MICROBIAL SOURCE TRACKING:
WATERSHED CHARACTERIZATION AND SOURCE IDENTIFICATION

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Water Quality and Fecal Contamination

Water quality has been a concern for numerous stakeholders 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 US rivers, lakes, and estuaries continue to be classified as failing to meet their designated use due to high levels of fecal bacteria (US 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 as well as 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 waters 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 non-point sources that are diffuse in the environment and may be difficult to identify (e.g., agriculture, forestry, wild-life, 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) (Figure 1).
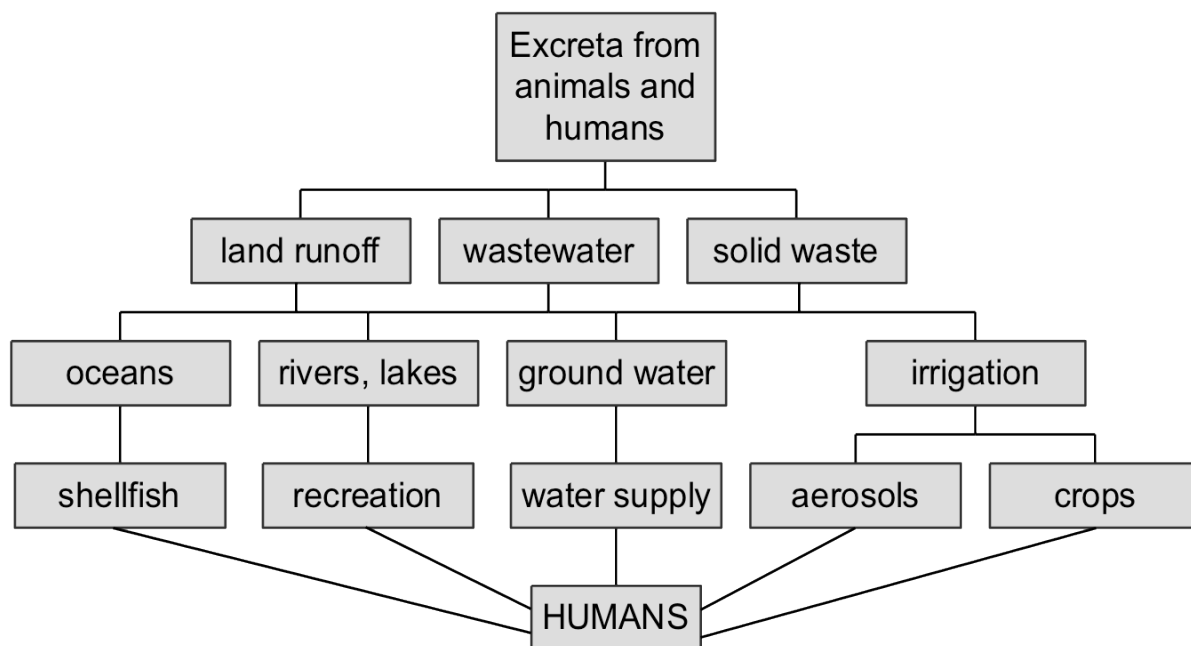

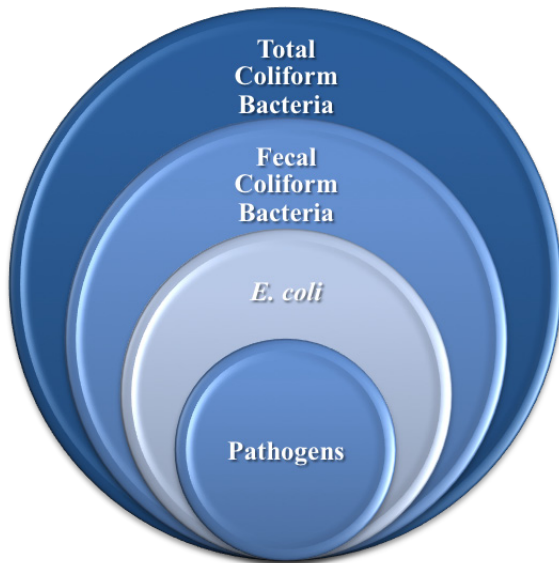**Figure 1**. Waterborne transmission of pathogens.

Figure 2. Relationship between indicators and pathogens.

