DEPARTMENT OF
ECOLOGY
State of Washington

Review and Critique of Current Microbial Source Tracking (MST) Techniques

November 2011
Publication No. 11-03-038

Publication and Contact Information

This report is available on the Department of Ecology's website at
www.ecy.wa.gov/biblio/1103038.html

The Activity Tracker Code for this study is 11-064.

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Review and Critique of Current Microbial Source Tracking (MST) Techniques

by

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Table of Contents

Abstract

Microbial source tracking (MST), also referred to as bacterial or fecal source tracking, is a method used to determine the sources of fecal indicator bacteria in the environment. MST techniques attempt to determine whether fecal bacteria are being introduced into waterbodies through human, wildlife, or domestic animal sources.

The purpose of this document is to describe some of the common MST techniques and to provide a discussion of the pros and cons of the techniques. This document also provides a discussion of quality assurance issues and the future direction for MST quality assurance.

Information in this document will be used by natural resource managers, decision-makers, Washington State Department of Ecology staff, regulators, and stakeholders.

Acknowledgements

The authors of this report thank the following people for their contributions to this study:

- Dr. Valerie J. Harwood, University of South Florida, who offered many valuable comments. Her review is gratefully acknowledged.
- Dr. Stephanie Harris, U.S. Environmental Protection Agency, U.S. Public Health Service.
- Washington State Department of Ecology staff:
  - Document reviewers: Nuri Mathieu, Ralph Svrjcek, Sally Lawrence, Dave Garland, Dale Norton, Will Kendra, Bob Cusimano, Rob Duff, George Onwumere, Gary Arnold, and Melissa Gildersleeve.
  - Joan LeTourneau, Jean Maust, and Cindy Cook for editing assistance.
  - Elisa Sparkman for assistance in preparing the references and bibliography.

Executive Summary

The purpose of this document is to provide a review and critique of current microbial source tracking (MST) techniques. This should enable natural resource managers to make informed decisions about the use of MST techniques and their results.

The review and critique resulted in the following key findings for MST techniques:

- MST techniques are experimental science. Users of these techniques need to demonstrate that these techniques produce acceptable levels of accuracy and reproducibility to meet project goals. At this time no MST technique is capable of determining all possible fecal sources accurately. A weight-of-evidence approach is necessary to link fecal pollution to its source.

- Currently there are no standardized and promulgated methods for molecular and biochemical MST techniques. Promulgated regulatory methods for MST are needed, and a certification program for MST laboratories is needed.

- Standard operating procedures (SOPs) for MST techniques are lacking. This situation has contributed to an MST literature that is highly technical, confusing, and full of ambiguous results. For any MST testing to be used in a regulatory context, formal SOPs conforming to U.S. Environmental Protection Agency (EPA) guidance must be prepared and available for ruggedness testing and evaluation.

- Due to the experimental nature and cost of many MST techniques, these methods should be used as a last resort to determine sources of fecal bacterial contamination. Conventional sampling techniques to identify fecal sources should be used first. Conventional techniques include bacteria source tracking methods, such as targeted instream monitoring for bacteria and sanitary and watershed surveys for fecal sources.

- Because the quality assurance (QA) status of these experimental techniques is often problematic, real-world use of the molecular and biochemical MST techniques must include the following QA sample types:

    - Field duplicates (50% of field samples duplicated to develop reproducibility information). 50% of samples.
    - Blind field positive controls (fecal material of all potential source organisms). A minimum of one per each potential source per study.
    - Blind field negative controls (field blanks). 20% of samples.

    Studies should include a sufficient number of QA samples to allow full evaluation of data usability. QA criteria for acceptability of results are not advanced at this time.

- Due to concerns about the experimental nature of MST techniques, we recommend use of multiple techniques for the same source to increase confidence in identification. This also allows for more potential source types to be investigated.

- For molecular and biochemical MST techniques, library-independent methods are recommended over library-dependent methods. Library-dependent methods are not recommended due to the expense, difficulty in building a sufficiently robust library, and their poor performance in MST comparison studies.

- Before conducting an MST study, source feces from the study area should be tested to confirm the source-specific MST indicator or marker is present. Source-specific markers can vary over time and depend on genetics, location, diet, and environment.

- Chemical source tracking methods show promise but need to be evaluated against performance criteria before these methods are adopted at a regional scale.

- Less expensive source tracking techniques such as *in-situ* monitoring for optical brighteners should be considered for supporting evidence for the presence of human sewage.

Introduction

In the Washington State Water Quality Standards, *fecal coliform bacteria* is used as an indicator of fecal contamination. In 2008 there were over 400 waterbodies in Washington that did not meet designated use criteria due to fecal coliform levels that exceed (do not meet) water quality standards. In recent years, nonpoint sources of bacterial pollution have surpassed point sources as the major source of fecal contamination to surface water (EPA, 2005). Identifying bacterial sources is an important first step in controlling fecal contamination and managing microbial risks.

Identifying nonpoint sources of bacterial pollution is generally not easy. In the past several years, numerous experimental techniques to determine the source(s) of bacterial pollution have been proposed. The goal of these experimental techniques is to identify sources contributing to fecal contamination in the environment to help regulators and stakeholders prevent, control, or remediate fecal pollution. These techniques are referred to as microbial source tracking (MST), also referred to as bacterial or fecal source tracking.

The purpose of this document is to provide natural resource managers the information they need to make informed decisions about the use of MST techniques and MST results.

History of MST Techniques

In the 1960s early attempts to track bacterial sources included the use of fecal coliform-to-fecal streptococci ratios (EPA, 2005). While this approach is no longer considered informative due to widely varying survival rates of the bacterial groups in the environment, scientists were encouraged to develop and evaluate new tools to discriminate among different sources of bacterial pollution.

The Washington State Department of Ecology's (Ecology's) first review of MST techniques was published in 1999 (Sargeant). Since then the MST field has continued to develop, new methods have been proposed, and other methods have proven to be less useful than originally thought.

The MST field is still an emerging body of science; recently published techniques have their own sets of strengths and weaknesses. A variety of MST techniques are actively being used in field studies, and new techniques are under development. Although this field is mainly experimental at this phase of its development, there is strong pressure on natural resource managers to use these techniques to identify bacterial sources of pollution.

In retrospect, many proposed MST techniques have failed to fulfill the early promise of initial testing after further validation, usually because of issues with specificity (defined as the frequency of true-negative results). Additionally, field validation and blind studies using the library-based techniques illustrate that the development and maintenance of libraries is expensive and may not be useful across geographic or temporal ranges.

Literature Sources for MST Techniques

There are excellent literature sources that provide an overview of MST principles and techniques including several EPA documents (EPA, 2005 and 2010).

In addition, leading researchers in the field have recently published documents that include an overview of MST techniques and applications:

- *Microbial Source Tracking: Methods, Applications, and Case Studies*. Published in 2011, edited by Charles Hagedorn, Anicet R. Branch, and Valerie J. Harwood. (Hagedorn et al., 2011)
- *Microbial Source Tracking: Current and Future Molecular Tools in Microbial Water Quality Forensics.* By Jorge W. Santo Domingo, Regina Lamendella, and Nicholas J. Ashbolt. Chapter 10 *in* Environmental Microbiology: Current Technology and Water Applications, editors Keya Sen and Micholas J. Ashbolt. Published in 2011 by Caister Academic Press, Norfolk, U.K. (Santo Domingo et al., 2011)
- *Final Report: Validation of Rapid Methods for Enumeration of Markers for Human Sewage Contamination in Recreational Waters*. Water Environment Research Foundation, 2011, by Valerie J. Harwood, Kristin V. Gordon and Christopher Staley. (Harwood et al., 2011)
- *Identification of primary sources of faecal pollution.* By J.W. Santo Domingo and T.A. Edge. Chapter 5 *in* Safe Management of Shellfish and Harvest Waters, editors G. Rees, K. Pond, D. Kay, J. Bartram, and J. Santo Domingo. Published in 2010. (Santo Domingo and Edge, 2010).

Critique and Recommendations for MST Techniques

This document provides an overview and critique of some of the common MST techniques being used. Performance of MST methods is discussed including sensitivity (how frequently a method detects a source when it is present) and specificity (the ability to rule out a source when it is absent). Poor sensitivity tends to lead to false-negative results (failure to detect the source when present), while poor specificity tends to lead to false-positive results (detection of the source when it is not present). MST techniques are also discussed in relationship to standard laboratory techniques and scientifically acceptable data.

This document provides recommendations on techniques that are promising and quality assurance (QA) measures that are essential when using MST to determine the validity of MST results. Presently there is no single MST technique that is preferred by a federal regulatory agency, and no single technique is capable of determining all possible fecal sources accurately.

Results from MST techniques can provide important insight into the sources of bacterial pollution. MST can provide another piece of evidence, along with traditional bacterial source tracking techniques, to assist natural resource managers in achieving control of bacterial sources. However, it is critical that natural resource managers using these techniques have a good understanding of the limitations of MST as well as the steps that should be taken to ensure defensible results. To provide valid results, an appropriate MST technique must be chosen, the study must be carefully designed, and verification of results in the form of adequate quality control (QC) sampling is needed.

Types of MST Techniques

MST techniques can be divided into two categories:

1. *Molecular (genotypic) and biochemical (phenotypic) techniques* rely on the close association of certain microorganisms (generally bacteria or viruses), with a specific host, and genetic or phenotypic differences that allow host-specific microbes to be discriminated from others.
2. *Chemical methods* generally rely on the detection of chemicals associated with anthropogenic activities.

Molecular and Biochemical Techniques

Molecular and biochemical MST techniques can be divided into two broad categories: library-dependent and library-independent.

- Library-dependent techniques identify fecal sources from water samples based on a library or database of bacteria isolated from known fecal sources. The library is developed by collecting microbial isolates from known potential sources. The molecular or biochemical pattern of the individual microbial isolates is sometimes referred to as a *fingerprint*. These identifying patterns can be discerned by a variety of methods, e.g., ribotyping (molecular) and antibiotic resistance analysis (biochemical).
    - Molecular (genotypic) techniques are based on the genetic makeup of a cell or organism, e.g., ribotyping, pulsed field gel electrophoresis, rep- polymerase chain reaction (PCR).
    - Biochemical (phenotypic) techniques use observable characteristics or traits of an organism such as biochemical or physiological properties, e.g., antibiotic resistance analysis, carbon source utilization.
- Library-independent techniques do not require the development of a source library database. These techniques rely on a species-specific genotype or characteristic.

A variety of bacteria and viruses have been used for MST. It is important to consider the MST indicator survival rate and abundance in the environment. Survival of microbial indicators depends on a variety of factors including their physiology, exposure to radiation, temperature, salinity, predation and competition, amount of organic matter present, and the type of sediments available (Harwood, 2011). Some indicators more closely correlate with fecal indicator bacteria (e.g., *E. coli* and fecal coliforms).

MST 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 (EPA, 2011). At best some methods like quantitative polymerase chain reaction (qPCR) can provide a gross quantitative estimate of source contribution for the sample time period.

Library-Dependent Techniques

A library is a database of fingerprints from individual bacterial isolates, obtained from potential fecal pollution sources. Bacteria for a source library are normally recovered from animal feces, though bacteria from animal waste lagoons, septic tanks, and wastewater treatment plants can be used.

