CHAPTER I

INTRODUCTION

Rationale

Microbial quality of water has been a concern in the United States for around a century, demonstrated by the treatment of drinking water before use and treatment of wastewater prior to discharge into a receiving water body. Control of water pollution has been a federal responsibility since the Environmental Protection Agency (EPA) was formed and the Clean Water Act was authorized in the 1970s (Friends of the Rivers of Virginia (FORVA) 2001). The knowledge of waterborne diseases transmitted via the fecal-oral route has lead to monitoring the nation's waters for indicators of human fecal contamination and to the development of standards for the acceptable levels of indicators. An indicator is used as a measure of fecal pollution so that every possible pathogen need not be tested. The indicator organism used in Virginia is the fecal coliform, a subset of total coliforms (Virginia Department of Environmental Quality (DEQ) 2001a). Total coliforms include *Escherichia, Citrobacter, Enterobacter,* and *Klebsiella,* characterized by "aerobic and facultatively anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that produce gas upon lactose fermentation in prescribed culture media with 48 hours at 35^{o}C" (Gerba in Maier et al 2000). Fecal coliform bacteria include *Klebsiella. Citrobacter,* and *Escherichia,* and are distinguished from total coliforms by fermenting lactose with gas production at 44.5^{o}C (Gerba in Maier et al 2000). The majority of fecal coliforms are in the genus *Escherichia.* The Virginia recreational water standards are 1000 colony forming units (CFU) of fecal coliforms per 100 ml of water for a one-time sample or a geometric mean of 200 CFU per 100 ml if more than one sample is taken in a 30 day period (Virginia DEQ 2001a).

After 30 years of selective monitoring in Virginia, 2,321.6 km of stream have been designated as impaired due to fecal coliform contamination (FORVA 2001). The Virginia DEQ submits a report, called the 305(b) because of the Clean Water Act clause that mandates it, to the EPA every 2 years on the status of water quality in the state. Impaired waters go on a 303(d) list. Most of the impairments in Virginia are suspected to be caused by non-point source pollution. The 1972 Clean Water Act included a section on how to address both point and non-point source pollution, the total maximum daily load (TMDL) process. Briefly, TMDL is a way to describe a pollution problem in a stream and address the problem so that the stream segment may be taken off the impaired waters list. Even though this process has been part of the regulations for over 25 years, it is just now being put into action. Some states are voluntarily adopting TMDL programs, but others are doing it because of court orders. In Virginia, the American Canoe Association (ACA) sued the EPA for Virginia waters that did not meet water quality standards even after point source discharges were relatively under control (ACA 2001). The court sided with the ACA and Virginia developed a TMDL schedule for its impaired waters (DEQ 2001b). The schedule can be found on the DEQ website at http://www.deq.state.va.us/tmdl/10yrsch.html.

Fecal coliform counts that exceed standards are the cause of the majority of water quality impairments in Virginia (EPA 2001b). Therefore, most of the TMDL projects involve fecal pollution. Bacterial source tracking (BST) is a necessary tool for fecal coliform TMDL projects as it provides a semi-quantitative measure of fecal pollution from various sources, an integral part of the TMDL calculation (point source + nonpoint source + margin of safety + seasonal variation = TMDL limit) (EPA 2001a). After a TMDL is developed, best management practices are implemented to reduce the fecal coliform load (and hopefully the pathogen load). Knowing

the main sources and their relative contributions to the loading is the key to deciding which best management practices to apply.

There are many potential sources for fecal coliform contamination. Livestock are major contributors in rural watersheds. The image of a small stream flowing through a dairy farm pasture is common in the Blackwater. Wildlife contributes in rural, suburban and urban settings. Pets can be a problem, especially in urban areas where irresponsible pet owners and stray populations cause untreated waste to flow through storm water drains directly to the stream. Humans may contribute to fecal coliform loading through faulty septic systems, pipe discharges, or ineffective wastewater treatment plants.

Identifying sources of pollution can also help communities/states make risk assessments. Evidence of human contamination indicates human fecal pollution and an increased risk of human viruses such as enteroviruses (poliovirus, coxsakievirus A and B, echovirus and others) (Rotbart 1995). Hepatitus A virus, which causes fever, abdominal pain and nausea followed by jaundice, is stable in the environment and resistant to chlorine water treatment (Maier 2000). Besides viruses, some parasitic and bacterial diseases harbored in wildlife and livestock can invade the human body. *Cryptosporidia parvum* is a protozoal parasite that is transmitted via the fecal/oral route and causes gastroenteritis. *C. parvum*¸ the species that infects humans, has been detected in cattle, white-tailed deer, sheep, horses, pigs, cats, dogs, and other pet and wild mammals (Fayer 1997). *Campylobacter jejuni*, a waterborne bacterium, also causes gastroenteritis. Stern states "no single animal food source can be excluded as a potential vehicle for infection in humans" (Nachamkin et al 1992). This fact could be extended to conclude that many, if not all, animals can contaminate water and disseminate pathogens that could possibly infect humans. *Giardia lamblia*, the cause of "backpacker's disease," is the most encountered

intestinal parasite in the U.S. (Maier 2000). Wild and domestic animals, especially beavers and muskrats carry the parasite and shed vast quantities in the environment. Even a small number of cysts in a water supply can cause infections in humans. Every species of the genus *Salmonella,* a bacterium carried by waterfowl, are pathogenic to humans, and the numbers of cases of salmonellosis are rising in the United States (Maier 2000). Even though bacterial source tracking will not address problems with pathogens carried by humans and animals alike, using BST for restoring water bodies in the TMDL process will aid in reducing the risk of infection.

In light of the demonstrated need to identify the sources of fecal pollution for TMDL development and to estimate the risk of human disease, BST is the best tool currently available. The failure of the historic fecal coliform to fecal streptococcus ratio to indicate the presence of human pollution left mangers without a tool for gauging fecal pollution (American Public Health Association 1995). The recent attention to TMDL has led to the development of several types of methods for BST projects, including both molecular and nonmolecular procedures. Only a few methods have been published or researched in detail at present, including ribotyping, pulsed field gel electrophoresis, randomly amplified polymorphic DNA, fatty acid analysis, coliphage indicators, strain specific profiling, nutritional patterning and chemical detection of human-specific pollution (Bower 2001). Antibiotic resistance analysis (ARA) is a nonmolecular method first reported by Bruce Wiggins (1996), and is based on another method, multiple antibiotic resistance (MAR). The advantages of ARA include simple techniques, the ability to analyze large sample sizes within a relatively short time period, and comparable rates of correct classification with molecular methods. ARA has been the most widely used BST method to date, having been reported in TMDL projects in Florida, Kansas, Oregon, Tennessee, Texas and Virginia and was the method selected for the Blackwater River project.

OBJECTIVES

There were two primary objectives in the Blackwater River project. The first was to build a known source library from enterococcus isolates from fecal samples gathered in the watershed. The second was to compare isolate profiles from stream samples against the known source library to determine the sources of fecal pollution in the Blackwater River.

There were also four secondary objectives involved with the Blackwater BST project. The first was to compare ARA with the Biolog™ metabolic fingerprint system by profiling a set of isolates by both methods and comparing the predicted source of each isolate. The second was to test the geographic restriction of the Blackwater ARA library. This was done by comparing a Blackwater stream sample against a known source library constructed from sources in the Shenandoah Valley, Va, and against the Blackwater library and comparing the results from each library. The third secondary objective was to explore the optimum size of a known source library by creating smaller versions of the Blackwater library, running the discriminant analysis model and comparing the rates of correct classification. The fourth secondary objective was to test ARA in the framework of a TMDL project of a large watershed. The Blackwater watershed is around 11,518 ha, compared to an ARA study performed by A. K. Graves on a 2,665 ha watershed (2000), and another done by Hagedorn et al. in the Page Brook watershed (3,000 ha, 1999).

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CHAPTER II

LITERATURE REVIEW

Fecal Source Tracking

Scientists and regulatory officials have employed various methods, both historically and recently, to try to determine the sources of fecal pollution. These methods fall into three main categories: molecular (genomic), non-molecular (phenotypic) and chemical. Molecular methods involve DNA fingerprinting of a bacterial isolate, while non-molecular methods include all other biological methods. Generally, non-molecular methods measure some metabolic trait such as carbon source utilization antibiotic resistance. Both molecular and non-molecular generally require a library of known source isolates to be built in order to identify the sources of unknown isolates. Chemical methods rely on an abiotic characteristic or component of a water sample, such as caffeine or optical brighteners, to determine the source of fecal pollution.

Several molecular methods that measure different DNA patterns are under development for source tracking, primarily using *E. coli*. Ribotyping involves isolation of the highly conserved ribosomal DNA (rRNA genes), digestion with restriction enzymes, and electrophoresis on agarose gels. After the electrophoresis, ribotyping involves a Southern blot and probing of the blot membrane with rRNA sequences. Each isolate has a profile of DNA bands of various molecular weights. Parveen et al. (1999) performed ribotyping and analyzed the profiles with discriminant analysis to obtain separation between human and nonhuman isolates. The rate of correct classification (RCC) was 97% for nonhuman isolates and 67% for human isolates. Carson et al. (2001) also used ribotyping with discriminant analysis, but distinguished between human and seven types of animal isolates. The average rate of correct classification (ARCC) with eight categories was 73.6%, and when the animal isolates were

pooled into one category, the RCC for human was 95% and 99.2% for animal. Hartel et al. (1999) used ribotyping of *E. coli* to compare profiles (ribotypes) of isolates from cattle manure with isolates from a pasture stream and a wooded stream in order to find manure ribotypes in the pasture stream. The comparison was done by looking at the different profiles, not a specific statistical method. Eight different ribotypes were found. All eight were present in the pasture stream, six were found in the wooded stream, and six were present in the manure. Only one profile was common to manure and the wooded stream and not present in the wooded stream. He concluded that there were differences in ribotypes among the three sources, but that the small number of manure profiles did not allow differences between manure and pasture stream when the same ribotypes were present in the wooded stream (Hartel et al. 1999). Samadpour and Chechowitz (1995) performed ribotyping on *E. coli* in the Little Soos Creek watershed in Washington State. A library of known source ribotypes was constructed from 227 septic tank isolates and 823 animal isolates. Stream ribotypes (589) were compared to the library by finding a matching ribotype, then determining their source. In this study, 71% of the *E. coli* isolates were placed into a source category, leaving 29% with an unknown source (Samadpour and Chechowitz 1995). Ribotyping is advantageous because it uses ribosomal DNA that is very stable in a population of bacteria. This stability provides a limit to genotype variability that may overcome geographical restrictions, but a very large library may be required to include all the possible ribotypes. The disadvantages of ribotyping include high cost per isolate, a time consuming procedure, and the need for highly trained technicians with access to specialized equipment.

Simmons et al. (2000) used another molecular method, pulsed-field gel electrophoresis (PFGE), in the Four Mile Run watershed, Virginia, to profile *E. coli* DNA. PFGE was done by

first isolating the total DNA from *E. coli* isolates, then cutting with a restriction enzyme. DNA fragments were separated in 1% agarose gel run on a contour-clamped homogenous electric field at 14^{o}C for 20 h. After staining the gel with ethidium bromide, it was photographed and band patterns were recorded. The banding profile of each unknown isolate was compared to a library of known source profiles by a "band to band" percent similarity. Four libraries with a total of 843 isolates were used to identify the source of stream isolates. Of a total of 439 stream isolates profiled using PFGE, 30% did not match any profile in the library, 6% were matched at equal similarity to more than one source. Of the remaining 63% of the isolates that matched a profile in one of the libraries, 17% were identified as human, 37% as waterfowl, 9% as canine, 10% as deer, 15% as raccoon and 12% as other mammals. Simmons suggested that a disproportionately high contribution from wildlife to the fecal loading is not because there is more wildlife in the watershed, but that those sources have more direct contact with the water. He did not observe a seasonal variation of fecal coliform concentration or in the predominance of human or animal isolates in the stream. The advantage of using PFGE is only a few long fragments of DNA are separated on the gel, so Southern blots and probes are not needed, making PFGE faster than ribotyping. The disadvantages of PFGE are high cost per isolate and the need for highly trained technicians.

Bernhard and Field (2000) described the use of ribosomal DNA genetic markers from *Bifidobacterium* and the *Bacteriodes-Prevotella* group to differentiate sources of pollution in water. The method involves polymerase chain reaction followed by terminal restriction fragment length polymorphism analysis. This study found different species compositions from the two groups in human and cow feces that could be used for bacterial source tracking. The *Bifidobacterium* and *Bacteriodes-Prevotella* bacteria are anaerobic, making the analysis more

difficult than methods using aerobic or facultative bacteria, but the method does not involve the time-consuming step of culturing the bacteria before analysis (Bernhard and Field 2000)

Random amplified polymorphic DNA (RAPD) was used by Tseng (2001) for source tracking of *E. coli* isolates. RAPD analysis involves purifying DNA from isolates, adding arbitrarily selected primers of a certain DNA sequence, performing polymerase chain reaction (PCR) and electrophoresis. A library of profiles was constructed where DNA fingerprints were placed in source categories based on 70% similarity, and unknown isolate profiles were compared to the library and placed into a known category.

Nonmolecular, or phenotypic fingerprinting methods are also under development. The fecal coliform to fecal streptococcus ratio was used for many years, but has since has been abandoned because of different die-off rates of the two groups of organisms (American Public Health Association 1995). Multiple antibiotic resistance (MAR) is a method where profiles are determined by inoculating antibiotic-containing agar with isolates and recording growth patterns. MAR was used by Parveen et al. (1997) to distinguish between *E. coli* from point and non point sources using cluster analysis. Coliphages, viruses that attack *E. coli,* have been used for distinguishing between human and animal sources of fecal pollution based on the type of coliphage present (Stewart el al. 2001). Using this type of fecal source tracking, male-specific coliphages are isolated and placed into one of two categories: F+DNA or F+RNA. The F+RNA coliphages are then placed into one of four groups. Groups II and III are indicative of human contamination, Group IV is indicative of nonhuman inputs, and Group I has components of both (Stewart et al. 2001).

