IDENTIFYING SOURCES OF FECAL POLLUTION IN WATER AS A FUNCTION OF SAMPLING FREQUENCY UNDER LOW AND HIGH STREAM FLOW CONDITIONS

By

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(ABSTRACT)

Sources of fecal pollution were evaluated as a function of sampling frequency with stream samples from Mill Creek, Montgomery County, VA. Samples were collected monthly for one year, plus weekly for four consecutive weeks during seasonal high flows (March), and seasonal low flows (September-October), plus daily for seven consecutive days within the weekly schedules. Thirty stream samples were collected from each of two sites (60 total) in Mill Creek, and 48 isolates of *E. coli* per sample (total of 2,880 stream isolates) were classified by source using antibiotic resistance analysis (ARA) and comparing the resulting patterns against a known-source *E. coli* library (1,158 isolates). The same process was performed with *enterococci* isolates against an *enterococci* library (1,182 isolates). The average rate of correct classification (ARCC) for the *E. coli* library with a three-way split (human, livestock, and wildlife) was 89.0%, and the ARCC of the species-specific *E. coli* library (cattle, deer, goose, human, misc. wildlife) was 88.9%. The ARCC of the *enterococci* library with a three-way split was 85.3%, and the ARCC of the species-specific *enterococci* library was 88.1%. The results did not justify the need for daily or weekly sampling, but indicated that monthly was adequate (quarterly and every-other-month were not). There was a seasonal effect as the human signature was highest during high flow while the livestock signature dominated during low flow. The results also indicated that sampling should be done over a period of time that includes both seasonal wettest and driest periods (at least 8 months).

I dedicate this Dissertation

In honor of my daughter

CHLOE REBECCA GRAVES

And

In Memory of my Grandmother

ADDIE REBECCA GRAVES

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I. INTRODUCTION

Virginia's Non-point Source Pollution Management Program has two essential elements, water quality monitoring and nonpoint pollution control implementation. The state ambient water quality monitoring programs are governed by the Department of Environmental Quality (DEQ) and responsible for sustaining and tracking nonpoint source (NPS) pollution control implementation is Department of Conservation and Recreation (DCR). Both DCR and DEQ encourage resident monitoring efforts by people living in Virginia communities. A primary focus of Virginia's water quality monitoring program (coordinated by both agencies) is identifying water quality problems and the sources of impairment (http://www.dcr.state.va.us/sw/npsupdt.htm).

Pathogens are a major pollutant of water bodies nationwide according to many states' Clean Water Act 303(d) reports. Various sources contribute pathogens to contaminated waters, including fecal pollution from humans, wildlife, and livestock. Besides being potential pathogens, fecal bacteria such as *E. coli* can indicate the presence of other waterborne pathogens. Bacteria from human sources may indicate the presence of human viruses, while bacteria from wild and domestic animals may indicate the presence of protozoal parasites such as *Giardia* and *Cryptosproidium*. The presence of any fecal bacteria in drinking water is considered a health hazard. Knowing the source(s) of bacteria in a water body or water supply is of great value in the remediation and prevention of further bacterial contamination (http://www.epa.gov/). Fecal bacteria are the major cause of impairments in Virginia's waterways according to DEQ, 1998. Agriculture is listed as the primary source of contamination and nearly 65% of the impaired stream segments in Virginia are contaminated by fecal pollution. This fecal contamination results in increased health risks to persons exposed to the water, degradation of recreational and drinking water quality, shellfish bed closures, and nutrient loss from the watershed to the Chesapeake Bay (DEQ, 1998). Until recently the source(s) of fecal pollution often could not be readily determined. Knowledge of the type of pollution source could aid in the restoration of water quality, reduce the amounts of nutrients removed from watersheds, and reduce the danger of infectious disease from exposure to recreational waters (http://www.deq.state.va.us/water/98-

305b.html). However, it can be difficult to address water quality impairment effectively without a reliable method to determine the source of contamination. Bacterial Source Tracking (BST) is new methodology used to determine the source of fecal pathogen contamination in environmental samples (http://www.epa.gov/);(http://filebox.vt.edu/cals/cses/chagedor/CH.html).

There are more than 2000 monitoring stations, scattered throughout the estuarine and fresh waters in Virginia that are maintained by DEQ. Water quality monitoring stations furnish a continuing description of water quality and data for the Section 305(b) and Section 303(d) assessment reports that are submitted to EPA and Congress. DEQ monitors a set consortium of parameters including nutrients, toxic compounds, benthic community, and others as needed to establish water quality. DEQ monitors various water matrices as well, including surface waters, sediment, fish tissue, and ground water. (http://www.dcr.state.va.us/sw/npsupdt.htm).

DCR develops and implements all statewide NPS pollution control programs. The Non-point Source Advisory Committee (NPSAC) is also coordinated by DCR. Virginia's NPS pollution assessment (conducted by NPSAC), a virtual evaluation of the state's waters on a watershed basis, is managed by DCR as well. This assessment assists in targeting NPS pollution activities and it ranks all watersheds based on land use, animal density, forest harvesting, disturbed urban acres, best management practices (BMPs) implementation, and other related factors for NPS pollution possibilities. The rankings are then used as a guide to lead in the implementation of Virginia's NPS pollution control programs. (http://www.dcr.state.va.us/sw/npsupdt.htm).

DEQ and DCR negotiated an agreement with EPA to complete over 600 TMDLs within the next 12 years. Many of these TMDLs will be completed based on sporadic sampling of just a few months duration. It then becomes critical to know how "representative" the data for such TMDLs actually is. This frequency of sampling study will allow those responsible for TMDLs to compare TMDL data to the results from this dissertation and obtain some idea regarding the accuracy and seasonality of their data. Without such comparisons, there is no way to know if the limited sampling performed for many TMDLs in Virginia is anywhere close to accurate.

When developing a water quality monitoring study two questions always arise… "How many samples should be collected and how long should the monitoring persist?" Regrettably, to date

there is no best response. Several factors affect the frequency of sampling. These factors include the objectives of the study, the type of water body being studied, the data variability, and the available resources (National Handbook of Water Quality Monitoring-NHWQM, 1996).

Long intervals between samples are acceptable during long-term trend monitoring and programs that assess program efficiency on a watershed basis. Frequent sampling might be advantageous for a study designed to understand a mechanism controlling certain water quality changes (NHWQM, 1996).

The class of aquatic system being studied also has an effect on sampling frequency. In most cases, supplementary samples are desirable for studying streams rather than lakes since variance is greater. Groundwater is considered less variable than streams as well; however soil water samples have the potential to be highly variable. Financial assets characteristically limit the sampling frequency, while time, people, and laboratory capabilities can also limit sampling frequency. In spite of this, financial means should not be permitted to determine a sampling frequency (NHWQM, 1996).

The Mill Creek Watershed study was a Virginia DCR project in Montgomery County, Virginia. The Mill Creek watershed contains the community of Childress and Riner, and Mill Creek is the stream that flows through both. The stream is 9.14 km long and drains a 3,767 ha watershed (Figure 1). The watershed contains 2,277 ha improved pasture, 823 ha forest and 388 ha cropland among several other land use/cover entities (Table 1, Figure 2). There are 645 households in the Mill Creek watershed. Of the 645 households, 567 (or 88%) are on septic systems and the septic system failure rate is 1.5%. There are beef operations throughout the watershed, five active dairy operations, but no poultry operations in the area (Table 2).

This project had six objectives…

(1) Using antibiotic resistance analysis (ARA) to determine sources of fecal pollution in Mill Creek, Montgomery County, VA, as a function of sampling frequency under low and high stream flow conditions. Water samples from two locations in Mill creek were collected monthly for 12 months, weekly for 4 weeks and daily for 7 days within the 4

weeks, performing this sequence twice, once during periods of high flow (February-March) and once during periods of low flow (August-September).

(2) Building a library of at least 500 known source isolates of enterococci from the Mill Creek Watershed and to compare these known source isolates from the Mill Creek library to other enterococci libraries to determine how well the Mill Creek isolates fit these libraries.

(3) During this series of sampling, standard methods were used to perform bacterial monitoring, of enterococci and fecal coliform populations from all stream samples.

(4) Performing ARA on the enterococci from all stream samples was performed as well. Isolates were classified as human, wildlife, and livestock sources.

(5) Building an ARA library of at least 300 known isolates of *Escherichia coli (E. coli)*, from the Mill Creek watershed; compare the known source isolates from the Mill Creek library to our other *E. coli* library (developed under DCR project 319-1999-14-PT) to determine how well the Mill Creek isolates fit this library. ARA on *E. coli* was performed the same as enterococci (above).

(6) Determining the optimum number of isolates needed for source identification. Most investigators use 48 isolates for ARA source identification because it is convenient, as the microwell plates have 48 inoculating wells on each side of the tray. However, it is possible to save both time and money if fewer isolates can be used to obtain accurate source identification results.

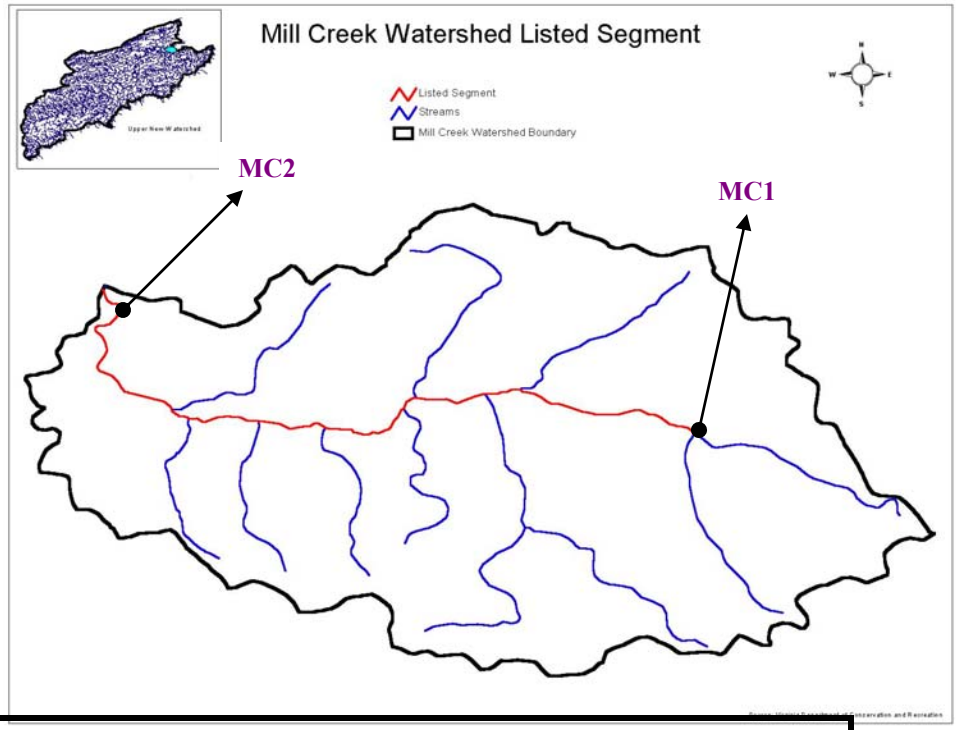
ARA results from enterococci and *E. coli* libraries will be compared to determine the level of similarity and identify where differences in source tracking results occurred. This project also allows for the determination of the optimum sampling frequency and optimum number of isolates needed for source identification, thus allows for better planning in the future regarding sampling plan designs that incorporates seasonal flow trend for other TMDL projects.

Results from this project can be used to identify nonpoint sources (NPS) of fecal pollution within the parameters of different sampling frequencies, and seasonality, and will allow more accurate development of TMDLs for fecal bacteria in surface waters, and enhance Virginia's overall NPS pollution prevention program.

Figure 1. Mill Creek Watershed Listed Segment

Based on the 1998 303(d) List

- Upstream Limit: 0.4 miles upstream of Rt. 8 Crossing, River Mile 5.68
- Downstream Limit: Mill Creek mouth on Meadow Creek, River mile 0.0
- This segment failed to meet the recreational use goal



Sampling Site	Location
MC 1	Rt. 8 Bridge Above Riner STP
MC 2	Rt. 669 Bridge Below Riner STP

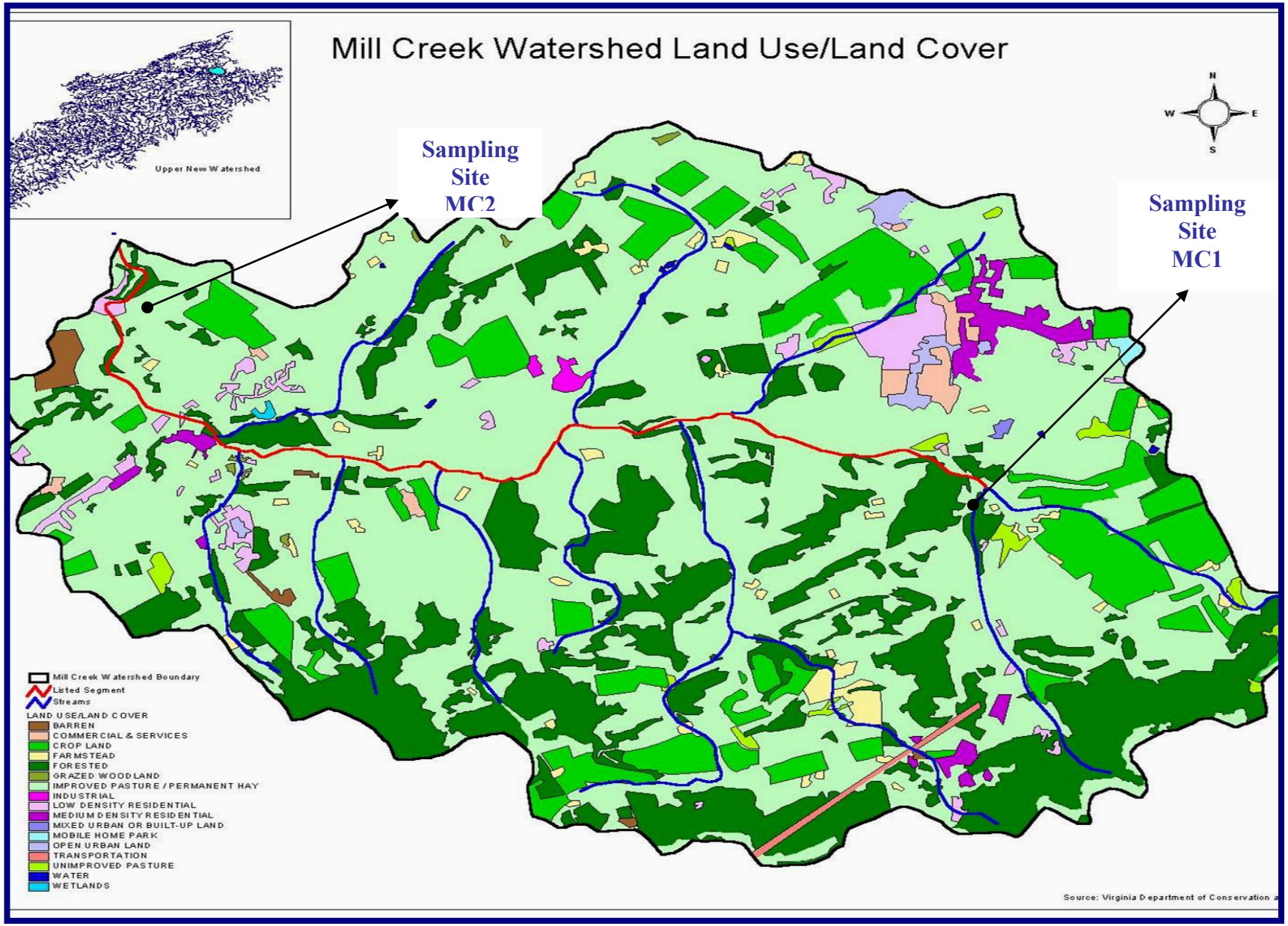
Table 1. Land Use/Land Cover Data for Mill Creek

Land Use/Land Cover	Acres	Hectare	%
Forest	2034.2	823.2	21.9
Water	6.2	2.5	0.1
Wetland	3.3	1.3	0.1
Barren	38.7	15.7	0.4
Commercial/Services	63	25.5	0.7
Industrial	14.8	5.9	0.2
Transportation	23	9.3	0.23
Urban Transition	3.7	1.5	0.1
Open Urban	46.2	18.7	0.5
Low Density Residential	176.1	71.3	1.9
Medium Density Residential	107.4	43.5	1.2
Mobile Home Park	9	3.6	0.1
Cropland	959.4	388.3	10.3
Farmstead	112.7	45.6	1.2
Grazed Woodland	8.6	3.5	0.1
Improved Pasture	5627.4	2277.4	60.5
Unimproved Pasture	68.2	27.6	0.7
Total	9301.9	3764.4	100.0

Table 2. Livestock Inventory in Mill Creek Watershed, VA.

Livestock Type	Number in Watershed
Beef Cattle	2,850
Dairy Cattle	950
Horse	34
Sheep	250
Total	4,184

Figure 2. Mill Creek Watershed Land Use/ Land Cover.



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II. REVIEW OF LITERATURE

A. Sanitary Significance of Fecal Coliforms in the Environment

The presence of certain microorganisms in water is used as an indication of possible contamination and an index of water quality. Heavy reliance has been placed on the presence of the coliform group of bacteria to determine the safety of potable water. Coliform bacteria and other organisms are used to signal conditions that could lead to adverse health consequences in recreational and shellfish-harvesting water. Although this practice is far from perfect, public health concerns have generally been well served by this system (Borrego et al., 2002).

The presence of coliform bacteria in potable water indicates unsuitable sanitation practices. Such occurrences may be a result of poor water treatment, plant design problems, improper operating procedures, inadequate hygienic practices in plant operation, or after growths in the distribution system (Geldreich, 1996). Nearly all-natural waters are habitats for coliform bacteria (Kott, 1977). The populations of coliforms should be noticeably curtailed by treatment, together with disinfection. Only a small number of the many fecal microorganisms found in wastewater and capable of contaminating drinking water could be considered as pollution indicators. These organisms include *Clostridium perfringens*, *Streptococcus faecalis,* the fecal coliforms, and *Escherichia coli* (Kott, 1977).

The presence of enteric pathogens in drinking and recreational waters is of great concern. Therefore, it is important to determine the microbiological safety of these waters. The ideal manner for doing this would be to analyze the waters for the presence of specific pathogens of concern. However, it is impractical to look for every pathogen potentially present in water because there have been hundreds of different microorganisms associated with waterborne diseases (Borrego, et al., 2002). As a result, analyses for the presence of waterborne pathogens is multifaceted and does not ensure complete safety of water for the consumer. Thus, members of the following groups of indicator microorganisms are used to determine the biological safety of the water; total coliforms, fecal coliforms, *Escherichia coli*, fecal streptococci, *enterococci*, bacteriophages, as well as several other organisms (Borrego, et al., 2002).

Water is necessary to sustain life and therefore every attempt should be made to achieve a drinking and recreational water quality as high as feasibly possible (Gleeson and Gray, 1997). Failure to maintain high water quality exposes the population to the peril of disease, principally the very young, elderly, the sick and those who live in sub-standard sanitary surroundings. Microbial contamination is considered to be the most serious risk factor in drinking water quality because of the possible consequences of waterborne disease (American Water Works Association-AWWA, 1999).

The quality of recreational waters and their appropriateness for activities such as swimming, surfing, boating, and fishing is of great value to our society. In an effort to protect the recreational use of our rivers, lakes, and coastal waters, management of these entities is necessary (EPA, 1997). The microbiology of untreated waters is drastically different from that of treated drinking waters. Thus, strong emphasis should be placed on the use of the appropriate indicators. In the case of recreational waters it should be determined whether the recreational areas are subjected to fecal pollution. The presence of bathers in the water will result in fecal deposition, and thus fecal contamination is likely. Therefore it is extremely important to use appropriate indicators to determine risk of enteric or other types of disease. If the area in question is evaluated thoroughly, the risk can be accurately determined. One should note that all indicators are not appropriate for all conditions. There is a statistical correlation that total coliforms can be used as indicators in the analysis of recreational waters. However, *E. coli* and coliphages are much more reliable indicators of the presence of fecal pollution. If the recreational waters are part of a tropical rain forest then coliphages may be the best option since coliform regrowth is possible in tropical waters (Hazen and Toranzos, 1990). Parameters to be used for determining recreational water quality include:

- an assessment of bacterial water quality using fecal coliforms and enterococci as indicator organisms
- an assessment of the presence of nuisance organisms, such as algae
- an assessment of visual clarity and color
- a measurement of pH
- a visual assessment of surface films
- a measurement of temperature.

There are also guidelines that make a distinction between primary and secondary contact recreation. Primary contact recreation includes sports such as swimming and surfing, where the user comes into frequent direct contact with water. Secondary contact recreation includes activities such as boating and fishing, in which there is less frequent body contact with the water and there is little chance of swallowing water (CDC, 1998).

Algal blooms pose a particular issue for recreational waters. Algal blooms accumulate along shorelines and reduce water clarity, thus decrease the aesthetic value of waters. They may also pose a health risk to humans who have direct contact with affected waters. Some species, blue-green algae for example, are potentially toxic and may cause skin irritations, gastro-intestinal disorders and influenza-like symptoms. Although monitoring of recreational water quality does not address the problem of water contamination directly, it is vital for protecting public health by identifying areas of particular concern for correction and educating the community (EPA, 1997).

A.1 Health Hazards Associated with Contaminated Water

Existing approaches for controlling health risks posed by microbes in drinking waters are centered on a barrier methodology involving treatment of wastewater as well as the treatment of raw waters. This methodology consists of disinfection and the creation of acceptable limits for indicators of water quality. In 1997 roughly two-thirds of the world's population lacked access to potable water supplies and ample sanitation (Gleeson and Gray, 1997). The World Health Organization estimates that 80% of all illness in the world was attributable to insufficient water supplies or sanitation. Over 250 million new cases of waterborne diarrhea are reported worldwide each year, resultant in more than 10 million deaths. Today there are many recognized waterborne pathogens. All are present in large numbers in human or animal waste; sporadically both are commonly resistant to environmental decomposition. Many of these pathogens are proficient in causing infections even when ingested in extremely small numbers (Schlossberg, 1999).

Viruses, bacteria and protozoa are the three principal groups of microorganisms that can be transmitted via drinking water. They are all transmitted by the fecal-oral route, and so largely arise either directly or indirectly by contamination of water resources by sewage or possibly animal wastes (CDC, 1996).

From a human perspective, one of the most important aspects of water microbiology, is the fact that humans acquire numerous diseases from microorganisms found in water (Hurst and Murphy, 1996). Some of these diseases represent intoxications resulting from the ingestion of microbial produced toxins. One category of intoxications is linked to drinking water which contains toxins produced by cyanobacteria such as *Anabaena* and *Microcystis*. A second category of intoxications is associated with dinoflagellates such as *Gambierdiscus*, *Gonyaulax*, and *Ptychodiscus*. These aquatic microorganisms produce neurotoxins that can become biologically concentrated in the tissues of reef fish and shellfish. Humans acquire this second category of toxins by ingesting the affected animals (Hurst and Murphy, 1996).

The majority of human diseases associated with water are infectious in nature. The magnitude of human morbidity and mortality associated with these infectious diseases has led to the development of epidemiological surveillance studies (Payment et al., 1997). The associated pathogens include numerous bacteria, viruses, and protozoa. These water-related infectious hazards can be characterized according to various schemes (Swaminathan et al., 1999). The hazards can be divided into four categories based upon the source of the involved pathogen and the route by which human recipients come in contact with that pathogen. Those categories could be defined as follows.

1. Infections that are waterborne: These are recognized as resulting from physical contact with microbial contaminated water. Infection may occur during bathing or recreational or occupational aquatic activities or after ingesting contaminated water, ice that has been made from contaminated water, or food items that come into contact with either contaminated water or contaminated ice (Angulo et al., 1997).
2. Infections caused by aquatic organisms: These result from pathogenic microorganisms which naturally spend at least a part of their normal life cycle living directly in the water or within intermediate vertebrate or invertebrate hosts that reside in aquatic

environments. These diseases are acquired either by ingestion of or immersion in the water or by inadvertent ingestion of infested intermediate host animals (Grimes, 1991).

3. Infections which have water-related insect vectors: The associated pathogens are acquired as a result of humans being bitten by invertebrate vectors such as mosquitoes whose life cycle depends upon access to water. Thus, the incidence of the resulting diseases may increase during periods of land surface flooding and during monsoon seasons (Cotton, 1999).
4. Infections associated with inadequate hygiene; these are caused by pathogens which are acquired by either contact with or ingestion of microbial contaminated material. Usually the material is contaminated with fecal matter. This disease hazard can be improved using clean water for sanitation purposes, including bodily cleansing and the washing of plates and drinking glasses (Rosenberg et al., 1997).

A. 2 Risk Assessment

Any dialogue on drinking water quality standards should include some consideration of risk assessment. Risk assessment is an effort to compute the potential health consequences of human exposure to specific conditions (Gleeson and Gray, 1997). Risk to human health is defined as the probability or chance that a given exposure to a microbial pathogen may possibly bring about illness or damage to health of the exposed persons. In setting any standard with regard to public safety, a definite balance must be attained between the profits of safety and the expenditures of accomplishing it. When considering the risks presented by consumption of drinking water several factors must be carefully evaluated. Some of these factors include determining the current health risk posed by modern day water supplies to public health and whether it is an acceptable risk. Another issue of concern involves the cost of upholding stringent standards in economic and social terms (Gleeson and Gray, 1997).

When considering the risks to human health from consumption of water supplies a number of other factors must be evaluated. Microorganisms causing illness by the water route must be identified and characterized, and the occurrence and distribution of the organism must also be assessed (Calderon et al., 1991). The limits of analytical tools for pathogen and indicator

detection must also be evaluated. The risks involved in terms of human health and social costs can be determined using this type of information (Gleeson and Gray, 1997).

While risk assessment approaches have been useful in systematically identifying, analyzing, quantifying, and characterizing the risk of specific waterborne illnesses, we still face some challenges (Haas et al., 1999). These models are based on field data for the occurrence of specific microorganisms in raw and treated water supplies, as well as experimental dose-response curves. It is difficult to model variations in microbial virulence factors and host-specific characteristics, such as age and immune status, which may affect personal exposure and sensitivity to infection and disease (Sobsey et al., 1993). For infectious agents with multiple transmission routes, it may be difficult to determine the attributable risk associated with waterborne transmission compared to other routes of transmission, especially in areas where waterborne diseases are endemic (Briscoe, 1984).

A. 3 Sources of Water in the United States

Raw source waters, surface waters and ground water are the three major sources of water in the United States (Geldreich, 1996). A number of issues have entered into the options of best obtainable raw source water for potable supply. These considerations include adequate quantity during any seasonal variation in flow, water quality that is modifiable to cost-effective treatment, and some degree of watershed protection from domestic, industrial, and agricultural pollution. Water supply utilities have a greater burden placed on them due to increasing urban populations (Geldreich, 1996). This burden is in terms of the quantity demanded and in terms of contamination potential through the use of less sought-after raw water, resulting from polluted rivers, lakes, and groundwater. Wastewater treatment plants, urban and rural runoff, agricultural activities, and industrial chemical spills are repeatedly discharged to watercourses that may well be the source of a public water supply (Novotny, 1988). Many of these wastes contain pathogens and innumerable of other waterborne organisms. There are a few essential ingredients to protecting downstream use of water supplies such as waste minimization management, waste treatment of point source discharges, and controlled treatment of urban stormwater (Geldreich, 1996). There must be a concentrated attempt to make the most of public health protection

through a multiple treatment barrier concept and not force the water purveyor to either demonstrate a public health threat to make upstream treatment of waste mandatory or have utilities assume the full burden of treatment for poor-quality water resources (Novotny, 1988).

Raw surface water is subject to an assortment of bacterial contaminants introduced in stormwater runoff over its supply watershed and the upstream discharges of domestic and industrial wastes. These sources of pollution introduce a wide range of infectious agents to waters that may ultimately be used in water supply (Angulo, et al., 1997).

Receiving waters between the point of discharge of sewage effluents and water intakes may not just serve as a buffer to accidental spills and treatment bypasses but can add to water quality improvements by way of natural self-purification. Every stream, lake, and groundwater aquifer has some limited capacity to assimilate waste effluents and storm water runoff entering the drainage basin. This self-purification process is a multifaceted and ill-defined mechanism that includes bacterial adsorption with sedimentation, predation, dilution, hydrologic tributary effects, water temperature, and solar radiation (Swaminathan et al., 1997).

Rivers are subject to considerable fluctuations in water quality as a result of stormwater runoff and spills of municipal and industrial wastes. While these sources assure that quantity will be available to meet the needs of the growing community, these waters are not necessarily of the best quality (Geldreich, 1996).

A. 4. Waste Disposal in the United States

In today's society, clean water is recognized as a high amenity in the US. Often, agriculture is held responsible for increasing the eutrophication status of water bodies, and landspreading of animal manure in an indiscriminately manner is one of the practices that causes the biggest outcry (DiPaola, 1998). The Clean Water Act endorsed a no pollution Policy in the 1970's that caused an increased interest in land disposal of liquid wastes such as conventionally treated sewage effluents, processing plant wastes, animal wastes and feedlot runoff, and sewage sludge (Novotny and Olen, 1994).

When publicly owned treatment works (POTW) are not obtainable, discarding of human waste transpires through the use of a septic system, cesspool, privy or other analogous types of on-site wastewater treatment and disposal systems (OSWTDS). Septic tanks and privies account for the highest total volume of water discharged directly into groundwater and are frequently implicated as sources of groundwater contamination (DiPaola, 1998). The term septic system is commonly used to describe a subsurface, anaerobic sewage disposal that uses soil filtration and adsorption for attenuating the effluent. Approximately 20 million residents, or 29% of the U.S. population, dispose of their sewage by individual on-site systems. The total amount of sewage and wastewater discharged to the subsurface in the United States is $3 \times 10^9 m^3/yr$. Apparently, septic tanks represent the highest total volume of wastewater discharged directly to ground water and are the most frequently recorded sources of contamination of ground water and surface flow (Novotny and Olen, 1994).

Septic effluent entering aquifers used for drinking water are the most common ground water contamination problems reported from individual home sites (Geraghty and Miller, 1978). Contaminated groundwater used for human, domestic, or recreational purposes can be the cause of occurrences of waterborne disease (Craun, 1985). Contamination of surface or groundwater from the overflow or seepage of sewage from septic systems and cesspools has been known to cause high percentages of outbreaks of illnesses reported from contaminated, untreated groundwater supplies (Craun, 1981). Researchers have suggested preventing contamination of drinking water sources, appropriate treatment of contaminated sources, and monitoring to identify contamination as areas of emphasis in the quest to safeguard drinking water sources (DiPaola, 1998).

B. Exposure Pathways for Fecal Contamination

Water quality is most directly impacted by how nutrient management practices affect the environment (Brady and Weil, 1999). The principal water pollutants to be considered are nitrogen, phosphorus, and sediments as well as fecal indicator bacteria that can be found amongst these substances. The primary means to avoid damaging surface waters with excess nitrogen, phosphorous, and fecal contamination is to manage the rate at which these nutrients are applied

(Brady and Weil, 1999). Fecal contamination in water occurs from three main sources, livestock (including poultry), wildlife, and humans and their pets. The reservoirs for pathogenic microorganisms found in environmental waters can be humans, animals, or the environment itself (Fayer et al., 1998). However, it is generally assumed that many microorganisms which infect humans and are found in aquatic resources originate from human sources. This human-related contamination can occur by defecation in water or recreational activities conducted in water (Acher et al., 1994). Furthermore, domestic and onsite wastewater seems to be of particular importance as a contributor of the pathogenic contaminates found in aquatic environments (Dowd et al., 1998). Thus attendant public health concerns have resulted in the development of methods for studying and reducing the levels of pathogens in wastewater (Acher, et al., 1994). Wastewater treatment efforts may help to reduce the incidence of problems resulting form the discharge of wastewater into natural surface water (Hurst and Murphy 1996). These problems include illness among swimmers, contamination of drinking water, and disease that can occur when bivalve molluscan shellfish harvested from contaminated waters are subsequently consumed by humans (Shieh et al., 1999). Aquifer contamination is resultant either indirectly following the percolation of surface-applied wastewater into the subsurface or directly during subsurface injection of wastewater can be reduced with treatment (Deborde et al., 1999). The treatment of wastewater also is intended to reduce the occurrence of crop contamination that occur when wastewater is eventually discharged onto land surfaces (Acher et al., 1999).