Most library-dependent techniques require a cultivation step to obtain the bacterial isolates that will be used to generate the library (knowns) and the water bacterial isolates (unknowns) that will be compared against the library.

Library-dependent techniques rely on either molecular or biochemical discrimination of isolates. A number of different microbes may be used for library-dependent techniques including fecal coliforms, *Escherichia coli (E. coli)*, *Enterococcus* species (enterococci), or fecal streptococci (essentially enterococci, but includes several additional *Enterococcus* species).

Recently the trend in MST research has been to move away from library-based techniques. This is due in part to their performance in the Southern California Coastal Water Research Project comparison study (SCCWRP) (SCCWRP, 2003; Hagedorn et al., 2011). In addition, the need to develop large site-specific libraries has decreased the interest in using library-dependent approaches (Santo Domingo et al., 2011).

Below is a description of some common library-based techniques, both molecular and biochemical.

Molecular (Genotypic)

Molecular techniques are based directly on the genetic material of the bacterial or viral organism. Bacteria used for these techniques are usually *E. coli* or *Enterococcus* spp. The theory behind these MST techniques is that unique strains of a bacteria species are adapted to their known specific environment (intestines of a particular host species) and, as a result, differ genetically from other strains found in other host species.

A number of genotypic methods are used to type bacteria for library-dependent techniques including ribotyping, pulse field gel electrophoresis (PFGE), and repetitive (rep) polymerase chain reaction (PCR). These techniques are described below. It is important to note that (1) the genotypic techniques described below differ in discriminatory ability, and (2) bacterial isolates that are grouped into the same "strain" by one method may be separated into distinct strains by a more discriminatory method. The *Bibliography* section of this report includes studies that have used these techniques.

Ribotyping

Ribotyping has been one of the most widely used techniques in library-dependent MST applications (EPA, 2011). Ribotyping is based on the detection of genetic differences in the genomic sequences within or flanking the 16S and 23S ribosomal ribonucleic acid (rRNA) genes. These rRNA genes are highly conserved in bacteria (EPA, 2005).

For this method, the chosen bacterial group is cultured from fecal samples using standard techniques. *E. coli* or enterococci are isolated, and a few isolates are picked for genotypic characterization (generally a percentage of the bacterial count). Genomic DNA is isolated for each *E. coli* strain. Bacterial DNA is digested into fragments using restriction enzymes. DNA fragments are separated by size using gel electrophoresis. The fragments are transferred to a gel blot, and a labeled probe is used to hybridize to certain portions of the rRNA genes. Because the genome contains several copies of the rRNA genes dispersed throughout the chromosome, the binding of the probe to the DNA fragments which contain it creates a banding pattern that can be visualized by autoradiography or chemical development. These patterns can be used to discriminate among bacterial strains.

The banding pattern is captured using digital cameras. Difference in the size and location of the banding patterns can then be compared to known sources in the library database. Image analysis to compare banding patterns can be performed using commercially available software (Scott et al., 2002; Rees et al., 2010).

Variables in ribotyping include the type of fecal indicator bacteria used to form the library, as well as the type and number of restriction enzymes used to fragment the DNA. It has been suggested that two restriction enzymes should be routinely used to increase the technique's discriminatory ability (EPA, 2005).

Advantages

This method can be used to classify isolates from multiple sources. When performed by a skilled technician, it is highly reproducible.

Disadvantages

Ribotyping is a demanding procedure that requires multiple steps and specialized equipment. Also, the need for specialized training, high supply costs, and the time required to complete the procedure are disadvantages.

As with many genotypic techniques, lab-to-lab variation, issues of repeatability, gel variability, and analysis techniques often make comparison of results from different laboratories difficult.

Complex statistical analysis is often required to determine which sources are likely present. A good working knowledge of statistics is needed.

The database (library) size, geographic distribution of isolated bacteria, and the presence of replicate isolates in the bacterial source library affect the ability of ribotyping to differentiate among bacteria at the host-species level (EPA, 2005). In addition, both genotypic and phenotypic techniques would likely break down in complex watersheds with numerous sources (Rees et al., 2010; Harwood, 2011).

Pulsed Field Gel Electrophoresis (PFGE)

One of the most common techniques, PFGE, is similar to ribotyping. The difference is the whole DNA genome is used instead of the rRNA portion of the genome. Initial steps for obtaining bacterial isolates are the same as ribotyping.

PFGE uses infrequently cutting restriction enzymes on the entire DNA genome. The procedure for DNA isolation is crucial, as large genomic fragments are generated which must not be broken during sample preparation. The genomic fragments are then separated by alternately pulsed, perpendicularly oriented electrical fields, instead of using standard gel electrophoresis. After electrophoresis and staining of the gels, a banding pattern emerges. Patterns are compared to known sources in the library database.

Advantages

PFGE can be used to classify isolates from multiple sources, and it is among the most discriminatory genotyping methods. When performed by a skilled technician, the method is highly reproducible.

Disadvantages

PFGE requires a high degree of technical skill and specific equipment, is time consuming, and is relatively expensive (EPA, 2011). As with ribotyping, a large, geographically-specific source database (library) is required.

Complex statistical analysis is often required to determine which sources are likely present. A good working knowledge of statistics is needed.

Both genotypic and phenotypic techniques would likely break down in complex watersheds with numerous sources (Rees et al., 2010; Harwood, 2011).

Repetitive Palindromic Polymerase Chain Reactions (rep-PCR)

PCR allows for rapid amplification of target DNA sequences. PCR is used both in cultivation dependent and independent approaches.

For the rep-PCR technique, intervening sequences between certain repetitive portions of the microbial DNA are amplified using rep-PCR and one primer that targets each end of the repetitive, palindromic sequence. Repetitive DNA elements are scattered throughout the bacterial genome and are separated by distances which vary according to the bacterial species or strain, which forms the basis for the discriminatory patterns generated by rep-PCR. BOX-PCR is a variant of rep-PCR that uses a different primer in the PCR step.

The amplified DNA fragments are separated in agarose gels, producing a banding pattern or “fingerprint” that discriminates among bacterial strains. Bacteria having the same pattern are considered to be of the same strain.

Advantages

Rep-PCR can be used to classify isolates from multiple sources. Compared to the other library techniques, rep-PCR is quicker, easier to use, less expensive, and potentially has a faster turnaround time.

Disadvantages

Although relatively simple compared to PFGE and ribotyping, rep-PCR results tend to be somewhat less reproducible than PFGE or ribotyping (EPA, 2011). A highly trained technician is required to obtain reproducible results.

Complex statistical analysis is often required to determine which sources are likely present. A good working knowledge of statistics is needed.

As with the other library techniques, a large source database (library) is required that is geographically specific. In addition, both genotypic and phenotypic techniques would likely break down in complex watersheds with numerous sources (Rees et al., 2010; Harwood, 2011).

Biochemical (Phenotypic)

Biochemical techniques are based on observable physical or biochemical characteristics of an organism, as determined by both genetic information and environmental influences. Library-dependent biochemical techniques include antibiotic resistance analysis and carbon and nutrient utilization profiling. The *Bibliography* section includes studies that have used these techniques.

Antibiotic Resistance Analysis (ARA)

ARA uses patterns of antibiotic resistance for identifying sources of fecal contamination. The premise is that humans and animals are exposed to different types of antibiotics, and that this selective pressure will alter the antibiotic resistance profile of their fecal bacteria. These differences should be useful in discriminating among fecal bacterial sources.

For this method to be applied, a source library must be developed, using fecal samples from potential contributors in the watershed (e.g., human, livestock, wildlife). The known sources are analyzed for antibiotic resistance and patterns of resistance. Discriminant analysis (a form of multiple analysis of variance) or logistic regression (a model used to predict the probability of an occurrence) uses the antibiotic resistance patterns from known sources to generate the predictive equations that are used to classify unknown isolates by source.

Advantages

ARA is relatively simple and fast, requiring less technical expertise and expensive equipment than genotypic methods. These techniques can distinguish multiple sources including human and domestic animals.

Disadvantages

Complex statistical analysis is often required to determine which sources are likely present. A good working knowledge of statistics is needed.

A geographically-specific reference database is required because phenotypic techniques are geographic and temporally specific. In addition, both genotypic and phenotypic techniques would likely break down in complex watersheds with numerous sources (Rees et al., 2010; Harwood, 2011).

Carbon Utilization Profile (CUP) and Nutrient Utilization Pattern (NUP)

Both CUP and NUP are based on differences among bacterial uses of a wide range of carbon and nitrogen sources for energy and growth. For CUP and NUP, the BIOLOG system allows the user to rapidly perform, score, and tabulate 96 carbon or nitrogen source utilization tests per isolate. Like ARA, the patterns of known sources can be analyzed using discriminant analysis to generate predictive equations that are used to classify unknown isolates using a source library.

While CUP and NUP work well in the laboratory for pure culture characterization/identification, there are many environmental factors in a watershed that can affect bacterial nutrient requirements that may make this method impractical for field determination (Simpson et al., 2002).

Like ARA, the CUP method is relatively simple and allows for the analysis of hundreds of isolates in a short period of time.

Advantages

CUP is relatively simple and fast, requiring very little technical expertise. Equipment and supplies are expensive (Harwood, 2011). These techniques can distinguish multiple domestic animal sources. However, the CUP and NUP techniques have been tested on a small scale and therefore require more testing.

Disadvantages

Complex statistical analysis is often required to determine which sources are likely present. A good working knowledge of statistics is needed.

A geographically-specific reference database is required because phenotypic techniques are geographic and temporally specific. In addition, both genotypic and phenotypic techniques would likely break down in complex watersheds with numerous sources (Rees et al., 2010; Harwood, 2011).

Evaluation of Library-Dependent Techniques

Advantages

Many of the library-dependent techniques use typical fecal indicator bacteria. This can be an advantage when comparing MST results to fecal indicator concentrations.

Library-dependent techniques can be used to classify isolates from multiple fecal sources.

Disadvantages

Both molecular and biochemical library-dependent techniques are based on the assumption that subspecies or strains of specific bacteria are associated with specific animal species. Recent *E. coli* studies suggest that subspecies of this bacterium change considerably with respect to geography, time, rainfall, and habitat. For this reason, the library would need to contain a very large number of isolates (Kuntz et al., 2003). The stability of fecal indicator populations over time, geography, and differing environmental conditions is a major concern for all library-based techniques.

Various studies show that small known-source libraries (i.e., fewer than 2000 isolates) collected in an area have higher accuracy in the local area than when they are applied in other areas. In addition, temporal variability can contribute to errors in studies (Stoeckel and Harwood, 2007).

Furthermore, most *E. coli* and *Enterococcus* strains are not host-specific; rather they are "cosmopolitan" and occur in the gastrointestinal tract and feces of many host species (EPA, 2005; Stoeckel and Harwood, 2007). Very large libraries, while more representative than smaller libraries, tend to contain large proportions of cosmopolitan strains, which reduce the specificity of source identification (Stoeckel and Harwood, 2007). However, small libraries, which may well appear to be very accurate when compared only against themselves (library self cross), lack representativeness and cannot accurately classify isolates that are not part of the library (Harwood, 2011).

