Critical Review

Microbial Source Tracking: State of the Science

JOYCE M. SIMPSON,
JORGE W. SANTO DOMINGO,* AND
DONALD J. REASONER

U.S. Environmental Protection Agency, Office of Research and Development, Water Supply Water Resources Division, Cincinnati, Ohio 45268

Although water quality of the Nation's lakes, rivers and streams has been monitored for many decades and especially since the passage of the Clean Water Act in 1972, many still do not meet the Act's goal of "fishable and swimmable". While waterways can be impaired in numerous ways, the protection from pathogenic microbe contamination is most important for waters used for human recreation, drinking water and aquaculture. Typically, monitoring methods used for detecting potential pathogenic microorganisms in environmental waters are based upon cultivation and enumeration of fecal indicator bacteria (i.e. fecal coliforms, *E. coli*, and fecal enterococci). Currently, there is increasing interest in the potential for molecular fingerprinting methods to be used not only for detection but also for identification of fecal contamination sources. Molecular methods have been applied to study the microbial ecology of environmental systems for years and are now being applied to help improve our waters by identifying problem sources and determining the effect of implemented remedial solutions. Management and remediation of water pollution would be more cost-effective if the correct sources could be identified. This review provides an outline of the main methods that either have been used or have been suggested for use in microbial source tracking and some of the limitations associated with those methods.

Introduction: What Are Current Water Quality Issues?

Today, there are still waters in the U.S. that do not meet the 1972 Clean Water Act goal of "fishable and swimmable". For example, approximately 35, 45, and 44% of assessed rivers, lakes and estuaries, respectively, have been classified as impaired based on the pollutant levels (1). Achievement of this goal was expected to eliminate waterborne microbial disease as a leading cause of public health problems. However, in terms of the Clean Water Act, water quality designations in the United States still vary from unimpaired (pristine) to impaired (highly polluted). Water quality impairment can result from a wide array of pollutants/pollutant classes including temperature (thermal pollution), sediment, pathogens, nutrients, metals, dissolved oxygen, pH, pesticides and other organic chemicals, to name a few. These pollutants can be transmitted to the public via recreational waters, surface water and groundwater, thereby potentially putting entire communities at risk. Water quality standards under the Clean Water Act and amendments (2–4) are the State's goals for individual water bodies and provide the legal basis for control decisions under the Act. Water quality monitoring activities provide the chemical, biological and physical data needed to determine the present quality of a State's waters and to identify the pollutant sources to those waters. Section 303(d) requires that total maximum daily loads (TMDL) be established for the impaired waters by states, territories, and authorized tribes with oversight by the U.S. E.P.A. (5). Impairment of water quality may involve multiple pollutants or pollutant classes, requiring the implementation of one or more pollution control remedies (e.g. best management practices, BMPs) to address all the pollutants that contribute to the overall water quality impairment. Thus, a TMDL for the achievement of State water quality standards must be developed for each pollutant or pollutant class in waters designated as impaired. Control techniques (BMPs and/or effluent limits) must be implemented for both point and nonpoint sources to meet the TMDLs.

Chemical Issues. Chemical quality of the water varies primarily with the variety of industrial and agricultural sources found in the watershed. Agricultural sources contribute animal wastes, commercial fertilizers, pesticides and herbicides, and nutrients (phosphorus and nitrogen) to the water via storm event runoff. In some geographic areas, geological formations and soil may contribute inorganic chemical contaminants such as arsenic, iron, copper and sulfur compounds. The intent is to develop plans to reduce pollutant source loads that contribute nutrients (primarily nitrogen and phosphorus), reduce the biological oxygen demand (improve the dissolved oxygen budget), and minimize toxic substances that enter the water.

Physical Issues. Physical aspects of water quality involve primarily suspended solids that cause turbidity in the water and reduce light penetration, thus interfering with photosynthesis in aquatic plants. Suspended solids carried by surface runoff also settles out of the water column to create suffocating coatings on rooted plants, attached growth on rocks and other surfaces and interferes with essential life processes of aquatic plants and animals. The settled solids form sediments that may be contaminated with toxic materials, depending on the source(s) and types of contamination. Thermal pollution is another physical characteristic of waters used for industrial plant cooling or power plant cooling that interferes with essential life processes of many types of aquatic plants and animals. Thermal pollution must generally be mitigated to preserve the health of aquatic communities downstream from the thermal source.

Microbiological Issues. Of the designated uses of water listed in section 303(c) of the Clean Water Act, protection from pathogenic microbe contamination is most important for waters used for recreation (primary and secondary contact), public water supplies, aquifer protection, and

protection and propagation of fish, shellfish and wildlife. Microbiological impairment of water may be assessed by monitoring, usually for the presence of indicator bacteria such as fecal coliforms, *E. coli* or fecal enterococci. These microorganisms are associated with fecal material from humans and other warm blooded animals and their presence in water may also signal the presence of enteric pathogens.

Specific pathogen monitoring may also be used to assess impairment of water. The U.S. EPA has published a protocol for developing pathogen TMDLs that provides guidance for this process (*5*). Exceeding the criteria developed for *E. coli* and enterococci bacteria indicates that the water may contain human enteric pathogens (such as hepatitis A virus) that could cause illness in exposed persons. Detection of pathogens and tracking them to their source(s) is a topic of intense interest in view of the current TMDL requirements. Microbial Source Tracking (MST) is one approach to determining the sources of fecal pollution and pathogens affecting a water body. It is based on the assumption that, using an appropriate method and appropriate indicator bacteria, sources of the microorganisms can be found and characterized as to animal or human origin.

What Is a TMDL and Which Are the Problem Sources?

