Using Microbial Source Tracking to Support TMDL Development and Implementation

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Contents

Executive Summary ..... **vii**

Introduction **1**

1. Using MST in TMDL Development and Implementation **2**

2. Understanding the Types of MST Methods **6**
Library-Dependent Methods 11
Genotypic Methods 11
PFGE 11
Ribotyping 12
rep-PCR 13
Phenotypic Methods 13
Antibiotic Resistance 13
Carbon Utilization 14
Library-Independent Methods 14
Bacteriophage 14
Viral PCR 15
Bacterial PCR 15
Quantitative PCR 16

3. Deciding Whether to Use MST **17**

4. Selecting an MST Method **20**

5. Designing an MST Study **23**
What to Collect and Analyze? 24
Where to Collect Samples? 25
When to Collect Samples? 26
How to Collect Samples? 27

6. Understanding MST Results **29**

7. Examples of MST Use for TMDLs **30**
Panhandle Streams, Idaho 33
MST Study 33
Fecal Source Sampling 33
Water Sampling 34
MST Analysis and Results 34
TMDL Development 35
References 36
Tillamook Bay, Oregon 37
MST Study 38
References 40
Middle Rio Grande, New Mexico 41
MST Study 41

Sanitary Survey 42
Library Development 42
Water Sampling 42
MST Analysis 43
TMDL Implementation 44
References 45
Hays Creek, Virginia 46
MST Study 46
TMDL Development 47
References 48
Sand Dam Village Pond Town Beach, New Hampshire 49
MST Study 49
Source Identification 50
TMDL Development 51
Management Strategies 51
References 52
Ecorse River, Michigan 53
Preliminary Source Identification 54
MST Study 55
TMDL Development 57
References 57
Beaver Creek, South Dakota 58
MST Study 58
Source Identification 59
TMDL Development 59
Management Strategies 60
References 60

8. References 63

Tables

Table 1. Common Types of MST Methods 6
Table 2. Comparison of MST Methods for Use in TMDL Studies 9
Table 3. Advantages and Disadvantages of MST Methods 10
Table 4. Example Laboratories Currently Using MST for TMDL Studies 11
Table 5. Summary of Example MST Studies 32
Table 6. Sources Identified in Hauser Creek, Right Fork Hauser Creek, and Riley Creek PFGE Analysis (Number of Samples and Percent of Total Samples for Each Station) 35
Table 7. Species Identified by Ribotyping in Samples from Sand Dam Village Pond Town Beach 50

Figures

Figure 1. Types of MST methods. 7
Figure 2. Decisions to consider when deciding whether to conduct an MST study. 18
Figure 3. Level of detail provided by MST methods for identifying fecal sources in bacteria impaired waters. 21
Figure 4. Trends in *E. coli* monitoring data in the Tillamook Bay watershed. 38
Figure 5. Geometric mean E. coli counts (lines) and ruminant (gray bars) and human (black bars) frequencies at sampling stations throughout the Tillamook Bay watershed (Shanks et al. 2006) 39
Figure 6. Distribution of identified sources in the Tillamook Bay watershed. 40
Figure 7. Sources of E. coli identified by ribotyping in the MRG study area, based on all sites and sample dates. 43
Figure 8. Source composition at select stations in MRG, from upstream to downstream. 44
Figure 9. Virginia HUCs where MST has occurred, as of March 2009 (MapTech 2009). 46
Figure 10. MST results for each sample date in Hays Creek. 47
Figure 11. Distribution of isolates identified by ribotyping. 50
Figure 12. MST results for dry-weather samples in Ecorse River. 56
Figure 13. MST results for wet-weather samples in Ecorse River. 56
Figure 14. Relative percent of E. coli sources as determined from 23 tested isolates. 58

Acronyms

AMAFCA	Albuquerque Metropolitan Arroyo Flood Control Authority
ARA	Antibiotic Resistance Analysis
BMP	best management practice
CDC	Centers for Disease Control
cfu	colony-forming units
CSO	combined sewer overflow
CSO	combined sewer overflow
DEQ	Department of Environmental Quality
DNA	deoxyribonucleic acid
EPA	U.S. Environmental Protection Agency
E. coli	*Escherichia coli*
FDEP	Florida Department of Environmental Protection
HSPF	Hydrological Simulation Program – FORTRAN
IEH	Institute for Environmental Health
LA	load allocation
LTHIA	Long Term Hydrologic Impact Analysis
MPN	most probable number
MRG	Middle Rio Grande
MS4	municipal separate storm sewer system
MST	microbial source tracking
NHDES	New Hampshire Department of Environmental Services
NMED	New Mexico Environment Department
NPDES	National Pollutant Discharge Elimination System
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
QA	quality assurance
QC	quality control
qPCR	quantitative polymerase chain reaction
rep-PCR	repetative extragenic palindromic polymerase chain reaction
RNA	ribonucleic acid
SDDENR	South Dakota Department of Environment and Natural Resources
SWCD	Soil and Water Conservation District
TEP	Tillamook Estuaries Partnership
WLA	wasteload allocation
WRAS	Watershed Restoration Action Strategy

Executive Summary

The purpose of this document is to educate TMDL practitioners on Microbial Source Tracking (MST) and how it can be used to support TMDL development and implementation. The document covers:

- Potential uses of MST
- Brief descriptions of common MST methods
- Factors for selecting an MST method and designing an MST study
- Examples of MST studies used to support TMDL development or implementation

MST can support bacteria TMDL development and implementation during the assessment process, the allocation analysis, and/or development of an implementation plan. Specifically, MST can help to identify the sources that contribute to the observed impairment, determine which sources are likely the most dominant in the watershed of interest, and focus management actions through targeting implementation and identifying controls that are appropriate and relevant to the identified sources.

Developing accurate and implementable TMDLs relies on identifying and appropriately characterizing the pollutant sources causing the impairment. In a watershed where sources are not known or understood, MST techniques provide the opportunity to analyze water samples in a way that identifies the source of fecal bacteria in the sample, sometimes simply identifying whether the source is human versus animal and sometimes identifying the source down to the species (e.g., cow, dog, deer) or eliminating insignificant sources of fecal bacteria.

MST can also be useful for obtaining stakeholder buy-in for supporting management activities during TMDL implementation in watersheds with contentious issues or communities. MST can provide more acceptable or concrete evidence to stakeholders regarding their role in bacteria inputs and resulting impairments, possibly facilitating acceptance of responsibility and subsequent implementation of best management practices (BMPs).

It is important to understand the various types of MST methods to decide which methods are most useful and effective for the TMDL practitioner. The analytical methods used for MST most commonly include molecular analysis of genetic material (e.g., deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) to determine which human or animal source contributed the bacteria or viruses observed in the water sample. There are also some MST methods that do not require molecular analysis and rely on the identification of physical or biochemical characteristics in bacteria that are the result of exposure to different host species or identify species or strains of microbes or viruses that are unique to a specific fecal source.

MST methods are typically divided into library-dependent and library-independent methods, as shown in Table ES-1. Library-dependent methods identify fecal sources from water samples based on databases or “libraries” of genotypic or phenotypic fingerprints for bacteria strains of known fecal sources. Library-independent methods identify sources based on known host-specific characteristics of the bacteria or virus, without the need of a library.

Table ES-1. Common Types of MST Methods

Library-dependent		Library-independent	
Culture-dependent			Culture-independent
Phenotypic	Genotypic	Phenotypic or Genotypic	Genotypic
▪ Antibiotic resistance ▪ Carbon utilization	▪ Rep-PCR ▪ PFGE ▪ Ribotyping	▪ Bacteriophage ▪ Bacterial culture	▪ Host-specific bacterial PCR ▪ Host-specific viral PCR ▪ Host-specific quantitative PCR

The primary advantage of library-dependent methods is that they can identify multiple sources (e.g., human, pets, livestock, and wildlife) of indicator bacteria (e.g., *E. coli* or *Enterococcus*). However, these methods generally are more expensive and require more time than library-independent methods due to the additional labor required for developing a library that is temporally and geographically specific to a watershed. Recent developments in MST methods have primarily focused on host-specific library-independent molecular methods as equipment and techniques have become available, expanding the range of fecal sources that can be identified quickly and easily. These methods are explained in further detail in the report, particularly the advantages and disadvantages of each in Table 3 of this report.

The selection of an appropriate MST method primarily depends on the objectives of the study. In the context of TMDL development and implementation, the goals of the study typically relate to the necessary level of discrimination in identifying sources of fecal contamination. The focus of MST studies for TMDL development and implementation can be divided into three categories of increasing discrimination and complexity (Figure ES-1):

- Determining the presence or relative abundance of human fecal sources (e.g., municipal sewage, residential septic systems, or use of unsanitary human practices)
- Determining the presence or relative abundance of select non-human fecal sources other than wildlife (e.g., livestock or dog)
- Determining the presence or relative abundance of all fecal source sources (e.g., human, cow, horse, dog, cat, bird, waterfowl, deer, raccoon, rodent, etc.)

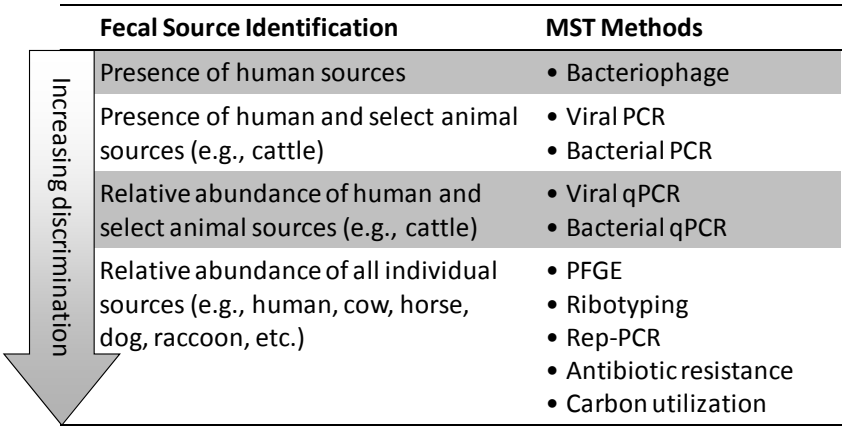

Figure ES-1. Level of detail provided by MST methods for identifying fecal sources in bacteria impaired waters.

An MST study should be designed to answer specific questions raised following thorough analysis of available bacteria monitoring and sanitary survey results and to address a list of prioritized study goals and objectives. A MST study for TMDL development or implementation should include multiple sampling locations, collection of multiple samples during a sampling event, and multiple sampling events during the period of interest (e.g., summer or winter) or hydrologic conditions (e.g., base or storm flow). Detailed information on designing the study is provided in this document.

While MST methods can be extremely useful in identifying bacteria sources in impaired watersheds, TMDL developers should be careful to use their results appropriately. MST methods should be used to supplement rather than replace current methods and tools for evaluating and identifying fecal bacteria sources—tools such as traditional monitoring of fecal bacteria indicators, sanitary surveys and watershed tours, and local knowledge. The results will likely be most useful to confirm the presence or absence of a particular source or to gain a qualitative understanding of the types and relative abundance of different sources. MST methods developed to date generally lack the accuracy required for quantifying all fecal bacteria sources or for definitively identifying the relative abundance of bacteria among multiple sources. Therefore, using MST quantitatively for source loading estimation, model calibration or distribution of load allocation is not recommended at this time. However, MST results can be effectively used to qualitatively identify those sources that are likely contributing more bacteria or are more abundant in the watershed and can therefore be prioritized for management or additional characterization.

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Introduction

Contamination of surface waters by pathogens is a major cause of water quality impairment in the United States. The presence of pathogens in waters designated for recreational use can cause gastrointestinal, respiratory, eye, ear, nose, throat, and skin diseases. Contamination of drinking water supplies can cause gastrointestinal disease, impairments of cells of the digestive tract and organs, and life-threatening infections in people with depressed immune systems. Pathogens may also be passed to humans through the consumption of raw or undercooked filter-feeding shellfish, such as clams, oysters, and mussels. Because the numbers of pathogenic organisms present in polluted waters are few and difficult to measure, indicator organisms are used to represent the potential occurrence of pathogens. Indicator organisms are generally nonpathogenic organisms that are used to determine the quality of waterbodies relative to presence of pathogens. Commonly used fecal indicator bacteria include total coliform, fecal coliform, *Escherichia coli* (*E. coli*), and fecal streptococci and enterococci.

State and federal surface water quality standards include criteria for three fecal indicator bacteria tests: *E. coli*, *Enterococcus*, and fecal coliform bacteria. Concentrations of fecal indicator bacteria exceeding water quality criteria indicate the probable presence of pathogens and present an exposure risk to water users and the possibility of illness. Under section 303(d) of the Clean Water Act, states, territories, and authorized tribes are required to develop total maximum daily loads (TMDLs) that calculate the maximum amount of pollutants, including fecal indicator bacteria, that a waterbody can receive and still meet water quality standards.

Understanding the types of sources contributing bacteria in a watershed is important for developing TMDLs as well as for effectively implementing them. In a watershed where sources are not known or understood, microbial source tracking (MST) techniques can provide the opportunity to analyze water samples in a way that identifies the source of fecal bacteria in the sample, sometimes simply identifying whether the source is human versus animal and sometimes identifying the source down to the species (e.g., cow, dog, deer).

The analytical methods used for MST most commonly include molecular analysis of genetic material (e.g., deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) to determine which human or animal source contributed the bacteria or viruses observed in the water sample. The underlying assumption of these types of MST methods is that there are characteristics unique to bacteria from a particular host that can be used to identify where the bacteria originated. Bacteria species can become adapted to a particular host (e.g., cows vs. humans), therefore resulting in bacteria strains with a genetic “fingerprint” unique to that host (e.g., human, animal species, or type of animal). These “fingerprints” can be isolated from the bacteria present in water samples and matched to those of known fecal sources in the watershed. There are also some MST methods that do not require molecular analysis and rely on the identification of physical or biochemical characteristics in bacteria that are the result of exposure to different host species. For example, the antibiotic resistance or carbon utilization will be different for bacteria from different hosts. Other methods identify species or strains of microbes or viruses that are unique to a specific fecal

Weighing the Benefits and Challenges of MST

While MST can provide useful information for identifying and understanding bacteria sources, there are a number of challenges that should be considered when deciding whether to conduct an MST study. Common challenges include

- Because the bacteria isolates analyzed from collected water samples represent a small portion of the population present in the sample (and an even smaller portion of the waterbody population), the results might not represent the actual relative presence of sources in the watershed.
- There is a lack of widely accepted and standardized techniques for the MST methods, raising questions about the reproducibility of results both within and across laboratories. The laboratory techniques have not been formally approved as an EPA method.
- The analytical precision and accuracy of MST techniques can vary greatly between methods, laboratories, and individual water samples.
- Depending on the method used, laboratory analysis can be very intensive and have a slow turnaround.
- For library-dependent methods, there is not clear or consistent guidance on appropriate library size.
- The transferability of existing libraries across time and geographical areas is not well understood.
- Field work is intensive because MST studies can require numerous water samples to capture different conditions and sources and, for library-dependent studies, fresh fecal samples from sources to build or supplement a DNA library.
- There are not uniform standards or reference materials for sampling and measurement designs.

Challenges related to using MST are discussed in more detail throughout the document, within the context of the relevant decisions and steps in the process (e.g., selecting an MST method, designing an MST study).

source. In addition, chemical testing – such as caffeine as an indicator of human waste- have been suggested as potential biomarkers.

The purpose of this document is to educate TMDL practitioners on MST and how it can be used to support TMDL development and implementation. The document begins with a summary of potential uses of MST and brief descriptions of common MST methods. Factors for selecting an MST method and designing an MST study are then discussed. Examples of MST studies used to support TMDL development or implementation are also provided.

1. Using MST in TMDL Development and Implementation

Developing accurate and implementable TMDLs relies on identifying and appropriately characterizing the pollutant sources causing the impairment. Using MST within a TMDL framework can support both TMDL development and implementation. The primary utility of MST is to identify sources contributing to the observed bacteria impairments. This can support setting TMDL allocations during the development phase, but the benefit of using MST will most likely be realized in the implementation phase by supporting targeted implementation and identification of controls that are appropriate and relevant to the identified sources. By identifying or at least confirming or prioritizing sources of fecal pollution, MST can support bacteria TMDL development and implementation at various points in the process, including.

- **Source identification—**The most obvious use of MST in the TMDL process is identifying those sources that are contributing to the observed impairment and should be included in the TMDL analysis. Depending on the type and outcome of the MST results, they can be used in subsequent steps of the TMDL process, including developing TMDL allocations and identifying management actions.

> ***For More Information on MST…***
>
> This document is intended to consolidate and distill the myriad of information and topics related to MST and put it in the context of the TMDL process and framework. The references cited throughout represent only a small subset of the existing literature on MST. A number of state and university websites, journal articles, and conference proceedings are available on a wide-range of topics related to MST and individual methods.

- **Allocation analysis—**When establishing load allocations (LAs) and wasteload allocations (WLAs), a number of allocation "scenarios" can result in the total load from all sources meeting the loading capacity, but with a number of different relative distributions among the sources. MST results can help to identify those sources that are likely the most dominant in the watershed or subwatershed that might be targeted for higher load reductions. The results can guide development and selection of more feasible or implementable allocation scenarios. Note that using MST quantitatively for source loading estimation or distribution of load allocations is not recommended at this time, but MST results can be used to qualitatively identify those sources that are likely contributing more bacteria or are more abundant in the watershed and can therefore be prioritized for management.
- **Development of implementation plan—**MST results can help to focus management actions on those sources identified or confirmed in the MST study. Even if the MST study is conducted after the TMDL is developed, it can be useful in targeting implementation activities to those sources expected to be contributing to the impairment, supporting identification of bacteria controls appropriate and relevant to those sources.

> ***For More Information on TMDLs…***
>
> This document is written for TMDL practitioners familiar with TMDLs and their associated requirements and development process. If you need additional information on developing TMDLs, please visit the U.S. Environmental Protection Agency (EPA) TMDL web site at www.epa.gov/owow/tmdl.

Conducting MST prior to TMDL development might lead to more specific and accurate allocations. For example, in a watershed where the fecal sources are unknown a TMDL developer might make assumptions to allocate the loading capacity among expected or potential sources, or the loading capacity might be allocated as a gross allocation to all nonpoint sources in the watershed. Having conducted an MST study, the TMDL developer might be able to allocate the loading capacity to specific sources identified or confirmed through the use of MST. For example, expected sources might include failing septic systems and runoff from grazing areas. Using MST could identify whether the sources are human versus animal, allowing the TMDL developer to determine how to target the load allocations and associated load reductions among the included sources. Virginia Department of Environmental Quality (DEQ) has used MST in determining TMDL allocations. For example, in the Carters Creek TMDL (VADEQ 2007), the relative percent of sources from the MST results was used to partition the calculated current load into the categories of wildlife, human, livestock and pets. Allocation scenarios were then developed, applying load reductions to each source category to meet the calculated loading capacity and necessary load reduction. However, this approach assumes that the relative

contribution of fecal bacteria from each of the four general sources shown in the MST results reflects the actual relative contribution of bacteria from the watershed. This is not likely accurate given that isolates in MST analysis represent a small portion of the bacteria in a water sample, and that sample in turn likely represents a small portion of the bacteria in the receiving waterbody. A TMDL developer should consider the amount and type of data available before deciding whether to use the MST results this directly in the allocation process (e.g., partitioning a load based solely on the MST results). Using the results more indirectly and qualitatively, the TMDL developer can use the MST results to identify sources to be included in the analysis and together with other relevant information (e.g., watershed studies, modeling assumptions, relevant literature) to characterize and calculate their load inputs.

Using Your MST Results Wisely: Qualitative vs. Quantitative Use of Results

While MST methods can be extremely useful in identifying bacteria sources in impaired watersheds, TMDL developers should be careful to use their results appropriately. The results will likely be most useful to confirm the presence or absence of a particular source or to gain a qualitative understanding of the types and relative abundance of different sources. MST methods developed to date generally lack the accuracy required for quantifying all fecal bacteria sources or for definitively identifying the relative abundance of bacteria among multiple sources. (Section 6 provides more detail on understanding and using your MST results.)