B.1 Livestock

Where animal and crop production are integrated on a farm, manure handling is not too much of a problem (Brady and Weil, 1999). The use of pasture can be maximized so that the animals themselves spread much of the manure while grazing. The manure from confined animals is produced in small enough quantities to be hauled daily to the fields or be stored under cover during periods when soil conditions are not favorable for spreading manure (Brady and Weil, 1999).

The problem of manure disposal takes precedence over its utilization where animals are concentrated in large confinement systems. Manure from such systems is handled by four

general management procedures. The manure can be collected and spread daily or the manure may be stored packed in a pile where it is allowed to partially decompose before spreading. Also, the manure can be stored in aerated ponds that are sufficiently shallow to permit fairly ready oxidation of the organic materials. Lastly, manure is stored in deep anaerobic lagoons where it is allowed to ferment in the absence of elemental oxygen, resulting in denitrification (Brady and Weil, 1999).

Water and excess nutrients can leach through soils to the groundwater and into streams and lakes, giving rise to contamination, algal blooms and eutrophication. Methods of manure handling that both prevent pollution and preserve nutrients would make a major contribution to ameliorating the manure problem for concentrated animal production enterprises (Brady and Weil, 1999).

B.2. Wildlife

Animals such as deer, geese, beavers, etc. contribute to fecal contamination in streams. Often it is hard to eliminate contamination from wildlife sources because of the inability to control the movement of the animals. Contamination from these sources is likely to be more severe in the summer months as the animals seek a means of cooling themselves and require greater amounts of water. Also, there may be a seasonal increase in wildlife contribution in fall and spring due to migratory waterfowl (Miner, 1990).

Most fecal contamination in watercourses occurs as a result of an animal defecating directly into the flowing water. Fecal material deposited on the stream banks will reach the water only under conditions of overland flow (Miner, 1990).

B.3. Human

Effluent form septic tanks that polluted natural soils and water by contaminating organisms is a serious concern (DiPaola, 1998). The number of families not serviced by sanitary sewage systems continues to increase with the development of suburban and rural areas. Septic tanks

and subsurface absorption fields are usually employed for the disposal of domestic wastes in the absence of centralized facilities (DiPaola, 1998). Over 32 million people in the United States utilize septic tanks and subsurface absorption fields for disposal of wastewater. Effluents from septic tanks should be disposed of in such a manner as not to result in bacteriological contamination of surface or ground waters (Graves, 2000). The Federal Water Pollution Control Administration (1968) has suggested that safe, clear, potable, aesthetically pleasing, and acceptable public water supplies can be obtained from raw surface water containing up to 10,000 coliform/100 ml or 2000 fecal coliforms /100 ml. The desired levels, however, are less than 100 coliforms or 20 fecal coliforms/100 ml (Graves, 2000).

Fecal coliforms characteristically inhabit the intestines of warm-blooded animals such as humans and are, therefore, more indicative of recent and potentially dangerous sources of pollution. Members of the total coliform count may be routinely detected in soil, on plants and insects, in old sewage, and in water polluted sometime in the past (DiPaola, 1998).

C. Bacterial Indicators for Drinking Water Quality

The absence of coliforms when a minimum of 100 ml is tested by the membrane filter (MF) technique or 50ml by the most probable number (MPN) method indicates drinking water of good bacteriological quality in the United States. Due to limitations such as media selection, temperature and incubation time, some ambiguity exists regarding the coliform group. Consequently, pathogens and secondary invaders are not recognized even though their numbers sometimes appear to exceed those of the coliform group (Ptak and Ginsburg, 1977).

The enterococci or fecal streptococci have been proposed as an indicator to the quality of drinking water, but data has shown that coliforms are better suited since the fecal streptococci die off very rapidly in water of good quality and are not as numerous (Cabelli, 1983). The enterococcus group is a subgroup of the fecal streptococci that includes *S. faecalis, S. faecium, S. gallinarum, and S. avium.* The enterococci are differentiated from other streptococci by their ability to group in 6.5% sodium chloride, at pH 9.6, and at 10˚C and 45˚C (http://soils1.cses.vt.edu/ch/biol_4684/mfstrep.html). In studies involving natural or fecally

polluted water or soils, fecal streptococci appear to survive longer than bacterial pathogens (Clausen et al., 1977). The enterococci might well be better indicators of pollution in wells swimming pools, and sewage treatment plants that are high in nutrient and low in oxygen. Similarly the fecal coliforms might be used in areas other than drinking water, since they are already included in the total coliform count and also die off rapidly in water of good quality (Ptak and Ginsburg, 1977).

The modern interpretation of the presence of the coliform group as a pollution indicator depends on the objectives of the pollution investigation (Geldreich, 1996). In some studies, the total coliform group will be the indicator of choice, whereas in another investigation with a different objective a portion of the group or even a single species may be utilized (Geldreich, 1996).

C.1. Criteria for Indicator Organisms

It was common during the early history of the United States for epidemics of diseases such as typhoid, shigellosis, cholera, and amebiasis to threaten society on a regular basis. Subsequently, it was determined that the primary source of these pathogens was sewage, which at that time was generally untreated and readily polluted environmental waters used for drinking and for swimming. Thus, the need to develop a test to determine when a body of water was polluted with sewage and sewage-borne pathogens was recognized. However, since methods for the detection of sewage-borne pathogens were not available, the approach was to develop a test to show that the water was contaminated with sewage (Rosenberg et al., 1997).

There were four important discoveries that led to our present-day system of evaluating the hygienic quality of water based on concentration of fecal bacteria. First, in 1885, Escherich (Hurst et.al., 2002; Klein and Houston, 1899) determined that a group of bacteria, which he called coliform bacteria, were always detected in high concentrations in human feces. Second, Klein and Houston (Hurst et al., 2002) reported that they could dilute sewage more than 10,000-fold and still detect the presence of coliform bacteria. Under the same conditions, all known physical or chemical tests could no longer detect the presence of sewage. Thus, the term

"indicator of sewage" was coined for coliform bacteria. Third, Scott (Scott, 1932) in 1932 took a pragmatic approach and proposed that concentrations (>1000/100ml) be used as Connecticut's marine bathing water quality standard. Fourth, in 1953 Stevenson conducted the first epidemiological study in the United States focusing on swimming-related diseases and reported a correlation between elevated concentrations (>2000/100ml) of coliform bacteria at freshwater beaches and increased incidences of all observable illnesses in swimmers (Stevenson, 1953). These four key studies led public health officials to accept the levels of coliform bacteria as the best standard for evaluating the hygienic quality of water (Hurst et al., 2002).

For an organism to serve as an indicator organism, it must meet several criteria. The organism must be of value as an assessor of fecal contamination and should be a member of the normal intestinal flora of healthy people. The indicator organism should also be exclusively intestinal in habitat and therefore exclusively fecal in origin if found outside the intestine (Dutka et al., 1974). Ideally they should only be found in humans and should be present when fecal pathogens are present. These organisms should be present in greater numbers than the pathogen they are intended to indicate. They should be unable to grow outside the intestine with a die-off rate slightly less than the pathogenic organism and be easy to isolate, identify and enumerate. Indicator organisms should also be non-pathogenic (Gleeson and Gray, 1997).

The concentration of indicator organisms should be related to the extent of fecal contamination and by implication to the concentration of pathogens and the incidence of waterborne disease. There is no absolute correlation between the numbers of the indicator organism present and the actual presence or numbers of enteric pathogens. The finding of an indicator organism in properly treated water indicates the presence of material of fecal origin and thus has been the principal means by which the sanitary quality of water has been determined (Gleeson and Gray, 1997).

C.2. MPN and Membrane Filtration

Assessment of microbiological drinking water quality by the detection and enumeration of coliform bacteria has existed in some manner since 1912 (Bancroft et al., 1989). The most-

probable-number (MPN) method is often referred to as the multiple-tube fermentation technique because it was first used to estimate the concentration of coliform bacteria by inoculating multiple tubes of liquid medium with 10-fold dilutions of the sample and observing them for growth based on turbidity and gas formation. In MPN, it is assumed that any sample volume with at least one viable bacterium will result in positive growth in the medium in the tube. Since the distribution of bacteria in the sample volume is based on a probability, this method uses probability statistics to determine the mean concentration of the bacteria being measured as the MPN per 100 ml. The MPN test is conducted in three sequential phases. These phases are presumptive, confirmatory, and completed, with each phase requiring 1 to 2 days of incubation. In the initial or presumptive phase, three volumes of water sample (10, 1, and 0.1 ml) are inoculated into sets of 3, 5, or 10 tubes containing bacteriological medium prepared to permit the bacteria of interest to grow. Tubes showing turbidity are considered presumptively positive for fecal streptococci or fecal or total coliforms. These readings are considered presumptive because false-positive and false-negative reactions often occur as a result of growth and interference by non-target bacteria in the growth medium (APHA, 1998).

Confirmation that fecal bacteria are growing in these tubed media is obtained by streaking subsamples from all presumptively positive tubes onto a more selective agar medium (such as enterococcus agar, for example, and observing brownish black colonies with brown halos after incubation for another 24h at 35°C). To confirm the presence of fecal bacteria, brownish black colonies with halos must be transferred and shown to grow in a tube of brain heart infusion broth containing 6.5% NaCl after 24h of incubation at 45°C as with enterococci. The confirmatory test is reliable evidence but not proof that fecal streptococci or enterococci have been detected. As a result, it is recommended that the completed test be conducted on 10% of positively confirmed samples by demonstrating the presence of gram positive cocci (APHA, 1998). It should be noted that the use of the MPN method to estimate the concentrations of enterococci will take at least 3 days and will require a lot of tubes and media. The same is true for fecal/total coliforms (Hurst et al., 2002).

The MPN method can also be used for the direct detection of coliforms and *E. coli* by using enzyme-specific tests. The characteristic color and/or fluorescent end points specified by

the manufacturer should be used to determine the presence or level of the target bacteria. The enterolert system is commercially available to detect enterococci. The U.S. Environmental Protection Agency has indicated that confirmation is not needed with the commercially available 4-methylumbellifery-β-D-glucuronide (MUG) based media. The same inoculation series used for enterococci is applied to *E. coli* as well. Media used in the presumptive test include lactose broth, lauryl tryptose broth or EC medium. Media used in the confirmation procedures for *E. coli* include brilliant green lactose bile broth and lauryl tryptose broth as well as EC medium.

Membrane filtration (MF) is possibly the method most widely used to detect coliform bacteria in water in North America and Europe. Membrane filtration is based on the theory that if a water sample containing the target bacteria is passed through a membrane with a pore size (e.g., 0.45 um) small enough then the target bacteria will remain on the filter surface while allowing the water to filter through the membrane. Usually, a vacuum is used to draw the water sample through the filter. The membrane filter is then placed onto a solid growth medium (agar) that is selective for the target bacteria. The membrane filter used in this assay must be made of material such as cellulose nitrate, which will allow nutrients from the agar medium to pass from the underside to the surface of the membrane and thus allow the viable bacteria trapped on the surface of the membrane to grow and to form a visible colony. After incubation for 1 to 2 days at a favorable temperature, the target bacteria will grow into a characteristic visible colony which can be counted. The number of target colonies is directly related to the concentration of viable bacteria in the water. Colony forming units (CFU) is used as the unit because although this test assumes that each colony resulted from the growth of one viable bacterium, the discrete unit or particle from which the colony formed may be aggregates of several bacteria. It is important that water samples be mixed well so that individual cells are suspended in the water before the sample is filtered. There are several advantages and disadvantages to the use of the membrane filter technique for evaluation of the microbial quality of water (APHA, 1998). Some advantages include 1) good reproducibility 2) single-step results often possible 3) filters can be transferred between different media 4) large volumes can be processed to increase assay sensitivity 5) time savings are considerable 6) ability to complete filtrations on site 7) lower total cost in comparison with the MPN procedure. Some disadvantages include i) high-turbidity waters limit

volumes sampled ii) high populations of background bacteria can cause overgrowth iii) metals and phenols can adsorb to filters and inhibit growth (Prescott et al., 1996).

The presence-absence test (P-A test) can be used for coliforms. This is a modification of the MPN procedure, in which a larger water sample (100ml) is incubated in a single culture bottle with a triple-strength broth containing lactose broth, lauryl tryptose broth and bromcresol purple indicator. The P-A test is based on the theory that no coliforms should be present in 100 ml of drinking water. A positive test results in the production of acid (a yellow color) and represents a positive presumptive test requiring affirmation (Prescott et al., 1996).

To test for both coliforms and *E. coli* the related Colilert defined substrate test can be used. A water sample of 100 ml is added to a specialized medium containing O-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl- β-D-glucuronide (MUG) as the only nutrients. The medium will turn yellow within 24 hours at 35˚C due to cleavage of the ONPG in the presence of coliforms. To test for *E. coli* the medium is viewed under long-wavelength UV light for fluorescence. The MUG is modified to yield a fluorescent product when *E. coli* is present. If the test is negative for the presence of coliforms, the water is considered acceptable for human consumption (Prescott et al., 1996).

C.3. Fecal Coliforms as Indicators of Pollution

The single most important indicator of public health hazard from infectious agents is the occurrence of fecal coliform bacteria in water (McFeters and Stuart, 1972). The concept of using fecal coliforms as indicator organisms revolutionized the whole approach to public health microbiology. In the early history of the United States, each individual state held the responsibility for protecting the public's health. Water quality guidelines and standards varied among states and even among counties within a state. Yet, the predominant recreation water quality guideline used was 1000 total coliforms per 100 ml. In 1972 congress passed the Clean Water Act (Public Law 92-500), which incorporated the formation of the EPA and provided directives to establish common, national regulations for all states to follow. EPA recognized that many bacteria that tested positive by the total-coliform test were from environmental sources

such as soil or plants. These bacteria were not specifically associated with feces of warm-blooded animals (Cabelli, 1983). By now, a test had been developed to measure the concentration of thermotolerant coliforms. The thermotolerant subgroup of coliform bacteria was shown to be more specifically related to coliform bacteria recovered from feces of humans and warm-blooded animals and it was called the fecal coliform group. It was consequently resolved that populations of fecal coliforms made up approximately 20% of the total coliform population, and the EPA in 1976 revised the recreational water quality standard from a geometric mean of 1000 total coliforms per 100 ml to a geometric mean of 200 fecal coliform per 100ml (Dufor, 1984).

However, there remained a serious limitation in the use of fecal coliform levels to establish recreational water quality standards. The absence of data correlating increased concentration of fecal coliforms in a body of water with increased concentration of pathogens or with increased incidences of diarrheal diseases among swimmers using that water posed as a limitation. Nevertheless, since feces and sewage are the sources of most waterborne pathogens and since fecal coliform bacteria are accepted as reliable indicators for the presence of sewage, public health officials accepted the dogma that higher concentrations of fecal coliforms in recreational water are indicative of greater sewage pollution (Hurst et. al, 2002).

The considerable decline in the number of reported waterborne outbreaks of classical communicable disease such as cholera and typhoid fever can be attributed to the effectiveness of the fecal coliform index. While numerous studies have shown that the presence of thermotolerant coliforms generally indicates that fecal contamination has occurred, their presence in water does not always imply a health hazard. Also, the absence of fecal coliforms does not necessarily indicate that the water is safe for consumption, in particular from enteric viruses and pathogenic protozoans (Gleeson and Gray, 1997).

C.4. *E.coli* and Enterococci as Indicators of Pollution

As early as 1972, the EPA recognized the limitations of the fecal coliform standards (see above paragraph) and initiated a 10-year project (Cabelli et al., 1982) to develop more reliable

recreational water quality standards. The introductory phase of this investigation was to develop more specific tests for different groups of bacteria such as *E. coli*, enterococci, and *Clostridium perfringens*. These organisms were presumed to be superior to coliform bacteria as indicators of fecal contamination. The development of these test methods was a prerequisite for completing a comprehensive water monitoring phase of a research study. The research study was implemented to evaluate which of nine different microbial water quality indicators in marine waters would best predict incidences of diarrheal diseases among swimmers. The results showed that of all the microorganisms measured, only the concentrations of enterococci in marine waters correlated positively with incidences of diarrheal disease among swimmers (Cabelli, 1983).

Fecal streptococci and enterococci, which are gram positive bacteria, have received widespread acceptance as useful indictors of microbiological water quality, because (i) they show a high and close relationship with health hazards, mainly gastrointestinal symptoms, associated with bathing in aquatic environments; (ii) they are not as ubiquitous as coliforms; (iii)they are always present in feces of warm-blooded animals; (iv) they are unable to multiply in sewage-contaminated waters; and (v) their die off is less rapid than that of coliforms in water, and persistence patterns are similar to those of potential waterborne pathogenic bacteria (Richardson et al., 1991). Fecal streptococci comprise species of different sanitary significance and survival characteristics; in addition, the numbers of species of this group are not the same in animal and human feces (Pourcher et al., 1991). Therefore a clearer definition of fecal streptococci is necessary to establish a specific standard methodology of enumeration. The taxonomy of this group has been subject to extensive revision (Holt et al., 1993). According to Borrego and Figueras (1999) the following species of the *Enterococcus* and *Streptococcus* genera may be included in the fecal streptococci group: *Enterococcus faecalis, Enterococcus faecium, Enterococcus durrans, Enterococcus hire, Enterococcus avium, Enteroccoccus gallinarum, Enterococcus cecorum, Streptococcus bovis, Streptococcus equines, Streptococcus alactoyticus, Streptococcus intestinalis, Streptococcus hyointestinalis, and Streptococcus acidominimus*.

Although fecal streptococci may not prove to be an ideal indicator in all circumstances, the use of streptococci may be advised in several situations. For instance, when assessing the

quality of reservoirs, drinking water, and other waters in which viral contamination is particularly undesirable. Also, when determining the quality of organically rich water, especially those waters receiving chlorinated sewage effluent. Finally, when attempting to locate the sources of fecal pollution (Clausen et. al, 1977).

C. 5. Assessment of Current Microbial Indicators

One of the most compelling reasons for the selection of various fecal bacterial indicators in the past has been the availability of an economical and feasible method. Based on all the studies conducted, there is clear evidence that bathers who swim in sewage-polluted waters have increased incidences of diarrheal diseases. Moreover, the concentrations of fecal streptococci and enterococci are the best predictors of disease incidence in marine waters. However, the concentration of this group of bacteria in water is not always a prediction of the anticipated outcome because the source of these bacteria is not always the same and because environmental conditions in different places vary considerably. As a result, several other microorganisms are currently being evaluated as alternative indicators of water quality such as *Bacteroides/Prevotella* and *Psuedomonas* (http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/279/report/0 ; http://ecommerce.hach.com/stores/hach/pdfs/literature/L7015.pdf).

D. Watershed Studies

Historically, the burden of controlling pathogens in drinking water has been placed on treatment plant operations. A more comprehensive approach is the use of multiple barriers. One of the first barriers in place should be the ability to control the entrance of microbes into source waters via watershed protection. At the national level, the Safe Drinking Water Act and the Clean Water act both directly regulate the introduction of contaminants into the nation's surface waters and groundwater. At the state and local levels, regulatory programs can vary but are usually designed to protect source waters and groundwater. At the state and local levels, regulatory programs can vary but are usually designed to protect source waters by restricting and/or regulating certain activities which could degrade their quality (Margolin, 2002).

In the last decade, the EPA's Watershed Protection Approach (WPA) has focused on organizing and managing the states' major watersheds. In this approach, activities such as water quality monitoring, planning, and issuing permits are coordinated on a set schedule. The WPA has four major features: targeting priority problems, a high level of stakeholder involvement, integrated solutions that make use of the expertise and authority of multiple agencies, and measuring success through monitoring and other data gathering (EPA, 1997).

Managing a watershed can be divided into nine subcategories: management units, management cycles, stakeholder involvement, strategic monitoring, assessment, prioritization and targeting, development of management strategies, basin or watershed plans, and implementation. Management units are areas that are delineated by each state and contain many watersheds. Management cycles concern the monitoring of the management units. Each unit is examined cyclically so that all of a state's management units are examined in a set period, typically 5 years. Stakeholder involvement allows for more individuals and groups to become involved in the outcome of one or more watersheds. The more involvement there is, the greater the number of individuals who have concern about water quality, the more likely it is that there will be an allied effort to preserve that water quality (EPA, 1997).

Recreation has become a major part of life in the United States. There is steady demand for multiple uses of reservoirs, streams, and tributaries which make up any watershed. Studies have shown that there is a greater possibility that pathogens will be introduced into waterways when they are subjected to increased use for recreational activities (Margolin, 2002). There is a need for local agriculture to limit or restrict the introduction of farm animal waste into the watershed. This approach to watershed management addresses both the introduction of pathogens into the watershed and the individual farmer's operational and financial circumstances.

Microbial monitoring remains the foundation of any watershed quality assessment. Nevertheless, there are several aspects of a monitoring program which need to be considered before the program can make a substantial contribution. The program design should include frequencies for sampling analysis, protocols, and the types of analysis needed (Margolin, 2002).

The detection of pathogens alone is not an adequate monitoring tool. Pathogens ordinarily appear in low concentrations, and the analytical techniques available for their detection are often not very reliable or sensitive. While monitoring of pathogens is important it must be combined with the evaluations of several different organisms. Also, since any source water is a dynamic body and because pathogens usually occur in low numbers, a monitoring schedule covering at least 1 year, and preferably more should be adhered to and results must be evaluated at the end of that period. The monitoring program should be designed to take into account the changing properties of the source water. To illustrate, if there is an increased use of water for recreational activities during the summer months, the frequency of monitoring should reflect the increased usage. The same may hold true for dry-versus wet-weather events. Discharges and runoff may occur only during heavy rains, and the pathogens that they bring may not be detected if monitoring is done only during the dry period (Hurst et al., 2002).

Watershed protection that involves evaluating all the parameters of any given watershed, breaking these parameters down into different parts, and using efforts to try to minimize source water contamination completely are more beneficial and cost-effective than water treatment alone (Hurst et. al. 2002).

D.1 The Regulatory Process and TMDLs

The Refuse Act passed by congress in 1899 was the first statutory environmental law passed by congress. It stated that it was unlawful to place any material, except sewage and runoff, into a navigable waterway or tributary thereof without a permit. Until 1972, this dated statute was used to control water pollution from industrial sources, although the primary purpose of the act was to protect navigation not water quality (Novotny and Olen, 1994).

Passage of the Public Health Service Act of 1914 was the beginning of legislation to protect bacteriological quality of drinking water in the USA (Gleeson and Gray, 1997). Initially these regulations were not mandatory for all public water supplies. However regulations did become mandatory in 1974 with creation of the Safe Drinking Water Act (SDWA). This particular act required the US-EPA to establish national standards for drinking water quality. All public water

systems serving 25 or more persons or that included 15 service connections were now federal responsibility. “EPA first regulated chemicals in drinking water by establishing maximum contaminant levels (MCLs) and sampling requirements for nine inorganic chemicals (IOCs), and six synthetic organic chemicals (SOCs) in the Interim Primary Drinking Water Regulations of 1975” (http://www.epa.gov). “In accordance with the Safe Drinking Water Act Amendments of 1986, EPA began adding to its list of regulated chemicals. In 1987, EPA adopted standards for eight volatile organic chemicals (VOCs) in the Phase I Rule. From that point on, regulations for contaminants in drinking water have been referred to as National Primary Drinking Water Regulations (NPDWRs)” (http://www.epa.gov)

Interim regulations were published in 1975 that provided for a minimum number of samples to be examined each month and established the maximum number of coliforms (Maximum Contaminant Level or MCL) allowable per 100 ml of finished water (Gleeson and Gray, 1997). MCLs are based on the presence or absence of coliforms in a sample. For systems where at least 40 samples per month are analyzed more than 5% of the monthly samples may be coliform positive. The MCLs for those systems collecting less than 40 samples per month is no more than one coliform positive sample per month. If the system is found to be in violation of the MCL for total coliforms then, the public must be notified (Novotny and Olen, 1994).

The SWDA was amended in 1986, significantly expanding the original act. Under the 1986 amendment disinfection was required for all water systems . Also the use of lead in any pipe, solder, flux or fittings in any public water system was prohibited with the amendment as well . Enforcement procedures were streamlined, which included raising penalties up to $25,000 per day for infringements (Gleeson and Gray, 1997).

In July 1989, USDA began the Water Quality Initiative (WQI) to promote both traditional and new land treatment and agrichemical management practices that would reduce pollutant loading to ground and surface water (USDA, 1997). The Rural Clean Water Program (RCWP), initiated in 1980 and ended in 1995, was an experimental program that addressed agricultural nonpoint source pollution in 21 U.S. watersheds. The RCWP was administered by the Agricultural Stabilization and Conservation Service in consultation with EPA (USDA, 1997).

Setting Total Maximum Daily Loads (TMDLs) has been required for years. It was mandated in 1972 by Section 303(d) of the Clean Water Act (CTIC, 200o). At the time TMDLs were mandated, people had a more limited idea of what constituted pollution than we have now (US EPA, 1999). Recently TMDLs actually became part of water quality management in the United States. The TMDL Federal Advisory Committee was formed by the EPA office of Water in 1996. The purpose of this committee was to review TMDL requirements. The EPA proposed revisions to the TMDL program based on the recommendation of the Committee in August 1999 (EPA 2001b). As a result the EPA posed the TMDL Final Rule. The Final Rule was announced in July 2000 and included five main points.

- The first is identification of polluted waters, which requires states to compile a complete list of all polluted waters, but reducing the report of impaired waters from every 2 years to every 4 years and not requiring threatened water bodies to be included on the list.
- The second component requires states to devise a schedule for implementing the TMDL within the 10 years after a water is listed as impaired and give a high priority for TMDL development for waters with endangered species or that serve as drinking water supply.
- The third section of the Rule includes six elements of a TMDL
    - (1) problem statement, (2) endpoints, (3) source assessment, (4) endpoint and source linkage, (5) allocation, and (6) monitoring.
- The fourth section lists requirements of an implementation plan.
- The fifth section requires "reasonable assurance" that non-point source load allocation process was applied to the pollutant, implemented swiftly, carried out effectively, and supported by state funding.

The last section provides state with flexibility on how to set water quality standards and how to efficiently implement the TMDL process (EPA, 2001b). Congress immediately blocked the rule for a public review period until October 2001 and EPA made that the effective date for the rule (EPA, 2001d).

For many years after the Clean Water Act, the TMDL section was practically ignored while point source pollution was addressed. Since EPA mandated the 1992 TMDL regulations, many environmental and recreational groups have sued the EPA because surface waters failed to

meet water quality standards. After the EPA loses a case in a state, it is the state's responsibility to begin the TMDL process. The state is then under pressure to establish TMDLs state-wide, but if it fails to do so, the EPA will intervene. The EPA has been issued court orders to establish TMDLs for 18 states (EPA, 2001c). Virginia is included in the 18 states with court orders to establish TMDLs. In 1998, American Canoe Association (ACA) and American Littoral Society (ALS) sued EPA for VA waterways that did not meet Water Quality Standards-WQS (ACA, 2001). It was settled in 1999 with a consent decree to establish a state-wide TMDL development and implementation schedule. Virginia has scheduled 301 impaired segments for TMDL development by 2010 with the goal to eventually remove segments from the impaired waters list (Virginia DEQ, 2001b). Water segments can be eligible for removal from the impaired waters list if monitoring data show that the impairment no longer exists (EPA, 2001a).

The TMDL process is virtually a cycle, beginning with monitoring. In Virginia, this is usually done by the DEQ. They make assessments and create the 303(d) list with impaired waters that require TMDLs to be established. It is sent to the EPA according to the reporting requirements under the Clean Water Act. The TMDL is written and submitted to EPA for approval and implementation plans are then developed. Finally, management strategies and control measures are implemented and more monitoring is conducted. Public participation is a key element in the process of improving water quality (FORVA, 2001). Public meetings allow citizens to be involved in the decision making process at several stages within the TMDL process (Booth, 2001).

TMDL is a process to improve water quality beyond point source controls, mandated by the Clean Water act and brought to the forefront in VA by a lawsuit from the American Canoe Association. Elements of a TMDL include identifying name, location and impairment, determining the maximum loading allowed and the reductions needed to meet standards, identifying sources of pollution, including a margin of error, allowing for seasonal variation and future growth and an implementation plan. The TMDL process is still relatively new in water quality legislation today. Although there is a long way to go, the TMDL process is under way in Virginia and across the country (Booth, 2001).

In addition to setting a TMDL, the responsibility for reducing pollution among both point sources (pipes) and non-point sources such as run-off, septic systems, and damage to a riparian area is still a priority (CTIC, 2000). TMDLs are critical for achieving water quality standards and are used to determine impaired waters. The key to success in restoring impaired waters is implementing TMDLs (US EPA, 1999).

A major concern nationwide is the failure to meet water quality standards. It is estimated that about 21,000 river segments, lakes, and estuaries have been identified by states as being in violation of one or more standards. Over the last 30 years there has been a drastic reduction in pollutant loading from point sources such as sewage treatment plants. Thus the successful implementation of most TMDLs will require controlling non-point source pollution (National Research Council, 2001).

D.2. Fecal Sourcing in Watersheds

The Virginia DEQ most recent water-quality assessment (1998) states the following about bacterial contamination in Virginia: 'In general fecal coliform bacteria exceedances are the leading cause of non or partial support of designated uses in rivers and streams. Agricultural practices appear to be one of the primary sources causing the loss of designated use support. Indications are present that uncontrolled agricultural and pasture land use result in much of the fecal coliform bacteria and nutrient contamination in Virginia's waters' (Keeling, 2000). The presence of fecal coliforms in water supplies indicates that the waste of humans, other mammals, or birds has contaminated the water. This contamination can cause health risks to people exposed to the water. Drinking and recreational water quality is diminished by fecal coliform contamination and can lead to closure and bans on recreational water use (Hagedorn, 2000). In the past, sources of fecal bacterial pollution in water could not be accurately or reliably determined.

Fecal coliform bacteria are the most widespread problem in Virginia's rivers and streams (EPA, 1995). Fecal coliform populations that exceed water quality standards have caused over one-half of the stream segments in Virginia, that have been evaluated to date to be listed as

impaired waters (Hagedorn et. al, 1999). The ability to develop realistic TMDLs for fecal bacteria, and implementation of cost-effective best management practices (BMPs) for watershed restoration will largely depend on using the new bacterial source tracking (BST) methodology to accurately identify sources of fecal pollution in water (Hagedorn et al., 1999).

While most emphasis on bacterial indicators of water quality has been placed on fecal coliforms, there are several good reasons to consider the fecal streptococci and enterococci as well (Wiggins, 1999). There has been little success in determining the source of fecal coliforms using natural antibiotic resistance patterns as identifying markers (Wiggins, 1999). Conversely, these patterns do seem to have potential with the fecal streptococci. There is an observance of a higher level of antibiotic resistance in fecal streptococci that are isolated form sewage than those found in soil or on vegetation, when using fecal streptococci as indicators (Wiggins, 1999).

There are standards for recreational water quality with the enterococci (33 cells per 100 ml for fresh water), so these organisms are still useful as indicators (Hagedorn et al., 1999). The fecal streptococci tend to persist longer in the environment than fecal coliforms and this may be useful as an indicator for less recent fecal pollution. However for determining the sources of fecal pollution, an indicator with a longer survival time can be an advantage (Hagedorn et al., 1999). Lastly, there are some potential sources of fecal contamination (e.g. composted animal and poultry litters, and advanced-treatment Class B biosolids) where it is difficult to isolate fecal coliforms while there is no difficulty in isolating fecal streptococci. Fecal coliforms would not be suitable for sourcing contamination from these types of materials (Hagedorn et al., 1999).