A TMDL establishes the maximum pollutant load that a water body can receive and still meet water quality standards and provides the basis for establishing water quality controls. TMDLs are also used to establish waste load allocations among point and nonpoint pollutant sources. Nonpoint sources such as agriculture, forestry, wildlife and urban runoff are continuous sources of impairment to water quality (e.g. agricultural runoff after a rain event or unrestricted access of livestock and wildlife to rivers and streams). Yet it is exceedingly difficult to determine the origin of particular contaminants within these categories. Management and remediation of water pollution would be more cost-effective if the sources could be correctly identified and remediation efforts allocated proportionally.

Since bacteria are among the most common biological pollutants affecting assessed rivers and streams and can provide evidence of possible fecal contamination that may cause waters to be unsafe for swimming and other recreational activity, indicator organisms are frequently monitored (*1*). Although it is important to distinguish point vs nonpoint contributions to the problem (e.g. treatment plant [point] vs wildlife [nonpoint]), it is also meaningful to distinguish between various nonpoint animal sources. To alleviate the overall fecal pollution problem, it is critical to determine the origin of the bacterial contamination whether the source is farm animals, companion animals, or wildlife. Of particular interest are those of commercial agricultural significance, namely production facilities for cattle, swine, poultry, sheep, and horses due to the large number of animals present.

Agricultural and Concentrated Animal Feedlot Operations (CAFO). Agricultural based fecal contamination of streams, rivers and groundwater has several origins, including runoff from manure deposited on grazed pastures, runoff from the application of fecal slurries to fields (both surface spread and injection) (*6*−*8*), and runoff/discharge from CAFO fields and barns. Cattle grazing increases fecal coliform levels in agricultural runoff as compared with background levels, and it has been reported that 80% of water samples from areas surrounded by pastures exceed water quality standards (*9*, *10*).

Urban Runoff and Septic Systems. Urban stormwater fecal coliform concentrations often far exceed the concentrations considered safe for water contact (*11*). However, fecal coliform sources in stormwater discharges are not always easily attributable to broken sanitary sewer lines, sewer overflows, leaking septic tanks or illegal discharges (*12*). A complication is that while the runoff from urban watersheds originates from both pervious and impervious surfaces (e.g. lawns and sidewalks respectively), the impervious surfaces are almost always more hydrologically active because their water storage capacity is minimal. Additionally, the amount of fecal pollutants washed off by rainfall depends on the amount of feces that accumulated during the preceding dry period and the volume and velocity of the runoff during a rain event. Moreover, varying contributions from residential, commercial and industrial areas into connecting storm drain systems can further complicate the issues regarding originating source.

Wildlife. Nonpoint source contributions from wildlife can be substantial, are difficult to identify and even harder to remediate. Typical wildlife which have been linked to fecal problems include deer, dogs, raccoons, cats, waterfowl, and to a lesser extent, rats (*13*, *14*). Once sources are identified, remediation can consist of instructing owners in responsible pet care and employment of licensed trappers/hunters to remove overpopulated wildlife. Fencing, establishment of riparian zones, and other practices that limit access of wildlife to streams might reduce wildlife loading rates. However, since restricting access of wildlife to streams and rivers is not practical, better wildlife management practices need to be developed to ensure that ecosystems are not out of balance.

Best Management Practices for Remediation of Water Quality Issues

Once a TMDL is developed, plans must be made and implemented for reducing the loading of the target pollutant. The BMP treatment method(s) chosen to attempt to reduce pollutant loading will depend on the type and nature of the pollutant and the characteristics of the watershed. For example, if reduction of nutrients (phosphorus and nitrogen from agriculture), sediments and bacteria is the goal, exclusion of livestock, stream bank protection and riparian restoration practices might be evaluated (*15*). On the other hand, if pathogen removal from animal wastewater (*16*) or municipal wastewater (*17*) is the goal, constructed wetlands (surface or subsurface flow) might be chosen as the most appropriate BMP. Other BMPs are available depending on the nature of the pollutant but will not be addressed in this review. Several researchers have begun to examine the effects of BMP implementation on reduction of fecal contamination from pastures and feedlots; the reader is referred to ref *18* for further review.

Microbiological Methods Used in TMDL Studies

The microbiological examination of surface waters has obvious implications pertinent to two watershed issues. First, the introduction of bacteria relevant to public health into watersheds is indirectly regulated using TMDL criteria. Monitoring of a microbiological TDML is normally achieved by determining the densities of bacterial indicators of fecal contamination, (e.g., fecal coliforms, *E. coli* and fecal enterococci). Second, the identification of fecal pollution sources impacting watersheds is increasingly being performed using MST methods (Table 1). Those methods that use bacteria, in particular, as target organisms are also known as bacterial source tracking (BST) methods. Most MST methods rely on the fingerprint profiles of fecal bacteria, although viruses, protozoa, and chemical markers have been suggested as capable of discriminating between human and animal fecal sources. As described by Sinton et al. (*19*), investigations into the use of microbes for fecal source tracking have mainly involved four approaches: 1) speciation − finding species indicative of the source; 2) biochemical reactions − biochemical tests to differentiate sources; 3) assemblages and ratios − comparison of two or more fecal