Alternatively, conducting an MST study subsequent to TMDL development serves to further define or understand the sources for determining management measures and activities to reduce bacteria loading. Load allocations for a TMDL might generally be assigned by land use based on pollutant loading modeling or assumptions or assigned as a gross allotment to an entire drainage basin. Regardless of how the TMDL allocation is expressed, MST analyses can provide information to further identify specific sources for implementation purposes. Depending on the method chosen, the analyses can be used to generally distinguish sources as human or non-human to confirm or eliminate as possibilities any potential or expected sources or to identify specific sources within a known category of sources. For example, in a watershed where waterbodies receiving runoff from agricultural areas consistently exhibit higher bacteria concentrations, a TMDL allocation and associated load reduction might be assigned to agricultural land uses as a group. MST can be used to further define what sources or animal-related activities within the agricultural areas (e.g., cattle grazing areas versus horse farms) are contributing bacteria loads and should be targeted for management.

MST can also be useful for obtaining stakeholder buy-in for supporting management activities during TMDL implementation in watersheds with contentious issues or communities. MST can provide more acceptable or concrete evidence to stakeholders regarding their role in bacteria inputs

Example: Using MST Results to Target Effective Source Controls in Virginia's Page Brook Watershed

The Page Brook Watershed in Virginia has been monitored for MST analyses since 1996, and an ARA of *Enterococcus* isolates identified cattle as the major source of fecal bacteria (>78% of isolates) in the impaired stream segment. Guided by these results, stream fencing was installed on 12 of 17 farms in the watershed from 1996 to 1997. Fecal coliform were reduced at the three monitoring sites by an average of 94%, from pre-fencing average populations of 15,900 per 100 mL to post-fencing average populations of 960 per 100 mL. After fencing, less than 45% of fecal streptococcus isolates were classified as cattle.

Source: Hagedorn et al. (1999)

and resulting impairments, possibly facilitating acceptance of responsibility and subsequent implementation of best management practices (BMPs). For example, MST results showing that cattle are a more significant source than humans (e.g., septic systems) or wildlife might lead to ranchers recognizing the impact of their operations and becoming more engaged in management decisions and actions.

Decisions of which MST method to use and when to conduct an MST study will likely depend on a number of technical and programmatic factors. The following sections discuss the various methods available for MST, the factors affecting method selection, and considerations for designing an MST study.

Terminology:

Fecal source refers to a human or animal host where a microbe originates in the fecal waste of that host. Depending on the specificity of an MST method, a fecal source might refer to a general group of hosts (e.g., all humans, all animals, or a group of animals such as canines, birds, rodents, or grazing animals), a type of human fecal waste (e.g., municipal sewage, residential septage, or an individual human), or a specific animal host (e.g., cattle, dogs, ducks, beaver, or raccoons).

Microbial strain is a genetic variant or subtype of a microorganism (e.g., virus or bacterial species).

Genotypic (aka molecular) methods distinguish among bacterial or viral strains (or subspecies) based directly on their unique genetic makeup and are often referred to as "DNA fingerprinting."

Phenotype (aka biochemical) methods distinguish samples based on observable characteristics of the isolated bacteria that might have been acquired from exposure to different host species or environments, such as resistance to certain antibiotic or profiles of carbon utilization. These methods are based on an effect of an organism's genes that actively produce a biochemical substance. The type and quantity of these substances is what is measured during the laboratory analysis.

Library-dependent methods identify fecal sources from water samples based on databases of genotypic or phenotypic fingerprints for bacteria strains of known fecal sources. These libraries are often geographically specific.

Library-independent methods identify sources based on known host-specific characteristics of the bacteria or virus, without the need of a library.

Culture-dependent methods rely on bacteria or viruses from water samples being grown or cultured in a lab.

Culture-independent methods isolate and identify DNA directly from a water sample without first having to grow or culture the bacteria or viruses in the sample.

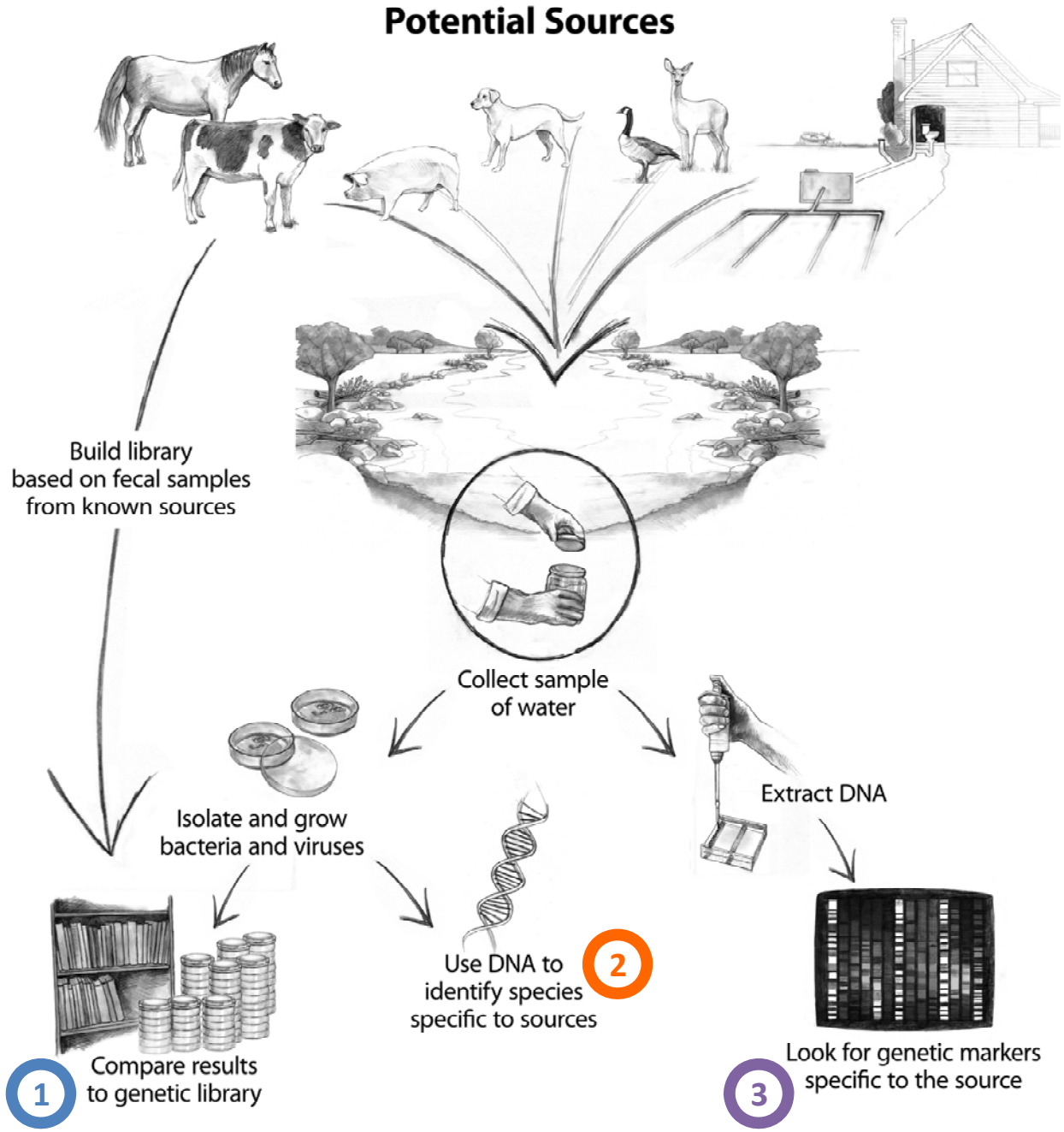
2. Understanding the Types of MST Methods

MST methods are typically divided into library-dependent and library-independent methods (Table 1). Figure 1 illustrates the different types of methods for MST and the general process used for each. Library-dependent methods require the development of databases or "libraries" of genotypic or phenotypic "fingerprints" for bacterial strains—fingerprints either of the genetic makeup of the bacteria strain through DNA analysis or of some biochemical process (e.g., antibiotic resistance). Bacteria strains are isolated from water samples collected from the study watershed and results of the necessary analyses (e.g., DNA analysis, antibiotic resistance) are compared to the library to match to a strain of a known fecal source. Library-independent methods typically extract bacterial or viral DNA/RNA directly from water samples and analyze the extract for the presence of a source-specific genetic marker using polymerase chain reaction (PCR) methods. Library-independent methods also include culture of source-specific bacteria or viruses without the need for DNA analysis.

Table 1. Common Types of MST Methods

Library-dependent		Library-independent	
Culture-dependent			Culture-independent
Phenotypic	Genotypic	Phenotypic or Genotypic	Genotypic
▪ Antibiotic resistance ▪ Carbon utilization	▪ Rep-PCR ▪ PFGE ▪ Ribotyping	▪ Bacteriophage ▪ Bacterial culture	▪ Host-specific bacterial PCR ▪ Host-specific viral PCR ▪ Host-specific quantitative PCR

Library-dependent methods are based on the assumption that different strains of bacteria have adapted to living within a particular host or environment due to reasons such as differences in pH and nutrient availability and that when these strains replicate they produce genetically identical progeny. Therefore, bacteria strains within a particular type of host (e.g., human, cow) would have identical genetic fingerprints—fingerprints that differ from bacteria strains adapted to a different host. Libraries to support these methods are created using fecal material collected from known sources, usually associated with the watershed being evaluated. Water samples are then collected from the study area and the bacteria strains isolated from those samples are compared to the library to identify the source from which the bacteria originated. The primary advantage of library-dependent methods is that they can identify multiple sources (e.g., human, pets, livestock, and wildlife) of indicator bacteria (e.g., *E. coli* or *Enterococcus*). However, these methods generally are more expensive and require more time than library-independent methods due to the additional labor required for developing a library that is temporally and geographically specific to a watershed. This is one of the main concerns with the library dependent methods: they can be very temporally and geographically specific and therefore perhaps not as generally applicable as the library independent methods.



Types of MST Methods:

1. Library-dependent, culture-dependent
2. Library-independent, culture-dependent
3. Library-independent, culture-independent

Figure 1. Types of MST methods.

Library-independent methods generally share the advantage of being less expensive and faster than library-dependent methods and are generally able to detect the presence of multiple markers in a single sample. However, they are limited to detection of a subset of sources based on current technology. Library-independent methods typically detect the presence of bacteria or viruses that are not used as the indicator bacteria used in water quality criteria and TMDLs and these microbes might have different survival and distribution characteristics than the indicator bacteria. Library independent methods generally are less temporally and geographically specific, making the methods universally applicable for use.

Recent developments in MST methods have primarily focused on host-specific library-independent molecular methods as equipment and techniques have become available, expanding the range of fecal sources that can be identified quickly and easily. These methods are cost-effective, rapid and potentially more specific than library-independent methods. Studies using library-independent methods can be considerably cheaper and faster because they do not require the investment in library development. These methods also have the potential for greater accuracy, since they focus on a specific trait rather than attempting to pattern-match a large number of isolates, some of which may be transient among species (Stewart et al. 2003). It is anticipated that these host-specific molecular methods will continue to develop with emphasis on those methods using the quantitative polymerase chain reaction (qPCR) technique that measures the amount of DNA present in a water sample rather than simply detecting a presence or absence of microbial DNA (Santo Domingo et al. 2007). Quantifying the amount or relative amount of DNA from fecal sources can provide insight into the relative magnitude of the different sources in a watershed.

MST methods used historically are briefly described separately below for those that do and do not require a library. Potential advantages and disadvantages are identified for each method with respect to source specificity, accuracy, cost, data turnaround time, and lab availability. MST method attributes are summarized in Table 2, and advantages and disadvantages of each are summarized in Table 3. Example MST laboratories and contacts are presented in Table 4.

Chemical Methods for MST

Some MST studies have used the presence of chemicals associated with sanitary sewage or animal waste to identify expected fecal sources. For example, the presence of caffeine in water samples might indicate that human sewage has been discharged to the waterbody, and chemicals found in laundry detergents (e.g., whitening agents, optical brighteners) have been used to indicate human impact, but might not necessarily indicate the presence of sewage or fecal pollution. The presence and type of fecal sterols and stanols can also indicate the potential origin of fecal contamination, such as domestic wastewater or livestock. Chemical indicators are primarily used to determine the presence of human-derived discharges, and they do not provide a direct link to the presence of pathogens or indicator bacteria. Therefore, they are not discussed here as an MST method for supporting bacteria TMDL development and implementation.

Table 2. Comparison of MST Methods for Use in TMDL Studies

Method	Library	Culture	Common Targets	Human and Animal Sources Identified	Accuracy	Cost	Time Required[c]
PFGE	Yes	Yes	*E. coli*	All species/groups	High with large library	$100/isolate (e.g., 100 isolates/site) [a]	2-4 days
Ribotyping	Yes	Yes	*E. coli*	All species/groups	High with large library	Similar to PFGE	1-3 days
Rep-PCR	Yes	Yes	*E. coli*	All species/groups	High with large library	Similar to PFGE	1 day
Antibiotic Resistance	Yes	Yes	*E. coli* Fecal enterococci Fecal streptococci	All species/groups	Moderate with large library	Lower than PFGE if library is developed	4-5 days
Carbon Utilization	Yes	Yes	Enterococcus	All species/groups	Moderate with large library	Lower than PFGE if library is developed	2-5 days
Bacteriophage	No	Yes	F+ coliphage	Human, animals	Low-High depending on source/experience	Low (<$100/ sample)	1-3 days
Viral PCR and qPCR	No	No	Human enterovirus and polyomavirus, bovine enteroviruses, pig teschoviruses	Human, cow, pig	Moderate-High	$400/source/ sample [b]	6-8 hours (1-3 hours for qPCR)
Bacterial PCR and qPCR	No	No	Bacteroides, Enterococcus	Human, ruminants, cow, horse, dog, pig	Moderate-High	$400-600/source/ sample	6-8 hours (1-3 hours for qPCR)

a. Approximate unit costs from the Institute for Environmental Health, Seattle, Washington
b. Approximate unit costs from Source Molecular, Miami, Florida
c. Time after enrichments or isolation performed. Time for isolation dependent on target and method used for isolation and confirmation can vary considerably. Also, time required for data analysis for library-dependent methods are not included because it is highly variable and dependent on available gel and data analysis software. (USEPA 2005)

Table 3. Advantages and Disadvantages of MST Methods

Method	Advantages	Disadvantages
PFGE	▪ Highly reproducible ▪ Sensitive of minute genetic differences ▪ May discriminate isolate from multiple host groups	▪ Labor-intensive ▪ Requires cultivation of target organism ▪ Requires specialized training of personnel ▪ Requires reference library ▪ Libraries may be geographically specific ▪ Libraries may be temporally specific
Ribotyping	▪ Highly reproducible ▪ Can be automated ▪ May discriminate isolate from multiple host groups	▪ Labor-intensive (unless automated system used) ▪ Requires cultivation of target organism ▪ Requires reference library ▪ Requires specialized training of personnel ▪ Variations in methodology ▪ Libraries may be geographically specific ▪ Libraries may be temporally specific
rep-PCR	▪ Highly reproducible ▪ Rapid; easy to perform ▪ Requires limited training ▪ May discriminate isolate from multiple host groups	▪ Requires reference library ▪ Requires cultivation of target organism ▪ Libraries may be geographically specific ▪ Libraries may be temporally specific
Antibiotic Resistance	▪ Rapid; easy to perform ▪ Requires limited training ▪ May discriminate isolate from multiple host groups	▪ Require reference library ▪ Requires cultivation of target organism ▪ Libraries geographically specific ▪ Libraries temporally specific ▪ Variations in methods in different studies
Carbon Utilization	▪ Rapid; easy to perform ▪ Requires limited training	▪ Require reference library ▪ Requires cultivation of target organism ▪ Libraries geographically specific ▪ Libraries temporally specific ▪ Variations in methods in different studies ▪ Results often inconsistent
Bacteriophage (F+ coliphage)	▪ Distinguishes human from animals ▪ Subtypes are stable characteristics ▪ Easy to perform ▪ Does not require a reference library	▪ Requires cultivation of coliphages ▪ Sub-types do not exhibit absolute host specificity ▪ Low in numbers in some environments
Host-specific bacterial PCR	▪ Host specific ▪ Does not require cultivation of target organism ▪ Rapid; easy to perform ▪ Does not require a reference library ▪ Can identify multiple sources from same sample	▪ Little is known about survival and distribution in water systems ▪ Primers currently not available for all relevant hosts
Host-specific viral PCR	▪ Host specific ▪ Does not require cultivation of target organism ▪ Easy to perform ▪ Does not require reference library	▪ Often present in low numbers; requires large sample size ▪ Not always present even when humans present

Based on information in USEPA (2005), Seurinck et al. (2005), Scott et al. (2002), Simpson et al. (2002), and Ahmed (2007)

Table 4. Example Laboratories Currently Using MST for TMDL Studies

Laboratory	MST Methods	Contact
EPA Region 10 Manchester Laboratory	*Bacteroides* PCR	Dr. Stephanie Harris Harris.stephanie@epa.gov; (360) 871-8710
Institute for Environmental Health (Seattle, Washington)	*E. coli* PFGE *Bacteroides* PCR	Dr. Mansour Samadpour ms@iehinc.com; (206) 522-5432
Source Molecular Corporation (Miami, Florida)	Viral PCR and qPCR Bacterial PCR and qPCR	Thierry Tamers ttamers@sourcemolecular.com; (786) 268-8363
University of California, Davis, College of Civil and Environmental Engineering (Davis, California)	*Bacteroides* qPCR	Dr. Stefan Wuertz swuertz@ucdavis.edu; (530) 754 6407
University of Minnesota, Department of Microbiology (Minneapolis, Minnesota)	rep-PCR *Bacterioides* qPCR	Dr. Michael Sadowsky sadowsky@umn.edu; (612) 624-2706
University of North Carolina at Chapel Hill, Institute of Marine Sciences (Morehead City, North Carolina)	Viral PCR and qPCR Bacterial PCR and qPCR	Dr. Rachel Noble rtnoble@email.unc.edu; (252) 726-6841 ext. 150
University of Southern Mississippi, Department of Biological Sciences (Hattiesburg, Mississippi)	Gel electrophoresis for human *Bacteroides*, *Methanobrevibacter smithii*, *Faecalibacterium*	Dr. R.D. Ellender rudolph.ellender@usm.edu; (601) 266-4720
University of South Florida, Department of Integrative Biology (Tampa, Florida)	PCR and qPCR for human polyomaviruses, *Bacteroides*, and *Methanobrevibacter smithii*	Dr. Valerie Harwood vharwood@cas.usf.edu; (813) 974-1524

Library-Dependent Methods

For most library-dependent MST methods, the target organisms are cultured from water samples that are collected from the waterbody of interest, and a source identifier is used to determine the animal source of the target organism. The source identifier is typically a genotypic or phenotypic "fingerprint" or pattern that requires the use of an MST library of known sources to determine the animal source(s) in the waterbody. Genotypic methods include molecular analysis of genetic material (e.g., RNA or DNA). Three commonly used genotypic methods are described below: pulsed field gel electrophoresis (PFGE), ribotyping, and repetative extragenic palindromic polymerase chain reaction (rep-PCR). Two commonly used phenotypic methods are described below: antibiotic resistance analysis (ARA) and carbon utilization. USEPA (2005) provides additional information about these and other library-dependent methods.

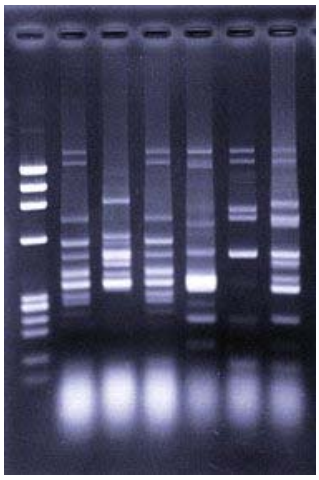
Genotypic Methods

PFGE

PFGE involves the extraction and purification of bacterial DNA, digestion of the DNA using an infrequently cutting restriction enzyme, and the separation of the digested DNA using gel electrophoresis. Because the digestion of the DNA results in relatively large fragments which are too large to migrate through a standard gel unit, the electric field used during electrophoresis is oriented in different directions, for different periods of time, and at different voltages to allow the fragments to orient in a banding pattern unique to the bacterial strain.

