC sampling machine. Ion chromatography allowed sampling for multiple ions to be performed at the same time. An anion separator column can test for concentrations of nitrate, nitrite, phosphate, sulfate, chloride, bromide, and fluoride. Ion chromatography works by injecting the sample into a stream of bicarbonate eluent and passing it through ion exchangers. The anions become separated according to their affinity for a low capacity, strongly basic anion exchanger. The anions are then converted into a highly conductive form within a fiber suppressor column. The carbonate–bicarbonate eluent is converted into carbonic acid, which is a poor conductor. Different anions remain on the column for different amounts of time, and it is known which peak corresponds to which ion. The area under the peak is a measure of the anion concentration (Greenberg et al., 2005).

Total Kjeldahl nitrogen (TKN) is a measure of organic nitrogen and ammonia. TKN was analyzed by using the LACHAT QuickChem Method 10-107-06-2-E (Diamond, 1992). In this method, water samples were prepared by block digestion and analyzed by colorimetry. This method works best for water with nitrogen levels between 0.1 and 5.0 mg L^{-1} . 25mL of raw creek water from each site was placed in test tubes and digested in a block digester. Sulfuric acid, mercuric oxide and potassium sulfate were added to raise the boiling point of the solution. During digestion organic nitrogen was converted to ammonium. After digestion, 20 mL of deionized water was added to the

sample and thoroughly mixed. After which, the sample was analyzed in a SKALAR spectrophotometer (Norcross, GA). The SKALAR apparatus automatically mixed the reagents salicylate and hypochlorite to heat the ammonium, producing a blue color. The intensity of the blue color was related to the concentration of ammonium. Concentration of the sample was determined by comparing the absorbance of the sample to a standard absorbance curve. The standard curve was made from concentrations of 3.0, 2.0, 1.0, and 0.5 mg N L⁻¹.

Total phosphorus (TP) analysis is similar to TKN, but uses a slightly different method, LACHAT QuikChem Method 10-115-01-1-C (Liao, 1993). This particular method can be used for TP levels between 0.01 – 5.0 mg P L⁻¹. The detection limit is 0.005 mg P L⁻¹. The same water samples used for TKN are used for TP. Therefore, they are also prepared by block digestion and analyzed by colorimetry. During digestion, organic phosphorus is converted to orthophosphate. A complex is formed when it reacts with ammonium molybdate and antimony potassium tartrate. This complex is reduced by ascorbic acid, and it forms a blue complex that absorbs light at 880 nm. Comparing colorimetric absorbance to a standard absorbance curve can be used to determine the amount of TP in the sample (Liao, 1993).

Total solids (TS) and suspended solids (SS) were measured in accordance with Standard Methods for the Examination of Water and Wastewater 21st Edition, using methods SM2540C and SM2540D (Greenberg et al., 2005). Dissolved solids (DS) were estimated by subtracting SS from TS. Total solids are the residues remaining in a dish after being dried at 180°C. An aluminum weighing dish was weighed on an analytical balance before adding 50mL of creek water to the dish. The dish was weighed again after

the water had evaporated. The difference in weight was the weight of the residue, or total solids per 50 mL. Suspended solids were residues that did not pass through a 2.0 μm pore glass filter.

Load Duration Curves

Development of Load Duration Curves (LDC) is based on the work of Bruce Cleland (Cleland, 2002a; Cleland, 2002b). The first step of creating a LDC is to produce a flow duration curve (FDC), which requires reliable streamflow data. Pond Creek is an ungauged stream. Discharge data gathered during monthly sampling trips from this study combined with data from another study (Sasser, 2003) yielded only 24 events.

Additionally, peak flows were not captured, because sampling trips were conducted without concern for streamflow conditions or antecedent rainfall. Previous studies have suggested that a regional flow duration curve based on gauged streams can be used to develop a FDC for an ungauged stream that is similar in size and located in the same physiographic region (Cigizoglu, 2000; Logan and Walker, 2005). Gauged and ungauged streams are related through drainage area, topography, and precipitation (Cigizoglu, 2000). Logan and Walker (2005) reported that normalizing gauged and ungauged streams solely on drainage area produces representative FDCs for ungauged streams in the Ridge and Valley physiographic region.

The objective of a flow duration curve is to plot historic streamflow data (flow in $\text{m}^3 \text{s}^{-1}$ at each exceedance probability) against drainage area in hectares, the independent variable. A linear regression can then be performed on every exceedance probability to generate a linear equation predicting streamflow at any flow duration interval and for any

size drainage area, between 259 hectares and 25,9000 hectares in this case. The terms “exceedance probability” and “flow duration interval” are used interchangeably in this study. Creating a FDC for Pond Creek first required an examination of all gauged streams in the Ridge and Valley physiographic region of Tennessee. Average daily discharge data for those streams with drainage areas between 259 hectares and 25,9000 hectares was compiled (Table 5).

Historic streamflow data were entered into a spreadsheet developed by Bruce Cleland (Cleland 2002a; Cleland 2002b) to generate flow duration curves for each gauged stream, which produces streamflow values at each flow duration interval. Then, the data were plotted in Microsoft Excel (Redmond, WA) to generate linear regressions for each data series (flow duration interval) by using the “add a trendline” function in Excel. R-square values for the regressions at every flow duration interval are shown in Figure 15.

Table 5. Attributes of Gauged Streams under 25,900 hectares in the Ridge and Valley physiographic region of Eastern Tennessee that were used to create a regional flow duration curve for Pond Creek, TN (HUC 06010201013). Data was collected from USGS National Water Information System Web Interface.

Name	ID #	Area (Hectares)	Dates of Operation	Number Recorded
First Cr. Mineral Springs	03496000	3082.1	1945-1963	6727
First Cr. Powers Ave.	03496200	3470.6	1963-1970	2557
First Cr. Fifth Ave.	03496500	4299.4	1932-1958	5632
Sweetwater below	03520045	6837.6	1970-1981	4201
Sweetwater near	03520050	7303.8	1964-1970	2086
Baker	03519640	4144.0	1965-1975	3574
Wolftever	03566420	4869.2	1964-1989	9374
Chestuee above Englewood	03565040	3833.2	1944-1957	5022
Bullrun Cr. Near Halls Crossroads	03535000	17741.4	1957-2003	11414
Poplar Creek Near Oak Ridge	03538225	21367.4	1960-1989	10622
Bear Cat Hwy 95 Near Oak Ridge	03538270	1124.1	1985-2000	5745
Bear Cr. Near Wheat	035382673	828.8	1986-1991	1826
Bear Cr. At Pine Ridge	03538273	1295.0	1986-1991	1832
Whiteoak Cr near Wheat	03536380	543.9	1986-1995	3226
Whiteoak Cr at ORNL	03536500	538.7	1950-1955	1870
Whiteoak below ORNL	03537000	937.6	1950-1964	4383

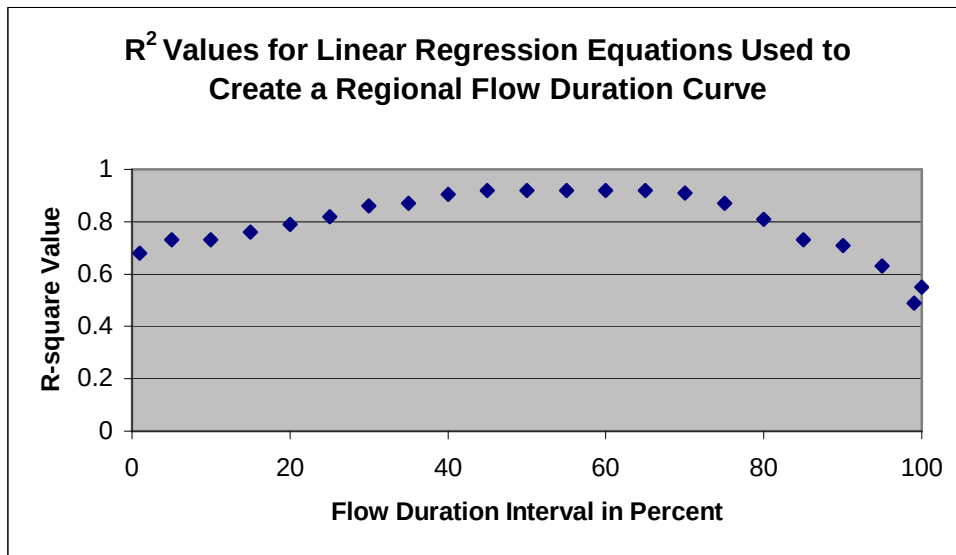


Figure 15. R^2 values for linear regression equations used to create a regional flow duration curve for Pond Creek, TN (HUC 06010201013).

Log-log transformations and semi-log transformations of the data series were investigated but did not yield better results. There was good correlation from the 25 percent to 80 percent flow duration intervals. R-square values ranged from .82 to .92. The weakest correlations were observed at the highest and lowest intervals (1 percent, 99 percent, and 100 percent). The linear regression produced an equation where drainage area was the independent variable. Streamflow can then be estimated if the drainage area is known. Drainage areas were determined by using the “Tabulate Area” function in ArcMap 9.2 (Redlands, CA). Streamflow values at each flow duration interval were calculated for Pond Creek using the drainage area as the x variable and solving for y. It is important to remember that the drainage area at each sample site is the sum of all drainage areas upstream. For example, in Pond Creek, the drainage area at Site 5 is the sum of the drainage areas at Sites 5, 6, 7, and 8.

After all predicted flows are calculated (Table 6), these values are entered into Bruce Cleland's Flow Duration Curve Spreadsheet Template to create Flow Duration Curves for every sample site. An example is presented in Figure 16. The solid line represents predicted streamflow based on the regional flow duration curve. The triangles mark flow duration curves based on measured streamflow values gathered during two years of sampling.

The low number of sampling events and the failure to capture storm events produced a relatively flat FDC (triangles in Figure 16), resulting in divergence at high and low flows between the FDCs based on predicted and observed data. They are similar, however, during moist and mid-range flows. The FDC based on regional streamflow data is likely more representative for the Pond Creek watershed, and therefore was used to calculate load duration curves in this study.

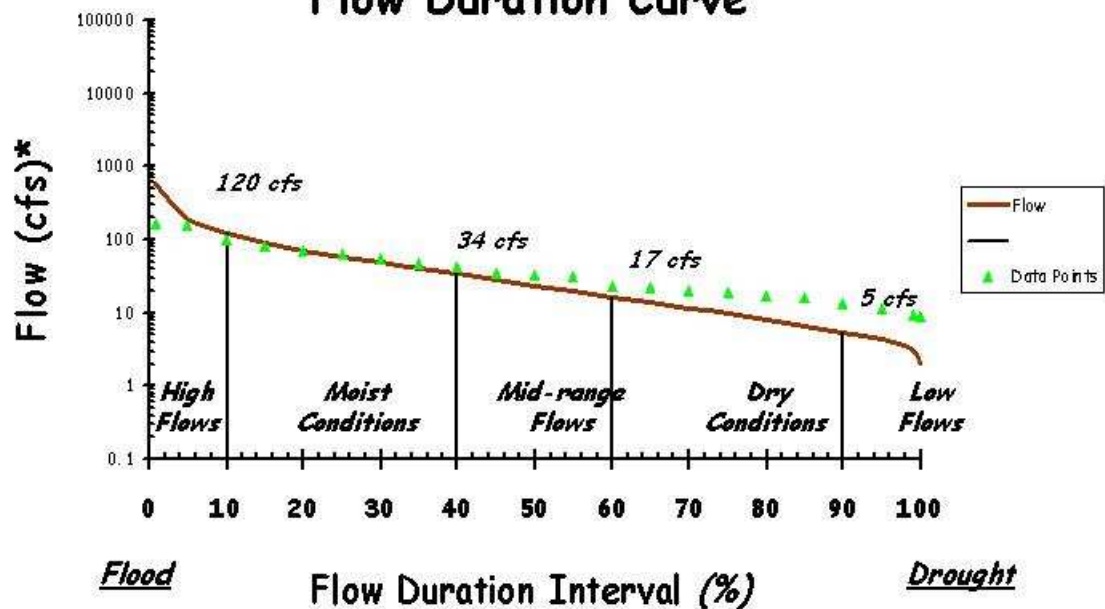
It is also possible to estimate the percentile flow rank for any sampling date by comparing observed streamflow ($\text{m}^3 \text{s}^{-1}$) with the percentile rank and corresponding streamflow on the predicted FDC.

In this study, the percentile rank was estimated to the nearest 5 percent. Additionally, flow conditions (high, moist, mid-range, dry, or drought) for the sampling data were estimated by calculating an average percentile rank for all sample sites on the day it was sampled. Based on these analyses for the period of November 2005 through November 2006, sampling was conducted mostly during dry periods (7 of 13 events). On three occasions mid-range conditions were encountered, while three other trips were during moist conditions (Table 7).

Table 6. Calculated Streamflow Values for all eight sample sites in Pond Creek, TN (HUC 06010201013) based on subwatershed area and a regression equation developed from a regional flow duration curve for watersheds less than 25,900 hectares in the Eastern Tennessee Ridge and Valley physiographic region.

	Site Number							
Flow Duration Interval	1	2	3	4	5	6	7	8
	Flow in Cubic Meters per Second							
1	17.45	16.27	15.6	13.83	11.92	10.47	5.76	6.22
5	5.78	5.41	5.21	4.67	4.08	3.64	2.2	2.34
10	3.53	3.31	3.18	2.85	2.5	2.23	1.35	1.44
15	2.63	2.46	2.37	2.12	1.85	1.64	0.98	1.04
20	2.09	1.95	1.87	1.67	1.45	1.28	0.74	0.79
25	1.71	1.59	1.53	1.36	1.18	1.03	0.58	0.63
30	1.43	1.33	1.27	1.13	0.97	0.85	0.46	0.5
35	1.2	1.12	1.07	0.95	0.81	0.71	0.38	0.41
40	1.01	0.94	0.89	0.79	0.67	0.59	0.3	0.33
45	0.84	0.78	0.75	0.66	0.56	0.49	0.25	0.27
50	0.71	0.65	0.63	0.55	0.47	0.41	0.21	0.23
55	0.59	0.55	0.52	0.46	0.4	0.35	0.19	0.2
60	0.49	0.46	0.44	0.39	0.33	0.29	0.16	0.17
65	0.41	0.39	0.37	0.33	0.28	0.25	0.13	0.14
70	0.34	0.32	0.31	0.27	0.24	0.21	0.12	0.12
75	0.28	0.27	0.25	0.23	0.2	0.18	0.1	0.11
80	0.24	0.22	0.21	0.19	0.17	0.15	0.09	0.1
85	0.19	0.18	0.17	0.16	0.14	0.12	0.08	0.08
90	0.16	0.15	0.14	0.13	0.12	0.1	0.07	0.07
95	0.13	0.12	0.12	0.1	0.09	0.08	0.06	0.06
99	0.09	0.08	0.08	0.08	0.07	0.06	0.04	0.04
100	0.06	0.06	0.05	0.05	0.05	0.04	0.03	0.03

Based on Regional Pond Creek, TN Site #1 Flow Duration Curve



USGS Flow Data

31.3 square miles

Figure 16. Flow Duration Curve for Site #1 in Pond Creek (HUC 06010201013) comparing curve shape of the regional flow duration curve with curve shape of a flow duration curve developed from 24 data points.*CFS stands for $\text{ft}^3 \text{s}^{-1}$. $1 \text{ ft}^3 \text{s}^{-1}$ equals $0.0283 \text{ m}^3 \text{s}^{-1}$.

Table 7. Estimation of Flow Percentile based on presumptive flow duration curves calculated for all eight sample sites in Pond Creek, TN (HUC 06010201013) between November 2005 and November 2006.*

	PC#1	PC#2	PC#3	PC#4	PC#5	PC#6	PC#7	PC#8	Mean	Range
Nov. 2005	60	80	90	60	95	95	100	100	85	Dry
Dec. 2005	40	50	50	55	80	50	100	100	66	Dry
Jan. 2006	55	25	25	25	50	35	50	100	46	Mid Range
Feb. 2006	20	25	15	25	30	45	40	65	33	Moist
April 3, 2006	25	35	25	25	50	50	70	90	46	Mid Range
April 27, 2006	5	5	5	15	15	50	15	35	18	Moist
May 2006	50	55	45	60	60	65	100	100	67	Dry
Jun. 2006	60	65	60	65	75	55	90	100	71	Dry
Jul. 2006	70	70	65	75	90	75	100	100	81	Dry
Aug. 2006	75	85	80	70	95	90	99	100	87	Dry
Sept. 2006	20	20	15	25	25	30	45	50	29	Moist
Oct. 2006	50	50	50	45	65	55	95	100	64	Dry
Nov. 2006	40	35	40	35	55	50	85	85	53	Mid Range

*Stagnant conditions were reported as the detection limit of the flow meter ($0.00283 \text{ m}^3 \text{ s}^{-1}$), which equates to 100 percent exceedance probability.

Pollutant loads can be calculated if both the concentration of the pollutant and streamflow are known. In this study concentrations of *Escherichia coli* and *Bacteroides* are known for all sample dates. *Escherichia coli* loads are calculated in Bruce Cleland's Water Quality Duration Tool spreadsheet with the following formula:

$\text{Load (CFU day}^{-1}\text{)} = \text{Concentration (CFU 100 mL}^{-1}\text{)} * \text{Flow (cfs)} * 283.17 * 60 * 60 * 24$
Bacteroides and other parameters with concentrations in parts per million (mg L⁻¹) were converted into pollutant loads with the following equation:

$$\text{Load (kg day}^{-1}\text{)} = (\text{Concentration (mg L}^{-1}\text{)} * \text{Flow (cfs)} * 5.38) / 2.2$$

Target pollutant loads are calculated by multiplying the recreational water quality standard of 126 CFU 100 mL⁻¹ by flow values generated by linear regressions developed from the regional flow duration curve (Table 6). The critical concentration of 126 CFU 100 mL⁻¹ is used as the target load rather than 941 CFU 100 mL⁻¹, the value we used to check compliance with water quality standards, because TMDL guidelines call for more conservative standards. Due to the fact our samples were collected monthly, 941 CFU 100 mL⁻¹ was selected as the water quality standard. However, for recreational use, the desirable *Escherichia coli* concentration in water samples is less than 126 CFU 100 mL⁻¹.¹ In fact, the geometric mean of at least five samples collected in 30 days must be less than 126 CFU 100 mL⁻¹.

Observed discharge values from sampling events are then rounded to the nearest 5 percent flow duration interval and plotted with the associated *Escherichia coli* load on the LDC. It is then evident if *Escherichia coli* loads are over or under the permissible limit.

Bacteroides data were utilized to attempt to differentiate the *Escherichia coli* load into *Escherichia coli* attributable to bovine and *Escherichia coli* attributable to human

sources. This procedure was accomplished by multiplying the *Escherichia coli* load by the percentage of feces attributable to bovines (percent BoBAC) or the percentage attributable to humans (percent HuBAC) for the sample date. If the percentage is >100 percent it is assumed to be 100 percent. The weighted loads are then paired with the appropriate percentile flow rank for the sample date and plotted on the LDC. Load duration curves are displayed in Power Point slides. Separate graphs are needed for *Escherichia coli* attributable to human and *Escherichia coli* from bovine sources. There are inherent assumptions when using this method, the first being that all animal fecal sources have equivalent concentrations of *Escherichia coli*. Second, *Escherichia coli* concentration is proportional to *Bacteroides* concentration.