TABLE 1. Example Case Studies of MST Techniques Used in Watersheds

technique	library	target	description	ref
Molecular Methods				
ribotyping	yes	*E. coli*	Genotypic method used to determine origin of fecal bacteria in water from Idaho and Georgia to evaluate method ability to discriminate not only source but also geographical variation.	(*62*)
repetitive PCR (rep-PCR)	yes	*E. coli*	Used to generate DNA fingerprints from *E. coli* isolated from human and animal sources. Multivariate analysis successfully differentiated isolates into corresponding groups. Data indicated that it was possible to differentiate between human and nonhuman sources.	(*71*)
Pulsed Field Gel Electrophoresis (PFGE)	yes	*E. coli*	*E. coli* isolates from seasonally varied streams and sediment samples were compared to isolates from known fecal sources. Human vs nonhuman distinctions were made and evidence suggested that *E. coli* regrowth perpetuated elevated fecal coliform densities without regard to specific hosts.	(*14*)
Length Heterogeneity PCR (LH−PCR) and Terminal Restriction Fragment Length Poly-morphism (T-RFLP)	no	*Bacteroides-Prevotella*	Identified human and cow-specific genetic markers in fecal samples. Host-specific patterns suggested that there are species composition differences in *Bifidobacterium* and *Bacteroides-Prevotella* populations of human and cow feces.	(*63,64*)
Denaturing Gradient Gel Electrophoresis (DGGE)	yes	*E. coli*	Evaluated strain level differences in *E. coli* from bovine, poultry and human sources. Isolates from nearby streams yielded unique patterns with a few patterns matching those of the known sources. High diversity among environmental isolates made it difficult to ascribe more strains found in water specific hosts.	(*77*)
Nonmolecular Methods				
Antibiotic Resistance Analysis (ARA)	yes	Fecal Streptococci	Determined reliability and repeatability of method for identifying sources of fecal pollution. Patterns of isolate resistance to nine antibiotics were analyzed by discriminant analysis. ARA patterns differed among isolates from various sources of fecal pollution and could be used to identify these sources.	(*47,48*)
ARA	yes	*E. coli*	ARA patterns in *E. coli* were analyzed by cluster analysis and used to identify sources of fecal pollution in Apalachicola Bay. Sets of *E. coli* isolates were obtained from water, sewage, humans, andwild-animal feces. Data indicated that wildlife species were influencing water quality and further investigation was necessary.	(*50*)

bacteria population ratios; and 4) DNA profiles − fingerprints using genomic DNA or specific phylogenetic genes of fecal bacteria. While all these approaches have been used with different levels of success in the U.S., the use of phenotypic profiles, specifically antibiotic resistance analysis (ARA), has been the most commonly used in small watersheds. However, the application of phenotypic profiles is not as effective when larger geographical areas are under investigation. Since genotypic profiles may be more stable than phenotypic profiles and, theoretically, are capable of discriminating between different animal sources, DNA-based fingerprinting techniques have received increasing attention as potential tools for fecal-pollution source tracking. This review focuses on the DNA profiles and only briefly describes some of the other options available today.

Microbial Source Tracking Methods

The methods used in source tracking studies can be divided into two groups: those based on molecular methods and those based on biochemical methods. In addition, MST techniques may also be categorized as library dependent and library independent (Table 1). In this particular case a "library" is a collection of microorganisms from different potential sources as well as from the watershed under study. Most MST techniques used thus far have depended on the development of comprehensive libraries. Although the latter libraries are often made of several hundreds of isolates per potential source impacting the particular watershed (i.e., human, wildlife, and livestock), the minimum number of

isolates needed to perform statistically sound studies has not been determined. Most methods have not been thoroughly tested, although several have been successfully applied. For example, the states of Virginia and North Carolina have funded several studies where researchers used antibiotic resistance analysis (ARA), carbon utilization profiling (CUP), and ribotyping to pinpoint the primary sources of fecal contamination (*16*, *17*, *20*, *21*). Some of the methods and approaches used in source tracking will be described in more detail in the following sections.

Source Tracking Target Selection

One of the most important decisions in MST studies relates to target selection. In a broad sense, targets can be of microbial origin (e.g., *E. coli*) or of chemical origin (e.g., caffeine). The microorganisms suggested as adequate targets have included bacterial groups, protozoan oocysts and viruses (*22*). Two criteria that influence the approach to be used are as follows: 1) the number of sources contributing to the contamination and 2) the type of discriminatory power needed on a particular case (e.g., human vs animals or animal specific). Essentially, an approach that uses a target that is only capable of discriminating between animal and human contamination might not be adequate if it is necessary to discriminate between different animal sources.

Bacterial Targets. Four bacterial genera thus far have been used as target organisms in MST studies: *Escherichia*, *Enterococcus*, *Bacteroides*, and *Bifidobacterium*. Species belonging to these bacterial groups are normally present in

the feces of higher mammals and birds. MST studies involving *E. coli* and fecal enterococci have been performed with bacterial strains isolated from different fecal sources, while research based on nonculturing approaches have targeted *Bacteroides* spp. and *Bifidobacterium* spp. Thus far, while studies using *E. coli* isolates dominate the scientific literature, the use of fecal enterococci as target organisms has gained some popularity. One advantage of using *E. coli* and fecal enterococci is that they are relatively easy to grow. Other bacterial indicators, like anaerobic fecal bacteria (e.g. *Bacteroides* spp.) require special culturing equipment that is not common in most laboratories.

Protozoan Targets. Research with *Cryptosporidium spp.* oocysts has shown that there are genotypes specific to different animals (e.g., cow, dogs, and humans) and for this reason oocysts have been used to identify the probable origins of fecal pollution in stormwaters (*23*). Using this approach Xiao et al. (*24*) reported that humans and cattle feces were primarily responsible for the contamination of the surface water samples, although several genotypes associated with wild animals were also obtained. However, the levels of *Cryptosporidium spp.* oocysts in natural waters are relatively low, which translates into the need to concentrate oocysts from 40 to 100 L of water before a signal can be detected. Concentration of microbial biomass from large volumes of water increases the concentration of organic material that can interfere with nucleic acid manipulation. Therefore, failure to concentrate oocysts and ineffective nucleic acid extraction from environmental samples has precluded the use of this approach in most natural waters with modest levels of fecal contamination.