PFGE has been used by the Centers for Disease Control (CDC) to track food-borne illnesses, and the CDC has developed a network for health agencies to use for quick comparisons of PFGE data. Use of PFGE in an MST setting is more limited. In a review of DNA fingerprinting MST methods, PFGE was found to have identification rates of 88 percent for human and 81 percent for all sources, and had relatively low (21 percent) false positive rates (Myoda et al. 2003). The PFGE method requires some technical skill, specific equipment, is time consuming, and is relatively expensive. The method is very discriminatory, and requires a large library to resolve differences between a large diversity of sources. As a result of the specific equipment required, the length of time needed to conduct the analysis, and the requirement for a large library, the method is considered to be expensive.

The only known laboratory that currently maintains a library and uses the PFGE method for MST is the Institute for Environmental Health (IEH) located in Seattle, Washington. IEH currently maintains over 50,000 DNA fingerprints of known *E. coli* sources from human, sewage, and animal fecal source samples collected throughout North America, with most from the Pacific Northwest. *E. coli* isolates are developed from membrane filter cultures of water samples at a cost of $100/isolate, and it is recommended that at least 100 isolates be developed from at least 20 water samples for each sampling site to ensure detection of all potential major sources. To increase the identification rate, fecal source samples are collected from a watershed and isolates of known *E. coli* sources are developed from those sources by IEH at no additional cost.



DNA results representing "DNA fingerprint" of a bacteria isolate.

Ribotyping

The ribotyping method involves the digestion of bacterial DNA, separation of the fragments using gel electrophoresis, and hybridization of certain portions of the fragments by radiolabeled probe. The orientation of the probes can be visualized using autoradiography or color formation, which creates the banding pattern that is unique to each bacterial strain.

Ribotyping has been one of the most widely used methods in MST (USEPA 2005, Woodruff and Evans 2003). Ribotyping was used in a 2001 study in Henderson Inlet in Puget Sound, Washington. Results indicated that 86 percent of the sources collected were correctly matched to the library (Samadpour et al. 2002). A more recent study conducted in Seattle, Washington, found 94 percent of the samples matched to the library (Herrera 2007). One drawback of the method is the geographic specificity of the library, which is remedied by the inclusion of source samples collected from the specific study area. Due to the time consuming nature of the method and the requirement for a large library, the method is considered relatively expensive. Cursory research conducted for this document did not identify a laboratory that is currently maintaining a library and using ribotyping for MST studies. Ribotyping had been performed by various university and commercial laboratories that are now using other MST methods.

rep-PCR

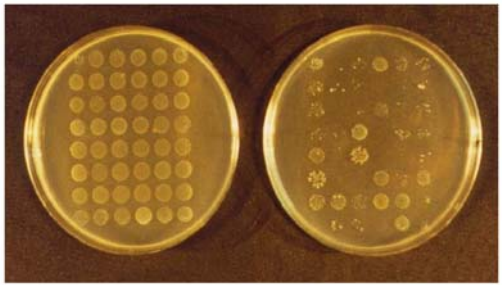
The rep-PCR technique uses the PCR and commercially available primers to amplify certain repetitive portions of the microbial DNA. The amplified DNA fragments are then separated in agarose gels, producing a banding pattern or "fingerprint" that is unique to each bacterial strain. Bacteria having the same pattern are considered to be of the same strain.

The rep-PCR method is quick, easy, and has the potential for a fast turnaround time (Woodruff and Evans 2003). Rep-PCR was used in a study of fecal contaminated streams in Minnesota (Dombek et al. 2000) and had an identification rate of 63 percent for environmental samples. The relatively low identification rate was attributed to a small library. A DNA fingerprinting method comparison study (Myoda et al. 2003) found that rep-PCR had high sensitivity and low false positive rates when identifying samples with human sources, and high sensitivity when identifying samples with many sources, but had a high false positive rate with the same multiple source samples. Although relatively simple when compared to PFGE and ribotyping, rep-PCR is a moderately expensive MST method due to the laboratory equipment needs and the requirement for a large library to distinguish diverse groups of sources. Cursory research conducted for this document identified one university research laboratory that is currently maintaining a library and using rep-PCR for MST studies (see Table 4).

Phenotypic Methods

Antibiotic Resistance

Antibiotic resistance use in MST is based on the premise that intestinal bacteria demonstrate antibiotic resistance when the host animal is treated with that antibiotic (USEPA 2005). The antibiotic resistance technique is carried out by first developing a database of antibiotic resistance patterns for bacteria from known human and animal sources. The bacteria are isolated, replicated, and then grown on a battery of antibiotic containing media which can contain multiple concentrations of a single antibiotic or a single concentration of different antibiotics. Based on the response of the bacteria, resistance patterns are developed and the patterns of bacteria from known sources are compared to create the predictive equations that are used to classify the unknown or target organisms.



Cultured E. coli isolates after ARA—the left plate represents the control plate with no antibiotics, allowing the bacteria to grow, and the right plate shows bacteria isolates that did not grow because they are susceptible to the tested antibiotic.

ARA has been commonly used in the past for MST (USEPA 2005) because it is rapid, relatively simple, and inexpensive to moderately expensive depending on the status of the database or library. ARA has been used in numerous studies with a variety of animals (Woodruff and Evans 2003) and has been used in many TMDL studies, yielding identification rates of approximately 72 percent for environmental samples. Because ARA requires a large library for high rates of identification, costs increase if there is no library, or if the existing library is not comprehensive or has not been recently developed from sources in the watershed. The method works well with smaller watersheds with simple bacterial contamination patterns and sources. Cursory research conducted for this document did not identify a laboratory that is currently maintaining a library and using ARA for MST studies. Laboratories formerly using ARA are now

primarily using PCR methods based on results of method comparison studies (Hagedorn 2009). ARA methods for MST studies have often varied and used nonstandard antibiotic resistance methods, leading to irreproducible results. In addition, comparative studies have given ARA methods low ratings (Field and Samadpour 2007).

Carbon Utilization

The use of this method in MST is based on the differential use of carbon substrates by different target organisms. Substrate use can be measured, due to the formation of a purple color caused by the reduction of a tetrazolium dye in the growth media, to create substrate use patterns. A database of substrate use patterns is developed for bacteria from known animal sources, and the patterns are compared to determine the substrate combination that best differentiates each animal source. Substrate use patterns developed for unknown target organisms are compared to the known use patterns to identify the source organism.

Carbon utilization is also a relatively easy and rapid method which requires little technical expertise (USEPA 2005). A study conducted in Virginia found a classification rate of 92 percent for human versus non-human sources, and a classification rate of 80 percent when comparing groupings of animals (Hagedorn et al., 2003). However, in an MST study comparison (Griffith et al., 2003), carbon utilization fared poorly in identifying the dominant sources in blind samples, and also exhibited high false positive rates. As with ARA, a high diversity of sources increases the size of the library needed to discriminate each source, which increases cost. Cursory research conducted for this document did not identify a laboratory that is currently maintaining a library and using carbon utilization for MST studies. Laboratories formerly using carbon utilization are now primarily using PCR methods based on poor performance in method comparison studies (Hagedorn 2009).

Library-Independent Methods

Library-independent MST methods require either the direct culture of microbes present in water samples or molecular analysis of genetic material present in water samples to identify specific bacteria or viruses that that are unique to a human or animal fecal source. Two common groups of culture methods are described below: bacteriophage and bacterial culture. Molecular methods assay specific marker genes using the PCR technique and are commonly referred to as “host-specific PCR” methods. Three common groups of host-specific PCR methods are described below: viral PCR, bacterial PCR, and qPCR. USEPA (2005) provides additional information about these and other library-independent methods.

Bacteriophage

A bacteriophage is a group of viruses that infect specific bacteria, usually causing their disintegration or dissolution. The presence of the F+RNA coliphage (bacteriophage of *E. coli*) in water samples can help distinguish between human and non-human fecal contamination based on determining which of four groups the coliphage belongs to. First, F+RNA coliphages are isolated in the presence of RNA to distinguish them from F+DNA coliphages. Then, the phages are either serotyped or genotyped to identify the particular group that the phage belongs to. Serotyping involves using specific antisera that inhibit infection. Genotyping involves using group specific labeled probes.

Bacteriophage typing can only be used to determine whether human and animal fecal sources are present, and cannot distinguish between various animal groups or species. This method is typically used to identify sewage contamination. Additionally, because bacteriophage distribution in the environment is irregular, this method is more accurate when fecal sources contain multiple individuals (e.g., sewage) and might be problematic when dealing with individuals (e.g., septage) (Field and Samadpour 2007). The method is fairly quick and relatively inexpensive, due both to limited equipment needs and the direct identification of the fecal source without the use of a library.

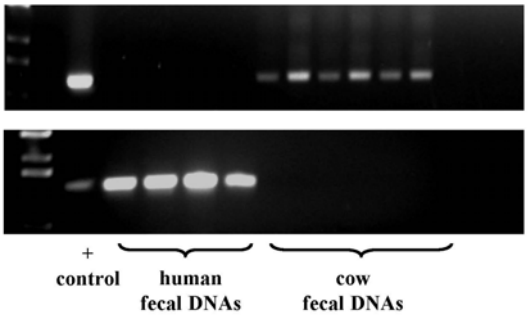
Viral PCR

Viruses with a limited host range can be used to differentiate sources of fecal contamination in water. To accomplish this, viruses are isolated from water sources, and then the viral DNA is extracted and amplified using PCR techniques. The viral DNA can then be confirmed using computer sequencing software. Human specific adenoviruses and enteroviruses have been used as indicators of human fecal contamination. Bovine enteroviruses and bovine and porcine adenoviruses have also been used as indicators of livestock fecal contamination.

This method has been used successfully to identify human and bovine fecal contamination in marine waters (USEPA 2005). However, another study found that bovine enteroviruses are not species specific and have been observed in other animals, including horses and geese (Field and Samadpour 2007). A method comparison study (Griffith et al., 2003) found that the human virus detection method failed to identify fecal contamination from individual humans but did identify fecal contamination from sewage. This result shows that the low rate of viral infection in the human population might be problematic in identifying human fecal sources. Viral PCR is currently being conducted by several universities and a private laboratory (see Table 4). These methods are important because some directly detect viral pathogens, which are not well correlated with indicator bacteria (e.g., fecal coliform, *E. coli*) that are usually measured in monitoring and assessment programs; therefore, viral methods give some information on pathogen status that is not provided by indicator counts (Field and Samadpour 2007).

Bacterial PCR

Various techniques have been developed for extraction, amplification, and analysis of bacterial DNA using PCR. Bacteria commonly analyzed using PCR include *Bacteroides*, *Enterococcus*, and *Methanobrevibacter smithii*. A conventional PCR method might be used that identifies the presences or absence of DNA specific to a fecal source or group of fecal sources. qPCR methods, described below, have been more recently developed and are commonly used for measuring the quantity of DNA present in a water sample extract. This quantifies the amount and relative amount of DNA from the fecal sources, rather than simply confirming their presence or



Results of bacterial PCR using Bacteroides, using cow primers to detect ruminant feces and human primers to detect human feces. Source: Field (2007)

absence. This can provide valuable information on the potential dominance or relative magnitude of the different sources.

Bacteroides is a genus of anaerobic fecal bacteria that are abundant in the gut of mammals. Although *Bacteroides* is present in feces at higher concentrations than indicator bacteria, cultivation of these bacteria is difficult and time consuming because they are anaerobic. However, human and other host-specific markers have been developed for *Bacteroides* to allow analysis of DNA using PCR techniques. PCR techniques are used to amplify specific segments of the bacterial DNA that are isolated from water samples using DNA primers of known bacteria. The unknown bacterial DNA segments are then amplified and analyzed to determine the source of the fecal contamination. Water samples are typically filtered to exclude extracellular DNA from dead and lysed cells. A method has been developed to discriminate between viable and dead *Bacteriodes* bacteria, and microcosm studies and decay models have shown the extracellular DNA persists in the environment for up to one week compared to one day for live cells (Bae and Wuertz 2009). Analysis of intact cells and the relatively short survival of these anaerobic bacteria in the environment yield results that represent recent fecal contamination.

Bacteroides PCR techniques have been used in several studies to identify fecal contamination sources. During a study in Tillamook, Oregon (Bernhard et al. 2003) genetic markers were developed for cattle and human sources and the markers were successfully used to identify sources in environmental samples. *Bacteroides* PCR has since become a popular MST method and genetic markers have been developed by various laboratories for humans and ruminants (which include cattle, sheep, goats, deer, and elk), while *Bacteroides* genetic markers are less developed for dog, horse, and pig. One drawback of this method is that markers for cattle are also found in other ruminants (Harris 2009). This lack of specificity, and the lack of markers for other animal sources, limits the use of this method to differentiating between human and non-human sources. *Bacteroides* PCR is currently being conducted by several universities and private laboratories (see Table 3).

> ***Terminology…***
>
> *Bacteroides PCR* is also referred to as *Bacteroidales* PCR or *Bacteroidetes* PCR because the analysis includes genetic material from *Prevotella* species, which is another member of the *Bacteroidales* order and *Bacteroidetes* class of bacteria.

Various *Enterococcus* species can be analyzed using PCR, generally following the method described for *Bacteroides* PCR. Genetic markers have been identified for specific strains of one species associated primarily with humans (*E. faecium*), cattle (*E. hirae*), and birds (*E. faecalis*) (Source Molecular 2009). *Enterococcus* PCR is currently being conducted by two universities and a private laboratory (see Table 3). *Methanobrevibacter smithii* can be analyzed using PCR as an indicator of human fecal source.

Quantitative PCR

qPCR MST techniques use the same conventional PCR techniques used in viral and bacterial PCR to amplify certain host-specific genetic markers. In addition, qPCR allows quantification by using fluorescent probes that are released during the amplification process. The fluorescent signal from the probes can be measured, and the strength of the signal related to the number of markers that were amplified. Thus, qPCR measures the concentration of DNA, which is likely related but not necessarily

directly proportional to the number of bacteria cells present in the original water sample. However, a method has recently been developed to estimate the concentration of *Bacteroides* indicator bacteria from qPCR results (Stefan Wuertz, University of California, Davis, personal communication). This new method was used in the Calleguas Creek, California, MST study (UC Davis 2006).

qPCR is primarily being used to detect human and cattle fecal contamination. Because it is a library- and culture-independent method, it is conveniently and cost-effectively used for single sample applications where an MST library would not be economical, such as for determining if human fecal sources are present in separated storm drain systems or in swimming areas (Tamers 2009). The qPCR method might also be used for long-term watershed studies where large numbers of samples are analyzed. qPCR is known as "real-time PCR" because results can be obtained within hours of sample collection, potentially before indicator bacteria enumeration results are obtained, as compared to months for library-dependent methods.

3. Deciding Whether to Use MST

Because MST studies can be expensive and resource-intensive it is important to carefully weigh the benefits, needs and goals against the expected expense. Figure 2 presents a decision tree highlighting major factors to consider before deciding whether to pursue an MST study.

Before using MST to identify bacteria sources, the TMDL developer should consider whether sources can be identified through other means or whether MST is necessary given the watershed uses and sources. MST should not be used before traditional bacteria source tracking methods (e.g., targeted in-stream monitoring for bacteria enumeration and sanitary or watershed surveys) have been used. Evaluating available data on in-stream bacteria concentrations along with watershed characteristics such as land use and/or conducting a watershed survey might be enough to identify likely sources or at least "hot spots" of elevated concentrations to focus management or further investigation. If sufficient data have not been collected to evaluate bacteria concentrations throughout the watershed, it might be most appropriate to conduct additional monitoring prior to deciding whether to use MST. Not only can it support the decision-making process, but if MST is selected, the relevant field data will be essential in designing an effective study.

Keeping MST in Perspective

A common recommendation for using MST is that MST methods should be used to supplement rather than replace current methods and tools for evaluating and identifying fecal bacteria sources—tools such as traditional monitoring of fecal bacteria indicators, sanitary surveys and watershed tours, and local knowledge.

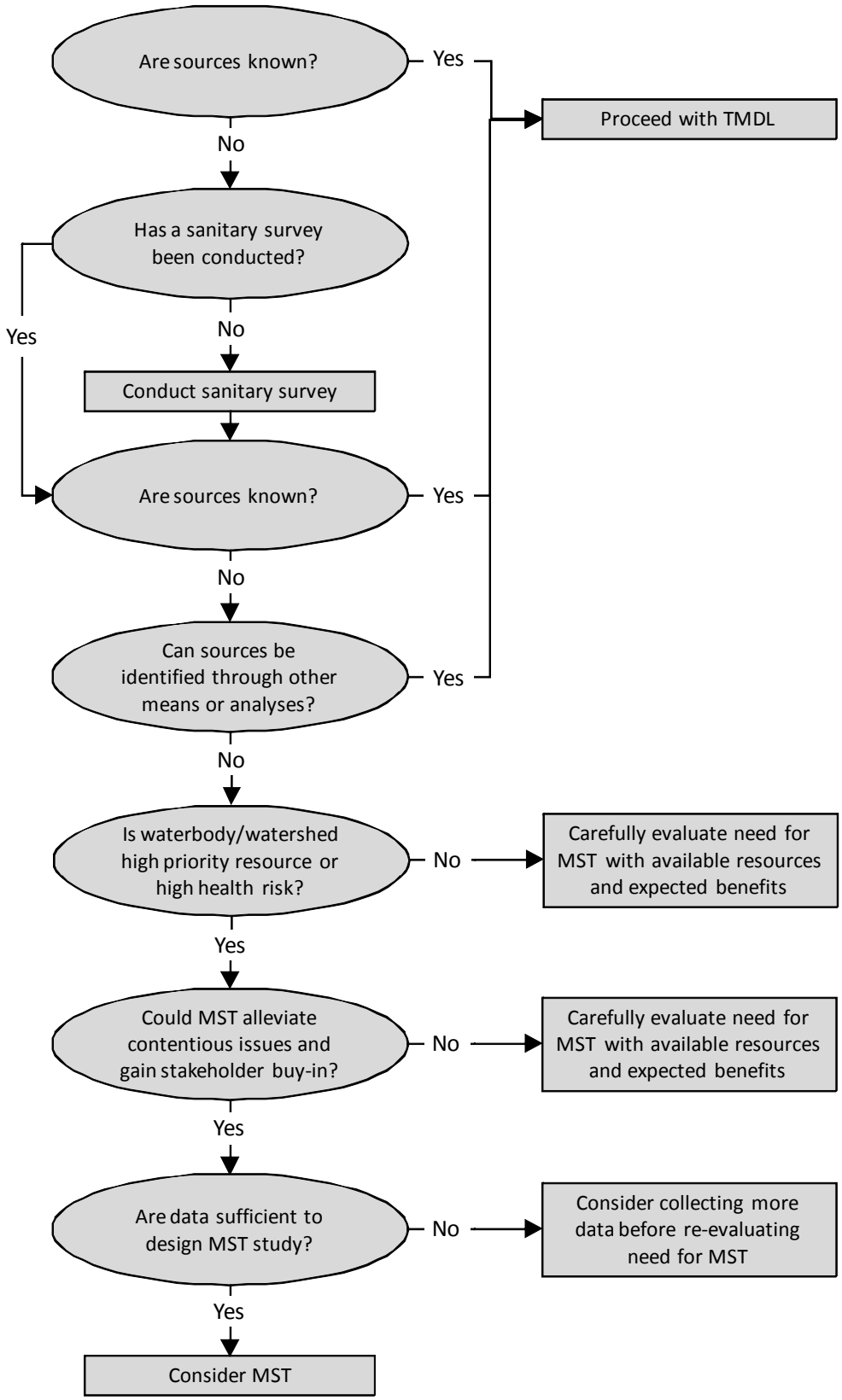


Figure 2. Decisions to consider when deciding whether to conduct an MST study.

Other ways of identifying sources might include outreach to state or local agencies or stakeholders. An active watershed group might have local knowledge of potential sources, either eliminating the need for MST or at least focusing the study better. Similarly, MST should not be undertaken without first discussing potential sources with counterparts in the state nonpoint source group, contacts at local planning or health departments, local residents, watershed groups, or staff at other locally relevant agencies (e.g., soil conservation districts).

Another consideration for deciding when or where to use MST is the priority of the impaired waterbody given its use or expected sources of fecal contamination. For example, resources for MST would likely be better spent on a study for a lake with high recreational use than a small headwater stream that is not used for contact recreation or consumption. Waterbodies where the bacteria impairment poses a higher health risk can certainly support the need for MST. MST can also be useful for obtaining stakeholder buy-in for supporting management activities and therefore might be prioritized for watersheds with contentious issues or communities.