Other disadvantages of the MST techniques that use a library are: the need for large library sizes, the complexity of statistical analyses for some techniques, and, for techniques that require bacterial culture, species identification is necessary (Rees et al., 2010). There is no consensus about the minimum number of fecal isolates needed for reliable source identification in a library. The number is likely quite large. Jenkins et al. (2003) suggested that a library of anywhere from 900-2000 fecal isolates would be needed to represent the number of transient and resident *E. coli* ribotypes for two cattle herds. If multiple fecal sources are present, the library size should be in the thousands in order to obtain a good representation of the fecal isolates present in a study area.

Performance

A few studies evaluated the performance of library-dependent MST techniques.

In 2002 SCCWRP conducted an MST comparison study (SCCWRP, 2003). Twenty-two researchers employing different techniques were given sets of identically prepared water samples. Each sample contained one to three of five possible fecal sources (human, dog, cattle, seagull, or sewage), and the fecal source was blinded to the researchers. In addition, source material was provided for the libraries (Griffith et al., 2003). For this study, several of the genotypic library-based techniques performed better than the phenotypic techniques.

Performance of the genotypic library-based techniques for this 2002 study was described by Myoda et al. (2003). PFGE, rep-PCR, and ribotyping were compared. While all the techniques identified the dominant sources in the samples, there was a wide range in the sensitivity (the percentage of true-positives that are reported as positive). The range was greatest among the ribotyping techniques, which ranged from 38-67% overall sensitivity. Sensitivity was higher for samples containing only human fecal sources.

Phenotypic library-based techniques from the SCCWRP 2002 study were evaluated by Harwood et al. (2003). Results of both phenotypic techniques, ARA and CUP, showed a number of false positives. Correct positive identification of unknowns ranged from 67-100% for ARA, and 73-93% for CUP, depending on the fecal bacteria used (Table 1). No data on the accuracy of NUP is available at this time.

Table 1. Predictive accuracy of phenotypic MST techniques in a controlled study (Harwood et al., 2003).

Lab Test	Target	Percentage of True-positives (sensitivity)	Percentage of False-positives	Specificity*
ARA	*E. coli*	86.7	42.4	57.6
	Fecal coliform	66.8	54.6	45.5
	Enterococcus sp.	80.0	54.6	41.9
	Fecal streptococci	100.0	39.4	60.6
CUP	*E.coli*	73.3	66.7	33.3
	Fecal streptococci	93.3	51.5	48.5

*Specificity has a greater accuracy and predictive value. When true-positive, positive predictive value and negative predictive value are high, near 100.

In a review of the performance of MST techniques, Stoeckel and Harwood (2007) evaluated the performance of a number of MST techniques based on specificity of the method (the number of true-negative results divided by the total number of samples tested that should not contain the target) and sensitivity of the method (the number of true-positive results divided by the total number of samples tested that should contain the target). Table 2 describes the specificity and sensitivity of library-based techniques.

Multiple Antibiotic Resistance (MAR), ARA, and CUP are phenotypic library techniques. Ribotyping, PFGE, and Box-PCR are genotypic library techniques. Most techniques were able to detect a fecal source if the bacteria was present (sensitivity), but the ARA using fecal coliforms had very low sensitivity. Most techniques lacked specificity, which means they detected a source even though the source was not present (Table 2).

Table 2. Performance statistics when MST techniques were tested with reference samples to determine the ability or failure to detect the sole source of fecal contamination (Stoeckel and Harwood, 2007).

Test	Target	Host Category	Sample Type	Sensitivity[1]	Specificity[2]
ARA	*E. coli*	Human	Blind	1.00 (n=7)	0.80 (n=5)
	Fecal coliform	Human	Blind	0.43 (n=7)	1.00 (n=5
	Enterococci	Human	Blind	0.75 (n=4)	0.25 (n=8)
	Fecal streptococci	Human	Blind	1.00 (n=4)	0.38 (n=8)
MAR[3]	*E. coli*	Human	Blind	1.00 (n=7)	0.00 (n=5)
CUP	*E. coli*	Human	Blind	1.00 (n=7)	0.20 (n=5)
	Fecal streptococci	Human	Blind	1.00 (n=4)	0.25 (n=8)
Ribotyping (2 trials)	*E. coli*	Human	Blind	0.88 (n=6) 1.00 (n=8)	0.00 (n=1) 0.50 (n=4)
Ribotyping	*Enterococci*	Human	Blind	1.00 (n=8)	0.00 (n=4)
PFGE	*E. coli*	Human	Blind	0.88 (n=8)	0.50 (n=4)
Box-PCR (3 trials)	*E. coli*	Human	Blind	1.0 each (n=8 each)	0.00-0.50 (n=4)

[1] Sensitivity is the ability to detect a source when it is present (calculated by dividing the number of true-positive results by the number of samples that should contain the target).
[2] Specificity is the ability to detect a source when it is not present (calculated by dividing the number of true-negative results by the number of samples that should not contain the target).
[3] Multiple Antibiotic Resistance (similar to ARA).

The Stoeckel and Harwood (2007) study also looked at performance statistics for various known-source categories. Table 3 presents results for the library-based techniques. Correct classification of human sources was poor; classification of non-human sources was better for most techniques.

Table 3. Performance statistics for library-based techniques of various known-source categories (adapted from Stoeckel and Harwood, 2007).

Test	Target	Sample Type	Human Source		Nonhuman Source	
			Proportion of true-positive classifications	n	Proportion of true-positive classifications	n
ARA	*E. coli*	Blind isolates	0.24-0.27	17-44	0.83-0.86	53-133
	Enterococci	Blind isolates	0.66	44	0.55	55
CUP	*E. coli*	Blind isolates	0.12	17	0.98	126
BOX-PCR	*E. coli*	Blind isolates	0.31	16	0.95	133
BOX-PCR HFERP	*E. coli*	Blind isolates	0.54	210	0.94	1321
REP-PCR	*E. coli*	Blind isolates	0.60	10	0.94	83
PFGE	*E. coli* (NotI)	Blind isolates	0.67	6	0.91	34
Ribotyping	*E. coli* (HindIII)	Isolates from reference feces	0.85	84	0.79	317
	E. coli (HindIII)	Blind isolates	0.06	17	0.81	53

Library-Independent Techniques

Library-independent techniques do not require development of the fecal source library as do the library-dependent techniques. Bacteria and viruses from environmental samples (water or sediment) are known to be from specific hosts or sources of fecal contamination, so there is no need to compare the results to a library. Library-independent techniques require culturing microbes present in environmental samples, or they may rely on direct molecular analysis of the genetic material present in the samples, in order to identify specific bacteria or viruses. Some of the library-independent techniques are described below.

Bacteriophages

A bacteriophage is a virus that uses a bacterial cell as its host. These viruses are highly specialized and usually infect bacteria of a particular species, and may even be strain-specific. A coliphage is a virus that is a parasite of *E. coli*. The coliphage genome can be made up of either RNA or DNA, which is used to categorize these viruses into broad groups. Another distinguishing factor is the cellular target of attachment for coliphages; some adsorb to the bacterial cell wall prior to infecting the bacteria and are termed somatic coliphages. Others target the *E. coli* sex pilus, and are called F+ (F-specific) coliphages.

The presence of F+RNA coliphage has been used to broadly distinguish human and animal fecal contamination by using serotyping or genotyping. First, F+RNA coliphages are isolated in the presence of the enzyme DNAse to distinguish them from F+DNA coliphages. Then, phages are either serotyped or genotyped to identify the particular group that the phage belongs to. Serotyping relies on specific antisera that are produced by vertebrate animals to inhibit infection. Genotyping relies on group-specific, labeled probes, or PCR (EPA, 2011).

There are four antigenically and genetically distinct groups of F+RNA coliphages, and those predominating in humans (groups 2 and 3) differ from those predominating in animals (groups 1 and 4).

Advantages

Advantages of bacteriophage are (1) the two-step approach is relatively simple and can be performed within two working days (EPA, 2005), and (2) a library database is not needed.

Disadvantages

F+RNA coliphage typing can be useful for determining whether human and animal fecal sources are present, but cannot distinguish between various animal groups or species.

Another disadvantage is different survival characteristics of the subgroups. Particularly poor survival of F+ RNA coliphages in warm waters has been observed (Harwood, 2011).

In addition, Schaper et al. (2002) noted that exceptions to the groupings can occur. A recent study showed that group 3 is also common in swine feces (Rees et al., 2010). The fact that these coliphage groups are present in more than one animal type suggests that these groups are not completely host-specific; therefore, assays based on these phage groups might not conclusively be able to discriminate between fecal pollution of human and animal origin.

Bacterial PCR

A number of host-specific indicator bacteria have been proposed for use in MST. These are discussed below. For most of these methods, PCR techniques are used to amplify specific segments of the bacterial DNA that are isolated from water samples. Water samples are typically filtered prior to PCR to exclude extracellular DNA from dead and lysed cells and to concentrate the sample (EPA, 2011).

Bacteroidales, *Bacteroidetes,* and *Bacteroides*

Some of the most promising results for MST marker development have been obtained with the members of the order *Bacteroidales*, family *Bacteroidetes*, using the 16S rRNA gene. The genus *Bacteroides*, the family, and the order comprise anaerobic fecal bacteria that are abundant in the intestines of mammals. *Bacteroides* are far more abundant in the gastrointestinal tract and feces of many animals than conventional indicator bacteria such as *E. coli* and enterococci, but we do not know how to culture many of them. They are obligate anaerobes and do not survive long in the water column but may do much better in anaerobic sediments (Harwood, 2011).

Currently there are methods developed for host-specific *Bacteroidales* markers for human and ruminant (e.g., cattle, sheep, deer, goat, and elk). There are also *Bacteroidales* markers for dog, horse, pig, cattle, and some birds, but they are less broadly tested.

In a study by Harwood et al. (2009), three human-specific sewage markers were tested, including *Bacteroidales,* from a variety of fecal sources at three different laboratories. *Bacteroidales* exhibited the most false-positive results but showed overall high specificity at 96%. False positive results were obtained for chicken and dog markers at one of the labs (the only lab that tested commercially raised poultry layers) and for dog and seagull markers at another lab. *Bacteroidales* were detected in all human sewage samples, showing 100% sensitivity.

Bifidobacterium species

Bifidobacterium species have been evaluated for use as a human marker. Using 16S rRNA PCR assays, nine human-specific markers of *Bifidobacterium* species were examined by Bonjoch et al. (2004). The assays were challenged against cattle, swine, poultry, and human fecal sources. The results showed that *B. adolescentis* and *B. dentium* were found only in human sewage samples.

However, in a study done by Lamendella et al. (2008), several *Bifidobacterium* species previously suggested as indicators of human fecal pollution were found to be broadly distributed in different animals. These species included *Bif. adolescentis, Bif. bifidum, Bif. dentium,* and *Bif. catenulatum*.

Methanobrevibacter species

Methanobrevibacter ruminantium and *M. smithii* have been tested for possibilities as ruminant and human markers, respectively. Ufnar et al. (2007) targeted the *nifH* gene of *M. ruminantium* as a fecal pollution marker from domestic ruminants, including sheep, goat, and bovine.

In a study by Harwood et al. (2009), *M. smithii* was tested as a human sewage marker from a variety of fecal sources at three laboratories. The *M. smithii* assay was 98% specific (false positive results from one lab for cow, dog, and seagull). The *M. smithii* marker was detected in all human sewage samples, showing 100% sensitivity.