Chemical methods do not directly measure any type of biological component associated with fecal pollution, but rather are indicative of human activity (Hagedorn 2001). A few

chemicals with possible use in fecal source tracking include optical brighteners and caffeine. Optical brighteners are a stable, long-lasting chemicals present in laundry detergent, urine, and fecal sterols that can be detected by long wave UV light. If sterols or brighteners are present in a stream receiving septic waste, then human pollution may be occurring. The advantage of optical brighteners is the test is low-cost and simple, but a disadvantage is that the persistent nature of optical brighteners may be due to prior contamination. Caffeine, primarily only used by humans, is not broken down in the human digestive tract. Therefore, caffeine present in stream samples may indicate human contamination. However, some plants also contain caffeine that could confuse readings. Caffeine tests, conducted with gas chromatography, are very expensive and because soil bacteria can degrade it, a negative result from the test may not rule out human contamination (Hagedorn 2001).

Wiggins (1996) first reported a method inspired by MAR called antibiotic resistance analysis (ARA) that uses several concentrations of each antibiotic instead of just one. ARA involves isolating bacteria and observing growth patterns on a battery of antibiotics. This method has been used by several researchers to determine sources of fecal pollution in water by building a library of known source profiles and comparing water sample isolate profiles against the library. ARA studies have been performed on enterococci and *E. coli*, using discriminant analysis (DA) and cluster analysis (CA) for building a library. Wiggins used fecal streptococcus (enterococcus) instead of *E. coli* for three main reasons. First, MAR studies with enterococcus at the time were more successful than with *E. coli.* In addition, enterococcus is present in some types of fecal matter that lack *E. coli* (Wiggins 1999). Wiggins (1996), using five drugs and 1,436 isolates, distinguished among six sources (beef cattle, chicken, dairy cattle, human, turkey and wild) with an ARCC of 72%, among four sources (cattle, human, poultry, wild) with ARCCs

of 82% and 84% (depending on the set of antibiotics used for analysis) and between human and animal with an ARCC of 95%. In another study, Wiggins et al. performed ARA on a larger set of isolates collected over a four-year period and tested different drugs (1999). With five different drugs and 5,990 isolates, the ARCC was 64%. A combination of five drugs from the first study and the new drugs on 2,635 isolates gave an ARCC of 66%. When six drugs were used on 2,844 isolates, the ARCC was 65%. A final set of nine drugs gave an ARCC of 78% on 3,032 isolates. It was suggested that a larger set of drugs should result in a higher ARCC and that bacterial source tracking projects use a library of "recently collected, local samples" (Wiggins 1999).

Harwood et al. used ARA and DA on *E. coli* to distinguish between human and animal isolates using 32 drug/concentration combinations (2000). When the library was split into human and animal categories, the RCC was 75.5% for human and 72.4% for animal. When the library was split among six sources, the ARCC was 62.3%. In the same study, enterococcus isolates were also analyzed with ARA and DA using 36 drug/concentration combinations. The two-category library had RCC of 69.3% for human and 78.4% for animal. When the library was categorized by the six sources, the ARCC was 63.9%. This article outlined the expected frequency of misclassification, a method to quantify the level of significance for stream samples. The expected frequency of misclassification is the percentage of isolates in a library that are from all sources except one, but are placed into that one source mistakenly by DA, or a false positive. Wiggins suggested that libraries periodically be updated with new known sources and that there is a trade-off between a high RCC and the ability of the library to capture enough representative profiles to be useful.

A study performed by Hagedorn et al. (1999) used ARA with enterococcus isolates from two geographical regions. One library was constructed with 7,058 isolates collected from Montgomery County, Virginia. When isolates were placed into six categories, the ARCC was 87%. When isolates in the library were placed into either human or animal categories, the RCC was 96% for human and 98% for animal isolates. For a library constructed of 892 isolates from Clarke County, Virginia, with four categories, the ARCC was 88%. In a separate library made two years later from 642 isolates in the same watershed, the ARCC was 82%. This study used both DA and CA to analyze ARA patterns and concluded that both statistical methods were useful in their own ways. DA allows for the RCC to be calculated and obtain a useful measure of the correctness of the library. CA allows inspection of isolates' relationship to each other and for identifying profiles that do not fit into any group, but are distinctive from the other profiles. Hagedorn suggested testing isolates from a new area with an existing library to ensure the library could be used in the new geographical area. He also mentioned that a library should be periodically updates with new known source isolates.

Bower (Glasoe and Bower 2001) performed ARA on enterococci in the Tillamook Bay, Oregon. Since the suspected major sources of pollution in the watershed were human, dairy and wildlife, the library of 830 isolates was split into three categories. The RCCs were 73% for human isolates, 88% for wildlife, and 89% for dairy, with an ARCC of 83%. BST performed on stream isolates showed that human and dairy sources dominated the sample with wildlife contributing only a small portion.

There is a need for BST method comparison studies in order to cross validate one method with another (Hagedorn 2001). Even this type of comparison may not result in one ultimate method for BST. Probably the best way to choose a BST method is to determine which

questions need to be answered, and then use a "toolbox" approach to select a method or a combination of different types of methods. A possible combination is a BST method with a low cost per isolate, such as ARA, or a chemical method combined with a molecular method where a subset of the isolates are profiled (Hagedorn 2001).

Total Maximum Daily Load

The total maximum daily load (TMDL) can be referred to as a "pollution budget" (EPA 2001a). The term is used in practice in two main ways: a process to clean up impaired waters to meet water quality standards and a numeric value of the maximum amount of contamination a waterbody can receive without exceeding water quality standards (EPA 2001a). The numerical load is calculated by adding point source inputs, nonpoint source inputs, and a margin of safety to account for errors. Point source inputs are also called the wasteload allocation. The load allocation includes nonpoint sources, unpermitted storm water discharges, atmospheric deposition, groundwater inputs, and background levels. A TMDL is developed for one pollutant at a time. In a stream segment with two impairments, like nutrients and fecal coliforms, separate TMDLs must be developed for each (FOVRA 2000). TMDL was mandated by section 303(d) of the Clean Water Act of 1972 and was reissued by the EPA in 1985 and 1992 regulations (EPA 2001a). It was not until recently that TMDL became part of water quality management in the United States.

In 1996, the EPA Office of Water determined that the TMDL requirements needed review, so the TMDL Federal Advisory Committee was formed (EPA 2001a). In August 1999, the EPA proposed revisions to the TMDL program based on the recommendation of the Committee (EPA 2001b). The resulting action was the TMDL Final Rule, announced in July 2000. The Final Rule 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 waterbodies 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 and the fourth section lists requirements of an implementation plan. The fifth section requires "reasonable assurance" that nonpoint 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 more than 25 years after the Clean Water Act, the TMDL section was virtually 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 TMDL 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 if the state does not take responsibility (EPA 2001c). Nine states have had groups file litigation suits to the EPA, and in two states they have issued intent to sue. In eleven states the cases were dismissed, some resulting in settlement agreements. In all, 37 states and the District of Columbia have been involved at one time or another in TMDL

litigation (EPA 2001c). The numbers here do not add to 38 because some states are included in more than one category.

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 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 removed 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 ALS is a national, nonprofit, public-interest organization comprised of professional and amateur naturalists organized in 1961, with headquarters in Sandy Hook, New Jersey. ALS seeks to encourage a better scientific and public understanding of the environment and provide a unified voice advocating protection of the delicate fabric of life along the shore (ALS 2001).

The first of the six required elements of a TMDL is the name and geographic location of the impaired water, pollutants responsible for impairment and the water quality standards for that pollutant (EPA 2001b). The second element is the amount of pollutant allowed in the water body without exceeding standards, the loading reduction to meet the standard, allocation of waste to point and nonpoint sources and an implementation plan. Third is a margin of safety to allow for seasonal variation and future loading. The fourth section is a transitional period to phase in the TMDL process and the fifth is public participation and comment on methods, lists of impaired waters, schedule of development and implementation of TMDLs before being

submitted to the EPA for approval. The last section of a TMDL is support from the EPA for development of TMDLs (EPA 2001b).

The TMDL process is a long-term goal for improving water quality. It requires three distinct processes: water quality assessment, developing the TMDL and implementing best management practices to reduce loading. Water quality assessment pulls together several components. The data gathered from water sampling is combined with historical data from state agencies or university studies to get a long-term picture of stream conditions. Standards must be evaluated to ensure they are strict enough so that when the water meets standards it supports uses and to ensure that they are not too strict that they are unattainable. For the fecal coliform TMDLs, bacterial source tracking may be used to determine the major source of contamination for more efficient use of resources for best management practices (Hagedorn et al. 1999). In the case of fecal coliforms, the standard is 1000 CFU per 100 ml for a single sample taken in a 30-day period. When two or more samples are taken in a 30-day period, the geometric mean of colonies per 100 ml must be below 200 (Virginia DEQ 2001a). The second component is TMDL development. This entails evaluating watershed parameters like flow and contaminant concentration for the entire watershed and predicting those parameters in the future. TMDL development involves determining how much each source must be reduced in order to meet the target load, a process known as waste load allocation. Several scenarios may be presented and stakeholders will usually decide which will work best for them. Implementation of the TMDL involves public participation and agency support. The local or state government may update water quality management plans to account for the loading reductions needed. The next step is to set goals and develop a timeline in which to reach them. Stakeholders then decide which source and how much each source must reduce to attain a certain loading. Best management practices

will be developed and implemented. Finally, water quality is monitored to determine progress. If satisfactory progress is not made, the implementation process will start at the beginning and continue again until the water is restored (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 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.

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.

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CHAPTER III

BUILDING A KNOWN SOURCE LIBRARY USING ANTIBIOTIC RESISTANCE ANALYSIS: ISSUES RELATED TO LIBRARY SIZE, GEOGRAPHICAL RESTRICTIONS AND COMPARISON WITH A DIFFERENT BACTERIAL SOURCE TRACKING METHOD (BIOLOG)

INTRODUCTION

Bacterial source tracking (BST) using discriminant analysis of antibiotic resistance patterns was first reported by Wiggins (1996). Antibiotic resistance analysis (ARA) was based on multiple antibiotic resistance (MAR) analysis, a method to differentiate between sources of pollution using antibiotic resistance patterns on a number of antibiotics. ARA differs from MAR because several concentrations of antibiotics are used instead of one.

Several researchers have successfully utilized ARA as a BST method with different numbers and concentrations of drugs and known source libraries and watersheds of varying sizes. A measure of the success of a known source library is the average rate of correct classification (ARCC). This percentage is an average of the rates of correct classification (RCC) for each category in the library. High RCCs and ARCCs mean that the discriminant analysis model used to statistically analyze the profiles of antibiotic resistance place most of the known source isolates in the correct category. Wiggins (1996), using five drugs and 1,436 isolates, distinguished among six sources (beef cattle, chicken, dairy cattle, human, turkey and wild) with an ARCC of 72%, among four sources (cattle, human, poultry, wild) with ARCCs of 82% and 84% (depending on the set of antibiotics used for analysis), and between human and animal with an ARCC of 95%. In another study, Wiggins et al. performed ARA on a larger set of isolates collected over a four-year period and tested different drugs (1999). With five different drugs and 5,990 isolates, the ARCC was 64%. A combination of five drugs from the 1996 study and the 1999 study on 2,635 isolates gave an ARCC of 66%. When six drugs were used on 2,844

isolates, the ARCC was 65%. A final set of nine drugs gave an ARCC of 78% on 3,032 isolates. It was suggested that a larger set of drugs should result in a higher ARCC and that bacterial source tracking projects use a library of "recently collected, local samples" (Wiggins el at. 1999).

Harwood et al. used ARA and DA on *E. coli* to distinguish between human and animal isolates using 32 drug/concentration combinations (2000). When the library was split into human and animal categories, the RCC was 75.5% for human and 72.4% for animal. In the library that was split among six sources, the ARCC was 62.3%. In the same study, enterococcus isolates were also analyzed with ARA and DA using 36 drug/concentration combinations. The two-category library had RCC of 69.3% for human and 78.4% for animal. When the library was categorized by the six sources, the ARCC was 63.9%. This article outlined the expected frequency of misclassification, a method to quantify the level of significance for stream samples. The expected frequency of misclassification is the percentage of isolates in a library that are from all sources except one, but are placed into that one source mistakenly by DA, or a false positive. Harwood (2000) suggests that libraries periodically be updated with new known sources and that there is a trade-off between a high RCC and the ability of the library to capture enough representative profiles to be useful.

A study performed by Hagedorn et al. (1999) used ARA with enterococcus isolates from two geographical regions. One library was constructed with 7,058 isolates collected from Montgomery County, Virginia. When isolates were placed into six categories, the ARCC was 87%. When isolates in the library were placed into either human or animal categories, the RCC was 96% for human and 98% for animal isolates. For a library constructed of 892 isolates from Clarke County, Virginia, with four categories, the ARCC was 88%. In a separate library made two years later from 642 isolates in the same watershed, the ARCC was 82%.

Bower (Glasoe and Bower 2001) performed ARA on enterococci in the Tillamook Bay, Oregon. Since the suspected major sources of pollution in the watershed were human, dairy and wildlife, the library of 830 isolates was split into three categories. The RCCs were 73% for human isolates, 88% for wildlife, and 89% for dairy, with an ARCC of 83%.

Figure 1 shows the relationship between ARCC and library size for 11 enterococci-based ARA studies reported above. Only libraries with at least 3 categories were used in this figure because libraries with 2 categories tend towards very high ARCCs (the Blackwater ARA library had 3 categories). The ARCC ranged from 64% to 88% and the library size ranged from 830 to 7,058 isolates. Since the R^2 value is close to zero (.09), the variability among the ARCC values is likely not due to change in the number of isolates. Put another way, only 9.5% of the variability in the ARCC is explained by the change in number of isolates.