When designing an MST study, it is important to have sufficient monitoring data to support sample design. Observed bacteria concentrations and associated flow and waterbody data are critical to determine where and when to collect samples for MST analysis to most effectively identify fecal sources. Sufficient bacteria data should be available to target sampling at locations of expected source activity or observed impairment and during times and conditions exhibiting impairments. If sufficient data are not available for this, it might be beneficial to first conduct additional water monitoring for indicator bacteria before proceeding with the MST study.

Alternatively, an initial MST study could focus on only a few stations or priority conditions identified through analysis of the available data or information to identify potential sources or areas of concern. For example, the study might first target only select downstream and upstream locations to identify if human or livestock sources are present at those key locations and then use that preliminary information to conduct sanitary surveys and additional source tracking to determine where those sources are present in portions of the watershed contributing high bacteria loadings. Thus, a phased or tiered approach could be used to include an initial source characterization at key locations in the watershed, a second characterization at selected locations exhibiting high bacteria concentrations, and a third characterization to determine the effectiveness of source control activities.

Example: Prioritizing Needs for MST Analysis in Northern Idaho

In the Panhandle of Idaho, the state agency used a process to prioritize the use of MST analysis for a few targeted streams based on such considerations as observed impairment, designated use, stakeholder concerns, TMDL schedule, and current understanding of sources. (Details are provided in the summary included in Section 7, Examples of MST Use for TMDLs.)

Example: Using Multiple Methods and Tools for Identifying Fecal Bacteria Sources in Florida

Florida Department of Environmental Protection (FDEP) has implemented a watershed management approach for restoring and protecting state waters and addressing TMDL Program requirements. The approach includes development of a Basin Management Action Plan. As part of the action plan development in the Lower St. Johns River Basin, FDEP used multiple methods of fecal bacteria source identification, including targeted MST in priority subwatersheds after initial data analysis and watershed surveys. The process included:

- Detailed and comprehensive analysis of GIS and existing technical reports and information to identify most probable sources for each of the 53 impaired waterbodies
- "Maps on the table" workshop
- Field reconnaissance ("Walk the WBID") with representatives from each Tributary Assessment Team with the most field knowledge of the basins (>20 participants, 8 days over two events)
- MST in 10 tributaries using qPCR to analyze human-specific *Bacteroides* marker, PCR to analyze ruminant- and horse-specific *Bacteroides* markers, qPCR to analyze human-specific marker of *Enterococcus faecium* and human-specific marker of *Human Polyomavirus* (HPyV)
- Thermal imaging for four waterbodies to identify inputs that are warmer than the surrounding water, possibly indicating inputs from stormwater outlets, failing septic tanks, or illicit connections.
- "Flexible" sampling stations that accommodated targeted and/or more detailed MST analysis based on high in-stream concentrations measured during traditional bacteria monitoring.

For more information see the Lower St. John's River Basin action plan at www.dep.state.fl.us/water/watersheds/docs/bmap/bmap-lsjt2.pdf or a presentation from the September 3, 2009 meeting of the Lower St. Johns Technical Advisory Committee (www.lsjr.org/pdf/pdf2009meetings/C.WapnickPresentation_ColiformSourceID_9-3-09%20(2).pdf).

4. Selecting an MST Method

The selection of an appropriate MST method primarily depends on the objectives of the study. In the context of TMDL development and implementation, the goals of the study typically relate to the necessary level of discrimination in identifying sources of fecal contamination. The focus of MST studies for TMDL development and implementation can be divided into three categories of increasing discrimination and complexity (Figure 3):

- Determining the presence or relative abundance of human fecal sources (e.g., municipal sewage, residential septage, or use of unsanitary human practices)
- Determining the presence or relative abundance of select non-human fecal sources other than wildlife (e.g., livestock or dog)
- Determining the presence or relative abundance of all fecal source sources (e.g., human, cow, horse, dog, cat, bird, waterfowl, deer, raccoon, rodent, etc.)

Increasing discrimination

Fecal Source Identification	MST Methods
Presence of human sources	• Bacteriophage
Presence of human and select animal sources (e.g., cattle)	• Viral PCR • Bacterial PCR
Relative abundance of human and select animal sources (e.g., cattle)	• Viral qPCR • Bacterial qPCR
Relative abundance of all individual sources (e.g., human, cow, horse, dog, raccoon, etc.)	• PFGE • Ribotyping • Rep-PCR • Antibiotic resistance • Carbon utilization

Figure 3. Level of detail provided by MST methods for identifying fecal sources in bacteria impaired waters.

Whether for use in developing a TMDL or for supporting subsequent implementation, a practitioner should decide the minimum level of source identification that will meet the study goals. Is it sufficient to determine the presence of human versus non-human sources, or is it necessary to have a pet-livestock-human distribution, or even a more comprehensive classification of all species? The most important consideration for making this decision is the expected use of the results in supporting potential management activities. The level of source identification should be comparable to the level of source distinction or "grouping" for management. For example, will your management approach be different if you know the distribution of horse, cows, pigs, sheep and other livestock versus if you more generally know the distribution of livestock versus other non-livestock sources in the watershed?

Conventional viral and bacterial PCR methods are likely the most cost effective tools for identifying the presence of human fecal sources at one or more sites. Viral PCR methods are effective for detecting the presence of municipal sewage, but might not be as effective as bacterial PCR for detecting the presence of residential septage or use of unsanitary human practices because of the small number of humans contributing those sources. Conventional *Bacteroides* PCR is a commonly used and cost effective method for detecting all human sources because the genetic markers for humans are very specific and well developed. Using both viral and *Bacteroides* PCR methods in combination provides an added benefit of confirming results from each method and might be used to identify if the observed human fecal sources originate from municipal sewage or individual human waste. The relative frequency of human source detection might be used to compare the relative abundance of human bacteria among the sampling locations and conditions.

Quantitative viral and bacterial PCR methods are more expensive than conventional PCR methods, but have the advantage of measuring the relative amount of DNA from human fecal sources, which might be used to estimate the relative abundance of those sources. *Bacteroides* qPCR also might be used to identify the presence or relative abundance of selected animal fecal sources. Genetic markers for cattle have been well developed by most laboratories, but an abundance of deer or other ruminants in the watershed might result in overestimation of cattle sources. Currently, genetic markers are less developed for dog, horse,

and pig, such that this method might not be able to detect the presence of those sources if they are not abundant.

As described in several MST review documents (USEPA 2005; Stoeckel 2005; Stoeckel and Harwood 2007; Field and Samadpour 2007), MST studies with objectives of identifying a diverse collection of specific fecal sources would require the use of a DNA library method to take advantage of the high discrimination that these methods offer among diverse sources. The three DNA library methods include PFGE, ribotyping, and rep-PCR. Method selection should consider numerous factors specific to the laboratory that performs the method (e.g., quality control protocols, accuracy of blind test results, laboratory availability, size of the library for each source of interest, data turnaround time, and costs of sample analysis and library development).

While the study goals will be the major consideration in selecting an MST method, factors related to cost, schedule, and potential uses of the results will also affect the decision. If cost is a limiting factor in the ability to conduct an MST study, a method should be chosen that meets the minimum goals within the available budget. For example, if a TMDL developer simply needs to distinguish between a few expected sources and identifying the relative presence of human and non-human sources will meet that goal, there is likely no need to use a detailed method such as PFGE that can identify specific species but is considerably more expensive than methods such as bacteriophage. Alternatively, if the available budget does not support a method that provides the level of discrimination or information needed to support the project goals, the TMDL developer should consider whether an MST study is warranted or should be scheduled as a future project.

Library-independent methods typically have a lower cost and provide results much faster (e.g., days versus months) than library-dependent methods. Among the library-independent methods, laboratory costs and the potential use of the results increases as the level of detail increases. Comparing unit sample costs for a commercial laboratory as an example (Source Molecular 2009), analysis for the presence of human *Bacteroides* using PCR costs $375/sample, analysis for the presence of four types of *Bacteroides*

Using a Toolbox Approach for MST Studies

A number of researchers in the MST community suggest using a “toolbox” or “tiered” approach for MST studies, moving from general to specific and from less to more expensive (Field and Scott 2007; Stoeckel and Harwood 2007; Stewart et al. 2003; Field and Samadpour 2007; Santo Domingo et al. 2007). As discussed in Section 3, it is recommended that prior to using MST, the first step in identifying sources of fecal bacteria should rely on sanitary surveys, watershed tours, traditional indicator bacteria monitoring, and data analysis as much as possible. Once it has been decided to use MST, the process might start with less expensive methods focusing on one or a few suspected or manageable sources, such as using chemical methods to identify the presence of human sources or host-specific PCR to identify select individual sources. First-tier results might provide sufficient information to move forward with management decisions. If not, they can be used to identify the need for more expensive and more comprehensive methods (e.g., library-based methods to identify all species) and support effective study design. For example, initially using less expensive methods (e.g., molecular instead of non-molecular) might allow for the collection and analysis of more samples, thereby providing greater spatial coverage and diversity in sample representation. This can be useful not only in better understanding sources and targeting management but also in designing more effective and targeted follow-up sampling should it be decided to conduct more comprehensive MST analyses.

(e.g., human, cow, horse and a general category for all sources combined) costs $1,200/sample, and costs for quantification of DNA from two types of *Bacteroides* (e.g., human and cow) using qPCR costs $1,090/sample.

Library-dependent methods provide a higher level of source discrimination than library-independent methods, but are typically too expensive to be used to examine short-term temporal variation in those sources. For an equivalent laboratory cost and sampling effort, a library-dependent method can detect all sources present at a location without determining how the frequency of those sources varies between sampling events, while a library-independent method can detect how the frequency of one source varies between sampling events. Thus, all study goals and constraints should be considered by a TMDL writer in the selection of an MST method(s) and design of an MST study.

5. Designing an MST Study

An MST study should be designed to answer specific questions raised following thorough analysis of available bacteria monitoring and sanitary survey results and to address a list of prioritized study goals and objectives. Due to the expense, MST should only be used if it is determined that those goals cannot be answered by conducting additional bacteria monitoring or sanitary surveys. It is also important to recognize the inherent inaccuracies associated with MST results and to design an MST study that collects enough data to account for those inaccuracies. Thus, an MST study for TMDL development or implementation should include multiple sampling locations, collection of multiple samples during a sampling event, and multiple sampling events during the period of interest (e.g., summer or winter) or hydrologic conditions (e.g., base or storm flow).

Designing an MST Study

Designing an effective MST study includes many of the same decisions as any sampling program, including answering the questions of:

- What?
- When?
- Where?
- How?

For an MST study, the sampling program should be designed to provide the most insight into the sources that are affecting observed bacteria impairments. Therefore, it is important to first understand the impairment and the associated areas, timing and conditions. Existing bacteria data should be analyzed to understand such things as

- Magnitude and frequency of elevated bacteria concentrations and water quality criteria exceedances
- Spatial variation in bacteria concentrations and exceedances
- Temporal trends in bacteria concentrations and exceedances
- Flow conditions under which exceedances occur (e.g., baseflow vs. storm flow)

This information can help to answer the questions of what, where and when to sample for the most effective and meaningful MST study.

What to Collect and Analyze?

For MST sample design, it is necessary to identify what types of samples are to be collected as well as how many samples of each and what laboratory analyses will be conducted for each. Water samples should be collected for use in both bacteria enumeration and the laboratory analysis associated with the chosen MST method. It is important to measure the concentration of the indicator bacteria used in water quality criteria (i.e., fecal coliform or *E. coli*) corresponding to the results of the MST analysis and what sources are identified for the respective samples. For example, some waters samples might be dominated by human bacteria while others show bacteria from cattle. However, without the corresponding bacteria concentrations of those samples, it is unknown which water samples are exceeding water quality criteria and which sources could be contributing to those exceedances. In the example, bacteria concentration data might show that all the human-dominated samples have low levels of bacteria; however the livestock-dominated samples have concentrations exceeding criteria. This indicates that while both human and livestock sources of bacteria exist in the watershed, it's likely that the livestock sources are causing the elevated concentrations that are leading to exceedances of water quality criteria. This type of analysis can have significant implications on the controls and management strategies designed for the watershed.

Often more difficult than identifying what type of samples will be collected and analyses performed is the decision of how many samples to collect as well as the subsequent decisions of when and where. Ideally, the number of samples collected for MST analysis would be determined using statistical procedures (i.e., power analysis). Power analysis determines the minimum number of samples required to detect significant differences in results based on the anticipated variability of those results (Zar 1984). However, a statistical approach is seldom used to determine the number of samples needed for MST studies due to a lack of data on the variance of fecal sources in water samples. Surface waters typically exhibit a high variance in bacteria concentrations and numerous samples are required to detect relatively rare bacteria sources. In general, a minimum of 10 samples should be collected from each site for each condition of interest regardless of the MST method used. For example, if the period of interest is during high recreational use throughout the summer, then at least 10 samples should be collected on various dates throughout the summer. If a study goal is to determine how the target source varies during all hydrologic and seasonal conditions, then at least 10 samples should be collected during each condition (e.g., summer base, summer storm, winter base, winter storm). In addition, a study goal might be to determine how the target source varies during a storm event or between ranges of storm sizes to select appropriate BMPs. Thus, the number of samples will increase with the level of detail, certainty, and understanding needed.

Using Your MST Results Wisely: The Real-World Context of Your Results

MST data's usefulness relies on their scientific defensibility and representativeness of actual conditions. When analyzing and using the results within the TMDL analysis or for making decisions, remember MST results represent a snapshot of the time and conditions during the sampling event. If samples are only collected during certain seasons or conditions (e.g., summer vs. winter, storm events vs. baseflow), then the results can be assumed to only represent sources actively contributing bacteria during those times or through those pathways.

Collecting Data to Build a Library

Water samples to be analyzed for the indicator bacteria or viruses used in the MST analysis method will be collected for all MST studies. In addition, studies using library-dependent methods should include collection of fecal material from known sources to build the library. The sample design should identify whether fecal material will be collected in addition to water samples, and if so, where, when and how those samples will be collected. Existing libraries at labs currently supporting MST studies can be used, however it is important to collect fecal samples within the study watershed to supplement the existing library and capture sources specific to the watershed and timing of sampling. The issues of library size and transferability across studies are topics of continuing study and debate in the MST community. It is unknown what the effect of temporal or geographic stability has on the applicability of existing libraries to other watersheds or to later studies. Temporal concerns surround the underlying assumption of MST methods that the genetic composition of bacteria strains is stable through time, with bacteria strains indefinitely reproducing to create "cloned" strains with identical genetic composition. Therefore, strains of bacteria isolated from one animal and included in a library would theoretically match a strain of bacteria isolated from that same animal years later. Similarly, geographic stability concerns relate to the question of whether host-specific strains from one animal in one region could be matched to the same type of animal in another geographic region. Because of these concerns, it is essential to collect fecal samples from the study watershed to build a new library or supplement an existing library to minimize the effects of spatial and temporal instability and improve the source classification accuracy. It is recommended that colleagues, contractors or academics knowledgeable of and experienced with MST be consulted regarding study design for library development.

Where to Collect Samples?

Where data are collected is critical to meeting project goals and obtaining information to answer management questions. For an MST study, the sample locations should be targeted to areas that will provide the most insight into the presence of sources that are affecting observed bacteria impairments. Therefore, it is important to first understand the impairment and the associated areas, timing and conditions. The sample locations should be targeted to sites that have exhibited elevated concentrations of bacteria and exceedances of water quality criteria. In addition to sites where high bacteria concentrations have been observed, it is a good idea to include one or more background sites located upstream where low bacteria concentrations have been observed (or are expected). Results for background sites can be used to verify the absence of target fecal sources or estimate the relative abundance of non-target fecal sources. For example, detection of a human source at a frequency or amount that is similar for a low-level upstream site and a high-level downstream site suggests that animal sources are primarily responsible for the high bacterial concentrations observed at the downstream site.

Factors to Consider When Identifying Sampling Locations

- Location of known or expected sources
- Areas with high bacteria levels
- Land use
- Historical monitoring data locations
- Confluence of tributaries
- Priority subwatersheds
- Type or number of targeted sources

Other considerations for identifying specific sampling locations include:

- **Land Use Type and Distribution.** Because bacteria sources often vary by land use, it would be helpful to include sites that drain primarily one land use of interest. For example, a site might be located in a subwatershed dominated by agricultural and livestock operations or alternatively by residential areas to identify the frequency or abundance of sources associated with the respective land use. For large basins with varied land use conditions, additional sites could be added between downstream and upstream sites to determine how target fecal sources vary in frequency or abundance between those sites.
- **Areas of Known or Expected Sources.** Collecting samples downstream (and upstream if resources allow) of areas of expected source activity can help to confirm the existence or influence of expected sources.
- **Confluence of Tributaries/Subwatersheds.** Locating sampling sites at the mouth of or confluence with major tributaries essentially breaks the watershed into smaller subwatersheds for more targeted analysis. Collecting samples at these sites can potentially provide results for understanding or evaluating the spatial distribution or influence of sources. The effect of bacteria contributed by sources in subwatersheds can extend to downstream segments. By evaluating the watershed as smaller units, the distribution or location of potential sources can be better understood to more effectively target management activities.
- **Level of Discrimination in Source Identification.** The number and location of sampling sites are highly dependent on the selected MST method and the corresponding level of discrimination needed in source identification. (See previous section on selecting an MST method.) If the study is being designed to identify the presence and abundance of a variety of sources, the number and location of sampling locations will likely differ from those in a study designed only to confirm the presence or absence of a specific single source.

Ideally, as many locations as necessary could be sampled to answer study questions and goals. However, that is often not feasible due to resource, time or logistical constraints; therefore, locations should be targeted to maximize the amount of information or insight they provide within the available budget and resources and consistent with the current understanding of the watershed and the observed impairment.

When to Collect Samples?

Bacteria concentrations can be highly variable within a receiving waterbody, especially across varying flow conditions. Depending on the types and characteristics of bacteria sources in a watershed, concentrations might be elevated during low flow or storm flow conditions or both. Fecal sources and resulting bacteria concentrations might vary between base and storm flow conditions, within and between storm events, and among seasons due to changes

Factors to Consider When Identifying Sampling Timing or Schedule

- Conditions (e.g., dry or wet weather) or times (e.g., season) exhibiting high bacteria levels
- Conditions or times of highest risk to uses (e.g.. summer for recreation)
- Times of expected source activity

in weather and resulting runoff patterns or source activity, such as changes in sewer/septic system function, wildlife populations, livestock grazing/distribution or seasonal residents or activities. For example, elevated bacteria concentrations during base flow suggests a source that contributes bacteria directly to a stream or is otherwise not dependent on surface runoff for delivery of bacteria—sources such as leaking sewers, failing septic systems, National Pollutant Discharge Elimination System (NPDES)-permitted discharges or livestock or wildlife with access to waterbodies. Alternatively higher concentrations during high flow indicate bacteria delivered through surface runoff during storm events—bacteria from sources such as wildlife, livestock operations or grazing areas, manure application on land surfaces, regulated stormwater or combined sewer overflows (CSOs).

Therefore, samples for MST analysis should be collected during times and conditions of elevated bacteria concentrations to identify those sources contributing to the observed impairment. In addition, the fecal sources identified through the results of the MST analysis can be viewed within the context of the sample timing and conditions to even further identify the bacteria sources. For example, detection of a human source at a higher frequency or amount in base flow than storm flow might suggest leaking sewers are more important than precipitation-driven runoff or sewer overflows. Conversely, detection of a human source at a higher frequency in storm flow than base flow suggests that septic system failures from saturated ground conditions might be contributing to bacteria loadings. Finally, samples for MST analysis should be collected on numerous separate occasions representing each condition of interest to verify the repeated presence or absence of a bacteria source.

When targeting MST sample collection to those times or conditions exhibiting bacteria impairment, it is important to thoroughly and accurately document the field and environmental conditions during sample collection. Documenting the preceding and current weather conditions, streamflow, waterbody characteristics, and nearby watershed conditions or activities is essential to evaluating the sample results in the context of source activity, flow conditions, weather patterns and other spatial or temporal variations that can affect bacteria loading and waterbody levels. This is helpful in better understanding or characterizing the sources identified through the MST analysis.

How to Collect Samples?