Table 8 presents the percentage values calculated from the real-time PCR assays. Samples that had a greater recovery of either HuBAC or BoBAC than AllBAC, were marked as 100 percent. For example, Site 1 on November 2005 reported 100 percent for both HuBAC and BoBAC. On this date concentrations were near the detection limit for all *Bacteroides* values, causing both HuBAC and BoBAC to be over 100 percent. It is possible to have values over 100 percent due to large standard deviation between the triplicate samples. Additionally, on many dates the percentages do not add to 100 percent. This gap was likely due to measurement variability, but it also could have resulted from the failure to characterize all the bovine and human-associated *Bacteroides*. The uncharacterized portion may represent an unknown source, measurement variability, or an undetected source. An undetected source, for example, could result from *Bacteroides* spp. being present in the creek water sample that belonged to a *Bacteroides* cluster that the BoBAC or HuBAC assays did not detect. A cluster refers to a grouping of closely

Table 8. Percent of total *Bacteroides* concentration calculated by dividing the concentration of either HuBAC or BoBAC by AllBAC for all eight sample sites in Pond Creek, TN (HUC 06010201013) during a period from November 2005 through November 2006.*

	Site 1		Site 2		Site 3		Site 4	
	% HuBAC	% BoBAC	% HuBAC	% BoBAC	% HuBAC	% BoBAC	% HuBAC	% BoBAC
11/14/2005	100	100	100	23.5	24.9	35.5	6.6	25.8
12/20/2005	5.1	25.5	9.5	83.7	5.7	26.3	1.8	75.7
1/26/2006	5	36	1.9	100	10.8	100	4.8	68.7
2/23/2006	4.1	89.6	1.1	100	0.7	73.5	1.4	77.7
4/3/2006	8.7	4.1	1.2	25.2	11.2	25.4	11.3	67.8
4/27/2006	0.2	100	0.1	100	0.5	100	1	100
5/24/2006	2.7	12.5	1.9	8.2	8.1	20.8	20.5	56.3
6/29/2006	1.3	5	0.7	32	5.9	17.8	3	7.5
7/27/2006	26.4	17.7	11.4	59.5	1.9	2.4	25	47.4
8/24/2006	29.6	11.7	9.7	37.2	6.8	36.6	8.9	13.7
9/28/2006	27.5	22.9	6.4	100	1.8	57.3	19.7	10.2
10/25/2006	4	20.2	3.9	46.1	3.6	73.6	2.7	23.9
11/28/2006	2.9	33.6	10.5	28.8	5.1	23.2	1.2	20.5
Average	16.7	36.8	12.2	57.2	6.7	45.6	8.3	45.8
	Site 5		Site 6		Site 7		Site 8	
	% HuBAC	% BoBAC	% HuBAC	% BoBAC	% HuBAC	% BoBAC	% HuBAC	% BoBAC
11/14/2005	15.2	76.5	11.9	10.8	22.2	44.9	0.6	52.3
12/20/2005	24.2	90.5	3.9	100	10.3	98.6	3.4	100
1/26/2006	3.7	73.4	3.5	40.8	5.7	100	2	87.8
2/23/2006	0.8	69.5	3.5	98.9	13.5	100	1.9	100
4/3/2006	6.3	30	37.9	100	19.2	84.3	5.2	100
4/27/2006	1.7	100	1.2	100	3.8	100	2.6	100
5/24/2006	4.9	66.8	19.4	15.3	11.2	24.4	1.1	39.2
6/29/2006	5.4	57.7	12.7	100	4.5	96.6	1.5	22.7
7/27/2006	27.7	100	6	89.1	66.4	17.5	3.1	21.6
8/24/2006	19.2	100	2.4	99.3	2	100	0.8	28.2
9/28/2006	10.7	62.7	28.9	100	28	13.2	2.4	52.7
10/25/2006	3	33.3	6.7	14.2	3.2	24.2	1.3	34.7
11/28/2006	4	81.5	8.1	35.8	42.2	26.2	11.8	27.8
Average	9.8	72.5	11.2	69.6	17.9	63.8	2.9	59

*Site 8 is a headwaters sites. Site 1 is the most downstream site.

related species of *Bacteroides*, as determined by their relative proximity on a phylogenetic tree. Assays were designed to pick up *Bacteroides* belonging to a cluster known to be associated with either human or bovine feces. However, analysis of *Bacteroides* from fecal samples showed 2 distinct clusters for bovine feces and 3 or 4 clusters for human feces. The assays used in this study were designed to capture only one cluster for both humans and for bovines. It is possible that *Bacteroides* species belonging to other clusters were present in creek samples, which would cause estimates of either human or bovine associated fecal contamination to be underrepresented.

The LDCs were also used to characterize the nature of fecal inputs into flow dependent or flow independent sources. Data used to generate human and bovine LDCs were analysed to determine if their slope differed from zero. Groups of data whose slope did not differ from zero were said to be flow independent. Conversely, if the slope was not zero, the source was said to be flow dependent.

Statistical Analysis

All statistical analysis utilized SPSS 15.0 (Chicago, IL). Concentration data was skewed (not normally distributed), so statistical analysis used the values obtained by taking the natural logarithm of concentration data. The ln-transformed values varied over a more narrow range, producing a parametric distribution. An analysis of variance (ANOVA) was performed at the $\alpha=0.05$ level on ln-transformed concentrations of fecal indicator organisms to detect differences between sampling sites and sampling dates. Tukey Honestly Significant Difference (HSD) Post Hoc Tests were run to investigate mean separations. Correlation analysis was performed to determine if certain parameters

behave similarly at the $\alpha=0.05$ level of significance. The Pearson Correlation coefficient was determined for the dependent variables (ln-transformed concentration values for fecal indicator organisms HuBAC, BoBAC, AllBAC, *Escherichia coli*, and *Enterococcus*) and for independent variables total solids, streamflow, total organic carbon, ammonia, nitrate, total Kjeldahl nitrogen, chloride, sulfate, orthophosphate, total phosphorus, and 5-day biological oxygen demand.

***Escherichia coli* Water Quality Standards**

Bacteriological water quality standards for Pond Creek must meet limits established for recreational use. The following is adapted from *State of Tennessee Water Quality Standards, Chapter 1200-4-3, General Water Quality Criteria, October, 2007*:

The concentration of the *E. coli* group shall not exceed 126 colony forming units per 100 ml, as a geometric mean based on a minimum of 5 samples collected from a given sampling site over a period of not more than 30 consecutive days with individual samples being collected at intervals of not less than 12 hours. For the purposes of determining the geometric mean, individual samples having an *E. coli* concentration of less than 1 per 100 ml shall be considered as having a concentration of 1 per 100 ml. Additionally, the concentration of the *E. coli* group in any individual sample taken from a lake, reservoir, State Scenic River, Exceptional Tennessee Water or ONRW (1200-4-3-.06) shall not exceed 487 colony forming units per 100 ml. The concentration of the *E. coli* group in any individual sample taken from

any other waterbody shall not exceed 941 colony forming units per 100 mL.

Grab samples were collected monthly, so the one time sampling maximum value of 941 CFU 100mL⁻¹ was used as the water quality standard for *Escherichia coli*.

Confusingly, a value of 126 CFU 100mL⁻¹ is used as the “target load” for *Escherichia coli* when creating Load Duration Curves. The apparent difference is due to the fact that we performed monthly sampling trips, not 5 or more trips per month. The desirable level of *Escherichia coli* in a waterbody is less than 126 CFU 100mL⁻¹.

CHAPTER IV

RESULTS AND DISCUSSION

Fecal Indicator Organisms

Our findings confirm reports of cattle being the main source of fecal contamination in Pond Creek. Summaries of *Escherichia coli* and *Bacteroides* concentrations are presented in Tables 9 and 10. *Escherichia coli* values exceeded the one time sampling maximum value of 941 CFU 100mL⁻¹ for 60 percent (64 of 104) of all samples. There was variability between sites as well. Site 8, a headwaters location, was in compliance with water quality standards on only two dates, whereas the most downstream site, Site 1, exceeded limits only twice. Generally, Sites 2 through 7 exceeded standards; noncompliance at Sites 2 through 7 ranged from 54 percent to 76 percent of sampling events. The actual average *Escherichia coli* concentrations are likely much higher, because samples were not diluted to fall within the range of IDEXX Quanti-Tray®. To compute the average, any values greater than 2419 were considered to be 2419. Even though this reduces the estimate of fecal contamination, the average values are still more than 10 times the legal limit.

Tables 11 and 12 summarize *Escherichia coli* and *Bacteroides* load data. *Escherichia coli* load duration curves indicate that *Escherichia coli* loads are much higher than permissible levels, and they are flow dependent (Appendix A). Observed loads fluctuated with streamflow. *Escherichia coli* concentrations were greatest at high flows and decreased as flows decreased.

Table 9. Summary of *Escherichia coli* and *Bacteroides* concentrations for Site 1 through Site 4 in Pond Creek, TN (HUC 06010201013) for all sampling dates between November 2005 and November 2006. Shaded Values exceeded water quality criteria for *Escherichia coli* (one time sampling maximum value of 941 CFU 100mL⁻¹).

	Site 1				Site 2			
Date	<i>E. coli</i>	HuBAC	BoBAC	AllBAC	<i>E. coli</i>	HuBAC	BoBAC	AllBAC
	CFU/100 mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹	CFU/100mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹
11/14/2005	88	1	0.5	0.5	816	8.6	0.9	4
12/20/2005	248	0.1	0.5	2	1046	0.7	6.5	7.8
1/26/2006	345	2.6	19	52.6	1203	1.4	77	75.4
2/23/2006	>2419	4.02	88.2	98.4	>2419	1.35	150	123.1
4/3/2006	613	1.85	0.9	21.3	>2419	1.63	34.6	137.6
4/27/2006	>2419	0.43	383.7	216.6	>2419	0.1	466.1	137.6
5/24/2006	276	0.2	0.9	7.5	866	0.2	0.7	8
6/29/2006	222	0.1	0.5	10.1	1733	0.1	4.5	14.2
7/27/2006	157	0.74	0.5	2.82	687	0.45	2.37	3.98
8/24/2006	138	1.27	0.5	4.29	579	0.52	1.98	5.33
9/28/2006	700	1.76	1.47	6.4	1414	0.35	8.47	5.45
10/25/2006	161	0.1	0.5	2.47	866	0.1	1.18	2.56
11/28/2006	205	0.1	1.14	3.39	435	0.61	1.68	5.84
Geomean	340.5	0.5	2.1	8.5	1125	0.5	7.6	14.3
Mean	614.7	1.1	38.3	33	1300.2	1.2	58.2	40.8
σ	820.7	1.2	106.6	61.9	723.7	2.3	130.1	55.9
Min	88	0.1	0.5	0.5	435	0.1	0.7	2.6
Max	2419	4	383.7	216.6	2419	8.6	466.1	137.6
	Site 3				Site 4			
Date	<i>E. coli</i>	HuBAC	BoBAC	AllBAC	<i>E. coli</i>	HuBAC	BoBAC	AllBAC
	CFU/100 mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹	CFU/100 mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹
11/14/2005	1414	0.6	0.9	2.4	387	0.1	0.5	1.9
12/20/2005	488	0.4	2	7.5	488	0.1	5.5	7.2
1/26/2006	345	3.1	31.1	28.7	921	5.8	82.9	120.6
2/23/2006	>2419	0.79	86.6	117.7	>2419	1.3	73.3	94.2
4/3/2006	>2419	2.92	6.6	26	1986	6.91	41.5	61.2
4/27/2006	1986	0.63	197.4	116.7	>2419	2.51	377.2	244.4
5/24/2006	1120	0.7	1.8	8.8	770	1.1	3.1	5.4
6/29/2006	1046	0.5	1.5	8.5	>2419	0.2	0.5	6.7
7/27/2006	1414	0.4	0.5	20.78	>2419	1.63	3.09	6.51
8/24/2006	1300	0.81	4.34	11.87	816	0.54	0.84	6.11
9/28/2006	1120	0.15	4.62	8.06	1553	2.14	1.11	10.88
10/25/2006	1046	0.1	2.06	2.8	770	0.1	0.88	3.69
11/28/2006	770	0.55	2.51	10.84	1046	0.1	1.68	8.19
Geomean	1138.1	0.6	4.9	14.1	1187.1	0.7	5.1	14.4
Mean	1299	0.9	26.3	28.5	1416.4	1.7	45.5	44.4
σ	648.5	1	56.7	40.2	809.5	2.2	103.8	71.7
Min	345	0.1	0.5	2.4	387	0.1	0.5	1.9
Max	2419	3.1	197.4	117.7	2419	6.9	377.2	244.4

Table 10. Summary of *Escherichia coli* and *Bacteroides* concentrations for Site 5 through Site 8 in Pond Creek, TN (HUC 06010201013) for all sampling dates between November 2005 and November 2006. Shaded Values exceeded the water quality criteria for *Escherichia coli* (one time sampling maximum value of 941 CFU 100mL⁻¹).

	Site 5				Site 6			
Date	<i>E. coli</i>	HuBAC	BoBAC	AllBAC	<i>E. coli</i>	HuBAC	BoBAC	AllBAC
	CFU/100 mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹	CFU/ 100 mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹
11/14/2005	>2419	1.4	6.9	9	1203	0.5	0.4	3.9
12/20/2005	411	1.8	6.6	7.3	461	0.3	8.2	7.3
1/26/2006	1203	5.8	117	159.4	291	0.7	8	19.7
2/23/2006	>2419	1.3	85.6	123.1	>2419	3.46	98.7	99.8
4/3/2006	>2419	6.91	33.1	110.3	1300	1.37	6.4	3.6
4/27/2006	1986	2.51	187.4	122.3	>2419	0.72	99.1	62.2
5/24/2006	1553	1.1	6.7	10	687	1.4	1.1	7.1
6/29/2006	>2419	0.2	16.3	28.3	1553	0.6	7.7	4.8
7/27/2006	2419	1.63	16.24	12.89	>2419	0.29	4.24	4.75
8/24/2006	921	0.54	12.05	7.13	1553	0.36	15	15.11
9/28/2006	1733	2.14	14.08	22.46	1553	2.42	8.96	8.37
10/25/2006	613	0.1	3.49	10.48	285	0.2	0.5	3.52
11/28/2006	>2419	0.1	47.9	58.8	727	0.69	3.04	8.48
Geomean	1550.4	1	20.9	27.9	1036	0.7	6	9.8
Mean	1883.4	2	44.2	54.6	1377.4	1.1	20.6	19.9
σ	755.5	2.1	55.5	55.7	786	1	35.2	28.9
Min	411	0.1	3.5	7.1	285	0.2	0.4	3.5
Max	2419	6.9	187.4	159.4	2419	3.5	99.1	99.8
	Site 7				Site 8			
	<i>E. coli</i>	HuBAC	BoBAC	AllBAC	<i>E. coli</i>	HuBAC	BoBAC	AllBAC
Date	CFU/ 100mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹	CFU/100mL	mg L ⁻¹	mg L ⁻¹	mg L ⁻¹
11/14/2005	>2419	5.3	10.8	24	>2419	0.3	25.7	49.1
12/20/2005	1733	1.1	10.8	10.9	>2419	46.5	2955.7	1370
1/26/2006	1203	2.6	85.8	45	1414	3.3	145.6	165.7
2/23/2006	>2419	4.54	52.7	33.7	>2419	12.96	887.9	686.9
4/3/2006	>2419	3.16	13.9	16.5	>2419	6.31	184.5	122.3
4/27/2006	>2419	1.57	51	41.7	>2419	0.87	59.3	33.9
5/24/2006	727	0.9	1.9	7.9	>2419	33.4	1154	2945.2
6/29/2006	488	0.7	14.1	14.6	>2419	1.4	20.8	91.5
7/27/2006	411	1.9	0.5	2.86	>2419	2.68	18.84	87.12
8/24/2006	1414	0.28	30.07	14.43	>2419	1.68	58.22	206.2
9/28/2006	1553	2.8	1.32	9.97	1120	0.21	4.54	8.62
10/25/2006	435	0.2	1.47	6.07	687	0.1	2.77	7.97
11/28/2006	548	2.06	1.28	4.88	345	0.69	1.62	5.82
Geomean	1139.3	1.5	7.8	13.2	1709.5	2	56.3	96.3
Mean	1399.1	2.1	21.2	17.9	1949	8.5	424.6	444.6
σ	829	1.6	26.5	14	770.7	14.6	845.4	844.5
Min	411	0.2	0.5	2.9	345	0.1	1.6	5.8
Max	2419	5.3	85.8	45	2419	46.5	2955.7	2945.2

Table 11. Summary of *Escherichia coli* and *Bacteroides* loads for Site 1 through Site 4 in Pond Creek, TN (HUC 06010201013) for all sampling dates between November 2005 and November 2006.

Date	Site 1				Site 2			
	E. coli	HuBAC	BoBAC	AllBAC	E. coli	HuBAC	BoBAC	AllBAC
	Organisms per Day	kg/Day	kg/Day	kg/Day	Organisms per Day	kg/Day	kg/Day	kg/Day
11/14/2005	3.38E+10	38.4	19.2	19.2	1.60E+11	168.7	17.7	78.5
12/20/2005	2.03E+11	8.2	41.0	163.8	6.17E+11	41.3	383.1	459.7
1/26/2006	1.62E+11	122.1	892.1	2469.7	1.67E+12	194.8	10714.3	10491.6
2/23/2006	3.98E+12	660.6	14494.3	16170.5	3.39E+12	189.2	21018.7	17249.3
4/3/2006	9.31E+11	280.9	136.7	3234.7	2.23E+12	149.9	3181.4	12652.2
4/27/2006	9.65E+12	171.4	152946.3	86338.7	9.14E+12	37.8	176103.2	51988.4
5/24/2006	1.53E+11	11.1	50.0	416.3	4.13E+11	9.5	33.4	381.5
6/29/2006	9.45E+10	4.3	21.3	429.8	5.64E+11	3.3	146.4	461.8
7/27/2006	4.72E+10	22.3	15.0	84.8	1.92E+11	12.5	66.1	111.0
8/24/2006	3.07E+10	28.3	11.1	95.5	9.77E+10	8.8	33.4	89.9
9/28/2006	1.25E+12	313.8	262.1	1141.0	2.36E+12	58.5	1414.7	910.3
10/25/2006	8.90E+10	5.5	27.6	136.5	5.19E+11	6.0	70.7	153.4
11/28/2006	1.67E+11	8.1	92.8	276.1	3.80E+11	53.3	146.7	509.8
Geomean	2.51E+11	39.2	153.1	624.2	7.49E+11	34.3	503.9	953.3
Mean	1.29E+12	128.8	13000.7	8536.7	1.67E+12	71.8	16410.0	7349.0
σ	2.74E+12	192.6	42235.8	23781.7	2.47E+12	74.9	48374.0	14634.7
Min	3.07E+10	4.3	11.1	19.2	9.77E+10	3.3	17.7	78.5
Max	9.65E+12	660.6	152946.3	86338.7	9.14E+12	194.8	176103.2	51988.4
Date	Site 3				Site 4			
	E. coli	HuBAC	BoBAC	AllBAC	E. coli	HuBAC	BoBAC	AllBAC
	Organisms per Day	kg/Day	kg/Day	kg/Day	Organisms per Day	kg/Day	kg/Day	kg/Day
11/14/2005	1.63E+11	6.9	10.3	27.6	1.26E+11	3.3	16.3	61.8
12/20/2005	2.38E+11	19.5	97.3	365.0	1.96E+11	4.0	220.6	288.8
1/26/2006	4.26E+11	382.8	3840.7	3544.3	1.10E+12	689.3	9852.6	14333.2
2/23/2006	4.88E+12	159.4	17471.6	23746.0	2.54E+12	136.4	7689.9	9882.5
4/3/2006	2.64E+12	318.5	719.8	2835.7	2.07E+12	719.9	4323.3	6375.6
4/27/2006	7.77E+12	246.5	77237.2	45661.5	4.43E+12	459.1	68997.4	44705.6
5/24/2006	5.73E+11	35.8	92.0	449.8	2.41E+11	34.4	97.0	169.0
6/29/2006	3.69E+11	17.6	52.8	299.3	7.10E+11	5.9	14.7	196.6
7/27/2006	3.91E+11	11.1	13.8	574.2	4.56E+11	30.7	58.2	122.6
8/24/2006	2.13E+11	13.3	71.1	194.5	1.74E+11	11.5	17.9	130.0
9/28/2006	2.02E+12	27.1	834.9	1456.6	1.92E+12	265.0	137.4	1347.1
10/25/2006	5.30E+11	5.1	104.3	141.7	3.96E+11	5.1	45.2	189.5
11/28/2006	5.44E+11	38.9	177.4	766.1	8.19E+11	7.8	131.5	640.9
Geomean	7.31E+11	37.8	313.0	901.8	6.68E+11	37.7	289.0	807.9
Mean	1.60E+12	98.6	7748.0	6158.6	1.17E+12	182.5	7046.3	6034.1
σ	2.30E+12	133.0	21421.3	13478.1	1.27E+12	268.1	18908.1	12488.2
Min	1.63E+11	5.1	10.3	27.6	1.26E+11	3.3	14.7	61.8
Max	7.77E+12	382.8	77237.2	45661.5	4.43E+12	719.9	68997.4	44705.6

Table 12. Summary of *Escherichia coli* and *Bacteroides* loads for Site 5 through Site 8 in Pond Creek, TN (HUC 06010201013) for all sampling dates between November 2005 and November 2006.

