Chapter 14

Microbial Source Tracking

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14.1 WATER QUALITY AND FECAL CONTAMINATION

Water quality has been a concern for numerous stakeholders including wastewater utilities, local governments and members of the community, and has been monitored for many decades—in particular since the enactment of the Clean Water Act in 1972. However, more than 30 years after the Clean Water Act was implemented, a significant fraction of U.S. rivers, lakes and estuaries continue to be classified as failing to meet their designated use due to high levels of fecal bacteria (U.S. EPA, 2005). As a consequence, protection from fecal contamination is one of the most important and difficult challenges facing environmental scientists, regulators and communities trying to safeguard public water supplies and waters used for recreation (primary and secondary contact). Traditional water quality monitoring has helped improve water sanitation to protect public health but has also led to economic losses due to closures of recreational beaches, lakes and rivers. Additionally, solutions to contamination are not always readily apparent and easily identifiable. The ability to discriminate between sources of fecal contamination is necessary for a more defined evaluation of human health risks and to make water safe for human use.

The potential sources of fecal contamination causing these impairments can be classified into two groups: point sources that are easily identifiable (e.g., raw and treated sewage and combined sewer overflows) and nonpoint sources that are diffuse in the environment and may be difficult to identify (e.g., agriculture, forestry, wildlife and urban runoff) (Okabe *et al*., 2007). Understanding the origin of fecal contamination is paramount in assessing associated health risks as well as identifying the actions necessary to remedy the problem (Scott *et al*., 2002). As a result, numerous methods have been developed to identify fecal contamination as well as differentiate between these sources of pollution. Accurately identifying these sources can help to facilitate the elimination of waterborne microbial disease as a leading threat to public health (Simpson *et al*., 2002).

14.2 MICROBIAL SOURCE TRACKING METHODS

Microbial source tracking (MST) methods are intended to discriminate between human and nonhuman sources of fecal contamination, and some methods are designed to differentiate between fecal contamination originating from individual animal species (Griffith *et al*., 2003). MST is an active area of research with the potential to provide important information to effectively manage water resources (Stoeckel *et al*., 2004).

MST methods are typically divided into two categories (Table 14.1). The first category is called library dependent, relying on isolate-by-isolate identification of bacteria cultured from various fecal sources and water samples and comparing them to a "library" of

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TABLE 14.1 Common Types of Microbial Source Tracking Methods

Library Dependent		Library Independent	
Culture Dependent			Culture Independent
Biochemical	Molecular	Biochemical or Molecular	Molecular
– Antibiotic resistance – Carbon utilization	– Rep-PCR – PFGE – Ribotyping	– Bacteriophage – Bacterial culture	– Host-specific bacterial PCR – Host-specific viral PCR – Host-specific quantitative PCR

Adapted from U.S. EPA (2011).

TABLE 14.2 Commonly Used Terms

Biochemical (phenotypic) methods refer to the ability to physically observe a characteristic of the isolated bacteria that might have been acquired from exposure to different host species or environment. Examples may be the resistance to certain antibiotics or utilization of carbon or nutrient source.
Culture-dependent methods rely on bacteria from water samples being grown or cultured in a lab.
Culture-independent methods isolate and identify DNA directly from a water sample without first having to grow or culture the bacteria from the sample.
Fecal source refers to a human or animal host where a microbe originates in the fecal waste of that host. Depending on the specificity of an MST method, a fecal source might refer to a general group of hosts (e.g., all humans, all animals or a group of animals such as ruminants), or a specific animal host (e.g., cattle, elk, dogs, etc.).
Library-dependent methods identify fecal sources from water samples based on databases of genotypic or phenotypic fingerprints for bacteria strains of known fecal sources.
Library-independent methods identify fecal sources based on known host-specific characteristics of the bacteria without the need of a library.
Microbial source tracking (MST) refers to a group of methods intended to discriminate between human and nonhuman sources of fecal contamination. Some methods are designed to differentiate between fecal contamination originating from individual animal species.
Microbial strain is a genetic variant or subtype of a microorganism (e.g., bacterial species).
Molecular (genotypic) methods utilize variations in the genetic makeup or the DNA of each individual organism or bacterium. This is often referred to as "DNA fingerprinting."