Generally, the MST study design should include those same quality assurance and quality control (QA/QC) procedures required for bacteria monitoring. Study design should be completed in coordination with experienced and knowledgeable laboratory and sampling technicians to ensure appropriate sampling techniques and sample processing are used. Things to consider for study design and QA/QC include:

- **Sample Collection and Handling Methods.** For example, all sampling and analysis should be conducted using sterile techniques. Collection of several samples from multiple locations is preferred to single-dip samples to include more of the cross-sectional area or volume of the waterbody. Water samples should be stored at 4 °C between collection and analysis, whereas membrane filtered samples where cultures will be grown and used for analysis by a DNA library method should be stored at room temperature and shipped overnight to the MST laboratory. The maximum sample holding time

for water samples might need to be extended from the required 6 hours to 24 hours to allow for overnight shipment to the MST laboratory.

- **Use of Replicate, Blind, and Blank Samples.** An MST study should also include replicate analysis of samples to verify method precision and analysis of blind or spike samples containing known fecal sources and blank samples containing no fecal sources to verify method accuracy. These quality control sample analyses are critical for assessing precision and accuracy of the MST method and should comprise at least 10 percent of the project samples. However, because of the naturally high variability in bacteria and the concerns regarding reproducibility and accuracy of some MST methods, it might be necessary to include a higher percentage of quality control samples depending on the study goals and design. While consistent guidance on QA/QC is not available for all MST methods, USEPA (2004) does provide recommendations for laboratory QA/QC practices for molecular methods.

Asking for Help

Because MST is a continually evolving science, there is currently a lack of consistency in protocols and methods, and there are no concrete guidelines on things such as minimum number of samples or isolates needed, size of library, etc. Therefore, it can be important to seek guidance or support from other TMDL practitioners, labs, contractors or academics with experience in designing and conducting MST studies.

- **Number of Isolates per Sample.** An MST study using a library-dependent method should consider the number of isolates analyzed in each sample. One water sample is often dominated by relatively few fecal sources and the diversity of sources observed at a sampling location increases with the number of samples analyzed. Therefore, a high number of samples and a low number of isolates per sample (e.g., less than 10) will provide a better estimate of the sources present at the sampling location than a low number of samples and a high number of isolates per sample.

Considering Costs and Resources

Finally, the MST study design needs to fit within the project budget while still meeting study goals. Detailed costs should be developed for study design and plan preparation, sample collection and laboratory analysis, and data analysis and reporting. Laboratory costs for MST analysis can range from $10,000 to $20,000 per sampling site for a typical study design. For example, the following study designs could be applied to a library-independent or library-dependent method based on an MST analysis budget of $10,000 for 10 sampling events at one site:

1. Library-independent design: *Bacteroides* qPCR analysis of 10 water samples (one sample/event) for quantification of human and cow sources at a unit cost of $1,000/sample.
2. Library-dependent design: PFGE analysis of 100 *E. coli* isolates developed from membrane filter cultures of 20 water samples (two samples/event), and analyzing 5 isolates/sample at a unit cost of $100/isolate.

All other costs associated with these design examples would be similar with the exception of additional bacteria enumeration costs associated with the 10 additional water samples collected for PFGE analysis and additional field costs associated with fecal source sampling for PFGE library development.

6. Understanding MST Results

Use and interpretation of MST results require caution and consideration of inaccuracies associated with a small sample size and the analytical procedure. As previously noted, bacteria concentrations and sources are often highly variable and the collected water samples represent a very small portion of the populations present in the waterbody. Also, MST procedures are not 100 percent accurate and the analytical precision and accuracy can vary greatly between methods, laboratories, and individual water samples. Therefore, it is important to interpret MST results in the context of the conditions sampled, evaluate how MST results vary among the replicate samples and sampling events, and use quality control sample results to document accuracy of the analytical procedure.

MST results are reported in various formats depending on the method and laboratory. For the library-dependent methods, one bacteria source is typically reported for each isolate analyzed. However, the degree of source specificity might vary for each isolate, requiring the grouping of related sources for interpretation. For example, one isolate might be identified as crow while another isolate is designated as a more general category of avian (bird). In addition, some isolates might be designated as unknown because they did not match a known source in the library or might be designated as multiple sources (e.g., cat/dog) because they have been observed in multiple sources in the library. Interpretation of results can be particularly challenging when an isolate has been observed in different kinds of sources (e.g., source identified as canine might have originated from coyote or dog representing wildlife or pets, respectively).

For the library-independent methods, results for each sample analyzed might include one or more detected sources or might include no detected sources. The relative abundance of detected sources might also be reported for each sample, either as an organism concentration for a culture method or as a DNA concentration for a qPCR method. As noted for library-dependent methods, positive results might be reported for a group of sources that include different kinds of sources (e.g., ruminants detected using *Bacteroides* PCR might have originated from either cow or deer). In this situation, a watershed survey might be used to identify dairy farm as the likely source, therefore input from deer is less likely than cows. It is also important to recognize that an MST method might be able to detect one bacteria source more readily than another source. For example, more markers have been established for cow than horse using *Bacteroides* PCR. Thus, data compilation and analysis will vary depending on the method used and how the results are reported.

While MST methods can be extremely useful in identifying bacteria sources in impaired watersheds, TMDL developers should be careful to interpret and use their results appropriately. TMDL developers should be mindful of the methods used and the data's representativeness of actual conditions when interpreting results to draw conclusions about watershed sources. For example, comparison of multiple sources observed at one site should consider the varied ability of a method to detect each source. Comparison of sources between sites or types of sampling events should consider the relative number of observations. For example, the absence of a human source at a site or differences among sites in the frequency of a detected human source might not be significant if relatively few samples were analyzed. Significant differences in sources between sites or events should be tested using appropriate statistical

procedures. A contingency table using chi-square analysis (Zar 1984) is an example of an appropriate test for determining significant differences in the frequency of bacteria source detection among multiple sampling sites or types of sampling events.

In addition to how results are interpreted, how they are used can affect a TMDL analysis or subsequent management decisions. The results will likely be most useful to confirm the presence or absence of a particular source or to gain a qualitative understanding of the types and relative abundance of different sources. MST methods developed to date generally lack the accuracy required for quantifying all fecal bacteria sources or for definitively identifying the relative abundance of bacteria among multiple sources. For example, while some library-dependent methods provide the number of isolates "matched" to different hosts, the total number of isolates from a water sample is just a small portion of the total population of bacteria in the sample. So, while the majority of the isolates might be matched to a particular animal as the origin of the bacteria, that does not necessarily equate to that animal being the primary source of bacteria in the watershed. While having the majority of isolates matched to a particular source means that source is active in the watershed and likely more abundant than others, it might not be appropriate to use the specific "percentages" or relative abundances of the different sources for quantitative analyses.

For example, some TMDLs have calibrated watershed models to ensure that the simulated relative distribution of bacteria loads among individual bacteria sources is equivalent to the relative abundance of host-specific isolates shown in the MST results. This can be a dangerous assumption because the MST results might capture only a subset of sources active during the time of sampling. A number of studies recognize the merits of qualitative uses of MST data, but have concluded that the ability of any MST method to quantitatively determine the relative contributions of fecal contamination in a water sample has not been convincingly demonstrated (Benham et al. 2010; Keeling et al. 2005; Stoekel and Harwood 2007; Field and Samadpour 2007; Santo Domingo 2007; Seurinck et al., 2005). Therefore, using MST quantitatively for such things as source loading characterization, model calibration or distribution of load allocation is not recommended at this time. Inappropriate use of the MST results might mislead management decisions and result in ineffective implementation planning and possibly wasted resources.

7. Examples of MST Use for TMDLs

MST has been used in several states to support TMDL development and implementation. This section summarizes a number of MST studies used to support source identification, model development, allocation development and identification of priority management activities within the TMDL framework. The following MST and TMDL studies are summarized in this section:

- Panhandle Streams, Idaho
- Tillamook Bay, Oregon
- Middle Rio Grande, New Mexico
- Virginia TMDLs

- Sand Dam Village Pond Town Beach, New Hampshire
- Ecorse River, Michigan
- Beaver Creek, South Dakota

Table 5 summarizes the example MST studies in this section. The examples represent a range of MST methods, geographic settings, source types and uses of the MST results within the TMDL process. The examples are expected to be a small subset of MST studies conducted to support TMDLs. They are not intended to represent the “right” or “wrong” way to do an MST study for TMDL purposes, but simply to provide examples of how some states are incorporating MST into their TMDL programs. The majority of the studies were conducted prior to TMDL development, while two were conducted after TMDL development to better define sources and support TMDL implementation. Of those studies conducted prior to TMDL development, most of them used the results qualitatively to identify potential sources and support the identification of appropriate management strategies. Two of the examples (those in Virginia) used the MST results directly in the TMDL calculation analysis, including for partitioning the loading capacity into source allocations and for calibrating watershed models.

Table 5. Summary of Example MST Studies

Waterbody	Year of MST Study	Sampling Summary	Sources Evaluated	MST Method	MST Indicator	Use in TMDL Context
Panhandle Streams, ID	2009	▪ Summer ▪ Dry and wet weather ▪ 10 events ▪ 5 sites	▪ All species	▪ PFGE ▪ Bacterial PCR	▪ *E. coli* ▪ Bacteroides	▪ Source identification ▪ Development of TMDL implementation plan
Tillamook Bay, OR	2001–2003	▪ Bimonthly for 2 years ▪ 30 sites	▪ Ruminant ▪ Human	▪ Bacterial PCR	▪ Bacteroidales	▪ TMDL implementation
Middle Rio Grande, NM	2002–2004	▪ Dry and wet weather ▪ 5 events ▪ 30 sites	▪ All species (riboyping) ▪ Human, livestock, wildlife (ARA)	▪ Ribotyping ▪ ARA	▪ *E. coli*	▪ TMDL implementation plan development
Hays Creek, VA	2004–2005	▪ Monthly for 1 year ▪ 1 site	▪ Human ▪ Pet ▪ Livestock ▪ Wildlife	▪ ARA	▪ *E. coli*	▪ Source identification ▪ Model calibration
Sand Dam Village Pond Town Beach, NH	2005	▪ Summer ▪ Dry weather ▪ 6 events ▪ 2 sites	▪ All species	▪ Ribotyping	▪ *E. coli*	▪ Source identification ▪ Identification of management activities
Ecorse River, MI	2007	▪ Dry and wet weather ▪ 1 wet event and 1 dry event ▪ 10 sites	▪ Human	▪ Bacterial qPCR	▪ Bacteroidetes ▪ Enterococcus	▪ Source identification
Beaver Creek, SD	2003–2005	▪ Monthly and during rain events ▪ MST on samples above 50/100 mL *E. coli* ▪ 1 site	▪ All species	▪ PFGE	▪ *E. coli*	▪ Source identification ▪ Development of management strategies

Panhandle Streams, Idaho

MST was chosen to support TMDL development for 13 streams in the Panhandle region of northern Idaho that are listed on the state's 303(d) list as impaired by *E. coli*. Unlike other regions in the state, the source of bacteria is not visibly obvious in these watersheds, and Idaho DEQ chose MST to identify sources of bacterial contamination in streams and to focus appropriate source control activities.

Year TMDL Developed:	Planned
Applicable Water Quality Criteria:	• Geometric mean of *E. coli* not to exceed 126 organisms/100 mL • Single sample maximum[a] of *E. coli* of 406 /100 mL (primary contact recreation) • Single sample maximum[a] of *E. coli* of 576 /100 mL (secondary contact recreation)
Year of MST Study:	Summer 2009
MST Method:	• PFGE • Bacterial PCR
MST Indicator:	• *E. coli* • *Bacteroides*
TMDL Calculation Approach:	n/a (not yet developed)
Use in TMDL Context:	• Source identification • Development of TMDL implementation plan
Reference:	Herrera (2009, 2010); Keith and Steed (2010)

a. Single sample maximum is not used to determine violations of water quality standards, but to initiate collection of additional water samples to calculate a geometric mean.

MST Study

Idaho DEQ ranked the 13 impaired streams for priority need for MST analysis based on observed bacteria concentrations and criteria exceedances, designated use, stakeholder concern, known sources, TMDL priority, and downstream recreational uses. For example, five streams designated for primary contact recreation received a higher priority for the "designated use" category than those designated for secondary contact recreation. The three streams chosen for MST analysis were: Riley Creek, Hauser Creek, and Right Fork Hauser Creek. All of these creeks are designated for primary contact recreation and have experienced high *E. coli* concentrations. They also flow into popular recreation lakes (Pend Oreille Lake and Hauser Lake). Hauser Creek and Right Fork Hauser Creek watersheds also have an active watershed group and known stakeholder concerns.

Because there were no known sources of bacteria in these three watersheds, DEQ wanted to use an MST method that is sensitive to various sources. The primary objective was to determine if humans and livestock are major sources, and a secondary objective was to determine if domestic pets and wildlife are important sources.

PFGE was chosen as the MST method, and Bacteroides PCR was selected as a secondary method for quality assurance of human and ruminant sources identified by the PFGE analysis.

Fecal Source Sampling

Idaho DEQ field personnel collected fecal source samples to update the lab's existing *E. coli* DNA library with local animal and human sources in the northern Idaho study area to increase the frequency of source identification (matching). Idaho DEQ field personnel collected a total of 29 animal scat samples from the study region, with between one and five source samples collected from each of three suspected major

fecal sources (sewage, cow, and waterfowl) and nine suspected minor fecal sources (alpaca, bear, chicken, coyote, elk, deer, dog, goat, horse, and turkey).

Water Sampling

One monitoring station was located in each of the three subwatersheds at downstream stations where, historically, water quality criteria have been exceeded. Additional upstream monitoring stations were located on Riley Creek and Hauser Creek to identify fecal sources in the upper portion of each of these subwatersheds. Water samples were collected by the Idaho DEQ and analyzed for the following parameters by the indicated laboratories:

- *E. coli* enumeration and isolation by the University of Idaho, Coeur d'Alene
- *E. coli* DNA analysis by the Institute for Environmental Health (IEH)
- Bacteroides DNA analysis by the EPA Region 10 Laboratory

Sampling occurred at the five stations for 10 events, and stream stage and flow measurements were taken during each event. Four grab samples were collected from each station approximately every two weeks between June 1 and the end of September 2009 – a time period where recreational activities occur in the creeks. To capture delivery of *E. coli* from storm runoff, samples were collected within 24 hours following a rain event (greater than 0.2 inches within 24 hours). A total of 10 sample events occurred during the study — two of which were following a rain events within both project watersheds.

MST Analysis and Results

The MST project goal was to develop an average of 2.5 *E. coli* strains (isolates) from each of the 40 samples collected from each station, resulting in 100 *E. coli* isolates from each station and 500 *E. coli* isolates in total. The project goal was exceeded, with between 118 and 120 *E. coli* isolates analyzed for each station and a total of 596 isolates analyzed for all five stream stations. Fecal source samples were used to prepare three blind isolates from each of the following five sources: sewage, cow, dog, goose, and horse. These 15 duplicate (known and blind) isolates were prepared by the University of Idaho and submitted to IEH for updating the existing *E. coli* DNA library with the known isolates and then matching the blind isolates to the updated library. Only 9 percent of the 596 isolates analyzed were identified as unknown, with 91 percent of isolates matching a known source in the updated DNA library. Twenty-four individual sources were identified across the subwatersheds using the PFGE method (Table 6).

The genetic fingerprinting showed that greater than 10 percent of the total *E. coli* colonies isolated for the sample period were from dogs in upper Hauser, lower Hauser, and lower Riley Creeks, and cats were almost 20 percent in upper Hauser Creek. In addition, there were two days on lower Hauser Creek when Idaho's primary contact water quality criterion for *E. coli* was exceeded, during which dogs were the source of over 40 percent of the isolates. Horses and cattle each did not exceed 10 percent of the total *E. coli* isolates in the sample period; however, horses were greater than 15 percent of the *E. coli* isolates from Right Fork Hauser Creek when Idaho's criterion was exceeded. Although humans made up 11 percent of the total *E. coli* colonies isolated within the project period on Right Fork Hauser Creek, only

one *E. coli* colony was isolated from water samples collected on days when the water quality criterion was exceeded.

Table 6. Sources Identified in Hauser Creek, Right Fork Hauser Creek, and Riley Creek PFGE Analysis (Number of Samples and Percent of Total Samples for Each Station)

Fecal Source	Upper Hauser Creek		Lower Hauser Creek		Right Fork Hauser Creek		Upper Riley Creek		Lower Riley Creek		All Stations	
Avian	16	13%	34	29%	24	20%	16	13%	23	19%	113	19%
Bear	4	3%	1	1%	0	0%	2	2%	3	3%	10	2%
Beaver	0	0%	0	0%	0	0%	19	16%	0	0%	19	3%
Bovine	1	1%	0	0%	2	2%	3	3%	1	1%	7	1%
Canine	19	16%	0	0%	2	2%	6	5%	2	2%	29	5%
Cat	0	0%	0	0%	0	0%	0	0%	1	1%	1	0%
Cow	1	1%	6	5%	0	0%	5	4%	4	3%	16	3%
Coyote	0	0%	1	1%	2	2%	2	2%	5	4%	10	2%
Crow	0	0%	1	1%	0	0%	0	0%	1	1%	2	0%
Deer	9	8%	15	13%	28	23%	7	6%	5	4%	64	11%
Dog	7	6%	6	5%	13	11%	2	2%	11	9%	39	7%
Duck	0	0%	0	0%	0	0%	2	2%	3	3%	5	1%
Elk	3	3%	6	5%	3	3%	5	4%	1	1%	18	3%
Feline	0	0%	0	0%	4	3%	1	1%	1	1%	6	1%
Feral cat	0	0%	0	0%		0%		0%	6	5%	6	1%
Goose	6	5%	2	2%	5	4%	3	3%	5	4%	21	4%
Horse	3	3%	8	7%	5	4%	1	1%	7	6%	24	4%
Human	3	3%	13	11%	3	3%	11	9%	8	7%	38	6%
Poultry	0	0%	0	0%	1	1%	0	0%	0	0%	1	0%
Rabbit	0	0%	0	0%	0	0%	0	0%	1	1%	1	0%
Raccoon	16	13%	16	14%	1	1%	12	10%	2	2%	47	8%
Rodent	11	9%	4	3%	11	9%	19	16%	2	2%	47	8%
Skunk	0	0%	0	0%	0	0%	4	3%	0	0%	4	1%
Squirrel	1	1%	0	0%	0	0%	0	0%	1	1%	2	0%
Turkey	12	10%	0	0%	1	1%	0	0%	1	1%	14	2%
Unknown	8	7%	5	4%	15	13%	0	0%	24	20%	52	9%
Total	120	100%	118	100%	120	100%	120	100%	118	100%	596	100%

The general marker for Bacteroides was present in 7 of the 38 samples analyzed, but none of the samples tested positive for the human-specific or ruminant-specific markers. The Bacteroides PCR results generally supported the PFGE results that wildlife was the predominant source of fecal bacteria in the sampled streams.

TMDL Development

TMDLs are scheduled for development for Hauser Creek, Right Fork Hauser Creek and Riley Creek, and MST results are expected to support identification of appropriate management activities.

While the MST results provide insight into the active (and not so active) sources in the watersheds, Idaho DEQ feels the only confident conclusions from the MST study data are that wildlife is the dominant *E. coli* source in the Hauser Creek and Riley Creek watersheds and that they did not take enough samples to rule out additional sources. Idaho DEQ has indicated that the low abundance and frequency observed in non-wildlife sources (such as human, livestock, and pets) would likely equate to too much uncertainty in a TMDL meant to prescribe reductions in *E. coli*. Therefore, Idaho DEQ decided to write a watershed-wide TMDL for Hauser and Riley Creeks with generalized load reductions that are not based on land-use or specific sources (Keith and Steed 2010).