	Site 5				Site 6			
Date	<i>E.coli</i>	HuBAC	BoBAC	AllBAC	<i>E.coli</i>	HuBAC	BoBAC	AllBAC
	Organisms per Day	kg/day	kg/day	kg/day	Organisms per Day	kg/day	kg/day	kg/day
11/14/2005	1.83E+11	10.6	52.3	68.2	8.54E+10	3.5	2.8	27.7
12/20/2005	5.83E+10	25.5	93.6	103.5	1.51E+11	9.8	268.7	239.2
1/26/2006	4.68E+11	225.5	4549.3	6197.9	1.63E+11	39.2	448.0	1103.2
2/23/2006	2.04E+12	109.7	7221.9	10385.7	1.05E+12	150.6	4296.3	4344.2
4/3/2006	9.35E+11	267.0	1278.9	4261.8	4.74E+11	49.9	233.2	131.2
4/27/2006	3.04E+12	383.6	28642.4	18692.4	8.40E+11	25.0	3441.3	2159.9
5/24/2006	4.29E+11	30.4	185.1	276.3	1.45E+11	29.4	23.1	149.3
6/29/2006	3.61E+11	3.0	243.2	422.2	4.56E+11	17.6	226.0	140.9
7/27/2006	2.07E+11	14.0	139.0	110.3	3.73E+11	4.5	65.3	73.2
8/24/2006	7.21E+10	4.2	94.3	55.8	1.47E+11	3.4	141.6	142.6
9/28/2006	1.63E+12	201.0	1322.2	2109.1	1.14E+12	178.1	659.5	616.1
10/25/2006	1.48E+11	2.4	84.5	253.7	8.30E+10	5.8	14.6	102.4
11/28/2006	7.40E+11	3.1	1464.2	1797.4	2.33E+11	22.1	97.4	271.7
Geomean	4.18E+11	27.6	562.8	753.0	2.82E+11	19.1	162.1	266.3
Mean	8.25E+11	100.6	3533.4	3499.0	4.33E+11	42.9	775.4	751.4
σ	8.48E+11	120.2	7312.3	5173.5	3.46E+11	52.8	1307.1	1153.3
Min	5.83E+10	2.4	52.3	55.8	8.30E+10	3.4	2.8	27.7
Max	3.04E+12	383.6	28642.4	18692.4	1.14E+12	178.1	4296.3	4344.2
	Site 7				Site 8			
	<i>E.coli</i>	HuBAC	BoBAC	AllBAC	<i>E.coli</i>	HuBAC	BoBAC	AllBAC
Date	Organisms per Day	kg/day	kg/day	kg/day	Organisms per Day	kg/day	kg/day	kg/day
11/14/2005	3.79E+10	8.3	16.9	37.6	5.92E+09	0.1	6.3	12.0
12/20/2005	3.05E+10	1.9	19.0	19.2	3.85E+10	73.9	4698.2	2177.7
1/26/2006	3.09E+11	66.8	2203.1	1155.5	3.46E+09	0.8	35.6	40.5
2/23/2006	1.00E+12	187.6	2178.0	1392.8	3.20E+11	171.1	11725.1	9070.8
4/3/2006	3.20E+11	41.7	183.6	217.9	1.42E+11	37.0	1082.8	717.8
4/27/2006	2.80E+12	181.6	5899.2	4823.4	8.34E+11	30.0	2044.7	1168.9
5/24/2006	2.31E+10	2.9	6.0	25.1	5.92E+09	8.2	282.2	720.2
6/29/2006	3.22E+10	4.6	93.1	96.4	5.92E+09	0.3	5.1	22.4
7/27/2006	9.05E+09	4.2	1.1	6.3	5.92E+09	0.7	4.6	21.3
8/24/2006	4.84E+10	1.0	102.9	49.4	5.92E+09	0.4	14.2	50.4
9/28/2006	4.71E+11	84.9	40.0	302.3	2.37E+11	4.4	96.1	182.6
10/25/2006	2.55E+10	1.2	8.6	35.6	8.07E+09	0.1	3.3	9.4
11/28/2006	4.42E+10	16.6	10.3	39.4	2.36E+10	4.7	11.1	39.9
Geomean	9.56E+10	12.2	65.1	111.0	2.52E+10	3.0	83.0	142.0
Mean	4.04E+11	47.3	832.9	639.4	1.28E+11	25.8	1545.6	1105.8
σ	7.76E+11	67.1	1721.0	1339.2	2.37E+11	48.9	3344.1	2481.2
Min	9.05E+09	1.0	1.1	6.3	3.46E+09	0.1	3.3	9.4
Max	2.80E+12	187.6	5899.2	4823.4	8.34E+11	171.1	11725.1	9070.8

Concentrations of *Bacteroides* detected by the three assays (AllBAC, HuBAC, and BoBAC) at all sample sites and for all sample dates were summed and are presented in Table 13. Summed BoBAC and HuBAC concentrations were examined as a percentage of the total concentration of detectable *Bacteroides* (AllBAC). Concentrations of *Bacteroides* associated with cattle compose 99 percent of detectable *Bacteroides*, while human associated *Bacteroides* were only 3 percent of the total. It is obvious that the sum of these percentages add to greater than 100 percent. The most likely explanation is measurement variability, but other reasons could be the failure of the AllBAC assay to fully characterize all species of *Bacteroides* present in Pond Creek or the cross-amplification of another source of fecal pollution (wildlife) with either the human or bovine assays (Layton et al., 2006). An assay's ability to detect the *Bacteroides* can be affected by the organism's diet, changes in the microbial fauna in the intestines, and environmental stresses (USEPA, 2005b). These changes cause the previously dominant cluster of *Bacteroides* in feces the assay was originally designed to detect to become less prolific (USEPA, 2005b).

Table 13. Sum of *Bacteroides* concentrations from all eight sample sites in Pond Creek, TN (HUC 06010201013) for 13 sampling dates between November 2005 and November 2006 and percent of total *Bacteroides*.

	Total Concentration mg L ⁻¹	Percent of Total AllBAC Concentration
HuBAC	246.0	2.8
BoBAC	8798.0	99.4
AllBAC	8850.0	

Spatial Variability

Table 14 and Table 15 show mean fecal indicator concentration and load values in addition to statistical differences between sample sites. Interpretation of spatial differences is heavily influenced by the end use of the data, regulation or TMDL development. Regulators typically rely on concentration data to assess stream water quality, whereas TMDL developers utilize load data. Streamflow inversely affects pollutant concentrations by diluting pollutants as streamflow increases, if all other factors remain the same. This explains why Site 8, a headwater site, usually had significantly higher fecal indicator concentrations than many downstream sites and Site 1, the most downstream site, had the significantly lower concentrations of AllBAC, BoBAC and *Escherichia coli* compared to many upstream sites (Table 14). *Escherichia coli* concentration was lowest at Site 1. BoBAC concentrations were significantly higher at Site 8 compared to Sites 1, 3, and 4 (Table 14). AllBAC concentrations were significantly higher at Site 8 compared to other sites, except Site 7 (Table 14). There were no statistically significant differences between sites for HuBAC and *Enterococcus* concentrations (Table 14). Additionally, the landuse changes from an agricultural to a forested setting downstream of Site 2, which reduces fecal inputs from cattle to Pond Creek.

Our results were consistent with another study in Kentucky. Brush Creek in Kentucky also had lower *Escherichia coli* concentrations at the most downstream sampling site in their study; landuse in the area draining to that site was 84 percent forest (KDEP, 2006). They cited direct fecal deposition by cattle and overland flow from

Table 14. Mean ln transformed fecal indicator concentrations and Tukey HSD statistical differences for all eight sample sites in Pond Creek, TN (HUC 06010201013) collected from November 2005 through November 2006.*

	BoBAC	AllBAC	HuBAC	E. coli	Enterococcus
Site	ln mg L ⁻¹			ln CFU 100mL ⁻¹	
1	0.732a	2.133a	-0.61a	5.831a	5.408a
2	2.024ab	2.662a	-0.675a	7.026b	6.5a
3	1.582a	2.644a	-0.526a	7.037b	6.207a
4	1.634a	2.666a	-0.364a	7.079b	6.177a
5	3.039ab	3.33a	0.548a	7.346b	6.119a
6	1.787ab	2.282a	-0.35a	6.962b	5.088a
7	2.051ab	2.583ab	0.368a	7.038b	5.47a
8	4.031b	4.568b	0.702a	7.444b	5.954a

*Means in a column followed by the same letter are not different by Tukey Honestly Significant Difference at $\alpha=0.05$.

Table 15. Mean ln transformed fecal indicator loads and Tukey HSD statistical differences for all eight sample sites in Pond Creek, TN (HUC 06010201013) collected from November 2005 through November 2006.*

	BoBAC	AllBAC	HuBAC	E. coli	Enterococcus
Site	ln kg day ⁻¹			ln organisms day ⁻¹	
1	5.8208a	7.2216a	4.4782b	26.2493bc	5.408a
2	7.0123a	7.6504a	4.3133b	27.3438c	6.5a
3	6.5357a	7.5974a	4.4269b	27.3206c	6.207a
4	6.4565a	7.488a	4.4583b	27.2314c	6.177a
5	7.1266a	7.4179a	4.6363b	26.7642bc	6.119a
6	5.882a	6.3771a	3.7456ab	26.3873bc	5.088a
7	4.9777a	5.5104a	3.2956ab	25.2955ab	5.47a
8	4.9935a	5.5298a	1.664a	23.7362a	5.954a

*Means in a column followed by the same letter are not different by Tukey Honestly Significant Difference at $\alpha=0.05$.

agricultural lands in upper portions of Brush Creek as the sources of fecal contamination at the upper sites (KDEP, 2006).

From a regulatory standpoint, the significantly lower *Escherichia coli* concentrations at Site 1 compared to all other sites makes it tempting to conclude that Pond Creek is not as contaminated in the lower reaches and is actually becoming cleaner lower in the watershed. However, load data indicated otherwise. Significantly higher *Escherichia coli* loads were observed at Sites 2,3, and 4, but Site 1, the most downstream site was not significantly different than these sites (Table 15). HuBAC loads increased downstream; Sites 1, 2, 3, 4, and 5 were significantly higher than Site 8, a headwater site (Table 15). There were no statistical differences between sites for BoBAC and AllBAC. Although Site 1 had significantly lower concentrations of BoBAC, AllBAC, and *Escherichia coli*, that fact that loads were not significantly lower suggests that fecal pollution from upstream sites was transported by Pond Creek and was likely discharged into Watts Bar Reservoir. TMDL developers are concerned about the amount of pollution Pond Creek discharges into Watts Bar Reservoir, not necessarily if a sample site exceeds standards. Intensive livestock production in the middle and upper portions of Pond Creek appear to be the main source of the fecal pollution.

Several studies have shown a strong correlation between the presence of cattle with pollution of adjacent waterbodies or increases in fecal indicator organisms from background levels after cattle are introduced. Stephenson and Street (1978) found a direct relationship between fecal coliforms and cattle grazing. Doran and Linn (1979) reported that rainfall runoff from an area grazed by cattle contained 5 to 10 times more fecal coliforms than runoff from an ungrazed area. *Escherichia coli* from livestock feces can

survive on grass for at least 5 to 6 months (Avery et al., 2004), so rainfall events can carry *Escherichia coli* that has been residing in manure deposited on pastures into waterbodies (Doran and Linn, 1979). Vinten et al. (2004) reported that for a 7 mm rainfall event, 14 percent of daily fecal deposits by grazing cattle were transported to a creek.

One of the objectives of the study was to identify areas that contribute disproportionate amounts of fecal indicator organisms to Pond Creek. The results were inconclusive in this regard. Instead, the results showed extensive fecal pollution throughout the watershed. Bovine-associated *Bacteroides* were dominant to human sources, but no statistical differences in loads were detected between sites for both BoBAC and AllBAC loads (Table 15). Statistical differences in loads between the headwaters and downstream sites were observed for human-associated *Bacteroides* loads (Table 15), but they were only a minor component of total *Bacteroides*. The spatial differences observed for HuBAC are possibly the result of a relatively higher population density near Pond Creek at Sites 1, 2, 3, 4, and 5 compared to Site 8 and comparative lower streamflow in the headwaters than downstream.

One possible approach to identify areas for targeting remediation efforts is to examine subwatershed livestock stocking densities. Gary et al. (1983) reported high livestock densities (0.93 cows ha⁻¹) were causing elevated fecal coliform levels in a Colorado stream. Moreover, Evans et al. (2004) found that *Escherichia coli* concentrations in a Mississippi stream increased as stocking densities increased. In Pond Creek, water quality was impacted at lower stocking densities (Table 16). A clear pattern between stocking density and fecal indicator loads could not be established. Intensive

Table 16. Cattle inventory in Pond Creek Watershed (HUC 06010201013) as of August 2007. The numbers are estimates provided by Lena Beth Carmichael, Pond Creek Watershed Coordinator and University of Tennessee Extension Agent.

Subwatershed ID	Drainage Area (ha.)*	Description	#	Total	# animals / ha.
1	743.4	Beef	0	0	0
		Milking	0		
		Other	0		
2	416.7	Beef	240	240	0.57
		Milking	0		
		Other	0		
3	1112	Beef	125	255	0.23
		Milking	80		
		Other	50		
4	1200.5	Beef	0	900	0.75
		Milking	700		
		Other	200		
5	916	Beef	140	400	0.44
		Milking	110		
		Other	150		
6	1443.5	Beef	150	150	0.10
		Milking	0		
		Other	0		
7	1227.7	Beef	65	475	0.39
		Milking	360		
		Other	50		
8	1516.2	Beef	110	450	0.30
		Milking	190		
		Other	150		

livestock production leads to elevated fecal indicator organisms throughout the watershed. Other possible explanations could be due to hydrology, karst topography, landuse patterns and cattle spending more time in the riparian zone.

Further conclusions can be made from the livestock inventory. It helps with choosing appropriate best management practices. Agricultural BMPs for livestock production are conservation practices that aim to reduce pollution to waterbodies. Several examples of BMPs for livestock production include vegetative filter strips, riparian exclusion, nutrient management, and rotational grazing. Table 16 can assist selection of appropriate BMPs. For example, BMPs in Subwatershed 2 should focus on beef cattle, whereas Subwatershed 4 should consider practices for dairy cattle such as manure storage systems and following nutrient management plans. Site 8 was subject to localized effects of beef cattle. Potentially, fecal inputs in Subwatershed 2 and 8 could be reduced by improving the conditions of pastures or implementing one or more of the following BMPs for beef cattle: vegetative filter strips, exclusion with careful riparian grazing, alternative water sources, dedicated stream crossings, and management of livestock stocking density.

HuBAC

There were no significant differences between sites for human-associated *Bacteroides* concentrations using the Tukey HSD Post Hoc Test (Table 14). Concentrations were considerably lower than bovine and total *Bacteroides* concentrations across the watershed. In contrast, HuBAC loads at Sites 1, 2, 3, 4, and 5 were statistically

greater than at Site 8, though they were still much lower than the AllBAC or BoBAC loads (Table 15). The spatial differences observed for HuBAC are possibly the result of a relatively higher population density near Pond Creek at Sites 1, 2, 3, 4, and 5 compared to Site 8 and comparative lower streamflow in the headwaters than downstream.

BoBAC

Sites 1, 3, and 4 had significantly lower average BoBAC concentrations than Site 8. However, average bovine-associated *Bacteroides* concentrations at Site 8 were not higher than Sites 2, 5, 6, and 7 (Table 14). BoBAC loads were not statistically different between sample sites (Table 15). The diluting effect of increased streamflow explains why downstream sites (1,3, and 4) had lower significantly lower concentrations than Site 8, the most upstream site (Table 14). No statistical differences were observed in BoBAC loads (Table 15) suggesting extensive bovine fecal pollution throughout the watershed. No single area can be implicated as the principal source of bovine-associated *Bacteroides*.

AllBAC

For total detectable *Bacteroides* (AllBAC), Site 8 had the highest average concentration and was significantly higher than all other sites, except Site 7. However, Site 7 was not different from the other sites (Table 14). AllBAC loads were not statistically different across the watershed (Table 15). Bovine sources make up approximately 99 percent of AllBAC (Table 13), so these spatial differences are likely

explained by the same factors that affect BoBAC, downstream dilution and extensive livestock production throughout the watershed.

Enterococcus and *Escherichia coli*

There was no significant difference in concentrations or loads between sites for *Enterococcus* (Table 14 and Table 15). *Escherichia coli* concentration at Site 1, the most downstream site, was significantly lower than all other sites in the watershed (Table 14). It is possible that significant differences would have been observed had the samples been diluted. *Escherichia coli* loads were significantly lower at Site 8 compared to Site 1 through Site 6 (Table 15). Sites 2, 3, and 4 had significantly higher *Escherichia coli* loads than Sites 7 and 8 (Table 15).

Temporal Variability

Temporal variability of all fecal indicator organisms were examined for trends in concentration and load data. Temporal variability of HuBAC, BoBAC, AllBAC, and *Escherichia coli* loads showed a clear pattern of being significantly greater during wetter months and lower during drier months (Figures 18, 20, 22, and 26). Additional trends possibly could have been observed had the samples been diluted to more accurately quantify *Escherichia coli*. Fecal indicator concentrations did not display a clear pattern (Figures 17, 19, 21, and 25). Although the maximum concentrations for all fecal indicator organisms occurred during wet months and the lowest concentrations were observed during dry months, middle values were not predictable; sometimes dry months had higher

concentrations than wetter months. This was true for all fecal indicator organisms. Similarly, no trends were observed for *Enterococcus* (Figure 23 and 24).

HuBAC

Concentrations of human-associated *Bacteroides* were significantly lower in October, 2006, a dry month, compared to wetter months of July, February, January, and April of the same year (Figure 17). HuBAC loads were significantly greater during the wet months of September, January, April, and February of 2006 than drier months such as October, August, and June of 2006 in addition to November 2005 (Figure 18).