From U.S. EPA (2011).

bacterial strains from known fecal sources. Library-dependent methods require the development of biochemical (phenotypic) or molecular (genotypic) fingerprints for bacterial strains isolated from suspected fecal sources (U.S. EPA, 2005). These fingerprints are then compared to developed libraries for classification (see Table 14.2). The use of fecal bacteria to determine the host animal source of fecal contamination is based on the assumption that certain strains of fecal bacteria are associated with specific host animals, and that strains from different host animals can be differentiated based on phenotypic or genotypic markers (Layton *et al.*, 2006). Library-dependent methods tend to be more expensive and require more time as well as the use of experienced personnel to complete the analysis due to the time it takes to develop a library. Additionally, one of the major disadvantages of library-dependent methods is that libraries tend to be temporally and geographically specific. While this can be useful for a specific location, they are generally not as applicable on a broader watershed scale, or on statewide issues (Information Box 14.1).

The second category is called library independent, and is based on the detection of a specific host-associated genetic marker or gene target identified in the molecular material isolated from a water sample. These methods can help identify sources based on a known host-specific characteristic (genetic marker) of the bacteria without the

Information Box 14.1 Use of Microbial Source Tracking to Identify Pollution Sources in Oak Creek Canyon, Arizona

Federal and state regulations require that a TMDL be established for the impaired waters with oversight by the U.S. Environmental Protection Agency (Simpson *et al.*, 2002). As a result several state departments of environmental quality are looking towards alternative methods to determine the sources of pollution across their states' watersheds. According to the 2010 assessment, the state of Arizona has 21 impaired waters due to *E. coli* levels higher than the set standards (U.S. EPA, 2011). It is anticipated that the number of impaired watersheds will increase by the year 2015. In watersheds where sources are not known or understood, MST techniques can help to identify and also eliminate potential sources of fecal bacteria. In a study conducted by the University of Arizona (Rivera and Rock, 2010), MST methods were chosen within regions across Arizona due to the anticipated source(s) of bacteria not visibly obvious in these watersheds. More specifically, molecular methods were selected to differentiate between human and animal sources of *Bacteroides* present in water samples collected by volunteers across the state using host-specific 16S rDNA (Shanks *et al.*, 2010). Each of the five different watersheds included in this study has unique land-use characterization (urban vs. rural) and potential inputs of pollution within their area. One of the watersheds that has been extensively studied in the state of Arizona is the Oak Creek Watershed near Sedona, Arizona. Oak Creek is specifically known for its frequently visited recreation areas including Slide Rock State Park. Since 1973, *E. coli* bacteria in the water of Oak Creek have been a concern. Southam *et al.* (2000) used DNA fingerprinting to identify the relative contributions of *E. coli* from source mammals. Human-related sources (from humans, pets, livestock, septic system effluent) accounted for about 33% of all *E. coli* found in Oak Creek, with perhaps a few more percentages attributable to wild animals that are present near the creek foraging on human food waste. The remainder of *E. coli* in Oak Creek was attributed to wildlife including: raccoons (31%), skunks (11%), elk (8%), white-tailed deer (6%), beaver (6%) and other mammals. While the contribution of human influence was significant, such a diverse number of wildlife contributors makes it a challenge to address dispersed nonpoint source pollution with comprehensive and complete measures to reduce *E. coli* loads to acceptable levels. Results of this study indicated both human and bovine inputs across multiple watersheds were causing the water quality impairments. More specifically, of the total 171 surface water samples that were analyzed using molecular methods, 37% were positive for human molecular markers for *Bacteroides*. Because of this research, best management practices or BMPs were implemented to reduce runoff from communities surrounding the creek, and failing septic systems leaching into rivers and lakes were repaired to help reduce contamination. This is one example of how the use of MST methods to identify the sources of fecal pollution can help to empower local regulatory agencies to work with stakeholders within the community to monitor and remediate locations contributing to contamination with the ultimate intent to delist impaired waters.

need of a "library." One of the most widely used library-independent approaches utilizes polymerase chain reaction (PCR) to amplify a gene target that is specifically found in a host population (Shanks *et al.*, 2010). PCR provides the ability to screen genetic material from bacteria (e.g., deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) isolated from a water sample for a specific sequence or target in a relatively short amount of time. These methods do not depend on the isolation of DNA directly from the original source, although some methods often require a pre-enrichment to increase the sensitivity of the approach (U.S. EPA, 2005).