Bacterial Source Tracking is a revolutionary technology used to identify fecal coliforms in water polluted sources. Reliable identification of fecal contamination sources will help direct efforts and expenditures to the correct source(s) of the impairment (Harwood, 2000). Potential sources of fecal bacteria are generally grouped into three major categories; human, livestock, or wildlife. In more urban watersheds, a fourth category of pets or dogs may be added. Each source produces unique, identifiable strains of fecal bacteria because the intestinal environments and selective pressures to which the bacteria are subjected differ from source to source (http://www.epa.gov). A methodology having the ability to turn nonpoint sources into point

sources is how BST has been described (Hagedorn, 2000). Fecal sourcing, fecal typing and molecular source tracking are other aliases for BST that are found in scientific literature. The recent implementation of the TMDL concept by US-EPA is the main driving force behind BST development in Virginia (Hagedorn et al., 1999).

There are three basic groups of BST methods: molecular, biochemical, and chemical. Molecular (genotype) are all referred to as DNA fingerprinting and are based on the unique genetic makeup of different strains, or subspecies, of fecal bacteria (Graves, 2000). Biochemical (phenotype) methods are based on an effect of an organism's genes that actively produce a biochemical substance. The type and quantity of these substances produced is what is actually measured. Chemical methods are based on finding chemical compounds such as detergents and optical brighteners that are associated with human wastewaters (Hagedorn, 2000). BST may use one of several methods to differentiate between potential sources of fecal contamination, all of which follow a common sequence of analysis. First, a differentiable characteristic, or fingerprint (such as antibiotic resistance patterns or DNA), must be selected to identify various strains of bacteria. A representative library of bacterial strains and their fingerprints must then be generated from the human and animal sources that may impact the water body. Indicator bacteria fingerprints from the polluted water body are compared to those in the library and assigned to the appropriate source category based on fingerprint similarity. BST methods can be grouped as molecular or non-molecular methods, according to the characteristic used to identify or finger print the bacteria (Table 3) (http://www.epa.gov).

Table 3. Classification of Bacterial Source Tracking Methods.

(Molecular methods)	
	Randomly Amplified Polymorphic
	DNA (RAPD) Analysis
	Ribotyping (RT)
	Polymerase Chain Reaction (PCR)
	Pulsed-field gel electrophoresis (PFGE)
	DNA Microarray
	DNA signature sequences
(Non-Molecular methods)	
	Biochemical methods
	Antibiotic Resistance Analysis (ARA)
	Cell wall fatty acid methyl ester (FAME)
	F-specific coliphage typing
	Carbon utilization (BIOLOG)
	Chemical methods
	Caffeine detection
	Optical brightener/Fluorescent detection

Molecular methods or DNA fingerprinting have received the greatest publicity, but to date there are at least ten or so different methods described in the scientific literature that show potential (Hagedorn, 2000). BST development is so new that no research comparing BST methods or identifying their relative strengths and weaknesses has yet been completed. It is most reasonable to assume that some combination of BST methods will be needed to provide the most accurate and reliable source identification answers (Wiggins et al., 1999). It is doubtful that any one BST method will emerge as the "best" method for all situations. Molecular methods offer very precise source identification, however they are limited by cost, detailed and time-consuming procedures, and are not yet suitable for assaying large numbers of samples in a reasonable time frame (Kern et al., 2000). Biochemical methods may or may not be as precise, but are simpler, quicker, less costly, and allow large numbers of samples to be assayed in a short period of time. Chemical methods are based on finding chemical compounds that are associated with human wastewaters, and are very experimental at present. Perhaps the best approach is to use a biochemical and/or chemical method(s) to run large numbers of samples, and then confirm both the method(s) and the results by assaying some subset of samples by a molecular procedure if desired or warranted (Hagedorn et. al, 1999).

Presently, BST can reliably determine if fecal bacteria are from human or animal sources (Hagedorn, 2000). If the bacteria are from animal sources, BST can also tell if the animals are livestock or wildlife, but less reliably than the human vs. animal separation. The key to correct existing fecal bacterial pollution and to prevent further problems is by reliably and accurately finding the contamination source(s). Therefore, BST should be heavily used in the future as a tool much like routinely used watershed computer models (Hagedorn, 2000).

E. Non-Genomic Techniques in Fecal Typing

Non-molecular methods use non-genetic characteristics as the fingerprint or basis to differentiate the source of fecal bacteria, and may be further subdivided between biochemical and chemical methods. Biochemical methods are based on the ability of an organism's genes to actively produce a biochemical substance. The type and quantity of the substance(s) produced

form the bacterial fingerprint. Antibiotic resistance analysis (ARA) is currently the most commonly used biochemical BST method. Other biochemical methods, such as cell wall analysis of fatty acid methyl ester (FAME), F-specific coliphage typing and carbon source utilization (BIOLOG system) are under development (http://www.epa.gov and Table 3). Chemical methods do not detect the presence of fecal bacteria, but rely on the identification of compounds that co-occur with fecal bacteria in human wastewater to differentiate the source of fecal pollution. Thus, chemical methods can only determine whether or not the source of fecal pathogens is human (Hagedorn, 2001) Examples of compounds used in chemical BST include caffeine and optical brighteners commonly used in laundry detergents. Several attempts have been made to develop methods to determine the sources of fecal pollution (Wiggins et al., 1999). Initially, the ratio of fecal coliforms to fecal streptococci was used as an indicator of the source: a high ratio (>4) was considered to indicate an animal source, while a low ratio (<0.7) suggested a human source. This ratio has since proven unreliable, and the method has been abandoned (APHA, 1995).

ARA is a biochemical (phenotypic) BST method pioneered around 1996 initially by Bruce Wiggins at James Madison University (Wiggins, 1996). The ARA method is based on the patterns of antibiotic resistance found in bacteria from human and animal sources. The principle behind the method holds that human fecal bacteria will demonstrate greater resistance to certain antibiotics, and animal fecal bacteria will be resistant to others. Humans are exposed to a different set of antibiotics, primarily through prescriptions than are farm animals. Although agricultural species routinely receive antibiotics as additions in commercial feed, those for cattle differ from those for pigs, which differ from those for poultry or horses, and so on. Wild animals receive relatively little exposure to antibiotics, thus their fecal bacteria should not exhibit substantive antibiotic resistance. ARA researchers use isolates of fecal streptococcus (including enterococci) and *E. coli*. To determine characteristic antibiotic resistance patterns, fecal bacterial isolates from different sources (human and animal) are tested on a battery of antibiotics (30 to more than 70 different antibiotic/concentration combinations). Following bacterial incubation, technicians score isolates for "growth/no growth" for each concentration of an antibiotic. These results form an organism-specific resistance pattern that can be used to identify individual sources of bacterial contamination (Hagar, 2001).

To carry out the ARA method, it is important to build a database of antibiotic resistance patterns of indicator bacteria from known sources. Once the database contains enough resistance patterns of different bacterial sources to sufficiently represent the area under investigation, it can be used to predict the animal or human sources of bacterial contamination in the water. Antibiotic resistance patterns are determined, and these patterns are analyzed through discriminant analysis. Discriminant analysis is a multivariate statistical technique that, finds out which group of patterns from known sources is closest to the pattern of unknown isolates (Bowman et al., 2000, Graves et al., 2002)

Sample turnaround time with ARA is about one week. The results are considered very reproducible within a sample. ARA researchers have conducted a number of experiments to test the stability of antibiotic resistance patterns (Harwood et al., 2000). Some professional criticism of the ARA technique questions whether the statistical analysis uses independent variables (e.g., antibiotic concentrations) and acceptable model design. ARA researchers point out that many people are unfamiliar with discriminate analysis, which they assert is particularly applicable to the variables used in ARA (Hagar, 2001, Hagedorn et al., 1999). ARA is a database-dependent method and might be location-specific, as its ability to correctly identify sources may decline when very large databases are constructed across geographically distinct sources. Since this is not a molecular method there is no genetic linkage to source animals. Additionally, the antibiotics used on livestock can change by region, which may require unique isolate libraries for each region of the state or country (Wiggins et al., 1999). ARA was chosen for use in this research and details regarding the ARA procedure are located in the materials and methods section.

F-specific (F+ or FRNA) coliphage is a biochemical method that is used to source track *E. coli* (Miesfeld, 1999). Bacteriophages are viruses that infect bacteria, and coliphages are bacteriophages that are specific pathogens of *E. coli*. Because coliphages almost always come from fecal material, their presence in water bodies can indicate bacterial contamination. *E. coli* cells can be male or female, and male-specific coliphages infect only male *E. coli* cells through bacterial appendages called "pili," which are unique to male cells. Male *E. coli* cells transfer genetic information to female cells through these pili. An "F+" designation–"F" signifying

"fertility"–represents the genetic information responsible for producing the pili on male *E. coli*. There are four groups of F+ coliphages: Group 2 is the human-specific coliphage group in North America; Group 4 is the animal-specific coliphage group, virtually never found in human wastes; Group 3 contains human-specific coliphages common in other parts of the world; and Group 1 consists of coliphages found in both humans and animals (Hagar, 2001).

Coliphages collected in water samples grow in a Petri dish on a "lawn" of *E. coli*. Coliphages can be spotted in circular areas called "plaques," where phages have lysed bacterial cells. The number of plaques represents the number of coliphages contained in the volume of water put into the Petri dish. The method is fairly sensitive, giving a clear number of coliphages affecting *E. coli*. Some of the phages are removed from the plaques and placed in bacterial suspension, then incubated again in Petri dishes on lawns of *E. coli*, where they form lysis zones, discrete areas of lysed cells that contain many coliphages. From each individual plaque and single drop of the suspension, lab workers can create multiple lysis zones. The area of lysis can be picked up by a piece of filter paper and identified by using a DNA probe with a visual tag. The coliphage typing approach is most useful when a question arises of whether fecal contamination in a water body originates from human or animal sources, but it is not capable of distinguishing particular animal sources. Coliphage genotyping is very simple and inexpensive to perform. The gene probes–short lengths of synthetic DNA–are widely available. The method can be performed fairly rapidly, producing results in about two days. Coliphage typing is not database dependent or location specific, only "waste-source specific," to human or animal contamination (Hagar, 2001).

Considered more indicative of viral contamination, FRNA coliphage is a pathogen of *E. coli* infecting the pilus of the male *E. coli*. These coliphages can be differentiated using serology (Miesfeld, 1999). There are four antigenically distinct serogroups of FRNA coliphages, and those predominating in humans (groups II and III) differ from those predominating in animals (groups I and IV). Consequently, it may be possible to distinguish between human and animal wastes by serotyping FRNA coliphage isolates. However, there is a problem with separation between human serotypes and serotypes associated with pigs, which also contain group II. Additionally not all animals have FRNA coliphage associated with their respective *E. coli*

(Miesfeld, 1999). The coliphage is persistent in the environment for less than a week and survival is a function of sunlight and water temperature. Ultraviolet light denatures the virus and below 25˚C F-pilus synthesis ceases. The coliphage does not replicate in the environment but only in the presence of F-pilus *E. coli*, and is not found in sediments, but rather just in the water column (Miesfeld, 1999). However, one major weakness associated with this method involves the rapid die off rate of coliphages in warm water (above 20˚C). This is a big weakness because most people use recreational water when it is warm.

Sterols or fatty acid analysis involves the constituents fatty acids in cell walls and membranes. This method is currently under development, however there are hopes to differentiate between the types and quantities of sterols in human *E. coli* cell walls and membranes verses those in other animals (Keeling, 2000). The MIDI Sherlock Microbial Identification System (MIS) identifies microorganisms based on the unique fatty acid pattern of each strain. The MIDI System features large databases of environmental and clinical organism, superior identification compared to biochemical test systems. It is around $2.00 per sample but one has to include the cost of high-end gas chromatography equipment and trained technicians so ultimately the cost is more than $2.00 per sample (http://www.midi-inc.com/pages/products.html).

A nutritional pattern is a technique based on differences among bacteria in their use of a wide range of carbon and nitrogen sources for growth. This method works well in the laboratory (Hagedorn, 2000). The BIOLOG System is currently under review for testing the nutritional patterns. The Biolog System can rapidly identify over 1,900 species of aerobic and anaerobic bacteria, yeasts, and fungi (http://www.biolog.com/mID_product.html).

Fecal-bacteria ratios are based on the ratios of many different types of stomach and intestinal bacteria, not just fecal coliform bacteria and enterococci. This method is not considered reliable for accurate source identification, but can be used as a general indicator of human verses non-human fecal bacterial contamination (Hagedorn, 2000).

Bacteriophages other than coliphages have also been proposed as indicators of the source of fecal pollution. However the usefulness of bacteriophages as an indicator is limited because only a small percentage of fecal samples contain phages (Wiggins, 1999). Other studies have shown that there are differences in the species composition of fecal streptococci among various types of animals as seen in different percentages of various fecal streptococci in the feces of poultry, cattle, and other animals.

E.1. Multiple Antibiotic Resistance Patterns (MAR)

One of the tools at researchers' disposal is called a multiple antibiotic resistance (MAR) test. It's based on *E. coli* hosts' exposure to antibiotics – patterns of resistance arise largely from prior exposure to antibiotics, and the bacteria's natural tendency (survival instinct) to try to build up resistance against them. However, little was known about applying the MAR test to distinguish between *E. coli* isolated from human, livestock, and wildlife. Until recently, science had not advanced to the point where a confident assessment could be made. Rapid advances in technology that can reveal small differences among related bacteria have now been developed. Researchers set out to determine if indeed they can use this new technology to identify specific *E. coli* sources. Results show the MAR test may indeed be useful in differentiating *E. coli* between livestock, wildlife, and humans. Results have demonstrated that wildlife *E. coli* bacteria were readily distinguished from others. That is because they normally receive little exposure to antibiotics. As a result, *E. coli* in their bodies would not be heavily engaged in developing antibiotic resistance. On the other hand, swine and poultry *E. coli* isolates had high indices. In production agriculture, these animals receive relatively high levels of antibiotics. So, *E. coli* in their bodies are active in forming resistance to the antibiotics. It was difficult for the researchers to distinguish between human and cattle *E. coli* isolates because they showed similar resistance levels. Further studies are underway to fine-tune the testing procedure using additional technology, in order to conclusively determine *E. coli* from different sources (Wiggins, 1999). Although there have been many studies that have measured antibiotic resistance of fecal isolates from various sources, it has been difficult to use that information to identify the sources of fecal pollution (Parveen et al., 1997; Harwood, 2000).

F. Genomic Techniques for Fecal Typing

Molecular methods are also referred to as "DNA fingerprinting" and are based on the unique genetic makeup of different strains of fecal bacteria. Molecular methods rely on genetic variation as the fingerprint to identify the source of fecal contamination. Three molecular BST methods are commonly used, including ribotyping (RT), polymerase chain reaction (PCR), and pulsed-field gel electrophoresis (PFGE). Procedures for the RT and PFGE methods are relatively similar among multiple studies, but substantially different variations are reported when using PCR methods (Hagedorn, 2001). The use of DNA Microarrays and DNA signature sequences are emerging as well. The success of DNA microarrays in BST will greatly depend on the bioinformatics tools available. Bioinformatics in the DNA Microarray field starts with fully automated and batchwise working image analysis programs and should cover all aspects of statistical analyses (reproducibility of experiments, background determination, clustering, etc.) and their link to gene regulation and function (Grigorenko, 2001).

The fecal bacteria in any two animals (including humans) are genetically the very similiar (Hagedorn, 2000). There are unique differences, but the differences are only in a small percentage of an organism's total DNA. The key to molecular BST is finding these differences amongst a large amount of similarity (Parveen et al., 2001). The distinctions among fecal bacteria of different animal species occur because of the different milieu found in the animal's intestines. These strains of microorganisms (generally *E. coli*) are isolated using standardized laboratory procedures before fingerprinting the organisms DNA. Typically 20 to 50 isolates are obtained from each water sample (Hagedorn, 2000). However, one should note that there is considerable of controversy involving the use of genetic concepts with *E.coli* for bacterial source identification. Studies suggest that geographical structure or host specificity accounts for little of the genetic diversity. Furthermore, evidence indicates that there is little temporal stability in clonal composition of *E.coli* populations. Thus, attempting to identify the source of coliform contamination that focuses on commensal isolates of E. coli appear to be invalid according to Gordon (2001).

Pulse Field Gel Electrophoresis (PFGE) is one method that has been used to differentiate sources of *E. coli*. This method is considered very accurate in differentiating between strains of the same microorganism and for DNA analysis or fingerprinting. Pulse field gel electrophoresis (PFGE), a technique used in the field of genetics, is a molecular BST method that provides DNA fingerprints of sources of fecal bacterial contamination in a water body (Simmons et al., 2002). PFGE is similar to ribotyping, although ribotyping analyzes ribosomal RNA genes of *E. coli* strains, whereas PFGE works with the whole DNA genome of *E. coli* strains. As with ribotyping, PFGE uses restriction enzymes to cut *E. coli* DNA at specific locations. The resulting segments are then run through electrophoresis to generate banding patterns that can be compared against known patterns.

Modifications incorporated by the PFGE method set it apart from other approaches to electrophoresis. A specially designed gel setup, called the Genepath apparatus, sends electric current through a gel in different directions for several hours, which allows for superior band separation. Bacterial DNA analyzed through PFGE are embedded in agarose plugs. These plugs are placed in hollow combs of the electrophoresis gel, where they become part of the gel as the gel moves over the combs. Following electrophoresis, banding patterns become apparent after the gels are stained. Embedding the DNA in the agarose plugs essentially eliminates the potential for sample contamination, a common problem with molecular BST approaches (Simmons et al., 2002).

PFGE is a database-dependent methodology, as researchers employing the technique seek to subtype isolates of water and bacterial sources by matching them with previously identified isolates stored in an established library. Because sources of bacterial contamination may vary from place to place, PFGE appears to be location specific in terms of requiring isolate libraries to represent *E. coli* strains specific to each sampling region (Hagar, 2001).

PFGE is a highly sensitive BST method, which enhances its effectiveness but can increase costs incurred through subtyping and identifying the many isolates that the method can detect. The method's sensitivity also makes it prone to detect random mutations, which are not likely to be useful additions to an isolate database. Still, PFGE maintains an accuracy rate of

70% or better. Sample turnaround time for PFGE analysis remains relatively slow: up to a month or longer. As with other molecular BST methods, PFGE can be costly, and it requires personnel with specific laboratory training. Sample volume representation is relatively low, with small samples (100 ml) and only a few strains are usually tested per sample (Hagedorn, 2000).

The ability of the PFGE method to accurately represent which *E. coli* strains are in a given watershed depends on the number of strains identified relative to the total number of *E. coli* in the sample and the number of samples taken. These factors also affect the ability of this method to be considered quantifiable (Hagar, 2001).

Reports show a good average rate of correct classification (ARCC >80%), however, about 88% has been the best ARCC result to date for PFGE (Simmons, 1994). All genomic methods for source tracking depend on number of mutations in the DNA, number of strains of *E. coli* any source animal may have, and the amount or degree of crossover from one strain to another (Hagedorn, 2000). PFGE method is relatively slow and expensive, costing about $30 to $75 per isolate. Time for analysis depends on staffing levels and generally takes days to weeks to process samples.

Randomly Amplified Polymorphic DNA (RAPD) is another method that is accurate and quick for conducting DNA analysis and bacterial differentiation. This method involves a total genomic digestion that focuses on the unique polymorphisms within the DNA of an organism (Donbeck et al., 2000). It is suspected that these polymorphisms are responsible for things such as antibiotic resistance. Newer analytical devices are creating very high-resolution digital photos of the separation gels and there are software packages that will automatically add and compare results to existing databases or libraries (Dombeck et al., 2000). This method is currently faster than either PFGE or ribotyping although it is comparable in expense. This method is still under development but is showing very promising results (Hagedorn, 2000).

Ribotyping is a molecular method first applied to human disease outbreaks about 20 years ago and to bacterial source detection 11 years ago. Ribotyping (RT) is a method that has non-PCR and PCR variations that can be used with it (Parveen et al, 1999). RT is being used in

the USA by other investigators with ARCC's between 68 and 82%. This method looks at the genetic differences in ribosomal RNA genes and is considered a very useful method of bacterial strain identification. RT is slow and expensive taking several weeks to months to get results and costs about $75 per isolate (Bernhard et al., 2000).

The RT method generates genetic fingerprints of *E. coli* strains, indicators of fecal coliform, collected from contaminated sites. The genetic fingerprint comes from genes that code for deoxyribosomal ribonucleic acids (rRNA) of *E. coli*. These genes are known to be highly conserved in microbes, meaning that the genetic information coding for rRNA will vary much less within bacteria of the same strain than it will between bacterial strains. This characteristic allows for a greater ability to distinguish between different bacterial strains (Hartel et al., 1999).

In ribotyping, technicians use one or more restriction enzymes to cut the DNA–the genes coding for rRNA–into pieces, and electrophoresis separates the pieces by size through a gel. Genetic probes then visualize locations of different-size fragments of DNA in the gel, which appear as bands. The banding pattern of DNA fragments corresponding to the relevant rRNA is known as the ribotype. Characteristic banding patterns identify bacterial strains (Bernhard et al., 2000).

As with many other BST techniques, ribotyping relies heavily on first establishing a database of known bacterial types, in this case the ribotype information for each bacterial strain analyzed. These are the "knowns" to which one compares unknown samples to determine contamination sources of *E. coli.* Without a substantial library of the genetic fingerprints of *E. coli* isolates from contaminated sites and potential contaminant sources in that project area, source identification remains impossible. Ribotyping, as with other BST methods, uses the clonal population structure of bacteria to identify and classify source organisms through their genetic fingerprints. Because the organisms that may impact bacterial contamination vary from region to region and require the collection of representative isolates in whatever region being sampled, ribotyping also appears specific to each geographical location (Graves, 2000).

In terms of ribotyping's sensitivity, ribotyping can quite accurately detect sources of bacterial contamination, perfectly matching isolates from humans and many different animals. Ribotyping sometimes provides little quantitative information, a shortcoming (and challenge) faced by many BST methods. An advantage of ribotyping is that new isolates can be, and are, constantly added to the database (Hagar, 2001).

Ribotyping is somewhat labor-intensive and time-consuming, but the level of expertise required to run the technique is not as great as for some of the other molecular approaches. Sample volume representation remains fairly low, as the volume tested is estimated to be 100 ml. Sample analyses takes a couple of weeks to complete. Costs per sample prove comparable to those of many other BST methods, but the per-isolate cost may be higher than for a biochemical method such as ARA. In an ideal study, many samples should be taken to increase accuracy, which of course also raises costs (Carson et al., 2001).

Many researchers criticize ribotyping's sampling methods with only a few *E. coli* strains analyzed per sample–as failing to accurately reflect local bacterial populations. A more common approach would be to analyze more strains from samples containing more bacteria (but this is usually not done due to costs).

F. 1. Polymerase Chain Reaction

One of the most important developments in applied molecular genetics in the last decade was the invention of an amazingly simple DNA amplification strategy called the PCR (Miesfeld, 1999). The logic of the PCR protocol follows directly from well-understood principles of nucleic acid biochemistry. The basic components of a PCR reaction are one or more molecules of target DNA, oligonucleotide primers, thremostable DNA polymerase and deoxynucleotide triphosphates (dNTPs). This reaction mix is repeatedly heated and cooled to 95, 55, and 72°C, in that order, a total of 25-35 times to produce a $>10^6$ – fold amplification of the target DNA (Miesfeld, 1999). An alternate approach involves using PCR to amplify quantities of DNA, to obtain large numbers of copies of small DNA sequences. PCR utilizes thermal cycling to disassociate DNA sequences at high temperatures (95˚C), then at low temperatures to use

specially designed primers to bracket the targeted piece of DNA (55˚C). A polymerase enzyme allows the primers to extend and copy the DNA sequence (72˚C). The entire disassociation-copying process occurs repeatedly, each time duplicating all DNA strands, original and copies (Dombek et al., 2000).

The advantages of PCR are that it can copy a very small amount of genetic information-usually a specific region of DNA-through rapid, geometric increases so that the volume of DNA obtained increases dramatically. In ribotyping, the amplified DNA sequences are then cut into pieces with restriction enzymes and run through gel electrophoresis. The resulting large concentrations of DNA obtained through PCR can enhance visualization of the DNA fragments. Although the PCR reaction is relatively simple, the biochemical and kinetic parameters that affect PCR specificity and sensitivity are actually quite complex and it can sometimes be troublesome to define a reaction condition that generates reproducible results. In fact, during the first few years after PCR was described in the literature, researchers encountered numerous problems, primarily with regard to reaction specificity and sample contamination. At the time, these drawbacks seem to indicate that PCR applications may not be well suited for routine diagnostic procedures (Miesfeld, 1999). This dire prediction did not hold up as continued refinement of the technique solved the most serious problems. Nevertheless, it is important to keep in mind that each time a new DNA target or PCR primer pair is designed for a specific research objective, a number of key parameters such as determining whether the primers (or the target sequence itself) have a significant degree of complementary to known repeat elements will have to be optimized (Dombek et al., 2000).

G. Applicability of BST in the Field.

Many BST techniques are undergoing intensive research that leads to rapid change in existing methods and the creation of new methods. BST technologies are quickly becoming proven and should be used by federal and state regulatory agencies to address sources of fecal bacterial pollution in water. Although they are still experimental, BST methods represent the best tools available to determine pathogen TMDL load allocations and TMDL implementation plan

development. The following are examples of BST technique performance in specific watershed studies.

Antibiotic Resistance Analysis Method

Holmans Creek, Virginia

Holmans Creek watershed was listed on the Commonwealth of Virginia's 1998 303(d) TMDL Priority List of Impaired Waters based on violations of the fecal coliform bacteria water quality standard. There are several potential fecal coliform sources in this watershed, including the non-point sources of wildlife, livestock, individual residential seweage systems, and land application of manure and litter. Beef cattle, poultry, and dairy are the major livestock operations in the Holmans Creek watershed. Residential sewerage in the watershed consists of direct discharges from straight pipes (homes without facilities to treat their waste discharge), privies, and failing septic systems. BST analysis using the ARA method was used to classify sources of the fecal bacteria found in the polluted water. Results of the BST analysis suggest that the primary source of fecal pollution is human, constituting just under half of the total fecal coliform deposited into the waters of Holmans Creek. Wildlife and cattle sources each contribute approximately one fourth of the total fecal coliform loads in the watershed. Poultry were determined to be a minor contributor to fecal coliform pollution in Holmans Creek, contributing one-tenth of the total fecal load.

Stevenson Creek, Florida

The Stevenson Creek basin encompasses approximately 2,428 ha in central Pinellas County, Florida. In keeping with the objectives of the Stevenson Creek Watershed Management Plan, a BST study was initiated to identify the dominant source(s) of fecal contamination to Stevenson Creek in Clearwater, Florida. The ARA method was chosen because it can assess the source of indicator organisms based on a much larger subset of the bacterial population than molecular methods can. The dominant sources of fecal coliform over the course of the study were wild animal, dog, and human, with the overall trend indicating that wild animal isolates comprised the majority of fecal coliforms obtained when colony forming units (CFU) counts exceeded the acceptable limit of 200 CFU per 100 mL. While human input was not the major

cause of elevated fecal coliform levels for most of the samples analyzed for this study, the domination of some small populations by human isolates suggests that human sources contribute to low-level background contamination. This occurs when fecal coliform populations are low, near the transition to dry season, and perhaps few isolates are washed into surface waters from draining storm water. Lowering water tables may also draw wastewater from small, otherwise innocuous leaks. Overall, there was little evidence of acute human fecal contamination on a large scale; however, human sources may influence two sampling sites, detectable despite the presence of fecal coliforms from other sources. The human input alone for these two sites in one month was high enough to violate water quality standards.

Spout Run, Virginia

Bacterial Source Tracking (BST) methodology was used to determine sources of fecal pollution in Millwood, VA. Millwood consists of 71 homes, all served by individual septic systems of indeterminate age and performance, and a stream (Spout Run) passes through the center of the community. Stream and well samples were collected monthly starting in 5/99 and ending in 5/00, and analyzed for fecal coliforms and enterococci. Twelve percent of the well samples and 92% of the stream samples were positive for fecal coliforms, and 26% of the stream samples exceeded the recreational water standard (1000 fecal coliforms/100ml). Antibiotic Resistance Analysis (ARA) was preformed on enterococci recovered from Spout Run in upper, middle, and lower Millwood. Isolates of human origin appeared in upper, middle, and lower Millwood and ranged from 2.0% to 42% of the sourced isolates over sampling sites and seasons. The percentage of human origin isolates was highest in upper and middle Millwood, and declined downstream from that point. These results were obtained by comparing the antibiotic resistance profiles of stream isolates against a library of 1,174 known source isolates with correct classification rates for the known sources of 94.6% for human isolates, 93.7% for livestock isolates, and 87.8% for wildlife isolates. The main impairment in water samples was fecal coliforms, as no other problems were identified based on water chemistry analyses for both well and stream water samples. The sporadic instances where well samples were positive for fecal coliforms appeared mainly during the very dry fall sampling period.

Pulsed-Field Gel Electrophoresis (PFGE) Method

Eastern Shore, Virginia

DNA fingerprinting using PFGE proved helpful when an oyster farmer on Virginia's Eastern Shore was faced with the closure of his shellfish beds due to elevated levels of *E. coli*. Failing septic tanks were assumed to be the primary source of the fecal pollution, but a survey of septic systems in the sparsely populated watershed indicated that they were not the cause, and it became necessary to identify other potential sources. The highest levels of coliform bacteria were measured in the small tidal inlets and rivulets of the wetlands located upstream of local houses, shifting suspected sources from human to other sources. Researchers collected fecal samples from raccoon, waterfowl, otter, muskrat, deer, and humans in the area and used DNA fingerprinting to confirm the suspicion that the source was not anthropogenic in nature. Comparing *E. coli* from the shellfish beds against the fingerprints of known strains in the DNA library, the researchers linked the in-stream *E. coli* to deer and raccoon (mostly raccoon). Several hundred animals, including 180 raccoon, were removed from areas adjacent to the wetlands. *E. coli* levels subsequently declined by 1 to 2 orders of magnitude throughout the watershed, allowing threatened areas of the tidal creeks to be reopened to shellfishing.

Four Mile Run, Virginia

Four Mile Run is listed on the Commonwealth of Virginia's 303(d) listing for elevated levels of fecal coliform bacteria. The Northern Virginia Regional Commission is currently developing a TMDL for the Four Mile Run watershed, with the final draft to be submitted to Virginia Department of Environmental Quality by March 1, 2002. Four Mile Run is an urban stream with no agricultural runoff. The watershed is home to 183,000 people, just over 9,000 per square mile. The dominant land use in the watershed is medium to high density residential housing. Seven central business districts exist within this 20 square mile watershed, and two high-capacity interstates pass through the watershed along with numerous primary and secondary roadways. The watershed is approximately 40 percent impervious. A large pet population accompanies the dense human population in the watershed. As to potential fecal sources, there is little manufacturing industry to generate point source discharges and there are no combined sewers in the majority of the watershed. Sanitary sewers serve more than 99.9 percent of the

watershed population. The number of septic systems in the watershed is believed to be less than 50. The PFGE method of BST analysis was conducted on *E. coli* DNA from seasonally varied stream and sediment samples in the watershed. Results of the analysis show that waterfowl contribute over one-third (38 percent) of the bacteria, humans and pets together account for over one-fourth (26 percent), and raccoons account for 15 percent of the contamination, with deer (9 percent) and rats (11 percent) also contributing. The predominant non-human sources include wildlife species with intimate association with the waterways.

Ribotyping (RT) method

Little Soos Creek, Washington

A BST survey was designed to help characterize sources of fecal coliform bacterial contamination in Little Soos Creek in southeast King County, Washington, in response to the impact of existing and anticipated urban development in the area. Little Soos Creek has historically been categorized as a Class A stream, but violates fecal coliform standards for this classification. The goal of the BST survey was to help determine the contribution to contamination of the stream from two potential sources: livestock on hobby farms and ranches adjacent to the stream and on-site septic systems close to the stream in highly permeable soils. Other animal sources were also considered. Genetic fingerprinting (using ribosomal RNA typing or RT) was performed on *E. coli* isolates to effectively match specific strains of *E. coli* from a contaminated site in the stream to its source. The intent was to provide information to support implementation of specific source controls. The study identified the sources of approximately three-fourths of the fecal coliform contamination, with the primary sources determined to be cattles, dogs, and horses. Although septage was identified as a contributor to the contamination problem, it was not indicated as a major source. However, even low levels of contribution from septage suggest the potential for Little Soos Creek to harbor a number of human viral, bacterial, and parasitic pathogens associated with human sources. For this reason, further investigation of the contribution from septic systems and of human exposure (particularly children) to the stream may be warranted.