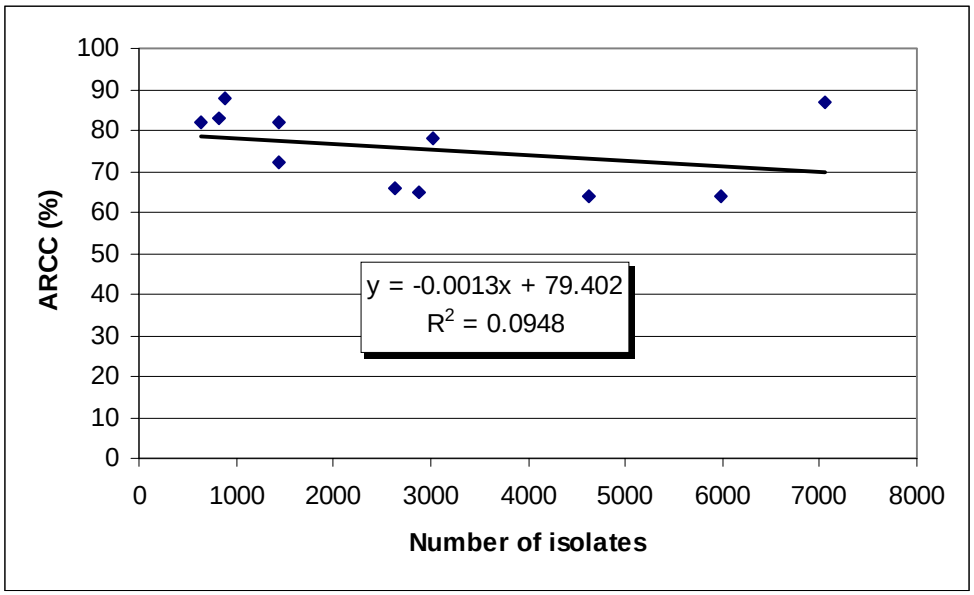

Figure 1: ARCC vs. Number of Isolates in Library From Literature

The objectives of this study were to build a known source library isolate profiles from Franklin County, Virginia, compare BST results from ARA and Biolog, a metabolic bacterial

identification system, compare BST results between two libraries from different geographical areas, and explore the size requirement of a known source library.

MATERIALS AND METHODS

Building the Known Source Library

Sample Collection: Isolates from thirteen different sources were collected in Franklin County, Virginia from September 1999 to April 2000 to build a known source library. These sources included beef cattle, dairy cattle, chicken, horse, goat, deer, raccoon, muskrat, goose, duck, coyote, wild turkey and human. Livestock fecal samples (cow, horse, chicken, goat) were collected from local farms. Wildlife fecal samples (deer, raccoon, muskrat, goose duck, coyote and wild turkey) were collected in areas where wildlife had been observed and scats could be identified by qualified persons. Human fecal samples were collected from septic tank pump-out trucks in Franklin and Clarke Counties, Virginia. Solid fecal samples were placed in sterile Whirlpac bags or petri plates and liquid samples were collected in sterile polystyrene bottles. All samples were placed on ice for transport to the laboratory.

Isolation of Enterococci: Isolation of enterococci from known sources was done by adding 10.0 g of solid fecal material or 10.0 ml of liquid fecal material to 90 ml of sterile phosphate buffer to make a 1:10 dilution. Fecal material from the same source and sampling date were combined to make a composite sample when possible. Three additional 1:10 dilutions were made, resulting in 1:100, 1:1,000 and 1:10,000 dilutions. Each dilution was spread-plated on mEnterococcus agar (BBL, Cockeysville, MD) in 15x100 mm petri dishes and incubated at 37^{o} C for 48 h. An individual colony was transferred to one well in a 96-microwell plate containing 0.2 ml Enterococcosel broth (BBL, Cockeysville, MD) using a sterile toothpick. Up to 48 colonies from each fecal sample were transferred to microwells. Microwell plates were incubated at 37^{o}C

for 24 h. Confirmation of enterococci was determined by a color change in the Enterococcosel broth after incubation, from yellow to black, caused by esculin hydrolysis.

Antibiotic Resistance Analysis: Thirty treatments of a combination of nine antibiotics in various concentrations were used to determine antibiotic resistance patterns of the enterococci isolates. Antibiotic stock solutions were prepared from commercial antibiotic powders (Sigma Chemical Co., St. Louis, MO) as indicated below (Table 1):

Table 1: Concentration and solvent of antibiotic stock solutions

Antibiotic	Commercial formulation	Solvent	Stock Conc (mg/ml)
Chlortetracycline	Chlortetracycline HCl	1N sodium hydroxide	10
Oxytetracycline	Oxytetracycline HCl	1:1 water-methanol	10
Streptomycin	Streptomycin sulfate	Distilled water	10
Cephalothin	Cephalothin	Distilled water	10
Erythromycin	Erythromycin	1:1 water-ethanol	10
Tetracycline	Tetracycline HCl	Methanol	10
Neomycin	Neomycin sulfate	Distilled water	10
Vancomycin	Vancomycin HCl	1:1 water-ethanol	10
Amoxicillin	Amoxicillin	1:1 water methanol	2.5

Antibiotic agar was prepared by adding an aliquot of stock solution to a flask of autoclaved Trypticase Soy Agar (TSA, BBL, Cockeysville, MD) cooled to 50^{o}C to achieve the desired concentration (Table 2), mixed until uniform, and poured into sterile 15x100 mm petri dishes so that each dish contained one antibiotic concentration. A control plate containing no antibiotic was included. Isolates were transferred from the microwell plate using a stainless steel 48-prong replica plater (Sigma Chemical Co., St. Louis, MO) so that each petri dish with one antibiotic concentration was inoculated with 48 isolates. The inoculant was allowed to soak into the agar and the plates were incubated at 37^{o}C for 48 h. Resistance to an antibiotic was determined by comparing the growth of each isolate on the antibiotic plate to the growth on the control plate. A one (1) was recorded if the isolate grew (a complete circle, mostly filled) and a zero (0) was recorded for any other case. This was repeated for each isolate on each of the thirty antibiotic plates.

Table 2: Concentrations of antibiotic treatments in TSA plates

Antibiotic	Plate concentrations (μg/l)
Chlortetracycline	60, 80, 100
Oxytetracycline	20, 40, 60, 80, 100
Streptomycin	40, 60, 80, 100
Cephalothin	10, 15, 30, 50
Erythromycin	10, 15, 30, 50
Tetracycline	10, 15, 30, 50, 100
Neomycin	40, 60, 80
Vancomycin	2.5
Amoxicillin	.625

Statistical Analysis: The statistical analysis was performed on 1,451 known source isolates using the discriminate analysis model (DA) in JMP-In statistical software (version 3.2.6 for Windows, SAS Institute, Inc.). Known source profiles are used to build the known source library by comparing the profile of an isolate to every other isolate in the library. Each isolate was assigned to the livestock, wildlife or human category based on the type of source from which it came. The DA model was run on the isolates and JMP placed the isolate in the appropriate category based on its profile. The number of isolates in a model-assigned category divided by the number of isolates in that category based on the type of source is the percent of correct classification for that category.

Manipulations were made to the library to group isolates in different categories from the human, livestock and wildlife mentioned above to observe changed in correct classifications. Each isolate was grouped according to the source from which it came. DA was performed again on the library and a percent of correct classification was recorded for each category.

Biolog comparison

Using the Biolog system for identification: Isolates from known sources were identified using the Biolog microbial identification system (Biolog Inc., Hayward, CA). The Biolog system exploits a "microbes ability to use particular carbon sources to produce a … metabolic

fingerprint" of an isolate, then the fingerprint is compared to the Biolog "library of species" for identification to genus and species (Biolog user guide, p. 2.1). Isolates previously profiled by ARA were cultured from microwells containing Enterococcosel broth. Isolates came from geese, deer, cow, human, dog, chicken, horse, cat, wild turkey, muskrat, goat, pig, and duck collected in the Holston River, North River, Blackwater River, and Clarke County watersheds and Tennessee. The Biolog method for gram positive microbes was used to obtain an identification and a profile of growth on the battery of carbon sources. A loop of broth was inoculated onto mEnterococcus agar and incubated 24-48 h at 37^{o}C until sufficient growth occurred. Isolates were transferred to BUG (Biolog universal growth) agar containing 5% sheep blood (by volume) with a sterile cotton swab and incubated at 37^{o}C for 24 h. Enterococci from the blood agar plates were transferred to a tube of gelling inoculating fluid (Biolog) with a sterile swab until 20% transmittance (+/- 3%) was achieved in the tube. The solution was inoculated into a 96 microwell plate containing a water blank well and 95 different dried carbon sources. The plates were incubated at 37^{o}C for 24 h and read using a Biolog plate reader. The isolate uses some carbon sources and not others. Growth in a well by an isolate was recorded as a 1 (one) and absence of growth was recorded as a 0 (zero). This signature of ones and zeroes created a profile for that isolate that was compared to the Biolog library for identification.

Use of Biolog for Bacterial Source Tracking: Thirty of the most relevant wells (Table 3) were chosen to create a known source Biolog library like the antibiotic resistance library described previously. The thirty wells were chosen by eliminating those wells where enterococci always or never grew, then, with the remaining wells, running discriminant analysis on many combinations of wells until the highest correct classifications were obtained. The profiles of 300 enterococci isolates were analyzed using discriminate analysis in JMP-In (version 3.2.6 for Windows, SAS

Institute, Inc.) where the software compares the profile of each isolate to the other profiles and assigns the isolate a predicted source in one of the three categories, human, livestock or wildlife.

Table 3: Biolog wells and carbon sources used in bacterial source tracking

Well Number	Carbon Source
A4	Dextrin
A11	N-acetyl-D-mannosamine
A12	Amygdalin
B1	L-arabinose
B7	D-galactose
B9	Gentiobiose
B10	D-gluconic acid
B11	α-D-glucose
C1	α-D-lactose
C2	Lactulose
C3	Maltose
C5	D-mannitol
C6	D-mannose
C7	D-melezitose
C8	D-melibiose
C10	B-methyl D-galactoside
D4	D-psicose
D5	D-raffinose
D7	D-ribose
D8	Salicin
D11	Stachyose
E2	D-trehalose
E12	α-keto valeric acid
F6	Methyl pyruvate
G12	Glycerol
H1	Adenosine
H5	Uridine
H7	Thymidine-5'-monophosphate
H9	Fructose-6-phosphate
H11	Glucose-6-phosphate

A stream sample from the Blackwater River sourced using the Blackwater antibiotic resistance known source library was also compared to the Biolog known source library. Thirty isolates from a sample taken March 2001 were used for the comparison, ten human isolates, ten

wildlife isolates, and ten livestock isolates. The predicted source from antibiotic resistance analysis and from Biolog was recorded as either the same or different.

Change in correct classification of known source library with added isolates

The size of a known source library is an integral question in the development of bacterial source tracking methods. In order to attempt to find the optimum size of a library, the Blackwater library was manipulated after completion. The Blackwater known source library was created from isolates collected on eight separate events: August 1999, September 1999, October 1999, December 1999, February 2000, April 2000, June 2000 and a set added from Roseville Run in Clarke County, Virginia in August 2000. More isolates were added periodically to build the library that changed the rates of correct classification in each category. The correct classification of each category was recorded for the first set of known source isolates and again after each addition of known source isolates.

Demonstration of geographical constraint of a known source library

In order to determine the usefulness of a known source library outside of the area where its isolates were collected, the Blackwater known source library was compared to a library from a different geographical area. The Shenandoah Valley library was created in January 2001 with 1430 isolates from human, goose, horse, cow, sheep, muskrat, deer, raccoon, and chicken sources. There are 203 human isolates, 910 livestock isolates and 317 wildlife isolates. The correct classifications are 95.1% for human, 86.7% for livestock and 84.9% for wildlife. The two libraries were combined into one library with 2,881 isolates and the correct classification rates were observed. The two libraries were also compared by comparing 48 profiles from a Blackwater stream sample with each library. The predicted source of each isolate from the two libraries was recorded as either the same or different. The stream isolates from the Blackwater

were compared to the combined library as well to observe how the rates of correct classification changed.

RESULTS AND DISCUSSION

Antibiotic resistance analysis known source library

The rates of correct classification (RCC) for categories in the Blackwater antibiotic resistance known source library were 82.3% for human, 86.2% for livestock and 87.4 for wildlife with an ARCC of 85.3% (Table 4). The discriminate analysis model identified 82% of the isolates as human that came from human fecal samples. Table 4 illustrates these data with the percentages of isolates assigned to each predicted source and the number of isolates in parentheses. These correct classifications are much higher than a random distribution of isolates into three categories, a classification of 33.3%. These correct classification rates are comparable to other bacterial source tracking studies using antibiotic resistance analysis and other methods. Hagedorn et al. performed ARA and DA on 7,058 isolates with correct classification rates of 86% for beef cattle, 85% for chicken, 87% for dairy cattle, 84% for deer, 93% for human and 90% for waterfowl (1999). In a study by Parveen et al (1999), multiple antibiotic resistance was performed on human and animal isolates to obtain a correct classification of 82% and 68%, respectively. The same study yielded correct classifications of 97% for human and 67% for animal when isolates were analyzed using ribotyping. Carson et al analyzed 287 isolates using ribotyping, obtaining RCCs of 95% for human and 99.2% for animal, but an ARCC of 73.3% when the library was used to classify individual sources (2001).

Table 4: Blackwater antibiotic resistance analysis database of 1,451 known source isolates

Predicted Source	Percent of known source isolates assigned to each predicted source (no. of isolates)		
	Human	Livestock	Wildlife
Human	**82.3** (349)	7.1 (46)	2.4 (9)
Livestock	10.1 (43)	**86.2** (557)	10.2 (39)
Wildlife	7.5 (32)	6.7 (43)	**87.4** (333)
Total	424	646	381

*the ARCC is 85.3%

The significant percentage of a source in a stream sample was determined for human, livestock and wildlife by calculating the expected frequency of misclassification using a variation of the method described by Harwood et al. (2000). Expected frequency of misclassification is the percentage of isolates from a known source that are placed in the wrong predicted source category. A stream sample with less than this percentage of contamination from any source category could be attributed to error in the database. The significant percentage of human was determined by adding the number of livestock and wildlife isolates that were placed in the human predicted source category, then dividing by the total number of livestock and wildlife isolates (Table 4). The percentage of non-human isolates that were misclassified as human was 5.4% (average of 7.1 % of the livestock isolates misclassified as human and 2.4% of the wildlife isolates misclassified as human). A stream sample with greater than 5.4% human can be treated as having significant human contamination because 5.4% percent of non-human isolates were misclassified by discriminant analysis as human. Similarly, the percentage of misclassification of livestock was determined to be 10.2% by adding the numbers of livestock isolates that were placed in human and wildlife categories and dividing by the total number of human and wildlife isolates. The significant level of wildlife contamination was calculated at 7.0% by adding the total number of human and livestock isolates misclassified as wildlife and dividing by the total number of human and livestock isolates.