Recently, there has been an effort to better understand the various types of MST methods available as well as which methods are most useful for the goals of source identification and watershed characterization. According to the U.S. EPA, while there has been significant progress in the past 10 years towards method development, 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 (U.S. EPA, 2005). Comparison studies have shown that no single method is clearly superior to the others (U.S. EPA, 2005). Therefore, no single method has emerged as the method of choice for determining sources of fecal contamination in all fecal-impaired water bodies. However, using the appropriate method and appropriate indicator, sources of fecal contamination can be found and characterized as being from either animal or human origin (Simpson *et al.*, 2002). MST based on identification of specific molecular markers can provide a more complete picture of the land uses and environmental health risks associated with fecal pollution loading in a watershed than is currently possible with traditional indicators and methods (Jenkins *et al.*, 2009). MST methods have the ability to identify "who" is contributing to the pollution, whereas traditional culture-based methods only tell you "if" and "when" fecal contamination is present. Table 14.3 describes existing MST methods that are currently being used and the general purposes for each.

There are several detection methods available for library-dependent and library-independent MST. Included in library-dependent MST are methodologies for phenotypic and genotypic analysis. Antibiotic-resistant analysis and carbon source utilization are two commonly used methods for phenotypic analysis. Antibiotic-resistant

TABLE 14.3 Comparison of Molecular Microbial Source Tracking Methods Used for Watershed Experiments

Method	Description	Advantages	Disadvantages	References
Ribotyping	Southern blot of genomic DNA cut with restriction enzymes; probed with ribosomal sequences; discriminates species	Highly reproducible; classifies isolates from multiple sources	Complex; expensive; labor intensive; geographically specific; database required; variations in methodology	Samadpour and Chechowitz, 1995; Farber, 1996; Tynkkynen *et al.*, 1999; Parveen *et al.*, 1999; Farag *et al.*, 2001; Hager, 2001a; Carson *et al.*, 2001; Hartel *et al.*, 2002; Samadpour, 2002; Scott *et al.*, 2003
Pulse-field gel electrophoresis (PFGE)	DNA fingerprinting with rare-cutting restriction enzymes coupled with electrophoretic analysis; discriminates species	Extremely sensitive to minute genetic differences; highly reproducible	Long assay time; limited simultaneous processing; database required	Tynkkynen *et al.*, 1999; Simmons *et al.*, 2000; Hager, 2001b; King and Stansfield, 2002
Denaturing-gradient gel electrophoresis (DGGE)	Electrophoresis analysis of PCR products based on melting properties of the amplified DNA sequences; discriminates species	Works on isolates	Technically demanding; time consuming; limited simultaneous processing; not good on environmental isolates; database required	Farnleitner *et al.*, 2000; Buchan *et al.*, 2001
Repetitive DNA sequences (Rep-PCR)	PCR used to amplify palindromic DNA sequences coupled with electrophoretic analysis; discriminates species	Simple and rapid	Reproducibility a concern; cell culture required; large database required; variability increases as database increases	Dombek *et al.*, 2000; Holloway, 2001
Length heterogeneity PCR (LH-PCR)	Separates PCR products for host-specific genetic markers based on length	Does not require culturing or a database	Expensive equipment; technically demanding	Suzuki *et al.*, 1998; Bernhard and Field, 2000a,b
Terminal restriction fragment length polymorphism analysis (T-RFLP)	Uses restriction enzymes coupled with PCR in which only fragments containing a fluorescent tag are detected	Does not require culturing or a database	Expensive equipment; technically demanding	Bernhard and Field, 2000a,b
Host-specific 16S rDNA	Combine LH-PCR and T-RFLP methods on fecal anaerobes (*Bacteroides* and *Bifidobacterium*); discriminates human and cattle; other markers being developed	Does not require culturing or a database; indicator of recent pollution	Expensive equipment; technically demanding; little known about survival of *Bacteriodes* spp. in environment	Bernhard and Field, 2000a,b

Adapted from Meays et al. (2004).

analysis is a method based on the premise that host animals/humans exposed to antibiotics will release bacteria resistant to those antibiotics, and on the assumption that this selective load would be a mechanism for distinguishing among fecal bacteria from different hosts (U.S. EPA, 2005). This method is labor intensive and time consuming as it requires culturing a large number of antibiotic-resistant isolates, as well as determining which antibiotics are involved with the resistance (Field *et al.*, 2003). Field *et al.* (2003) reported that when sets of isolates from a single type of feces have been evaluated, rates of correct classification have ranged from approximately 64 to 87%; however, when individual isolates from mixed fecal sources were analyzed, rates of correct classification were lower. As a result, using this method to determine fecal contamination by analyzing resistance to antibiotics has low accuracy. According to Griffith *et al.* (2003), carbon source utilization is similar to antibiotic-resistant analysis, but instead relies on growth patterns created when fecal bacterial isolates are exposed to a number of antibiotics or grown on different carbon sources. While this method can work in the laboratory for