Lower Boise River, Idaho

The Lower Boise River watershed from Lucky Peak Reservoir to the Snake River near Parma contains almost one-third of Idaho's population and four major municipalities, including the city of Boise. An arid climate (approximately 10 inches of annual rainfall) makes irrigation a requirement on most farmland. This irrigation coupled with reuse of pasture water on irrigated fields results in the contribution of non-point discharge of fecal coliform bacteria to the Lower Boise River. In 1994, the Idaho Department of Environmental Quality (IDEQ) placed the Lower Boise River on the 303(d) list for impairment of primary and secondary contact designated uses because fecal coliform levels exceeded state standards. A draft TMDL was completed and submitted to the USEPA on December 1998 and approved on January 2001 with implementation plan due July 2001. The TMDL indicates that bacteria discharge loads will require more than 95 percent reductions from non-point source bacteria loadings to meet the primary contact bacteria standard. A DNA fingerprinting of coliform bacteria was conducted to focus bacteria reduction improvement. *E. coli* cultures were grown from fecal samples of cattles, sheep, humans, ducks, and geese, and DNA from these samples was identified. The major bacteria sources in the watershed identified using the RT method were waterfowl, humans, pets, and cattle/horses. Waterfowl were clearly the largest source. The major advantage of using the DNA fingerprinting tool is the ability to develop accurate control measures (BMPs) in terms of bacterial sources.
Prior to this study, IDEQ knew there were bacteria problems, but did not know where to focus control measures. The results of the BST analysis identify the major sources, allowing IDEQ to strategically place BMPs.

University of Georgia/USDA RT comparison

BST methods, including RT, rely on a database of known source fingerprints to identify environmental isolates of fecal bacteria. It is not well understood to what degree these known source fingerprints are biogeographically variable. This is important because a fingerprint database developed for one state or region may or may not be applicable to another. The objective of a University of Georgia/USDA study (Hartel et al., 2002) was to use the RT method of BST analysis to determine the geographic variability of the fecal bacterium, *E. coli*, from one location in Idaho and three locations in Georgia for four animals: cattle, horse, swine, and poultry. The study identified distance from the source sample to the watershed as a key variable

for cattle and horses, but not for swine and poultry. When the *E. coli* ribotypes among the animals were compared at one location, the relative percent difference between them was 86, 89, 81, and 79 for each of the four locations, suggesting good ribotype separation among host animal species at one location. Achieving a high degree of accuracy in matching environmental isolates of fecal bacteria to a host origin database depends on having a large number of isolates for comparison and using a distance of 175 km or less (at least for certain host animal species).

H. Summary of Literature Review

Monitoring and detection of indicator and disease-causing microorganisms are a key element of microbiological studies of water quality. By chlorinating drinking water supplies, most of the principal disease-causing microbes have been eliminated. Additional problems caused by contaminated surface run-off at recreational bodies of water and areas where shellfish are harvested pose a pollution problem as well. At such sites water treatment barriers against contamination may not be present, and the risk of transmission of microbial diseases is higher (Prescott et al., 1996).

Watershed conditions are affected by sometimes indeterminate and always multifaceted environmental trends. The watershed approach to long-term research and monitoring of natural and remote areas within the national parks and related preserves offers significant data on ecosystem processes and relations for identifying both spatial and temporal changes in water environments.

Watershed ecosystem studies are designed for the compilation of long-term baseline data on the ecosystem health of park and equivalent reserves. Watershed level research and monitoring procedures have been a factor in the accumulation of important baseline information on deposition, hydrology, ecosystem performance, and biology. The data collections permit the separation of cause and effect relationships of ecological change within watersheds. They also function to meet both reference and early warning objectives in correlation to natural ecosystems transformation (Prescott, et al., 1996).

Differentiation of sources of fecal contamination of ground and surface waters is an important problem, especially for waters receiving mixed agricultural and human waste (Wiggins, 1999). In most contaminated water the presence of fecal indicator organisms can be demonstrated even though the source of the pollution is unknown (Hagedorn, 2000). The contamination of natural water with untreated fecal material may result in an increased risk of transmission of diseases to the humans who use those waters. Because risk to humans may be greater from human than from animal waste, the knowledge of the source of the pollution is an important factor in determining the degree of risk (Wiggins, 1999). It would thus be desirable to be able to determine the source of the fecal material, both to assess the risk to the people who are exposed to the waters and to assist in the location of the sources of pollution (Wiggins, 1999). BST will help with the development and implementation of realistic TMDLs and will be applied widely by regulatory agencies to investigate violation of fecal bacteria stands in the nation's waters (Hagedorn et al., 1999).

Although the incidence of waterborne diseases has been greatly reduced in areas with effective water treatment, control of waterborne transmission of infectious agents continues to be an important challenge for public health research. Methods to detect many of these agents in clinical and environmental samples must be developed or improved. There is a critical need for rapid methods to detect waterborne pathogens (Hurst et al., 2002). There is also a need to develop simple methods to detect fecal contamination in water that can be readily used in developing countries with limited laboratory facilities and resources. Also, information on the occurrence and persistence of enteric and aquatic pathogens in various types of water supplies should be updated. We need to advance our knowledge about the efficiency of various water treatment processes and disinfectants in removing and or inactivating these microorganisms. Furthermore, current events bear witness to the progress in microbiology geared to alleviate humankind's suffering by minimizing and controlling infectious diseases, has been subverted by biological weapons designed to bring about mass destruction. In the past 15 years, the threat of biological weaponry has taken on a new face: that of biological terrorism. In some respects biological terrorism is an even greater threat to biological warfare; it is more difficult to detect and control and can strike without warning (Krasner, 2002). Therefore, the indications of bacterial source tracking can go far beyond fecal sourcing in a watershed but to detecting agents

such as *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, *Bacillus anthracis* and others. Bioterrorism and Homeland security is of major concern. Finally, better regulation of waterborne diseases would lead to earlier recognition and examination of epidemic and endemic waterborne disease. Because the health risks posed by some emerging waterborne infectious agents are still unfamiliar, water utilities and public health professionals need to work together to maintain vigilance for any indication of waterborne transmission of disease in their community (Begue et al., 1998).

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III. MATERIALS AND METHODS

A. Project Objectives

This project had six objectives…

(1) Using antibiotic resistance analysis (ARA) to determine sources of fecal pollution in Mill Creek, Montgomery County, VA, as a function of sampling frequency under low and high stream flow conditions. Water samples from two locations in Mill creek were collected monthly for 12 months, weekly for 4 weeks and daily for 7 days within the 4 weeks, performing this sequence twice, once during periods of high flow (February-March) and once during periods of low flow (August-September).

(2) Building a library of at least 500 known source isolates of enterococci from the Mill Creek Watershed and to compare these known source isolates from the Mill Creek library to other enterococci libraries to determine how well the Mill Creek isolates fit these libraries.

(3) During this series of sampling, standard methods were used to perform bacterial monitoring, of enterococci and fecal coliform populations from all stream samples.

(4) Performing ARA on the enterococci from all stream samples was performed as well. Isolates were classified as human, wildlife, and livestock sources.

(5) Building an ARA library of at least 300 known isolates of *Escherichia coli (E. coli)*, from the Mill Creek watershed; compare the known source isolates from the Mill Creek library to our other *E. coli* library (developed under DCR project 319-1999-14-PT) to determine how well the Mill Creek isolates fit this library. ARA on *E. coli* was performed the same as enterococci (above).

(6) Determining the optimum number of isolates needed for source identification. Most investigators use 48 isolates for ARA source identification because it is convenient, as the microwell plates have 48 inoculating wells on each side of the tray. However, it is possible to save both time and money if fewer isolates can be used to obtain accurate source identification results.

B. Research Site Description

The Mill Creek Watershed is located in Montgomery County, Virginia. The watershed contains two communities (Childress and Riner) and a stream, Mill Creek, runs through both communities. Mill Creek is 9.14 km long and drains a 3,767 ha watershed. The Mill Creek watershed contains 2,277 ha improved pasture, 932 ha forest, and 388 ha cropland among several other land use/land cover entities (Table 1, Figure 2). There are 645 households in the Mill Creek Watershed. Of the 645 households, 567 or 88% are on septic systems, the remaining 78 homes or 12% are connected to a public sewer in Riner. The septic system failure rate is 1.5% and the fecal coliform concentration from failed septic systems is 10,000 colony forming units (CFU) per 100 ml (VDH, 2001). Beef operations (2,850 beef cattle) occur throughout the Mill Creek Watershed and there are five dairy (950 dairy cattle) operations as well

C. Experimental Setup

Stream water samples were collected and analyzed on a monthly, weekly, and daily schedule from the same two sample collection sites (Section C). Flow rates and water chemistry analyses were conducted as described in Section D. Fecal bacteria were detected, enumerated, and recovered from the water samples as described in Section E, and ARA was determined as described in Section F. Computations were carried out on a per-sample basis (48 isolates per sample), using analysis of variance (ANOVA) to conduct tests of significance. The data was then evaluated using discriminate analysis (Section G). ANOVA and contingency tables were also used to determine the optimal sampling site, sampling frequency and the number of unknown source isolates needed to best determine sources of fecal pollution (Graves et al., 2002).

D. Sample Collection

Sample collection began in January 2001 and ended in December 2001. Items prepared before leaving the laboratory included a cooler filled with ice, one set of latex gloves per sample to be collected, flame-sterilized spatulas (one for each known sample, in a whirl-pac bag), a

sterile Whirl-Pac bag for each sample, and boots, waders, or galoshes (if necessary). The Whirl-Pac bags were labeled prior to taking any samples so samples and labels did not become mixed up. For livestock, mixtures of at least 3-4 fresh "patties" were placed in Whirl-Pac bags using a flame-sterilized spatula. A fresh sample is defined as one that is very wet without a dry top layer or one that is witnessed to be fresh. For human samples, a 250ml sample was obtained from septic tank pump-out trucks, and stored in a sterile bottle. For wildlife samples, mixtures of several identified droppings were placed in Whirl-Pac bags using a flame-sterilized spatula. For stream water samples a sterile bottle was placed in the water with the opening upstream to prevent any contact of the sampler's hand and the water. This ensured that any bacteria that may be on the sampler's glove did not enter the bottle. For all sampling, latex gloves were used to avoid direct contact with the sample. The filled Whirl-Pac bags and bottles were placed on ice in a cooler and taken back to the laboratory for analysis. Samples were filtered within 6 hours of collection when possible, but no later than 24 hours after collection. Refrigeration of the samples maintained the viability of fecal bacteria and samples were routinely kept at 4°C until enough isolated colonies had been obtained to perform ARA. The samples were then discarded (Graves et al., 2002). There were two sampling sites in Mill Creek. Site MC-1 was located just above the confluence of Mill Creek with Meadow Creek and Site 2 was located upstream approximately half the length of the stream.

E. Water Monitoring

E.1 Flow Rates

Flow rates in Mill Creek were determined by a Global Water flow meter model FP201 (Global Water, Inc., Gold River, CA). "The Flow Probe is a highly accurate water velocity instrument for measuring flows in open channels and partially filled pipes. The Flow Probe consists of the protected Turbo-Prop positive displacement sensor coupled by the expandable probe handle to the digital readout display. The Flow Probe incorporates true velocity averaging for the most accurate flow measurements. The Flow Probe is ideal for storm water runoff studies, measuring flows in rivers and streams as well as ditches and sewers" (www.globalw.com/flow.html). Results from the flow meter were recorded as feet/sec and then converted to liters/min.

E.2. Turbidity

Turbidity measurements were made by the nephelometric method using a Hanna HI93703 Microprocessor Turbidity Meter (Hanna Instruments Inc., Woonsocket, RI), with a range from 0.00 to 1,000 formazin turbidity units (FTUs). The method is based on a comparison of the intensity of light scattered by the sample under defined conditions as compared to the intensity of light scattered by standard reference suspensions under the same conditions. The standards were used to reference and calibrate the turbidity meter. The higher the intensity of scattered light, the higher the turbidity. Results were reported as nephelometer turbidity units, or NTUs.

E.3. Temperature and Dissolved Oxygen

The temperature and dissolved oxygen (DO) measurements were evaluated using the YSI Model 95 Handheld DO and Temperature System (YSI incorporated, Yellow Springs, Ohio http://www.ysi.com). This system is a microprocessor based, digital meter with an attached YSI microelectrode array (MEA) DO probe. The MEA sensor eliminates the need for stirring in most environmental applications allowing measurement of DO in the field without an external stirring device. It also reduces measurement errors caused by insufficient or inconsistent stirring. The YSI system can be easily calibrated with the touch of a few buttons, thus a standard was not necessary.

F. Microbial Detection and Recovery

For membrane filtration, a known volume of a water sample was passed through a membrane filter that is then placed on M-Endo, M-FC or LES-Endo agar (Becton Dickinson, Cockeysville, MD) for 24 h incubation at 37˚C or 44.5˚C, depending on the medium used (APHA, 1998). All organisms that produce red colonies with a metallic sheen within 24 h of incubation are considered members of the coliform group on M-Endo agar at 37˚C, or dark blue colonies on m-FC agar at 44.5˚C for detection and enumeration of fecal coliforms. A benefit of the membrane filter method over the Most Probable Number (MPN) technique is that results are available within 24 h instead of the 3 to 4 days required for confirming MPN results, although

confirmation of colonies observed after membrane filtration is advisable. The membrane filter technique is as effective as the MPN test for detecting bacteria of the coliform group (APHA, 1998).

F.1. Presence-Absence Test

The Colilert system (IDEXX Laboratories Inc., Westbrook, ME) was developed to enumerate total coliforms and *E. coli* from drinking and source waters without the need for confirmatory tests. No equipment other than an incubator and ultraviolet light (366 nm) is necessary. All that is required is to add water from the sampling sites to the powdered formula in a test tube or vessel. A colorless solution results. After incubation at 35°C for 24 hours, yellow color indicates the presence of total coliforms, and fluorescence under ultraviolet light indicates the presence of *E. coli* (Prescott et al., 1996). Colilert uses the patented Defined Substrate Technology (DST) to simultaneously detect total coliforms and *E.coli* . Colilert is a specially designed reagent formulation of salts, nitrogen, and carbon sources that are specific to total coliforms. It provides specific indicator nutrients: ONPG (O-Nitrophenyl-ß-dgalactopyranoside) and MUG (4-Methylumbelliferyl-ß-glucuronide) for the target microbes, total coliforms and *Escherichiacoli*. As these nutrients are metabolized, yellow color (from ONPG) and fluorescence (from MUG) are released confirming the presence of total coliforms and *E. coli*, respectively. These nutrients can be metabolized by the coliform enzyme β- galactosidase and the *E. coli* enzyme β-glucuronidase respectively. As coliforms grow in Colilert, they use β- galactosidase to metabolize ONPG and change it form colorless to yellow. *E.coli* use β-glucuronidase to metabolize MUG and create fluorescence. Since most non-coliforms do not have these enzymes, they are unable to grow and interfere (http://www.idexx.com). Non-coliform bacteria are suppressed and cannot metabolize the indicator nutrients. Consequently, they do not interfere with the specific identification of the target microbes during the test incubation period.

Colilert is a primary water test (i.e., a test directly from a water sample) that requires no confirmation. (Prescott et al., 1996). The Colilert system was used to determine if samples were positive or negative for coliforms. Only positive samples were membrane filtered.

F.2. Membrane Filtration for Fecal Coliforms

Water samples (100 ml) were filtered through a 0.45 um pore-size membrane filter. The filters were then transferred to a 50-mm petri dish containing 5 ml of m-FC agar. The filters were incubated for 24 h at 44.5°C. Dark blue colonies were counted and the fecal coliform density was reported as the membrane filter count per 100 ml (APHA, 1998).

F.3. Membrane Filtration Method for Enterococci

Water samples (100 ml) were filtered through a 0.45 um pore-size membrane filter (APHA. 1998). The filters were then transferred to a 50-mm petri dish containing approximately 5 ml of m-Enterococcus (m-ENT) agar (Becton Dickinson, Cockeysville, MD). The filters were incubated for 24-48 h at 37°C. After 48 h of incubation, red to burgundy colored colonies were counted and the enterococci density was reported as the membrane filter count per 100 ml.

F.4. Filtration for Solids

Fecal bacteria from manure, septage, and scat samples were isolated after the samples were collected, and then mixed in a Whirl-Pac bag by massaging the bag. After sample collection, 0.1 - 0.2 g of sample was added to 50 ml of sterile saline buffer (SB) in a 125 ml screw-cap bottle and then shaken for 30 sec. Heavy solids were allowed to settle before pipetting. For all materials, 0.1, 1.0, and 3.0 ml of buffered sample were normally filtered, although sometimes other volumes were necessary. Since fecal and septage samples can be difficult to filter, prefiltration using a very fast flowing filter paper (e.g., grade 363) was useful (Graves et al., 2002). Petri dishes with 5 ml of m-ENT agar were labeled with the sample number and volume filtered in triplicate. The autoclaved filter towers were placed in the filtration manifold, and sterile membrane filters were added by using sterile filter forceps (tips are dipped in 95% ethanol and flamed). Five milliliters of 95% ethanol was added to each tower to check for leaks in the filters. An aliquot of each volume of sample was pipetted into each of the three towers (for a total of 3 filters/volume tested). After the vacuum pump was turned on and the sample was filtered, 5-10 ml of saline buffer were then added to wash the filter and the

sides of the tower. Sterilized forceps were used to transfer the filter to the 50-mm petri dishes containing the agar. After all three volumes are filtered (9 plates total), the petri dishes were placed in plastic bags, labeled with the sample number and date, and incubated at 37°C for 48 hours (Graves et al., 2002).

G. Antibiotic Resistance Analysis of Enterococci

Samples were obtained from several types of known sources: human, cattle, deer, horse, waterfowl, and small mammals for the development of a known source database. After enterococci had been enumerated and colonies were available on membrane filter plates, sterile toothpicks were used to transfer 48 individual colonies per sample to 96-well microtiter plates. No set number of colonies was taken from any one plate, but rather colonies were selected randomly and equally (where possible) from the plates that constituted one sample. No more than 48 colonies were taken from any one composite known sample. If 48 colonies were not available, then less than 48 were used. The 96-well plates (pre-sterilized) were first filled with Enterococcosel broth (Becton Dickinson, Cockeysville, MD) using an 8-channel multi-well pipetter to add 0.2 ml of broth to each of the wells. Care was taken to ensure that each colony picked was separate and distinct. Each isolate was scraped from the filter with the toothpick and thoroughly inoculated into one well of the 96-well plate. The 96-well plate was placed in a plastic container to prevent the microwells from drying up, and then incubated at 37˚C for 24 to 48 h. Isolates were confirmed as enterococci when the enterococcosel broth turned black. The black color was a result of esculin hydrolysis by the bacteria. Only the wells what turned black were counted and used in ARA analysis.

The antibiotic-containing plates were prepared by adding filter-sterilized stock solutions (10 mg/ml) in sterile water (ampicillin, neomycin, oxytetracycline, streptomycin), 1:1 water: ethanol (chlorotetracycline, erythromycin, tetracycline, vancomycin), and 1:1 water: methanol (amoxicillin,), to autoclaved and cooled Trypticase Soy Agar (TSA; (Becton Dickinson, Cockeysville, MD) at initial concentrations of 2.5, 5, 10, 20, 40, 60, 80, and 100 μg/ml. The isolates were transferred with a stainless steel 48-prong replica-plater (Sigma Chemical Company, St. Louis, MO) from the Enterococcosel-containing microwells to a set of 30

Tryptocase Soy Agar (TSA) plates containing the various concentrations of each antibiotic to be tested, and to a control plate containing no antibiotic (Table 4). The plates were incubated at 37^{o}C for 48 h and growth of each isolate on each concentration of every antibiotic was determined. An isolate was considered to be resistant to a given concentration of antibiotic if growth comparable to the controls occurred on that plate. When recording this information the isolate is recorded as "1". Any isolate that did not grow on the control plates (containing no antibiotic) was not used in the analysis and recorded as "0". A data set for one sample was 48 isolates x 30 antibiotics/concentrations (Hagedorn et al., 1999).

G.1. Data Analysis

Data on the ability of each of the known isolates to grow in the presence of each concentration of the antibiotics were analyzed with SAS-JMP statistical software (version 5.0, SAS Institute Inc.), by the procedure DISCRIM (prior probabilities, equal; covariance matrix, pooled). Several variants of discriminant analysis were performed by varying the combination of antibiotics and the level of pooling of source types. The classification table produced by the DISCRIM procedure was used to calculate the percentages of misclassified isolates and determine the average rate of correct classification (ARCC). The table is a source-by-source matrix in which the numbers and percentages of correctly classified isolates are found on the diagonal. The ARCC for a given combination of antibiotics was computed by averaging the percentages along the diagonal. The percentage of misclassified isolates for a given source (false negatives) was determined by adding the percentages of misclassified isolates in the appropriate row of the table (excluding the value in the diagonal). The percentage of isolates from other sources that were misclassified as a given source (false positives) was determined by taking the average of the percentages in the appropriate column (excluding the value in the diagonal). Results are recorded in laboratory notebooks and in JMP computer files prior to evaluation by discriminate analysis (Hagedorn et al., 1999).

G.2. Antibiotic Resistance Analysis of *E. coli*

Samples were obtained from several types of known sources: human, cattle, deer, and horse for the development of a known database. After fecal coliforms had been enumerated and colonies were available on membrane filter plates, sterile toothpicks were used to transfer 48 individual colonies per sample to 96-well microtiter plates. No set number of colonies was taken from any one plate, but rather colonies were selected randomly and equally (where possible) from the plates that constituted one sample. No more than 48 colonies were taken from any one composite known sample. If 48 colonies were not available, then less than 48 were used. The 96-well plates (pre-sterilized) were first filled with colilert using an 8-channel multi-well pipetter to add 0.2 ml of broth to each of the wells. Care was taken to ensure that each colony picked was separate and distinct. Each isolate was scraped from the filter with the toothpick and thoroughly inoculated into one well of the 96-well plate. The 96-well plate was placed in a plastic container to prevent the microwells from drying up, and then incubated at 37˚C for 24h. After the 24h incubation the microwells were viewed under ultraviolet light to confirm the presence of *E .coli.* If the wells did not fluoresce then *E.coli* was not present the well number was documented as not to interfere with ARA.

The antibiotic-containing plates were prepared by adding filter-sterilized stock solutions (10 mg/ml) in sterile water (cephalothin, neomycin, oxytetracycline, streptomycin), 1:1 water: ethanol (erythromycin, tetracycline, vancomycin), and in methanol (rifampicin, tetracycline), to autoclaved and cooled Trypticase Soy Agar (TSA; Becton Dickinson, Cockeysville, MD) at initial concentrations of 0.65, 5, 7.5, 10, 15, 25, 35, 60, 70, 90 and 100 μg/ml. The isolates were transferred with a stainless steel 48-prong replica-plater (Sigma Chemical Company, St. Louis, MO) from the Colilert broth-containing microwells to a set of 30 Tryptocase Soy Agar (TSA) plates containing the various concentrations of each antibiotic to be tested, and to a control plate containing no antibiotic (Table 5). The plates were incubated at 37^{o}C for 24 h and growth of each isolate on each concentration of every antibiotic was determined. An isolate was considered to be resistant to a given concentration of antibiotic if growth comparable to the controls occurred on that plate and was recorded as “1” in notebooks and JMP computer files. Any isolate that did not grow on the control plates (containing no antibiotic) was not used in the

analysis and was recorded as “0” in notebooks and JMP computer files. A data set for one sample was 48 isolates x 30 antibiotics/concentrations (Hagedorn et al., 1999).

Data on the ability of each of the known isolates to grow in the presence of each concentration of the antibiotics were analyzed with SAS-JMP statistical software (version 5.0, SAS Institute Inc.), by the procedure DISCRIM (prior probabilities, equal; covariance matrix, pooled). Several variants of discriminant analysis were performed by varying the combination of antibiotics and the level of pooling of source types. The classification table produced by the DISCRIM procedure was used to calculate the percentages of misclassified isolates and determine the average rate of correct classification (ARCC). The table is a source-by-source matrix in which the numbers and percentages of correctly classified isolates are found on the diagonal. The ARCC for a given combination of antibiotics was computed by averaging the percentages along the diagonal. The percentage of misclassified isolates for a given source (false negatives) was determined by adding the percentages of misclassified isolates in the appropriate row of the table (excluding the value in the diagonal). The percentage of isolates from other sources that were misclassified as a given source (false positives) was determined by taking the average of the percentages in the appropriate column (excluding the value in the diagonal). Results are recorded in laboratory notebooks and in JMP computer files prior to evaluation by discriminate analysis (Hagedorn et al., 1999).

Table 4. Antibiotics and their concentrations used in Antibiotic Resistance Analysis of Enterococci.

Antibiotic/Abbreviation	Concentrations (μg/ml)
Amoxicillin, AMX	0.65
Cephalothin, CEP	10, 15, 30, 50
Chloratetracycline, CTC	60, 80, 100
Erythromycin, ETC	10, 15, 30, 50
Oxytetracycline, OTC	20, 40, 60, 80, 100
Streptomycin, STR	40, 60, 80, 100
Tetracycline, TET	10, 15, 30, 50, 100
Neomycin, NEO	40, 60, 80
Vancomycin, VAN	0.65

Table 5. Antibiotics and their concentrations used in the Antibiotic Resistance Analysis of *E. coli*

Antibiotic/Abbreviation	Concentrations (μg/ml)
Erythromycin, ERY	60, 70, 90, 100
Neomycin, NEO	2.5, 5.0, 10
Oxytetracycline, OTC	2.5, 5.0, 7.5, 10, 15
Streptomycin, STR	2.5, 5.0, 7.5, 10, 15
Tetracycline, TET	2.5, 5.0, 7.5, 10, 15
Cephalothin, CEP	15, 25, 35
Rifampicin, RIF	60, 75, 90

H. Statistical Methods

Analysis of variance (ANOVA) performs comparisons like the t-Test, but for an arbitrary number of factors (factor or a treatment is a property, or characteristic, that allows us to distinguish the different populations from one another). For example, in this study factors included monthly, weekly, and daily evaluations. Each factor can also have an arbitrary number of levels. In this study the levels were the human, livestock, and wildlife fecal sources. Furthermore, each factor combination can have any number of replicates. ANOVA works on a single dependent variable while, the factors must be discrete. The ANOVA can be thought of in a practical sense as an extension of the t-Test to an arbitrary number of factors and levels. It can also be thought of as a linear regression model whose independent variables are restricted to a discrete set (Sall and Lehman, 1996).

When performing discriminate analysis (SAS-JMP statistical software, version 5.0 SAS Institute, Inc.), the software program first performs ANOVA on the data set and tests for significance by subjecting the data to chi-square, the Likelihood Ratio test, and the Pearson test, and generates a kappa value to measure the degree of agreement within the data set. If the data set fails the chi-square test, discriminate analysis is not performed.

H.1. Discriminant Analysis (DA)

Variables for the analyses include the number of drugs used and the degree of pooling of sources. Each analysis produces a classification set for every known source isolate. The correct classification rates were calculated by using one set of antibiotic resistance patterns (ARPs) both to establish the classification rule and as test subjects (Harwood, 2000). The number of isolates from a given source that are placed in the correct source category by discriminant analysis is termed the rate of correct classification. The average rate of correct classification (ARCC) for the database is obtained by averaging the correct classification percentages for all sources (Harwood, 2000). The holdout method of cross validation, in which isolates from known sources are randomly removed form the data set and treated as test subjects, was used as a more

rigorous test of the predictive power of the databases (Harwood, 2000). To determine whether the known databases were large enough or had ample representation, artificial clustering was used. Artificial clustering involves randomly assigning equal numbers of each source and applying discriminant analysis to determine the random ARCC. Our database contains 3 source types, human, livestock and wildlife. The random ARCC should be approximately 33.3% for each source. Thus, any percent significantly greater than the 33.3% ARCC indicates that the known source database is not representative.

H.2. Comparison of Means

H.2.a. The t –Test

The t test was used to compare the means of two groups MC1 versus MC2. However, to test the means of more than two groups (sampling frequencies and number of isolate comparisons), the t-test is not adequate. One-way ANOVA must be used to compare more than two groups and the key to ANOVA is the F- test.

The t- test is used in situations where comparisons are made between just two populations. The t-test is used to determine the level of confidence that two populations have significantly different means. In the case of the t-test, the null hypothesis tested is that two populations have the same mean (Sall et al., 2001).

H.2.b. One-Way Analysis of Variance/F-test

ANOVA is like an extension of the t-test. ANOVA tests whether all the populations have the same mean. ANOVA can also be used to just compare two means. The idea behind ANOVA is that if several samples are taken from identical populations, the variance for these populations can be estimated in two different ways: (i) from the spread of values within the samples, and (ii) from how spread out the sample means are (Townend, 2001). The term one-way is used because the sample data are separated into groups according to one characteristic or

factor. When there are three or more samples to test it is not adequate to test just two samples at a time as with the t-test. This is because there would be several different hypothesis tests, so the degree of confidence could be lower. Also, as the number of individual tests of significance increase, the likelihood of find a difference by chance alone instead of a real difference in the means increases. The risk of a type I error, finding a difference in one of the pairs when no such difference actually exists, is far too high. ANOVA avoids that particular pitfall (rejecting a true null hypothesis) by using one test for equality of several means (Triola, 2001).

I. The Minimal Detectable Percentage

The minimal detectable percentage (MDP) is important to calculate and account for possible misclassification among fecal sources. For example, when using a three way split of human, livestock, and wildlife, the following misclassification could occur...

10% of wildlife misclassified as human

5% of livestock misclassified as human

In this scenario the MDP for human would be the average of the two. Thus, for a human signature to be considered as a real threat, the MDP should be greater than 7.5% (Harwood et al., 2001).

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IV. RESULTS AND DISCUSSION

A. Water Quality Monitoring

Monitoring the water resource in a watershed is necessary to identify and record pollution. Monitoring is also essential to constantly evaluate water quality and the health of the water resource. The most dependable way to ascertain if changes in land-based activities have affected water quality is to monitor the land and the water resource before, during, and after a change in land management or restoration (Graves, 2000).

One of the most important aspects of water microbiology, from a human perspective, is the fact that numerous diseases are acquired from microorganisms found in water (Hurst and Murphy, 1996). The reservoirs for pathogenic microorganisms found in environmental waters can be humans, animals, or the environment itself (Frayer et al., 1998).

It is commonly presumed that many microorganisms that infect humans and are found in aquatic resources that originate from human sources. This human-related contamination can occur during either defecation in water or recreational activities conducted in water (Hurst and Murphy, 1996). In addition, domestic wastewater seems to be of particular importance as a contributor of the pathogenic contaminants found in aquatic environments (Dowd et al., 1996). The attendant public health concerns have resulted in the development of methods for studying and reducing levels of pathogens in wastewater (Acher et al., 1994).

Waterborne transmission is a highly effective means of spreading infectious agents to a large portion of the population. Large quantities of enteric organisms can be introduced into the aquatic environment by contamination of the water by feces of infected persons (or animals) that is discharged into sewers or unprotected waterways (Ewald, 1994).

The presence of certain microorganisms in water is used as an indication of possible contamination and an index of water quality, and the presence of enteric pathogens in drinking

and recreational waters is of great concern. As a result of the danger to public health due to the presence of these pathogens it is important to determine the microbiological safety of these waters. The ideal manner for doing this would be to analyze the waters for the presence of specific pathogens of concern. However, hundreds of different microorganisms (especially viruses) have been shown to be involved in waterborne-disease outbreaks; thus, it is impractical to look for every pathogen potentially present in water samples. Culture methods are usually used for bacteria and cell culture techniques for the detection of viruses, while microscopic methods are used for protozoa. However, target bacteria may not grow in culture media since they are frequently injured by exposure to environmental stressors such as disinfectants used during water treatment. Additionally, several enteric viruses cannot be cultured in the laboratory, and methods for the detection of protozoan pathogens are notoriously inefficient (Graves, 2000).