Rhodococcus coprophilus

Rhodococcus coprophilus has been suggested as an indicator of nonhuman fecal contamination, with preferential distribution in herbivores, due to its frequent isolation in animal feces and common absence in human feces. PCR techniques for *R. coprophilus* have been developed. High survival rates can diminish their value in cases of recent fecal contamination events (Rees et al., 2010).

Escherichia coli (*E. coli*)

Escherichia coli toxin genes have been used in PCR assays to identify the presence of cattle and swine fecal pollution. LTIIa and STII PCR assays were found to be highly host-specific, after being tested against DNA extracts from different animal and human fecal sources. ETEC strains are normally found in low densities in environmental water, and a cultivation step is required to increase the sensitivity of the assays (Rees et al., 2010).

Enterococcus species

Various *Enterococcus* species and strains have been analyzed using similar PCR techniques, generally following the same method as *Bacteroides* PCR. Genetic markers have been identified for specific strains of one species associated primarily with humans (*Ent. faecium*), cattle (*Ent. hirae*), and birds (*Ent. faecalis*) (EPA, 2011; Source Molecular, 2011).

A human-specific PCR assay targets the *esp* gene, a virulence factor in *Ent. Faecium* (Scott et al, 2005). This method is similar to the LTIIa and STII PCR assays described above. A cultivation step is needed due to low densities of the *esp* gene of *Ent. faecium* in the feces and environment.

Viral PCR

Several techniques are available for the detection of viruses. In fact, monitoring for human viruses has been suggested as an alternate approach to assess human health risks in environmental waters. Viruses are generally highly host-specific, which is a positive characteristic for MST. On the other hand, pathogenic viruses generally infect a small percentage of any given population, making them relatively rare targets (and thus more difficult to detect). Certain nonpathogenic viruses, such as human polyomaviruses (HPyVs) have a wider distribution in human populations than pathogenic viruses.

Viruses cannot replicate themselves. They need a host organism to replicate their genetic code and produce their proteins and lipids. Viruses contain either RNA or DNA as their genetic material (also known as genome).

Viruses with a limited host range can be used to differentiate sources of fecal contamination in water. Viruses first must be isolated from water sources, then the viral genome is extracted and amplified using PCR techniques. Human-specific adenoviruses and enteroviruses have been used as indicators of human fecal contamination. Bovine enteroviruses, and bovine and porcine adenoviruses, have been used as indicators of livestock fecal contamination (EPA, 2011). Although Field and Samadpour (2007) found that bovine enteroviruses are not species specific and have been observed in other animals, including horses and geese.

Harwood et al. (2009) tested the human sewage marker, human polyomaviruses (HPyVs), from a variety of fecal sources at three different laboratories. The HPyVs were detected in human sewage samples showing 100% sensitivity. The HPyVs assay was 100% specific, showing no cross-reactivity to dog, cow, cat, bird, or wild animal feces among >300 samples from three Gulf Coast regions.

Evaluation of Library-Independent Techniques

Advantages

The most significant advantage of library-independent techniques is that they do not require development of a library database; this saves time and resources. Furthermore, the techniques that do not require culture of microorganisms can be quite rapid (i.e., completed and reported in the same day in which sampling occurred).

Disadvantages

One of the major limitations of library-independent techniques is the lack of techniques for host species beyond humans and a few domestic animal species. While some of the markers have been thoroughly vetted, and their error rate is fairly well-established (e.g., human *Bacteroides* HF183), other markers require much more substantial validation before they can receive general use (Harwood, 2011).

The distribution of many markers has not been thoroughly characterized; the expectation that host-associated markers are present in a specific host population and not present in non-host populations should be confirmed.

In addition, the relative abundance of many of these MST molecular markers has not been assessed. Validation studies need to include assessment of the abundance of these markers in the environment.

All markers (with the exception of HPyVs) tested in validation studies do show some cross-reactivity with non-target host species (Harwood, 2011). Validation studies would enable quantification of cross-reactivity, thus helping to determine usability of MST data.

Performance

In 2002 SCCWRP conducted an MST comparison study (SCCWRP, 2003). Twenty-two researchers employing different techniques were given sets of identically prepared water samples. Each sample contained one to three of five possible fecal sources (human, dog, cattle, seagull, or sewage), and the fecal source was blinded to the researchers.

For this study, the library-independent techniques were tested including: human-specific *Bacteroidetes sp*. (several laboratories) and the *E. coli* toxin gene. Both techniques correctly identified a majority of the human fecal sources (no method identified < 75%). For samples that did not contain a human source of contamination, *Bacteroidetes* performed better than the toxin gene. The toxin gene method incorrectly identified 50% of these samples (Field et al., 2003).

For the same 2002 study, Noble et al. (2003) assessed the performance of viruses (human pathogens and coliphages) as an MST tool. Adenoviruses, enteroviruses, and F+ specific coliphages were tested. These markers only discriminate between human and non-human sources. False positive rates for the virus-based techniques (0-8%) were among the lowest of MST techniques tested in the 2002 study, but sensitivity was low (high false negative rate). Generally virus-based techniques are not as effective when applied to individual human fecal contamination (individual on-site sewage treatment source) versus sewage (Noble et al., 2003).

In a review of the performance of MST techniques, Stoeckel and Harwood (2007) evaluated method performance based on (1) specificity of the method (the number of true-negative results divided by the total number of samples tested that should not contain the target) and (2) sensitivity of the method (the number of true-positive results divided by the total number of samples tested that should contain the target). Table 4 describes the specificity and sensitivity of the library-independent techniques.

Generally the host-specific, library-independent markers were detectable in most proficiency samples that represented fecal material from many individuals, but the distribution was patchier among individuals. The sensitivity and specificity of the markers varied greatly depending on the geographic location and research lab, highlighting the need for local validation of method performance (Stoeckel and Harwood, 2007).

Table 4. Performance statistics for tests in which library-independent MST techniques were tested with reference samples to determine the ability or failure to detect the sole source of fecal contamination (adapted from Stoeckel and Harwood, 2007).

Marker Detection	Host Category	Sample Type	Sensitivity[1]	Specificity[2]
Bacteroides thetaiotaomicron, PCR	Human	Individual feces Wastewater	0.78, 0.92 (n=9,25) 1.00 (n=20)	0.98,0.76 (n=241,71) NR
Bacteroidales, PCR	Human	Blind samples Individual feces Wastewater	0.70-1.00 (n=10,14) 0.20-0.85 (n=7-25) 1.00 (n=41)	1.00,1.00 (n=6,7) 0.85-1.00 (n=46-73) 1.00 (n=75)
	Ruminants	Blind samples	1.00 (n=7,9)	0.89, 0.92(n=9,12)
	Cattle	Individual feces	1.00, 1.00 (n=19,19)	0.73, 0.70 (n=40,40)
	Ruminants	Individual feces	0.97,1.00 (n=31,20)	1.00,1.00 (n=20,28)
	Ruminants	Wastewater	1.00 (n=75)	0.93 (n=14)
	Dog	Blind samples	0.40 (n=15)	0.86 (n=7)
Bacteroides fragilis phage	Human/ nonhuman	Wastewater Fecal samples	1.00 (n=36) 0.13 (n=90)	0.90 (n=20) 1.00 (n=145)
Bifidobacterium adolescentis PCR	Human	Wastewater	1.00 (n=22)	0.84 (n=60)
Bifidobacterium adolescentis Colony hybridization	Human	Individual Feces Wastewater	0.92 (n=12) 0.67 (n=3)	1.00 (n=85) 1.00 (n=3)
Enterococcus faecium enrichment, PCR	Human	Septic system Wastewater	0.80 (n=10) 1.00 (n=55)	1.00 (n=59) 1.00 (n=43)
Escherichia coli toxin gene	Human Cattle	Blind samples Wastewater	0.75 (n=15) 0.87 (n=31)	0.33 (n=7) 1.00 (n=207)
Escherichia coli Enrichment, PCR	Swine Swine	Wastewater Individual feces	0.90 (n=31) NR	1.00 (n=217) 1.00 (n=224)
Adenovirus Nested, PCR	Human Human Swine Cattle	Wastewater Blind samples Individual feces Individual feces	0.92 (n=12) 0.50 (n=8) 0.74 (n=23) 0.75 (n=8)	1.00 (n=31) 1.00 (n=3) 1.00 (n=20) 1.00 (n=35)
Enterovirus RT-PCR	Human Cattle Cattle, deer	Blind samples Individual feces Individual feces	0.38 (n=8) 0.76 (n=95) 0.63 (n=145)	1.00 (n=4) 0.63 (n=54) 0.75 (n=4)

[1] Sensitivity is the ability to detect a source when it is present (calculated by dividing the number of true-positive results by the number of samples that should contain the target).

[2] Specificity is the ability to detect a source when it is not present (calculated by dividing the number of true-negative results by the number of samples that should not contain the target).

What Makes a Good MST Marker?

In the past 20 years, various MST markers have been proposed and discarded. Early efforts focused on library-based MST techniques. Due to concerns with library-based techniques, recent research has focused on library-independent markers. Harwood and Stoeckel (2011) detailed the characteristics of an ideal marker (Table 5).

Table 5. Characteristics of an ideal marker versus a useful MST marker from Harwood and Stoeckel (2011).

Characteristic	Ideal Marker	Useful Marker
Specificity.	Found only in target host species.	Differentially distributed among host species.
Distribution in host population (contributes to sensitivity of marker).	Found in all members of all populations of host	Consistently found in host species whose feces impact the target area.
Evenness.	Each individual host has the same amount of the marker.	The amount of marker in aggregate sources (e.g., sewage, animal waste lagoons) is similar.
Temporal stability in host.	Does not change over time in the host.	Frequency and concentration does not change over time at the population level.
Geographic range/stability.	Present in all geographic regions for the specific host.	Consistently detected in different geographic regions
Environmental persistence.	Consistent decay rate in various habitats (e.g., freshwater, marine water) and matrices.	Predictable decay rate in various habitats and matrices.
Quantitative assessment.	Can be accurately quantified.	Accurately indicates presence/absence of contamination source.
Relevance to regulatory parameters or fecal indicator bacteria (FIB).	Derived from an organism that is a regulatory tool or FIB.	Correlated with an organism that is a regulatory tool or FIB.

Specificity

An MST marker should be host specific; it should not be found in non-host species. Specificity is the ability to rule out a target when it is absent. Specificity is measured as the portion of non-target samples that test negative by the method (true-negative results divided by all non-target samples). The formula for specificity is 1 minus the proportion of non-target fecal samples in which the marker is detected, or 1 minus the false positive rate (Harwood and Stoeckel, 2011). Calculations are specified as a percentage, so the calculation above would be multiplied by 100.

Determining the appropriate number of non-target samples to include for specificity testing is not standardized, but tests should be based on the geographic area of the study. The 2005 EPA document recommends that at least 10 animals per host type are sampled for specificity. Composite samples would be an efficient means of testing. For example, test 5-10 animals (same species) from several different farms in the study area, or test several septic systems at various locations in the study area.

Presently there is no universal criterion for a specificity measure, but it is generally agreed that methods with less than 80% specificity are not useful in most cases. The majority of recently published studies use methods which have 90% or greater specificity in the geographic area of interest (Harwood and Stoeckel, 2011).