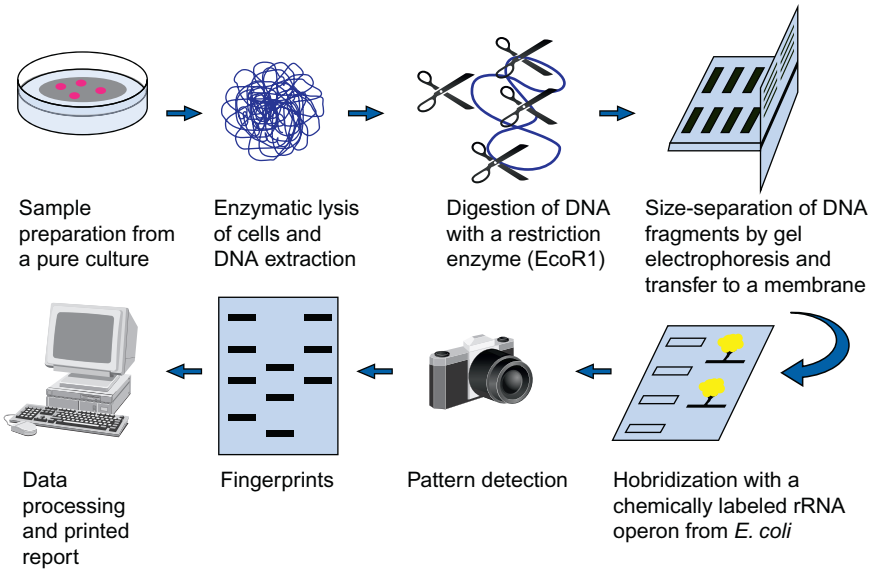

FIGURE 14.1 Ribotyping procedure. From Meays *et al.* (2004).

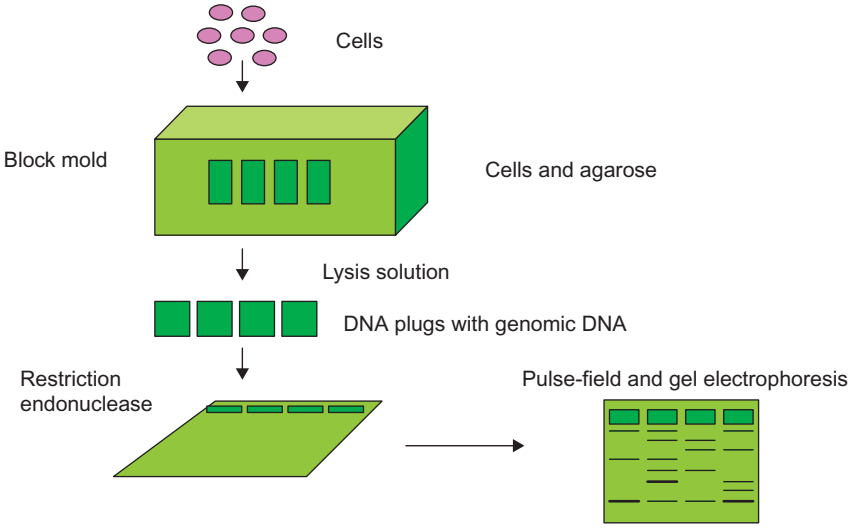

FIGURE 14.2 Pulse-field gel electrophoresis. From Meays *et al.* (2004).

analysis of pure cultures, there are numerous factors in an environmental water body that can influence bacterial nutrient needs that may make this method inconvenient for field determination (Simpson *et al.*, 2002).

Ribotyping, pulsed-field gel electrophoresis (PFGE) and rep-PCR are MST methods that have been used to quantify genotypic characteristics (Griffith *et al.*, 2003). Ribotyping is a technique that involves digestion of restriction enzymes from genomic DNA (Figure 14.1). Fragments are then separated by gel electrophoresis and transferred to a nylon membrane by Southern transfer and subsequent hybridization using a labeled probe of the *E. coli* rRNA genes or the entire operon (U.S. EPA, 2005). According to Field *et al.* (2003), ribotyping is excellent for accurately differentiating between human and animal fecal isolates, but is less successful at pinpointing the animal source and takes approximately 2 weeks to complete. The PFGE method (Figure 14.2) consists of pure culture bacterial cells placed in agarose plugs where the DNA is broken down by using a series of restriction enzymes (Griffith *et al.*, 2003). Both ribotyping and PFGE methods need extensive local collections of strains for comparison. Finally, by using rep-PCR, amplification of a genetic fragment can be visualized by using specific primers that target the region, followed by matching to a library of known sources (Stoeckel *et al.*, 2004). This method requires the establishment of a library containing identified strains. In addition, it is necessary to screen a large geographic region that includes known isolates (Scott *et al.*, 2002).