A.1. Monitoring Results for Fecal Coliforms

Fecal coliforms are the subset of the more comprehensive coliform or total coliform group that is more definitive as an indicator of fecal contamination (Geldreich, 1967). Bacteria in this coliform subgroup have been found to have an excellent positive correlation with fecal contamination from warm-blooded animals (Hurst and Murphy, 1996). The EPA designates three levels of fecal coliform contamination for recreational waters. These levels are termed as low, moderate or high based on the values of the fecal coliform (FC) counts. Low is designated for FC counts less than 100, moderate is characteristic of FC values of 100-1000 and high levels of contamination are defined as FC values greater than 1000 (the regulatory standard for recreational waters based on monthly grab samples)(Graves, 2000).

A.1.a. Monthly Monitoring Observations

Monthly observations of fecal coliforms included the months of January 2001-December 2001 (Table 6). For site MC-1, four samples were low (<100 FC per 100ml, January, February, March, and November), four samples were moderate (100-1000 FC per 100ml, June, August, September, and October) and four were high (>1000 FC per 100ml, April, May, July,

and December). For Site MC-2, three samples were low (<100 FC per 100ml, January, February, and March), five were moderate (100-1000 FC per 100 ml, April, June, September, October, and November), and four were high (> 1000 FC per 100ml, May, July, August, and December) (Table 6). Based on fecal coliform counts, the recreational standard (1,000 colony forming units (CFUs)/100ml) was exceeded, for monthly sampling (12 samples per site), a total of eight times, 4 each for sites MC-1 and MC-2, for a 25% standard violation for each site. The annual average fecal coliform counts (monthly sampling, Table 6) were 658 CFUs/100ml for MC-1 and 872 CFUs for MC-2.

A.1.b. High Flow Monitoring Observations

High flow observations of fecal coliforms included the dates of February 27, 2001 thru March 20, 2001 (Table 7). For site MC-1 five samples were low (<100 FC per 100ml, 2/27/01, 3/5/01, 3/6/01, 3/7/01, and 3/20/01), four samples were moderate (100-1000 FC per 100ml, 3/2/01, 3/3/01, 3/4/01, and 3/13/01), and one sample was high (>1000 FC per 100ml, 3/8/01). For site MC-2, three samples were low (<100 FC per 100 ml; 2/27/01, 3/8/01, 3/20/01), seven samples were moderate (100-1000 FC per 100 ml; 3/2/01, 3/3/01, 3/4/01, 3/5/01, 3/6/01, 3/7/01, and 3/13/01) and none were high (>1000 FC per 100ml) (Table 7). The 1,000 standard was exceeded only once during the 4-week and 7-day daily sampling period during high flow (Table 7). The 4-week fecal coliform average (weekly sampling) for high flow was 107.5 colony forming units (CFUs)/100ml for MC-1 and 125 CFUs for MC-2 (Table 7). The 7-day fecal coliform average (daily sampling) for high flow was 662.9 CFUs/100ml for MC-1 and 214 CFUs for MC-2. The high daily average for MC-1 was due to a very high reading on 3/8/01 (3,480 CFUs). If this value is removed the 6-day average for MC-1 then becomes 193 CFUs. The monthly monitoring for March 2001 was taken on 3/20/01, and was 70 CFUs for both MC-1 and MC-2 (Table 6). This value (70) is numerically lower than the 4-week and 7-day averages for both sites under high flow conditions (especially if one includes the high 3/8/01 reading in the 7-day average for MC-1).

A.1.c. Low Flow Monitoring Observations

Low flow observations of fecal coliforms included the dates of September 14, 2001 thru October 6, 2001 (Table 8). For Site MC-1, no samples were low (<100 FC per 100ml), six samples were moderate (100-1000 FC per 100ml; 9/21/01, 9/24/01, 9/25/01, 9/30/01 and 10/6/01) and four were high (>1000 FC per 100ml; 9/24/01, 9/26/01/, 9/27/01, 9/28/01 and 9/29/01) (Table 8). The 1,000 standard was exceeded nine times (4 for MC-1 and 5 for MC-2) during the 4-week and 7-day sampling period during low flow (Table 8). The 4-week fecal coliform average (weekly sampling) for low flow was 1630.0 colony forming units (CFUs)/100ml for MC-1 and 890 CFUs for MC-2 (Table 8). The 7-day fecal coliform average (daily sampling) for low flow was 1398.6 CFUs/100ml for MC-1 and 2235.7 CFUs for MC-2. The monthly monitoring for September 2001 was taken on 9/21/01, and was 460 CFUs for MC-1 and 130 CFUs for MC-2 (Table 6). These values (460 and 130) are numerically lower, in some cases by an order of magnitude, than the 4-week and 7-day averages for both sites under low flow conditions.

A.1.d. Fecal Coliform Results in relation to the TMDL Program

In an effort to improve water quality beyond point sources the TMDL process was implemented (Literature Review, Section D. 1.). Based on the fecal coliform counts from the Mill Creek stream, this stream is a worthy candidate for TMDL Implementation. Pastureland serves as the major land use (Table 1) and is an indication of the numerous recreational standard (1000 CFU/100ml) exceedances, eight times for the monthly sampling (Table 6) and nine times for low flow sampling. Low flow averages exceeded the state standard and cattle were suspect even before BST was performed.

A.2. Monitoring Results for Enterococci

Enterococci have received widespread acceptance as useful indicators of microbiological water quality because (i) they show a high and close relationship with health hazards, mainly gastrointestinal symptoms, associated with bathing in aquatic environments; (ii) they are not as

ubiquitous as coliforms; (iii) they are always present in feces of warm-blooded animals; and (iv) their die-off is less rapid that that of coliform in water and persistence patterns are similar to those of potential waterborne pathogenic bacteria (Cabelli et al., 1982). These microorganisms are more closely related to the presence of human feces than are the fecal streptococci (Figueras, 1996).

Currently, the United States does not have a standard for enterococci in freshwater because of possible regrowth, but there is a standard of 33 for marine waters; however Canada has 35 enterococci per 100ml recreational standard (Hurst et al., 2002). Based on this Canadian standard for enterococci, both MC-1 and MC-2 exceeded 35 enterococci per 100 ml every month. During high flow (February 27, 2001-March 20, 2001) the Canadian standard was exceeded at site MC-1 each sampling date with the exception of 2/27/01. For site MC-2 the Canadian standard was exceeded each sampling date except for 9/14/01 (Table 6, 7, and 8).

A.3. Stream Flow Results

Stream flow was measured monthly January 2001 thru December 2001 (Table 6). Flow measurements for MC-1 ranged from 454 L/min to 20,141 L/min. For site MC-2, flow measurements ranged from 68 L/min to 2,877 L/min (Table 6). Stream flow measured during high flow (February 27, 2001 thru March 20, 2001) ranged from 8,744 L/min to 16,409 L/min at MC-1. For site MC-2, flows measured at 1,064 L/min to 2,093 L/min (Table 7).

Stream flow measurements during low flow (September 14, 2001 thru October 6, 2001) for site MC-1 measured at 371L/min to 3,581 L/min. For site MC-2, measurements ranged from 114 L/min to 723 L/min (Table 8). Average stream volume was 22 times greater at high flow for MC-1 than at low flow, and 9 times higher for MC-2 at high flow than at low flow (Tables 7 and 8). The low flows must be regarded as unusual due to the severe drought that occurred between August and December 2001. Heavy rains in early December resulted in a substantial runoff event, reflected in both increased flow rates and higher fecal coliform counts for the final monthly sample in December (Table 6). Site MC-1 averaged 3.4 meters wide, a

high flow depth of 11.2 cm. and a low flow depth of 10.2cm, while site MC-2 averaged 1.1 meters wide, a high flow depth of 8.6 cm. and a low flow depth of 5.3 cm.

A.4. Temperature, Turbidity, and Dissolved Oxygen

Temperature, turbidity, and DO measurements were all taken according to Materials and Methods Section E. Optimum readings for temperature for warm water should not exceed 89°F, and for cold water should not exceed 68°F, as high temperatures contribute to eutrophication. Turbidity measurements of 60 FTU is indicative of waters that are relatively clear. Turbidity measurements higher than 60 FTU are indicative of a significant amount of sediment. DO measurements below 5.0 mg/L leads to aquatic stress and is an indication of eutrophication. All of the samples taken during this project had temperatures, dissolved oxygen and turbidity measurements of normal status and were not problematic (Tables 6, 7, and 8).

Table 6. Monthly Monitoring Data, Mill Creek Watershed.

Date	Site* Number	Dissolved Oxygen mg/l	Temp. ˚C	Turbidity (FTU)	FC/100ml**	Ent/ ** 100ml	Flow L/min
1/10/2001	1	12.8	1.1	20.2	65	120	6745
	2	11.5	3.2	3.22	50	42	666
2/15/2001	1	10.2	13.7	89	70	50	10648
	2	9.61	13.2	11.3	90	122	1927
3/20/2001	1	11.9	6	25.65	70	96	8744
	2	11.1	6.8	4.62	70	36	1514
4/28/2001	1	8.55	16.1	24.9	2100	262	11610
	2	9.01	14.9	9.66	290	348	2657
5/8/2001	1	9.77	17.9	62.25	17400	220	11212
	2	8.87	16.9	10.6	1490	808	1351
6/11/2001	1	10.2	18.7	90	490	206	20142
	2	11.9	17.8	25.6	740	516	2877
7/19/2001	1	14.5	24.8	22.9	1040	1216	1450
	2	16.8	20.8	78	2850	576	1064
8/14/2001	1	12.4	20.2	18.3	360	852	2271
	2	14.3	21.2	6.4	1040	1216	1476
9/21/2001	1	15.6	19.8	6.74	460	400	7574
	2	17.9	16.7	9.5	130	400	723
10/6/2001	1	18.9	16.1	35	380	116	575
	2	21.6	17.2	56	240	176	106
11/6/2001	1	17.6	13.8	2.1	60	126	454
	2	15.3	12.5	6.5	690	214	68
12/11/2001	1	12.3	7.1	59	1056	1264	13108
	2	10.2	8.7	17.9	2784	3504	1018
Yearly Avg	1	11.6	14.6	38	578	411	7877
	2	13.2	14.2	19.9	872	663	1283

* MC1-Sampling site at Rt. 8 bridge above Riner;MC2 sampling site at Rt. 669 bridge where Mill Creek and Meadow Creek joins.

** FC= fecal coliforms, Ent=Enterococci.

Table 7. High Flow Monitoring Data, Mill Creek Watershed.

Date	Site* Number	Dissolved Oxygen mg/l	Temp. ˚C	Turbidity (FTU)	FC/100ml**	Ent/ ** 100ml	Flow L/min
2/27/2001	1	11.9	10.1	20.2	60	22	10693
	2	10.4	10.8	3.22	30	70	1064
3/2/2001	1	10.2	7	89	710	54	16031
	2	10.2	7.8	11.3	420	127	1147
3/3/2001	1	11.1	8.3	25.65	200	130	16409
	2	10.6	7.1	4.62	330	158	1332
3/4/2001	1	11.3	8.3	24.9	100	50	13551
	2	10.2	8.2	9.66	240	190	1983
3/5/2001	1	11.8	7.1	62.25	80	174	15944
	2	10.9	6.8	10.6	120	128	1923
3/6/2001	1	13.4	1.6	90	60	186	13847
	2	12.4	2.3	25.6	150	86	2093
3/7/2001	1	11.9	1.8	22.9	10	60	13729
	2	11	3.7	78	190	100	1722
3/8/2001	1	12.7	5.7	18.3	3480	50	10928
	2	10	7.6	6.4	50	80	1722
3/13/2001	1	11.3	12.2	6.74	220	70	8744
	2	9.9	13	9.5	280	154	1628
3/20/2001	1	11.9	6.5	35	70	96	8744
	2	11.1	6.8	56	70	36	1650
7 day-Avg	1	11.8	5.7	38	663	101	14346
	2	10.8	6.2	19.9	214	124	1703

* MC1-Sampling site at Rt. 8 bridge above Riner;MC2 sampling site at Rt. 669 bridge where Mill Creek and Meadow joins.

** FC= fecal coliforms, Ent=Enterococci.

Table. 8. Low Flow Monitoring Data, Mill Creek Watershed.

Date	Site* Number	Dissolved Oxygen mg/l	Temperature C	Turbidity (FTU)	FC/100ml*	Ent/100ml*	Flow (L/min)
9/14/01	1	25.6	18.7	32.67	1,200	500	3581
	2	32.1	16.9	4.75	30	558	329
9/21/01	1	27.8	19.8	6.74	460	400	1518
	2	32.6	16.7	9.5	130	400	723
9/24/01	1	31	16.8	25.82	250	1,904	371
	2	34.7	18.9	92	3,640	2,080	163
9/25/01	1	28.8	18.6	12.45	430	568	727
	2	33.1	16.3	8.74	540	572	197
9/26/01	1	28.2	17.9	15.76	250	1,904	556
	2	33.3	16.8	39.84	3,640	2,080	185
9/27/01	1	28.5	19.4	17.6	3,000	6,000	1045
	2	29.6	16.5	26.8	3,000	2,160	360
9/28/01	1	27.4	18.6	12.36	4,480	1,072	575
	2	30.4	16.2	24.8	3,160	488	140
9/29/01	1	29.3	19.1	1.86	1,240	504	575
	2	32.2	17.4	22.62	1,030	828	140
9/30/01	1	17.6	15.4	26.7	140	162	651
	2	20.5	17.4	29.8	640	424	114
10/6/01	1	18.9	16.1	35	380	116	575
	2	21.6	17.2	56	240	176	106
7 Day-Avg.	1	27.3	18.0	16.1	1,399	1,731	644
	2	30.5	17.1	34.9	2,235	1,233	185

*MC1-Sampling site at Rt. 8 bridge above Riner; MC2 sampling site at Rt. 669 bridge where Mill Creek and Meadow Creek Joins.

** FC= Fecal Coliforms, Ent=Enterococci.

B. Bacterial Source Tracking-*E. coli*

Antibiotic resistance analysis of *E.coli* was used to determine the sources of fecal contamination in Mill Creek. The sources fell into one of three categories: human, livestock, or wildlife.

B.1. Summary of Project Results Based on Source Tracking with *E. coli*.

B. 1.a. *E. coli* Known Source Libraries

The ARCC for all four libraries (large and small, both 3-way human, livestock, wildlife split, and specific source split) were excellent and ranged from 87.0% to 98.4% (Table 9). The small library (384 isolates) was from known sources collected entirely within the Mill Creek watershed and the large library (1158 isolates) consisted of the 384 isolates from Mill Creek plus 774 isolates from the Blacksburg area, Holston River, and Blackwater River watersheds. The high ARCC of the large library demonstrates that isolates from different regions can be put together and it is not necessary to build a new large library with every new watershed.

By assigning the sources randomly to the isolates, artificial clustering appears in the small libraries (Table 10). The ARCC for the 3-way small library is 48.2% where it should be 33.3% based on random distribution. The large libraries had ARCCs within 1% of the random classifications. Examining the ARCC values based on random assignments of sources cannot be used to determine the representativeness of a library, but can be used as a measure of library size. When the ARCC of random assignments equals that of the calculated random ARCC, the library size is sufficient. The artificial clustering of sources in the small libraries indicates that these libraries are not adequate for classifying stream (unknown source) isolates.

When viewing the monthly classifications of human, livestock and wildlife (Table 11), it quickly becomes apparent that there are months where the small library provided results that are both open to question and differed from the results provided by the large library (e.g. see 1/10/01 in Table 11, 100% human for MC-1, small library, and 0.0% human, MC-1, large library). Such

differences occur many times in the monthly (Table 11), daily (Tables 12 and 13), and weekly (Table 14) source classifications. Such results indicate that the small library is not representative, as well as not large enough as demonstrated by the artificial clustering in Table 10. Therefore, the large library should be used to present results for *E. coli* source tracking.

Table 9. Rates of correct classification by source based on discriminant analysis for the *E. coli* libraries
***ARCC=Average Rate of Correct Classification.**

Small Human-Livestock-Wildlife Library, Mill Creek Only

Source	Number of Isolates	% Correctly Classified	
Human	144	95.8	
Wildlife	144	99.3	
Livestock	96	100	
Totals	384	98.4	(ARCC)*

Small Specific Source Library, Mill Creek Only

Source	Number of Isolates	% Correctly Classified	
Human	144	95.8	
Wildlife	48	97.9	
Cattle	96	100	
Deer	96	82.3	
Totals	384	94.0	(ARCC)*

Large Human-Livestock-Wildlife Library, Mill Creek + Other Areas

Source	Number of Isolates	% Correctly Classified	
Human	307	89.6	
Wildlife	429	87.0	
Livestock	422	84.4	
Totals	1158	87.0	(ARCC)*

Large Specific Source Library, Mill Creek + Other Areas

Source	Number of Isolates	% Correctly Classified	
Human	307	90.0	
Wildlife	48	100	
Cattle	326	84.3	
Deer	336	76.5	
Goose	45	86.7	
Horse	96	95.8	
Totals	1158	88.9	(ARCC)*

Table 10. Rates of artificial clustering based on discriminant analysis and random assignment of isolates to sources *E. coli* libraries.

Small Human-Livestock-Wildlife Library, Mill Creek Only

(3 sources, random ARCC = 33.3%)

Source	Number of Isolates	% Correctly Classified
Human	128	50.8
Wildlife	128	52.3
Livestock	128	41.4
Totals	384	48.2 (ARCC)*

Small Specific Source Library, Mill Creek Only

(4 sources random ARCC = 25%)

Source	Number of Isolates	% Correctly Classified
Human	96	30.2
Wildlife	96	40.6
Cattle	96	42.7
Deer	96	40.6
Totals	384	38.5 (ARCC)*

(3 sources, random ARCC = 33.3%)

Large Human-Livestock-Wildlife Library, Mill Creek + Other Areas

Source	Number of Isolates	% Correctly Classified
Human	386	40.7
Wildlife	386	33.9
Livestock	386	28.2
Totals	1158	34.3 (ARCC)*

(6 sources, random ARCC = 16.7%)

Large Specific Source Library, Mill Creek + Other Areas

Source	Number of Isolates	% Correctly Classified
Human	193	27.5
Wildlife	193	8.3
Cattle	193	29.5
Deer	193	22.8
Goose	193	5.7
Horse	193	10.4
Totals	1158	17.3 (ARCC)*

* Average Rate of Correct Classification.

Table 11. Monthly source classifications (%) for *E.coli* small and large libraries, Human, Livestock, and Wildlife Categories.

Category	Small Library (n = 384)			Large Library (n=1158)	
	MC1	MC2		MC1	MC2
			1/10/2001		
Human	100	6.3		0	0
Livestock	0	18.7		33.3	41.7
Wildlife	0	75		66.7	58.3
			2/15/2001		
Human	77	14.6		19.2	6.2
Livestock	11.5	6.2		53.9	77.1
Wildlife	11.5	79.2		26.9	16.7
			3/20/2001		
Human	52.1	0		12.5	2.3
Livestock	47.9	100		54.2	79.5
Wildlife	0	0		33.3	18.2
			4/28/2001		
Human	100	69.6		0	0
Livestock	0	0		50	73.9
Wildlife	0	15.2		50	26.1
			5/8/2001		
Human	37.5	15.2		2.1	4.4
Livestock	54.2	82.6		60.4	82.5
Wildlife	8.3	2.2		37.5	13.1
			6/11/2001		
Human	79.2	54.2		8.3	2.1
Livestock	20.8	45.8		87.5	91.6
Wildlife	0	0		4.2	6.3
			7/19/2001		
Human	0	0		0	2.1
Livestock	87.2	60.4		100	89.6
Wildlife	12.8	39.6		0	8.3
			8/14/2001		
Human	29.2	22.4		6.6	0
Livestock	56.3	55.2		50	93.7
Wildlife	14.5	22.4		43.4	6.3
			9/21/2001		
Human	0	16.7		0	0
Livestock	16.7	44.4		62.5	91.9

Table 11 Continued

Wildlife	83.3	38.9	37.5	8.1
		10/6/2001		
Human	100	0	27.1	0
Livestock	0	0	37.5	70.8
Wildlife	0	100	35.4	29.2
		11/6/2001		
Human	61.9	0	0	4.2
Livestock	38.1	0	47.6	83.3
Wildlife	0	100	52.4	12.5
		12/11/2001		
Human	42.6	77	12.8	2.1
Livestock	21.2	4.2	51	83.3
Wildlife	36.2	18.8	36.2	14.6
		12-Month Average		
Human	56.6	19.7	8.3	2
Livestock	29.5	40.6	56.5	79.9
Wildlife	13.9	39.7	35.3	18.1

Table 12. Daily source classifications (%) for *E.coli* small and large libraries*, Human, Livestock and Wildlife categories, High Flow conditions, winter 2001.

Day	Category	Small Library		Large Library	
		MC1	MC2	MC1	MC2
1	Human	25	2.1	4.2	12.5
	Livestock	0	50	75	41.7
	Wildlife	75	47.9	20.8	45.8
2	Human	0	12.5	0	10.4
	Livestock	62.5	64.6	72.9	66.7
	Wildlife	37.5	22.9	27.1	22.9
3	Human	25	0	27.1	4.2
	Livestock	22.9	95.8	37.5	64.5
	Wildlife	52.1	4.2	35.4	31.3
4	Human	2.1	25.5	17	0
	Livestock	46.8	10.7	63.8	85.2
	Wildlife	51.1	63.8	19.2	14.9
5	Human	0	0	22.9	25
	Livestock	25.5	43.8	45.8	37.5
	Wildlife	87.5	56.2	31.3	37.5
6	Human	14.6	56.3	0	4.2
	Livestock	33.3	27.1	70.8	77.1
	Wildlife	52.1	16.6	29.2	18.7
7	Human	53.3	30	14.9	2.3
	Livestock	0	70	60	79.5
	Wildlife	46.7	0	25.1	18.2
7-day Avg.	Human	20	18.3	12.3	8.4
	Livestock	28	51.7	60.8	64.6
	Wildlife	52	30	26.9	27

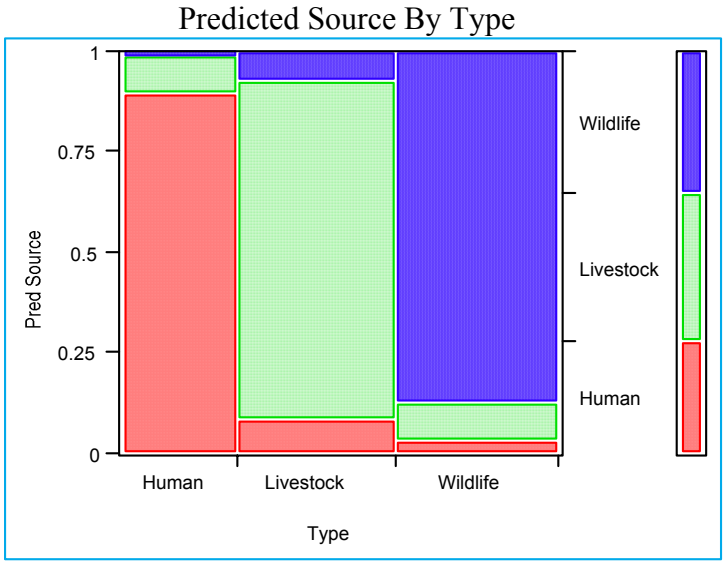
*Small library=384 isolates, large library=1158 isolates.

Table 13. Daily source classifications (%) for *E.coli* small and large libraries*, Human, Livestock and Wildlife categories, Low Flow conditions, fall 2001.

Day	Category	Small Library		Large Library	
		MC1	MC2	MC1	MC2
1	Human	6.3	0	10.4	0
	Livestock	43.7	81.2	62.5	100
	Wildlife	50	18.8	27.1	0
2	Human	4.4	100	8.3	0
	Livestock	0	0	66.7	72.9
	Wildlife	95.8	0	25	27.1
3	Human	80	58.3	0	8.3
	Livestock	20	41.7	75.6	68.8
	Wildlife	0	0	24.4	22.9
4	Human	100	89.6	20.8	8.3
	Livestock	0	0	41.7	56.3
	Wildlife	0	10.4	37.5	35.4
5	Human	91.6	89.6	6.3	10.4
	Livestock	6.3	0	64.6	56.3
	Wildlife	2.1	10.4	29.1	33.3
6	Human	100	95.8	0	0
	Livestock	0	2.1	70.8	91.7
	Wildlife	0	2.1	29.2	8.3
7	Human	100	100	0	0
	Livestock	0	0	62.5	100
	Wildlife	0	0	37.5	0
7-day Avg.	Human	68.9	76.1	6.5	3.9
	Livestock	10	17.9	63.5	78
	Wildlife	21.1	6	30	18.1

*small library=384 isolates, large library=1158 isolates

Figure 3. Three-way human, livestock, and wild life library-printout for *E. coli* from Discriminant Analysis (DA).

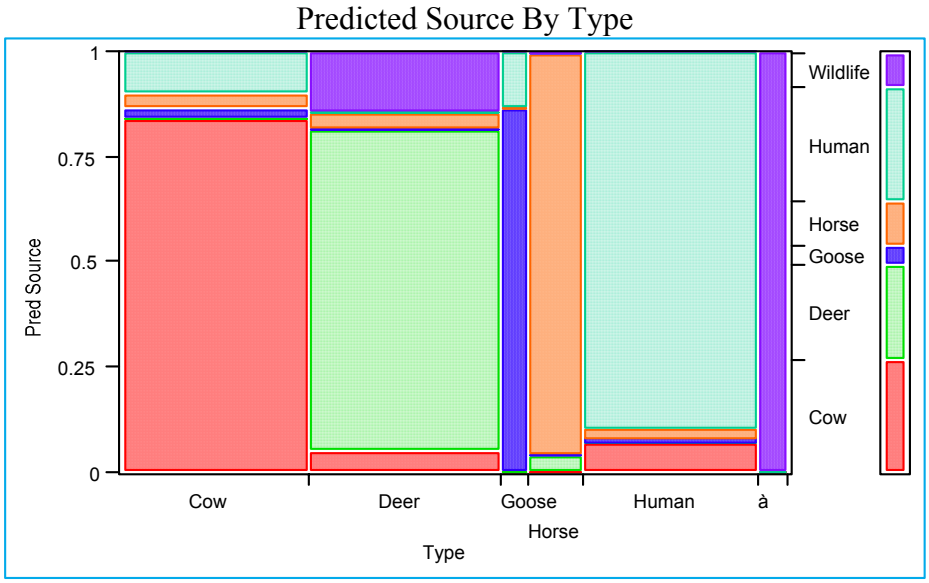


Predicted Source in DA Model Type*

Count Col %	Human	Livestock	Wildlife	Total
Human	275 **89. 58**	3 8.53	15 3.50	326
Livestock	30 9.77	356 **84.36**	41 9.56	427
Wildlife	2 0.65	30 7.11	373 **86.95**	405
				1158

*The predicted source in the DA Model Type is denoted by the numbers in bold and represents the percent of isolates correctly classified.

Figure 4. Specific source library, *E. coli*, from Discriminant analysis (DA).

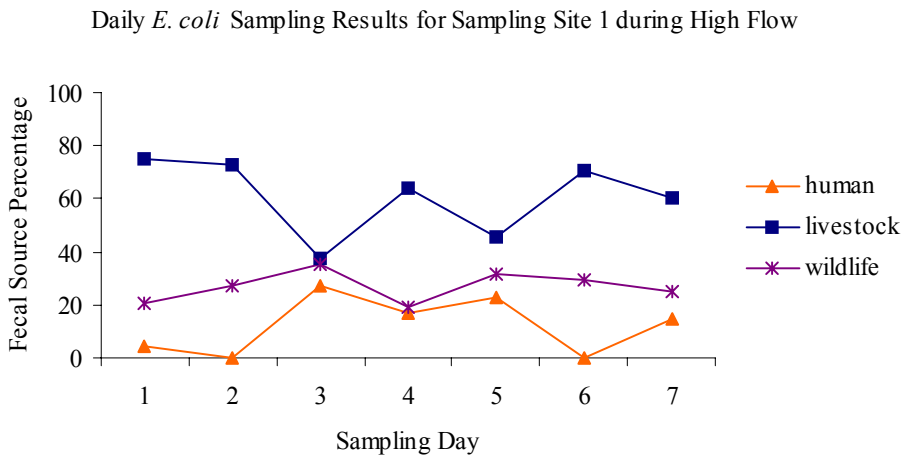


Predicted Source in DA Model* **Type**

Count Col %	Cattle	Deer	Goose	Horse	Human	Wildlife	Total
Cattle	274 **84.31**	17 5.06	0 0.00	0 0.00	23 7.47	0 0.00	314
Deer	0 0.00	257 **76.49**	0 0.00	4 4.17	0 0.00	0 0.00	261
Goose	8 2.46	0 0.00	39 **86.67**	0 0.00	2 0.65	0 0.00	49
Horse	11 3.38	14 4.17	0 0.00	92 **95.83**	6 1.95	0 0.00	123
Human	32 9.85	0 0.00	6 13.33	0 0.00	277 **89.94**	0 0.00	315
Wildlife	0 0.00	48 14.29	0 0.00	0 0.00	0 0.00	48 **100.00**	96
							1158

* The predicted source in the DA Model Type is denoted by the numbers in bold and represents the percent of isolates correctly classified

Figure 5. Daily E.coli Observations for Mill Creek Sampling Site MC 1 during High Flow and Low Flow from Tables 12 and 13.



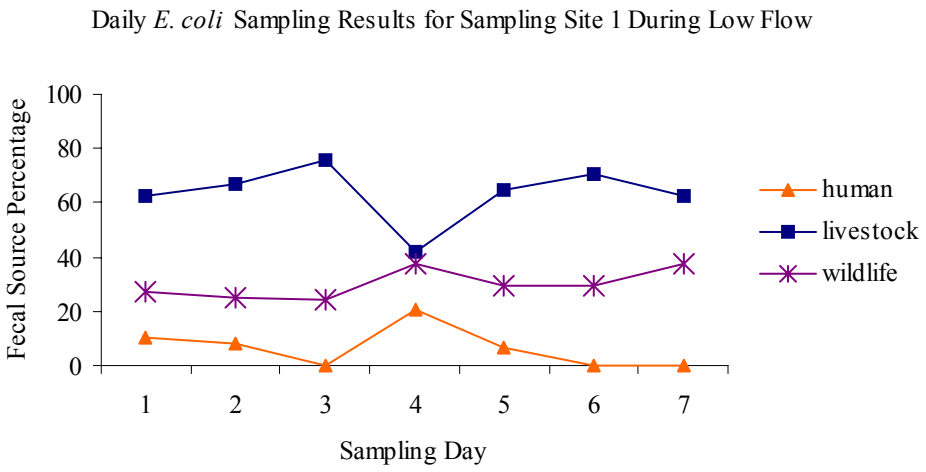
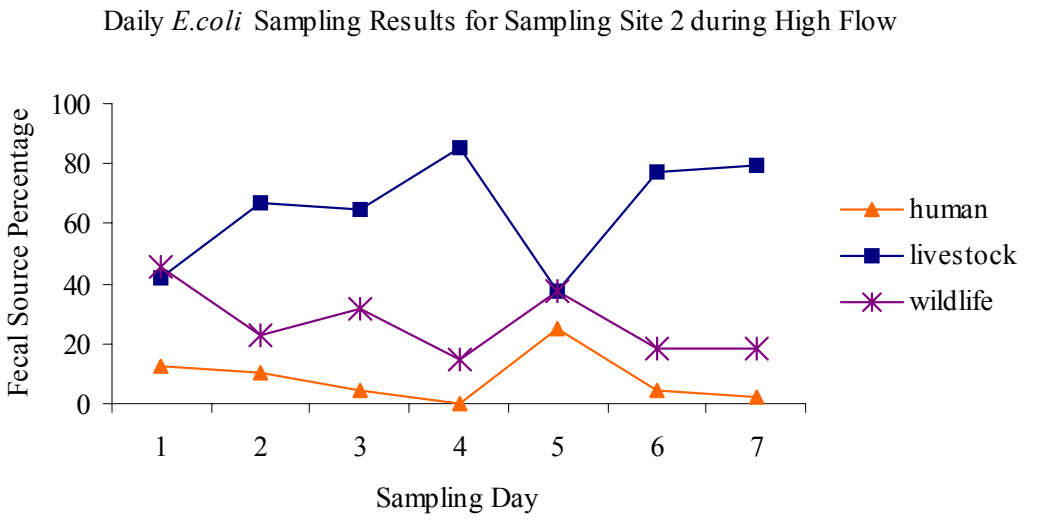
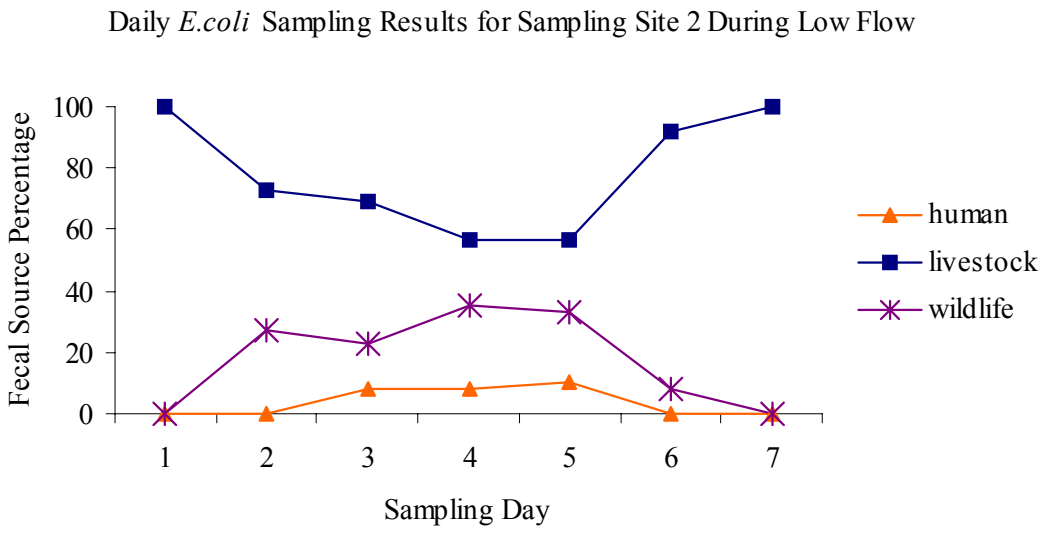


Figure 6. Daily *E.coli* Observations for Mill Creek Sampling Site MC 2 during High Flow and Low Flow From tables 12 and 13.