Sensitivity and Distribution

The distribution of a marker in the feces of individual members of the host species is the main factor in method sensitivity. A more sensitive marker will be more frequently detected in a polluted water sample than a less sensitive marker. Sensitivity is measured as the proportion of positive-control fecal samples that produce a positive result. The formula is: divide the number of true-positive results by the number of samples that should contain the target, then multiply by 100 to express as a percentage.

The number of samples needed to determine sensitivity has not been determined but recent studies have included 20 or more samples. Key to a sensitivity determination is obtaining fecal samples from the study area to determine sensitivity of the marker you propose to use. This should be done in advance of water testing to ensure the marker is detectible in the study area.

Selecting an MST Technique

When selecting an MST technique, it is important to consider monitoring goals and the performance characteristics of MST markers. No MST marker or technique meets all the criteria described in Table 5. Some markers achieve some of the characteristics of a useful marker (Table 5).

EPA (2005) recommends a 'toolbox' approach, where the best MST method is selected after considering each method's cost, reproducibility, discriminatory power, ease of interpretation, and ease of performance.

The following EPA reports should be reviewed before selecting an appropriate MST tool. These EPA documents provide step-by-step guidance for choosing the appropriate MST tool for a project.

- Using Microbial Source Tracking to Support TMDL Development and Implementation (EPA, 2011).
- Microbial Source Tracking Guide Document (EPA, 2005).

Current peer-reviewed literature and MST performance studies should be reviewed for performance characteristics before selecting a MST technique. There are a few comparison studies and reviews that provide information on MST techniques; one was conducted by SCCWRP in 2002 (SCCWRP, 2003) and another by Stoeckel and Harwood (2007). These studies include data on performance of some of the methods and laboratories.

Currently EPA is working with SCCWRP to conduct another comparison study. The study includes 64 blind samples of 12 source types submitted to 29 laboratories; 23 MST techniques are being tested (Peed, 2011). The results of this comparison study will be published in 2012.

Generally, initial testing for newer methods is more limited, but more information is available as a method is used and more comprehensive studies are developed. It is highly recommended that markers with limited or unknown specificity and sensitivity be avoided unless you are willing to conduct that assessment (Harwood and Stoeckel, 2011).

Due to concerns about the experimental nature of MST techniques, some researchers recommend use of multiple MST techniques to increase the confidence in bacterial source identification. This also allows for more potential source types to be investigated (Wapnick et al., 2009). In Wapnick et al. (2009), several library-independent techniques were used to discern sources including: human polyomaviruses, the virulence gene for *Enterococcus faecium*, as well as *Bacteroidetes* human-, ruminant-, and horse-specific markers.

MST studies that are limited to restricted geographic areas will be easier to interpret, and in all probability provide more accurate results, than studies conducted over a wider geographic range (Harwood, 2011). The current state of the science may not be able to resolve complex multi-host discrimination questions over large temporal and spacial scales (Rees et al., 2010). Larger source tracking studies should be broken down using a targeted sampling approach.

Before conducting an MST study obtain fecal samples for testing to determine if the proposed MST marker is specific to the target host in your study area. MST markers may not be specific to a geographic region. Also, presence of MST markers may change over time.

In addition, EPA has developed quality control (QC) steps for laboratories performing PCR analysis: Quality Assurance/Quality Control Guidance on Environmental Samples (EPA, 2004).

Studies evaluating MST techniques found that the methods evaluated had issues with false positives (identifying a source when it was not there) and false negatives (not identifying a source when it was present) (SCCWRP, 2003). Extensive QC sampling to assess precision and accuracy of results should be included in all MST studies. For example, field and laboratory QC should include: spiked blind samples, 50% field sample replication, and blind blank samples. Past MST studies often did not include adequate QC sampling, creating difficulty in the evaluation of these methods.

Conclusions and Recommendations for Molecular and Biochemical MST Techniques

Conclusions

A significant issue associated with molecular and biochemical MST techniques is the absence of standardized methods. This is an important problem considering the various steps associated with molecular and biochemical MST techniques, and the potential for error associated with each step. Each step in laboratory analysis should follow validated SOPs and include controls (Santo Domingo et al., 2011).

Recently, SOPs for qPCR of human-associated *Bacteroides* HF183 and human polyomaviruses were published (Harwood et al., 2011). SOPs for conventional PCR of human-associated *Bacteroides* HF183, *M. smithii,* and human polyomavirus are available (Harwood et al., 2010).

Because of the difficulty of building a sufficiently robust library, the complexity of data analysis, and the poor performance of MST in the SCCWRP and USGS method comparison studies, the focus of much recent MST research has shifted to the development of library-independent techniques (Rees et al., 2010; Hagedorn et al., 2011). At this time, library-independent molecular techniques are much more widely used over library-dependent techniques.

Very few existing MST studies include adequate QC sampling to assess precision and accuracy of results. MST comparison studies and reviews have found differing sensitivity (ability to detect a source when it is present) and specificity (ability to detect a source when it is not present) for MST techniques analyzed at different laboratories or with different target organisms (Stoeckel and Harwood, 2007). This highlights the need for standardized techniques and SOPs.

While molecular and biochemical MST techniques are experimental, they can provide some important insights into the complexity of fecal pollution and, in some cases, even direct us to choose adequate management practices (Santo Domingo et al., 2011). However, a good understanding of the nature of the fecal contamination and the limitation of MST techniques is required before selecting an appropriate method and conducting a useful MST study.

Recommendations for Using Molecular and Biochemical MST Techniques

Library-independent MST techniques are recommended over library-dependent methods.

MST techniques that have been more frequently used or tested are preferred over newer methods.

For determining the presence\absence of human sources, these markers are recommended:

- Human-associated *Bacteroidales*: Bacteroidetes is prevalent in the human intestine; the limit of detection for tested laboratories is equivalent to 10 cfu/100 mL *enterococci*, with excellent sensitivity (100%). Specificity is adequate (some false positives found) ranging from 88-100%. (Harwood et al., 2009).
- Human polyomaviruses (HPyVs) or *Methanobrevibacter smithii*: Both of these methods have a higher limit of detection (may produce false negative results). Both markers have greater host specificity than *Bacteroidales*, with 100% specificity at tested laboratories (Harwood et al., 2009).

Before conducting an MST study, obtain fecal samples for testing to determine if the proposed MST marker is specific to the target host in the study area. This should be done in advance of water testing. MST markers may not be specific to a geographic region. Also, the presence of MST markers may change over time.

Due to widely varying survival rates of the bacterial groups in the environment, fecal indicator bacteria sampling (e.g. fecal coliforms) should occur concurrent with MST sampling. This will allow for comparison of fecal indicator concentrations and MST results.

Finally, MST studies should include adequate QA. QA sampling should include field and laboratory duplicates, field positive controls or blind spiked samples, and field and laboratory blank samples. Recommended QA protocols are described in the *Quality Assurance* section of this document. Laboratories performing PCR analysis on environmental samples for MST techniques should follow EPA's guidance (2004). QA results are essential for assessing usefulness and accuracy of the MST data.

Chemical Methods

Chemical source tracking methods for fecal source tracking are based on the detection of a chemical that is related to a specific fecal source but is not found in unpolluted waters. In some cases the chemical is directly associated with feces, while in others the chemical is discharged together with feces in wastewaters. More than 35 chemical compounds have been identified as being specific to either human or animal sources of fecal waste (Hagedorn et al., 2005, Harwood and Stoeckel, 2011).

The advantages of using chemical methods to track sources of bacteria are: many of the methodologies are standardized, analysis can be less expensive than MST methods, and results can be obtained sooner than some MST methods that require culture of microorganisms. The disadvantages of these methods are: many of these chemicals are ubiquitous in the environment, and concentrations of some chemicals are often below detection limits once they enter surface waters. Despite these concerns, certain chemical methods have demonstrated their usefulness, in conjunction with traditional fecal detection methods.

In the process of this evaluation, three publications were identified that provide a comprehensive and comparative evaluation of current chemical source tracking methods. Two publications (Cimenti 2007; Hagedorn and Weisberg, 2009) assessed the performance of several chemical methods to several performance criteria. The third was a report published by EPA (2007d).

The report published by EPA was the result of a five-day workshop which included 43 national and international experts who discussed the state of the science on recreational water quality research and implementation. The goal of the workshop was to identify research and science needs for developing new or revised water quality criteria for recreation in the near future (EPA, 2007d). As part of this workshop, indicators of fecal and pathogen contamination were examined to identify critical research and science needs. Discussion of chemical methods was part of the workshop deliberations.

For the purposes of this guidance document, methods and conclusions highlighted in these three publications, as well as more recent studies, are compared and summarized below. These studies were all used to make recommendations on the uses of these chemical methods for fecal source tracking in Washington State.

Types of Chemical Methods

Four general classes of chemical compounds were identified as having the most potential as source tracking tools for fecal bacteria. These include:

1. Fecal sterols and hormones
2. Caffeine
3. Optical brighteners
4. Pharmaceuticals

Below is a brief summary of these four classes as well as the general advantages and disadvantages for each method.

Fecal Sterols and Hormones

Fecal sterols are a group of cholestane-based sterols that are formed in the digestive tracks of animals from the metabolism of sterols. The metabolic end products vary in concentration between animal groups based on diet and intestinal flora. These end products can be analyzed with high-resolution gas chromatography and mass spectrometry (GC/MS).

Both concentrations and ratios of sterols have been used to attribute fecal source contributions from humans, herbivores, and birds (Ashbolt and Roser, 2003; Leeming et al., 1997; Leeming et al., 1998; Roser and Ashbolt, 2007; Shah et al., 2007). Fecal material from cattle, horses, and sheep contains a greater proportion of the sterols 5b-campestanol and 5b-stigmastanol (Leeming et al., 1996). Cholesterol was found to be dominant in dog feces, while bird feces contained predominantly cholesterol and sitosterol (Leeming et al., 1997).

Ratios of certain stanols have been used in field studies both to exclude and validate specific sources of fecal pollution. Noblet et al. (2004) compared sterol ratios from samples collected from raw and processed sewage to surface water samples collected in the Santa Ana River. Steroid ratios in the Santa Ana River differed from those found in raw and treated sewage. The authors used multivariate statistical analysis to show that bird fecal steroids were the most abundant steroids in the stream sample.

Although several studies have correlated direct measures of fecal sterols with traditional fecal indicators, correlations may be seasonally dependent (Isobe et al., 2004). Also, some sterols have been found to be ubiquitous in trace amounts in both soil and sediments (Bull et al., 2002).

The fecal sterol technique offers many diagnostic and quantitative advantages when used in conjunction with traditional techniques for detecting bacteria pollution. Fecal sterol analysis has resolved problems of source identification in urban and rural environments. This was not possible with use of traditional fecal indicator bacteria and MST methods (Roser and Ashbolt, 2007).

With the recent evidence of the reproductive effects of endocrine disrupting compounds (EDCs) on reproductive processes in aquatic organisms, runoff from cattle operations has been studied as a potential source of EDCs (Parks et al., 2001; Sorensen et al., 2005). Several endogenous

steroid hormones have been detected in surface waters associated with confined animal feeding operations (CAFOs) as well as rangeland cattle grazing (Matthiessen et al., 2006; Kolodziej and Sedlak, 2007; Zheng et al., 2008).