BoBAC

Bovine-associated *Bacteroides* concentrations for the wetter sampling dates in January, February, and April of the year 2006 were significantly greater than during drier months of October, July, and November of 2006 and November of 2005 (Figure 19). Moreover, BoBAC concentration on April 27, 2006 was much higher than all other dates except for January, February, and April 3 of the year 2006. Bovine sources of fecal pollution are expected to increase during wet months, because precipitation and runoff are higher (Collins et al., 2005; Kistemann et al., 2002). BoBAC loads were significantly lower in the drier months of July and October of 2006 and November 2005 than September, January, February, and April 2006, which were the wettest months (Figure 20). This suggests runoff is the main pathway for bovine-associated *Bacteroides* to enter Pond Creek.

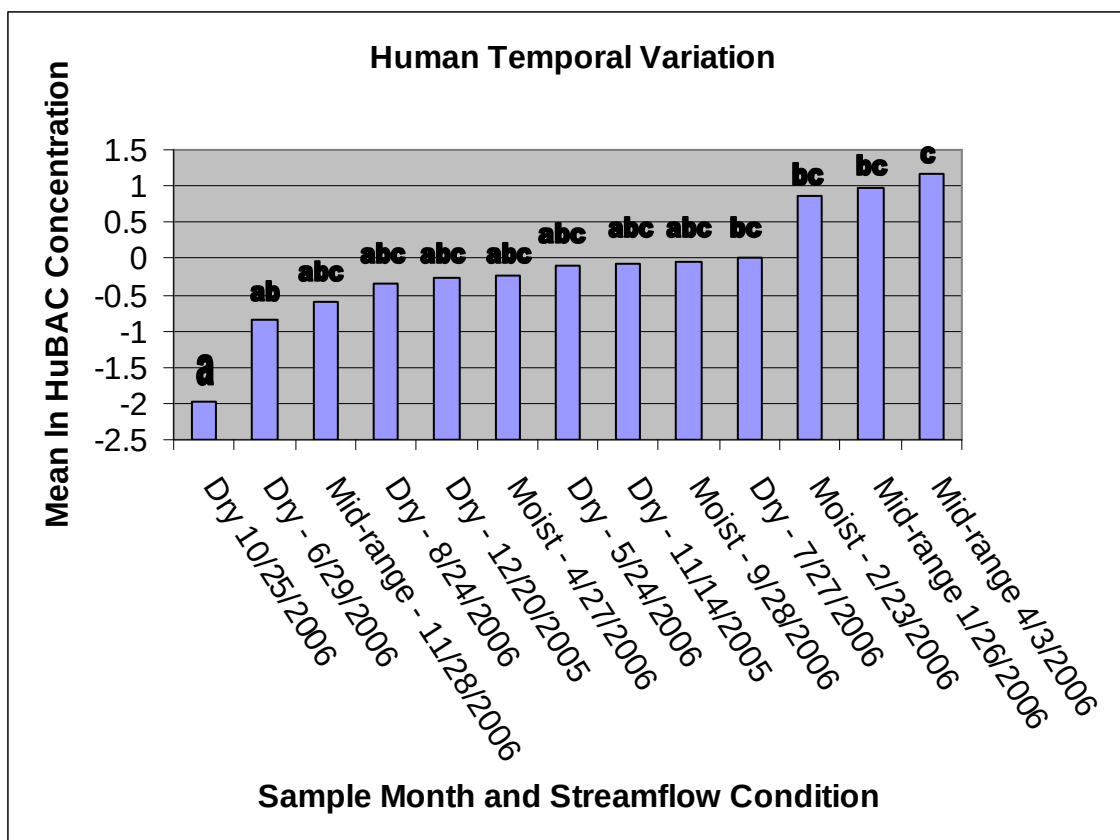


Figure 17. Mean ln HuBAC concentrations from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

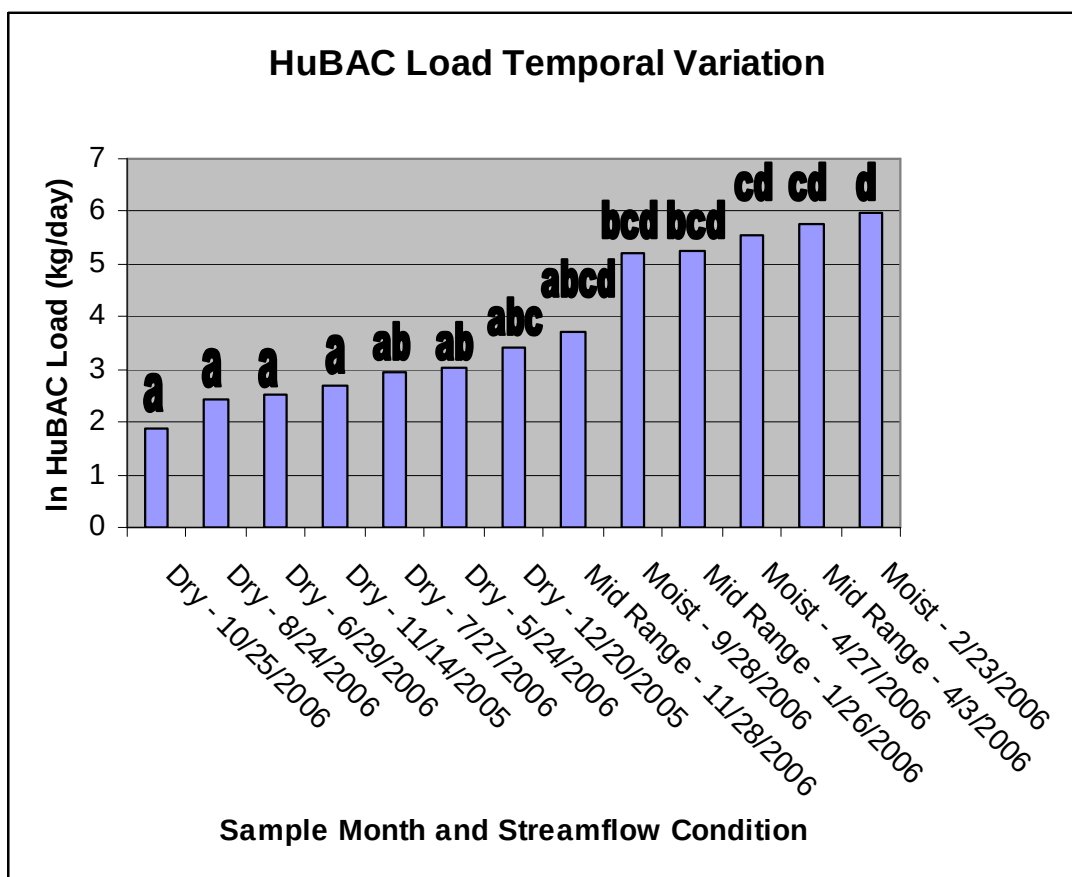


Figure 18. Mean ln HuBAC load from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

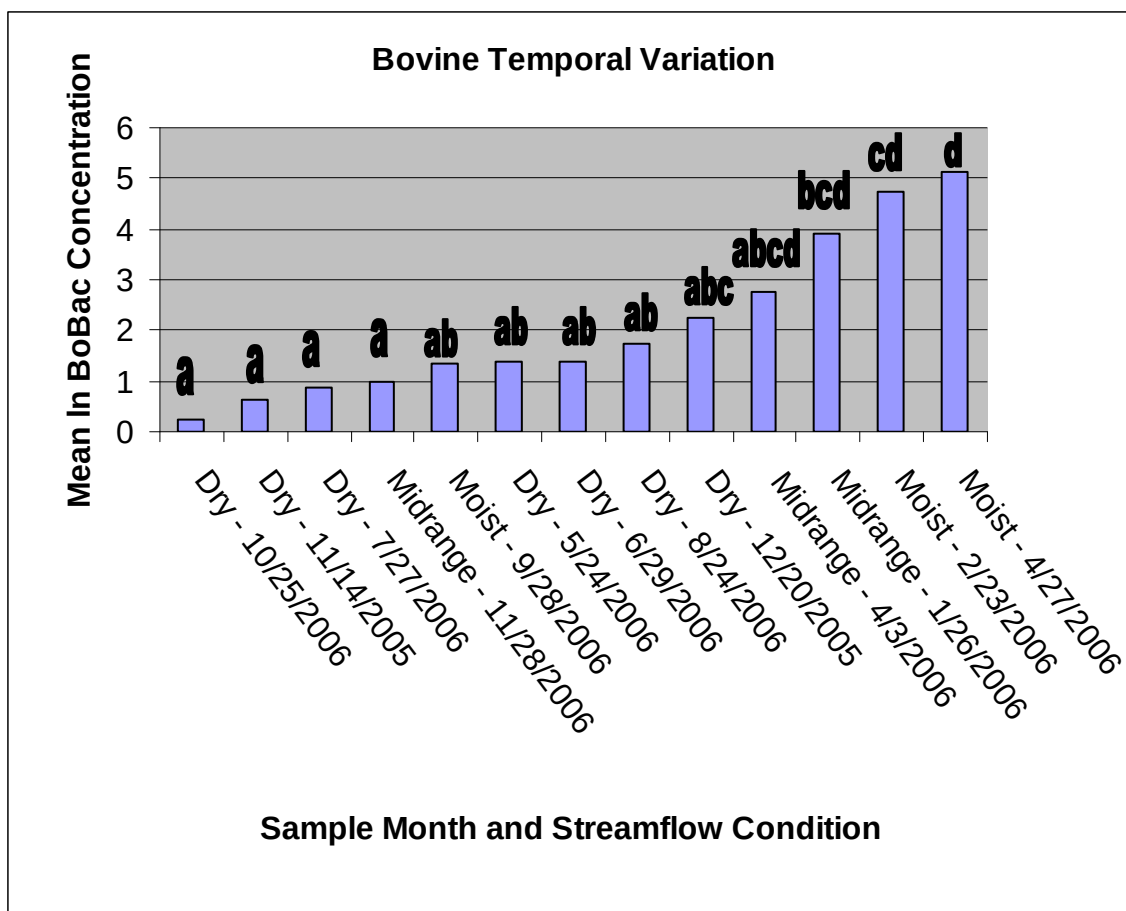


Figure 19. Mean ln BoBAC concentrations from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

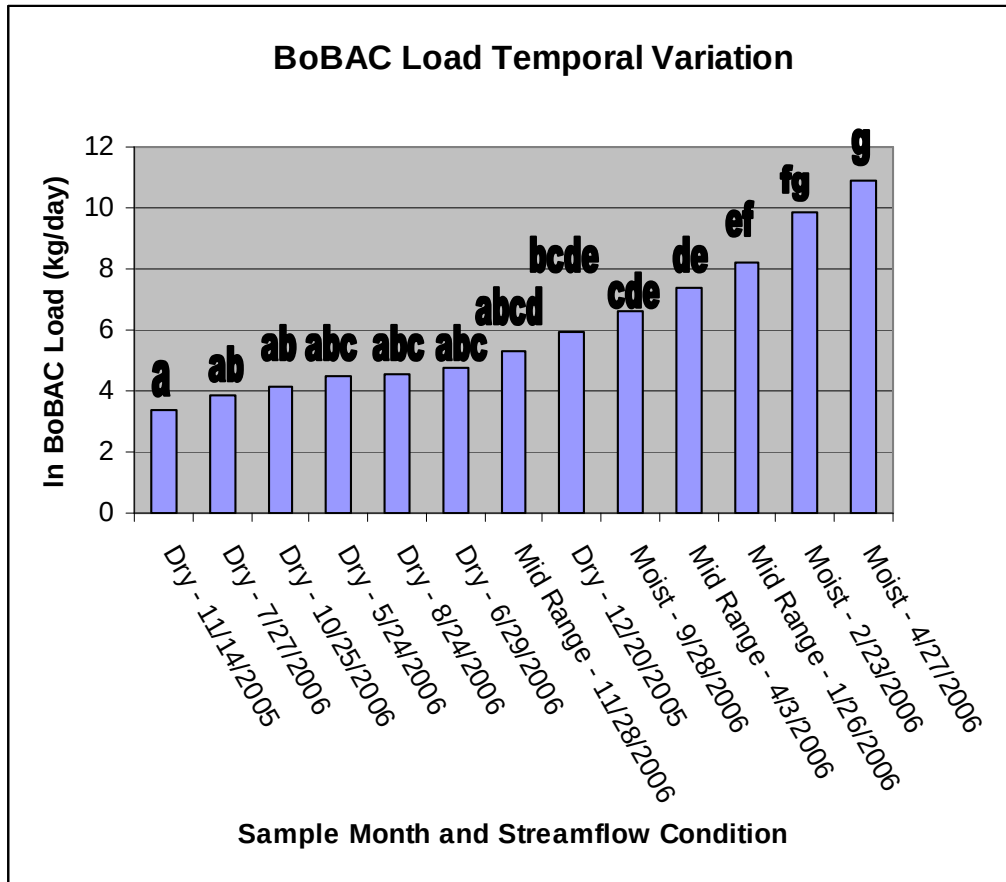


Figure 20. Mean In BoBAC loads from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

AllBAC

Our results show that bovine sources make up approximately 99 percent of total *Bacteroides* in the Pond Creek watershed. Therefore, AllBAC temporal patterns should follow BoBAC patterns. Concentrations of *Bacteroides* detected by the AllBAC assay were significantly lower in October 2006, a dry month, than for sampling events in January and February 2006 and for two additional dates in April 2006, which were wetter months. The February 2006 event was much higher than eight other sampling dates

(Figure 21). Correlation analysis confirmed that these two variables behave similarly; the Pearson correlation coefficient is 0.903. AllBAC loads were significantly lower in July, August, and October of the year 2006 and November 2005 than during wetter months of January, February, and April of 2006, suggesting runoff carries the majority of all detectable *Bacteroides* into Pond Creek (Figure 22).

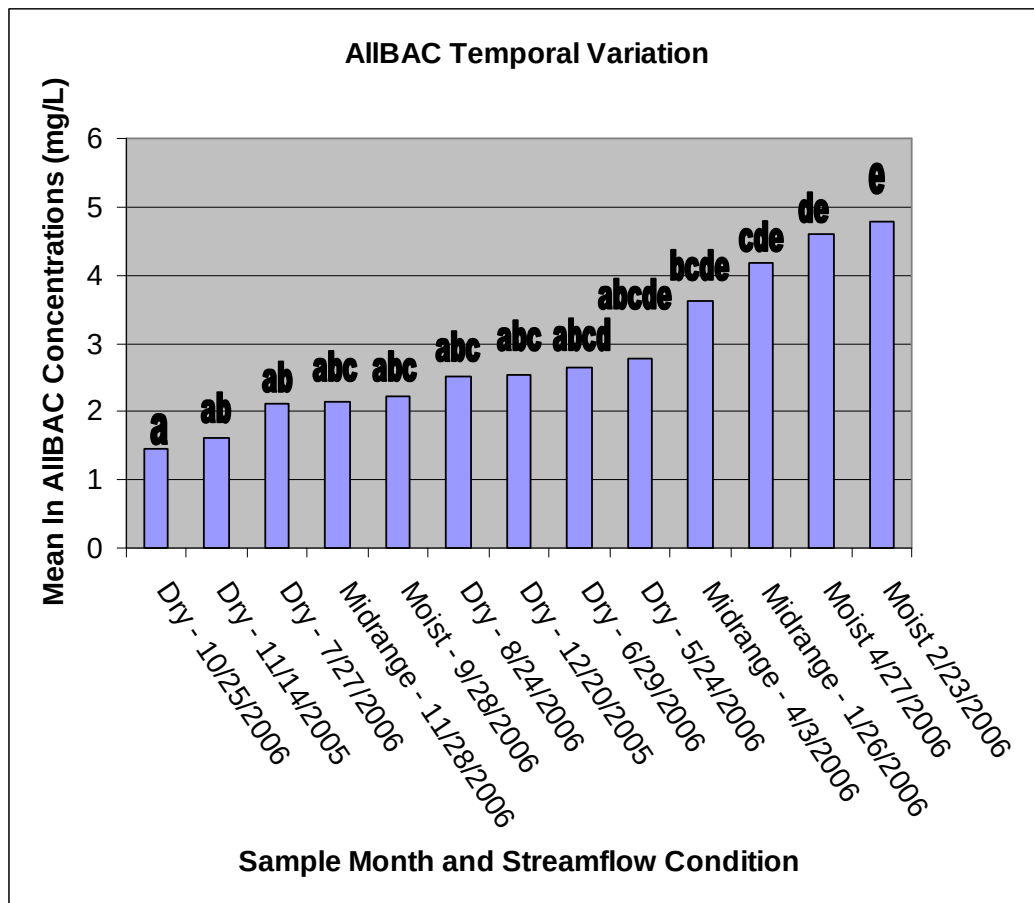


Figure 21. Mean In AllBAC concentrations from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

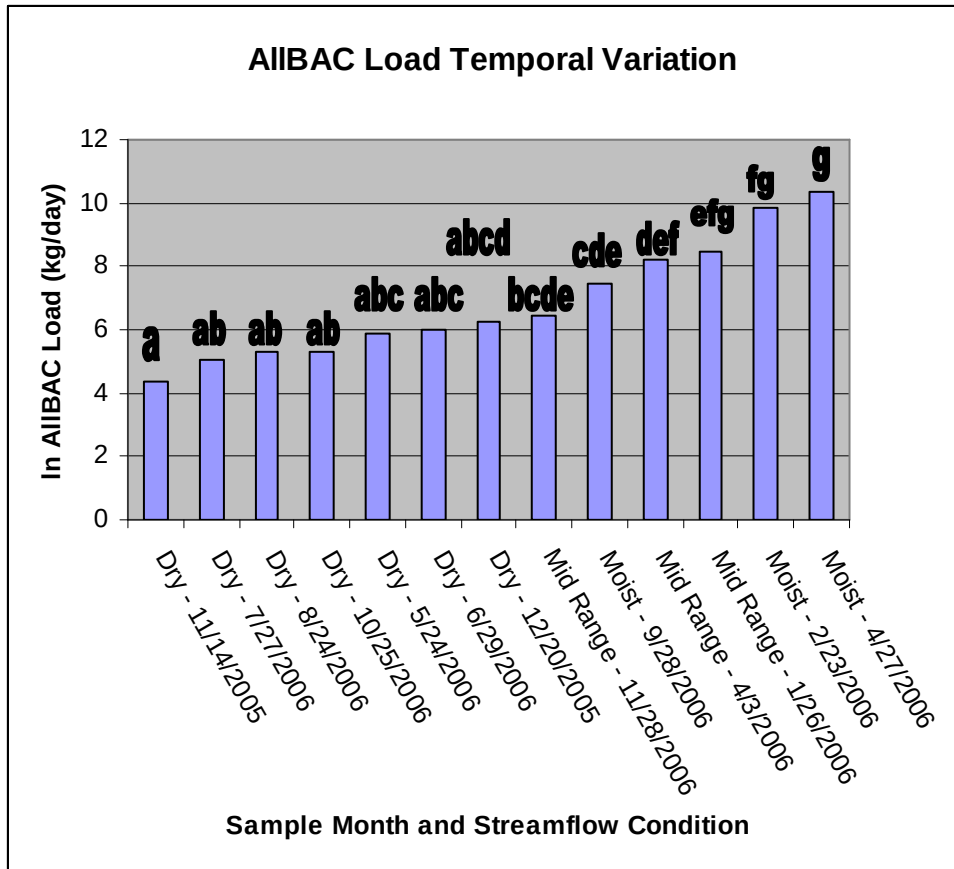


Figure 22. Mean In AllBAC load from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

Enterococcus and Escherichia coli

No trends in *Enterococcus* load or concentration data were observed (Figure 23 and Figure 24). For *Escherichia coli*, October 2006 was the only month significantly lower than months where the highest average concentrations were observed, twice in April and once in February, 2006 (Figure 25). *Escherichia coli* loads were significantly higher in February and April of 2006 than the drier months of August 2006 and November 2005 (Figure 26). It is possible that additional trends could have been observed for *Escherichia coli* had the samples been diluted to fall with the range of the

IDEXX kits. Again, the results are similar to other studies that show increases of fecal indicators during wet months (Collins et al., 2005; Kistemann et al., 2002).