For the 3-way human, livestock, wildlife large library (Figure 3), the wildlife is misclassified as human 3.5% of the time, and livestock is misclassified as human 8.5% of the time. Averaging these together equals 6 % average misclassification for human. Using a similar approach, the average misclassifications for livestock are 9.7% for livestock and 3.9% for wildlife. For the following tables (11-14) that cover the classification of *E. coli* stream isolates, to determine the level of classification with the misclassifications removed, subtract 6.0% from human, 9.7% from livestock, and 3.9% from wildlife classifications. These may be important for those samples that contain a human signature, as the human signatures are usually much lower than livestock or wildlife and some samples do contain a human signature that is less than 6.0%.

For the 6-way specific source library (Figure 4), human is misclassified 2.0% of the time based on averaging the classifications of human from the other 5 sources. Applying the same approach to the other sources, the average misclassifications were 3.1% for cattle, 4.7% for deer, 2.7% for waterfowl (Canada goose), 0.8% for horse, and 0.0% for miscellaneous wildlife.

B. 1.b. Source Tracking Results for *E. coli* with Stream Isolates, H-L-W Classifications

Unlike the fecal coliform monitoring counts, where monthly sampling was lower than weekly or daily averages, the source tracking classification averages (large library) were remarkably similar (Table 14). As specified in the project proposal, 30 stream samples were collected from each of two sites (60 total), and 48 isolates of both *E. coli* and *Enterococcus* per sample were analyzed by ARA, for a total of 2,880 stream isolates of each.

The monthly averages, Table 11, the daily averages from Tables 12 and 13, and the weekly averages from Table 14 were all derived with both libraries for purposes of comparison (Table 15). For the large library, site MC-1 had a higher human signature than MC-2 and site MC-2 had a higher livestock signature than MC-1. The higher human signature at site MC-1 may be a result of homes being within close proximity to the streams. Both sites were very similar for their wildlife signature. There was little difference between high flow and low flow, and the daily and weekly averages for each site were very close to the 12-month average. For example, the monthly sampling for March (high flow) was taken on 3/20/01 (week 4, Table 14)

and the results were 12.5% human, 54.2% livestock, and 33.3% wildlife for MC-1 and 2.3% human, 79.5% livestock, and 18.2% wildlife for MC-2. These results are very close to the daily and weekly averages for high flow, both sites, (Table 15) and indicate that monthly sampling was adequate. The monthly sampling for September (low flow) was taken on 9/21/01 (week 3, Table 14) and the results were 0.0% human, 75.6% livestock, and 24.4% wildlife for MC-1 and 8.3% human, 68.8% livestock, and 22.9% wildlife for MC-2. These results were likewise very close to the daily and weekly averages for low flow, both sites, (Table 15) and also tended to indicate that monthly sampling was adequate. The one exception is that the small human signature for MC-2, low flow, would have been missed if the monthly sample alone was used (human = 0.0%) and the human signature for MC-1 (15.1% weekly low flow) would have been underestimated if just the monthly result (8.3% human for September) had been used. However, if one subtracts the misclassifications (6% for human), then the human signature is essentially erased from all the MC-2 averages except daily, high flow. Even with the 6% misclassification removed for MC-1, there does appear to be a small but legitimate human signature present during daily high flow and weekly low and high flow that would not have been picked up with monthly sampling (Table 15).

The livestock signature was large and persuasive over all sites and seasons. There was the distinct possibility that livestock so overwhelmed all other sources, when the cattle inventory in Mill Creek (approximately 3,800 animals) was considered, that the lack of seasonal variation was simply a function of the high land use pattern devoted to pasture and cattle in the Mill Creek watershed (Table 1).

Table 14. Weekly source classifications (%) for *E.coli* small and large libraries*, Human, Livestock and Wildlife categories, High Flow conditions, winter 2001 and Low Flow Conditions fall 2001.

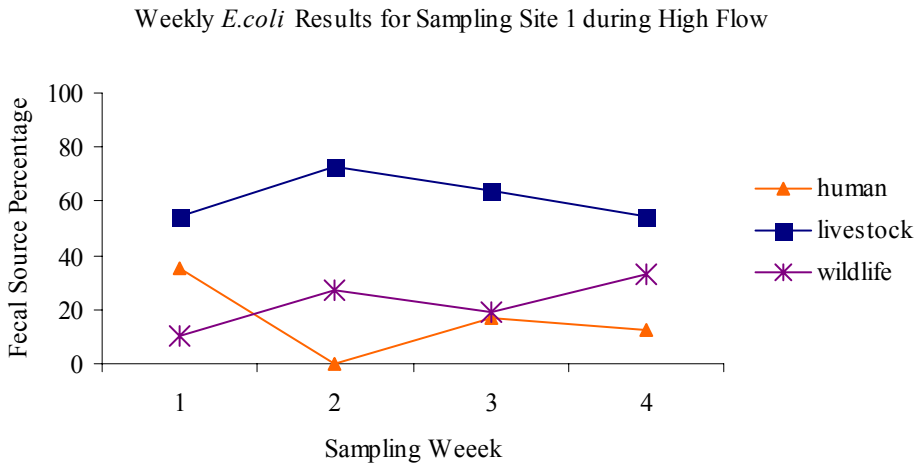
Week	Category	Small Library		Large Library	
		MC1	MC2	MC1	MC2
High Flow					
1	Human	87.5	14.6	35.4	6.3
	Livestock	4.2	8.3	54.2	58.3
	Wildlife	8.3	77.1	10.4	35.4
2	Human	0	12.5	0	10.4
	Livestock	37.5	64.6	72.9	66.7
	Wildlife	62.5	22.9	27.1	22.9
3	Human	51.1	63.8	17	0
	Livestock	2.1	25.5	63.8	85.1
	Wildlife	46.8	10.7	19.2	14.9
4	Human	52.1	0	12.5	2.3
	Livestock	47.9	100	54.2	79.5
	Wildlife	0	0	33.3	18.2
4-Wk Avg.	Human	47.7	22.7	16.2	4.7
	Livestock	22.9	49.6	61.3	72.4
	Wildlife	29.4	27.7	22.5	22.9
Low Flow					
1	Human	100	64.9	33.3	16.2
	Livestock	0	29.7	54.2	43.2
	Wildlife	0	5.4	12.5	40.6
2	Human	0	16.7	0	0
	Livestock	83.3	44.4	62.5	91.9
	Wildlife	16.7	38.9	37.5	8.1
3	Human	80	58.3	0	8.3
	Livestock	20	41.7	75.6	68.8
	Wildlife	0	0	24.4	22.9

Table 14 continued.

4	Human	100	100	27.1	0
	Livestock	0	0	37.5	70.8
	Wildlife	0	0	35.4	29.2
4-Wk Avg.	Human	70	35	15	6.1
	Livestock	25.8	29	57.5	68.7
	Wildlife	4.2	36	27.5	25.2

*small library=384 isolates, large library=1158 isolates

Figure 7. Weekly *E. coli* Results for Sampling Site 1 during High Flow and Low Flow from Table 14.



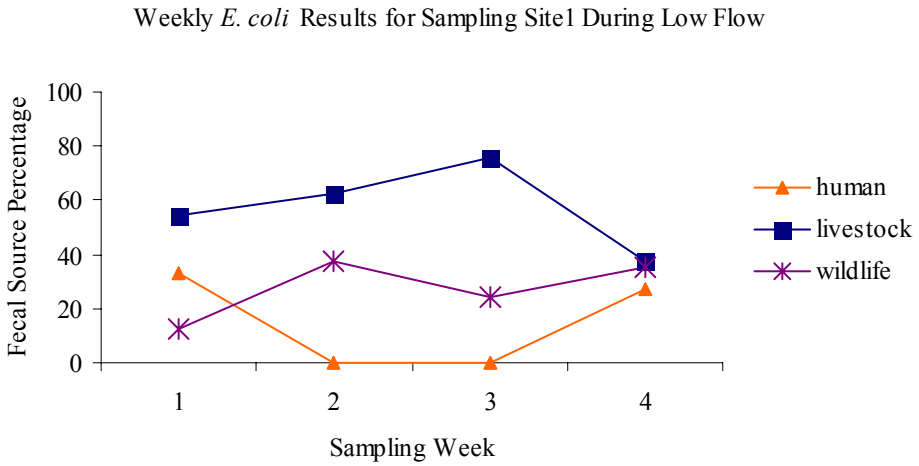
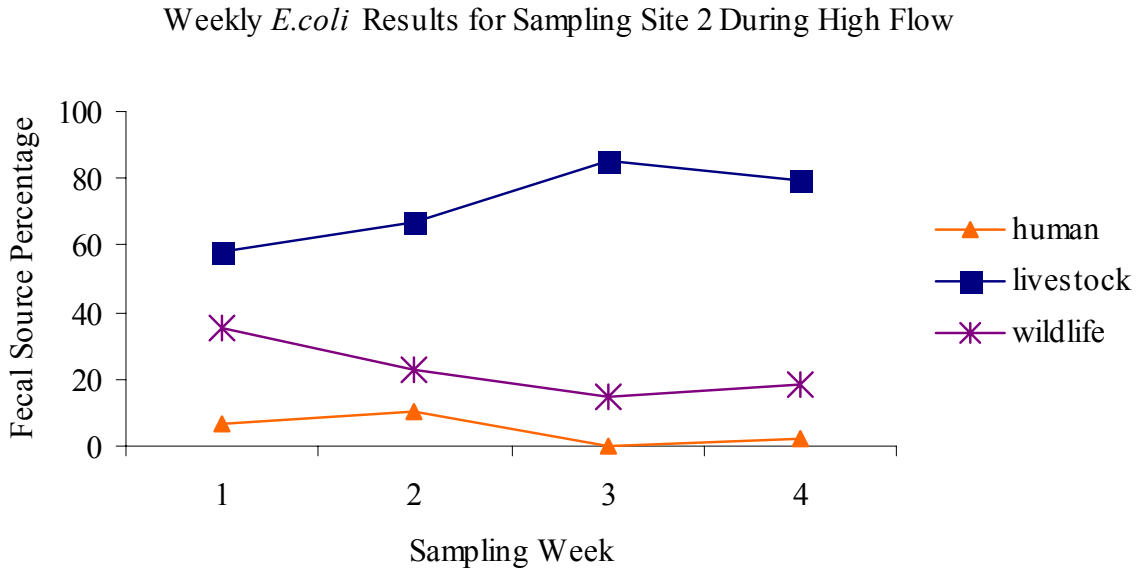


Figure 8. Weekly E.coli Results for Sampling Site 2 During High Flow from Table 14.



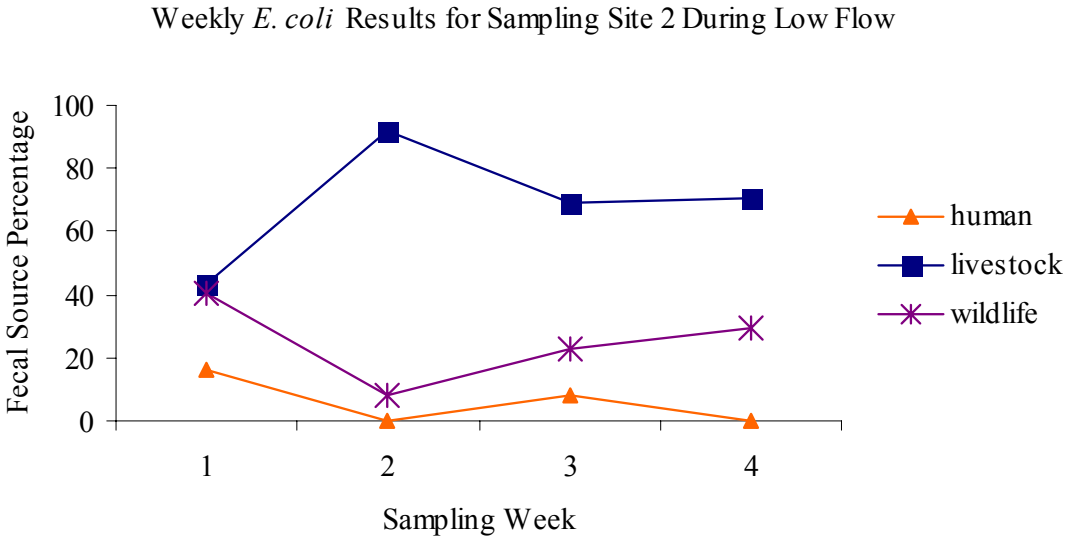


Table 15. Monthly, daily, and weekly human (H), livestock (L), and wildlife (W) source classification averages (%) for Mill Creek *E. coli* isolates.

Source	Monthly (12 months)		Daily (7 days) High Flow		Daily (7 days) Low Flow		Weekly (4 weeks) High Flow		Weekly (4 weeks) Low Flow	
(%)	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2
Large Library (1,158 isolates)										
H	8.2	1.9	12.3	8.3	6.5	3.9	16.2	4.8	15.1	6.1
L	56.5	79.9	60.7	64.7	63.5	78.0	61.3	72.4	57.5	68.7
W	35.3	18.2	27.0	27.0	30.0	18.1	22.5	22.8	27.4	25.2
Small Library (384 isolates)										
H	56.6	19.7	20.0	18.3	68.9	76.1	47.7	22.7	70.0	35.0
L	29.5	40.6	28.0	51.7	10.0	17.9	22.9	49.6	25.8	28.9
W	13.9	39.7	52.0	30.0	21.1	6.0	29.4	27.7	4.2	36.1

Table 16: Monthly, daily, and weekly human (H), cattle (C), horse (Ho), deer (D), goose (G) and wildlife (W) source classification averages (%) for Mill Creek *E. coli* isolates.

Source (%)	Monthly (12 months)		Daily (7 days) High Flow		Daily (7 days) Low Flow		Weekly (4 weeks) High Flow		Weekly (4 weeks) Low Flow	
	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2
Large Library (1,158 isolates)										
H	3.0	3.6	5.0	5.7	5.0	7.0	7.8	2.0	3.0	7.0
C	58.0	71.0	54.0	67.0	61.0	55.7	49.3	73.0	65.0	54.0
Ho	0.0	0.4	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D	25.0	19.0	31.0	21.0	25.0	27.0	38.2	24.0	20.0	26.0
G	0.0	1.0	0.0	0.3	0.0	0.3	0.0	0.0	0.0	0.0
W	11.0	5.0	9.0	5.7	8.3	10.0	4.7	1.0	12.0	13.0

B. 1.c. Source Tracking Results for *E. coli* with Stream Isolates, Species-Specific Classifications

The species-specific source classifications (Table 17, monthly; Table 18, daily high flow; Table 19, daily low flow; and Table 20, weekly) agree closely with the source classifications divided between human, livestock, and wildlife. This comparison is based on the large library as results from the small library were unreliable and species-specific classifications were not performed with it. By comparing Table 16 (species-specific) to the large library part of Table 15, the similarities are apparent.

The species-specific analysis (Table 16) tended to reduce the human and livestock signatures and increase the wildlife signature, but the reductions were not substantial. These reductions are probably related to the species-specific library having a miscellaneous wildlife category (in addition to goose and deer categories) that was not part of the human, livestock, and wildlife library. There was essentially no horse or goose signature in Mill Creek, and a substantial deer signature was present throughout the year (Table 16). The cattle signature in Table 16 was very close to the livestock signature in Table 15 (large library) and the deer signature plus miscellaneous wildlife signature in Table 16 was also very close to the wildlife category in Table 15.

The human, livestock, wildlife classifications indicated that the human signature was highest in MC-1 (Table 15), while the source-specific classifications indicated that the human signature was similar at both sites but slightly higher for MC-2 (except for weekly high flow). If the 2.0% average misclassification for human (Figure 5) is applied to Table 16, only the signature for MC-2, weekly high flow is subtracted to zero. The basic conclusion for the source-specific classification is that this approach demonstrated the same major result as the 3-way classification; that cattle (livestock) comprised an overwhelming signature that dominated over both the sampling scheme and seasonality. There was a human signature present for both analyses, but it was small, and deer dominated the wildlife signature (although this could be affected by the composition of the known source library). The species-specific library appears to

be an unnecessary contribution to this study, because there was such an overwhelming cattle signature. The species-specific library would be advantageous in a study with a more diverse fecal source background.

Table 17. Monthly source classifications (%) for E.coli large library, specific source Categories'

	Large Library (n=1158)		
Category	**MC1**		**MC2**
Human	0	1/10/2001	0
Cattle	68.8		79.2
Horse	0		0
Deer	31.2		18.8
Goose	0		0
Wildlife	0		2.1
		2/15/2001	
Human	7.7		10.4
Cattle	46.2		66.7
Horse	0		0
Deer	15.4		14.6
Goose	30.7		2.1
Wildlife	0		6.3
		3/20/2001	
Human	8.3		2.7
Cattle	43.8		67.6
Horse	0		0
Deer	37.5		27
Goose	0		0
Wildlife	10.4		2.7
		4/28/2001	
Human	5.9		0
Cattle	44.1		71.7
Horse	0		0
Deer	32.4		28.3
Goose	0		0
Wildlife	17.6		0
		5/8/2001	
Human	0		0
Cattle	47.9		80.4
Horse	0		2.2
Deer	31.3		10.9
Goose	0		0
Wildlife	20.8		6.5

Table 17. continued

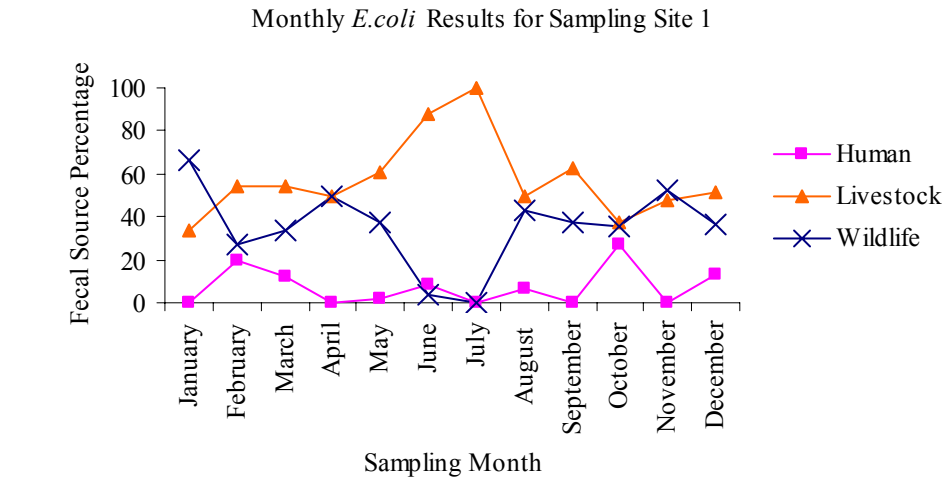
		6/11/2001	
Human	4.2		2.1
Cattle	64.6		60.4
Horse	0		0
Deer	29.2		35.4
Goose	0		0
Wildlife	2.1		2.1
		7/19/2001	
Human	0		0
Cattle	100		100
Horse	0		0
Deer	0		0
Goose	0		0
Wildlife	0		0
		8/14/2001	
Human	6.3		4.2
Cattle	45.8		72.9
Horse	0		2.1
Deer	37.5		18.8
Goose	0		0
Wildlife	10.4		2.1
		9/21/2001	
Human	0		4.2
Cattle	66.7		56.3
Horse	0		0
Deer	14.6		25
Goose	0		0
Wildlife	18.8		14.5
		10/6/2001	
Human	2.1		4.6
Cattle	45.8		43.2
Horse	0		0
Deer	27.1		34.1
Goose	0		0
Wildlife	25		18.2

Table 17 continued.

		11/6/2001	
Human	0		4.2
Cattle	57.1		70.8
Horse	0		0
Deer	42.9		16.7
Goose	0		0
Wildlife	0		4.2
		12/11/2001	
Human	4.2		4.2
Cattle	70.2		81.3
Horse	0		0
Deer	4.3		8.3
Goose	0		6.2
Wildlife	21.3		0
		12-month Avg., 2001	
Human	3		3.6
Cattle	58		71
Horse	0		0.4
Deer	25		19
Goose	0		1
Wildlife	11		5

*small library=384 isolates, large library=1158 isolates.

Figure 9. Monthly *E. coli* Observations for Mill Creek Sampling Site 1 and 2 from table 11.



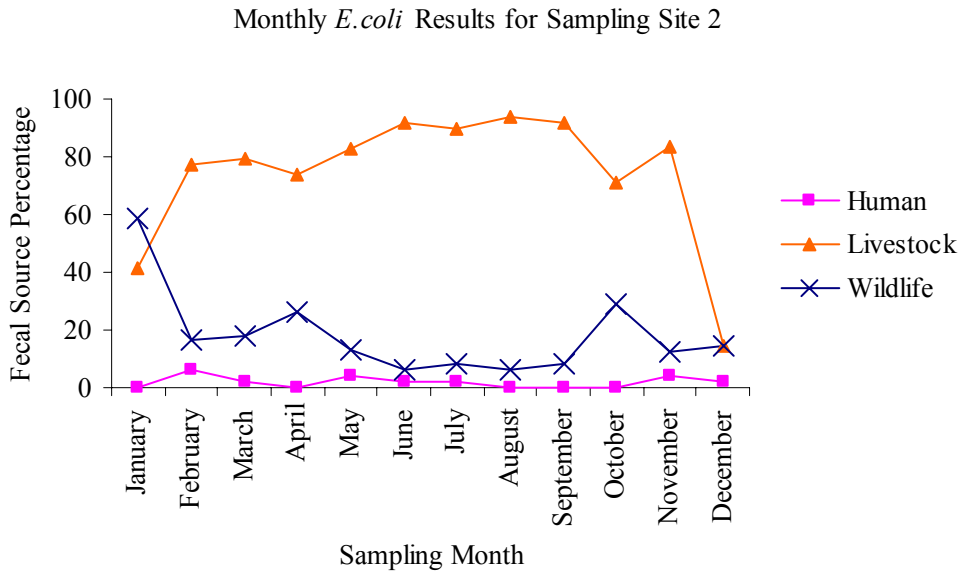


Table 18. Daily source classifications (%) for *E.coli* large library, specific source categories, High Flow conditions, winter 2001.

	Large Library (n=1158)		
Category	**MC1**		**MC2**
Human	0	Day 1	0
Cattle	79.2		83.3
Horse	0		0
Deer	20.8		14.6
Goose	0		2.1
Wildlife	0		0
		Day 2	
Human	6.2		0
Cattle	54.2		72.9
Horse	0		0
Deer	39.6		27.1
Goose	0		0
Wildlife	0		0
		Day 3	
Human	4		14.6
Cattle	47.9		64.6
Horse	4		0
Deer	35.9		20.8
Goose	0		0
Wildlife	8.2		0
		Day 4	
Human	0		10.2
Cattle	55.3		56.3
Horse	0		0
Deer	38.3		27.1
Goose	0		0
Wildlife	6.4		6.4
		Day 5	
Human	6.3		10.4
Cattle	43.7		52.3
Horse	0		0
Deer	25		24.8
Goose	0		0
Wildlife	25		12.5

Table 18. Continued.

		Day 6	
Human	10.4		8.3
Cattle	54.2		68.8
Horse	0		0
Deer	22.9		8.3
Goose	0		0
Wildlife	12.5		22.9
		Day 7	
Human	8.3		2.7
Cattle	43.8		67.6
Horse	0		0
Deer	37.5		27
Goose	0		0
Wildlife	10.4		2.7
		7-Day Avg.	
Human	5		5.7
Cattle	54		67
Horse	1		0
Deer	31		21
Goose	0		0.3
Wildlife	9		5.7

*Large library = 1158 isolates.

Table 19. Daily source classifications (%) for *E.coli* large library, specific source categories Low Flow conditions, fall 2001.

	Large Library (n=1158)		
Category	**MC1**		**MC2**
Human	22.9	Day 1	12.5
Cattle	70.8		41.7
Horse	0		0
Deer	6.3		39.5
Goose	0		0
Wildlife	0		6.3
		Day 2	
Human	6.3		12.5
Cattle	57.5		58.3
Horse	0		0
Deer	30		12.5
Goose	0		0
Wildlife	6.3		16.7
		Day 3	
Human	6.3		4.2
Cattle	50		59.6
Horse	0		0
Deer	37.4		14.2
Goose	0		0
Wildlife	6.3		17
		Day 4	
Human	4.2		2.2
Cattle	45.8		59.6
Horse	0		0
Deer	33.3		19.1
Goose	0		0
Wildlife	16.7		19.1
		Day 5	
Human	0		6.7
Cattle	67.9		55.8
Horse	0		0
Deer	23.8		31.3
Goose	0		2.1
Wildlife	8.3		4.1

Table 19. Continued.

		Day 6	
Human	0		8.3
Cattle	72.9		52.1
Horse	0		0
Deer	14.6		31.3
Goose	0		0
Wildlife	12.5		8.3
		Day 7	
Human	0		2.7
Cattle	62.5		63.2
Horse	0		0
Deer	29.2		34.1
Goose	0		0
Wildlife	8.3		0
		7-Day Avg.	
Human	5.7		7
Cattle	61		55.7
Horse	0		0
Deer	25		27
Goose	0		0.3
Wildlife	8.3		10

*Large library = 1158 isolates.

Table 20. Weekly source classifications (%) for *E.coli* large library, specific source categories, High Flow conditions, winter 2001 and Low Flow conditions, fall 2001.

	Large Library (n=1158)		
Category	**MC1**		**MC2**
		High Flow	
Human	10.4	Week 1	2.7
Cattle	43.8		67.6
Horse	0		0
Deer	37.5		27
Goose	0		0
Wildlife	8.3		2.7
		Week 2	
Human	6.2		0
Cattle	54.2		72.9
Horse	0		0
Deer	39.6		27.1
Goose	0		0
Wildlife	0		0
		Week 3	
Human	6.4		2.1
Cattle	55.3		83.3
Horse	0		0
Deer	38.3		14.6
Goose	0		0
Wildlife	0		0
		Week 4	
Human	8.3		2.7
Cattle	43.8		67.6
Horse	0		0
Deer	37.5		27
Goose	0		0
Wildlife	10.4		2.7
		4-Wk Avg.	
Human	7.8		2
Cattle	49.3		73
Horse	0		0
Deer	38.2		24
Goose	0		0

Table 20. Continued.

Wildlife	4.7		1
		Low Flow	
		Week 1	
Human	4.2		14.6
Cattle	95.8		56.3
Horse	0		0
Deer	0		25
Goose	0		0
Wildlife	0		4.1
		Week 2	
Human	0		4.2
Cattle	66.7		56.3
Horse	0		0
Deer	14.6		25
Goose	0		0
Wildlife	18.8		14.5
		Week 3	
Human	6.3		4.2
Cattle	50		59.6
Horse	0		0
Deer	37.4		19.2
Goose	0		0
Wildlife	6.3		17
		Week 4	
Human	2.1		4.6
Cattle	45.8		43.2
Horse	0		0
Deer	27.1		34.1
Goose	0		0
Wildlife	25		18.2
		4-Wk. Avg.	
Human	3		7
Cattle	65		54
Horse	0		0
Deer	20		26
Goose	0		0
Wildlife	12		13

Table. 21. Rates of correct classification by source based on discriminant analysis for the Enterococcus libraries.

Small Human-Livestock-Wildlife Library, Mill Creek Only.

Source	Number of Isolates	% Correctly Classified
Human	103	100
Wildlife	120	85.3
Livestock	277	95.7
Totals	500	93.7 (ARCC)*

Small Specific Source Library, Mill Creek Only.

Source	Number of Isolates	% Correctly Classified
Human	103	100
Wildlife	31	93.6
Cattle	277	87.0
Deer	89	97.8
Totals	500	94.6 (ARCC)*

Large Human-Livestock-Wildlife Library, Mill Creek+Other Areas

Source	Number of Isolates	% Correctly Classified
Human	392	90.8
Wildlife	261	83.9
Livestock	529	81.1
Totals	1182	85.3 (ARCC)*

Large Specific Source Library, Mill Creek+Other Areas

Source	Number of Isolates	% Correctly Classified
Human	391	89.3
Wildlife	32	96.9
Cattle	529	75.1
Deer	230	91.3
Totals	1182	88.2(ARCC)*

*ARCC=average rate of correct classification.

Table. 22. Rates of artificial clustering based on discriminant analysis and random assignment of isolates to sources for the Enterococcus libraries.

Small Human-Livestock-Wildlife Library, Mill Creek Only.

(3 sources, random ARCC=33.3%)

Source	Number of Isolates	% Correctly Classified
Human	167	35.9
Wildlife	167	21.0
Livestock	168	60.1
Totals	500	39 (ARCC)*

Small Specific Source Library, Mill Creek Only.

(4 sources, random ARCC=25.0%)

Source	Number of Isolates	% Correctly Classified
Human	125	32.8
Wildlife	125	45.6
Cattle	125	24.0
Deer	125	13.6
Totals	500	29.0 (ARCC)*

Large Human-Livestock-Wildlife Library, Mill Creek+Other Areas

(3 sources, random ARCC=33.3%)

Source	Number of Isolates	% Correctly Classified
Human	384	40.3
Wildlife	394	34.5
Livestock	394	28.2
Totals	1182	34.3 (ARCC)*

Large Specific Source Library, Mill Creek+Other Areas

(4 sources, random ARCC=33.3%)

Source	Number of Isolates	% Correctly Classified
Human	296	27.7
Wildlife	296	43.6
Cattle	295	26.1
Deer	295	20.3
Totals	1182	30.9 (ARCC)*

*ARCC=average rate of correct classification.

C. Summary of Project Results Based on Source Tracking with *Enterococcus.*

C.1.a. *Enterococcus* Known Source Libraries

Average rates of correct classification (ARCC) for all four libraries (large and small, both 3-way human, livestock, wildlife split and specific source split) were excellent and ranged from 85.3% to 94.6% (Table 21). The small library (500 isolates) was from known sources collected entirely within the Mill Creek watershed and the large library (1182 isolates) consisted of the 500 isolates from Mill Creek plus 682 isolates from the Blacksburg area, Holston River, and Blackwater River watersheds. The small library for enterococcus (500 isolates) was larger in comparison to the small library for *E.coli* (384 isolates) because the enterococcus isolates were more numerous and were easier to obtain from fecal samples. The high ARCC of the large library demonstrates that isolates from different regions can be put together and it is not necessary to build a new large library with every new watershed. The ARCCs for the *Enterococcus* libraries (Table 21) were very similar to those reported for *E. coli* (Table 9).