Three endogenous hormones – 17α-estradiol, 17β-estradiol, and estrone – were detected in dairy wastewater and lagoon water in California (Zheng et al., 2008). Concentrations of 17α-estradiol rapidly decreased along the wastewater disposal route, while estrone concentrations increased. This suggests 17α-estradiol is oxidized to the metabolite estrone. These results were consistent with hormone concentrations observed in fresh and piled manure (Zheng, 2008). Levels of 17α-estradiol decreased 87% while levels of estrone increased 23%.

Researchers in California used gas chromatography-tandem mass spectrometry (GC-MS-MS) to test for the presence of steroid hormones in surface waters associated with cattle grazing (Kolodziej and Sedlak, 2007). Out of 88 samples collected from 30 sites over a 12-month period, steroids were detected in 86% of the samples. The steroid hormones, estrone and 17α-estradiol, were most frequently detected immediately after rain events and at the beginning of the wet season. Although the main source of these hormones was linked to cattle in this study, their presence and concentrations did not correlate with nitrite and total coliform concentrations (Kolodziej and Sedlak, 2007).

Advantages

Sterols have been identified that are reasonably specific to humans and other animal sources, sterols are temporally and geographically applicable, and detection methods for sterols are adequately sensitive and repeatable.

Disadvantages

Natural sources or analogs of target stanols may exist, and the degradation rates of sterols in different matrixes are not well understood.

While estrone and 17α-estradiol in surface waters have been linked to livestock, there is little agreement in the literature about source specificity. However, GC/MS methods for detection are standardized, and rapid presence/absence tests are available for both chemicals (Lubliner et al., 2010). In certain circumstances, these indicators could be useful for determining if livestock are contributing to fecal pollution.

The instrumentation for fecal sterols analyses is expensive, and expertise necessary to conduct the analyses are extensive.

Caffeine

Caffeine has been extensively examined as a tool for assessing human influence on aquatic systems. Although caffeine is metabolized when consumed, a small amount (<10%) of ingested caffeine remains intact when excreted (Peeler et al., 2006). Most work in the past decade has focused on sewage systems and the efficiency of caffeine removal in sewage treatment plants.

However, with the improvements in techniques and detection limits, the scope of application has broadened to include stream, wetland, estuarine, and groundwater systems.

Caffeine has been successfully detected in freshwater streams and lakes, groundwater, marine waters, and stormwater outfalls (Buerge et al., 2003; Chen et al., 2002; Peeler et al., 2006). However, some studies reported a lack of correlation between fecal coliform or *Enterococci* concentrations with caffeine levels (Buerge et al., 2003). Also, some studies have detected caffeine in presumably uncontaminated waterways (Weigel et al., 2004; Peeler et al., 2006).

Advantages

Caffeine appears to be a ambiguous indicator of human waste in surface waters; however, it can be detected at low levels using solid-phase extraction and GC/MS (Peeler et al., 2006).

Disadvantages

A major disadvantage is that caffeine and its metabolites are often present in the urban environment associated with numerous plant species debris as well as from human "dumping" of coffee wastes. Further, the current methods used (specific extraction and GC/MS analysis) are relatively complex and expensive.

Optical Brighteners

Optical brighteners (OBs) are compounds added to laundry detergents and soaps. Because household plumbing systems mix effluent from washing machines and toilets together, OBs are associated with human sewage in septic systems and wastewater treatment plants. In the United States, 97% of laundry detergents contain OBs (Hagedorn et al., 2005).

There are three commonly used methods for detecting OBs:

- The fastest, most cost-effective approach uses dye-free cotton or charcoal pads and an ultraviolet light (Dixon et al., 2005). Cotton pads are exposed to surface waters for a period of time and are then exposed to ultraviolet light. If OBs are present, the cotton pad will fluoresce.
- The second method uses ion-pair high performance liquid chromatography to measure concentrations of OBs (Shu and Ding, 2005). This method is highly sensitive; however, its usefulness is limited by high cost.
- The third method uses a fluorometer to detect OBs (Hartel et al., 2007). The method is inexpensive and sensitive, and it can provide either real-time results or can be deployed in the field for continuous data logging.

No matter which OB method is used, it must be combined with measurements of fecal indicator bacteria and/or pathogens. For example, effluent from a wastewater treatment plant contains OBs, regardless of how effective the treatment processes have been at removing or inactivating pathogens. Thus, data on the presence of OBs, without accompanying data on viable fecal indicators, do not provide information on the potential health risk from pathogens.

Advantages

The biggest advantage in using OBs to identify human sources of fecal bacteria is the ability to detect OBs in real-time or logging data continuously. This can be advantageous as it applies to pinpointing an area(s) where leaking septic systems are a source of fecal bacteria.

Disadvantages

One concern with these OB methods is the presence of contradictory results when comparing fluorometry and bacterial counts. Although various reports have documented a strong fluorescent signal and high numbers of fecal indicators, studies have also reported no correlation between fluorometry and counts of fecal bacteria (Hartel et al., 2007).

Another concern is that numerous other natural and anthropogenic substances found in surface waters, such as humic acids and polyaromatic hydrocarbons, may interfere with the detection of OBs by fluorometry (Dickerson et al., 2007). This is a particular issue in waters with substantial contributions from tannic rivers, which are a feature of coastal Florida waters (V.J. Harwood, personal communication). In addition, the longevity of OBs in surface water is unknown and may be dependent on the detergent manufacturer (Cao et al., 2009).

Pharmaceuticals

The use of pharmaceutical chemicals to detect sewage-based human pollution has received considerable scrutiny since it was described by Buser et al. (1999). Chemicals used in the pharmaceutical industry have been examined as indicators of human wastewater pollution (Leeming and Nichols, 1996), as impacting on aquatic ecosystems including acute and chronic toxicity (Brun et al., 2006; Fent et al., 2006), and as agents affecting growth and reproduction (Binzcik et al., 2004).

Researchers have measured pharmaceuticals in groundwater near three high-volume septic systems in Ontario, Canada (Carrara et al., 2008). Several pharmaceutical and organic compounds were detected in wastewater samples collected from septic tanks, as well as in groundwater samples collected down-gradient of the infiltrations beds. Of the compounds analyzed, ibuprofen, gemfibrozil, and naproxen were observed at the highest concentrations and greatest distances from source areas (>60 ft). The extent of transport of these compounds was correlated with anoxic zones that developed in the wastewater plumes. Because many of these compounds are attenuated to soil particles, their presence in groundwater was found to be dependent on regional groundwater chemistries (Carrara et al., 2008).

The drugs found most frequently in surface waters and wastewaters include clofibric acid, carbamazepine, and salicylic acid (Weigel et al., 2002; Ternes, 1998; Lee et al., 2003). Clofibric acid has recently been proposed as a marker for anthropogenic contamination (Clara et al., 2004).

Antibiotics and other drugs used as growth promoters in CAFOs are also being used as indicators in Europe and North America (USGS, 2003; Boxall et al., 2003; Scribner et al., 2003). There is still uncertainty about the quantities of growth promoters currently used in CAFOs. However, some of the most persistent drugs have already been found in watersheds (Boxall et al., 2003) and could be used as tracers of CAFO fecal pollution.

Advantages

Advantages of using pharmaceuticals include the specificity of many of the synthetic chemicals to human wastewater, as there are no natural sources or known analogs of these compounds.

Disadvantages

The sensitivity of detection of pharmaceuticals in large waterbodies could be an issue, and the target compound must be used heavily enough within a given locality so that it can be reliably detected. This may limit application to larger cities where the target compounds are more prevalent than in smaller or rural communities.

Another disadvantage of using pharmaceuticals is the cost of analytical equipment necessary to analyze samples, and the expertise needed. This can result in high per-sample costs, and obtaining results in a rapid fashion will depend on the analytical lab.

Pharmaceuticals associated with CAFOs may be regionally or temporally specific. To be effective, CAFO operators would need to disclose the list of pharmaceuticals being used at the time of the study.

Finally, correlation to specific compounds and fecal indicator bacteria, and an understanding of environmental degradation rates or persistence especially in different matrices (and possible interference with detection), remain unresolved at this time for all candidate pharmaceuticals.

Conclusions and Recommendations for Chemical Methods

Chemical markers appear to be useful for researchers with the resources to conduct the testing and the expertise to interpret the results. Many of the publications acknowledge there is no one-size-fits-all method that can efficiently identify unknown sources of fecal bacteria. Researchers suggest that chemical methods could be used to augment more conventional fecal indicators such as fecal coliform and *Enterococci* (Cimenti et al., 2007; EPA, 2007d**)**. They also suggest understanding the fate and transport of pathogens is necessary before the appropriate indictor(s)/monitoring approach is chosen.

Of the current chemical-based, source tracking methods reviewed in this guidance document, three classes of indicators were consistently mentioned in the literature as having potential for source tracking:

1. Fecal sterols and hormones have been useful in successfully identifying multiple sources of fecal bacteria.
2. Optical brighteners have proven to be the most inexpensive and fastest way for indentifying human sources when optical brighteners are present in substantial levels and when waterbodies are free from inhibitors.
3. Pharmaceuticals, specifically carbamazepine and diphenhydramine, have been shown to be highly specific to humans.

Currently there is no chemical method that has been developed which provides direct links to a given fecal source (specifically who or what is responsible). Proving ownership of fecal sources will continue to involve a weight-of-evidence approach. This requires not only an understanding of fecal fate and transport, but also cooperation between regulators and landowners. It is important to consider the limitations of all methods before substantial resources are invested in tracking down fecal sources.

Advantages and Disadvantages

The main advantages in using chemical-based fecal MST methods include:

- Analytical methods for chemicals are better refined than methods requiring culture, often having standardized methods.
- Many of the chemical compounds have longer holding times than biological samples, allowing for composite samples to be collected over time.

The main disadvantages of using chemical techniques include:

- The persistence of some of these chemicals is unknown.
- Method detection limits for many of these chemicals are inadequate, leading to low sensitivity and analogs of some of these compounds may be naturally occurring.

Quality Assurance and MST Techniques

Quality assurance (QA) has been defined as efforts to *"monitor, improve and assess… scientific practices"* (Kammin, 2011), and also as *"a set of activities designed to establish and document the reliability and usability of measurement data"* (Kammin, 2010).

It is critical to assess all new analytical techniques, including molecular and biochemical MST techniques, through the lens of QA. Evaluating how any new method performs in the real world is essential to establishing the utility of that method.

For these MST techniques, important criteria for method assessment include:

- Method documentation and standardization
- Ruggedness testing
- Ease of adaptation/cost and required expertise
- Accuracy (specificity) for bacterial source identification
- Method precision/reproducibility
- Quantitation in MST
- Timeliness in MST reporting
- Method assumptions
- Study design

Following is a discussion of these issues in the context of molecular and biochemical MST techniques.

Introduction

What are the characteristics of the ideal MST method? The method should be:

- Inexpensive
- Quickly performed
- Infallibly accurate in the identification of source organisms or association with waste streams
- Formally published by EPA
- Must contain SOPs for sampling, positive control collection/spiking, and other field procedures
- Round-robin validated for ruggedness
- Accessible to labs with limited financial resources and academic expertise
- Widely available at commercial and government labs

However, the Washington State Department of Ecology (Ecology) has identified the following QA issues associated with currently used molecular and biochemical MST techniques:

- Techniques do not have regulatory approval.
- Techniques are still actively evolving.
- Many techniques can be reliably performed only by scientists with advanced training.