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Tillamook Bay, Oregon

Year TMDL Developed:	2001
Applicable Water Quality Criteria:	Shellfish Growing Waters: • A fecal coliform median concentration (or MPN) of 14 per 100 mL, with not more than 10 percent of the samples exceeding 43 per 100 ml. Recreational Contact in Water: • A 30-day log mean of 126 *E. coli* organisms per 100 mL • No single sample shall exceed 406 *E. Coli* organisms per 100 ml.
Year of MST Study:	2001–2003
MST Method:	Bacterial PCR
MST Indicator:	*Bacteroidales*
TMDL Calculation Approach:	n/a (MST study conducted after TMDL)
Use in TMDL Context:	TMDL Implementation
Reference:	Shanks et al. (2006)

The 572-mi^2 Tillamook Bay Watershed lies near the northwest corner of Oregon on the western side of the Coast Range Mountains. Aside from some small creeks and sloughs that drain directly to the Bay, the majority of water entering the Tillamook Bay is carried by five large rivers – Miami, Kilchis, Wilson, Trask, and Tillamook. Tillamook Bay supports commercial shellfish harvesting, and the rivers are used for recreational swimming and wading. Concentrations of bacteria in the waters of the rivers and the Bay are commonly too high to allow safe use for either of these activities. Sources of bacteria in the watershed include rural and urban residential development (many homes have failing septic systems), urban stormwater runoff, livestock management and other agricultural activities, and several wastewater treatment plants that discharge either to the rivers or the Bay.

In 1998, the lower reaches of the Miami, Klichis, Wilson, Trask, and Tillamook Rivers, the five major systems in the Tillamook Bay Watershed, were placed on the 303(d) list based on data collected by Tillamook Estuaries Partnership (TEP) local volunteers. Oregon DEQ developed a TMDL for *E. coli* bacteria in the Tillamook Bay Watershed in 2001.

Since the start of TEP monitoring in 1997, volunteers have collect 15,000 water samples from 43 locations in the five major river systems in the watershed. Through a partnership with DEQ beginning in 2006, TEP analyzed *E. coli* data from 1997 through 2008, indicating that four of the five rivers in the watershed still routinely violate Oregon's water quality criteria for recreational contact, while the Wilson River has improved and currently meets water quality criteria. The analysis also revealed that bacteria concentration increases as the major land uses switch from forestry in the upper watershed to urban and agriculture in the lower reaches. Bacteria data showed a strong correlation between high bacteria concentration and precipitation, mainly in the spring, summer, and fall. A trend analysis was completed for the 43 sites to determine whether *E. coli* concentrations were changing over time. Eighteen sites showed statistically significant trends, with the Tillamook River sites all indicating decreasing trends even though the concentrations in the river are still above recreational criteria (Figure 4). There are three locations where *E. coli* concentrations are increasing, Holden, Mill, and lower Bewley Creeks.

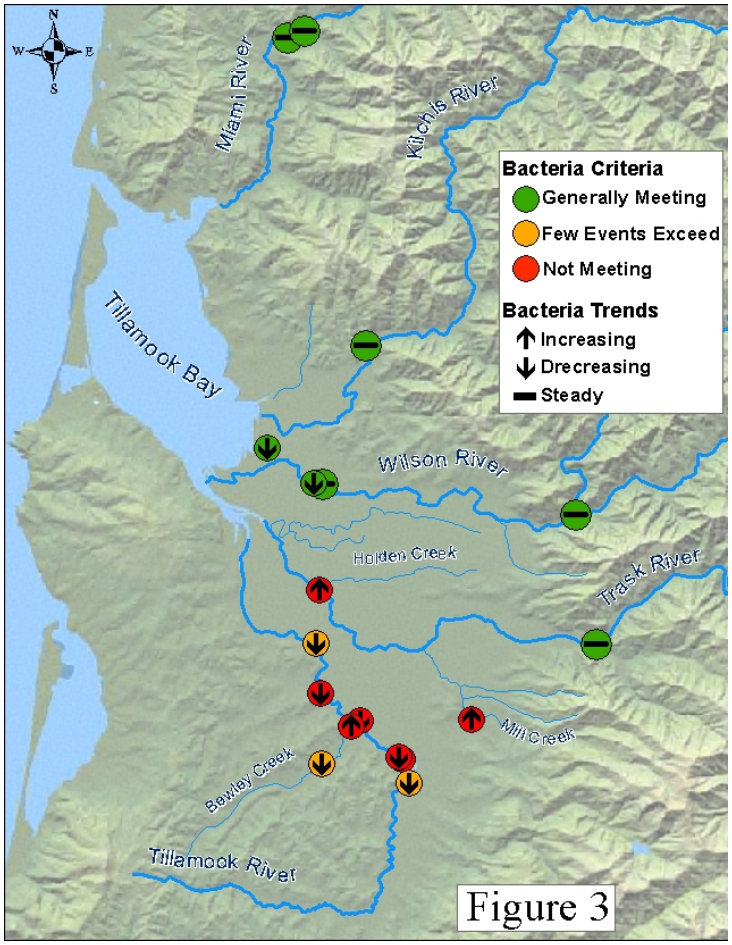

Figure 4. Trends in *E. coli* monitoring data in the Tillamook Bay watershed.

MST Study

To better identify bacteria sources, TEP contributed water samples from 30 sites collected in the Bay and tributaries from March 2001 through March 2003 for MST analysis led by Oregon State University researchers. *Bacteroidales* PCR assays were performed with general, ruminant-source-specific, and human-source-specific primers to identify fecal sources (Shanks et al. 2006). Because replicate sampling in large-scale studies can be expensive and time-consuming, researchers collected and analyzed 1 year of triplicate samples to test whether replication significantly increased the frequency of detection of high *E. coli* counts (>126 MPN/100 ml) and host-specific markers. They found no significant increase in the frequency of detection of either host-specific markers or samples with an *E. coli* count greater than 126 MPN/100 ml when replicate samples were analyzed (Shanks et al. 2006).

The objectives of this study were to evaluate spatial and temporal dynamics in source-specific *Bacteroidales* marker data across a watershed; to compare these dynamics to fecal indicator counts, general measurements of water quality, and climatic forces; and to identify geographic areas of intense exposure to specific sources of contamination.

To identify sites with elevated levels of fecal exposure, the observed frequencies of human and ruminant host-specific markers were calculated for each sampling site and plotted against the corresponding *E. coli* geometric means (Figure 5). Ruminant frequencies were higher than human frequencies at all sampling sites except the Trask-5 site. At eight sampling sites the average levels of *E. coli* did not meet the state recreational contact criterion (126 MPN/100 ml); these were the Bay-1 and -2, Kilchis-5, Trask-4, and Tillamook River-2, -3, -4, and -5 sites.

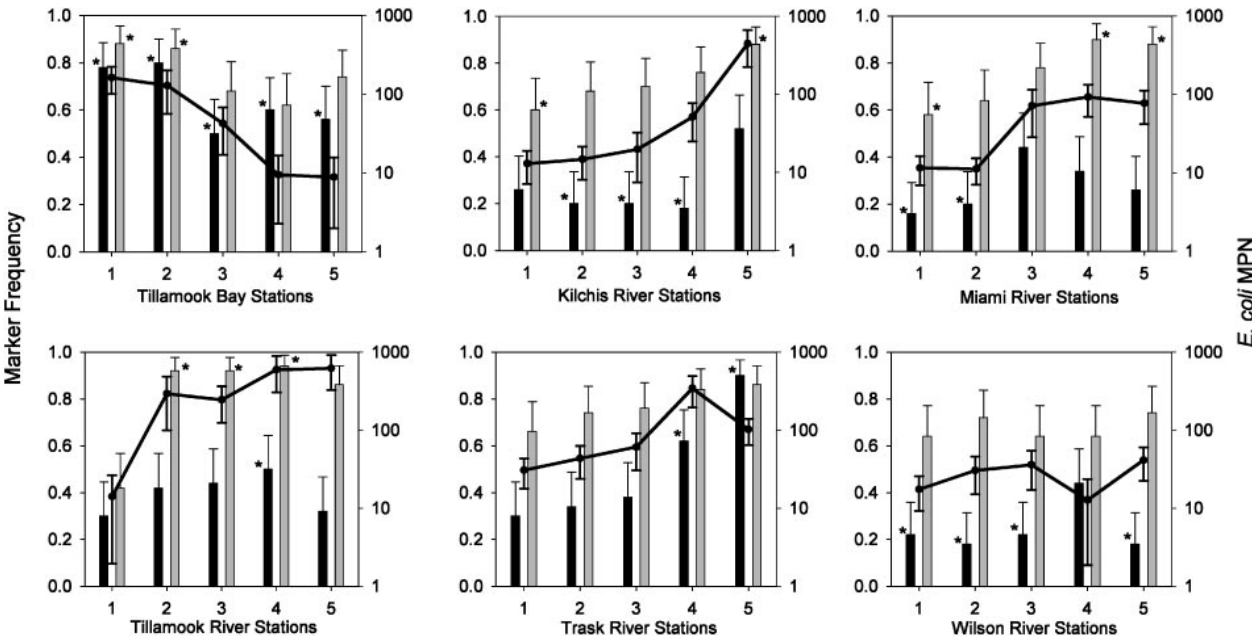

Figure 5. Geometric mean E. coli counts (lines) and ruminant (gray bars) and human (black bars) frequencies at sampling stations throughout the Tillamook Bay watershed (Shanks et al. 2006).

Results indicated widespread contamination from ruminants and, in certain river segments of the Trask, Miami, and Tillamook Rivers and Holden Creek, significant contamination from humans (Figure 6). Based on results from this monitoring and analysis effort DEQ can identify which stream reaches currently meet state bacteria criteria and which streams are still in violation. At these same locations, DEQ has determined whether bacteria concentrations are increasing or decreasing, and with information described in the MST study, contamination can be linked to human or ruminant (non-elk) sources. Knowledge gained though these efforts will help DEQ guide the direction of management in the Tillamook Bay Watershed to minimize the impact certain practices have on water quality as it relates to *E. coli* bacteria.

Shanks et al. (2006) includes detailed information on the study design, sampling techniques, laboratory analyses, QA/QC procedures and various statistical analyses of results.

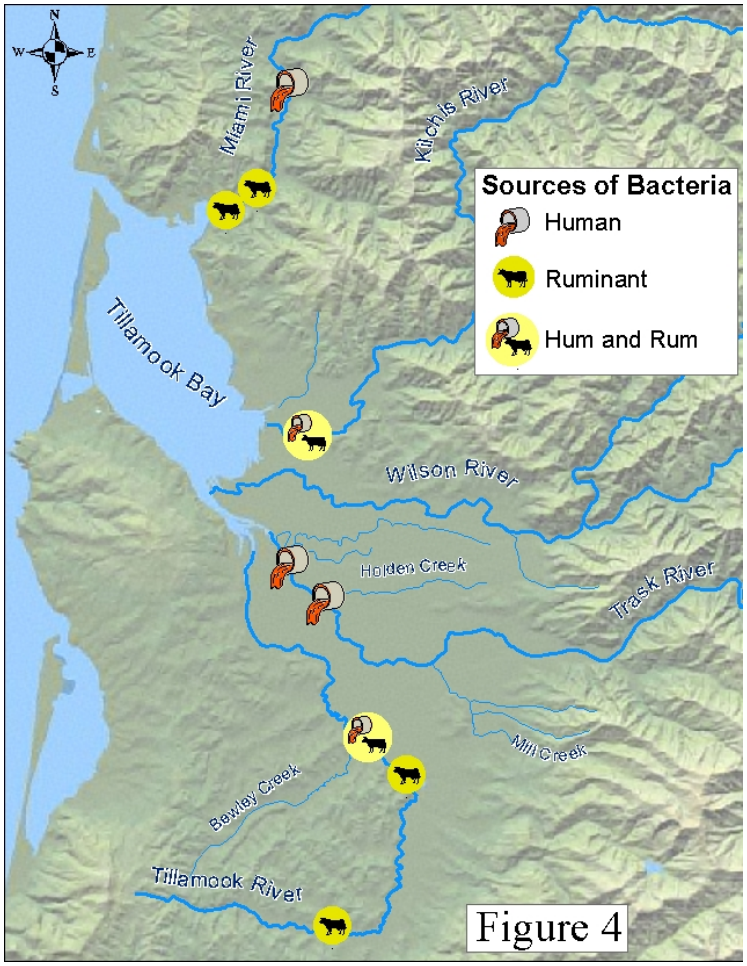

Figure 6. Distribution of identified sources in the Tillamook Bay watershed.

References

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Shanks, O.C., C. Nietch, M. Simonich, M. Younger, D. Reynolds, and K.G. Field. 2006. *Basin-Wide Analysis of the Dynamics of Fecal Contamination and Fecal Source Identification in Tillamook Bay, Oregon*. Applied and Environmental Microbiology, Aug. 2006, Vol. 72, No. 8, p. 5537–5546

Middle Rio Grande, New Mexico

The Middle Rio Grande (MRG) was included on New Mexico's 1998/2000 303(d) list for fecal coliform and a TMDL was developed to address the two impaired segments in 2002 (NMED 2002). Subsequently, the state changed their water quality criteria from including fecal coliform to *E. coli*. The New Mexico Environment Department (NMED) Surface Water Quality Bureau conducted monitoring in the Middle Rio Grande watershed in 2005 and results indicated the segment was impaired for *E. coli*. A TMDL was developed in 2010 to address the three impaired segments based on the new *E. coli* criteria (NMED 2010).

Year TMDL Developed:	2002
Applicable Water Quality Criteria:	Monthly geometric mean of fecal coliform not to exceed 200/100 mL No single sample to exceed 400/100 mL
Year of MST Study:	2002–2004
MST Method:	• Ribotyping • ARA
MST Indicator:	*E. coli*
TMDL Calculation Approach:	n/a (MST study conducted after TMDL)
Use in TMDL Context:	TMDL implementation plan development
Reference:	Parsons (2005)

After completion of the initial fecal coliform TMDL, an MST study was conducted, focusing on one of the two impaired segments addressed in the TMDL. The MST study was jointly funded by NMED, Albuquerque Metropolitan Arroyo Flood Control Authority (AMAFCA), and Bernalillo County and was designed to identify sources to support development of a TMDL implementation plan and more efficiently target resources toward effective BMPs.

MST Study

This project involved several steps:

- A sanitary survey of the watershed and a review of available data and literature to identify potential contributing sources of fecal bacteria to be considered.
- Development of watershed-specific libraries of ribotypes and antibiotic resistance profiles of *E. coli* isolated from fecal matter collected from known sources.
- Collection and culturing of a representative set of *E. coli* isolates from the waterbodies of concern under dry- and wet-weather conditions.
- Determination of the ribotypes and antibiotic resistance profiles of these waterborne *E. coli* isolates, followed by matching to those from the known source library to identify the sources of each *E. coli* isolate.
- Quantification of the accuracy and precision of the ribotyping and ARA source determinations.
- Estimation of the relative source contributions of *E. coli* in the MRG watersheds and the confidence of these estimates, based on the above measurements.

Sanitary Survey

The MST study began with a sanitary survey and reconnaissance tour of the watershed along with compilation of available data and literature to identify potential fecal bacteria sources in the watershed and support design of the subsequent MST sampling. Examples of existing data and literature reviews used to identify potential sources of fecal coliform included land use data; population and means of sewage disposal (e.g., sewer connection or on-site disposal); estimates of cat and dog populations using published per household data; and countywide livestock populations.

A reconnaissance tour, or sanitary survey, of the MRG watershed was performed in May and June 2002 to identify sources of fecal coliform that could potentially be missed by a review of available data and literature. The sanitary survey proved valuable as it provided greater understanding of the diversity of animal species, location, and condition of wastewater infrastructure, and hydrology (pollutant loading pathways) throughout the watershed. This step was important and influenced the sampling approach for collecting fecal samples for development of a local library of known isolates.

Library Development

Major potential sources were identified based on results of the watershed sanitary survey, review of existing data and information, and communications with stakeholders, and a plan was designed to develop libraries of *E. coli* isolated from fecal samples from these potential sources. The goal was to develop a local library of 1,000 *E. coli* isolates from approximately 500 samples with, on average, two *E. coli* collected from each known source sample. The sources sampled included sewage, wildlife, avian, pet, livestock, and exotic species from the Albuquerque Zoo. These locally collected isolates supplemented a library of more than 65,000 isolates ribotyped from hundreds of different species and sources, including many of the domestic and wild species found in the MRG watershed.

Water Sampling

Based on review of available water quality data, results of the sanitary survey and information contained in the 2002 TMDL, it was expected that contaminated runoff from land was an important source of fecal contamination. Therefore, it was important to evaluate land uses and potential fecal sources by individual subwatersheds contributing runoff to the MRG. The geography, complex hydrology, historical sampling stations, and an understanding of potential fecal sources as outlined in the 2002 TMDL influenced the sampling design of this MST study. NMED, AMAFCA, and Bernalillo County collaborated to recommend monitoring stations throughout the MRG watershed. After evaluating and prioritizing these recommendations, 30 sampling sites were identified, with sites on the MRG and a number of contributing subwatersheds with varying land uses and potential sources. The sampling design called for each location to be sampled on five dates (events). In addition, two “integrator” sites were to be sampled on 10 dates to provide more precise source contribution estimates. To identify fecal coliform sources associated with rain events, water samples were initially collected only during, or within 24 hours after, a rainfall event. Because drought conditions prevented obtaining the required number of samples, the monitoring plan was revised to include sample collection during February and March 2004 to quantify fecal coliform sources under dry-weather conditions. A total of 206 water samples were collected between July 2002 and July 2004 from the 30 sampling stations, with only 10 stations sampled during both dry-weather and storm

water runoff conditions due to a lack of rain. Parsons (2005) includes additional details on the sampling techniques, laboratory methods, and QA/QC procedures used in the study.

MST Analysis

Two MST methods were used for the MRG study—ribotyping and ARA—to quantify and compare the precision, accuracy, and specificity of the two methods. In addition to the usefulness of the direct head-to-head comparison, two methods were chosen to provide the additional following benefits to NMED and stakeholders:

- Application of two independent methods can validate the results, increasing stakeholder confidence in the outcome.
- Because any one method may not perform completely successfully in all samples of a given study, a second method provides back-up to ensure the study will generate useful results. For instance, if an *E. coli* ribotype from a water sample does not match a ribotype from a known source species, ARA may be able to at least indicate whether the source was wildlife, livestock, or human.
- Results may be more directly compared to other studies, including a 2002 ARA project conducted by the City of Albuquerque.

Ribotyping was performed on 1,620 isolates from the water samples, with the number of isolates by station ranging from 0 to 202. Overall, ribotyping results show the largest fraction of *E. coli* matched those found in avian sources, followed by canine, human/sewage, rodents, bovine, and equine (Figure 7). The source of approximately 9 percent of the *E. coli* could not be identified. The percentages shown in the figure measure the quantity of *E. coli* strains in the water at that particular station and time as percentage contribution of each species can vary with time and conditions. The larger the number of *E. coli* strains identified, the more confidence there is that the measured percent contribution is close to the actual percent contribution.

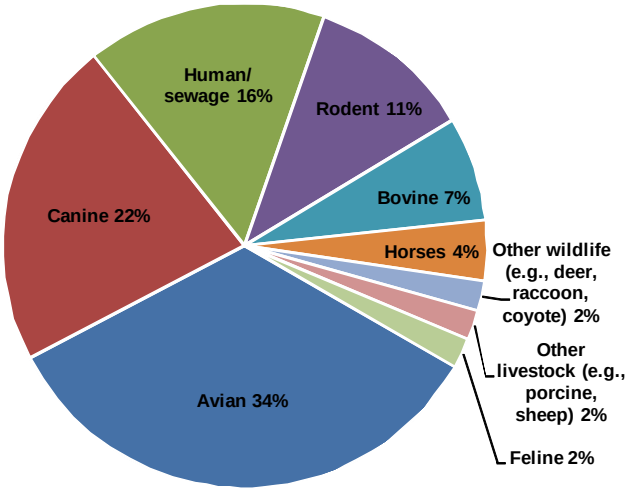

Figure 7. Sources of E. coli identified by ribotyping in the MRG study area, based on all sites and sample dates.

The study identified sources for each of the sampled stations to spatially evaluate sources of bacteria (Figure 8).

TMDL Implementation

The MST study report (Parsons 2005) discusses MST results for each of the 30 stations and provides recommendations on future studies and potential management actions for each as well as provides general recommendations for management of the major identified sources.