By assigning the sources randomly to the isolates, artificial clustering appears in the small libraries (Table 22). The ARCC for the 3-way small library was 39.0% where it should be 33.3% based on random distribution. The ARCC for the 4-way small library was 29.0% where it should be 25.0% based on random distribution. The large library (1182 isolates) had ARCCs within 1% to 5% of the random classifications. When the ARCC of random assignments equals that of the calculated random ARCC, the library size is sufficient. The artificial clustering of sources in the small *Enterococcus* library (500 isolates) was less than those for the small *E. coli* library (384 isolates), a further indication that artificial clustering decreases as the library size increases. Examining the ARCC values based on random assignments of sources cannot be used to determine the representativeness of a library, but can be used as a measure of library size. Based on the low level of random classifications, both the large and small *Enterococcus* libraries appeared to be appropriate for use with unknown source stream isolates.

When viewing the monthly classifications of human, livestock and wildlife (Table 22), it quickly becomes apparent that there are a few examples where the small library provided results that differed from the results provided by the large library (e.g. see February in Table 22, 100% wildlife for both MC-1 and MC-2, small library, vs. 83.3% livestock for MC-1 and 64.6% livestock for MC-2, large library). Such differences between the small and large libraries did not occur as many times with *Enterococcus* as with *E. coli* (Table 11), and was probably because the small *E. coli* library contained only 384 isolates. The closeness of the two *Enterococcus* libraries indicated that both were suitable for use with stream isolates, but the occasional sample where the small library gave results that were quite different from the large library means that the small library must be used with care. Over 12 monthly samplings, however, the 12-month averages for both libraries were very close, although the small library did under-represent the human signature (1.0% human, both sampling sites, small library vs. 2.0% human for MC-1 and 5.9% human for MC-2, Table 22). The small library also yielded a 4% to 8% lower livestock average for both sites, and an 8% to 9% higher wildlife average.

For the 3-way human, livestock, wildlife large library (Figure 10), the wildlife is misclassified as human 0.77% of the time, and livestock is misclassified as human 8.42% of the time. Averaging these together equals 4.6% average misclassifications for human. Using a similar approach, the average misclassifications for livestock are 9.5% for livestock and 8.1% for wildlife. For the following tables (23-26) that cover the classification of *Enterococcus* stream isolates, to determine the level of classification with the misclassifications removed, subtract 4.6% from human, 9.5% from livestock, and 8.1% from wildlife classifications. These may be important for those samples that contain a human signature, as the human signatures were much lower than livestock or wildlife and some samples do contain a human signature that is less than 4.6%. Using the 12-month averages as an example (Table 22), if 4.6% human (the average rate of misclassification) was subtracted, only MC-2 for the large library would still contain a human signature as the other 12-month averages were all below 4.6%.

For the 4-way specific source library (Figure 11), human is misclassified 3.6% of the time based on averaging the classifications of human from the other 3 sources. Applying the same approach to the other sources, the average misclassifications were 8.3% for cattle, 2.9% for deer, and 1.1% for miscellaneous wildlife.

C.1.b. Source Tracking Results for *Enterococcus* with Stream Isolates, H-L-W Classifications

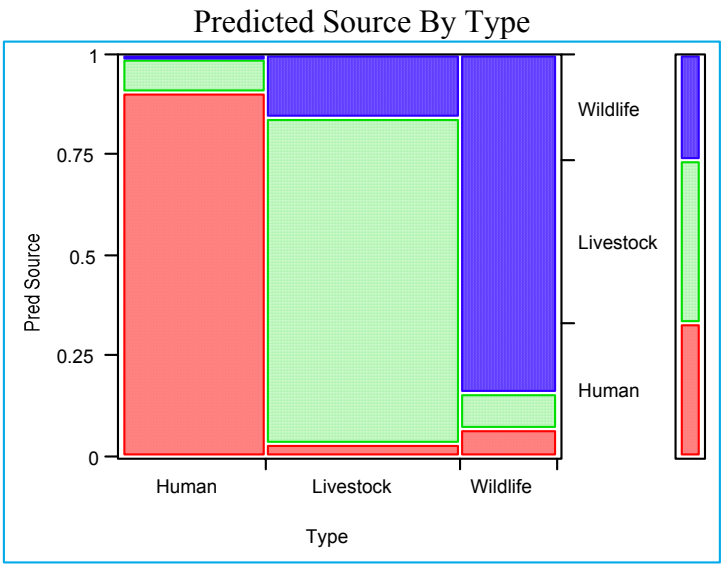
The source tracking classification averages (large library) were remarkably similar (Table 23). As specified in the project proposal, 30 stream samples were collected from each of two sites (60 total), and 48 isolates of both *E. coli* and *Enterococcus* per sample were analyzed by ARA, for a total of 2,880 stream isolates of each. As with the *E. coli* results, livestock was the dominant signature, followed by wildlife, and a very small human signature.

The monthly averages, Table 23, the daily averages from Tables 24 and 25, and the weekly averages from Table 26 were all derived with both *Enterococcus* libraries. For the large library, site MC-2 had a higher human signature than MC-1 based on the 12-month average, while site MC-1 had a higher human signature for the high and low flow daily averages and the high flow weekly average (Table 27). The human signature never exceeded 14.6% and little seasonality was evident. The small library yielded a smaller human signature at all times except for the 7-day average, high flow, both sites. The livestock signature was dominant over weekly, daily, and monthly averages and tended to be higher during low flow, especially for the weekly averages. The wildlife averages (large library) ranged from 0.0% (MC-1, weekly low flow) to 29.2% (MC-1, weekly high flow). The small library tended to reduce the livestock signature (especially during high flow conditions) as well as the human signature and enlarge the wildlife signature. While there was a wider range in the averages for *Enterococcus* (Table 27) than for *E. coli* (Table 15), the monthly averages (large library) for *Enterococcus* do appear to be representative, and therefore adequate, as far as sampling is concerned.

As with the *E. coli* results, when the cattle inventory in Mill Creek (approximately 3,800 animals) was considered, there was the distinct possibility that livestock so overwhelmed all

other sources, the lack of seasonal variation was simply a function of the land use pattern devoted to pasture and cattle in the Mill Creek watershed.

Figure10. Three-way human, livestock, wildlife library printout for *Enterococcus*.

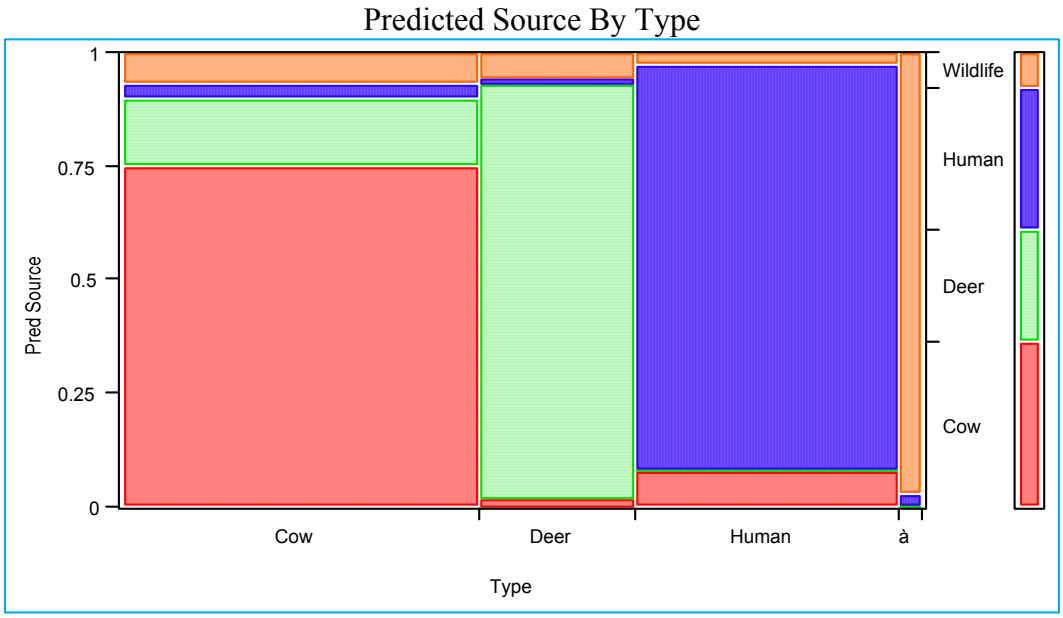


Predicted Source in DA Model **Type**

Count Col %	Human	Livestock	Wildlife	Total
Human	356 **90.82**	17 3.21	19 7.28	392
Livestock	33 8.42	429 **81.10**	23 8.81	485
Wildlife	3 0.77	83 15.69	219 **83.91**	305
Total	392	529	261	1182

*The predicted source in the DA Model Type is denoted by the numbers in bold and represents the percent of isolates correctly classified.

Figure 11. Four-way source classification library for *Enterococcus*.



Predicted Source in DA Model Type

Count Col %	Cattle	Deer	Human	Wildlife	Total
Cattle	397 **75.05**	4 1.74	31 7.93	0 0.00	432
Deer	79 14.93	210 **91.30**	1 0.26	0 0.00	290
Human	17 3.21	3 1.30	349 **89.26**	1 3.13	370
Wildlife	36 6.81	13 5.65	10 2.56	31 **96.88**	90
Total	529	230	391	32	1182

*The predicted source in the DA Model Type is denoted by the number bold and represents the percent of isolates correctly classified.

C. 1.c. Source Tracking Results for *Enterococcus* with Stream Isolates, Species-Specific Classifications

The species-specific source classifications (human, cattle, deer, miscellaneous wildlife, Table 23, monthly; Table24, daily high flow; Table 25, daily low flow; and Table 26, weekly) agree closely with the source classifications divided between human, livestock, and wildlife. This comparison is based on the large library as results from the small library appeared less reliable and species-specific classifications were not performed with it. By comparing Table 28(species-specific, this page) to the large library part of Table 27, the similarities are apparent. The predominant signature over all seasons was from cattle, followed by deer, with much smaller signatures from miscellaneous wildlife and human. While the species-specific library for *E. coli* included geese and horses (Table 16), and the species-specific library for *Enterococcus* did not, the trends were identical although the *E. coli* species-specific library tended to have a slightly lower cattle signature and a slightly larger deer signature. There were no geese or horses found in Mill Creek, and in an effort to test our confidence in the library we left these sources in the E.coli library to see if known source isolates would be misclassified.

Table 23. Monthly source classifications (%) for *Enterococci* large library, specific source categories.

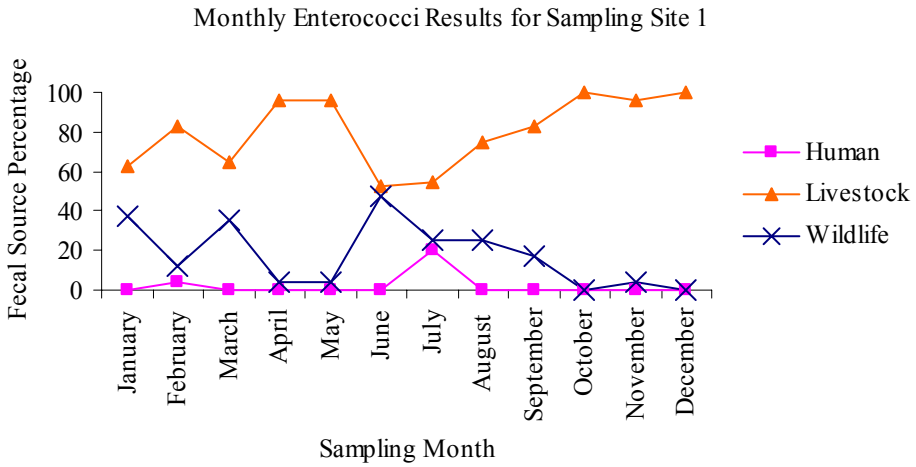
Category	Large Library (n=1182)	
	MC1	MC2
	1/10/2001	
Human	0	4.2
Cattle	64.6	52.1
Deer	31.2	39.5
Wildlife	4.2	4.2
	2/15/2001	
Human	4.2	0
Cattle	50	77.1
Deer	29.1	22.9
Wildlife	16.7	0
	3/20/2001	
Human	0	10.4
Cattle	58.3	77.1
Deer	27.1	12.5
Wildlife	14.6	0
	4/28/2001	
Human	0	2.1
Cattle	61.5	52.1
Deer	30.8	45.8
Wildlife	7.7	0
	5/8/2001	
Human	0	0
Cattle	81.2	97.9
Deer	0	0
Wildlife	18.8	2.1
	6/11/2001	
Human	0	14.6
Cattle	58.3	45.8
Deer	39.6	33.6
Wildlife	2.1	6
	7/19/2001	
Human	12.5	2.1
Cattle	58.3	61.7
Deer	25	19.2

Table 23. Continued.

Wildlife	4.2		17
		8/14/2001	
Human	0		0
Cattle	72.9		64.6
Deer	8.3		35.4
Wildlife	18.8		0
		9/21/2001	
Human	0		0
Cattle	83.3		64.6
Deer	16.7		14.4
Wildlife	0		21
		10/6/2001	
Human	0		0
Cattle	72.9		58.3
Deer	27.1		41.7
Wildlife	0		0
		11/6/2001	
Human	0		16.7
Cattle	91.7		45.8
Deer	4.1		31.2
Wildlife	4.2		6.3
		12/11/2001	
Human	0		0
Cattle	100		100
Deer	0		0
Wildlife	0		0
		12-month Avg., 2001	
Human	0		0
Cattle	100		100
Deer	0		0
Wildlife	0		0

*MC1=Mill Creek sample site #1, MC2=Mill Creek sample site #2.

Figure 12. Monthly Enterococci Observations for Mill Creek Sampling Site 1 and 2 from table 23 with Miscellaneous Wildlife and Deer combined.



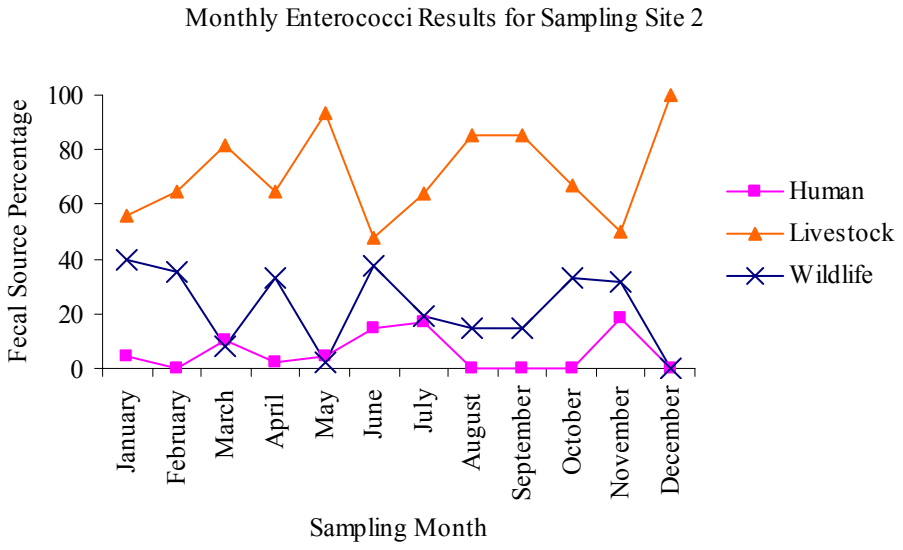


Table 24. Daily source classifications (%) for *Enterococcus* large library, specific source categories, High Flow conditions, winter 2001.

Category	Large Library (n=1158)		
	MC1		**MC2**
Human	7.9	Day 1	0
Cattle	62.1		83.3
Deer	20.0		14.6
Wildlife	10.0		0
		Day 2	
Human	2.1		0
Cattle	47.9		72.9
Deer	27.1		27.1
Wildlife	22.9		0
		Day 3	
Human	0		14.6
Cattle	58.3		64.6
Deer	27.1		20.8
Wildlife	14.6		0
		Day 4	
Human	0		10.2
Cattle	79.2		56.3
Deer	8.0		27.1
Wildlife	12.8		6.4
		Day 5	
Human	0		10.4
Cattle	58.3		52.3
Deer	41.7		24.8
Wildlife	0		12.5
		Day 6	
Human	4.2		8.3
Cattle	56.2		68.8
Deer	6.3		8.3
Wildlife	33.3		22.9
		Day 7	
Human	4.2		2.7
Cattle	60.4		67.6
Deer	31.2		27
Wildlife	4.2		2.7

Table 24. Continued

		7-Day Avg.
Human	15.8	4.8
Cattle	47.0	72.2
Deer	23.5	21.8
Wildlife	13.7	1.2

*Large library = 1158 isolates.

Table 25. Daily source classifications (%) for *Enterococcus* large library, specific source categories Low Flow conditions, fall 2001.

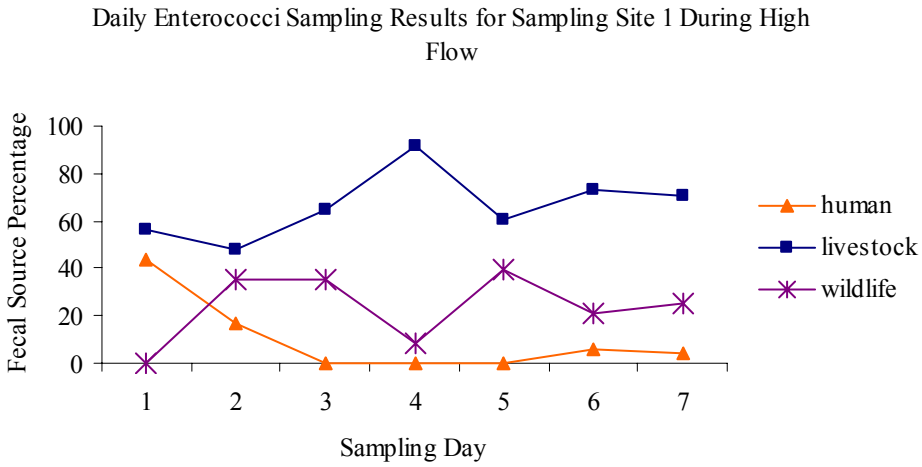
Category	Large Library (n=1158)		
	MC1		**MC2**
Human	0	Day 1	0
Cattle	83.3		64.6
Deer	16.7		20
Wildlife	0		15.4
		Day 2	
Human	0		0
Cattle	41.7		54.2
Deer	22.9		20
Wildlife	35.4		25.8
		Day 3	
Human	7.1		0
Cattle	72.9		100
Deer	20		0
Wildlife	0		0
		Day 4	
Human	0		0
Cattle	100		82.1
Deer	0		15.8
Wildlife	0		2.1
		Day 5	
Human	15		9.2
Cattle	43.7		70.8
Deer	31.3		10
Wildlife	10		10
		Day 6	
Human	10.4		10.4
Cattle	89.6		83.3
Deer	0		6.3
Wildlife	0		0
		Day 7	
Human	0		0
Cattle	70		68.3
Deer	15		11.6
Wildlife	15		20.1

Table 25. Continued

		7-Day Avg.
Human	12.2	12.5
Cattle	52.1	59.3
Deer	16.4	9.2
Wildlife	19.3	19

*Large library = 1158 isolates.

Figure 13. Daily Enterococci Observations for Mill Creek Sampling Site 1 during High Flow and Low Flow From tables 24 and 25 .



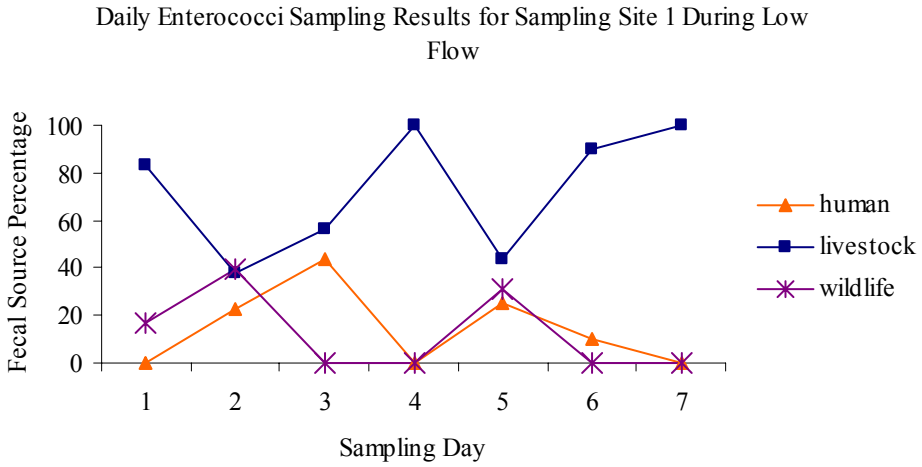
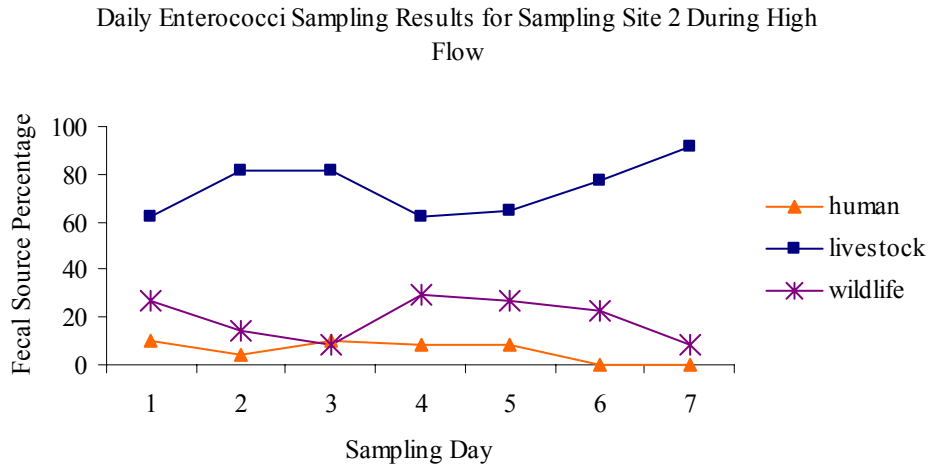


Figure14. Daily Enterococci Observations for Mill Creek Sampling Site 2 during High Flow and Low Flow From tables 24 and 25 .



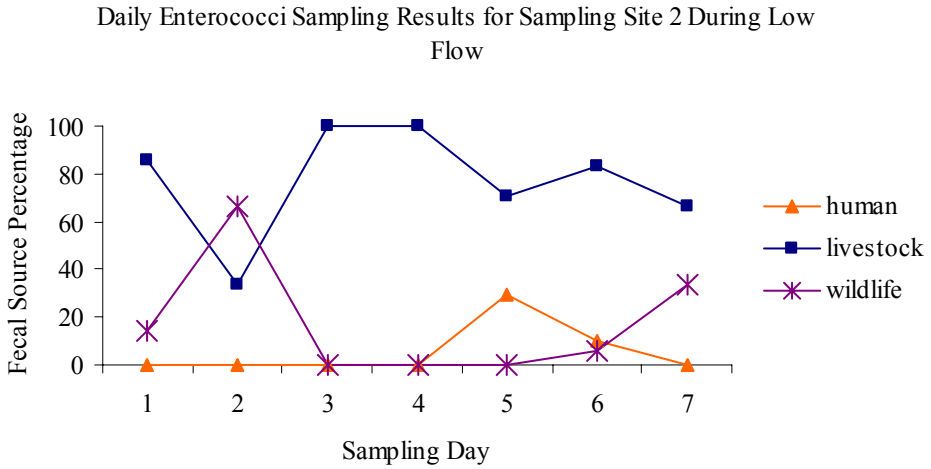


Table 26. Weekly source classifications (%) for *Enterococcus* large library, specific source categories, High Flow conditions, winter 2001 and Low Flow conditions, fall 2001.

Category	Large Library (n=1158)	
	MC1	**MC2**
	High Flow	
Human	6.3	0
Cattle	83.7	64.6
Deer	10	25
Wildlife	0	10.4
	Week 1	
Human	0	10.4
Cattle	58.3	77.1
Deer	27.1	12.5
Wildlife	14.6	0
	Week 2	
Human	0	8.3
Cattle	52.1	56.3
Deer	47.9	35.4
Wildlife	0	0
	Week 3	
Human	0	8.3
Cattle	81.2	68.8
Deer	18.8	22.9
Wildlife	0	0
	Week 4	
Human	1.6	6.8
Cattle	39	50
Deer	32.3	40.7
Wildlife	27.1	2.5
	4-Wk Avg.	
	Low Flow	
	Week 1	
Human	0	10.4
Cattle	83.3	79.2
Deer	0	0
Wildlife	16.7	10.4
	Week 2	
Human	0	0
Cattle	70.8	67.9

Table 26. Continued.

Deer	0		20
Wildlife	29.2		12.1
		Week 3	
Human	0		0
Cattle	100		82.1
Deer	0		15.8
Wildlife	0		2.1
		Week 4	
Human	0		0
Cattle	70		68.3
Deer	15		11.6
Wildlife	15		20.1
		4-Wk. Avg.	
Human	0		6.2
Cattle	63.5		54.2
Deer	0		21.9
Wildlife	36.5		17.7

Figure 15. Weekly Enterococci Results for Sampling Site 1 During High Flow and Low Flow from Table 26.

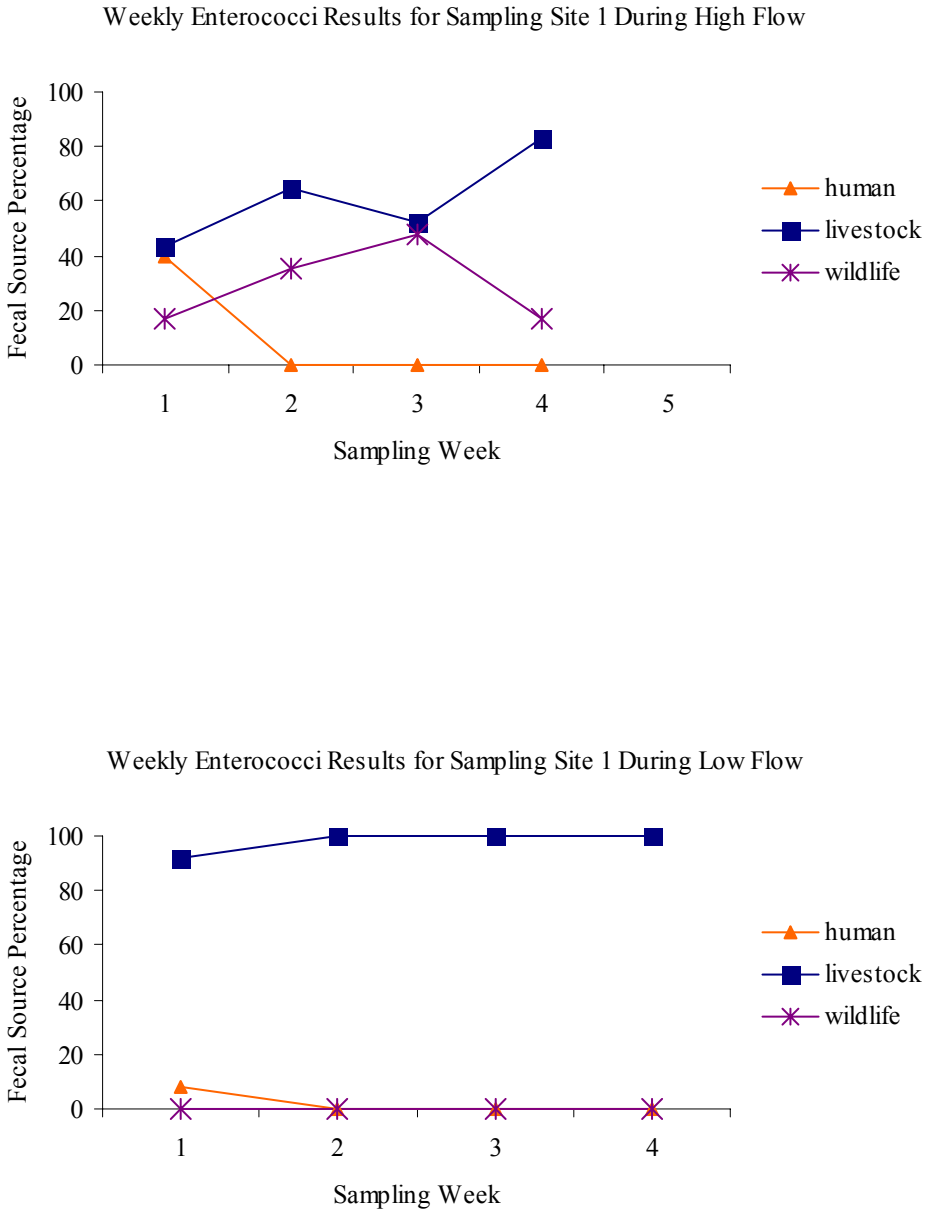
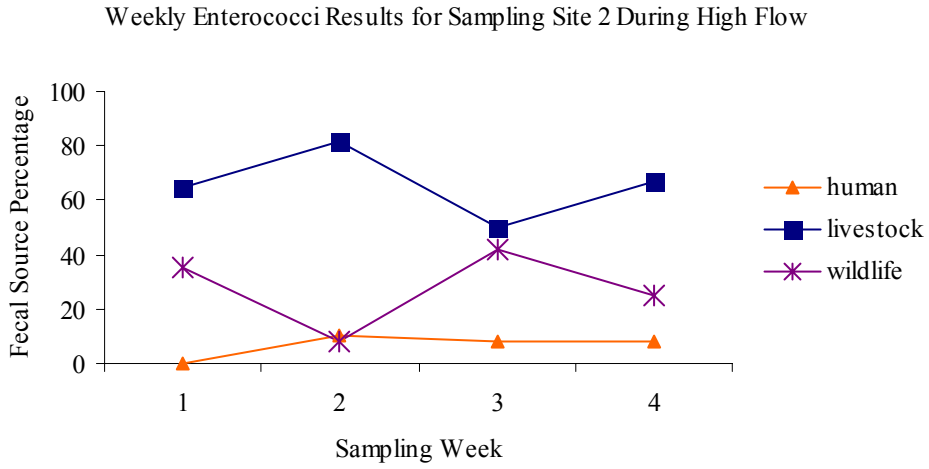


Figure 16. Weekly Enterococci Results for Sampling Site 2 During High Flow and Low Flow from Table 26.



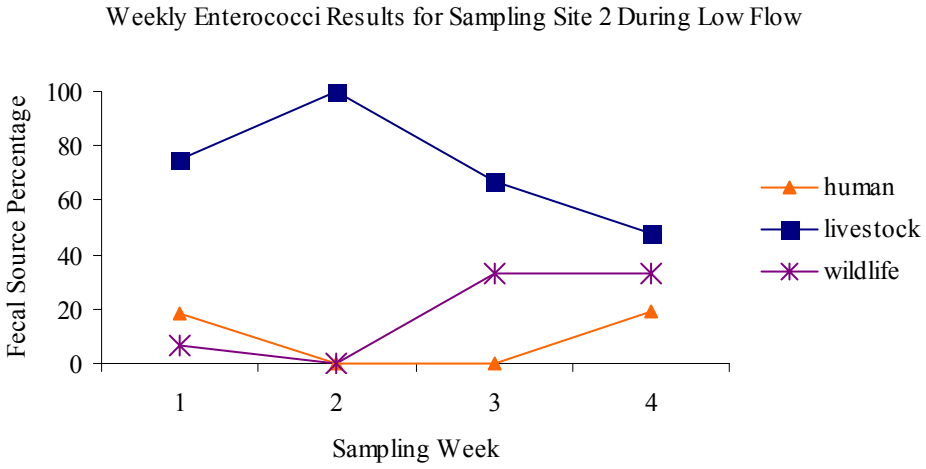


Table 27. Monthly, daily, and weekly human (H), livestock (L), and wildlife (W) source classification averages (%) for Mill Creek *Enterococcus* isolates.