With respect to time, bovine-associated *Bacteroides* showed the highest variability; they ranged over two orders of magnitude. The highest concentration detected by the AllBAC assay was 24 times greater than the lowest AllBAC value. In general Bovine associated concentrations trended in the same manner as AllBAC detected *Bacteroides*.

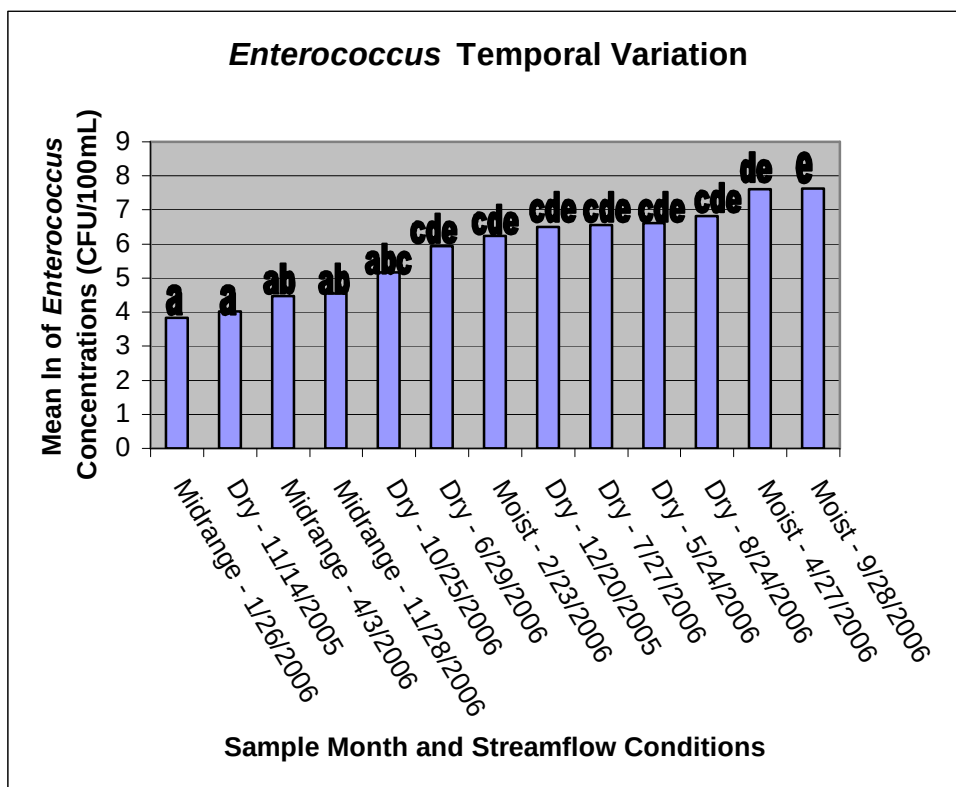


Figure 23. Mean ln *Enterococcus* concentrations from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

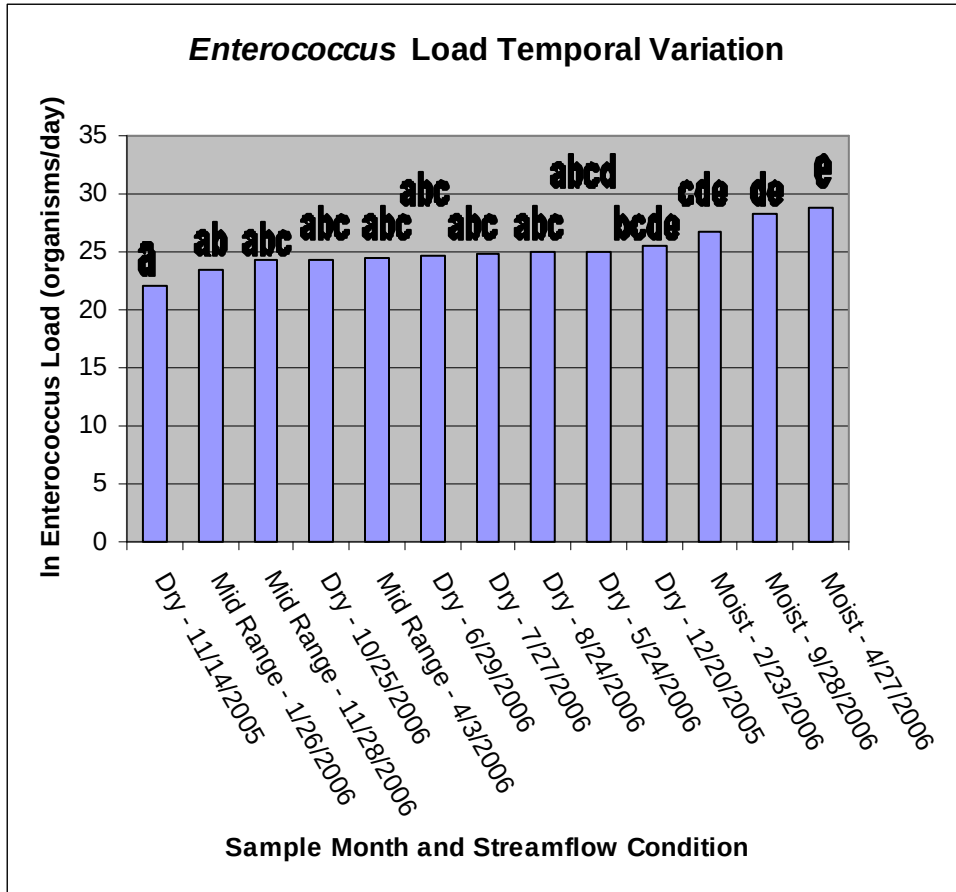


Figure 24. Mean ln *Enterococcus* loads from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

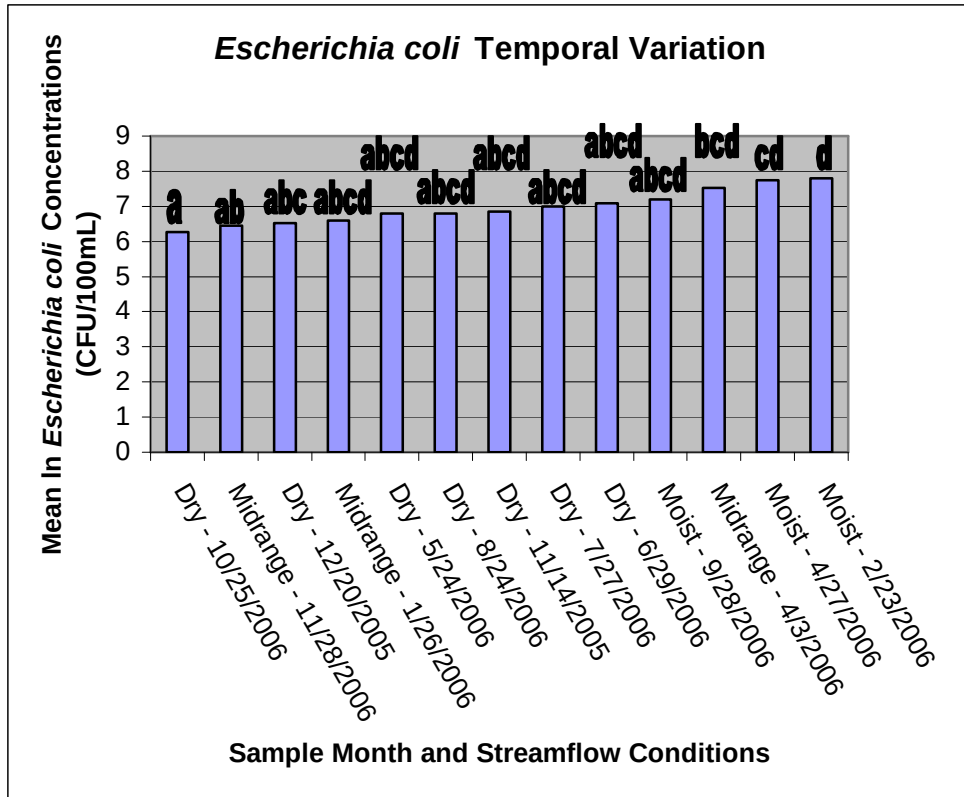


Figure 25. Mean ln *Escherichia coli* concentrations from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

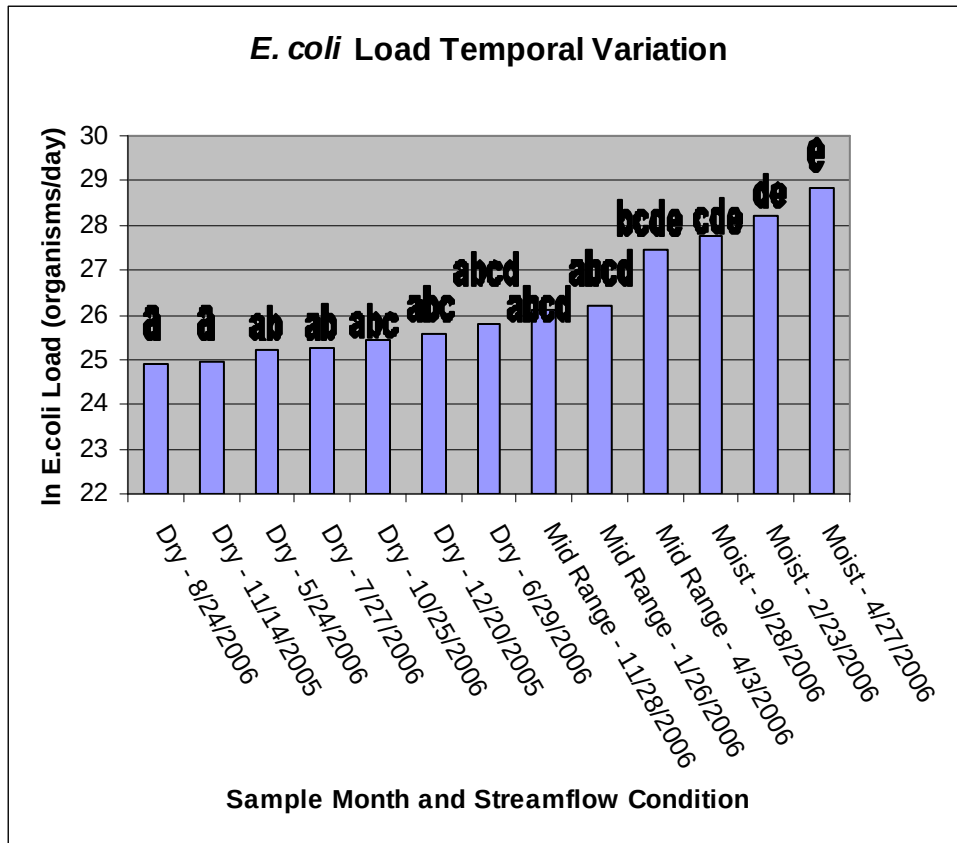


Figure 26. Mean ln *Escherichia coli* loads from all eight sampling sites showing Tukey HSD statistical differences between sampling dates during a period from November 2005 to November 2006.

Their concentrations were also very similar from November 2005 until May 2006; after which their concentrations were different by almost an order of magnitude.

From May through August, all sampling trips were during hot and dry summer conditions. It is unlikely that the difference was caused by another source, because bovine associated *Bacteroides* composed 99 percent of all *Bacteroides* detected. The difference is likely due to the assay's reduced ability to detect the bovine associated *Bacteroides*, not a reduction in bovine fecal inputs. It is speculated that differences in the cattle's diet, environmental stresses, or a change in the microbial fauna in the rumen

during the summer cause the previously dominant cluster the assay was designed to detect to become less prolific (USEPA, 2005b).

In contrast, concentrations of human associated *Bacteroides* were always much lower than either AllBAC or BoBAC detected assays, 100 times lower on one sample date. Also, for most of the study, human associated *Bacteroides* behaved inversely to AllBAC and BoBAC *Bacteroides*.

Correlations

HuBAC

Human-associated *Bacteroides* were significantly correlated with all parameters except for nitrate, flow, and total solids (Table 17). All significant correlations showed positive relationships. Five-day biological oxygen demand and total organic carbon showed the strongest relationship while sulfate, chloride, and orthophosphate were less strongly correlated. The absence of a correlation between streamflow and total solids with HuBAC is expected from human sources, because it is a flow independent, point source of pollution (USEPA, 2007; McPherson, 2002). Fecal matter is an organic material, so it is expected to have a positive correlation with BOD₅ (Viessman and Hammer, 1993) and TOC (EBMUD, 2001).

BoBAC

Bovine-associated *Bacteroides* were significantly correlated with all parameters except for nitrate (Table 17).

Table 17. Summary of Pearson Correlations using mean ln transformed concentrations of fecal indicator organisms and other measured parameters collected between November 2005 and November 2006 at all eight sample sites in Pond Creek, TN (HUC 06010201013).

Pearson Correlation		lnHuBac	lnBoBac	lnAllBac	lnentero	lnecoli
	lnHuBac	1	.551(**)	.593(**)	-0.014	.392(**)
	lnBoBac	.551(**)	1	.903(**)	.205(*)	.642(**)
	lnAllBac	.593(**)	.903(**)	1	.245(*)	.625(**)
	lnentero	-0.014	.205(*)	.245(*)	1	.306(**)
	lnecoli	.392(**)	.642(**)	.625(**)	.306(**)	1
	TS	0.174	.237(*)	.354(**)	.237(*)	.442(**)
	FLOW	-0.099	.327(**)	.285(**)	.301(**)	0.163
	TOC ppm	.393(**)	.549(**)	.647(**)	.336(**)	.541(**)
	NH3-N ppm	.250(*)	.345(**)	.462(**)	.198(*)	.310(**)
	NO3 ppm	-0.187	-0.152	-0.174	0.169	-.196(*)
	TKN ppm	.269(**)	.315(**)	.404(**)	0.047	.267(**)
	Cl ppm	.372(**)	.450(**)	.496(**)	.244(*)	.379(**)
	SO4 ppm	.334(**)	.474(**)	.447(**)	0.124	0.176
	PO4-P ppm	.284(**)	.476(**)	.589(**)	.358(**)	.352(**)
	TP ppm	.216(*)	.444(**)	.534(**)	.372(**)	.382(**)
	BOD 5- day	.431(**)	.667(**)	.705(**)	0.112	.369(**)

*Significant at the 0.05 level.

**Significant at the 0.01 level.

All other variables were positively correlated to BoBAC concentration. This is expected, because bovine fecal inputs are largely the result of overland flow. Many other studies have also demonstrated increased pollutant levels in waterbodies for pollutants carried by overland flow following precipitation events (Edwards et al., 1997; McPherson, 2002; Vinten et al. 2004). Five-day biological oxygen demand and total organic carbon showed a stronger relationship with BoBAC than for HuBAC. This also supports the belief that

bovine fecal inputs are from overland flow, because more organic material is washed into the stream during high flow conditions than during low flow conditions (McPherson, 2002). Most notably, BoBAC shows a .903 correlation coefficient with AllBAC (Table 17). This suggests that bovine-associated *Bacteroides* are the dominant source of fecal pollution in Pond Creek. Gentry et al. (2007) also found a strong correlation between BoBAC and AllBAC at several sampling sites and concluded bovine sources are the main source of fecal contamination at those sites.

AllBAC

Concentrations of total detectable *Bacteroides* were correlated with all measured variables except for nitrate (Table 17). The AllBAC assay was most strongly correlated with five-day biological oxygen demand and total organic carbon. AllBAC concentrations had the highest Pearson correlation coefficients compared to other fecal indicator organisms. Gentry et al. (2007) also found a high correlation between *Escherichia coli*, AllBac, and BoBac with the following variables, 7-day antecedent precipitation, flow, and turbidity. AllBAC was correlated with both streamflow and total solids, a parameter related to turbidity.

Enterococcus and *Escherichia coli*

Enterococcus was significantly correlated with total solids, streamflow, total organic carbon, ammonia, chloride, orthophosphate, and total phosphorus, with total and soluble phosphorus having the highest correlations (Table 17). *Escherichia coli* was the only fecal indicator that was correlated to nitrate; additionally, it is the only negative

correlation (Table 17). *Escherichia coli* was most strongly correlated with total organic carbon and total solids, but it was also correlated with streamflow. Collins et al. (2005) found that under heavy rainfall overland flow can transport substantial levels of *Escherichia coli* to streams. Kistemann et al. (2002) also showed how the bacterial load increases in waterbodies during extreme rainfall and runoff events.

In summary, the correlation analysis strengthened the case for cattle being the main source of fecal contamination in Pond Creek. Bovine-associated *Bacteroides* concentration values are very strongly correlated with total *Bacteroides* concentrations; the Pearson correlation coefficient was 0.903. Most of the variables were positively correlated. Only *Escherichia coli* and nitrate showed a significant negative relationship; however, it was fairly weak (-0.196). Also of interest, every variable except for human-associated *Bacteroides* was positively correlated with total solids. This is expected due to the flow independent nature fecal inputs from human sources. Conversely, bovine sources are primarily the results of overland flow or direct deposits by cattle into the stream, both of which are accompanied by elevated levels of sediment in the stream.

Load Duration Curves

Loads are highest during high flows and decrease as flow decreases. This indicates that *Escherichia coli* loads are heavily influenced by surface runoff. Observed *Escherichia coli* loads were consistently above the target load of 126 CFU 100mL⁻¹ at all sites and for all sample dates, except for one date at Site 1 and two dates at Site 8 (Appendix A), and likely would have been higher had the samples been diluted to fall within the range of the IDEXX kits. TDEC considers 126 CFU 100mL⁻¹ to be the

maximum *Escherichia coli* concentration acceptable for recreational use of a waterbody, and is the target value used for developing TMDLs.

Appendix B contains *Escherichia coli* load duration curves that differentiate the *Escherichia coli* load from human and bovine sources. Figures 27 and 28 are presented to show general trends among the sample sites.

When the load duration curves are separated into their bovine and human components, the *Escherichia coli* load duration curves are different from the more generalized LDCs in Appendix A. The human associated *Escherichia coli* load is usually flow independent (Table 18, Figure 28, Appendix B), which suggests point sources of contamination such as failing septic systems or untreated discharge of sewage into the creek (McPherson, 2002).

The bovine-associated *Escherichia coli* load is generally above the target load (Table 18, Figure 27, Appendix B). The human-associated *Escherichia coli* load is usually under the target load (Table 18, Appendix B). This indicates that remediation efforts should be focused on beef cattle and dairy operations in the watershed. Allocating resources to reduce human sources of *Escherichia coli* would not sufficiently reduce pathogen levels to meet TMDL guidelines.

It is speculated that reductions in pathogen loading rates will be obtained by implementing agricultural best management practices. Best management practices specifically targeted to reduce the effect of cattle on stream water quality include exclusion, riparian buffers, alternative water sources for livestock, providing additional shade for livestock, management of pasture stocking density, vegetative filter strips, and nutrient management (Agouridis et al., 2005; Coyne et al., 1995).