Viral Targets. Studies using coliphages, which are viruses that infect *E. coli*, have shown that there are four different groups of male specific (or F+) RNA coliphages. Group I coliphages are found in both human and animal feces, group II and III are predominantly associated with human fecal contamination, while group IV is predominantly associated with animal fecal waste (*25*). Recently, similar observations were made during a study investigating the prevalence of F+ RNA coliphages as indicators of animal fecal contamination (*26*). The detection of each of these groups in water samples can be performed using serological techniques or by using gene probes in hybridization assays. However, the genetic distinction between coliphages from different animals does not appear to be great enough to discriminate among various animal sources. Additionally, serotyping is expensive and time-consuming, thus the use of coliphage-based methods might be limited to screening for the presence/absence of animal and/or human fecal pollution. For further information regarding F+ RNA coliphage use in source tracking, the reader is referred to ref *19*. Other viral targets currently under investigation are the bovine enteroviruses (*27, 28*). These viruses have been isolated from cattle, deer, geese, and from the general environment encompassed within closed cattle lots. Viral variants were found to coexist in some areas, leading to the possibility that sequencing individual strains could provide clues to the origin of contamination.

Chemical Targets. The use of chemical targets has been suggested as an alternate approach to biological markers based on the premise that certain chemicals are only found in fecal samples. Different chemical compounds have been recently evaluated as tools to predict sources of fecal pollution (*29*). Most chemical markers have been used primarily to trace human contamination. For example, caffeine, fragrance materials, and fluorescent whitening agents (laundry detergent brighteners) have been under investigation due to their exclusive use by humans (*30*). Fecal sterols and fecal stanols found in humans are also promising sewage pollution markers. However, the long-term fate of these organic chemicals in environmental waters has not been studied in great detail. Additional studies to verify the host specificity of many of these compounds (e.g., fecal stanols, like coprostanol) are needed to confirm their potential value.

Immunological Targets. Another approach focuses on the identification of species-specific secretory immunoglobulin A (sIgA) in fecally impacted waters. sIgA is normally present in the feces of mammals, and it has been implicated in the protection of intestinal mucosa against enteric infections (*31*). Since sIgA excreted in relatively high concentrations by mammals, is associated with easy to concentrate particulate matter (e.g., feces), and can be detected using relatively easy to follow immunological procedures, its possible value as an indicator of fecal pollution of water is currently under investigation. Like any other chemical marker, the fate of sIgA once it enters a body of water is relevant to its potential usefulness. A long half-life would give the wrong impression of recent fecal contamination, while rapid degradation might underestimate the levels of fecal pollution. In addition, while the assays are relatively sensitive, the currently proposed approach requires concentrating a large volume of water (i.e., 100 gallons) which is cumbersome to perform in most field settings.

Use of Phenotypic Methods for Source Tracking

Most methods currently used to monitor microbiological TMDLs in watersheds depend on culturing bacterial indicators of fecal contamination (e.g., *E. coli* and fecal enterococci). Indicator organisms are used because they tend to occur in the same sources as pathogenic organisms (e.g. fecal material), are present in greater densities, and often are easier to identify using standard culture methods. While these methods provide an indication of the fecal pollution levels in surface waters, unfortunately, most culturing techniques tend to underestimate bacterial densities in environmental samples, especially when fecal bacteria are exposed to environmental conditions that can cause physical/physiological injury and stress (e.g., nutrient limitation and UV light). In addition, the identification of many bacterial water isolates is not always accurate via conventional phenotypic characterization. Although some environmental bacterial isolates can be identified using phenotypic traits, it can take several days before an accurate identification is achieved. Consequently, culturing methods cannot assess the microbiological quality of watersheds in a timely manner.

It is worth noting that methods that depend on culturing bacteria can introduce biases that only reflect trends for organisms that can be readily isolated from environmental waters. Thus, the level of diversity of an easy to culture species and the number of strains examined could play a role in the outcome of a MST study. For example, studies by Gordon and co-workers (*32, 33*) have shown that host specificity cannot explain the genetic variation of *E. coli* populations in rodents and other mammals. It has also been suggested that a large number of *E. coli* strains can be isolated from hosts of the same species living in close proximity and that the clonal diversity of *E. coli* changes as a result of adapting to conditions outside of the animal gut (*33*). The results from these studies argue against the use of enteric bacteria and other commensal bacteria in general, as target organisms in MST methods. Such a potentially high level of genetic diversity among commensal bacteria can seriously complicate the genetic analysis of isolates found in secondary habitats.

Fecal Coliform/Fecal Streptococci (FC/FS) Ratio. According to studies performed by Geldreich and co-workers (*34, 35*), human feces normally has a ratio of fecal coliform to fecal streptococci greater than or equal to 4.0, whereas ratios below 0.7 are associated with animal feces. Based on this criterion, several researchers have used the FC/FS ratio to differentiate between human and animal fecal pollution sources (*36, 37*). However the use of FC/FS ratio has been

strongly criticized, primarily due to the differences in fecal enterococci densities found in individuals with different diets (38) and the different effects that environmental factors (e.g., temperature and UV light) have on the survival of coliforms and streptococci bacteria (39−41). In fact, the environmental persistence of fecal enterococci in marine waters can directly affect the FC/FS ratio and, therefore, the interpretation of water quality data (42). Moreover, a recent study showed that the FC/FS ratio could not discriminate between human and domesticated animals fecal samples (10). Due to the inconsistent relationship between the ratio and pollution sources and the fact that the taxonomy of the fecal streptococci group was recently revised (43), it is not surprising that the use of the FC/FS ratio in source tracking has significantly decreased in the last two decades.

Antibiotic Resistance Analysis (ARA). A phenotypic based method uses antibiotic resistance patterns for fecal streptococci, enterococci, or *E. coli* to identify the fecal sources (Table 1). The premise is that human fecal bacteria will have greater resistance to antibiotics than those from animal sources (44). The fecal microbiota of animals should have significantly less and different resistance to the various antibiotics and concentrations used commercially (45). Fecal bacteria are grown in microtiter trays and then transferred onto a series of agar plates, each containing one specific antibiotic concentration. After incubation, each isolate is scored for growth or no growth, and a resistance pattern emerges that can be used in source differentiation. Since a replicator can be used to transfer bacterial cultures, hundreds of isolates can be processed in a week (45). In contrast with most molecular fingerprint studies, hundreds to thousands of isolates are typically analyzed in studies using ARA.