The library was divided into 13 categories by source and analyzed by DA. The correct classifications for each source, the number of isolates from each source, the organism that was misclassified as that known source in the highest percentage and the percent misclassification are listed in Table 5. The ARCC was 71.3%, ranging from 36.4% for dairy cow to 95.8 for sheep isolates. Random classification was 7.7% (100% divided by 13 sources). This drop in ARCC when a finer split of source categories was used is in agreement with a study performed by Wiggins (1996) with similar results. The number of categories used in DA is integral to the BST question at hand. There is a trade-off between a high ARCC with fewer categories and a lower ARCC with many categories. High ARCC leads to higher confidence in the results for stream isolates, but the number of categories used must be in accordance with the goals of the project. The Blackwater project did not need a library split into 13 categories, but some applications of ARA would require that level of separation.

Table 5: Blackwater known source library correct classifications split by source:

Source (no. of isolates):	Rate of Correct Classification (source with highest misclassification, percent misclassification):
Beef Cattle (79)	84.8 (Chicken, 15.2)
Chicken (48)	79.2 (Horse, 8.3)
Coyote (31)	38.7 (Duck, 32.3)
Dairy Cattle (269)	36.4 (Beef cattle, 22.3)
Deer (93)	79.6 (Beef cattle, 11.8)
Duck (21)	76.2 (Goat, 14.3)
Goat (96)	80.2 (Human, 11.5)
Goose (44)	61.3 (Wild turkey, 20.4)
Horse (106)	60.4 (Beef cattle, 19.8)
Human (424)	77.6 (Beef cattle, goose, 3.8)
Raccoon (96)	70.8 (Beef cattle, 5.2)
Sheep (48)	95.8 (chicken, dairy cattle, coyote, 2.1)
Wild Turkey (96)	86.5 (goat, 6.2)

Biolog comparison

The RCCs for the Biolog known source library were 88.6% for human, 78.5% for livestock, 84.6% for urban and 69.7% for wildlife, with an ARCC of 80.3% (Table 6). The urban category includes dog and cat isolates. These isolates were collected from a wide geographical range and are comparable to the Blackwater antibiotic resistance analysis library correct classifications of 82.3% for human, 86.2% for livestock and 87.4% for wildlife, with an ARCC of 85.3% (Table 4). The Biolog library also included 26 dog and cat isolates placed into the urban category. Thirty isolates from sample BW 64, taken in March 2001, were identified and profiled using the Biolog system (Table 7). Of ten isolates identified by the Blackwater antibiotic resistance analysis library as human, the Biolog library identified one as human, four as livestock, and five as wildlife. Of ten isolates identified by the ARA library as livestock, the Biolog library identified seven as livestock and three as wildlife. Of ten isolates identified by ARA as wildlife, one was identified as human, three as livestock and six as wildlife. The overall correct classification of Blackwater isolates in the Biolog library was 14 of 30 isolates, or 47%.

One explanation for the lower than expected correct classification is that there are not enough isolates in the Biolog library to adequately represent the variability shown by the Blackwater isolates. Some of the isolates in the Biolog library came from the Blackwater watershed. However, the number of Blackwater isolates in the library was probably not high enough for all the Blackwater stream isolates to be correctly identified.

Table 6: Biolog known source database

Predicted Source	Percent of known source isolates assigned to each predicted source (no. of isolates)			
	Human	Livestock	Urban	Wildlife
Human	**88.6 (93)**	4.3 (4)	7.7 (2)	3.9 (3)
Livestock	3.8 (4)	**78.5 (73)**	0 (0)	22.4 (17)
Urban	6.7 (7)	2.1 (2)	**84.6 (22)**	3.9 (3)
Wildlife	.9 (1)	15.0 (14)	7.7 (2)	**69.7 (53)**
Total	105	93	26	76

*The ARCC is 80.3%.

Table 7: Percentage and (number of isolates) from a Blackwater stream sample compared to the Biolog library

Biolog category	BW human	BW livestock	BW wildlife
Human	10.0 (1)	0 (0)	10.0 (1)
Livestock	40.0 (4)	70.0 (7)	30.0 (3)
Urban	0 (0)	0 (0)	0 (0)
Wildlife	50.0 (5)	30.0 (3)	60.0 (6)
Total	10	10	10

Size of known source library

The number of isolates needed for a bacterial source tracking library is integral to doing a BST project. The Blackwater known source library was constructed with isolates from 8 sampling events. Changes in RCCs with each addition of isolates to the library were recorded (Table 8).

The ARA library constructed from profiles of isolates collected in August and September 1999 in the Blackwater watershed had 758 isolates with correct classifications of 92.6% for human, 92.4% for wildlife and 88.4% for livestock with an ARCC of 91.1. The library

constructed in October 1999 had 834 isolates and correct classifications of 92.6% for human, 93.3% for wildlife and 88.9% for livestock with an ARCC of 91.6%. The library analyzed in December 1999 had 930 isolates with correct classifications of 92.6% for human, 91.8% for wildlife, and 88.1% for livestock with an ARCC of 90.8%. In February 2000, the library had 1088 isolates with correct classifications of 80.9% for human, 93.6% for wildlife, and 88.2% for livestock with an ARCC of 86.2%. The library constructed in April 2000 had 1184 isolates and correct classifications of 80.2% for human, 91.6% for wildlife, and 86.1% for livestock, with an ARCC of 85.2%. In June 2000, the library contained 1343 isolates with correct classifications of 76.2% for human, 87.9% for wildlife, and 87.4% for livestock, with an ARCC of 83.8%. The final library, constructed in August 2000, had 1451 isolates and correct classification rates of 82.3% for human, 86.2% for wildlife, and 87.4% for livestock with an ARCC of 85.3%. Figure 1 is a graphical representation of the relationship between ARCC and library size. It shows that the ARCC fell overall when more isolates were added to the library, but the lowest ARCC of 83.8 is higher than 82% (9 of 11) of the libraries in Figure 1 from libraries reported in the literature. The changes in correct classification when more isolates are added are likely also reflections on the types of isolates added to the library in addition to the number of isolates.

Table 8: Changes in rates of correct classification and (number of isolates) with increase in size of the Blackwater library

Month	No. of isolates	Human	Wildlife	Livestock	ARCC
September 99	758	92.6 (95)	92.4(189)	88.4(474)	91.1
October 99	834	92.6(95)	93.3(189)	88.9(550)	91.6
December 99	930	92.6(95)	91.8(285)	88.1(550)	90.8
February 00	1088	80.9(157)	93.6(381)	88.2(550	86.2
April 00	1184	80.2(157)	91.6(381)	86.1(646)	85.2
June 00	1343	76.2(316)	87.9(381)	87.4(646)	83.8
August 00	1451	82.3(424)	86.2(646)	87.4(381)	85.3

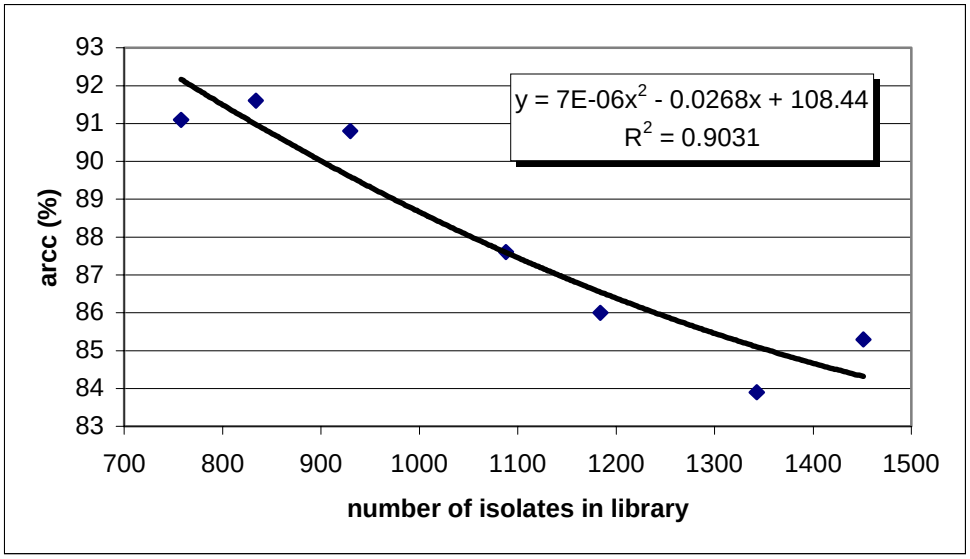

Figure 2: Changes in ARCC with increase in library size

Geographical constraint

The Blackwater library and the Shenandoah Valley library were combined into one large library to demonstrate the geographical constraint of an ARA library. Correct classification rates for the Blackwater are 82.3% for human isolates, 86.2% for livestock and 87.4% for wildlife, with an ARCC of 85.3% (Table 9). The correct classification rates for the Shenandoah library are 93.4% for human, 87.4% for wildlife and 86.0% for wildlife with an average rate of correct classification (ARCC) of 88.9%. These high correct classifications indicated that both libraries were good tools for source tracking. However, when the profiles from the two libraries were combined and DA was performed, the correct classifications changed to 72.4% for human, 85.0% for livestock, and 82.7% for wildlife with an ARCC of 80.0%. Each of the three categories had a lower correct classification when the two libraries were combined, indicating that isolates from a small geographical area results in a better library.

Table 9: Correct classifications of Blackwater, Shenandoah and Combined libraries

Category	Blackwater	Shenandoah	Combined
Human	82.3%	93.4%	72.4%
Livestock	86.2%	87.4%	85.0%
Wildlife	87.4%	86.0%	82.7%
ARCC	85.3%	88.9%	80.0%

ARA profiles from isolates from sample BW64, taken from the Middle Blackwater, were compared to the Shenandoah Valley library. The Blackwater library classified 34.0% of the isolates as human, 42.5% as livestock and 23.4% as wildlife (Table 10). The same set of isolates compared to the Shenandoah library classified 0% as human, 93.7% as livestock and 6.2% as wildlife. Twenty-two of 47 isolates (46.8%) were placed into the same category by both libraries. The stream sample BW64 was compared to the combined library with 16.7% of the isolates placed in the human category, 60.4% in livestock and 22.9% in wildlife. Although the percentages in each category are different, livestock was still the dominant source when the sample was compared to each library. Whether these libraries exhibit geographical restriction depends on the goals of the project. Regulatory agencies usually need the dominant source, but modelers for TMDL projects need the allocations from the major sources.

Table 10: Percentages of isolates in human, livestock and wildlife for sample BW64 compared against the Blackwater library, Shenandoah library and a combined library.

Category	Blackwater	Shenandoah	Combined
Human	34.0%	0.0%	16.7%
Livestock	42.5%	93.7%	60.4%
Wildlife	23.4%	6.2%	22.9%

SUMMARY

Antibiotic resistance analysis was used to obtain profiles of 1,451 enterococcal isolates collected from the Blackwater River basin in Franklin County, Virginia. The correct classification rates for this library were 82.3% for human isolates, 86.2% for livestock and 87.4% for wildlife, comparable to other bacterial source tracking projects. The library was constructed

with isolates added on eight separate events. As isolates were added, the ARCC fell, but even the lowest ARCC was high enough for use in a BST project. A similar library was constructed using Biolog metabolic profiles of 300 enterococci with correct classification rates of 88.6% for human, 78.5% for livestock, 84.6% for urban (dog and cat) and 69.7% for wildlife. A stream sample collected from the Blackwater was profiled using ARA and Biolog, with 47% of the isolates classified in the same category by both methods. The geographical restrictions of an ARA library were shown by comparing the Blackwater library with one constructed from isolate profiles from the Shenandoah Valley, Virginia. When the two libraries were combined to form one, the correct classifications of human, livestock and wildlife dropped. A stream sample from the Blackwater was compared to the Blackwater library, the Shenandoah library and the combined library. All three sets of classifications had different distribution of isolates in the human, livestock and wildlife categories, but livestock remained the dominant source for each.

The results from this study suggest that ARA is an effective tool for bacterial source tracking. While the ARCC of the library decreased as the number of isolates increased, a large library should be used for source tracking projects in order to get adequate representation from the watershed. Bacterial source tracking with Biolog looks promising due to the high rates of correct classification, but it did not give the same results as ARA in this example. A higher similarity of classification may be found with a larger sample size than 30 isolates. Given that the same stream isolates, when compared to three libraries, resulted in the same dominant source category, suggests that a rough estimate of the source of fecal contamination to a water source can be made with a library made with isolates from a large area. Bacterial source tracking results for a stream sample are probably similar to indicator bacteria counts in their highly variable

nature, even over a short time span. In this light, bacterial source tracking results are semi-quantitative, and should be viewed as one of the many tools for managing water quality.

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CHAPTER IV

DETERMINING SOURCES OF FECAL POLLUTION IN THE BLACKWATER RIVER USING ANTIBIOTIC RESISTANCE ANALYSIS

INTRODUCTION

Fecal bacteria are the major cause of impairments in Virginia's waterways according to the Virginia Department of Environmental Quality (DEQ), with agriculture listed as the primary source of contamination (Virginia DEQ 1998). Fecal coliforms, specifically *Escherichia coli* (*E. coli*), are indicator organisms used to demonstrate the potential presence of microbial pathogens and an increased risk of exposure to these pathogens in water. Knowing the sources of fecal pollution is fundamental to the total maximum daily load (TMDL) program currently being implemented nation-wide. Fecal coliforms, *E. coli*, and other enteric bacteria are present in warm-blooded animals and humans, as well as some cold-blooded reptiles and most birds (Harwood et al. 1999, Alderisio and DeLuca 1999). A simple enumeration of fecal coliforms is not sufficient to implement best management practices for bringing impaired stream segments into compliance, especially in a mixed-use watershed.