Pond Creek #2 *E. coli* attributable to Cattle Load Duration Curve

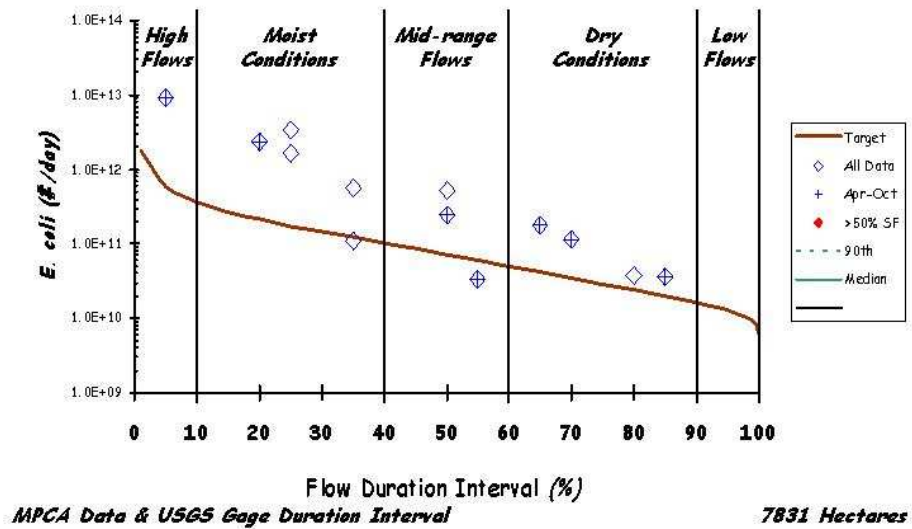


Figure 27. *Escherichia coli* load attributable to cattle at Site 2 for 13 data points collected between November 2005 and November 2006 in Pond Creek, TN (HUC 06010201013). Target *Escherichia coli* load is 126 CFU 100 mL⁻¹.

Pond Creek #2 ***E. coli* attributable to Humans** **Load Duration Curve**

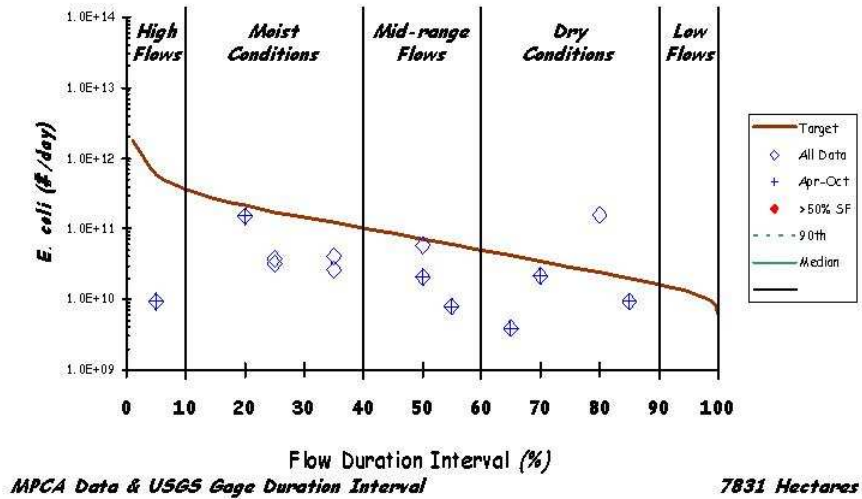


Figure 28. *Escherichia coli* load attributable to humans at Site 2 for 13 data points collected between November 2005 and November 2006 in Pond Creek, TN (HUC 06010201013). Target *Escherichia coli* load is 126 CFU 100 mL⁻¹.

Table 18. Characterization of bovine and human-associated *Escherichia coli* load in Pond Creek, TN (HUC 06010201013) using Load Duration Curves separated into bovine and human components and data collected between November 2005 and November 2006.

Site	Bovine <i>E. coli</i> Load		Human <i>E. coli</i> Load	
	% of Samples	LDC Type	% of Samples	LDC Type
	above Target Load		above Target Load	
1	77%	Flow Dependent	8%	Flow Independent
2	85%	Flow Dependent	8%	Flow Independent
3	85%	Flow Dependent	15%	Flow Independent
4	85%	Flow Dependent	31%	Flow Independent
5	100%	Flow Dependent	31%	Flow Independent
6	69%	Flow Independent	31%	Flow Independent
7	92%	Flow Dependent	46%	Flow Dependent
8	38%	Flow Dependent	0%	Flow Dependent

Line et al. (2000) reported that an off stream water source as a single practice did not reduce pollutant loads from grazed pastures in North Carolina. Moreover, exclusion and riparian buffers are not popular with producers due to the cost of installation, increased operating costs due to providing other water sources, and loss of grazing land (Agouridis et al., 2005). An alternative to absolute exclusion is the concept of a riparian pasture. A riparian pasture acts as a vegetative filter strip that reduces overland transport of pollutants while allowing stream-side areas to be grazed for short periods when the stream is least sensitive to the effects of grazing (Agouridis et al., 2005). Another study reported that a 95 percent reduction in *Escherichia coli* bacterial loads was possible if cattle feces are deposited at least 2.5 meters from the stream (Larsen et al. 1994). In this scenario, vegetation (grassland buffers) acts to slow runoff and filter pollutants out of runoff before it enters the stream. Coyne et al. (1995) also reports vegetative filter strips can remove 90 percent or more of pollutants from runoff. Rotational grazing can also reduce levels of fecal coliforms and turbidity in streams (Sovell et al., 2000; Lyons et al. 2000.) Gary et al. (1993) showed that maintaining stocking densities less than 1.75 cow/calf pairs ha⁻¹ can also help reduce bacterial loads in streams. An alternative to a single vegetative filter strip at the edge of the creek would be to make the entire pasture a filter strip by improving the condition of pastures. This can be accomplished by rotational grazing and managing livestock stocking densities.

Most of the literature examines the effect of a single BMP on water quality, but several studies have shown that combining several BMPs has a net improvement on water quality (Edwards et al., 1997; Brannan et al., 2000; Agouridis et al., 2005). Brannan et al. (2000) reported reductions in nutrients and sediment levels in a Virginia

watershed by implementing waste storage facilities, nutrient management, exclusion, water troughs, and stream crossings. Edwards et al. (1997) noted that nutrient management was the most probable cause of improved water quality.

In summary, a combination of BMPs will likely improve water quality in Pond Creek. Vegetative filter strips, nutrient management, vigilant riparian grazing, and careful rotational grazing and stocking density management are the most promising practices to achieve a reduction in *Escherichia coli* loading rates.

CHAPTER V

CONCLUSION

During the 13-month investigation of fecal indicator organisms in Pond Creek, *Escherichia coli* concentrations regularly exceed water quality standards. *Escherichia coli* values exceeded the one time sampling maximum value of 941 CFU 100 mL⁻¹ for 60 percent (64 of 104) of all samples. Furthermore, Load Duration Curves plainly show that *Escherichia coli* loads are consistently above the desirable level (126 CFU 100mL⁻¹). Pond Creek is clearly receiving excessive amounts of fecal pollution and potentially dangerous pathogens.

Analysis of Pond Creek water samples using host associated real-time PCR assays indicated that cattle were the dominant source of fecal pollution. Although, human-associated *Bacteroides* were detected, their concentrations remained relatively low across the watershed. Bovine-associated *Bacteroides* were very highly correlated ($r^2 = .903$) with AllBAC concentrations. These two variables were so highly related, because bovine-associated *Bacteroides* composed the majority (99 percent) of all detected *Bacteroides spp.* in the Pond Creek watershed. It is possible that undetected sources such as wildlife or *Bacteroides* belonging to a cluster the assays could not detect were present; however, it is safe to say that cattle were the dominant source of fecal pollution during the course of our study. An investigation of the *Escherichia coli* Load Duration Curves separated into human and bovine components also supported this idea (Appendix B). The human fraction of the *Escherichia coli* load rarely exceeded water quality standards.

Site 1, the most downstream site, was the only site with significantly lower *Escherichia coli* concentrations than the other sites. Site 8, a headwaters site, generally

had the highest mean fecal indicator concentrations. This site had significantly greater concentrations of both total and bovine-associated *Bacteroides* than all other sites. Fecal indicator loads, however, generally increased as you moved downstream. *Escherichia coli* and HuBAC loads at Site 1, the most downstream site, remained high, which suggests Pond Creek potentially discharges excessive levels of pathogens to Watts Bar Reservoir. No significant differences were observed for AllBAC and HuBAC loads between sample sites.

Temporal variation closely followed the seasonal weather patterns. Mean fecal indicator loads, except *Enterococcus*, were highest during the “wetter months” of January, February, and April, and additionally in September 2006 for *Escherichia coli*. The lowest average loads for all fecal indicators, except *Enterococcus*, occurred during dry months. No temporal pattern was established for fecal indicator concentrations.

Given that cattle were the main source of fecal contamination in Pond Creek, the temporal variation followed patterns typical of a flow dependent pollutant such as pathogens from manure. The majority of fecal pollution from cattle entered the stream as runoff. Precipitation increases in the wet months, which caused water to move over land transporting more fecal matter to surface waters.

To help Pond Creek meet TMDL pathogen reduction goals, our findings suggest that resource expenditures should focus on best management practices designed to reduce fecal inputs to streams from livestock. Possible best management practices include vegetative filter strips, rotational grazing, nutrient management, careful riparian grazing, and improving pastures. No statistical differences were observed between sites for bovine sources of pollution, the dominant source, therefore no recommendations can be made for

specific areas to target BMPs. Reducing human sources of fecal pollution will do little to reduce total pathogen levels, but would improve human safety by reducing levels of human pathogens.

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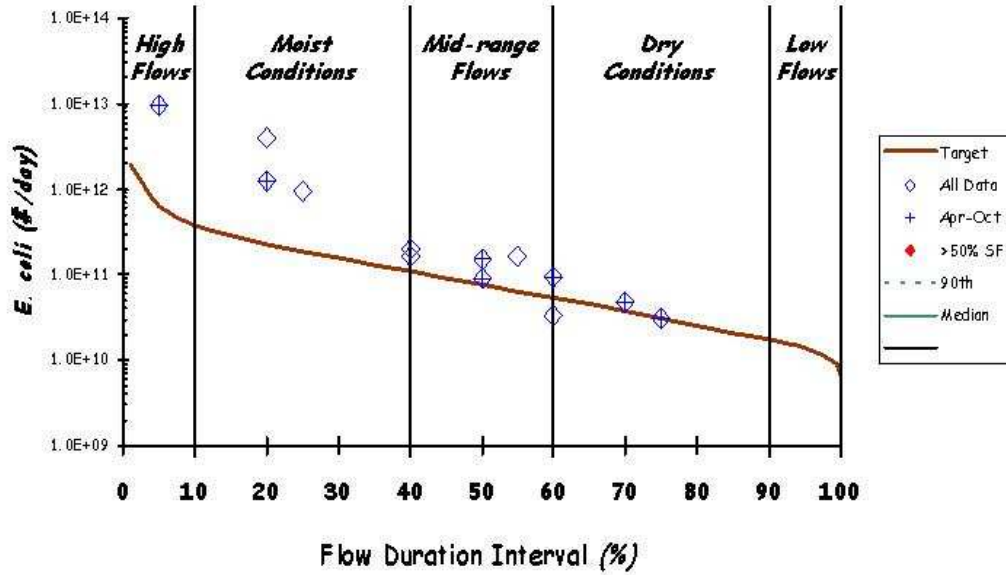
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APPENDIX

Appendix A

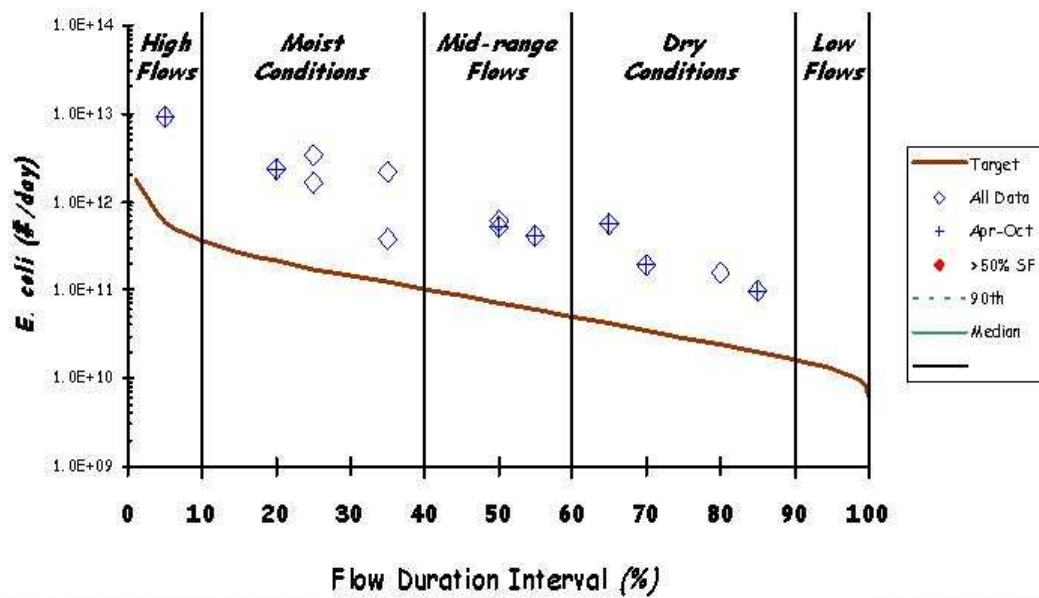
Pond Creek #1 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

8575 Hectares

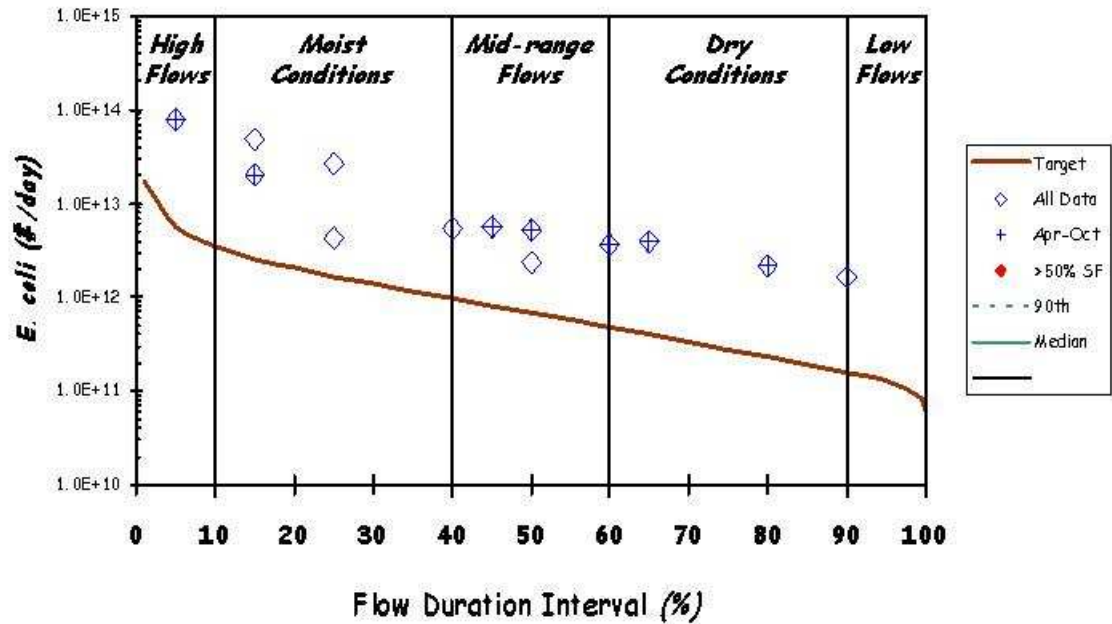
Pond Creek #2 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

7831 Hectares

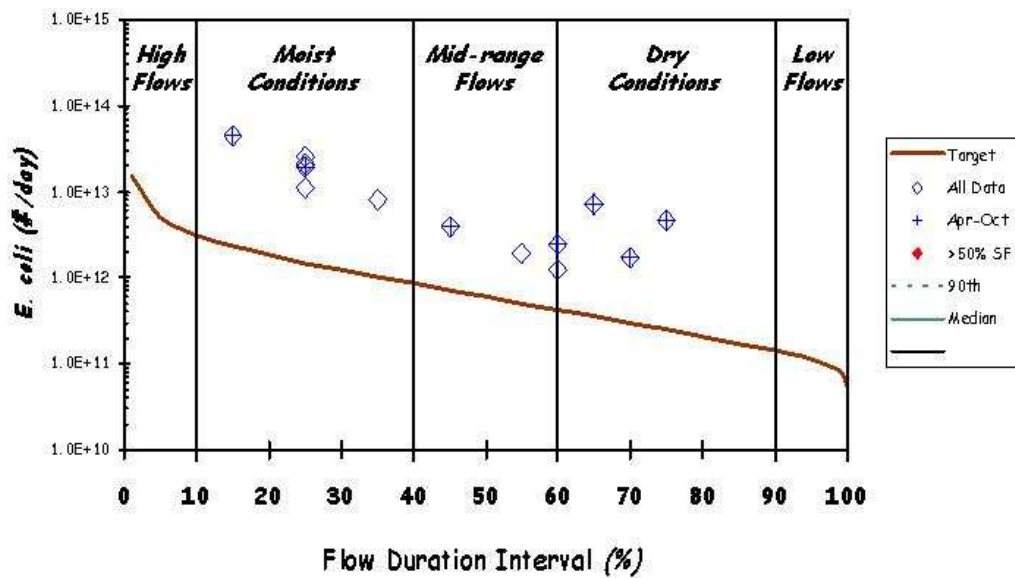
Pond Creek #3 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

7414 Hectares

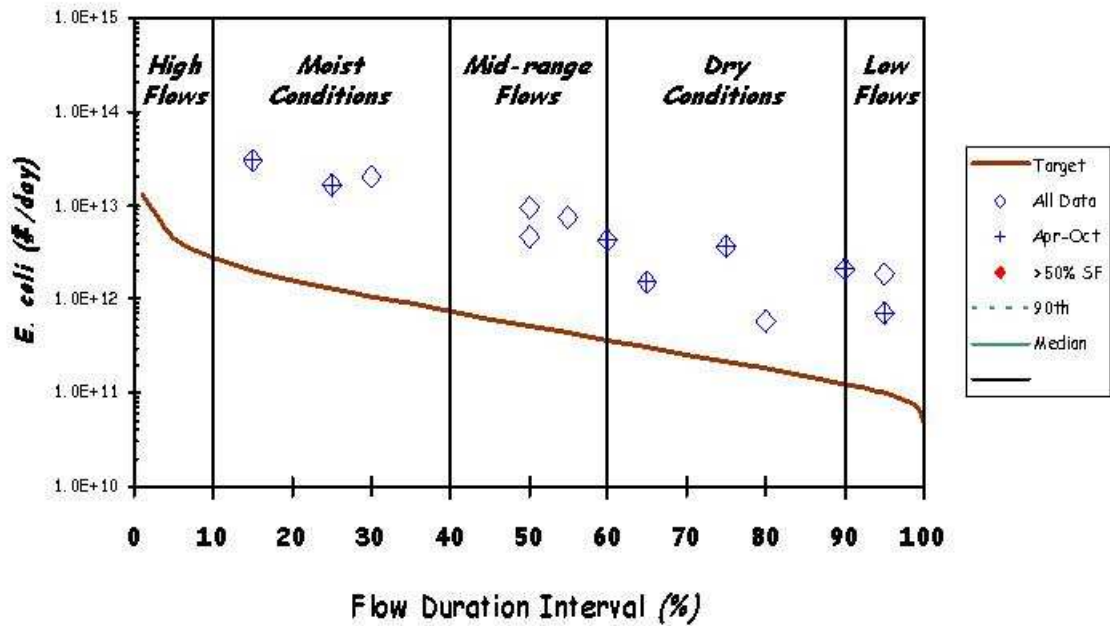
Pond Creek #4 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