Discriminant analysis of antibiotic resistance patterns for fecal enterococci (21, 46−49), *E. coli* (50), and fecal coliforms (46, 51) has been used to differentiate between human and animal sources. A variety of sources were included for comparison in these studies, including humans, sewage, cattle, chickens, turkeys, dogs, pigs, raccoons, wild birds, wild animals, and pristine waters. Typical results from these studies indicated that the average rate of correct classification (ARCC) ranged from 62 to 94% when individual species were compared. When comparisons included just two categories (human vs nonhuman) ARCC values increased about 10%. However, when unknown source isolates (water isolates) were compared with the databases, ARCC values decreased to around 72%.

While it appears that the ARA approach may be appropriate to MST analysis of simple watersheds, caution is advised with its application to large-scale, complex systems due to potentially lower ARCC values for unknowns and the array of genetic variability present in these organisms resulting from different exposures to antibiotics during carriage in their hosts. This is particularly relevant when multiple sources of pollution are contributing to watershed contamination at relatively similar levels. ARA could also be difficult to interpret in scenarios where agricultural practices involve application of fecal materials to land. Another issue to consider is that ARA is susceptible to bias due to the transfer of plasmids (and other mobile genetic elements) carrying multiple antibiotic resistance genes. Acquisition or loss of a plasmid can change the resistance profile of an isolate, thereby adding another layer of complexity to the analysis and/or library construction. On the other hand, gene transfer might play a significant role only when the environmental conditions considerably prolong the survival of the targeted-bacterial group.

Carbon Utilization Profile (CUP). Another phenotypic technique, CUP is based on differences among bacteria in their use of a wide range of carbon and nitrogen sources for energy and growth. The BIOLOG system allows the user to rapidly perform, score, and tabulate 96 carbon source utilization tests per isolate and is widely used in the medical field for identification of clinical isolates. While, this method works 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 (45). Like ARA, the CUP method is relatively simple and allows for the analysis of hundreds of isolates in a short period of time. CUP has been discussed as a potential new tool in fecal source tracking (20); however, no peer-reviewed publications concerning the successful use of this method were available at the time of this review. Since most commercially available kits for biochemical profiling (e.g., BIOLOG) have been developed for the identification of clinical bacterial isolates, the development of microtiter plates that contain substrates compatible with environmental enterococcus isolates seems to be a prerequisite for the application of CUP in MST studies.

Use of Nucleic Acid Based Molecular Methods for Source Tracking

Recently, nucleic acid-based methods have been used by microbial ecologists to circumvent many of the shortcomings of the culture-based methods. Of particular importance to TMDL development is the advancement of Polymerase Chain Reaction (PCR)-based methods that have the potential of detecting the presence of fecal bacteria without relying on culturing the target organisms. Furthermore, recently developed PCR methods that are coupled with fluorescence-based detection, are capable of near real time monitoring of bacteria present in environmental samples (52, 53). Although these methods are showing much promise, the most obvious problem of PCR-based methods relates to the removal of inhibitory substances (e.g., cations and humic acids) found in water samples. These problems are exacerbated when biomass concentration steps are required to increase the detection sensitivity. Improvements in the way water samples are concentrated and processed will be necessary to precisely quantify low bacterial numbers, as is currently required for regulatory decisions of TMDL programs. Additionally, there are biases inherent in the PCR technique itself, although using optimized conditions and parameters can minimize these biases. Despite some technological limitations, the main advantage of PCR techniques is the potential for tracking several different genes concurrently, thereby allowing for a greater level of certainty in source tracking and pathogen detection.

Several molecular methods used for MST have relied on universal genetic markers to discriminate between different bacterial strains (Table 2). Some examples of these are ribotyping, length heterogeneity-PCR (LH−PCR), and terminal restriction fragment length polymorphisms (T-RFLP). Other techniques rely on DNA sequences that are randomly distributed throughout the chromosome of many bacteria. LH−PCR and T-RFLP are considered library independent methods since they do not require the isolation of environmental strains. This eliminates any biases introduced by the culturing steps. However, the robustness of methods such as LH−PCR and T-RFLP depends on the extent to which DNA sequences from environmental strains are represented in the currently available molecular databases (e.g., GenBank (54) and the Ribosomal Database Project (55)). In the following section we summarize several of the most recent MST studies using molecular techniques.

Ribotyping. Ribotyping consists of fingerprint pattern generation for genomic 16S rDNA restriction fragment length polymorphisms (RFLP) [essentially, patterns based upon size

TABLE 2. Comparison of Molecular Typing Methods Which May Be Considered for MST[a]

method	description	advantages	disadvantages
RFLP	electrophoretic analysis where DNA is detected with probes after Southern blotting	reproducible most strains typeable	technically demanding many probes needed to achieve adequate discrimination
ribo-typing	southern hybridization of genomic DNA cut with restriction enzymes, probed with ribosomal sequences	works with most strains automated	slow complex procedure inconclusive results
phage typeing	testing for susceptibility to different types of phages	does not require electrophoresis high level of host specificity	need access to phage libraries not all strains typeable technically demanding inconclusive results
rep-PCR	PCR is used to amplify palindromic DNA sequences couple with electrophoretic analysis	discriminatory does not require knowledge of genomic structure reproducible	cell culture required requires large database of isolates variability increases as database increases
DGGE	electrophoretic analysis of PCR products based on melting properties of the amplified DNA sequence	works on isolates and total DNA community reproducible	technically demanding time-consuming limited simultaneous processing
LH−PCR	separates PCR products for host specific genetic markers based upon length differences	does not require culturing does not require database	expensive equipment required technically demanding
T-RFLP	uses restriction enzymes coupled with PCR in which only fragments containing a fluorescent tag are detected	does not require culturing does not require database	expensive equipment required technically demanding
PFGE	DNA fingerprinting using rare cutting restriction enzymes coupled with electrophoretic analysis	high discrimination works with most strains reproducible conclusive results	long assay time limited simultaneous processing
AFLP	DNA fingerprinting using both rare and frequent cutting restriction enzymes coupled with PCR amplification	high discrimination works with most strains reproducible conclusive results automatable	technically demanding expensive equipment required