The total maximum daily load (TMDL) is a process to clean up impaired waters to meet water quality standards. A TMDL is pollutant-specific, so for a stream segment with two impairments, like nutrients and fecal coliforms, separate TMDLs must be developed for each. The TMDL process is watershed based, meaning the influence of land surrounding the water body and the water body are viewed together as a whole. TMDL was mandated by section 303-d Clean Water Act of 1972, but that section was virtually ignored for nearly 25 years while point sources were controlled. TMDL projects are being pursued across the country mainly because although point source discharges are generally under control, many water bodies are still impaired. So far, nonpoint source pollution, including pathogens, has not been addressed by

Clean Water Act mandates and TMDL is the mechanism to do so. TMDL projects are being developed in many states because of court orders; in Virginia, the EPA was sued by the Virginia Canoe Association (DEQ 2001b). Section 303(d) of the clean water act requires states to follow several steps in the TMDL process. The first is to identify water bodies that do not meet state water quality regulations after point sources are controlled. After identification, states must develop the TMDL and submit to EPA for approval. If the TMDL is approved, implementation begins. If EPA disapproves, it will take over developing the TMDL for the state, as has been done in West Virginia and Georgia.

Identifying sources of pollution can also help communities/states make risk assessments. Evidence of human contamination indicates human fecal pollution and an increased risk of human viruses such as enteroviruses (poliovirus, coxsakievirus A and B, echovirus and others) (Rotbart 1995). Hepatitus A virus, which causes fever, abdominal pain and nausea followed by jaundice, is stable in the environment and resistant to chlorine water treatment (Maier et al. 2000). Besides viruses, some parasitic and bacterial diseases harbored in wildlife and livestock can invade the human body. *Cryptosporidia parvum* is a protozoal parasite that is transmitted via the fecal/oral route and causes gastroenteritis. *C. parvum*¸ the species that infects humans, has been detected in cattle, white-tailed deer, sheep, horses, pigs, cats, dogs, and other pet and wild mammals (Fayer 1997). *Campylobacter jejuni,* a waterborne bacterium, also causes gastroenteritis. Stern states "no single animal food source can be excluded as a potential vehicle for infection in humans" (Nachamkin et al 1992). This fact could be extended to conclude that many, if not all, animals can contaminate water and possibly infect humans. *Giardia lamblia,* the cause of backpacker's disease, is the most encountered intestinal parasite in the U.S. (Maier et al. 2000). Wild and domestic animals, especially beavers and muskrats carry the parasite and

shed vast quantities in the environment. Even a small number of cysts in a water supply can cause infections in humans. Every species of the genus *Salmonella,* a bacteria carried by waterfowl, are pathogenic to humans, and the number of cases of salmonellosis are rising in the United States (Maier et al. 2000). Even though bacterial source tracking will not directly address problems with pathogens carried by humans and animals alike, using BST for restoring water bodies in the TMDL process will aid in reducing the risk of infection.

Several bacteria source tracking (BST) methods are under development for use in determining sources of fecal pollution in a watershed. Both molecular methods such as ribotyping, pulse-field gel electrophoresis, and randomly amplified polymorphic DNA, and non-molecular methods such as antibiotic resistance analysis (ARA), nutritional patterning, cell wall fatty acid analysis, and strain specific coliphages have been used (and published) in source tracking projects (Hagedorn 2001). ARA has been developed for both fecal streptococci and *E. coli* (Harwood et al. 2000). ARA relies on different antibiotic resistance patterns that can be related to specific sources of fecal pollution. Variations of the ARA method used in this study have been successfully employed by other researchers and in other watersheds (Wiggins et al. 1999, Hagedorn et al. 1999, Glasoe and Bower 2001). Benefits of ARA include use of simple laboratory techniques, basic equipment requirements, and can be performed at a relatively low cost compared to some other methods. In addition, high levels of separation have been found comparable to those reported for molecular methods (Dombek et al. 2000).

The Blackwater River is one of Virginia's high priority areas for TMDL development. Monthly compliance monitoring is performed by DEQ at five stations in the Blackwater River watershed. During the 1993-1995 assessment period for the 1996 303(d) list, 4 of the 5 stations exceeded the 10% violation limit used for classification as impaired. For the 1998 303(d) list, a

longer assessment period was used (1992-1997), and the list incorporated the results from a five-year 319 funded study, resulting in seven segments being designated as impaired. These segments represent 180.2 stream km, with the suspected source of impairment identified as non-point source (NPS) agriculture.

The goal of this study was to develop and test BST methodology in the Blackwater River watershed, Franklin County, Va (Figure 1, Appendix A). The project objectives were (1) to use ARA as a BST method to develop a profile library of fecal bacteria from known sources in the watershed, and (2) compare profiles of fecal bacteria from stream in the watershed (unknown sources) against the profile library to determine the sources of the fecal bacteria.

This research is the part of the Blackwater River watershed project that will provide a proof-of-concept for BST methodology. Target audiences for BST results include the many small communities and the agricultural industry in the watershed, but the main target audience is regulatory agencies such as the Department of Conservation and Recreation (DCR), DEQ, and the EPA. The potential exists to provide agencies responsible for water quality and public health with a resource to determine sources of fecal contamination. Until sources of pollution are identified, risk to communities cannot be accurately assessed, and water quality improvements will remain a hit-or-miss affair.

MATERIALS AND METHODS

Study Area and Sampling Regime

The Blackwater River watershed is located in Franklin County, Virginia and is part of the Roanoke River basin (Appendix A, Figure 1). The watershed encompasses approximately 72,000 ha of rolling piedmont and mountain topography in the northern half of the county. The Blackwater River basin is divided into six main areas: South Fork, North Fork, Upper

Blackwater, Middle Blackwater, Maggodee Creek, and Lower Blackwater. This research project included the South Fork, North Fork, Upper and Middle Blackwater, an area of 28,463 ha (Appendix A, Figure 2). A 1999 survey estimated population of the project area at 5,756 people in several small communities and rural areas (Map Tech 2000). The upper mountainous areas are predominately forested, while most agriculture activities are located in the piedmont region of the watershed. Land use areas are estimated as 18,528 ha forest, 8,858 ha agriculture, and 1,077 ha urban (Map Tech 2000). The Blackwater River watershed contains several possible sources of fecal contamination including abundant wildlife populations, many private septic systems from rural homes, urban runoff, active dairy and beef production, and intensive row crop agriculture. Franklin County has the second highest population of dairy cows and the 6th highest population of total cattle in Virginia (Map Tech 2000). The Blackwater River supplies drinking water for the town of Rocky Mount and ultimately drains into Smith Mountain Lake, a 8,000 ha recreational and hydroelectric impoundment on the Roanoke River. The Roanoke River flows southeast below Smith Mountain Lake and empties into Abermarle Sound in North Carolina.

Thirteen water quality monitoring sites in the four subwatersheds were sampled throughout the project. Sites BSF001.15, GCR000.01, and GCR002.44 are located in the South Fork area. Sites BNR000.40, BNR009.36, and BNR004.36 are located in the North Fork Area. Sites BWR061.20, Subwatershed 8 outlet and BWR054.81 are located in the Upper Blackwater area. Sites LLE005.22, TEL001.02, BWR045.80, and BWR032.32, and are located in the Middle Blackwater area. Table 1 shows the sampling schedule for the BST project. From September 1999 to August 2000, sites were sampled periodically according to Map Tech's sampling regime. From October 2000 to April 2000, sites were sampled monthly according to the TMDL sampling plan DCR and Map Tech finalized.

Table 1: Blackwater River Sampling Sites and Dates Monitored

Site	9/99	10/99	12/99	3/00	8/00	10/00	11/00	12/00	1/01	2/01	3/01	4/01
BNR000.40	X	X	X	X	X	X	X	X	X	X	X	X
BNR009.36	X	X	X	*	X	X	X	X	X	X	X	X
BSF001.15	X	X	X	X	X	X	X	X	X	X	X	X
BWR032.32	X	X	X	X	X	X	X	X	X	X	X	X
BWR045.80	X	X	X	X	X	X	X	X	X	X	X	X
BWR054.81	X	X	X	X	X	X	X	X	X	X	X	X
BWR061.20	X	X	X	*	X	X	X	X	X	X	X	X
LLE005.22	*	*	*	X	X	X	*	X	*	X	*	X
TEL001.02	X	X	X	X	X	X	X	X	X	X	X	X
GCR002.44	X	X	X	*	*	X	X	X	X	X	X	X
BNR004.36	*	*	*	*	*	X	X	X	X	X	*	X
Rt.643sub8	*	*	*	*	*	X	X	X	X	X	X	X
GCR000.01	*	*	*	*	*	X	X	X	X	X	X	X

*Not sampled

Building the Known Source Library

Sample Collection: Enterococcus isolates from thirteen known sources were collected in Franklin County, Virginia from September 1999 to April 2000 to build the library. These sources included dairy and beef cow, chicken, horse, goat, deer, raccoon, muskrat, goose, duck, coyote, wild turkey and human. Livestock fecal samples (cow, horse, chicken, goat) were collected from local farms. Wildlife fecal samples (deer, raccoon, muskrat, goose, duck, coyote and wild turkey) were collected in areas where wildlife had been observed and scats could be identified by qualified persons. Human fecal matter was collected from septic tank pump-out trucks in Franklin and Clarke Counties, Virginia. Human samples from Franklin County were few, so isolates from Clarke County were added to make the percentage of isolates from the three categories more evenly distributed in the known source library. Solid fecal samples were placed in sterile Whirlpac bags or petri plates and liquid samples were collected in sterile polystyrene bottles. All samples were placed on ice for transport to the laboratory and processed within 24 h of collection.

Isolation of Enterococci: Isolation of enterococci from known sources was done by adding 10.0 g of solid fecal material or 10.0 ml of liquid fecal material to 90 ml of sterile phosphate buffer to make a 1:10 dilution. Fecal material from the same source and sampling date were combined to make a composite sample when possible in order to obtain fewer isolates from a larger number of samples. Three additional 1:10 dilutions were made, resulting in 1:100, 1:1,000 and 1:10,000 dilutions. Each dilution was plated in duplicate on mEnterococcus agar (BBL) in 15x100 mm petri dishes by spreading 0.1 ml on each plate and incubated at 37^{o}C for 48 h. An individual colony was transferred to 96-microwell plate with a sterile toothpick containing 0.2 ml Enterococcosel broth (BBL) in each well. Up to 48 colonies from each sample were transferred to microwells. Microwell plates were incubated at 37^{o}C for 24 h. Confirmation of enterococci was determined by a color change in the Enterococcosel broth after incubation, from yellow to black, caused by esculin hydrolysis.

Analysis of Stream Samples

Sample Collection: Water samples were taken according to the sampling schedule outlined in Table 1. Samples were collected in sterile polystyrene bottles and packed on ice for transport. Filtering took place within 24 h, usually on the same day as collection.

Enumeration and Isolation: Samples were screened for *E. coli* using the Colilert system (IDEXX Laboratories, Inc., Westbrook, Maine). Fecal coliform counts were obtained by filtering and placing the filter mFC agar (BBL, Cockeysville, MD) according to Standard Methods (American Public Health Association 1995). Enterococci counts from positive samples were obtained similarly, placing the filters on mENT agar (BBL) and incubating at 37^{o}C for 48 h. Results were recorded as colony forming units (CFU) per 100 ml.

Antibiotic Resistance Analysis

Thirty treatments of a combination of nine antibiotics in various concentrations were used to determine antibiotic resistance patterns of the known source enterococcus isolates. Antibiotic stock solutions were prepared from commercial antibiotic powders (Sigma Chemical Co., St. Louis, MO) (Table 2).

Table 2: Concentration and solvent of antibiotic stock solutions

Antibiotic	Commercial formulation	Solvent	Stock Conc (mg/ml)
Chlortetracycline	Chlortetracycline HCl	1N sodium hydroxide	10
Oxytetracycline	Oxytetracycline HCl	1:1 water-methanol	10
Streptomycin	Streptomycin sulfate	Distilled water	10
Cephalothin	Cephalothin	Distilled water	10
Erythromycin	Erythromycin	1:1 water-ethanol	10
Tetracycline	Tetracycline HCl	Methanol	10
Neomycin	Neomycin sulfate	Distilled water	10
Vancomycin	Vancomycin HCl	1:1 water-ethanol	10
Amoxicillin	Amoxicillin	1:1 water methanol	2.5

Antibiotic agar was prepared by adding an aliquot of stock solution to a flask of autoclaved Trypticase Soy Agar (TSA, BBL) cooled to 50^{o}C to achieve the desired concentration (Table 2), mixed until uniform, and poured into sterile 15x100 mm petri dishes so that each dish contained one antibiotic concentration. A control plate containing no antibiotic was included. Isolates were transferred from the microwell plate using a stainless steel 48-prong replica plater (Sigma). The inoculant was allowed to soak into the TSA and the plates were incubated at 37^{o}C for 48 hours. Resistance to an antibiotic was determined by comparing the growth of each isolate on the antibiotic plate to the growth on the control plate. A one (1) was recorded if the isolate grew (a complete circle, mostly filled) and a zero (0) was recorded for any other case. This was repeated for each isolate on each of the thirty antibiotic plates.

Table 3: Concentrations of antibiotic treatments in TSA plates

Antibiotic	Plate concentrations (μg/ml)
Chlortetracycline	60, 80, 100
Oxytetracycline	20, 40, 60, 80, 100
Streptomycin	40, 60, 80, 100
Cephalothin	10, 15, 30, 50
Erythromycin	10, 15, 30, 50
Tetracycline	10, 15, 30, 50, 100
Neomycin	40, 60, 80
Vancomycin	2.5
Amoxicillin	.625

Statistical Analysis

Statistical analysis was performed using the discriminant analysis (DA) model in JMP-In statistical software (version 3.2.6 for Windows, SAS Institute, Inc.). Known source profiles were used to build the known source library by comparing the profile of an isolate to every other isolate in the library. Each isolate was assigned to the livestock, wildlife or human category based on the type of known source from which it came. The DA model was run on the combined isolates and JMP placed each isolate into a category if the probability of a fit in that category was greater than 50%. The number of isolates in a model-assigned category divided by the number of isolates in that category based on the organism is the percent of correct classification for that category. For stream isolates, the profiles were placed into the JMP library and the model assigned them a predicted source by comparing each profile to the known source profiles. Usually 48 isolates from each stream sample were entered into the library and classified by source. The percentage and number of isolates from the stream sample in each category was developed in this matter.