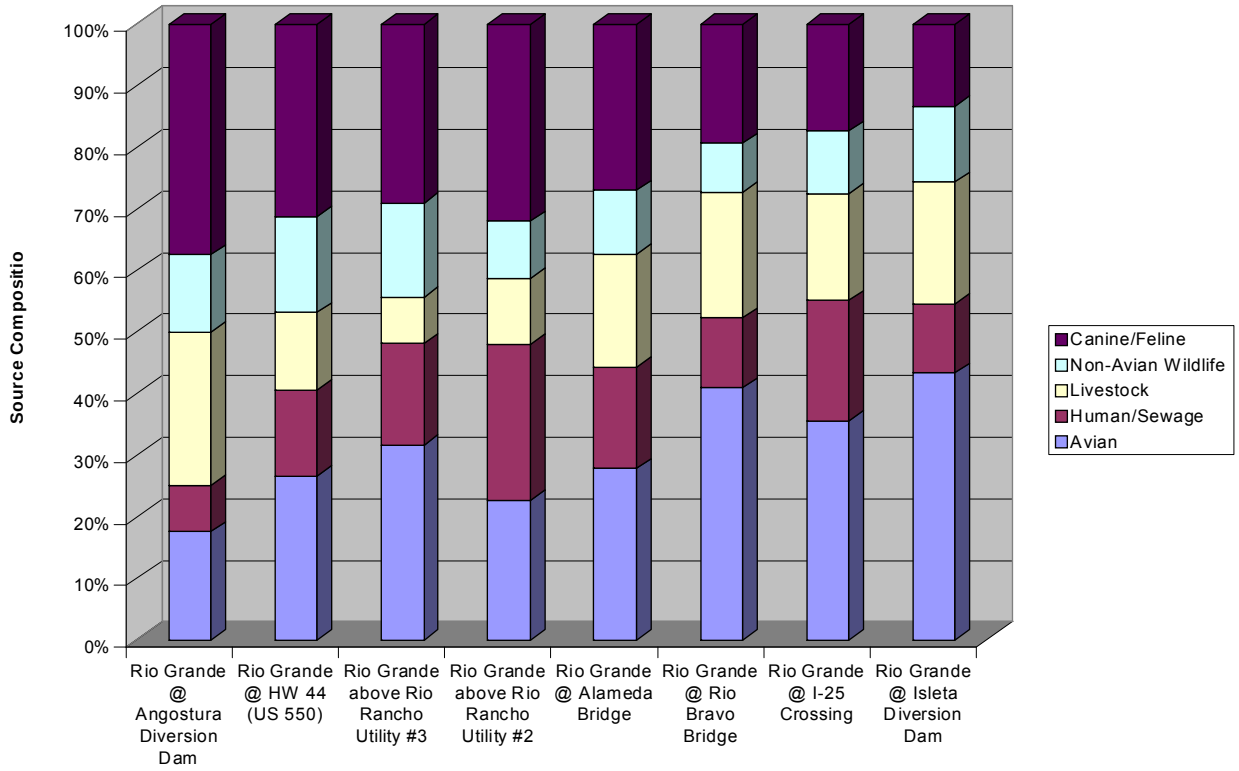

Figure 8. Source composition at select stations in MRG, from upstream to downstream.

Following completion of the MST study in 2004, Middle Rio Grande-Albuquerque Watershed Group of the Ciudad Soil and Water Conservation District (SWCD) developed a Watershed Restoration Action Strategy (WRAS). Development of the WRAS was the first step in formulating specific effective measures in support of the long-term environmental health of the watershed and the Middle Rio Grande MST Study (Parsons 2005) was a key reference in planning and writing the this strategy.

The Watershed Group chose to address the documented presence of fecal coliform as the focus of the watershed effort because fecal coliform is often used as an indicator of overall watershed health and many BMPs used to reduce the input of fecal coliform are also anticipated to result in the reduction of other pollutants that may enter the river. Detailed information on the proposed projects and implementation plans and schedules are included in the WRAS available online:
www.ciudadswcd.org/specialprojects/WRAS%20and%20Appendices%20December%2008a.final.pdf

References

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Parsons. 2005. *Middle Rio Grande Microbial Source Tracking Assessment Report*. Prepared for New Mexico Environment Department, Albuquerque Metropolitan Arroyo Flood Control Authority, and Bernalillo County. Prepared by Parsons Water & Infrastructure Inc., Austin, TX. www.nmenv.state.nm.us/swqb/Rio_Grande/Middle/MST/index.html

Hays Creek, Virginia

Virginia DEQ began using MST to identify fecal bacteria sources in the 1990s and has since implemented a statewide MST program to support TMDL development (Figure 9). The state has used different types of MST to identify sources of *E. coli* as well as the relative percentage contribution from source groups (i.e., livestock, wildlife, human and pets) to support the development of bacteria TMDLs throughout the state. The state has relied most heavily on ARA and PFGE, but has also used ribotyping, PCR, and the chemical method of detecting optical brighteners in detergents (fluorometry) (Hagedorn et al. 2009). MST results have been used to improve public awareness of the problem, improve model calibration/validation of bacteria densities, and provide a more equitable allocation of loads to source classes.

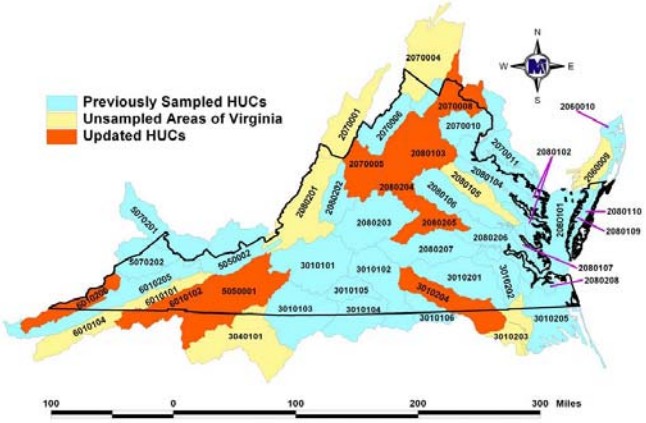

Figure 9. Virginia HUCs where MST has occurred, as of March 2009 (MapTech 2009).

A number of bacteria TMDLs have been developed by the state with supporting MST data. This summary for Hays Creek provides one examples of bacteria TMDLs and MST in Virginia.

The Hays Creek watershed, including tributaries Moffatts Creek, Walker Creek, and Otts Creek, is located in Augusta County and Rockbridge County. Hays Creek and Moffatts Creek were first listed on Virginia's 303(d) list as impaired for bacteria in 1998, and Otts Creek and Walker Creek were added in 2006.

There are two small point sources covered under general permits to discharge bacteria into the Hays Creek watershed. However, the majority of the bacteria load originates from nonpoint sources. The nonpoint sources of bacteria originate from livestock, wildlife, and humans. Significant bacteria loads come from cattle and wildlife directly depositing feces in the stream.

Year TMDL Developed:	2008
Applicable Water Quality Criteria:	• Calendar-month geometric mean of *E. coli* shall not exceed 126 cfu/100 mL • No single sample *E. coli* can exceed 235 cfu/100 mL
Year of MST Study:	2005–2006
MST Method:	ARA
MST Indicator:	*E. coli*
TMDL Calculation Approach:	HSPF
Use in TMDL Context:	• Source identification • Model calibration
Reference:	VADEQ (2008)

MST Study

As part of the TMDL effort, MST data were collected once a month at one station on Hays Creek from July 2005 to June 2006. The ARA method was used to analyze these samples. MST data reported in MapTech (2006) are shown in

Figure 10. Fluorometric analysis was also used to determine the concentration of optical brighteners. Optical brighteners are used in laundry and dishwasher detergent, as well as toilet paper. Their presence in high levels indicates the likely presence of human wastewater. The results for the fluorometric analysis were not included in MapTech (2006).

With the targeted sample size of 24 isolates in each sample, MapTech (2006) indicates a 90% confidence that the proportions measured in each sample are within 15% of the actual proportions in the sampled population (i.e., all bacteria in the stream at the time of sampling). Because a fixed-frequency sampling scheme was used, samples are not biased toward a particular flow regime; therefore it was assumed that combining the samples to estimate the proportions contributed by the different sources over the entire year provide greater precision (i.e., 90% confidence that the estimate is within 5% of the actual proportions) (MapTech 2006).

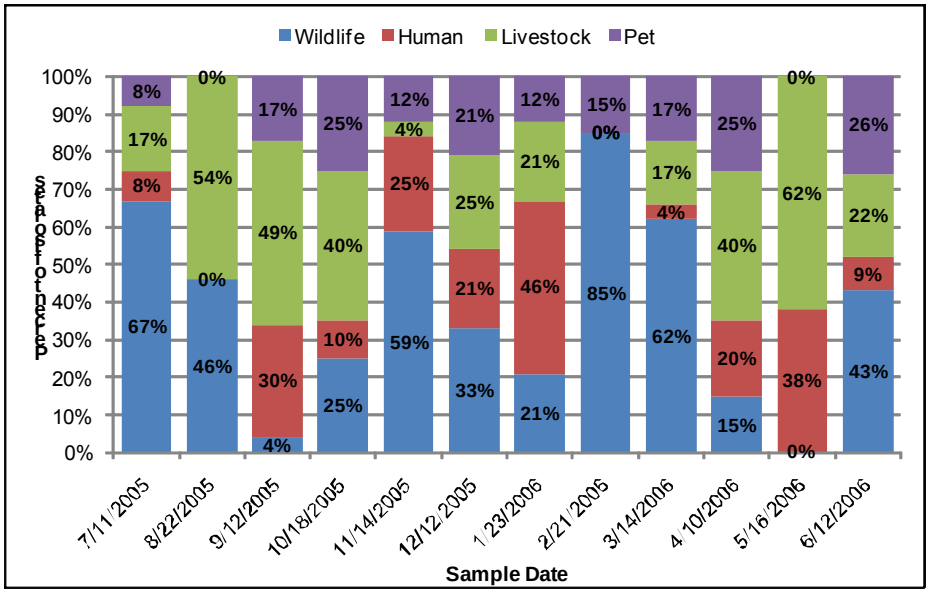

Figure 10. MST results for each sample date in Hays Creek.

TMDL Development

The Hydrological Simulation Program – FORTRAN (HSPF) was used to simulate the fate and transport of fecal coliform bacteria in the Hays Creek watershed. Contributions from various sources in the watershed were represented in HSPF to establish the existing conditions for a representative 6-year period that included both low- and high-flow conditions. There are two small permitted facilities allowed to discharge bacteria into Moffatts Creek. During future conditions, flow from these facilities was modeled at the facilities' design flows and bacteria concentrations were modeled at their permitted limits (126 cfu/100 mL). Model inputs for nonpoint source bacteria loadings were estimated and considered the following potential sources:

- Failing septic systems
- Straight pipes
- Pets
- Manure deposition from cattle in streams and on pastures

- Land application of liquid dairy and solid manure
- Poultry
- Sheep and goats
- Horses
- Wildlife

Estimations were based on information from VADEQ, Virginia Department of Conservation and Recreation, Virginia Department of Game and Inland Fisheries, Virginia Department of Agricultural and Consumer Services, Virginia Cooperative Extension, Natural Resources Conservation Service, Soil and Water Conservation Districts, public participation, watershed reconnaissance and monitoring, published information, and professional judgment.

MST data were also used to evaluate the model performance and calibration. MST results were used to identify the range of percent contributions from each of the four source groups during the sampling period of record. For comparison, model outputs from different bacteria sources were generated for the corresponding time period at the appropriate subwatershed outlet. The minimum and maximum observed and simulated values were compared to determine whether the model is producing output within the general expected range for the watershed based on MST results and known conditions.

The model was used to develop allocation scenarios by reducing different sources until in-stream simulated concentrations met applicable water quality criteria.

References

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Sand Dam Village Pond Town Beach, New Hampshire

Sand Dam Village Town Beach is a designated beach located in the town of Troy, New Hampshire, on the northeast corner of Sand Dam Village Pond. The drainage area of Sand Dam Village Pond is approximately 2,456 acres. The watershed is primarily forested with some residential land use and open land (i.e., ball fields) located in the vicinity of the pond.

Impairment of the beach by bacteria was determined using *E. coli* data collected by the New Hampshire Department of Environmental Services (NHDES) Public Beach Inspection Program. From 1991 – 2004 the beach was sampled one to four times per year. In 2005, more intensive monitoring was conducted in support of the TMDL.

Year TMDL Developed:	2006
Applicable Water Quality Criteria:	E. coli: Not more than a geometric mean based on at least 3 samples obtained over a 60-day period of 47 per 100 mL, or 88 per 100 mL in any one sample; unless naturally occurring.
Year of MST Study:	Summer 2005
MST Method:	Ribotyping
MST Indicator:	*E. coli*
TMDL Calculation Approach:	Concentration-based allocations equivalent to water quality criteria
Use in TMDL Context:	• Source identification • Identification of management activities
Reference:	NHDES (2006), Jones (2006)

E. coli concentrations from sampling data vary greatly under both wet and dry conditions with some samples being above and some below the criterion and the average wet-weather concentration slightly higher than the dry-weather (261 versus 202 cfu/100 ml). This suggests that restoring water quality in the Beach swimming area will require focus on both dry- and wet-weather sources of bacteria. Although violations occur during dry weather, data analysis showed that when precipitation exceeds approximately 0.25 inches of rain within the previous 24 hours, bacteria concentrations almost always exceed the single sample criterion of 88 cfu/100 ml.

MST Study

During the summer of 2005, University of New Hampshire conducted MST at three public beaches, including Sand Dam Village Pond Town Beach, to help determine the sources of bacteria using ribotyping.

Sampling occurred at two of the same stations that the NHDES Public Beach Inspection Program and TMDL Program had sampled. A total of six samples were collected for ribotyping although the 9/21/05 sample was not used due to the *E. coli* concentration being too low to effectively identify isolates. All of the samples were collected during dry weather, defined as days with less than 0.25 inches of rain in the previous 24 hours. Precipitation did occur in the previous 24 hours for the 8/30/05 sample but the amount was less than 0.25 inches.

Ribotyping was used to determine the source(s) of bacteria in a sample (i.e., human, waterfowl, pets, etc.) using *E. coli* isolated from the water samples. Two sources of known isolate patterns were used for this study for comparison purposes. One was the New Hampshire Regional Source Species database and the

second was a local source species library that was developed by collecting scat samples from known animals in the vicinity of the beach and then producing the ribopatterns for those animals. Since ribotyping involves a comparison analysis, a threshold similarity index is set to determine known isolates from unknown isolates. The use of the local source species ribopatterns for comparison turned out to be a very valuable asset in this study resulting in higher than average identification rate. For this study the similarity index target was set at 90% similarity, however, two isolates that matched at 89% were included in the known isolates.

Source Identification

Likely sources of bacteria identified by the ribotyping analysis are shown in Table 7 and Figure 11. As shown, ribotyping identified source species for 76% (19/25) of the *E. coli* isolates in the water samples. The remaining isolates (24%) could not be matched with certainty to patterns in the ribopattern database. Bacteria from four different species were identified at the Beach swimming area. Of the identified isolates, geese constituted the largest portion (52%) followed by livestock [sheep (12%) and cows (4%) for a total of 16%] and dogs (8%).

Table 7. Species Identified by Ribotyping in Samples from Sand Dam Village Pond Town Beach

Station	Date	E. coli (cfu/100 mL)	Total Isolates	Identified Isolates	Geese	Cow	Sheep	Dog
TROLF	7/21/2005	40	5	1		1		
	8/3/2005	36	5	5	3		2	
	8/30/2005	72	5	5	4		1	
TROCR	7/5/2005	68	5	3	2			1
	8/18/2005	420	5	5	4			1
Total			25	19	13	1	3	2

Ribotyping results indicate that the majority of the bacteria is from geese. These findings are supported by visual observations by NHDES field staff, noting goose droppings and sightings of numerous geese in the area. Livestock were not observed in the vicinity of the beach by field staff and are probably located further upstream in the watershed. It is also possible that the source of bacteria from sheep and cows might be from manure applied to agricultural fields or gardens in the watershed. Although no dogs were observed on the days of sampling, it is believed that dogs do frequent the beach and likely are present throughout the watershed.

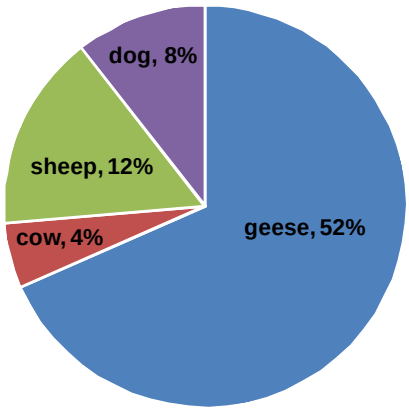

Figure 11. Distribution of isolates identified by ribotyping.

Based on water quality data analysis, field observations and MST results, NHDES ranked potential sources with their best estimate of which bacteria sources are most important relative to the amount of bacteria they likely contribute—“High”, “Medium” or “Low.” Bacteria originating from non-human sources (e.g., geese, dogs) and delivered through stormwater runoff

and direct deposition of fecal matter received the highest ranking as they are believed to be the major sources of bacteria to the Beach. Illicit connections and failed septic systems are not believed to be major sources of bacteria and were therefore given a low ranking. Direct deposition of bacteria from people recreating at the beach (i.e., swimming) was also give a low ranking as no people were observed swimming at the beach on the days that samples were taken for this study.

TMDL Development

The Sand Dam Village Town Beach bacteria TMDL is expressed as *E. coli* concentration (counts / 100 mL) equivalent to applicable water quality criteria. Two sets of allocations are provided—one for the single-sample criterion of 88 counts *E. coli* / 100 mL and one for the geometric mean criterion of 47 counts *E. coli* / 100 mL. WLAs and LAs are assigned to a variety of sources. The WLA for municipal separate storm sewer system (MS4) stormwater and LAs for non-MS4 stormwater, non-human direct discharges to surface waters, and people recreating in the water (i.e., swimming) are set equal to the water quality criterion, while LAs for unallowed discharges (illicit sewer connections, failed septic systems) and WLAs for nonexistent sources (WWTFs and CSOs) are set equal to zero. TMDLs are also presented as necessary reductions in concentration to meet applicable water quality criteria—85% single sample and 79% geometric mean.

The MST results were not used directly in the calculation of the TMDL, but rather to identify sources and support identification of management strategies.

Management Strategies

Based on watershed information and MST results, the TMDL identifies the following management strategies for implementing the TMDL and restoring water quality at the beach:

- *Waterfowl Management:* Goose droppings should be collected and disposed of away from the beach and in a manner that will prevent stormwater from coming in contact with them and transporting their bacteria to surface waters. The current method of raking the droppings to the corner of the beach and leaving them there does not appear to be effective. The Town should also investigate and implement methods to discourage geese (and other waterfowl) from frequenting the beach, pond and surrounding area.
- *Livestock Management:* Field reconnaissance conducted for this study did not identify any livestock in the immediate vicinity or just upstream of the beach although it's possible some could exist further upstream. It is recommended that the Town conduct investigations to determine the source of livestock (sheep and cow) bacteria. If livestock are found it is recommended that they be prevented from directly accessing surface waters tributary to Sand Dam Village Pond. In addition, manure deposited on the land should be properly managed to minimize contact with stormwater runoff and transport to the pond. Where feasible, vegetated buffers should be provided to help filter runoff and reduce bacteria loads before entering surface waters.
- *Pet Management:* While dog waste constituted only approximately 8% of the bacteria samples collected for the ribotyping analysis, it is a relatively simple source to reduce or eliminate. Therefore, it is recommended that the Town take steps to encourage people to clean up their dog's waste and to

dispose of it properly. There are a variety of products available for parks and beaches that dispense plastic bags to dog owners and provide a container for proper disposal of the waste. To help ensure compliance, the Town may want to adopt a "pooper scooper" ordinance and make it mandatory for people to clean up after their pets.

The TMDL also recommends that the Town conduct an illicit connection study in the watershed if water quality does not improve after implementing measures to address bacteria from geese, livestock and pets.

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Ecorse River, Michigan

Year TMDL Developed:	2008
Applicable Water Quality Criteria:	300 *E. coli* per 100 mL maximum concentration from May 1 to October 31 130 *E. coli* per 100 ml as a 30-day geometric mean concentration from May 1 to October 31 1,000 *E. coli* per 100 ml maximum concentration year-round
Year of MST Study:	2007
MST Method:	Bacterial qPCR
MST Indicator:	Bacteroidetes, Enterococcus
TMDL Calculation Approach:	Load duration curves; critical flow calculation
Use in TMDL Context:	Source identification
Reference:	MDEQ (2008)

The Ecorse River was originally placed on Michigan's 2008 303(d) list for *E. coli* in 1998. The source of the impairment was expected to be untreated sewage discharges. The Ecorse River watershed drains 43 mi^2 of Wayne County, and the watershed is home to approximately 160,000 people. There are two primary watercourses within the Ecorse River watershed. The North Branch of Ecorse Creek extends 17 miles in the northern portion of the watershed and the South Branch Ecorse Creek (also known as the Sexton-Kilfoil Drain) extends 13 miles draining the southern portion of the watershed. In addition to these two open watercourses, the LeBlanc Drain, an enclosed storm sewer, runs 9.6 miles and drains the central portion of the watershed.