Source (%)	Monthly (12 months)		Daily (7 days)				Weekly (4 weeks)			
			High Flow		Low Flow		High Flow		Low Flow	
	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2
Large Library (1,182 isolates)										
H	2.0	5.9	10.1	5.9	14.6	5.7	9.9	6.8	2.1	9.4
L	76.1	67.8	66.4	74.5	72.9	77.1	60.9	65.6	97.9	72.4
W	21.9	26.3	23.5	19.6	12.5	17.2	29.2	27.6	0.0	18.2
Small Library (500 isolates)										
H	1.0	1.0	11.4	12.2	0.0	0.0	4.1	3.2	0.0	0.0
L	68.9	63.2	46.7	57.0	83.6	71.4	47.4	31.2	100.0	90.1
W	30.1	35.8	41.9	30.8	16.4	28.6	48.5	65.6	0.0	9.9

Table 28: **Monthly, daily, and weekly human (H), cattle (C), deer (D), and miscellaneous wildlife (W) source classification averages (%) for Mill Creek *Enterococcus* isolates.**

Source	Monthly (12 months)		Daily (7 days) High Flow		Daily (7 days) Low Flow		Weekly (4 weeks) High Flow		Weekly (4 weeks) Low Flow	
(%)	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2	MC-1	MC-2
Large Library (1,182 isolates)										
H	1.4	4.2	2.6	4.8	4.6	2.8	1.7	6.8	0.0	2.6
C	71.1	66.4	60.3	72.2	71.6	74.8	68.8	66.7	81.0	74.4
D	19.9	24.7	23.1	21.8	15.2	12.0	25.9	23.9	3.7	11.8
W	7.6	4.7	14.0	1.2	8.6	10.4	3.6	2.6	15.3	11.2

The species-specific analysis (Table 28) tended to reduce the human signature (as compared to the human, livestock, and wildlife analysis), and the reduction did appear to be substantial for several of the averages. This reduction is probably related to the species-specific library having a miscellaneous wildlife category that was not part of the human, livestock, and wildlife library, and a substantial deer signature being present throughout the year (Table 28). The cattle signature in Table 28 was very close to the livestock signature in Table 27 (large library) and the deer signature plus miscellaneous wildlife signature in Table 28 was also very close to the broader wildlife category in Table 27.

The human, livestock, and wildlife classifications indicated that the human signature was highest in MC-1 (Table 27, daily low flow); while the source-specific classifications indicated that the human signature was highest for MC-2 (Table 28, weekly high flow). If the 4.6% average misclassification for human (based on human, livestock, and wildlife, Figure 10) is applied to Table 27 (large library), the signature is subtracted to zero for two of the ten averages, monthly for MC-1 and low flow weekly for MC-1. By comparison, if the 3.6% average misclassification for human (based on species-specific classification, Figure 11) is applied to Table 31, the signature is subtracted to zero for six of the ten averages (monthly MC-1, daily high flow MC-1 and daily low flow MC-2, weekly high flow MC-1, and both sites, weekly low flow). The basic conclusion for the source-specific classification is that this approach demonstrated the same major result as the 3-way classification; that cattle (livestock) comprised an overwhelming signature that dominated over both the sampling scheme and seasonality. There was a human signature present for both analyses, but it was small, and deer dominated the wildlife signature (although this could be affected by the composition of the known source library). Although the human signature was small, it does demonstrate a lot more variability. The difference between 1% and 2% is 100%. Large signatures, such as the one from cattle don't vary so much (60 to 81% Table 28).

D. Mill Creek Site 1 compared to Mill Creek Site 2

There were two sampling sites designated as MC1 and MC2. The null hypothesis was based on the fact that site MC1 would be sampled, and site MC2 was included to capture any bacterial variation. The question to be addressed was whether or not is was necessary to sample at both site MC1 and MC2 monthly, weekly, and daily within the weekly seasonal flow conditions to capture bacterial variation. The null hypothesis was that it was necessary to sample at both sites to capture bacterial variation.

MC1 and MC2 were treated as two independent groups. The goal was to estimate the group means and use the t-test to determine if there was a significant difference in the bacterial variation among *E.coli* and *Enterococcus* between the two sites. The t-Test was used because two populations (MC1 & MC2) were compared to determine if the two populations had different means. The result of the t-Test is the P-value (Townsend, 2001). The P-value or (probability value) is the probability of getting a value of the sample test statistic that is at least as extreme as the one found from the sample data, assuming that the null hypothesis is true (Triola, 2001). In other words if one wants to reject the null hypothesis, a small p value is desirable. The p value is the probability of being wrong if an effect to be non-null is declared; the probability of rejecting the true null hypothesis (Sall et al., 2001). In other words, the p value was evaluated to determine whether the sample result was unusual. The power of the test was the probability of being right when one says that there is a difference.

D.1. E. coli

When comparing the means of MC1 vs. MC2, there was not enough information to prove that there was a significant difference in the sources of isolates from the two sites. Thus, the null hypothesis was rejected and it was not necessary to sample at both sites (Table 29). There was no significant difference among this study's 24 observations (monthly, weekly, daily). The power detail generated within the JMP's oneway analysis function provides the ability to determine the amount of error associated with the sample size. Using the sample size of 24

observations the power detail (The sensitivity of our test or the possible error) report from the oneway analysis function reveals the following…

- A possible error as large a 2.67 for Human. If there is an error for the Human findings, it is 95% likely to be less than 2.67%.
- A possible error as large as 13.16 for Cattle. If there is an error for the Cattle findings, it is 95% likely to be less than 13.16%.
- A possible error as large as 0.5 for Horse. If there is an error for the Horse findings, it is 95% likely to be less than 0.5%.
- A possible error as large as 10.3 for Deer. If there is an error for the Deer findings, it is 95% likely to be less than 10.3%.
- A possible error as large as 5.4 for Goose. If there is an error for the Goose findings, it is 95% likely to be less than 5.4%.
- A possible error as large as 6.8 for Miscellaneous Wildlife. If there is an error for the Goose findings, it is 95% likely to be less than 6.8%.

The possible error values for *E. coli* supports the findings from the average misclassifications analysis (Figure 4). The average misclassifications for human were 2.0%, cattle was 3.1%, horse was 1.0%, deer was 4.7%, goose was 2.7% and 0.0% for miscellaneous wildlife. Each of the aforementioned values is lower than the possible error value determined in the power report.

D.2. Enterococci

When comparing the means of MC1 vs. MC2, there was not enough information to prove that the there was a significant difference in the sources of isolates from the two sites. Thus, the null hypothesis was rejected (Table 30). There was no significant difference among this study's 24 observations (monthly, weekly, daily). The power detail generated within the JMP's oneway analysis function provides the ability to determine the amount of error associated with the sample size. Using the sample size of 24 observations the power detail (The sensitivity of the test or the possible error) report reveals the following…

- A possible error as large as 4.3 for Human. If there is an error for the Human findings, it is 95% likely to be less than 4.3%.
- A possible error as large as 14.3 for Cattle. If there is an error for Cattle, it is 95% likely to be less than 14.3%.
- A possible error as large as 13.2 for Deer. If there is an error, it is 95% likely to be less than 13.2%.
- A possible error as large as 6.2 for Miscellaneous Wildlife. If there is an error for the Miscellaneous Wildlife findings, it is 95% likely to be less than 6.2%.

The possible error values for *enterococci* support the findings from the enterococcus known source library average rate of misclassification (Section C1.a, Figure 10). According to the average misclassifications for human was 3.6%, livestock was 8.3%, deer was 2.9, and 1.1% for miscellaneous wildlife. Each of the aforementioned values was lower than the possible error value determined in the power report.

Table 29. *E. coli* P values of MC1 compared to MC2 using the t-Test

	P value				
	Human	Cattle	Horse	Deer	Wildlife
Assuming Equal Variances	0.8928	0.0625	0.1523	0.2855	0.4825
Unequal Variances	0.8928	0.0628	0.1662	0.2862	0.4887

A P value of less than 0.05 is regarded as significant.

Table 30. Enterococci P values of MC1 compared to MC2 using the t-Test

	P value			
	Human	Cattle	Deer	Wildlife
Assuming Equal Variances	0.1934	0.5059	0.6102	0.3443
Unequal Variances	0.1964	0.5061	0.6102	0.3443

A P value of less than 0.05 is regarded as significant.

D.3. Summary of Mill Creek Site 1 compared to Mill Creek Site 2.

In statistics, showing that there is no significant difference is the same as saying results are inconclusive. Thus, it was necessary to provide a report detailing the sensitivity of the test. The sensitivity of the test provides the option to collect more data if there is a high margin of error. In most BST publications, databases with an ARCC of 80% or greater are widely accepted as an excellent database. This leaves a 20% margin of error. With the 24 observations of *E.coli* sources the error was 13.16% or less and for the *enterococci* the error was 14.3% or less. These two values are lower than the accepted 20% margin of error, thus there was no need to collect more data.

E. Monthly Sampling Versus Weekly Sampling Versus Daily Sampling

The two sampling sites designated as MC1 and MC2 were sampled monthly, weekly, and daily within the weekly seasonal flow conditions. The null hypothesis was based on the fact that sampling was performed monthly, weekly, and daily to capture any bacterial variation. The question to be addressed was whether or not it was necessary to sample at both site MC1 and MC2 weekly, and daily within seasonal flow conditions to capture bacterial variation. The null hypothesis was that it was only necessary to sample monthly to capture bacterial variation.

The monthly, weekly, and daily observations were treated as three independent groups. The goal was to estimate the group means and use one-way analysis to determine if there was a significant difference in the bacterial variation among the monthly, weekly, and daily observations.

E. 1. *E .coli*

When comparing the means of the monthly, weekly, and daily observations there were some significant differences in the sources of isolates from the sampling period. Thus, the null hypothesis was rejected (Table 31). Monthly and daily observations were only different for

cattle, thus daily sampling can't be justified. Monthly and weekly observations were different for human, horse, deer, goose, and wildlife. It would appear that weekly sampling would be best however, one has to consider the fecal source proportion for the area. Cattle are the overwhelming major fecal source contributor to the watershed and since there was not a significant difference among the cattle it would appear that monthly sampling was adequate.

E. 2. Enterococci

When comparing the means of the monthly, weekly, and daily observations there were no significant differences in the sources of isolates from the sampling period. Thus, the null hypothesis was accepted (Table 32). Clearly monthly sampling was adequate to capture a representative sample of fecal source isolates.

Table 31. *E. coli* Monthly versus Weekly versus Daily Means.

Sampling Period	Human	Cattle	Horse	Deer	Goose	Wildlife
Monthly	3.14B	64.65A	0.18B	22.55A	1.63B	7.71A
Weekly	12.5A	59.35AB	19.95A	6.23B	10.29A	0.51B
Daily	6.08B	47.2B	0.14B	25.94A	0.15B	8.46A

Means not connected by the same letter are significantly different.

Table 32. *Enterococci* Monthly versus Weekly versus Daily Means.

Sampling Period	Human	Cattle	Deer	Wildlife
Monthly	2.78A	68.7A	22.30A	6.16A
Weekly	2.73A	72.73A	17.98A	8.16A
Daily	3.71A	69.7A	16.38A	8.58A

Means not connected by the same letter are significantly different.

E. 3. Summary of Monthly Sampling Versus Weekly Sampling Versus Daily Sampling

Mill Creek is a worthy candidate for TMDL implementation based on the fecal coliform counts, however, Mill Creek may have not been the best candidate for a sampling frequency study. After, evaluating the percent pasture use and the number of cattle in Mill Creek it was obvious that one could predict the large cattle signature without performing the laboratory work. A watershed with a more mixed land use pattern would have been more appropriate.

F. Isolate Comparison-48, 36, 24, 12, and 6 isolates

The two sampling sites designated as MC1 and MC2 were sampled monthly, weekly and daily within the weekly seasonal flow conditions. ARA was performed on the samples using 48 randomly selected CFUs. Most researchers employing ARA use 48 CFUs because of convenience as the microwell plates have 48 inoculating wells on each side of the plate. The question to be addressed was whether or not it was necessary to use 48 isolates at both site MC1 and MC2 monthly, weekly, and daily within the weekly seasonal flow conditions to capture bacteria variation. One-way analysis was used to determine if the means of 48, 36, 24, 12, and 6 isolates from the daily samples (Days 1-6) from site MC1 and MC2 during the high flow sampling period and the low flow sampling period were significantly different. Day 7 was omitted from the analysis because there was an abundant growth of non-fecal coliform bacteria on the m-FC media during the high flow sampling period. The null hypothesis was based on the fact that 48 isolates were used to capture any bacterial variation. The null hypothesis was that is was not necessary to use 48 isolates to capture bacterial variation but it was not known how few isolates could be used.

Each set of randomly selected groups of isolates (48, 36, 24, 12, and 6) were treated as five independent groups. The goal was to estimate the group means and use one-way analysis to determine if there were significant differences in the bacterial variation among the five observations.

F. 1. *E. coli*

When comparing the means of the five observations there were no significant differences among the sources of isolates from either high flow sampling period or the low flow sampling period. Thus, the null hypothesis was rejected for both the high flow sampling period the low flow sampling period (Table 33 and 34). Even though a significant difference was not detected among the means, inspection of the individual days identified some situations where the use of less than 24 isolates was likely not to include some signature sources (Tables 37 and 38). For example, in Table 37, (sampling site 1, sampling day 3) even the predominant source cattle was missed as well as goose and horse when using either 6 or 12 isolates.

F. 2. Enterococci

When comparing the means of the five observations there were no significant differences among the sources of isolates from either high flow sampling period or the low flow sampling period. Thus, the null hypothesis was rejected for both the high flow sampling period the low flow sampling period (Table 35 and 36). Even though a significant difference was not detected among the means, inspection of the individual days identified some situations where the use of less than 24 isolates was likely not to include some signature sources (Tables 39 and 40). For example, in table 39, (sampling site 2, sampling day 6) signature sources deer, human, and wildlife were missed when using either 6 or 12 isolates.

F. 3. Summary of Isolate Comparisons-48, 36, 24, 12 and 6 isolates.

As Mill Creek may have not been the best candidate for a sampling frequency study, Mill Creek may not have been the best candidate for an isolate comparison study as well. After evaluating the percent pasture use and the number of cattle in Mill Creek it was obvious that one could predict the large cattle signature without performing the laboratory work. The overwhelming dominant cattle signature overpowered the analysis. Regardless of the number isolates used, cattle was always predominant. A watershed with a more mixed land use pattern

would have been more appropriate to actually see what numbers of isolates are adequate for source identification.

Table 33. Observed differences among the mean fecal source identification using 48, 36, 24, 12 and 6 randomly selected *E. coli* isolates during high flow.

Number of Isolates	Fecal Sources						
	Cattle	Deer	Goose	Horse	Human	Wildlife	Total
6	29.2 A	11.1 A	16.7 A	13.9 A	27.8 A	1.4 A	100
12	33.3 A	7.6 A	13.9 A	15.3 A	28.5 A	1.4 A	100
24	40.1 A	8.3 A	11.5 A	15.0 A	24.7 A	0.7 A	100
36	38.8 A	8.0 A	13.0 A	13.0 A	26.4 A	0.5 A	100
48	37.5 A	8.0 A	14.7 A	13.9 A	25.5 A	0.3 A	100

Means not connected by the same letter are significantly different.

Table 34. Observed differences among the mean fecal source identification using 48, 36, 24, 12 and 6 randomly selected *E. coli* isolates during low flow.

Number of Isolates	Fecal Sources						
	Cattle	Deer	Goose	Horse	Human	Wildlife	Total
6	55.6 A	16.7 A	1.4 A B	6.9 A	19.4 A	0.0 A	100
12	50.7 A	18.2 A	1.5 A B	7.6 A	21.9 A	0.0 A	100
24	48.5 A	11.7 A	0.7 A B	15.5 A	44.7 A	0.0 A	100
36	44.7 A	9.9 A	0.7 A B	14.1 A	30.5 A	0.0 A	100
48	47.5 A	8.9 A	0.2 B	10.4 A	34.7 A	0.0 A	100

Means not connected by the same letter are significantly different.

Table 35. Observed differences among the mean fecal source identification using 48, 36, 24, 12 and 6 randomly selected *Enterococci* isolates during high flow.

Number of Isolates	Fecal Sources				
	Cattle	Deer	Human	Wildlife	Total
6	68.1 A	20.8 A	8.3 A	2.8 A	100
12	67.4 A	20.1 A	10.4 A	2.1 A	100
24	59.3 A	20.5 A	12.5 A	7.7 A	100
36	57.8 A	21.6 A	11.1 A	9.5 A	100
48	57.2 A	22.8 A	11.6 A	8.3 A	100

Means not connected by the same letter are significantly different.

Table 36. Observed differences among the mean fecal source identification using 48, 36, 24, 12 and 6 randomly selected *Enterococci* isolates during low flow.

Number of Isolates	Fecal Sources				
	Cattle	Deer	Human	Wildlife	Total
6	52.8 A	11.1 A	18.1 A	18.1 A	100
12	50.7 A	13.9 A	16.7 A	18.8 A	100
24	53.5 A	10.8 A	16.3 A	19.4 A	100
36	52.5 A	12.5 A	19.4 A	15.6 A	100
48	53.8 A	12.1 A	17.3 A	16.9 A	100

Means not connected by the same letter are significantly different.

Table 37. Isolate Comparisons for *E.coli* during High Flow.

Number of Isolates	Site number	sampling date	Cattle	Deer	Goose	Horse	Human	Wildlife
6	1	1	66.67	0	0	0	33.33	0
12	1	1	33.33	0	0	0	66.67	0
24	1	1	20.83	0	0	0	79.17	0
36	1	1	19.44	0	0	0	80.56	0
48	1	1	20.83	0	0	0	79.17	0
6	2	1	0	66.67	0	33.33	0	0
12	2	1	33.33	33.33	0	25	8.33	0
24	2	1	41.67	29.17	4.17	20.83	4.17	0
36	2	1	38.89	25	5.56	16.67	13.89	0
48	2	1	41.67	27.08	6.25	12.5	12.5	0
6	1	2	33.33	33.33	0	0	33.33	0
12	1	2	58.33	25	0	0	16.67	0
24	1	2	54.17	33.33	0	0	12.5	0
36	1	2	50	41.67	0	0	8.33	0
48	1	2	54.17	39.58	0	0	6.25	0
6	2	2	50	0	33.33	0	16.67	0
12	2	2	50	8.33	16.67	16.67	8.33	0
24	2	2	62.5	4.17	12.5	12.5	8.33	0
36	2	2	58.33	5.56	8.33	13.89	13.89	0
48	2	2	58.33	6.25	6.25	16.67	12.5	0
6	1	3	0	33.33	0	0	66.67	0
12	1	3	0	16.67	16.67	0	66.67	0
24	1	3	37.5	8.33	8.33	8.33	37.5	0
36	1	3	30.56	5.56	11.11	5.56	47.22	0
48	1	3	35.42	4.17	8.33	4.17	47.92	0
6	2	3	16.67	0	33.33	0	50	0
12	2	3	8.33	0	16.67	0	75	0
24	2	3	25	0	8.33	0	66.67	0
36	2	3	38.89	0	5.56	0	55.56	0
48	2	3	37.5	0	6.25	0	56.25	0
6	1	4	83.33	0	16.67	0	0	0
12	1	4	66.67	8.33	25	0	0	0
24	1	4	50	8.33	41.67	0	0	0
36	1	4	47.22	8.33	44.44	0	0	0
48	1	4	38.3	6.38	55.32	0	0	0
6	2	4	50	0	16.67	33.33	0	0
12	2	4	50	0	8.33	41.67	0	0
24	2	4	60.87	4.35	4.35	30.43	0	0
36	2	4	60	2.86	17.14	20	0	0
48	2	4	59.57	2.13	19.15	17.02	2.13	0
6	1	5	33.33	0	16.67	0	50	0
12	1	5	41.67	0	8.33	8.33	41.67	0
24	1	5	37.5	4.17	12.5	4.17	41.67	0
36	1	5	27.78	2.78	25	2.78	41.67	0

Table 37 Continued

48	1	5	25	2.08	25	6.25	41.67	0
6	2	5	0	0	66.67	16.67	0	16.67
12	2	5	16.67	0	50	8.33	8.33	16.67
24	2	5	33.33	4.17	29.17	16.67	8.33	8.33
36	2	5	38.89	2.78	22.22	13.89	16.67	5.56
48	2	5	35.42	2.08	31.25	10.42	16.67	4.17
6	1	6	0	0	16.67	16.67	66.67	0
12	1	6	16.67	0	16.67	25	41.67	0
24	1	6	12.5	0	8.33	45.83	33.33	0
36	1	6	13.89	0	8.33	50	27.78	0
48	1	6	12.5	0	10.42	54.17	22.92	0
6	2	6	16.67	0	0	66.67	16.67	0
12	2	6	25	0	8.33	58.33	8.33	0
24	2	6	45.83	0	8.33	41.67	4.17	0
36	2	6	41.67	5.56	8.33	33.33	11.11	0
48	2	6	31.25	6.25	8.33	45.83	8.33	0

Table 38. Isolate Comparison For E. coli during Low Flow

No. of Isolates	Site number	Sampling Date	Cattle	Deer	Goose	Horse	Human	Wildlife
6	1	1	33.33	16.67	0	0	50	0
12	1	1	58.336	16.67	0	0	25	0
24	1	1	66.67	12.5	0	0	20.83	0
36	1	1	69.44	8.33	0	0	22.22	0
48	1	1	70.83	6.25	0	0	22.92	0
6	2	1	83.33	0	16.67	0	0	0
12	2	1	91.67	0	8.33	0	0	0
24	2	1	83.33	0	4.17	12.5	0	0
36	2	1	80.56	0	2.78	16.67	0	0
48	2	1	83.33	0	2.08	14.58	0	0
.48	1	2	37.5	25	6.25	25	6.258	0
6	1	2	66.67	16.67	0	16.67	0	0
12	1	2	41.67	25	8.33	25	0	0
24	1	2	33.33	29.17	4.17	33.33	0	0
36	1	2	30.56	27.78	5.56	33.33	2.78	0
6	2	2	50	0	0	0	50	0
12	2	2	58.33	0	0	0	41.67	0
24	2	2	70.83	0	0	0	29.17	0
36	2	2	75	0	0	0	25	0
48	2	2	72.92	0	0	0	27.08	0
6	1	3	100	0	0	0	0	0
12	1	3	91.67	0	0	0	8.33	0
24	1	3	95.83	0	0	0	4.17	0
36	1	3	94.44	0	0	0	5.56	0
48	1	3	91.67	0	0	0	8.33	0
6	2	3	50	50	0	0	0	0
12	2	3	58.33	41.67	0	0	0	0
24	2	3	75	20.83	0	0	4.17	0
36	2	3	63.89	19.44	0	0	16.67	0
48	2	3	64.58	14.58	0	0	20.83	0
6	1	4	50	16.67	0	33.33	0	0
12	1	4	25	50	0	25	0	0
24	1	4	20.83	25	0	54.17	0	0
36	1	4	13.89	25	0	55.56	5.56	0
48	1	4	16.67	33.33	0	45.83	4.17	0
6	2	4	100	0	0	0	0	0
12	2	4	91.67	8.33	0	0	0	0
24	2	4	45.83	8.33	0	20.83	258	0
36	2	4	30.56	5.56	0	13.89	50	0
48	2	4	27.08	6.25	0	10.42	56.25	0
6	1	5	33.33	33.33	0	16.67	16.67	0
12	1	5	41.67	25	0	25	8.33	0
24	1	5	29.17	16.67	0	25	29.17	0
36	1	5	33.33	11.11	0	19.44	36.11	0
48	1	5	27.08	8.33	0	20.83	43.75	0

Table 38 Continued

6	2	5	100	0	0	0	0	0
12	2	5	0	33.33	0	0	66.67	0
24	2	5	12.5	16.67	0	0	70.83	0
36	2	5	30.56	5.56	0	13.89	50	0
48	2	5	27.08	6.25	0	10.42	56.25	0
6	1	6	0	0	0	16.67	83.33	0
12	1	6	0	0	0	8.33	91.67	0
24	1	6	0	0	0	25	75	0
36	1	6	0	5.56	0	16.67	77.78	0
48	1	6	0	14.58	0	12.5	72.92	0
6	2	6	0	66.67	0	0	33.33	0
12	2	6	13.89	11.11	0	0	75	0
24	2	6	22.92	8.33	0	0	68.75	0

Table 39. Isolate Comparisons for Enterococci during High Flow

Number of Isolates	Site number	Sampling Date	Cattle	Deer	Human	Wildlife
6	1	1	0	100	0	0
12	1	1	0	100	0	0
24	1	1	62.5	0	37.5	0
36	1	1	66.67	0	33.33	0
48	1	1	83.33	16.67	0	0
6	1	2	50	33.33	0	16.67
12	1	2	75	16.67	0	8.33
24	1	2	54.17	25	0	20.83
36	1	2	44.44	25	2.78	27.78
48	1	2	52.08	0	47.92	0
6	1	3	66.67	33.33	0	0
12	1	3	66.67	33.33	0	0
24	1	3	41.67	45.83	0	12.5
36	1	3	36.11	44.44	0	19.44
48	1	3	47.92	27.08	2.08	22.92
6	1	4	100	0	0	0
12	1	4	91.67	0	0	8.33
24	1	4	66.67	0	0	33.33
36	1	4	75	0	0	25
48	1	4	27.08	58.33	0	14.58
6	1	5	83.33	16.67	0	0
12	1	5	66.67	33.33	0	0
24	1	5	62.5	37.5	0	0
36	1	5	63.89	36.11	0	0
48	1	5	79.17	0	0	20.83
6	1	6	16.67	0	66.67	16.67
12	1	6	16.67	0	75	8.33
24	1	6	8.33	8.33	62.5	20.83
36	1	6	5.56	5.56	58.33	30.56
48	1	6	58.33	41.67	0	0
6	2	1	50	33.33	16.67	0
12	2	1	50	16.67	33.33	0
24	2	1	50	29.17	20.83	0
36	2	1	50	36.11	13.89	0
48	2	1	4.17	6.25	56.25	33.33
6	2	2	83.33	16.67	0	0
12	2	2	91.67	8.33	0	0
24	2	2	83.33	16.67	0	0
36	2	2	75	25	0	0
48	2	2	60.42	29.17	10.42	0
6	2	3	100	0	0	0
12	2	3	100	0	0	0
24	2	3	83.33	4.17	12.5	0
36	2	3	80.56	5.56	13.89	0
48	2	3	72.92	22.92	4.17	0

Table 39. Continued

6	2	4	66.67	16.67	16.67	0
12	2	4	66.67	25	8.33	0
24	2	4	45.83	50	4.17	0
36	2	4	61.11	36.11	2.78	0
48	2	4	77.08	12.5	10.42	0
6	2	5	100	0	0	0
12	2	5	83.33	8.33	8.33	0
24	2	5	65.22	17.39	13.04	4.35
36	2	5	51.43	28.57	8.57	11.43
48	2	5	62.5	35.42	2.08	0
6	2	6	100	0	0	0
12	2	6	100	0	0	0
24	2	6	87.5	12.5	0	0
36	2	6	83.33	16.67	0	0
48	2	6	61.7	23.4	6.38	8.51

Table 40. Isolate Comparisons for Enterococci during Low Flow

Number of Isolates	Site number	Sampling Date	Cattle	Deer	Human	Wildlife
6	1	1	0	100	0	0
12	1	1	0	100	0	0
24	1	1	79.17	8.33	12.5	0
36	1	1	77.78	8.33	13.89	0
48	1	1	16.67	83.33	0	0
6	2	1	100	0	0	0
12	2	1	91.67	0	0	8.33
24	2	1	83.33	0	16.67	0
36	2	1	75	0	0	25
48	2	1	64.58	0	0	35.42
6	1	2	33.33	0	33.33	33.33
12	1	2	33.33	0	25	41.67
24	1	2	29.17	70.83	0	0
36	1	2	19.447	80.56	0	0
48	1	2	41.67	0	22.92	35.42
6	2	2	33.33	0	0	66.67
12	2	2	58.33	0	0	41.67
24	2	2	79.17	0	0	20.83
36	2	2	55.56	0	0	44.44
48	2	2	54.17	0	0	45.83
6	1	3	83.33	0	16.67	0
12	1	3	75	0	25	0
24	1	3	41.67	0	25	33.33
36	1	3	36.11	0	22.22	41.67
48	1	3	72.92	0	27.08	0
6	2	3	100	0	0	0
12	2	3	100	0	0	0
24	2	3	58.33	0	0	41.67
48	2	3	0	0	47.92	52.08
6	1	4	0	0	0	100
12	1	4	0	0	0	100
24	1	4	66.67	0	33.33	0
36	1	4	72.22	0	27.78	0
48	1	4	43.75	31.25	25	0
6	2	4	0	0	83.33	16.67
12	2	4	0	0	66.67	33.33
24	2	4	100	0	0	0
36	2	4	70.83	0	29.17	0
6	1	5	100	0	0	0
12	1	5	50	50	0	0
24	1	5	0	0	0	100
36	1	5	41.67	36.11	22.22	0
48	1	5	89.58	0	10.42	0

Table 40. Continued

6	2	5	100	0	0	0
12	2	5	58.33	0	41.67	0
24	2	5	0	0	62.5	37.5
36	2	5	0	0	55.56	44.44
48	2	5	83.33	6.25	10.42	0
6	1	6	33.33	0	66.67	0
12	1	6	66.67	0	33.33	0
24	1	6	50	50	0	0
36	1	6	86.11	0	13.89	0
6	2	6	50	33.33	16.67	0
12	2	6	75	16.67	8.33	0
24	2	6	54.17	0	45.83	0
36	2	6	61.11	0	38.89	0

V. CONCLUSIONS

Antibiotic resistance analysis (ARA) was successful in determining sources of fecal pollution in Mill Creek, Montgomery County, VA, as a function of sampling frequency under low and high stream flow conditions. There is a human signature in Mill Creek, however it is small compared to wildlife and the overwhelming proportion of isolates from livestock. Restricting livestock access to streams can dramatically lower fecal coliform counts during the unusually hot and dry periods. Reducing FC counts to below recreational water standards for Virginia (1000 per 100ml for any one sample) may be achievable, however, to maintain streams below standards may prove to be difficult, as Mill Creek is in an area where there are large populations of deer, and other wildlife, and will be hard to restrict these animals.

During the series of sampling, standard methods used to perform bacterial monitoring, of enterococci and fecal coliform populations from all stream samples supported Mill Creek being placed on the TMDL list. The knowledge of the sources of pollution is an important factor in determining the degree of risk for humans exposed to contaminated water. It is desirable to be able to determine the sources of fecal contamination, both to assess the risk to the people who are exposed to the waters and to assist in the development of BMPs to reduce fecal loading. Some recommended BMPs include stream fencing, establishing riparian buffers, as well as installing in-pasture watering stations.

Because the Mill Creek study area was heavily influence by cattle, Mill Creek was not the best choice to determine sampling frequency or the optimum number of isolates necessary to adequately identify sources of fecal pollution. A watershed with a more diverse land use pattern is more appropriate to adequately determine sampling frequency and the optimum number of isolates necessary for fecal source identification.

VITA

The author was born on July 12, 1976 to Alexander and Claretta Graves and raised in Yanceyville, NC. She graduated from Bartlett Yancey Senior High School in 1994 and began her undergraduate studies at Winston-Salem State University in August 1994. In 1998 the author received a Bachelor of Science in Biology with a minor in Chemistry from Winston-Salem State University.

In the fall of 1998 the author began her Master of Science in Crop and Soil Environmental Science at Virginia Tech under the guidance of Dr. Charles Hagedorn. She also worked with Dr. Hagedorn on determining sources of fecal pollution in water for a rural Virginia community and this project was sponsored by the Virginia Department of Conservation and Recreation. Her research project focused on the use of a new methodology, bacterial source tracking, to determine the source of fecal contamination in water.

After finishing all required coursework, the author was accepted to begin her studies in the Doctorate program, in the Department of Crop and Soil Environmental Sciences at Virginia Tech. Upon completion of the Doctorate program Alexandria Graves plans to pursue a career in academia.