Chemical and Physical Analysis

The Virginia Department of Environmental Quality regularly monitors sites along the Blackwater River for temperature, pH, conductance, flow, nutrients and other dissolved

constituents. Flow data were collected for sites BNR000.40 on the North Fork and BSF001.15 on the South Fork of the Blackwater River during August 1999 and monthly from January 2000 to April 2001. The results of this monitoring were obtained for August 1999, January 2000 through August 2000, October 2000 and April 2001 for sites BNR000.40, BSF001.15, BWR032.32, BWR045.80, BWR054.81, BWR061.20, LLE005.22 and TEL001.02.

RESULTS AND DISCUSSION

Bacterial Source tracking

Up to 48 isolates from each water sample (unknown sources), a total of 4,852 isolates, were sourced using ARA and DA. Tables 1-12 in Appendix B show the number of isolates, and source tracking results for each sample site during that sampling month.

The significant percentage of a source in a stream sample was determined for human, livestock and wildlife by calculating the expected frequency of misclassification using a variation of the method described by Harwood, et al. (2000). Expected frequency of misclassification is the percentage of isolates from a known source that are placed in the wrong predicted source category. A stream sample with less than this percentage of contamination from any source category could be attributed to error in the database. The significant percentage of human was determined by adding the number of livestock and wildlife isolates that were placed in the human predicted source category, then dividing by the total number of livestock and wildlife isolates (Table 4). The percentage of non-human isolates that were misclassified as human was 5.4%. A stream sample with greater than 5.4% human can be treated as having significant human contamination because 5.4% percent of non-human isolates were misclassified by discriminant analysis as human. Similarly, the percentage of misclassification of livestock was determined to be 10.2% by adding the numbers of livestock isolates that were placed in

human and wildlife categories and dividing by the total number of human and wildlife isolates. The significant level of wildlife contamination was calculated at 7.0% by adding the total number of human and livestock isolates misclassified as wildlife and dividing by the total number of human and livestock isolates.

Table 4: Antibiotic resistance analysis database of known source isolates

Predicted Source	Percent of known source isolates assigned to each predicted source (no. of isolates)		
	Human	Livestock	Wildlife
Human	**82.3** (349)	7.1 (46)	2.4 (9)
Livestock	10.1 (43)	**86.2** (557)	10.2 (39)
Wildlife	7.5 (32)	6.7 (43)	**87.4** (333)
Total	424	646	381

In September 1999, two sites of 9 had more than 5.4% human (Table 5). October 1999 had one site above 5.4% human. December 1999 had six of 7 sites with substantial human contamination. In March 2000, every site sampled had greater than 5.4% human, and in August 2000, every sample was greater than 5.4% human. In October 2000, all 13 samples had greater than 5.4% human. In November 2000, ten of 12 sites had a significant human contribution. Of thirteen samples taken in December 2000, all had more than 5.4% human. In January 2001, all 12 samples had a significant human percentage. In February 2001, all of the eleven samples that were sourced had a human contribution greater than 5.4%. In March 2001, only seven samples of 12 were sourced and all seven had a significant human contribution. Of 12 samples of 13 sourced in April 2001, all 12 had a human percentage greater than 5.4% (Table 5). A significant human signature was present at every sampling site at some point during the year and indicates that human pollution is an important source of fecal bacteria at the sampling sites included in this study.

Sites with greater than 10.2% of the isolates from livestock sources were designated significant. A site with less than 10.2% has a higher probability of being incorrectly classified.

In September 1999, all 9 sites had more than 10.2% livestock (Table 5). October 1999 also had all sites above 10.2% livestock. December 1999 had every site sampled with substantial livestock contamination. In March 2000, every site sampled had greater than 10.2% livestock, and August 2000 had 9 of 9 samples greater than 10.2%. All thirteen samples taken in October 200 had greater than 10.2% livestock percentage. In November 2000, all twelve samples had a significant livestock percentage. Of thirteen samples taken in December 2000, eleven had more than 10.2% livestock. Of twelve samples taken in January 2001, every sample had a significant livestock percentage. In February 2001, every one of the eleven samples had more than 10.2% livestock percentage. Of seven samples source in March 2001, six had a significant livestock percentage. In April 2001, 10 of 12 samples had a significant livestock percentage (Table 5). A clear livestock signature was present at every sampling site at some point during the year and indicates that livestock pollution is also an important source of fecal bacteria (as with human) at the sampling sites included in this study.

Sites with greater than 7.0% of the isolates from wildlife sources were designated as significant. A site with less than 7% has a higher probability of being incorrectly classified. In September 1999, all nine sites had more than 7% wildlife (Table 5). October 1999 had eight of nine sites above 7% wildlife. December 1999 had all 7 sites with significant livestock contamination. In March 2000, every site sampled had greater than 7% wildlife, and August 2000 had 9 of 9 samples greater than 7% wildlife. In October 2000, 11 of 13 samples had a significant wildlife percentage. All twelve samples taken in November 2000 had a significant wildlife percentage. Of thirteen samples taken in December 2000, all had a wildlife percentage greater than 7%. In January 2001, all twelve samples had a significant wildlife percentage. In February 2001, ten of eleven samples had greater than 7% wildlife percentage. Of seven

samples sourced taken in March 2001, all had a significant wildlife percentage. In April 2001, 11 of 12 samples had a wildlife percentage greater than 7% (Table 5). A clear wildlife signature was present at every sampling site at some point during the year and indicates that wildlife pollution is also an important source of fecal bacteria (as with human and livestock) at the sampling sites included in this study.

Of 121 samples taken during the sampling period, 103, or 85.1% had a significant contribution of human isolates, 116, or 95.9% had a significant percentage of livestock isolates, and 116, or 95.9% had a significant wildlife contribution (Table 5). This emphasizes the need to address all three source categories for success in meeting water quality standards.

Table 5: Number of samples with significant percentages of human, livestock and wildlife for each sampling month

Month	No. of samples	Human	Livestock	Wildlife
September 1999	9	2	9	9
October 1999	9	1	9	8
December 1999	7	6	7	7
March 2000	7	7	7	7
August 2000	9	9	9	9
October 2000	13	13	13	11
November 2000	12	10	12	12
December 2000	13	13	11	13
January 2001	12	12	12	12
February 2001	11	11	11	10
March 2001	7	7	6	7
April 2001	12	12	10	11
Total	121	103	116	116
Percentage		85.1%	95.9%	95.9%

Human, livestock and wildlife categories each dominated the samples at various sites and months. The category with the largest percentage was considered to be dominant for any one sample. In September 1999, livestock dominated approximately half of the samples (5 of 9) and wildlife the others (4 of 9, Table 6). In October 1999, livestock dominated 8 of 9 samples with wildlife dominating 1 of 9. In December 1999, livestock dominated two samples, and wildlife 4

of 7. One sample was split between wildlife and human at 41.7% each. In March 2000, livestock dominated 1 of 7, wildlife dominated three and human dominated 3 of 7. In August 2000, human dominated 1 of 9 samples, livestock dominated 8 of 9 samples, and wildlife dominated none. In October 2000, human dominated 5 of 13 samples, livestock 7 of 13 and wildlife 1 of 13 samples. Of twelve samples taken in November 2000, 10 were dominated by livestock and 2 dominated by wildlife, none dominated by human. In December 2000, human dominated 6 of 13 samples, livestock dominated 3 and wildlife dominated 4 samples. In January 2001, 4 of 12 samples were dominated by human, 6 samples dominated by livestock, 1 was dominated by wildlife, and 1 was split between wildlife and livestock. Of 11 samples taken in February 2001, 6 were dominated by human, 4 dominated by livestock, and 1 dominated by wildlife. In March 2001, 5 of 7 samples sourced were dominated by human, none by livestock and 2 by wildlife. Of 12 samples taken in April 2001, 6 were dominated by human, 1 by livestock, and 5 by wildlife (Table 6). Livestock dominated 46.3% of the samples over the entire sampling period, followed by human at 30.5% and wildlife at 24.8%. With a reasonably even distribution, all three source categories will require attention for BMP implementation.

Table 6: Number of samples dominant in each category for each sample

Month	No. of samples	Human	Livestock	Wildlife
September 1999	9	0	5	4
October 1999	9	0	8	1
December 1999	7	1	2	5
March 2000	7	3	1	3
August 2000	9	1	8	0
October 2000	13	5	7	1
November 2000	12	0	10	2
December 2000[a]	13	6	3	4
January 2001[b]	12	4	7	2
February 2001	11	6	4	1
March 2001	7	5	0	2
April 2001	12	6	1	5
Total	121	37	56	30
Percentage		30.5%	46.3%	24.8%

[a]One sample in December 1999 was split between wildlife and human.
[b]One sample in January 2001 was split between wildlife and livestock.

Source tracking comparisons generally cannot be made among sites due to large distances between sites and differences in land use patterns throughout the Blackwater basin. Therefore, the most sensible way to interpret BST data is to examine the results loosely as a function of the activities in the surrounding area, which does not always follow land use. A forested site may lend to having the source labeled as wildlife in the absence of BST, but a significant portion of the fecal loading may be human if there are faulty drainfields in the area. A site with pasture may be labeled as a source of livestock loading, but in reality the loading may come from a straight pipe or abundant wildlife populations. BST is the best tool available for answering these questions. Sampling sites the Blackwater basin have a variety of land uses immediately surrounding them. The sampling locations have forest, open space, pasture, cropland, urban, or a combination of two or three uses. For example, Site BNR000.40 on the North Fork of the Blackwater River is surrounded by grass and pasture. However, the dominant source of fecal pollution at that site was human, livestock and wildlife at various times during the sampling period. Table 7 shows the percentage and number of samples in the three source categories

divided by watershed. In the North Fork of the Blackwater River, the majority of the samples were dominated by human isolates, followed by livestock, then wildlife. Livestock is the predominant source in the South Fork, Upper, and Middle Blackwater. The relatively higher human signature in the North Fork may be the result of an absence of other sources or because the human loading is high. In the remaining three watersheds, the absolute human loading may be as high as in the North Fork, but a high livestock contribution to the stream would mask it. The land use surrounding the site does not immediately explain the BST results, but, rather, the BST data give a reasonable tool for BMP implementation. The results call for consideration to all three source categories, not just agriculture, the suspected source of fecal pollution, so that water quality standards can be met.

Table 7: Percentage (and number) of samples in the four Blackwater impairments dominant in each source category

	N. Fork	S. Fork	Upper	Middle
Human	46.2%(12)	33.3%(9)	29.0%(9)	20.0%(8)
Livestock	38.5%(10)	44.4%(12)	38.7%(12)	55.0%(22)
Wildlife	15.4%(4)	22.2%(6)	32.3%(10)	25.0%(10)
Total	26	27	31	40

When the sample months were divided into warm (May, June, July, August, September, and October) and cool (November, December, January, February, March, and April) seasons, a difference in the distribution of which source dominated the sample became evident. Of 40 samples that were collected during the warm months, human dominated 15.0%, livestock dominated 70.0%, and 15.0% were dominated by wildlife (Table 6). With the 81 samples collected during the cool months, the proportion of isolates in each category was more evenly distributed. Human dominated 38.3% of the samples, livestock dominated 34.6% of the samples and wildlife dominated 29.6% of the samples (Table 6). This predominance of livestock during the warm months (70%) compared to cool months (34.6%) could be due to two scenarios. The

first explanation is that the proximity of livestock to the stream during warm months causes an increase in the loading that is revealed in the BST results. In this explanation, the loading contributions from human and wildlife are constant throughout the year, and when the livestock are kept away from the stream bank during cool months, the human and wildlife signatures come through. The second plausible explanation is that the human and wildlife loadings actually increase during winter months. The actually scenario may be a combination of the two. Restricting livestock access during the warm months could lower livestock loadings. Monitoring and performing BST would confirm whether that action alone could bring the impaired segments back into compliance with water quality standards.

Monitoring

Biological: Fecal coliform counts were placed into three categories based on colony forming units (CFU) per 100 milliliters. Samples with less than 100 CFU/100 ml were designated as low, samples between 100 and 1000 CFU/100 ml were moderate and samples with fecal coliforms greater than 1000 CFU/100 ml were high. Samples in the high category of greater than 1000 CFU/100 ml exceed the Virginia recreational water use standard (Virginia DEQ 2001a). Table 8 shows the number of samples taken during each sampling month and the number of samples in each of the three categories. Of 131 samples taken from September 1999 to April 2001, 25 (19%) were low, 71 (54%) were moderate and 35 (27%) were high.

Table 8: Classification of fecal coliform counts into low, moderate and high categories

Month	No. of samples	Low	Moderate	High
September 1999	9	0	6	3
October 1999	9	1	3	5
December 1999	9	3	6	0
March 2000	7	0	5	2
August 2000	9	0	3	6
October 2000	13	0	3	10
November 2000	12	3	9	0
December 2000	13	1	8	4
January 2001	12	2	7	3
February 2001	13	5	7	1
March 2001	12	3	8	1
April 2001	13	7	6	0
Total	131	25	71	35

*Low = < 100 CFU/100 ml; moderate = 100-1000 CFU/100 ml; high = >1000 CFU/100 ml

Chemical and physical: Virginia Department of Environmental Quality periodically monitored eight sites in the upper Blackwater River watershed from August 1999 to April 2001. Temperature, pH, dissolved oxygen, conductivity, and nitrate levels (NO_3-N) were measured (Table 9).