An *E. coli* monitoring study was conducted in the watershed between May and October of 2007. Sampling occurred at 11 sites: four sites on the North Branch, three sites on the South Branch, three on the LeBlanc Drain and one on the mainstem of the Ecorse River near its outlet to the Detroit River. A total of 500 grab samples were collected in the watershed during the 23-week study with 7 of the 23 monitoring events during wet-weather conditions.

There were frequent, almost consistent, exceedences of water quality criteria on the Ecorse River during the 2007 study. Collectively, data from the North Branch and South Branches of Ecorse Creek exceeded Michigan's daily and 30-day criteria 92 percent and 100 percent of the time, respectively, and 73 percent of the daily geometric means were also greater than the partial body contact standard of 1,000 cfu/100mL. The LeBlanc Drain had higher *E. coli* concentrations than those found in the other branches of Ecorse Creek, with 95 percent of the samples above 1,000 cfu/100mL and 41 percent of the samples above 10,000 cfu/100mL.

Data were evaluated based on weather condition, showing that dry weather values were generally the same as the wet weather values at many sites. In most urban watersheds in southeast Michigan, dry weather values are much lower than wet weather values. Elevated concentrations in the Ecorse River during dry weather suggest the presence of potentially significant dry weather sources (e.g., illicit connections, failing septic systems, leaking sanitary sewer) upstream of these locations.

Load duration curves were also developed for each sampling location to assess under what stream flow conditions, ranging from low flows to peak flows, the daily target is most frequently exceeded (and by how much) and to help identify potential sources. The analysis showed that all sites commonly experience

water quality exceedences during low-flow (dry-weather) and high-flow (wet-weather) conditions, consistent with the results of the dry- and wet-weather analysis.

Preliminary Source Identification

The watershed is largely urbanized with potential sources of *E. coli* including illicit connections and discharges to storm sewers, domestic pets, wildlife, sanitary sewer overflows, storm sewer infiltration, sanitary sewer leaks, and failing on-site sewage disposal systems.

A combined sewer study of the Ecorse River was conducted by the Michigan Department of Natural Resources in 1980 and found fecal coliform counts as high as 380,000/100 mL in the North Branch, 280,000/100 mL in the Le Blanc Drain, and 690,000/100 mL in the South Branch. Combined sewer system outfalls have since been eliminated from the Ecorse River watershed and therefore should no longer be a source of *E. coli*.

Sanitary sewer overflow (SSO) events were fairly common in the Ecorse River prior to 2000, but the expansion of the Downriver Wastewater Treatment Facility in 2001 substantially reduced the number of SSO events. Since 2001 only one SSO was reported to Michigan DEQ, occurring in February 2005 and resulting in the discharge of 2.54 million gallons of partially treated sewage to the Ecorse River.

Illicit discharges were identified when the Wayne County Department of Environment conducted an illicit connection elimination project between 2002 and 2005 in partnership with nine Ecorse River watershed communities. The program, funded by a Clean Michigan Initiative grant, involved dye testing select businesses in the watershed for illicit connections and conducting an outfall survey on portions of the North and South Branches of Ecorse Creek for signs of illicit discharges.

The project resulted in the identification of 276 illicit connections and 4 illicit discharges from 79 facilities. Two-thirds of these illicit connections originated from a sanitary sewer that was mistakenly connected to the City of Southgate’s storm sewer. This sanitary sewer served 37 homes and received drainage from an estimated 37 toilets, 37 showers and 111 sinks. The remaining third of the illicit connections were comprised of connections from floor drains, interior trench drains, interior catch basins and sinks, process water lines, drinking fountains and a sump pump.

The illicit connections from 64 of the 79 facilities, including the ones carrying sanitary wastewater, were corrected by June 2005. The illicit connections at 11 additional facilities were subsequently corrected by February 2008. The corrections are pending at the remaining four facilities with illicit connections.

The county also identified several more areas in the watershed that showed signs of illicit discharges; however due to funding constraints, they were not able to locate the source(s) of these suspicious discharges and subsequently referred the problems to the local communities for follow-up investigations.

Although the number of failing on-site sewage disposal systems in the watershed is not known, it is suspected that there are few. Given the limited number of systems that are present, they are likely only a minor source of the *E. coli* problems in the watershed.

MST Study

A monitoring study was conducted in the Ecorse River between May and October of 2007 with the purpose of collecting *E. coli* and MST data to support TMDL development. The *E. coli* data were analyzed to determine compliance with the State of Michigan's water quality standards, and a subset of the *E. coli* samples were evaluated using MST to investigate sources of the *E. coli* detected throughout the watershed. The MST analyses were conducted using the Human Bacteroidetes ID™ Method and Human Enterococcus ID Method™, DNA-based methods that screen for the presence of specific genes in samples suspected of containing human fecal matter. MDEQ (2008) includes copies of the project QAPP and laboratory QAPP.

Generally, each site was sampled once during dry conditions and once during wet conditions. A "positive" result for either test indicates the presence of *E. coli* from human source(s) at a given monitoring site. Since only a limited amount of MST testing was performed, a "negative" result at any given site does not mean that human contamination is not present at that site, only that it was not present in that particular sample.

During dry conditions, the human biomarker was present at all sites on the North and South Branches and on LeBlanc Drain, except one site (Figure 12). To confirm the negative result initially found at EC7, the site was sampled two more times for MST analysis. The results were always negative for the human biomarker, giving a strong indication that *E. coli* from human sources was not impacting this site during dry conditions. Positive results for the other sites suggest that there are dry-weather sources of *E. coli* of human origin.

During wet conditions, fewer positive results were found in the watershed (Figure 13). Based on experience in other watersheds and the positive indicator during dry weather, it is likely that positive results would have been found at all sites if repeated sampling could have been performed during wet conditions.

These human sources of *E. coli* could include cross-connections between the sanitary and storm sewer systems, illicit discharges to storm sewers, failed on-site sewage disposal systems, and leaking sanitary sewers.

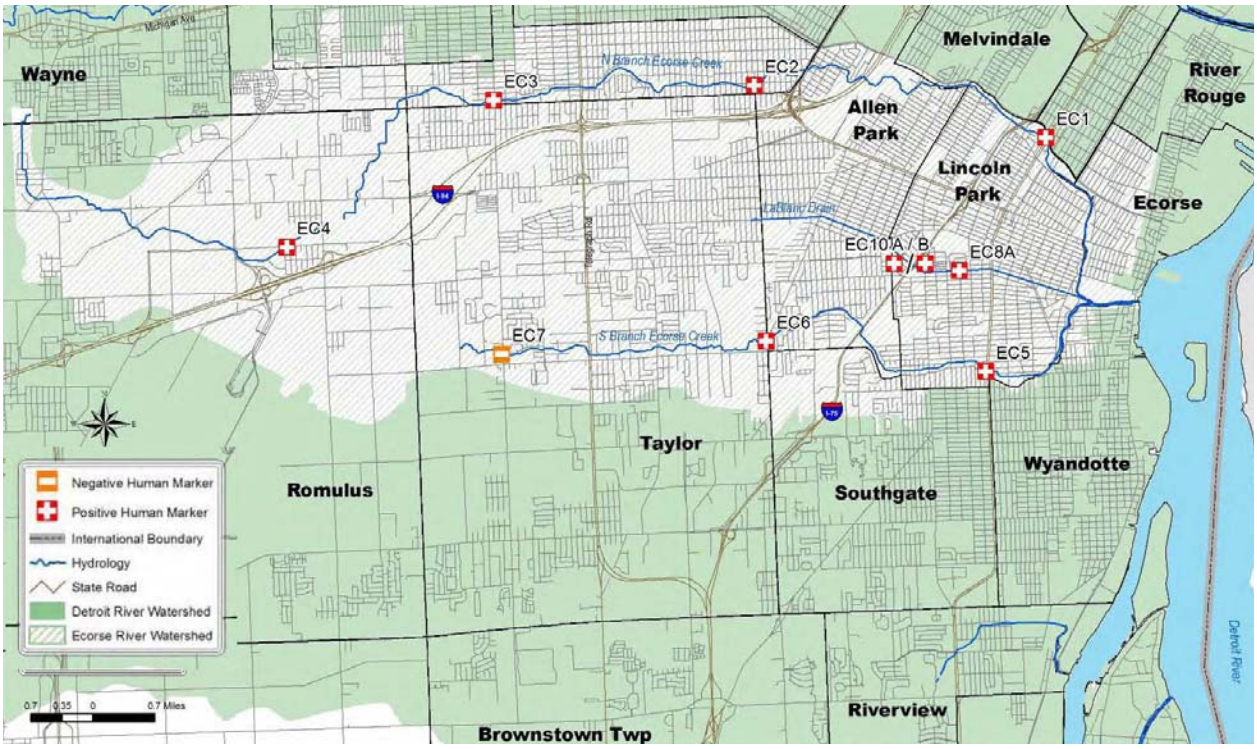

Figure 12. MST results for dry-weather samples in Ecorse River.

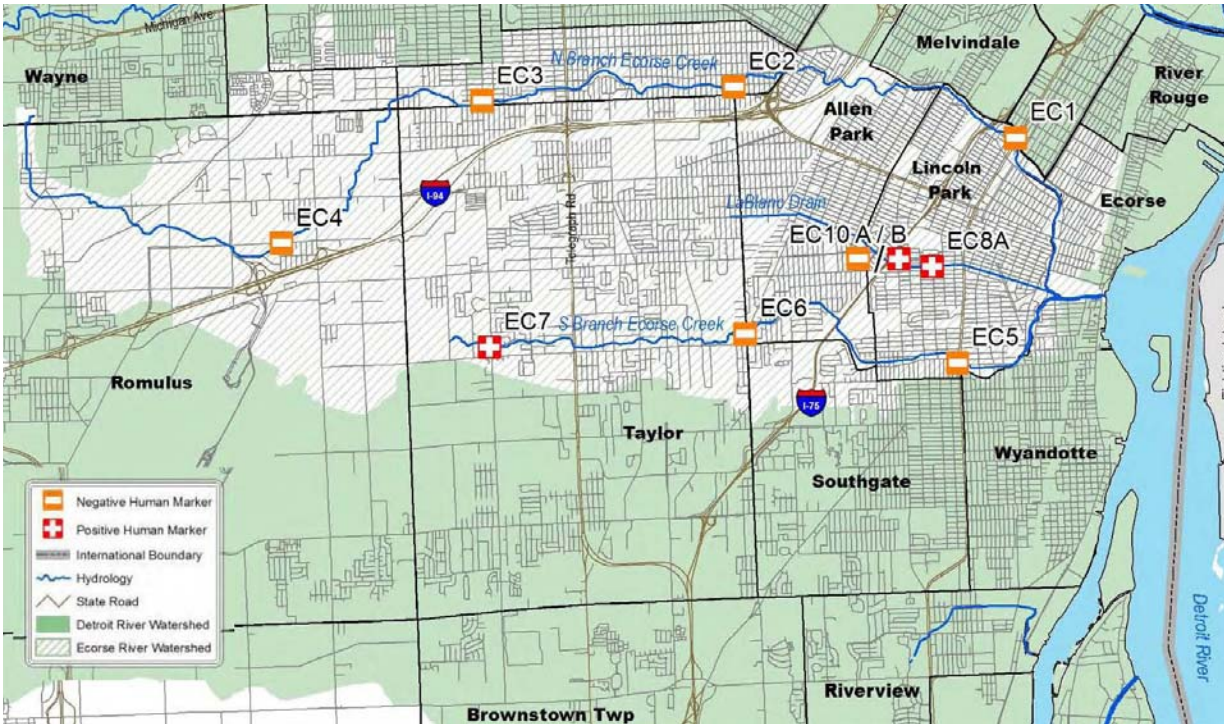

Figure 13. MST results for wet-weather samples in Ecorse River.

TMDL Development

The TMDL was calculated using the water quality criterion for a maximum *E. coli* concentration (300/100 mL) and five critical values from the flow duration curve. Loading capacities were calculated for the 5%, 25%, 50%, 75%, and 95% exceedance flows to represent high, moist, midrange, dry and low flow zones, respectively.

The loading capacity was divided among a margin of safety, a WLA for general industrial storm water permits and individual permits authorizing storm water and a WLA for general MS4 storm water permits. No LA was included because the entire watershed falls within the jurisdiction of municipal or industrial NPDES permits.

The WLA for industrial storm water permits was assigned based on the anticipated *E. coli* loading from storm water runoff associated with the industrial areas under various flow conditions. The Long Term Hydrologic Impact Analysis (LTHIA) web application developed by Purdue University was used to approximate *E. coli* loadings associated with industrial storm water runoff. LTHIA is a curve number-based model that uses land use and hydrologic soil group data to predict long term runoff and non-point source pollution from watersheds. The WLA for general MS4 storm water permits was calculated as the remainder of the loading capacity after allocation to the industrial permit and minus the margin of safety.

While the MST results were not used directly in the calculation of the TMDL, they supported identification of potential sources to support future management decisions.

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Beaver Creek, South Dakota

Beaver Creek was first listed on South Dakota's 2006 303(d) list as impaired for fecal coliform due to sample concentrations that exceeded the daily maximum criterion for the protection of the limited contact recreation use. South Dakota Department of Environment and Natural Resources (SDDENR) developed a TMDL in January 2010 to address the bacteria impairment.

Year TMDL Developed:	2010
Applicable Water Quality Criteria:	Fecal coliform: • Daily maximum of ≤ 2,000 CFU/100mL • Geometric mean of at least 5 samples over a 30 day period ≤ 1,000 CFU/100mL • (These criteria apply from May through September)
Year of MST Study:	2003–2005
MST Method:	PFGE
MST Indicator:	*E. coli*
TMDL Calculation Approach:	Load duration curve (with HSPF modeling to estimate source loads and evaluate management scenarios)
Use in TMDL Context:	• Source identification • Development of management strategies
Reference:	SDDENR (2010)

The watershed is located in southwestern South Dakota and drains 1,670 square miles, including parts of Pennington, Custer and Fall River Counties. Approximately 71% of the watershed is in Wyoming, draining much of the eastern portion of Weston County. The impaired segment of Beaver Creek is approximately 25 km long and begins at the Wyoming / South Dakota border and ends at the mouth of stream. Watershed land use is predominantly herbaceous rangeland (56%) and forest (38%) with a small amount of cropland (5%).

MST Study

An MST study was undertaken to identify sources contributing to the bacteria impairment. Samples were analyzed with PFGE. SDDENR sampled one site on the creek monthly and during rain events from September 2003 to August 2005 as part of the TMDL assessment project for the Upper Cheyenne River watershed. From each sample that contained at least 50 cfu/100ml, laboratory staff attempted to isolate five *E. coli* bacteria to test using the PFGE technique. A total of 23 *E. coli* isolates were successfully cultured from Beaver Creek samples. DNA from these isolates was compared to a reference database of known-DNA isolates from other samples collected in western South Dakota (Ecoregion 43). Of the 23 isolates that were tested, approximately 4% were unidentifiable. Among the isolates for which the source could be identified, 26% were equine (horse) and 30% were ovine (sheep). Other identified animal sources include porcine (pig), bovine (cow), canine (dog), feline (cat) and human (Figure 14).

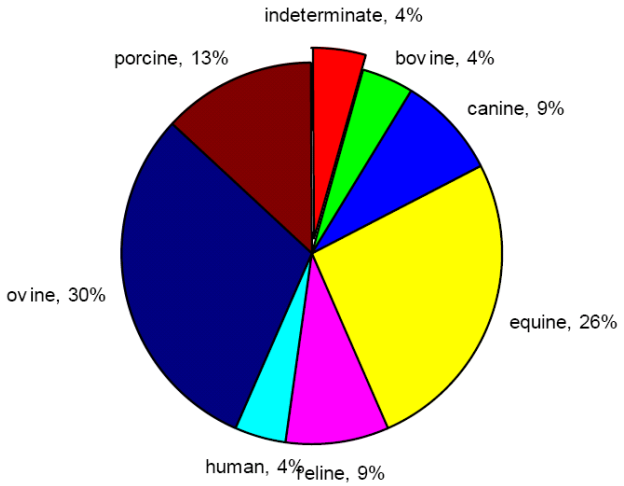

Figure 14. Relative percent of E. coli sources as determined from 23 tested isolates.

In developing the TMDL, SDDENR noted several cautions for interpreting the source tracking results. The small number of isolates successfully identified allows a high margin of error when identifying sources of E. coli. The average rate of correct classification of DNA when using the Ecoregion 43 library varies from about 55% (horses and human) to 90% (feline and canine). Also, when compared with the statewide DNA database, sources are identified much differently, with 39% beef cow, 17% sheep, 13% dog, 9% indeterminate, 9% cat, 9% horse and 4% human. These discrepancies suggest that source tracking technology is not perfected, and that results should not be taken as absolute. Increasing the size of the database would improve the average rate of correct classifications and reduce the number of indeterminate-source classifications. Increasing the number of bacteriological samples collected at Beaver Creek would increase the accuracy of source tracking results, and sampling multiple locations on Beaver Creek would help define spatial distribution of bacteriological contamination.

Source Identification

MST results were used to better identify potential bacteria sources in the watershed.

No permitted point source dischargers are located in the South Dakota portion of the Beaver Creek watershed. One permitted wastewater treatment facility (NPDES ID WY0020605) is located in Upton, Wyoming in Weston County. This facility has fecal coliform bacteria permit limits for one outfall. Because this discharge is more than 50 stream miles upstream of the Wyoming-South Dakota border, the associated bacteria load likely does not reach the impaired segment of Beaver Creek in South Dakota.

Based on review of available information and communication with state and local authorities, the primary nonpoint sources of fecal coliform within the Beaver Creek watershed include agricultural runoff, as well as wildlife and human sources. Using the best available information, loadings were estimated from each of these sources using the EPA's Bacterial Indicator Tool based on the density and distribution of animals (livestock and wildlife) and failing septic systems in the watershed.

Human fecal coliform bacteria were identified from MST tests. The Beaver Creek watershed is largely rural, with few centralized wastewater collection and treatment facilities. Thus, septic systems are assumed to be the primary human source of bacteria loads to Beaver Creek. Densities of septic systems in the watershed were derived from the 1990 U.S. Census septic data and the 2004 U.S. Census population data.

The HSPF model was used to determine the contribution of fecal coliform bacteria from identified sources in the Beaver Creek watershed and evaluate the implementation of BMPs to control these sources.

TMDL Development

The TMDL was developed using the load duration curve approach, resulting in a flow variable target that considers the entire flow regime within the recreational season (May 1 – September 30). The WLA is assigned a zero value, as no point sources of fecal coliform bacteria discharge into the impaired segment of Beaver Creek. The overall LA was determined by subtracting the WLA and Margin of Safety from the load duration curve. The load allocation was further divided to assign a portion of the load allocation to

South Dakota and Wyoming based on the proportion of the model-predicted current total load contributed by each state. Using the HSPF model, the contributions from Wyoming were estimated by simulating the removal of all loadings from the South Dakota portion of the watershed, and vice versa. Then, the estimated daily load contributions for each state were summed by flow zone, and the percent contribution from each state was used to determine flow zone load allocations. The LA was assigned as a gross allotment to all nonpoint sources in the watershed.

Management Strategies

Based on water quality monitoring, MST and HSPF model results, the recommended control measures to be implemented in South Dakota are expected to achieve the required load reductions and attain the TMDL goal. Four management scenarios were simulated using the HSPF model to evaluate management of sources identified through MST and other watershed information. Scenarios included: 1) reduced Wyoming bacteria loads to comply with South Dakota water quality criteria, 2) removal of septic system bacteria loads, 3) exclusion of cattle from streams, and 4) general rangeland management. Modeling indicated that implementation of scenarios 3 and 4 are expected to achieve the TMDL goal.

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