Other MST methods that do not rely on library databases include amplicon length heterogeneity PCR (LH-PCR), terminal-restriction fragment length polymorphism (T-RFLP) and host-specific PCR. These methods are library independent and distinguish between sources of fecal pollution by recognizing specific genetic sequences distinct to the host fecal bacteria (Griffith *et al*., 2003). These methods function by looking at the bacterial community as a whole rather than individually. Bernhard and Field (2000a) define LH-PCR and T-RFLP as methods used to explore changes in the sizes of gene fragments due to additions and removal, and to calculate the approximate relative quantity of each gene fragment. T-RFLP analysis is a method of comparative community analysis, and is based on the restriction endonuclease digestion of fluorescently end-labeled PCR products such as the 16S rRNA gene. The digested products are separated by gel electrophoresis and detected on an automated sequence analyzer. The method provides distinct profiles (fingerprints) dependent on the species composition of the communities of the samples. While useful, these methods have only been used against a small number of animal fecal samples and need additional evaluation using other possible sources of contamination (Simpson *et al*., 2002). In addition, these two methods require expensive equipment and are technically demanding.

There are also MST methods that are an alternative to the library-dependent and library-independent methods. These methods offer a direct measure of viruses found in humans, and aim to detect viruses present in human feces that are not present in other animals, including human enteroviruses, adenoviruses and F^+ coliphage (Griffith *et al*., 2003). While these methods can be useful at determining if human fecal contamination is present, they do not determine other fecal contamination within an impaired water body. Additionally, the isolation and detection of viruses can be cumbersome due to the extensive training, equipment and time needed to accurately determine their presence.

A recent review of the literature has identified an increase in library-independent methods available for watershed characterization. In particular, host-specific bacterial and viral PCR as well as host-specific quantitative PCR methods have recently been developed. In theory, host-specific PCR (library-independent MST) uses genetic marker sequences that are not only specific to fecal bacteria, but are also specific to the host species that produced the feces, allowing discrimination among different potential sources (Field *et al*., 2003). Host-specific PCR holds promise as an effective method for characterizing a microbial population without first culturing the organisms in question (Scott *et al*., 2002). Furthermore, these methods are cost effective, rapid and potentially more specific than library-dependent methods. It is anticipated that these host-specific molecular methods will continue to develop with emphasis on those methods using the quantitative polymerase chain reaction (qPCR) technique that measures the amount of microbial DNA present in the water sample rather than simply detecting a presence or absence of microbial DNA (Santo Domingo *et al*., 2007). By quantifying the amount of microbial DNA, comparisons can be made regarding the relative impacts of a specific source to a specific location within the watershed. In particular, one of the most widely cited bacteria analyzed for library-independent MST is *Bacteroides*.

14.3 COMMON BACTERIA USED IN SOURCE TRACKING STUDIES: *Bacteroides*

The genus *Bacteroides* contains Gram-negative, non-spore-forming, nonmotile, anaerobic rod bacteria generally isolated from the gastrointestinal tract (GI tract) of humans and animals (Smith *et al*., 2006). As members of the indigenous flora, they play a variety of roles that contribute to normal intestinal physiology and function. These include beneficial roles such as polysaccharide breakdown or nitrogen cycling (Smith *et al*., 2006). According to Smith *et al*. (2006) *Bacteroides* generally cause opportunistic infections that can occur any time the integrity of the mucosal wall of the intestine is compromised. Another important aspect of *Bacteroides* biology is their inability to proliferate in the environment as well as their potential to survive in the environment at a rate directly proportional to pathogens of concern. *Bacteroides* survival depends primarily on temperature and presence of predators, and they have been found to survive for up to 6 days under oxygen-stressed conditions (Field and Dick, 2004).

The abundance of this bacterium in human and animal feces has allowed for host-related analysis targeting genes present in the *Bacteroides* genome. Layton *et al*. (2006) suggested that bacteria belonging to the genus *Bacteroides* could be an alternative fecal indicator to *E. coli* or fecal coliform bacteria, because they make up a significant portion of the fecal bacteria population, have little potential for growth in the environment and have a high degree of host specificity that likely reflects differences in host animal digestive systems.