Table 9: Chemical and Physical Measurements

Parameter	Temperature (^oC)	pH	Dissolved Oxygen (mg/L)	(NO_3-N) (μg/ml)	Conductivity (μmhos/cm)
Mean	15.0	7.3	8.2	.63	73.8
Minimum	.2	6.0	5.42	.19	8.8
Maximum	27.2	8.7	12.9	1.13	112.7
Standard dev.	7.7	.6	1.6	.2	16.6

Chemical and physical analysis values were within acceptable ranges. Temperature ranged from 0.2^{o}C in January 2000 to 27.2^{o}C in August 1999 with an average of 15.0^{o}C +/- 7.7. The pH values averaged 7.3 +/- .6, ranging from 6.0 to 8.7. Dissolved oxygen averaged 8.2 mg/L +/- 7.5and ranged from 5.42 to 12.9 mg/L. Nitrate concentrations were low, averaging 0.63 μg/ml +/- .2 with a range of .19 to 1.13 μg/ml. Conductivity was relatively low, averaging 73.8 +/- 16.6 μmhos/cm, ranging from 8.8 to 112.7.

Stream flows were measured for two sites, one on the North Fork and one on the South Fork, for selected months during the study period in cubic meters per second by the Virginia DEQ (Appendix A, Figure 3). Flow was lowest during August 1999, a period of drought for the region. The highest measured flows occurred during March 2000. Generally the flow in the Blackwater basin peaks during the spring. The median flow is 1.4 cms.

SUMMARY

ARA has proven to be an effective tool in determining sources of fecal pollution in an impaired watershed, shown by the high rates of correct classification in the known source library. Samples from the Blackwater River were found to contain bacterial isolates from livestock, wildlife, and human sources in varying proportions throughout the sampling period. To improve water quality in the impaired stream segments, best management practices (BMPs) will be developed and implemented by regulatory and community officials to reduce fecal loading. Reliable identification of fecal pollution sources will ensure that BMP efforts and costs are directed at the correct sources. Before source tracking was made available, implementing BMPs to improve water quality was relative guesswork. Now, with source tracking methodology shown to work in a large watershed, a semi-quantitative tool is now available for use in BMP planning and implementation. BST results can also be used to assess the impacts of BMPs and to monitor the reductions and changes in the ratio of fecal bacteria from human, wildlife and livestock sources.

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CHAPTER V

SUMMARY

Blackwater Bacterial Source Tracking

There were two primary objectives in the Blackwater River watershed BST project and four secondary objectives. The first primary objective was to build a known source antibiotic resistance analysis (ARA) library from enterococcus isolates from fecal samples gathered in the watershed and the second was to compare isolate profiles from stream samples to the known source library to determine the source. ARA was done in the Blackwater project by isolating enterococci from a fecal or stream sample and observing a growth profile on 30 antibiotic plates of varying concentrations of nine antibiotics. A library of 1,451 enterococci isolates from thirteen sources was created. Each isolate was placed in either the human, livestock or wildlife category and discriminant analysis was performed on the profiles. The correct classifications were 82.3% for human isolates, 86.2% for livestock and 87.4% for wildlife, with an average rate of correct classification (ARCC) of 85.3%. Profiles were also determined for 48 isolates from 128 stream samples collected periodically from August 1999 thru April 2001 and compared to the known sources using discriminate analysis. A human signature was found at each site at least once during the year, ranging from 0.0 to 85.0% of the sample isolates. The livestock signature varied from 2.3% to 100% over sites and months, and the wildlife signature varied from 0.0% to 79.5%.

Comparison of ARA and Biolog

The first secondary objective was to compare ARA with the Biolog metabolic fingerprint system by profiling a set of isolates by both methods and comparing the predicted source of each isolate. The Biolog metabolic profiling procedure involves isolating enterococci, inoculating a

96-well plate containing a different carbon source in each well and recording the growth pattern of the isolate in 30 wells. A known source library was built using DA, similar to the ARA library. Thirty isolates from a Blackwater stream sample were profiled using Biolog. Of ten isolates identified by the Blackwater antibiotic resistance analysis library as human, the Biolog library identified one as human, four as livestock, and five as wildlife. Of ten isolates identified by the ARA library as livestock, the Biolog library identified seven as livestock and three as wildlife. Of ten isolates identified by ARA as wildlife, one was identified as human, three as livestock and six as wildlife. The overall correct classification of Blackwater isolates in the Biolog library was 14 of 30 isolates, or 47%. The lower than expected correct classifications may be explained by the fact that there are not enough isolates in the Biolog library to adequately represent the variability shown by the Blackwater isolates or that more than 30 isolates should be used for the comparison.

Optimum size of an ARA library

The second secondary objective was to explore the optimum size of a known source library by creating versions of the Blackwater library, running the discriminant analysis model and comparing the rates of correct classification. The Blackwater known source library was constructed with isolates from 8 sampling events. The library started with 758 isolates with RCCs of 92.6% for human, 92.4% for wildlife and 88.4% for livestock with an ARCC of 91.1%. ARCC fell to a low ARCC of 83.8% with 1,343 isolates, then rose slightly to 85.3% with 1,451 isolates. Overall, the ARCC fell when more isolates were added to the library, but even the lowest ARCC is within the values reported in published studies of ARA. The changes in correct classification when more isolates are added are likely also reflections on the types of isolates added to the library in addition to the number of isolates. Adding a set of profiles to the library

that are even slightly different from the existing library will diversify the library and cause ARCC to fall. There is a trade-off between including enough profiles to make the library representative of the watershed and having a high ARCC.

Geographical restraint of an ARA library

The third secondary objective was a test of the geographic restriction of the Blackwater ARA library. This was done using two steps. The step involved comparing a Blackwater stream sample against a known source library constructed from sources in the Shenandoah Valley, Virginia and against the Blackwater library, then comparing the predicted source for each isolates. The second step was to combine the Blackwater and Shenandoah libraries and observe the changes in RCCs. Correct classification rates for the Blackwater are 82.3% for human isolates, 86.2% for livestock and 87.4% for wildlife, with an ARCC of 85.3%. The correct classification rates for the Shenandoah library were 93.4% for human, 87.4% for wildlife and 86.0% for wildlife with an ARCC of 88.9%. These high correct classifications indicated that both libraries were good tools for source tracking. However, when the profiles from the two libraries were combined and DA was performed, the correct classifications changed to 72.4% for human, 85.0% for livestock, and 82.7% for wildlife with an ARCC of 80.0%. Each of the three categories had a lower correct classification when the two libraries were combined, indicating that isolates from a small geographical area results in a better library. ARA profiles from isolates from a Blackwater sample were compared to the Shenandoah Valley library. The Blackwater library classified 34.0% of the isolates as human, 42.5% as livestock and 23.4% as wildlife. The same set of isolates compared to the Shenandoah library classified 0% as human, 93.7% as livestock and 6.2% as wildlife. Twenty-two of 47 isolates (46.8%) were placed into the same category by both libraries. The stream sample was also compared to the combined

library with 16.7% of the isolates placed in the human category, 60.4% in livestock and 22.9% in wildlife. Although the percentages in each category are different, livestock was still the dominant source when the sample was compared to each library. Whether these libraries do in fact exhibit geographical restriction depends on the goals of the project. Regulatory agencies usually need the dominant source, but modelers for TMDL projects need exact numbers.

ARA in a Large Watershed

The fourth secondary objective was to test ARA in the framework of a TMDL project of a large watershed. The Blackwater watershed is around 11,518 ha. An ARA study performed by A. K. Graves was done on a 2,665 ha watershed (2000). Hagedorn et al. perform ARA in the Page Brook watershed, with 3,000 ha (1999). This objective was met by comparing the results from the known source library to other reported studies. The Blackwater ARA library had comparable RCCs with other libraries.