6302 Hectares

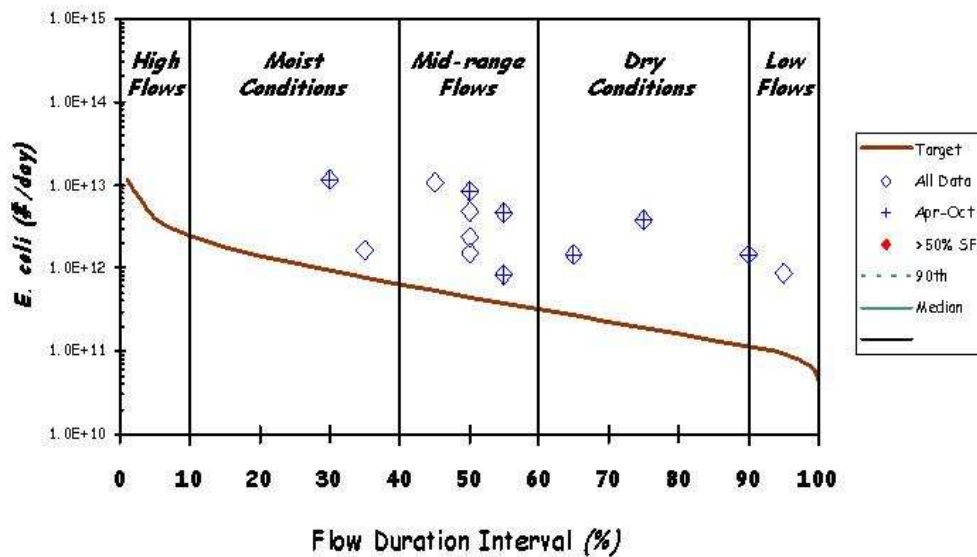
Pond Creek #5 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

5102 Hectares

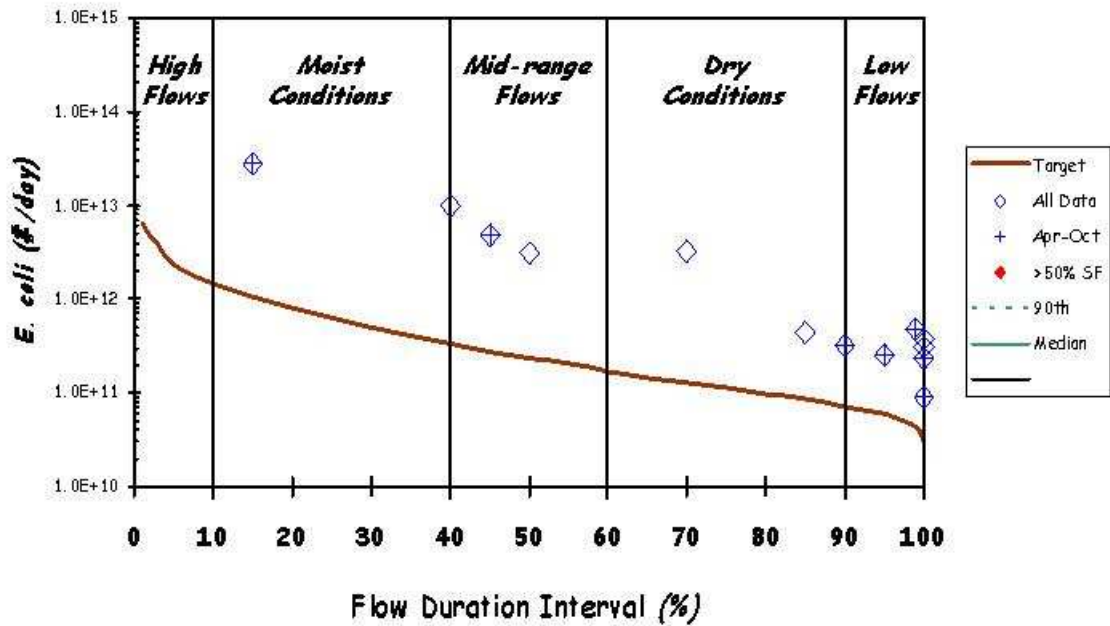
Pond Creek #6 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

4186 Hectares

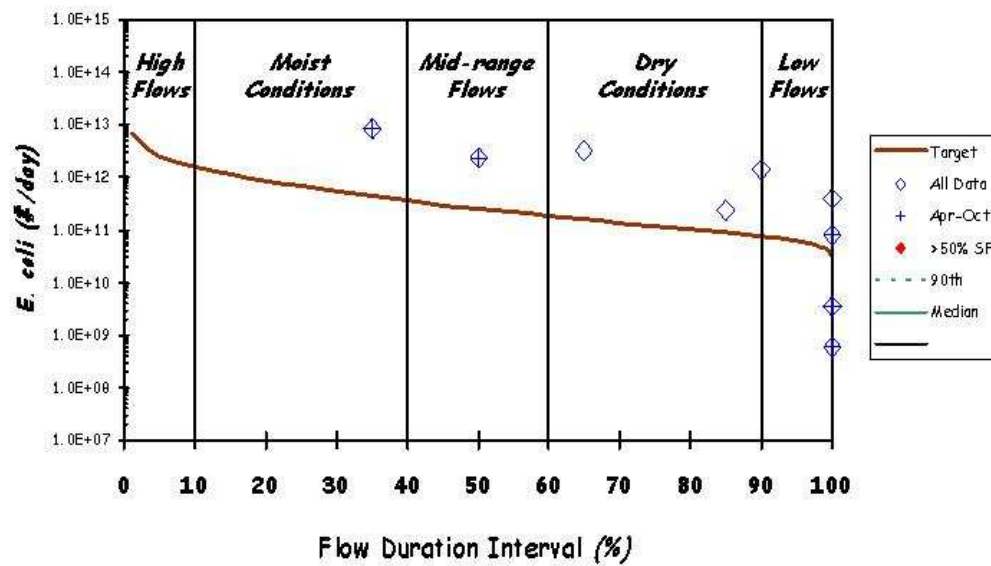
Pond Creek #7 *E. coli* Load Duration Curve



MPCA Data & USGS Gage Duration Interval

1228 Hectares

Pond Creek #8 *E. coli* Load Duration Curve

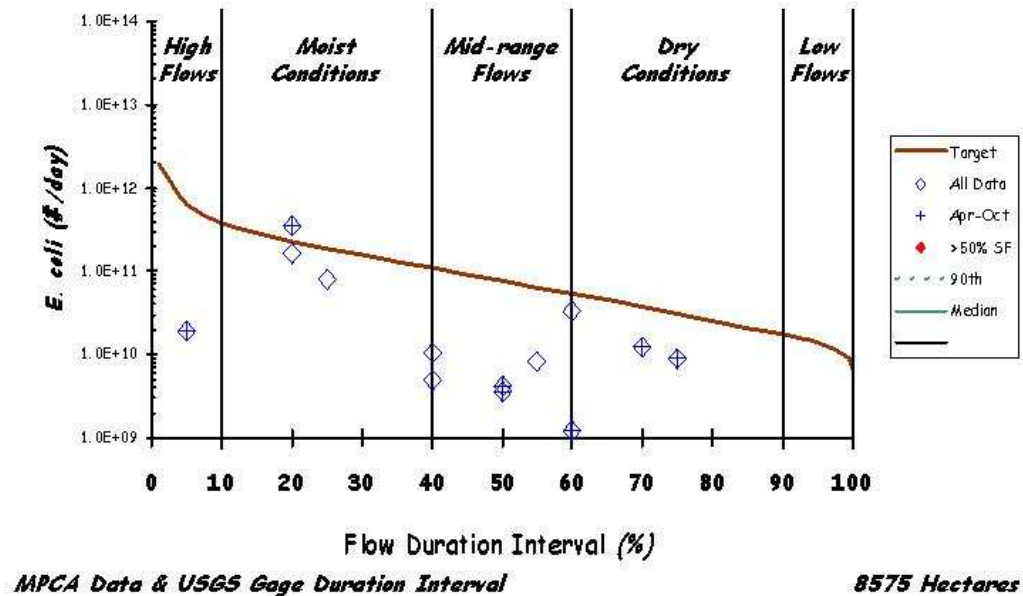


MPCA Data & USGS Gage Duration Interval

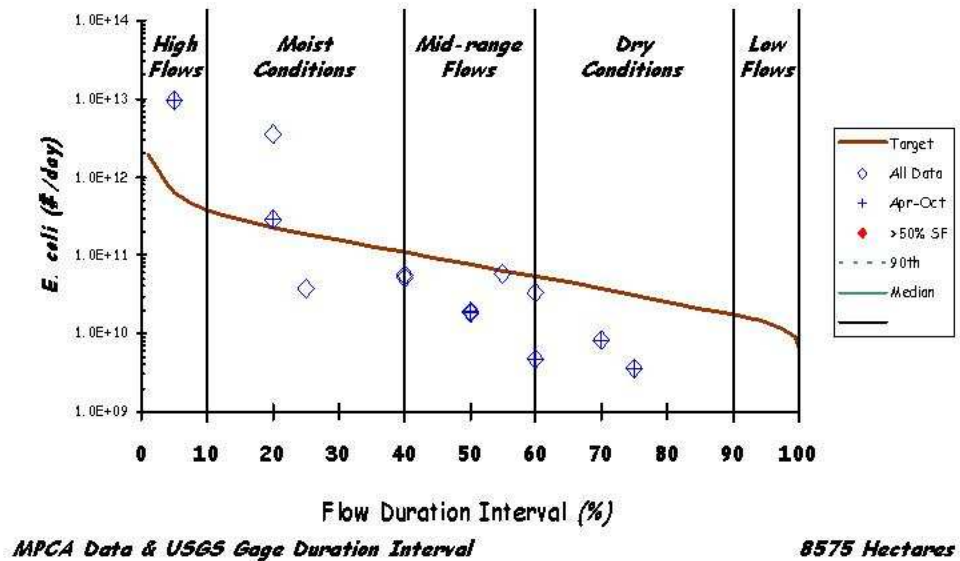
1515 Hectares

APPENDIX B

Pond Creek #1 *E. coli* attributable to Humans Load Duration Curve



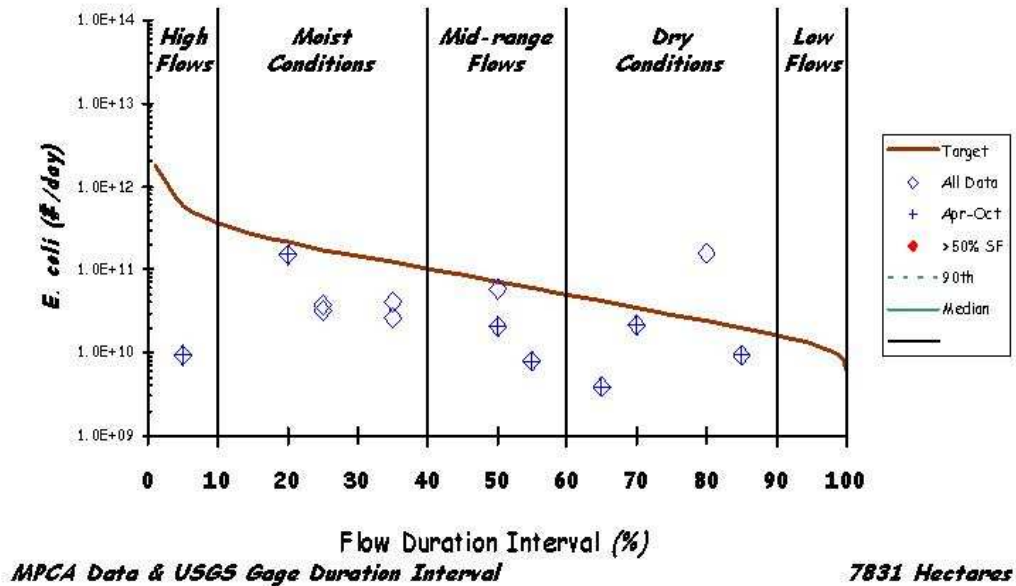
Pond Creek #1 *E. coli* attributable to Cattle Load Duration Curve



Pond Creek #2

E. coli attributable to Humans

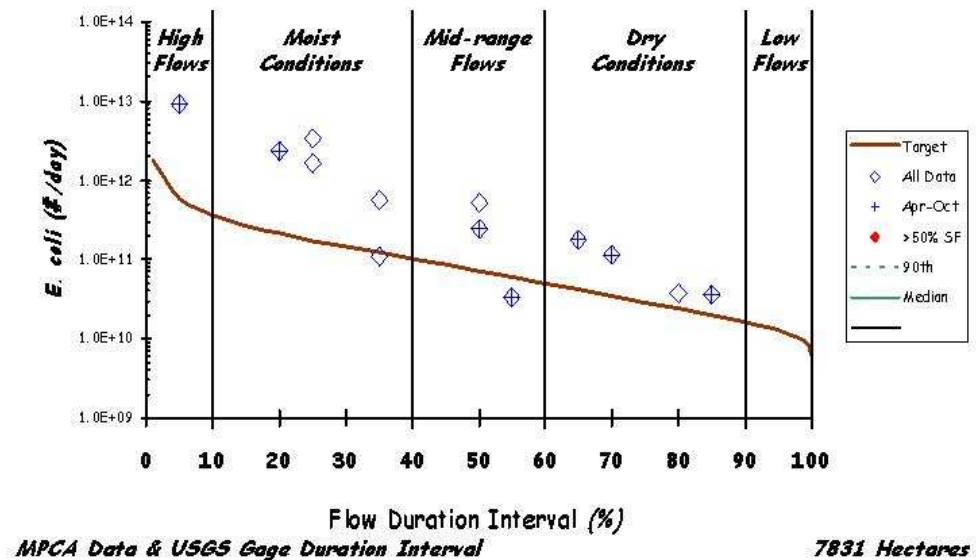
Load Duration Curve



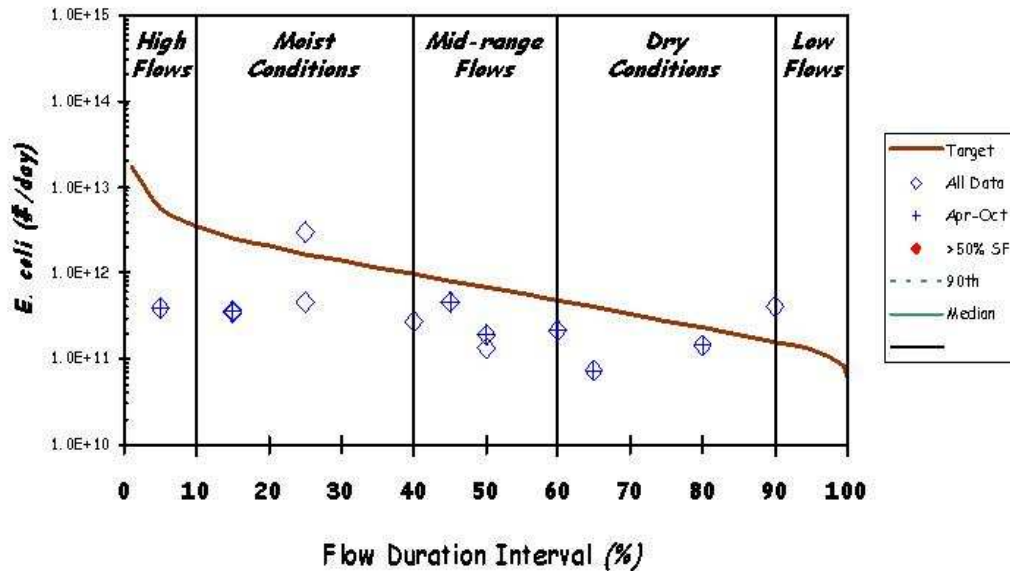
Pond Creek #2

E. coli attributable to Cattle

Load Duration Curve



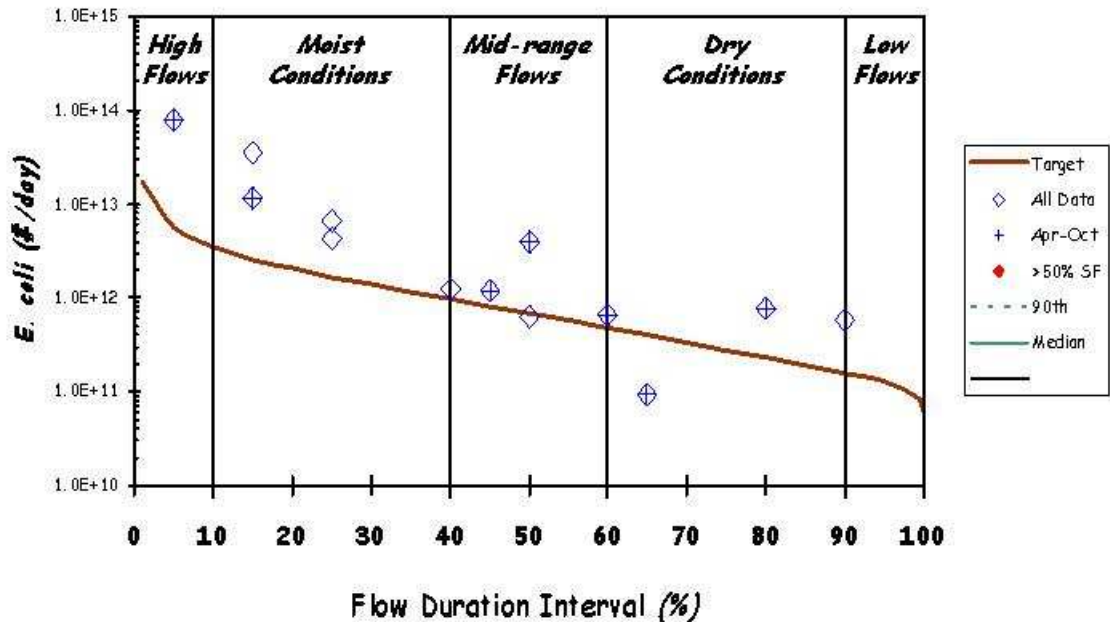
Pond Creek #3 ***E. coli* attributable to Humans** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

7414 Hectares

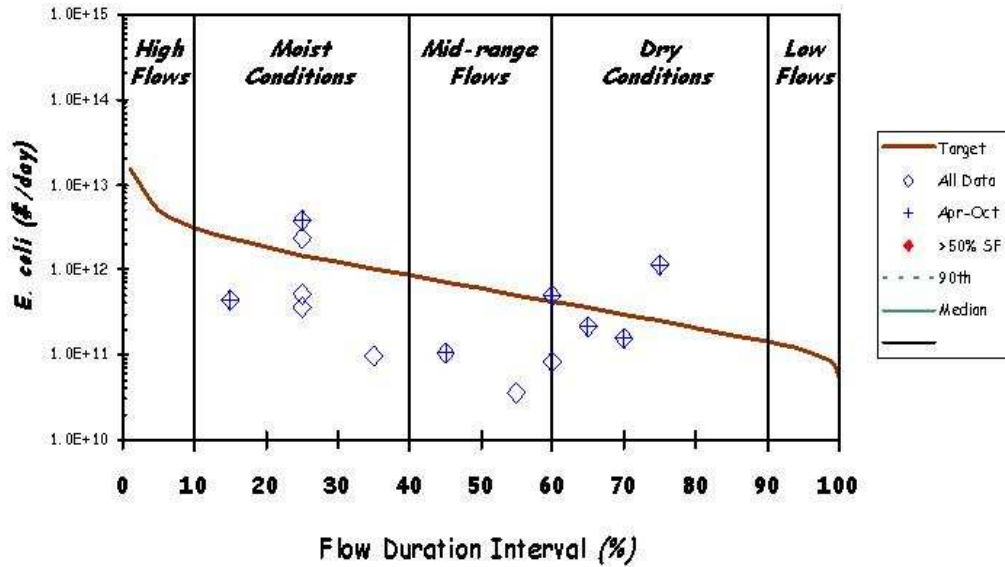
Pond Creek #3 ***E. coli* attributable to Cattle** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