Numerous methodologies have been designed to target specific diagnostic sequences within the *Bacteroides* 16S rRNA gene (which is vital for protein synthesis and therefore present in all bacteria) present in feces from different animals. Field and Dick (2004) developed 16S rRNA gene makers from *Bacteroides* to detect fecal pollution, and to distinguish between human and ruminant (e.g., bovine, goat, sheep, deer and others) sources by PCR. Developing MST methods specific to molecular markers

within the target gene will allow differentiation between human and ruminant-associated *Bacteroides*, therefore identifying the possible source of contamination. This approach offers the advantage of circumventing the need for a culturing step, which allows for a more rapid identification of target organism (Scott *et al*., 2002)

While progress has been made in identifying genetic markers that are useful for MST, few studies have evaluated how these molecular markers used as MST targets vary temporally and spatially following fecal contamination of surface waters (Bower *et al*., 2005). There are several studies that have used MST methods; in particular, host-associated PCR-based assays targeting *Bacteroides* genetic markers to investigate the sources and levels of fecal pollution in recreational water and watersheds. In a study conducted by Gourmelon *et al*. (2007), three estuaries were compared by PCR using human-specific *Bacteroides* markers in combination with human- and animal-specific targets. In this study PCR was found to be a reliable indicator of fecal contamination. *Bacteroides* was observed in 95% of fecal samples in all sewage treatment plant samples and pig liquid manure. A separate study targeting *Bacteroides* (Shanks *et al*., 2010) compared seven PCR and qPCR assays targeting *Bacteroides* genes reported to be associated with either ruminant or bovine feces. PCR-indicated prevalence ranged from 54 to 85% for all DNA extracts from 247 individual bovine fecal samples, and specificity (how well the PCR assay detected known bovine fecal samples) ranged from 76 to 100% for the assays studied. A previous study by Griffith *et al*. (2003) using blind samples demonstrated that *Bacteroides* source-specific MST methods identified fecal sources correctly when the sources comprised as little as 1% of the total fecal contamination in the samples. While a wealth of knowledge exists in the literature, there are still many ongoing MST studies targeting the 16S rRNA *Bacteroides* gene to improve detection and watershed characterization.

Although *Bacteroides* MST has been useful for pollution characterization, it is still an emerging science and research is currently being done to validate published methods and better understand the effectiveness of available technologies. Extensive field testing is ongoing to determine the efficacy of published assays and the geographic distributions of presumptively human-specific markers (McLain *et al*., 2009). Several recent studies have described testing of feces from domestic animals, livestock, bird and mammal wildlife as well as fish and other aquatic species for cross-amplification with human assays and molecular markers previously thought to be human specific (McLain *et al*., 2009). Therefore, it is critical that MST-based methods be evaluated on a watershed-by-watershed basis to ultimately understand the utility of the methods for accurate pollution characterization.

14.4 APPLICATION OF SOURCE TRACKING

A primary driver of microbial source tracking has been the U.S. Environmental Protection Agency's Total Maximum Daily Load or TMDL program. A TMDL is defined as the maximum amount of a pollutant the water body can receive, and still meet regulated limits for that pollutant. Under the 1972 Clean Water Act, all waters in the United States must be evaluated in the context of applicable water-quality standards, which include water quality criteria designed to protect the water's designated uses (e.g., swimming, fishing). Water bodies that do not meet water quality standards are classified as impaired. A TMDL is defined as the total pollutant (e.g., fecal bacteria, pesticides) load a water body can receive and still meet applicable water quality standards. Waters are designated as having pathogen impairment if fecal indicator bacteria concentrations exceed standards for the water use (e.g., swimming). One of the most common uses of MST is to identify sources of fecal bacterial indicator impairments (e.g., human, livestock, wildlife) for the purpose of prioritizing control. Where impairments are demonstrated as primarily from bacteria sources such as wildlife, which are not practical to control and are thought to pose less risk to human health, TMDL development and implementation may not be warranted.

Overall, while many approaches to source tracking have been developed, only recently has large-scale testing of the different approaches in multiple laboratories been conducted, and even this is limited to only a few contaminant sources. Clearly, there is a need to develop more standardized procedures for source tracking.

QUESTIONS AND PROBLEMS

1. What are the two major approaches used for microbial source tracking? What are the advantages and disadvantages of these methods?
2. What is Total Maximum Daily Load?
3. Can viruses be used in source tracking? How?

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