[a] Modified from ref 88.

differences of fragments of DNA related to the location of specific target sequences] (56). In this technique, total genomic DNA is extracted from pure cultures after which the DNA is enzymatically treated. The digested DNA fragments are first separated via agarose gel electrophoresis and transferred to nylon membranes. Southern blot hybridization analysis is then performed using rDNA probes, which results in a pattern composed of four to twelve bands. To increase the discriminatory power of this technique, several restriction enzymes can be independently used in separate analyses. Ribotyping methods have typically been used in epidemiological studies (57−59) but are now being applied to MST. Recently, Parveen et al. (60) used ribotyping to distinguish fecal E. coli of human origin from pooled fecal E. coli isolates of nonhuman origin. In this particular study, the ARCC for human and nonhuman isolates was 82%. Fecal E. coli ribotype patterns were also used by Carson et al. (61) to distinguish among fecal isolates associated with human and seven individual nonhuman hosts. While the results from the latter study suggest that the technique could discriminate between fecal bacteria of human and nonhuman origin, the ARCC values were poor when isolates from more than three fecal sources were analyzed. Compared to most MST studies using ARA, a smaller number of isolates were analyzed in these ribotyping studies. Since natural populations of E. coli exhibit a high diversity of genetic variation, future studies using E. coli ribotype patterns must substantially increase the number of isolates in order to better assess the applicability of this approach to MST. For example, in a recent study, Hartel et al. (62) used ribotyping to show that geographic variability exists for E. coli by comparing isolates from cattle, horses, swine and chickens located either in Idaho or Georgia. While Hartel and co-workers suggested that ribotyping could also be used to discriminate among host species at one location, they concluded that it would be contingent upon the use of a large number of isolates.

Length Heterogeneity-PCR (LH−PCR) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP). LH−PCR and T-RFLP are two other techniques which have recently been proposed and are based on the detection of fluorescently labeled 16S rDNA PCR products using an automated DNA sequencer (63, 64). These methods are used to analyze differences in lengths of gene fragments due to insertions and deletions, and once a suitable target sequence is identified, it can be followed easily through the automated process. One advantage that they have over other molecular and biochemical approaches is that they do not involve culturing bacteria from the environmental samples and hence the development of extensive culture-based libraries is not required. Another advantage is that these methods have been used to focus on the detection of rDNA sequences specific to anaerobic fecal bacteria inhabiting the animal gastrointestinal tract. This is relevant since anaerobic bacteria are present in animal feces in higher densities than conventional indicator bacteria. More importantly, most fecal anaerobes do not survive for long periods in the environment and their presence therefore represents recent fecal contamination events (19). MST studies performed by Bernhard and Field using LH−PCR and T-RFLP have specifically targeted the close relatives of *Bifidobacterium* and *Bacteroides* spp. (63, 64). Several important findings have been documented using these methods. First, a significant diversity of as yet undescribed *Bacteroides-Prevotella* 16S rDNA sequences is present in the feces of animals examined thus far. Second, the *Bacteroides-Prevotella* group was suggested to be a better indicator of source tracking in marine waters than *Bifidobacterium* because specific markers for the latter group were not always detected in bovine fecal samples or in coastal waters associated with fecal pollution. Third, *Bacteroides-Prevotella* fingerprints were capable of discriminating between cow and human fecal samples. However, since these methods have only been evaluated against a

limited number of animal fecal samples, a more extensive evaluation using other potential sources of contamination is currently underway.

Repetitive PCR (rep-PCR). Hundreds of studies have used conserved sequences in bacterial repetitive elements as PCR primers to distinguish among different strains of the same bacterial species. This typing approach, also known as rep-PCR, has been used to study many types of bacteria, including phyto-pathogenic bacteria, (65–67), human pathogens (68, 69), and animal pathogens (70). rep-PCR has also been used to examine fecal bacterial strains isolated from different sources of fecal pollution; however, only one MST study using rep-PCR has been published so far (71). There are three major repetitive elements in bacteria that have been used to generate bacterial fingerprints: REP, ERIC, and BOX. While these repetitive elements are present in most Gram negative bacteria tested, the BOX primers have been shown to be better suited for MST studies. For example, Dombek et al. (71) showed that a particular BOX-AR1 primer was more useful in separating E. coli human isolates from nonhuman isolates (geese, ducks, cows, pigs, chickens, and sheep) than REP primers. In this study, between 78% and 100% of the isolates were correctly assigned to the original source groups. However, only 19–29 isolates per source were used in the statistical analysis in this study, and, therefore, it is necessary to interpret these results with caution.