- Techniques commonly produce both false negatives and false positives.
- There is only a very small group of labs available to perform MST work.
- Lab timeliness in reporting (turnaround) has been a problem for Ecology molecular MST studies.
- Many MST "studies" are poorly designed and are inadequate in terms of frequency of sampling, number of samples, and sampling locations.
- SOPs generally have not existed for these techniques; this is beginning to change with the recent publication of two PCR/MST SOPs by Harwood et al. (2011).
- Due to lack of internal expertise, Ecology is currently unable to accredit MST techniques.
- For library-dependent techniques, DNA libraries become obsolete due to bacterial evolution and plasmid transfer.
- Some library-independent techniques (PCR, qPCR) amplify DNA from both viable and non-viable organisms.

All these issues must be addressed adequately before a molecular MST method can be considered robust, mature, and widely useable. Stoeckel and Harwood (2007) summarize:

> "Although there has been significant progress in the MST field over the last ten years, variability among performance measurements and validation approaches in laboratory and field studies has led to a body of literature that is very difficult to interpret, both for scientists and end-users."

Criteria for Assessing MST Techniques

Method Documentation and Standardization

As of the writing of this paper (November 2011), there are no promulgated methods for any of the molecular or biochemical MST techniques. Promulgation is the final step in the formalization of an EPA method and involves publication of that method in the Federal Register. Additionally, there are no draft or preliminary molecular or biochemical MST methods published by EPA or the Standard Methods (SM) committee. Currently, the status of these MST techniques is not acceptable for their use in a regulatory context.

Draft methods published by EPA are needed for several of the MST techniques. In this way we can begin to test the methods in the real world and provide iterative information to improve and stabilize the methods.

The disclaimer to the EPA (2005) *Microbial Source Tracking Guide* is of interest here. It states:

> "…EPA does not support or condone any of the uses of the MST data presented here… This document does not impose legally binding requirements on states, authorized tribes, or the regulated community and does not substitute for Clean Water Act (CWA) or Safe Drinking Water Act requirements, EPA's regulations, or the obligations imposed by consent decrees or enforcement orders."

Clearly, in 2005 EPA considered these techniques advisory.

Ruggedness Testing

Ruggedness testing is required for the evaluation of all molecular and biochemical MST techniques.

Huber defines ruggedness as "…a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst" (Huber, 2007). Ruggedness would usually be determined by a multi-laboratory study in which all labs would analyze identical sub-samples and results would be assessed. This type of study is normally referred to as "round-robin" testing. In molecular MST ruggedness testing, there are several criteria to assess. These include:

- Accuracy of the analytical method toward the target.
- False negative rate, defined as *frequency of failure to identify a source when it is present in a sample*.
- False positive rate, defined as frequency of *identifying a source when it is not present in a sample*.
- Reproducibility of test results between laboratories.

Unfortunately, the molecular and biochemical MST techniques are known to produce ambiguous results, false positives, and false negatives. These issues will be discussed in more detail later in this paper.

Ease of Adaptation/Cost and Required Expertise

Many of the library-dependent MST techniques are expensive due to the complexities of library development and maintenance. Because of these complexities, as well as ambiguity in results, these types of MST techniques are no longer widely used. A laboratory that conducts indicator bacteria testing could be set up to perform conventional PCR for about $15,000. However, the instrumentation required for the library-independent qPCR and PCR techniques is also very expensive, often costing more than $100,000 U.S.

In addition to the high cost of entry into molecular MST, the expertise required to run and interpret the results from these techniques is greater than that required to culture indicator bacteria. Advanced degrees would typically be required for generating results with some measure of reliability.

Simpson et al. (2007) comment extensively on cost and training issues related to MST.

> "The equipment and technical expertise necessary to engage in a MST study is often limiting. The start-up and running cost associated with the use of a given molecular technique is more expensive than for a biochemical profiling method. Another concern relates to the level of education or technical training needed for technical support personnel to perform the actual experiments. In general, biochemical methods are less

difficult or cumbersome than molecular methods, which do require a higher level of theoretical and practical training."

Pillai and Vega (in Santo Domingo and Sadowsky, editors, 2007) state the concerns of the food industry regarding the expanding use of MST, which are similar to the issues the environmental industry has:

"…the cost, the level of technical expertise required (to perform the test and interpret the test results), and the laboratory infrastructure required."

Accuracy (Specificity) in Bacterial Source Identification

Accuracy in the identification of target source species, also known as specificity, is critical for the effective use of MST. However, the molecular and biochemical MST techniques are known to produce both false positive and false negative results and to misidentify source organisms. The intent of this section is to highlight both historical and current concerns with specificity in MST testing.

Ecology has found problems with accuracy in identification and false negatives in testing molecular MST techniques. Mathieu (2010) discussed these issues in a communication with EPA Region 7. Mathieu noted that a contract lab conducting MST testing for Ecology determined all (3) positive blind controls to be negative. Without this necessary QA information, the study was determined to be unusable.

Stoeckel et al. (2004) conducted a study comparing seven molecular MST protocols using *E. coli*. They concluded that due to false positives, interpretations for most protocols would be hindered. They also concluded that, "Given the results described in this paper and those by other investigators, it is crucial that thorough reliability assessment of the chosen protocol be done before and during attempts to apply these protocols to a given environmental setting." Finally, they reported that five of the seven test protocols "would have made inaccurate reports of contributing sources."

Another example of a proposed protocol which was later discovered to produce ambiguous results is the use of the Enterococcal Surface Protein (*esp*) as a genetic marker. Byappanahalli et al. (2008) reports, "the differential occurrence of the *esp* gene in the environment and its association with enterococci from nonhuman sources may weaken its use as a reliable marker of sewage contamination."

McLain and co-workers found that "our results strongly demonstrated the potential for cross amplification of human-specific PCR assays with fish feces, and may call into question the results of studies in which these *Bacteroides*-specific molecular markers are used to quantify human fecal contamination in waters where fish contribute to fecal inputs." (McLain, 2009). This is an example of a thought-to-be specific molecular MST method later found to produce ambiguous results.

Method Precision/Reproducibility

One of the recommendations of this paper is to duplicate 50% of field samples. Performing duplicates at 50% frequency is equivalent to performing confirmatory samples at 50% frequency. It is similar to the use of dual column analysis of organic compounds by gas chromatography (GC) for the confirmation of detection capabilities. Because detection in GC can be ambiguous, samples are analyzed using two dissimilar columns, which yield unique retention times for compounds of interest. Detection is not verified unless a specific compound elutes at the correct retention time for both columns. For an example of a dual-column method, see EPA Method 8082 at www.epa.gov/wastes/hazard/testmethods/sw846/pdfs/8082a.pdf (EPA, 2007c).

Because the molecular and biochemical MST techniques often yield ambiguous results, false positives, and false negatives, 50% duplication of all samples can help to provide confirmatory evidence that the chosen method is both specific and reproducible. It can also provide evidence regarding unacceptable reproducibility and specificity.

Quantitation in MST

Harwood and Stoeckel (2007) succinctly summarize quantitation in MST:

> "… the ability of any MST method to quantitatively determine the relative contributions of fecal contamination has not been convincing demonstrated."

They advance the use of blind samples spiked with fecal material in known proportions to evaluate the quantitation performance of the MST method(s).

For library-dependent techniques, quantitation is confounded by:

- Survival and naturalization of target bacterial species in natural waters
- Misclassification of sample isolates *ibid.*

For library-independent techniques, issues associated with quantitation include:

- For EPA qPCR Method B (EPA, 2010), and qPCR techniques in general, possible method bias based on potential differences in the number of 16s rRNA gene copies was found in calibrator and target organisms. This bias would affect accuracy of quantitation for this "quantitative" method.
- Olson and Gedalanga (2009a, 2009b) discuss positive biases found in PCR and qPCR due to the known issue of amplification of non-viable DNA from injured or dead sources. This issue will cause over-estimation of bacterial concentration. They are treating samples with ethidium monoazide bromide (EMA) in an attempt to eliminate this interference.

Timeliness in Molecular MST Reporting

Timeliness and acceptable sample turnaround time remain a matter of concern for Ecology MST studies and have had impacts on the quality of our reports. Because of the constantly changing

temporal and spatial environmental conditions in riverine environments, it is crucial that source tracking data be developed as quickly as possible. High water events can alter sources of contamination and even change the course of a river, which may significantly change the microbial characteristics of the aqueous environment. These environmental changes can impact the usability of generated source data. Mathieu (2010) provides an example of problems with timeliness in reporting of MST results. Results for a *Bacteroides* ribotyping study were received between 363 and 477 days after the contractual 90-day requested turnaround time had expired.

Method Assumptions

Rochelle and De Leon (2005) in their MST review, speaking in the context of library-dependent methods, discuss big-picture assumptions upon which the use of MST techniques is predicated. The question they pose is:

> "Is the assumption of unique host-adapted strains valid? If this assumption is incorrect, or only correct in some instances, then no amount of technique refinement, technique comparison, or development of new techniques will improve the reliability of source tracking tools to the point where it can be categorically stated that a single particular source is responsible for any given contamination event."

This is a key assumption that has not been resolved.

Study Design

Study design is critical if MST techniques are to provide useful and useable data. It is very possible to design a MST study that, either unintentionally or deliberately, produces misleading or erroneous results or conclusions based on an inadequate sampling design. Critical design factors for molecular MST are discussed by many reviewers, including:

- EPA, 2011
- EPA, 2005
- Santo Domingo and Sadowsky, 2007a
- Santo Domingo, 2007b
- Rochelle and De Leon, 2005
- Field and Scott, 2007
  http://cws.msu.edu/documents/MicrobialSourceTrackingWhitePaper.pdf
- Stoeckel and Harwood, 2007

EPA (2011) discusses several critical factors to consider in MST study design. These include:

- Magnitude and frequency of elevated bacteria concentrations and water quality criteria exceedances.
- Spatial variation and temporal trends in bacteria concentrations and exceedances.
- Flow conditions under which exceedances occur (e.g. baseflow versus storm flow).
- Land use type and distribution.

Standard Operating Procedures

Santo Domingo et al. (2007b) discuss the lack of SOPs for MST techniques. They state:

> "To set performance standards for an individual method, it will be necessary to establish standard operating procedures (SOPs). SOPs will be needed for all the different steps of the experimental design (i.e., from sample collection to data analysis)." They continue:
>
> "Eventually a laboratory certification program will need to be developed, just as laboratories that analyze drinking water samples that are used for compliance testing in the US must be certified."

In a recently published report (Harwood et al., 2011) from the Water Environment Research Foundation (WERF), formal SOPs on two PCR techniques are presented. These SOPs conform in format and content to the published EPA SOP guidance (EPA, 2007b). This is a significant development and points to the continued evolution and formalization of these techniques.

Recommended Quality Assurance Protocols for Molecular and Biochemical MST Techniques

EPA (2005) provides a useful discussion of over-arching QA issues for molecular and biochemical MST techniques. They state:

> "These measures are organized into five QC issues including specificity, precision, control samples, QA documentation, and minimum number of controls."