Appendix A: Figures

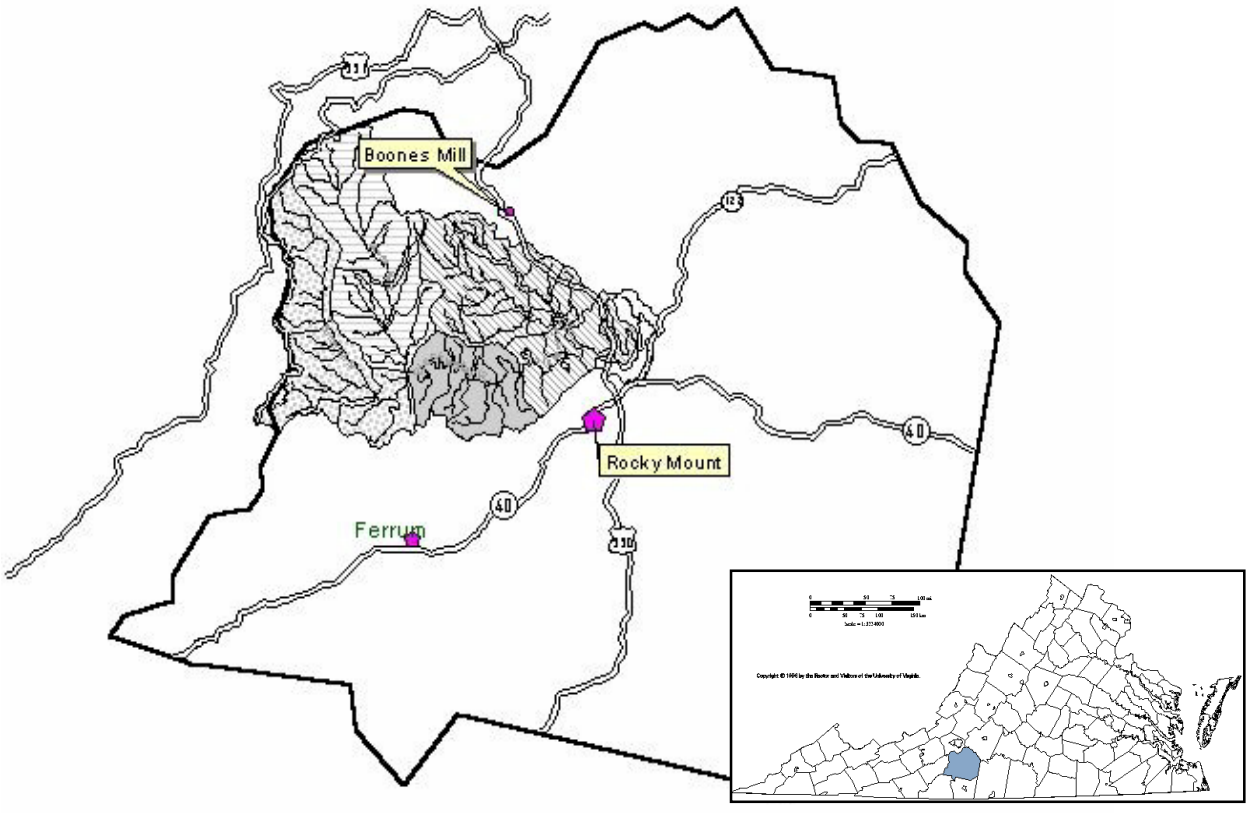

Figure 1: Franklin County, Virginia; Upper Four Impairments of Blackwater River

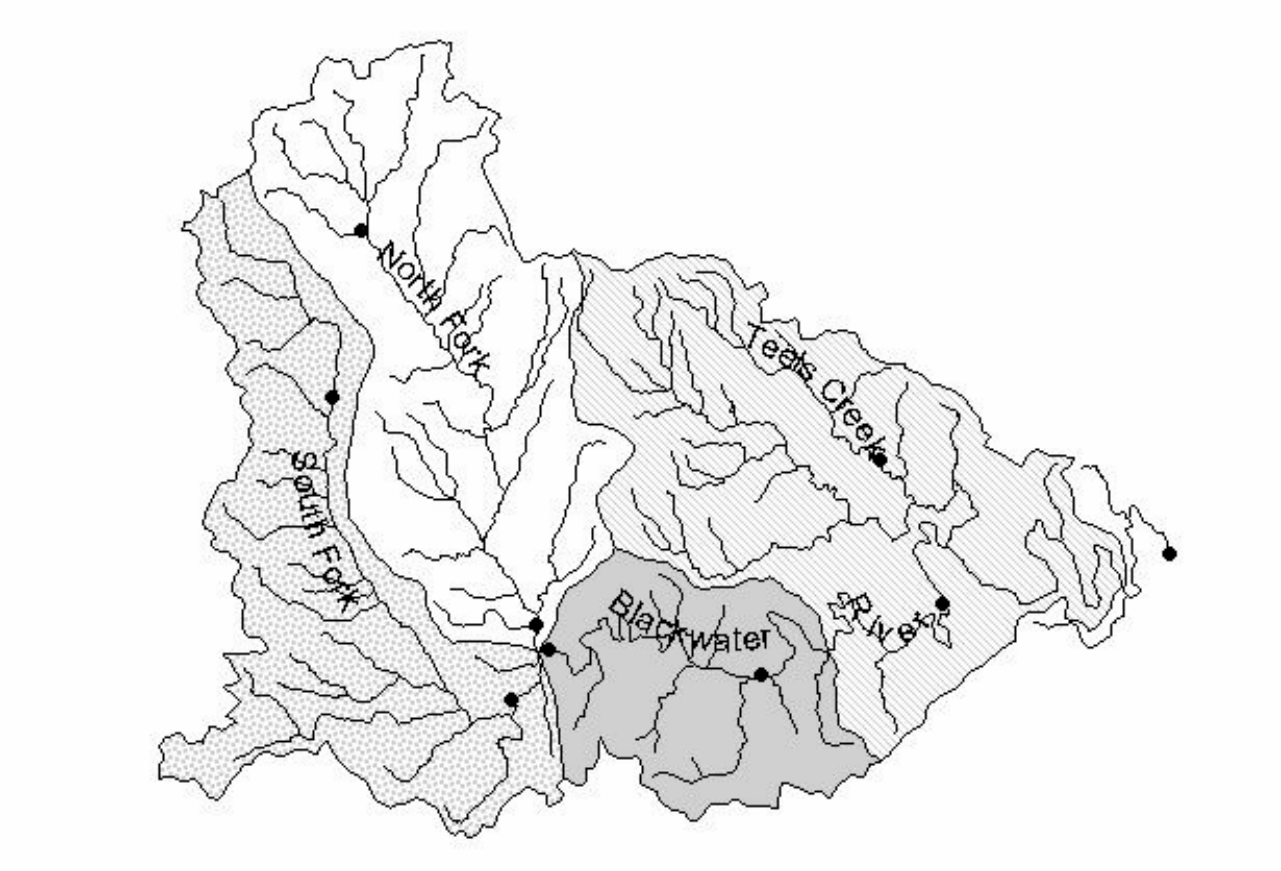

Figure 2: Upper Four Impairments of Blackwater River with Sampling Sites

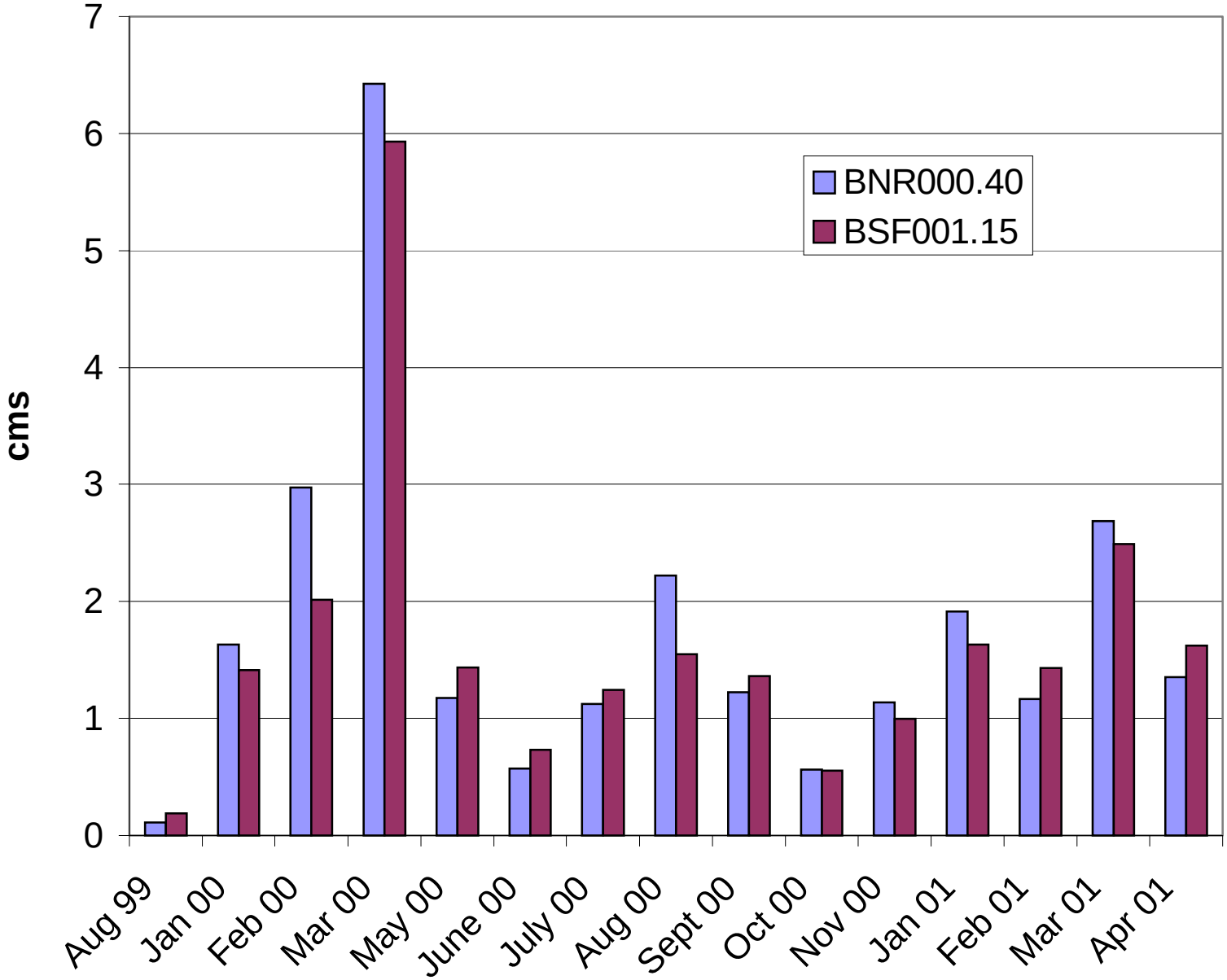

Figure 3: Flow (cms) for two sites in the Blackwater basin

APPENDIX B: BST RESULTS BY MONTH

Table 1: Antibiotic resistance analysis of enterococci for September 1999

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	48	14.6%	66.7%	18.7%
	BNR000.40	48	2.1%	29.2%	68.7%
South Fork	GCR002.44	45	0.0%	84.4%	15.6%
	BSF001.15	47	4.3%	66.0%	29.8%
Upper BW	BWR061.20	48	18.7%	39.6%	41.7%
	BWR054.81	48	4.2%	43.7%	52.1%
Middle BW	BWR045.80	47	0.0%	42.5%	57.4%
	BWR032.32	47	0.0%	62.8%	36.2%
	TEL001.02	48	0.0%	29.2%	70.8%

Table 2: Antibiotic resistance analysis of enterococci for October 1999

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	48	4.2%	91.7%	4.2%
	BNR000.40	47	10.6%	48.9%	40.4%
South Fork	GCR002.44	46	4.3%	87.0%	8.7%
	BSF001.15	48	2.1%	39.6%	58.3%
Upper BW	BWR061.20	47	2.1%	63.8%	34.0%
	BWR054.81	47	4.2%	72.3%	23.4%
Middle BW	BWR045.80	48	2.1%	58.3%	39.6%
	BWR032.32	48	2.1%	83.3%	14.6%
	TEL001.02	44	4.5%	87.0%	8.7%

Table 3: Antibiotic resistance analysis of enterococci for December 1999

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	*			
	BNR000.40	45	17.8%	40.0%	42.2%
South Fork	GCR002.44	*			
	BSF001.15	48	41.7%	16.7%	41.7%
Upper BW	BWR061.20	48	6.2%	22.9%	70.8%
	BWR054.81	48	18.7%	31.2%	50.0%
Middle BW	BWR045.80	48	18.7%	43.7%	37.5%
	BWR032.32	24	29.2%	12.5%	58.3%
	TEL001.02	48	4.2%	66.7%	29.2%

* Too few isolates for analysis

Table 4: Antibiotic resistance analysis of enterococci for March 2000

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	*			
	BNR000.40	32	56.2%	21.9%	21.9%
South Fork	GCR002.44	*			
	BSF001.15	48	37.5%	33.3%	29.2%
Upper BW	BWR061.20	*			
	BWR054.81	41	43.9%	31.7%	24.4%
Middle BW	BWR045.80	48	22.9%	31.2%	45.8%
	BWR032.32	45	20.0%	48.9%	31.1%
	TEL001.02	48	18.7%	29.2%	52.1%
	LLE005.22	48	25.0%	25.0%	50.0%

* Sample not taken

Table 5: Antibiotic resistance analysis of enterococci for August 2000

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	47	42.6%	46.8%	10.6%
	BNR000.40	47	51.1%	40.4%	8.5%
South Fork	GCR002.44	47	38.3%	46.8%	14.9%
	BSF001.15	47	17.4	58.7%	23.9%
Upper BW	BWR061.20	47	40.4%	51.1%	8.5%
	BWR054.81	47	31.9%	40.4%	27.7%
Middle BW	BWR045.80	46	28.9%	51.1%	20.0%
	BWR032.32	46	17.4%	54.3%	28.3%
	TEL001.02	47	38.3%	46.8%	14.9%

Table 6: Antibiotic resistance analysis of enterococci for October 2000

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	40	53.8%	41.0%	5.1%
	BNR004.36	43	7.0%	60.5%	32.6%
	BNR000.40	46	6.7%	71.1%	22.2%
South Fork	GCR002.44	45	59.1%	36.4%	4.5%
	GCR000.01	48	33.3%	37.5%	29.2%
	BSF001.15	35	14.7%	73.5%	11.8%
Upper BW	BWR061.20	48	8.3%	45.8%	45.8%
	BWR054.81	46	8.7%	87.0%	4.3%
	Sub 8 Outlet	45	15.6%	73.3%	11.1%
Middle BW	BWR045.80	47	75.0%	12.5%	12.5%
	BWR032.32	47	66.0%	19.1%	14.9%
	TEL001.02	45	56.8%	34.1%	9.1%
	LLE005.22	48	14.6%	60.4%	25.0%

Table 7: Antibiotic resistance analysis of enterococci for November 2000

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	45	12.2%	39.0%	48.8%
	BNR004.36	24	25.0%	70.8%	4.2%
	BNR000.40	30	6.7%	56.7%	36.7%
South Fork	GCR002.44	47	2.2%	52.2%	45.6%
	GCR000.01	31	19.3%	38.7%	41.9%
	BSF001.15	26	26.9%	65.4%	7.7%
Upper BW	BWR061.20	30	34.5%	51.7%	13.8%
	BWR054.81	39	7.9%	76.3%	15.8%
	Sub 8 Outlet	30	0.0%	100%	0.0%
Middle BW	BWR045.80	46	20.8%	50.0%	29.2%
	BWR032.32	27	33.3%	37.0%	29.6%
	TEL001.02	40	15.0%	77.5%	7.5%
	LLE005.22	*			

*Sample not taken

Table 8: Antibiotic resistance analysis of enterococci for December 2000

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	48	39.6%	33.3%	27.1%
	BNR004.36	48	51.1%	19.1%	29.8%
	BNR000.40	44	44.2%	25.6%	30.2%
South Fork	GCR002.44	48	51.1%	28.9%	20.0%
	GCR000.01	48	6.2%	50.0%	43.7%
	BSF001.15	48	37.5%	33.3%	29.2%
Upper BW	BWR061.20	48	32.6%	32.6%	34.8%
	BWR054.81	45	22.2%	24.4%	53.3%
	Sub 8 Outlet	47	42.5%	25.5%	31.9%
Middle BW	BWR045.80	48	25.0%	41.7%	33.3%
	BWR032.32	48	36.7%	47.7%	15.9%
	TEL001.02	48	22.9%	27.1%	50.0%
	LLE005.22	48	37.0%	13.0%	50.0%

Table 9: Antibiotic resistance analysis of enterococci for January 2001

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	48	58.3%	27.1%	15.6%
	BNR004.36	46	26.1%	60.0%	10.9%
	BNR000.40	48	28.9%	55.6%	15.6%
South Fork	GCR002.44	48	29.2%	56.2%	30.4%
	GCR000.01	48	45.8%	35.4%	18.7%
	BSF001.15	48	39.6%	22.9%	37.5%
Upper BW	BWR061.20	48	31.9%	40.4%	27.7%
	BWR054.81	48	33.3%	29.2%	37.5%
	Sub 8 Outlet	48	29.2%	35.4%	35.4%
Middle BW	BWR045.80	48	56.2%	33.3%	10.4%
	BWR032.32	48	20.8%	47.9%	31.2%
	TEL001.02	48	17.4%	52.2%	30.4%
	LLE005.22	*			

*Sample not taken

Table 10: Antibiotic resistance analysis of enterococci for February 2001

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	44	54.6%	11.4%	34.1%
	BNR004.36	48	64.4%	22.2%	13.3%
	BNR000.40	42	61.9%	35.7%	2.4%
South Fork	GCR002.44	22	40.9%	45.4%	13.6%
	GCR000.01	22	57.1%	28.6%	14.3%
	BSF001.15	48	47.9%	25.0%	27.1%
Upper BW	BWR061.20	48	43.7%	22.9%	33.3%
	BWR054.81	48	58.4%	15.2%	26.1%
	Sub 8 Outlet	48	50.0%	31.2%	18.7%
Middle BW	BWR045.80	47	27.7%	51.1%	27.3%
	BWR032.32	32	15.6%	40.6%	43.7%
	TEL001.02	48	35.4%	47.9%	16.7%
	LLE005.22	48	34.0%	42.5%	23.4%

Table 11: Antibiotic resistance analysis of enterococci for March 2001

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	*			
	BNR004.36	*			
	BNR000.40	48	81.2%	10.4%	8.3%
South Fork	GCR002.44	*			
	GCR000.01	*			
	BSF001.15	48	45.8%	22.9%	31.2%
Upper BW	BWR061.20	48	34.0%	17.0%	48.9%
	BWR054.81	48	21.9%	34.1%	43.9%
	Sub 8 Outlet	48	60.4%	29.2%	10.4%
Middle BW	BWR045.80	48	85.0%	5.0%	10.0%
	BWR032.32	*			
	TEL001.02	47	66.0%	19.1%	14.9%
	LLE005.22	#			

*Too few isolates for analysis
sample not taken

Table 12: Antibiotic resistance analysis of enterococci for April 2001

Stream	Rivermile	No. of isolates	Human	Livestock	Wildlife
North Fork	BNR009.36	*			
	BNR004.36	40	50.0%	30.0%	20.0%
	BNR000.40	44	22.7%	15.9%	61.4%
South Fork	GCR002.44	24	41.7%	8.3%	50.0%
	GCR000.01	28	22.2%	14.8%	63.0%
	BSF001.15	45	37.2%	20.9%	41.9%
Upper BW	BWR061.20	44	61.4%	2.3%	36.4%
	BWR054.81	48	75.0%	20.8%	4.2%
	Sub 8 Outlet	48	54.2%	35.4%	10.4%
Middle BW	BWR045.80	47	45.6%	26.1%	28.3%
	BWR032.32	48	31.8%	20.4%	47.7%
	TEL001.02	47	59.6%	19.1%	21.3%
	LLE005.22	48	33.3%	52.1%	14.6%

*Too few isolates for analysis

VITA

The author was born April 5, 1978 to Warren Bowman and Anne Herr. She grew up in Franklin County, Virginia, just south of the Blackwater watershed, and graduated from Franklin County High School in 1996. She graduated from Virginia Tech in 2000 with a bachelor of science in environmental science and a minor in chemistry.

During her fourth undergraduate year at Virginia Tech, the author began graduate coursework and research under Dr. Charles Hagedorn in the Crop and Soil Environmental Science department in the Environmental Science and Engineering major. She began working on a bacterial source tracking project in the Blackwater River, funded by MapTech, Inc. in Blacksburg, Virginia. This research was part of the development and implementation of a fecal coliform total maximum daily load, in conjunction with the Virginia Department of Conservation and Recreation.

Upon completion of all requirements for her degree, the author plans to live in Christiansburg, Virginia with her husband and work in the water quality field.