Denaturing Gradient Gel Electrophoresis (DGGE). The DGGE technique is capable of discriminating between different PCR products of similar size based on changes in electrophoretic mobility which are influenced by the melting properties of the DNA fragments (72, 73). DGGE has been used to characterize and compare fecal and gastrointestinal bacterial populations of both humans and animals, though typically as a direct sample, rather than a complex matrix component (74, 75). However, Farnleitner et al. (76) demonstrated that DGGE could be used to detect and differentiate E. coli populations from fecally impacted freshwater samples, but have not yet utilized their method to differentiate between sources. Buchan et al. (77) used DGGE to differentiate environmental E. coli isolates associated with bovine, poultry, and human sources. The results from this study revealed that DGGE patterns obtained for the samples analyzed could not be used to pinpoint the source of contamination in the watershed studied. Since the investigators only used 132 isolates for the analysis, it is possible that a larger database might be required for this particular method to be useful in source tracking. However, it should be noted that the gene targeted for DGGE analysis must contain enough sequence variability among the strains in order to find nucleotide substitutions that are specific to a bacterial strain inhabiting a particular host.

Pulsed-Field Gel Electrophoresis (PFGE). For PFGE analysis, pure culture bacterial cells are placed in agarose plugs where the DNA is digested using a series of restriction enzymes. These digested plugs are then imbedded into specialized electrophoresis gels and electrophoresed for an extended period of time (avg. 30 to 50 h) with alternating currents from different directions using specialized equipment. PFGE has been used extensively in clinical microbiology and for some clinical scientists it represents the gold standard (78). However, PFGE is time-consuming and the number of isolates that can be processed simultaneously is limited. Nonetheless, PFGE has been used for genotypic characterization of clinical and environmental E. coli and enterococcus isolates to determine sources of contamination (79–82).

Amplified Fragment Length Polymorphism (AFLP). The AFLP technique has two main advantages over other fingerprinting methods: 1) it has the ability to inspect an entire genome for polymorphisms and 2) it is highly reproducible. AFLP analysis has been applied to many different types of DNA samples (human, animal, plant, and microbial) thereby demonstrating its potential to become a universal DNA fingerprinting method (83). Indeed, AFLP has been used successfully in taxonomical, epidemiological, and ecological studies involving microbial parasites, fungi, and bacteria (84). AFLP combines the use of RFLP and PCR amplification to generate between 50 and 100 DNA fragments. DNA fragments are commonly analyzed using modern DNA sequencers containing fluorescence based detectors (85). Automatic loaders allow the analysis of over one hundred strains per day. Since fluorescent standards can be included with each of the samples, normalization is a relatively simple process. However, like other isolate fingerprinting methods, this is a library dependent method and relies on cultivation of the target organisms. Recently, Guan et al. (86) reported results of a comparison study which used AFLP, ARA and 16S sequencing to discriminate among fecal sources of E. coli. Their results indicated that AFLP was the most effective of the three methods considered. However, as only 105 isolates were analyzed by AFLP, more extensive field testing is necessary before this method is considered for routine monitoring.

Problematic Issues Associated with MST Methods

Theoretically most fingerprinting techniques could be used in MST studies. However, ARA, rep-PCR, AFLP and T-RFLP seem to offer the best possibilities to discriminate between different fecal bacterial strains. Examples of several methods which have been used to date in watersheds and the key advantages and disadvantages of some molecular typing methods that have been considered in MST are summarized in Tables 1 and 2. While results from MST studies could help significantly in the implementation of best management practices, there are a number of problems that need to be addressed (Table 3). Some of these problems relate to detection limits, temporal and spatial variability of markers, and reproducibility of the assays. A clear understanding of the role of each variable plays is necessary to obtain robust results and correctly interpret the data. Some of the major issues which need attention are described below.

The detection limit of many methods depends on the type of approach used for source tracking. To apply PCR methods to targets found in low levels, a concentration step is necessary. However, the biomass concentration steps often co-extract organic contaminants that can interfere with the amplification of targeted genes. Thus, a low level of sensitivity is often difficult to obtain for environmental samples. Commercially available kits are now including DNA purification steps that help eliminate many of the PCR inhibitors. One possible way to increase sensitivity of PCR methods for the appropriate detection of very low cell densities is to target genes such as the 16S rDNA that have multiple copies per cell or to increase the cell densities via a cultural enrichment step.

Spatial and temporal variations are factors that have not been incorporated in most MST studies, and therefore, their impact in source tracking is not well understood. However, it is reasonable to assume that the number and locations of the sampling sites within a particular watershed will have an impact on how representative the results are. Moreover, temperature and chemical changes associated with seasonal variation can significantly affect the survival of microorganisms targeted. Flow dynamics and rainstorm events could also affect the bacterial densities on a seasonal basis.

Reproducibility is relevant, in that the selected method should give the same type of answer if standard operating procedures are always followed. For example, a given method should always be able to discriminate between the most common animal sources of pollution on a particular watershed. These results should also be reproducible regardless

TABLE 3. Evaluation Criteria for Performance Appraisal of MST Methods[a]

category of criteria	specific evaluation criteria
tier 1: measurement reliability	reproducibility of results within and across laboratories
	accuracy of classification of isolates into the correct group of sources (for library dependent methods)
	confidence that an identified indicator is from the presumed source (for library independent methods)
	level of resolution, or ability to discriminate among sources (i.e., human vs nonhuman, livestock vs wildlife, nonhuman species level, cattle from separate farms)
	matrix stability (in what matrices, e.g., saltwater, freshwater, turbid water, humic acid environments, is the method applicable?)
	geographical stability (over what area is the method applicable?)
	temporal stability (over what time frame is the method applicable?)
	confirmation by peer review
tier 2: management relevance	relationship to actual source(s) of contamination
	relationship to public health outcomes
	relationship to commonly used water quality indicators
	ease of communication to the public
	ease of communication to management audiences
tier 3: cost and logistics	equipment and laboratory facilities required
	training required
	library size required (for library dependent methods)
	library development effort per "unit" required (library dependent methods)
	implementation time
	cost of ensuring results are legally defensible
	cost per sample, including all operations and maintenance overhead
	sample turnaround time

[a] Table derived from EPA sponsored MST Workshop, Feb 2002, Irvine, CA.

of fluctuations in the fecal loads from the different sources that contribute to the contamination.