7414 Hectares

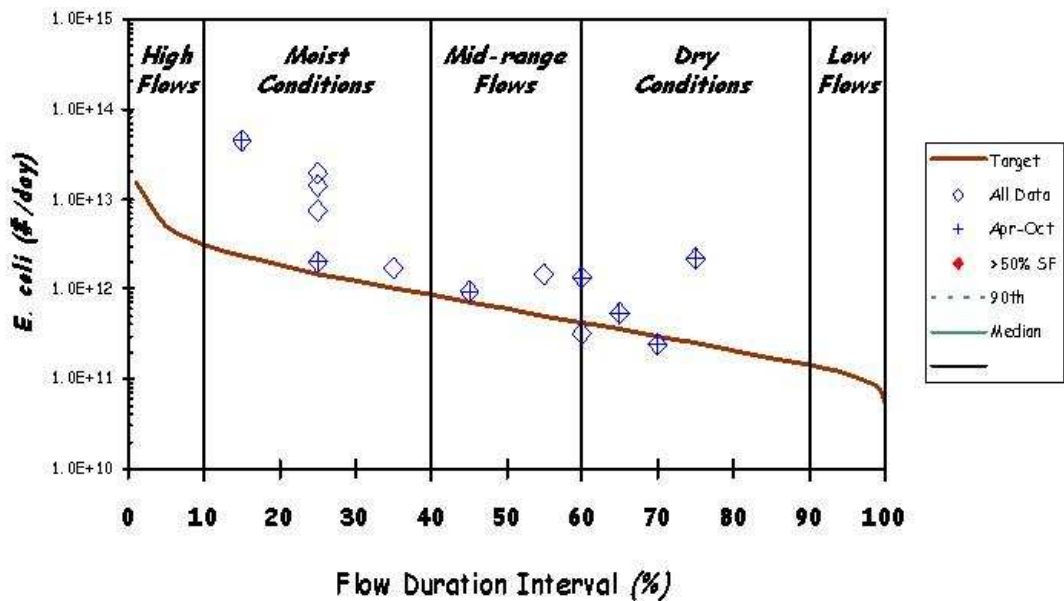
Pond Creek #4 ***E. coli* attributable to Humans** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

6302 Hectares

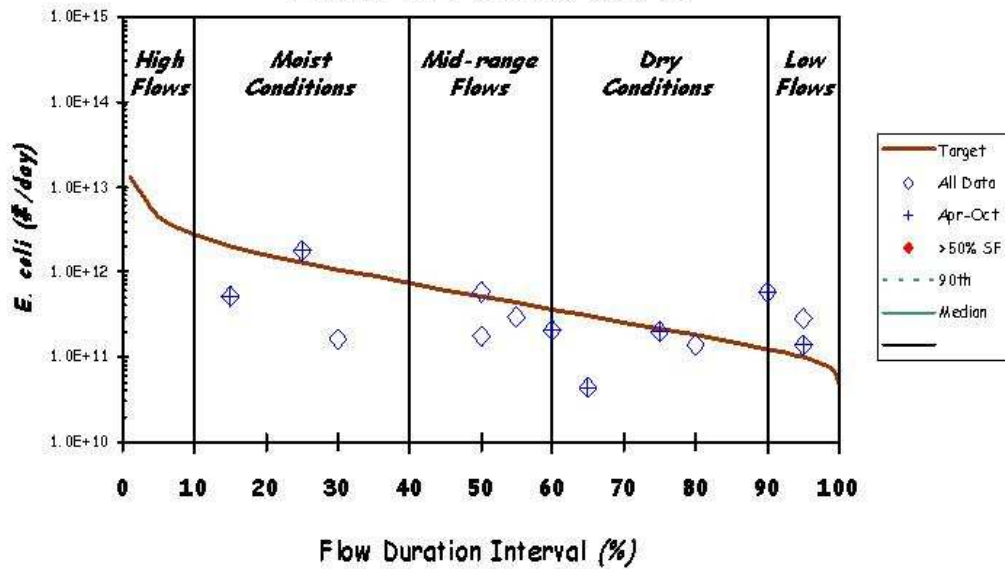
Pond Creek #4 ***E. coli* attributable to Cattle** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

6302 Hectares

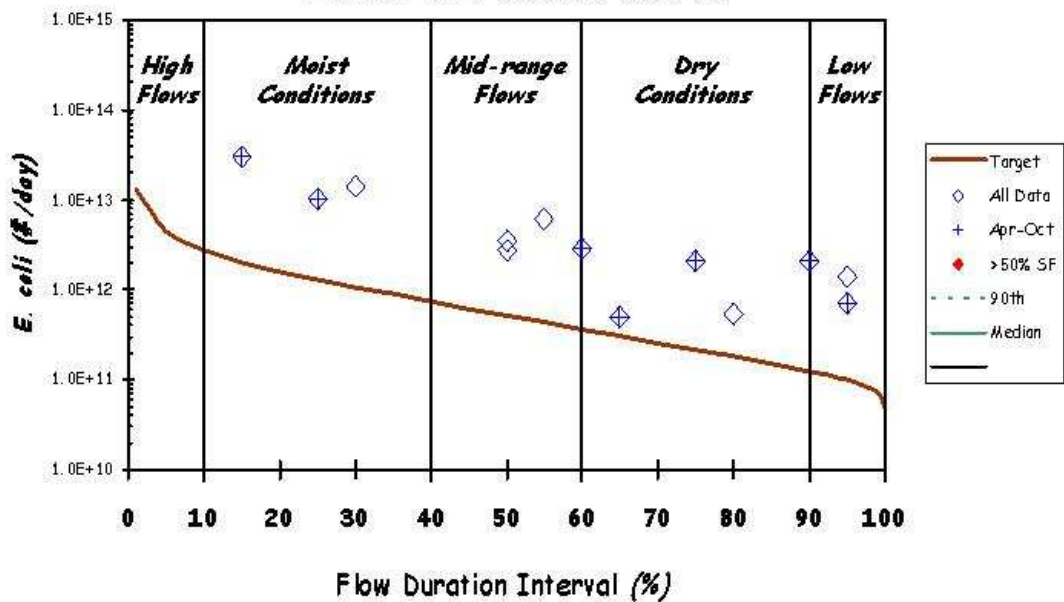
Pond Creek #5 ***E. coli* attributable to Humans** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

5102 Hectares

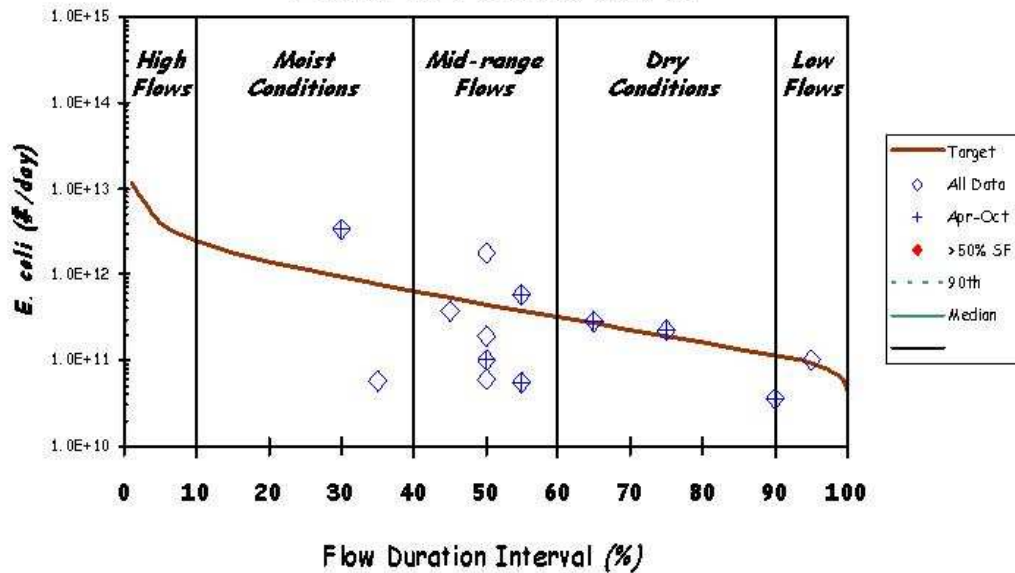
Pond Creek #5 ***E. coli* attributable to Cattle** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

5102 Hectares

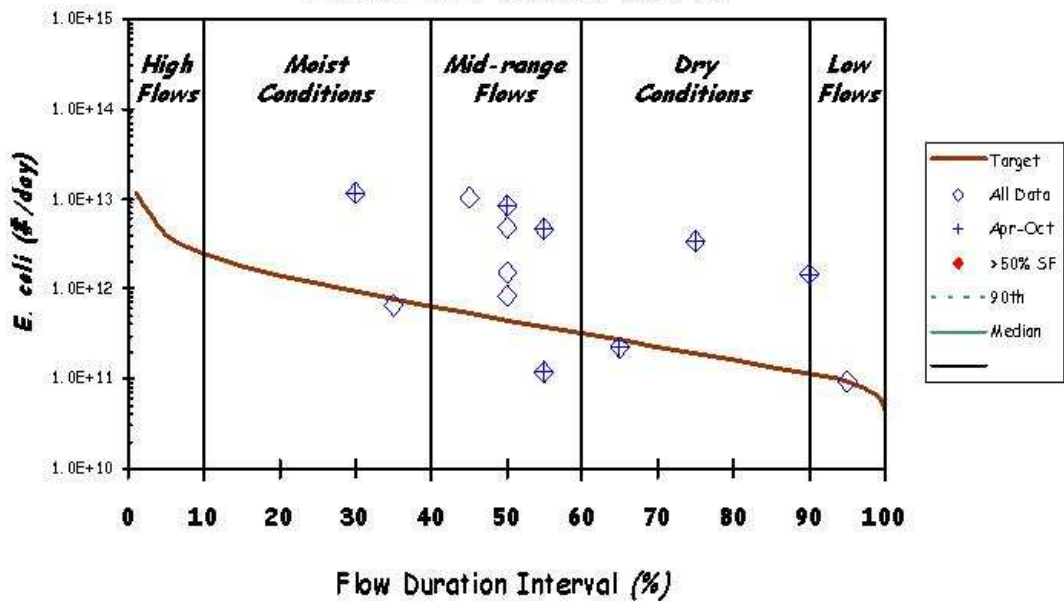
Pond Creek #6 ***E. coli* attributable to Humans** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

4186 Hectares

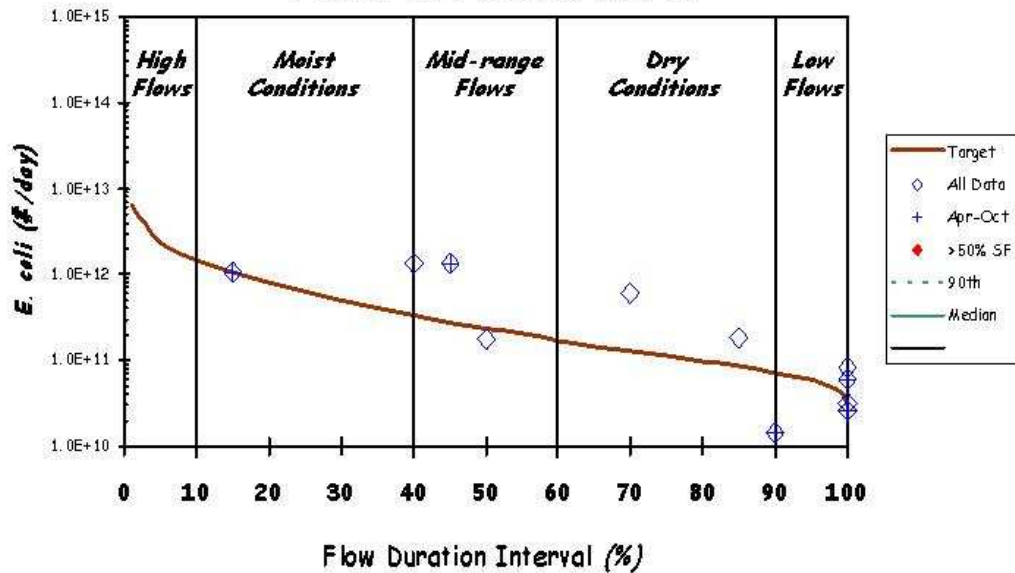
Pond Creek #6 ***E. coli* attributable to Cattle** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

4186 Hectares

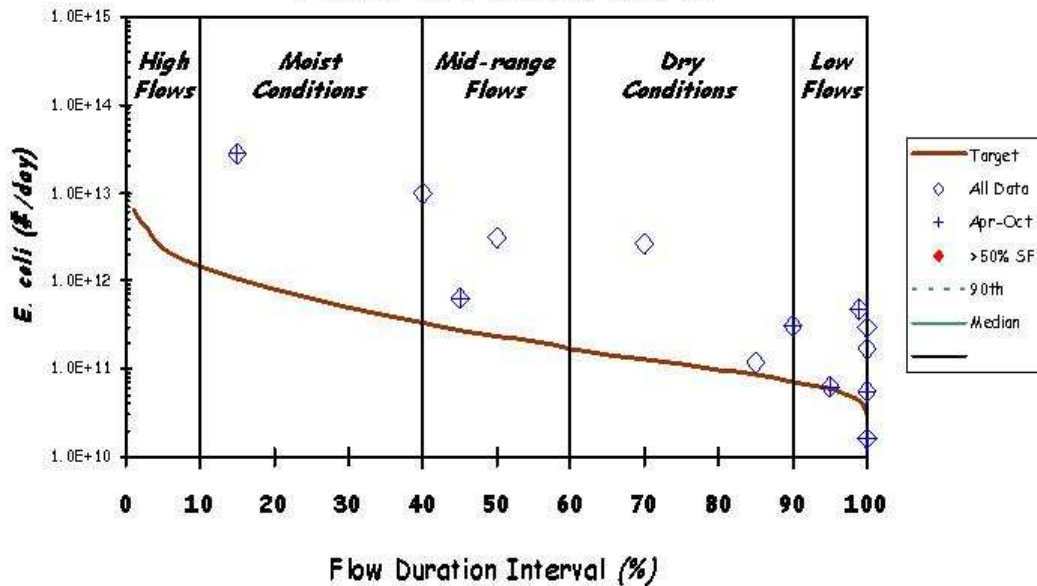
Pond Creek #7 *E. coli* attributable to Humans Load Duration Curve



MPCA Data & USGS Gage Duration Interval

1228 Hectares

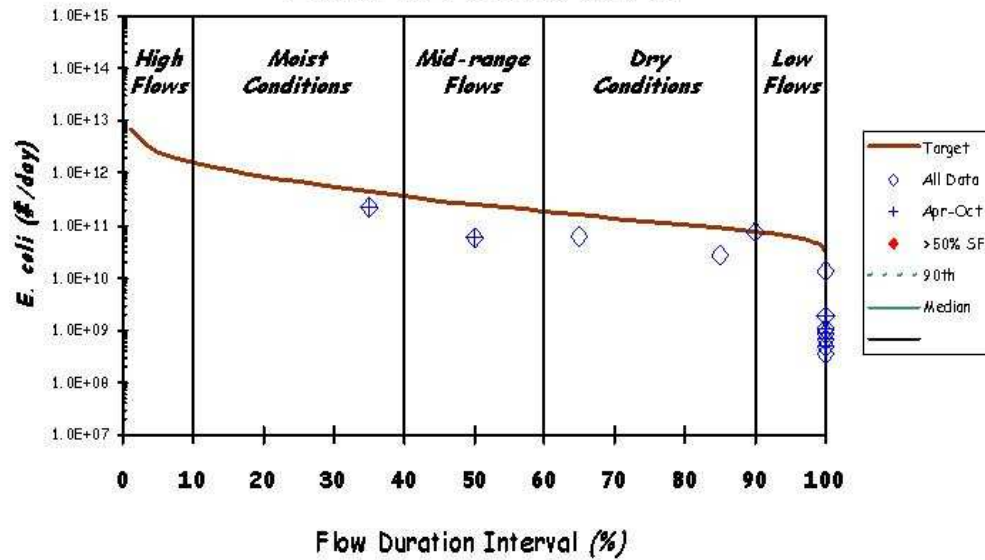
Pond Creek #7 *E. coli* attributable to Cattle Load Duration Curve



MPCA Data & USGS Gage Duration Interval

1228 Hectares

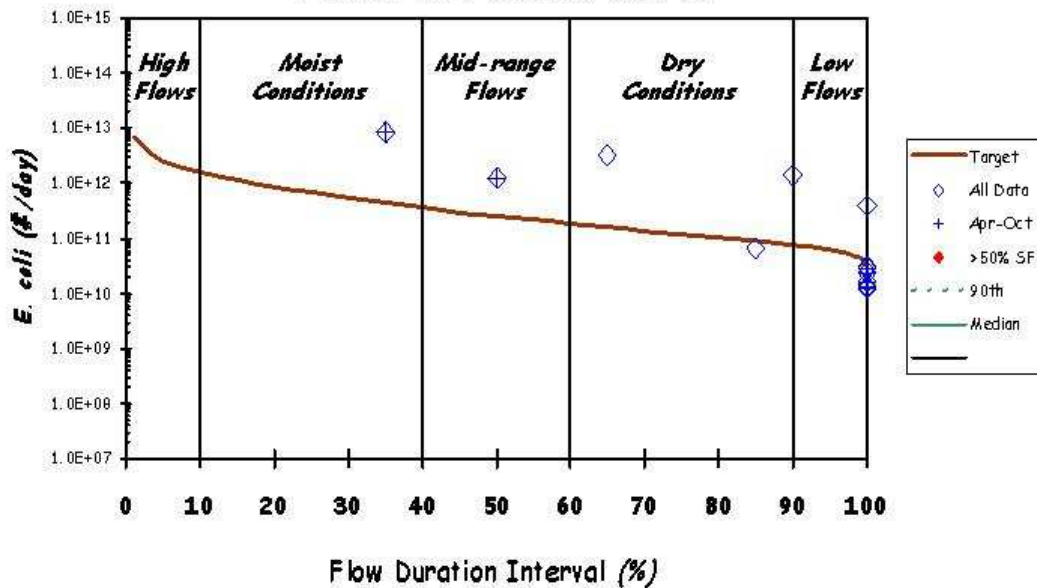
Pond Creek #8 ***E. coli* attributable to Humans** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

1515 Hectares

Pond Creek #8 ***E. coli* attributable to Cattle** **Load Duration Curve**



MPCA Data & USGS Gage Duration Interval

1515 Hectares

VITA

Ryan Michael Ragsdale was born in Chattanooga, Tennessee in 1979. He was raised in Chattanooga and graduated high school from The McCallie School in 1997. Four years later he received a Bachelor's of Science degree in Environmental Economics and Management from the University of Georgia. After college Ryan walked 1,800 miles of the Appalachian Trail and served two years as an Agroforestry Extension Agent in Paraguay for the United States Peace Corps. He also speaks Spanish and Guaraní. In graduate school at the University of Tennessee Ryan worked as a teaching assistant for an introductory soil science course. He received his Master of Science degree in Environmental and Soil Science from the University of Tennessee in December 2007.