The molecular and biochemical MST techniques have several QA issues associated with their performance. Because of this, an extremely rigorous QA regime is recommended when these techniques are used. This would include the following sample types and test techniques:

- 50% field duplicates (50% of field samples duplicated to demonstrate reproducibility).
- Blind field positive controls (fecal material of all potential source organisms).
  - SOP required for positive control sample collection and spiking process.
- Blind field negative controls (field blanks).
  - Requires DNA free water.
- Use of multiple MST techniques simultaneously ('toolkit' or 'toolbox' approach).
- Supplementation of MST with
  - Visual observations – otherwise known as a sanitary survey to establish potential sources.
  - Conventional coliform testing.

The intent of the QA requirements above is to provide sufficient QA information so that each study can be assessed on a stand-alone basis. Acceptance limits are not advanced at this point; rather, for methods associated with a published SOP or set of SOPs, performance information should be compiled and evaluated. It can be reasonably expected that as familiarity with the

methods increase, and method and lab performance is documented, these initial QA requirements may be scaled back.

Investigators should consider the use of several MST techniques, each one optimized for a particular source organism.

Harwood et al. (2009) are to be commended for their evolving and rigorous approach to QA in the testing of library-independent MST techniques in the Gulf of Mexico. They conclude,

> "MST techniques, however, require laboratory capabilities beyond those needed for culture-based enumeration of indicator organisms, and these methods must be standardized among state or regional laboratories if they are to be utilized beyond the research realm."

These researchers are developing regional SOPs for three high-specificity protocols for the detection of human sewage. Laboratories from Florida and Mississippi are participating in the validation of these protocols.

Quality Assurance and Chemical Source Tracking Methods

A brief mention should be made of the chemical source tracking techniques. These techniques are usually based on established and promulgated regulatory methods, and have rigorous and detailed QA protocols associated with them. See EPA Method 1694, for pharmaceuticals and personal care products, for an example of rigorous QA protocols in a complex analytical method (EPA, 2007a).

Before Conducting an MST Study

Before using MST to identify sources of fecal contamination, other means to identify sources should be considered. MST should not be used before conventional fecal source identification methods, such as targeted instream monitoring for bacteria, and sanitary and watershed surveys. A detailed account of such an approach is given in Propst et al. (2011). A common recommendation for using MST is that MST techniques should be used to supplement, rather than replace, current techniques and tools for evaluating and identifying bacteria sources (EPA, 2011).

Targeted Approach for Identifying Bacteria Sources

There are several *targeted approaches* recommended in various scientific papers. In Wapnick et al. (2009), the targeted approach includes:

1. Collect indicator bacterial organism data to prioritize locations with likely fecal contamination.
2. Conduct site-specific field surveys to identify the sources of contamination and assess their potential risk. This is a comprehensive source survey that may include review of historical data, field surveys, one-on-one interviews with local stakeholders, and field reconnaissance. Potential sources are identified, and management actions for removing the sources are developed.
3. Conduct water quality sampling including MST techniques. Due to the expense associated with MST, this final component is reserved for watersheds where other methods have failed to identify and prioritize bacterial sources.

Another targeted approach recommended by Hartel et al. (2008) and McDonald et al. (2006) includes the following steps:

1. Separate sampling into different environmental conditions (e.g., baseflow and storm flow; or flood and ebb tidal cycles). Generally different environmental conditions, like storm events, can produce increased fecal contamination.
2. Identify potential sources of fecal contamination by talking to stakeholders and looking at land use.
3. Combine this knowledge with targeted sampling of the contaminated waterway, collecting as many samples from the water body and tributary (as appropriate) in one day. Each site is identified using global positioning system (GPS) coordinates.
4. Place the data in a geographic information system (GIS) database to identify hotspots (areas of high bacteria levels) of fecal contamination on a map. The targeted sampling is repeated at the hotspots as needed to limit the area of interest to the smallest geographic area possible. Limiting the samples to a small geographic area reduces bacterial changes with geography and animal diet.
5. The last step may be to conduct MST. In most cases, persistent sources of fecal contamination are obvious, and MST is unnecessary.

Considerations for Using MST Techniques

While MST can provide useful information for identifying and understanding bacterial sources, there are a number of issues that are important to consider before deciding to use MST (EPA, 2011):

- There are no EPA standard methods for MST techniques. Reproducibility of results across laboratories is an issue due to lack of standardized laboratory techniques.
- The analytical precision and accuracy of MST techniques can vary greatly between techniques, laboratories, and individual water samples.
- Depending on the method used, laboratory analysis can be very intensive, expensive, and have a slow turnaround time.
- For library-dependent techniques, there is not clear or consistent guidance on appropriate library size.
- For library-dependent techniques, there are concerns about the transferability of existing libraries across time, differing environmental conditions, and geographic areas.
- Field work is intensive because MST studies can require numerous water samples to capture different conditions and sources, and library-dependent studies require fresh fecal samples from sources to build or supplement the DNA library.
- Bacteria isolates from collected water samples represent a small portion of the population present in the water sample (*and* an even smaller portion of the waterbody population). The relative presence of sources in the watershed is difficult to determine.
- There are not uniform standards or reference materials for sampling and measurement designs.
- The technology should be used only when other means of determining and removing fecal sources have not been successful in reducing bacteria in the waterbody.

Conclusions and Recommendations for MST Techniques

Microbial source tracking (MST) techniques are experimental science. Methodologies have not been formalized as documented methods with associated standard operating procedures (SOPs) and quality assurance (QA) protocols. Users of these techniques need to demonstrate, on an on-going and real-time basis, that these techniques produce acceptable levels of accuracy in identification of sources and reproducibility both within labs and between labs. At this time no single MST technique is capable of determining all possible sources accurately. Promulgated, regulatory methods are needed, and a certification program for MST labs is needed.

The Washington State Department of Ecology's (Ecology's) greatest concern about all current MST techniques is the quality of the data, especially considering the expense of MST studies. Currently no MST techniques have established EPA-approved methodologies. In addition, quality control (QC) sampling to assess precision and accuracy of results is not included in the majority of MST studies.

Due to the experimental nature and cost of MST techniques, these methods should be used as a last resort to determine sources of bacterial contamination. A targeted approach and/or conventional bacteria source identification techniques should be used before MST, keeping in mind the fact that (1) conventional indicator bacteria such as fecal coliforms may be contributed from almost any animal in the watershed, and (2) extended survival of these organisms in aquatic environments and even soil is possible. MST techniques, properly used, are another tool that may provide useful information on sources contributing to bacterial pollution.

Recommendations for using MST techniques include:

- An understanding of the possible fecal sources and pathogen transport mechanisms (e.g., storm events, tidal cycle) is necessary before the appropriate MST indictor(s)/monitoring approach is chosen.
- The limitations of MST techniques must be fully understood before proceeding with MST testing.
- Because the QA status of these experimental techniques is unacceptable, real-world use of the molecular and biochemical MST techniques must include the following QA sample types:
  - Duplicates (50% of field samples duplicated to demonstrate reproducibility). 50% of samples.
  - Blind field positive controls (fecal material of all potential source organisms). A minimum of one per each potential source per study.
  - Blind field negative controls (field blanks). 20% of samples.

- Due to concerns about the experimental nature of MST techniques, we recommend use of multiple techniques for the same source to increase confidence in identification. This also allows for more potential source types to be investigated.

- For molecular and biochemical MST techniques, library-independent methods are recommended over library-dependent methods. Library-dependent methods are not recommended due to their expense, difficulty in building a sufficiently robust library, and their poor performance in MST comparison studies.

- Before conducting an MST study, source feces from the study area should be tested to confirm the source-specific MST indicator or marker is present. Source-specific markers can vary depending on genetics, location, diet, environment, and over time.

- Less expensive source tracking techniques, such as *in-situ* monitoring for optical brighteners or presence/absence tests for fecal sterols, should be considered for supporting evidence for the presence of human sewage.

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Appendix. Glossary, Acronyms, and Abbreviations

Glossary

Anthropogenic: Human-caused.

Effluent: An outflowing of water from a natural body of water or from a man-made structure. For example, the treated outflow from a wastewater treatment plant.

Exceeds criteria: Does not meet criteria.

Fecal coliform: That portion of the coliform group of bacteria which is present in intestinal tracts and feces of warm-blooded animals as detected by the product of acid or gas from lactose in a suitable culture medium within 24 hours at 44.5 plus or minus 0.2 degrees Celsius. Fecal coliform bacteria are "indicator" organisms that suggest the possible presence of disease-causing organisms. Concentrations are measured in colony forming units per 100 milliliters of water (cfu/100 mL).

Fingerprint: A multi-parameter chemical signature (distinctive chemical pattern) used to characterize the source of contaminants in an environmental sample or to differentiate the sample from contaminants present in samples representing background conditions.

Library: Database of fingerprints from individual bacterial isolates, obtained from potential fecal pollution sources.

Microbial: Pertaining to viruses, bacteria, protozoa, and certain invertebrates such as helminthes.

Microbial Source Tracking (MST): Approach intended to identify the fecal sources impacting a water system. Other terms that relate to MST are bacterial source tracking and fecal source identification.

Nonpoint source: Pollution that enters any waters of the state from any dispersed land-based or water-based activities, including but not limited to atmospheric deposition, surface-water runoff from agricultural lands, urban areas, or forest lands, subsurface or underground sources, or discharges from boats or marine vessels not otherwise regulated under the NPDES program. Generally, any unconfined and diffuse source of contamination. Legally, any source of water pollution that does not meet the legal definition of "point source" in section 502(14) of the federal Clean Water Act.

Pathogens: Disease-causing microorganisms such as bacteria, protozoa, viruses.

Point source: Sources of pollution that discharge at a specific location from pipes, outfalls, and conveyance channels to a surface water. Examples of point source discharges include municipal wastewater treatment plants, municipal stormwater systems, industrial waste treatment facilities, and construction sites that clear more than 5 acres of land.

Pollution: Contamination or other alteration of the physical, chemical, or biological properties of any waters of the state. This includes change in temperature, taste, color, turbidity, or odor of the waters. It also includes discharge of any liquid, gaseous, solid, radioactive, or other substance into any waters of the state. This definition assumes that these changes will, or are likely to, create a nuisance or render such waters harmful, detrimental, or injurious to (1) public health, safety, or welfare, or (2) domestic, commercial, industrial, agricultural, recreational, or other legitimate beneficial uses, or (3) livestock, wild animals, birds, fish, or other aquatic life.

Promulgated: Published or issued.

Source tracking: The science of searching for an organism(s) or signal in the environment that can identify the contribution of a particular type of animal or human to fecal contamination in the environment.

Acronyms and Abbreviations

ARA	Antibiotic Resistance Analysis
CAFO	Confined animal feeding operation
CUP	Carbon Utilization Profiling
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
Ecology	Washington State Department of Ecology
EPA	U.S. Environmental Protection Agency
F+RNA coliphage	Male-specific coliphage
GC/MS	Gas chromatography-mass spectrometry
GIS	Geographic Information System software
MAR	Multiple Antibiotic Resistance
MST	Microbial Source Tracking
n	number
NUP	Nutrient Utilization Patterns
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoreses
QA	Quality assurance
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SCCWRP	Southern California Coastal Water Research Project
SOP	Standard operating procedures
spp.	Species
USGS	U.S. Geological Survey