Another relevant issue relates to the stability of the marker used in source tracking. While somewhat easier to track, phenotypic markers (e.g., antibiotic resistance) are often unstable if the selective pressure is not maintained. A similar argument can be used against molecular fingerprinting methods, like rep-PCR and AFLP. Since there is little selective pressure to maintain an intact genome structure, this genetic instability will increase the level of pattern complexity in and outside of the host. In this context, the fingerprint method should ideally depend on the use of stable markers that possess some ecological relevance, for example, genes that are associated with host-bacterial interactions. Such phenotypic and genotypic instability has a direct impact on the number of microbial strains that need to be examined in any particular study. As a consequence, substantial empirical evidence is necessary to establish the predictive value of methods that use unstable markers, particularly after the targeted bacterial indicator group enters the aquatic environment.

The target microorganism chosen for use in MST will influence the number of isolates that need to be examined in order to achieve a desirable level of certainty in the data analysis. For example, a larger culture collection (library) is required when an organism with a high level of genetic variation is used in a study. The number of isolates will also depend on the type of method used. Methods with a large number of distinct characters (e.g., band patterns) will require smaller culture collections. Unfortunately, the numbers of isolates and samples that are examined is often dictated by economics and not by a statistically sound experimental design. The fewer the number of fecal sources and the number of isolates per source, the less reliable the statistical analysis will be. Therefore, to have a good representation of the natural clonal diversity in any watershed, it is necessary to examine the largest possible number of isolates per fecal source and per water sample. The analysis of a relatively similar number of isolates per source is also useful for the overall predictive power of any method.

The portability of databases is an issue due to the lack of comparative studies, as it is currently unknown if any given MST method can be applied to different regions. It is possible that in most cases information gained will only be applicable to a specific watershed, and therefore, most markers will not transcend different geographical regions. At this time, the universal value of all fingerprint methods remains at best speculative.

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 technical training. Moreover, the application of methods such as PFGE for MST studies will be limited in that they are time-consuming and not suitable for rapid identification of a large number of isolates.

Summary and Future Prospects

Most MST methods have only been tested against a limited number of watersheds, and many of them will require further development before they can be considered appropriate for source tracking of fecal contamination. Thus far, ARA appears to be the most practical approach for source tracking in small watersheds, primarily because it is relatively inexpensive and simple to execute. Many of the molecular methods (e.g., AFLP, and T-RFLP) appear promising, but they need to be used in a larger number of studies that include a larger number of isolates before any firm conclusions can be reached on their applicability and usefulness. Similarly, the number of samples from each potential animal source and from the habitat under examination must be larger than those reported in most of the published studies to more accurately calculate the percentage of correctly classified isolates. To compare the results from MST studies, the index of correct classification or ARCC must be calculated using the same approach. It is important to recognize that none of the current MST methods have been systematically applied to evaluate the population dynamics of the targeted organism(s) on a spatial and temporal basis. Investigation is also needed as to how environmental and biological factors within the primary habitat (i.e., the animal gut) and the secondary habitats (i.e., watersheds) affect the stability of an organism's fingerprint profile.

A number of criteria for the accurate evaluation of different MST methods were recently proposed by a panel of experts that included scientists and engineers from Academia, government and private industry (Table 3). Variables such as library size, processing costs, and type of professional expertise required for data development and data interpretation will almost certainly restrict the use of some methods (e.g., molecular-based) on a case-by-case basis. Since the effectiveness of the MST method may also depend on the

size of the watershed and the number of potential pollution sources, the development of multidisciplinary research teams will be necessary to determine which method(s) should be used to discriminate between primary and secondary sources of pollution. As it is possible that indicator bacteria like *E. coli* and fecal enterococci are not the most adequate target organisms for MST, support is also needed for research that examines the population dynamics of different fecal organisms, including those that are difficult to culture. The sequencing analysis of comprehensive 16S rDNA molecular databases from different fecal sources may provide new bacterial groups with closer associations with the hosts than the currently used indicator organisms. The results from these research initiatives could result in the development of methods based on better indicators of fecal contamination. Furthermore, it is reasonable to propose that future developments in gene chip technology (*87*) and microbial genomics will provide the opportunity of finding alternate genetic markers in fecal bacteria that carry ecological meaning and that correlate with host specificity. Some of these genes are likely to be directly associated to host and microbial interactions, like virulence factors (e.g., cellular attachment and cell invasion) or to physiological traits that endow the bacterium with an advantage toward occupying a particular microbial niche in the gut of a particular animal group. If some of these bacterial groups, bacterial genes or gene sequences are found to be "host-specific", this information could be further used to develop assays such as real-time PCR to track contamination sources in a matter of hours. Using these tools, scientists will be able to study complex microbial communities impacted with different levels of fecal pollution and to better determine the risks associated with the recreational use of such bodies of water. This would permit development of an adequate microbiological framework to be used in risk management of U.S. recreational waters.

Acknowledgments

Nomenclature

16S	subunit of ribosomal DNA used for phylogenetic analysis of bacteria
BST	bacterial source tracking
database	collection of molecular fingerprints generated by various enzymatic means
DNA	deoxyribonucleic acid—DNA is the genetic repository for all cells
fingerprint	a pattern that is generated for an organism or population which is distinct to that organism or population based upon the technique selected − a unique identifiable pattern
library	collection of bacterial isolates to be used for fingerprinting analysis
MST	microbial source tracking
PCR	polymerase chain reaction − a method for copying specific sequences of DNA, with repeated replication of a sequence resulting in formation of millions of copies in a short period of time
RNA	ribonucleic acid − RNA is involved in all stages of protein synthesis and many play regulatory roles in the cell
rRNA	ribosomal RNA − RNA which is specific to ribosome genesis and its subunit 16S is used for phylogenetic analysis

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