Fecal coliform & *Escherichia coli*

Indicator bacteria are used to predict the presence/absence or minimize the potential risk associated with pathogenic microorganisms (Scott *et al*. 2002). Fecal coliform are a group of bacteria that originate in the feces of mammals and include the genera *Escherichia* and *Klebsiella* (Figure 2). These indicator bacteria are identified in the laboratory using certain tests to evaluate their ability to use lactose as a food source. *Escherichia coli* or *E.coli* are fecal coliform bacteria that have been extensively used to indicate the presence of human pathogens in water (Parveen *et al.* 2001). A pathogen is defined as a microorganism that has the potential to make a healthy individual sick. Methods such as the IDEXX Colilert and Colisure (IDEXX Laboratories Inc., Westwood, Maine) have been widely used by municipalities, regulatory agencies, researchers, and volunteers to evaluate the health and safety of water. These methods work by estimating the concentration or amount of *E.coli* in a water sample that is able to grow and produce a color change using specified media (Figure 3). *E.coli* is widely used as an indicator of fecal contamination due to the fact that cultivation and detection methods are relatively inexpensive, little training is needed to perform tests, and their presence may indicate the presence of pathogens. Due to the many health risks *E. coli* presence can pose, entities such as the US EPA and State Departments of Environmental Quality (DEQ) have implemented ways to assess and regulate waters containing *E.coli.* Regulatory levels of *E.coli* have been established to determine if a water is suitable for partial or full body contact based on an acceptable human health risk. According to the US EPA, partial-body contact (PBC) means the recreational use of surface water that may cause the human body to come into direct contact with the water, but normally not to the point of complete submergence. The use is such that ingestion of the water is not likely and sensitive body organs, such as the
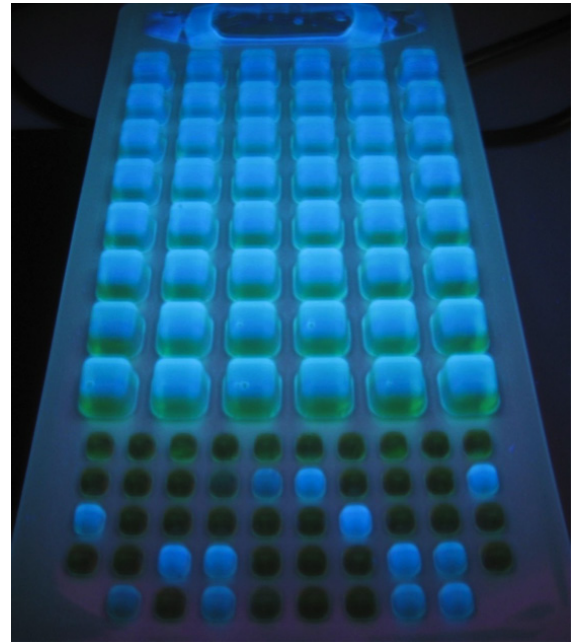

Figure 3. Visualization of a fecal contaminated water sample; cells fluorescing blue indicate the presence of *E.coli* in the water.

eyes, ears, or nose, will not normally be exposed to direct contact with the water. Full-body contact (FBC) means the use of surface water for swimming or other recreational activity that causes the human body to come into direct contact with the water to the point of complete submergence. The use is such that ingestion of the water is likely and sensitive body organs, such as the eyes, ears, or nose, may be exposed to direct contact with the water. Numerous epidemiology studies have been conducted worldwide to evaluate the association between recreational water quality and adverse health outcomes including gastrointestinal (GI) symptoms, eye infections, skin irritations, ear, nose and throat infections and respiratory illness, and have indicated that the rates of some adverse health outcomes are higher in swimmers compared with non-swimmers (Soller *et al.* 2010). Concentrations of *E.coli* cannot exceed 575 Colony Forming Units (CFU) per 100 mL for partial body contact (PBC) while full body contact (FBC) cannot exceed 235 CFU per 100 mL for human health protection and regulatory purposes. This regulatory value for FBC equates to the acceptable risk of approximately 8 cases of gastrointestinal illness (diarrhea) per 1000 swimmers per year (US EPA 2009).

Although the presence of *E.coli* in water indicates the presence of fecal contamination and potential pathogens, it has been established that most warm-blooded animals can release fecal coliform bacteria and *E.coli* to a body of water (Buchan, *et al.* 2001). Consequently, the presence of *E. coli* in water is not specific to human sources of pollution. Fecal coliform bacteria are found in both human and animal feces and thus, may present a unique tool for tracking sources or contamination. Tracking and monitoring the source of contamination is critical for problem identification and remediation (Fong, *et al.* 2005). The most widely used

method for measuring fecal pollution is to quantify viable fecal coliform bacteria by culturing them. However, culture based methods do not identify the source of fecal contamination (Field and Bernhard 2000).

What is Microbial Source Tracking?

Microbial Source Tracking (MST) methods are intended to discriminate between human and non-human 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. 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 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 (US EPA 2005). These fingerprints are then compared to developed libraries for classification. 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 and experienced personnel completing the analysis due to the time it takes to develop a library (Figure 4). Additionally, one of the major disadvantages to library-dependent methods is that libraries tend to be temporally and geographically specific.
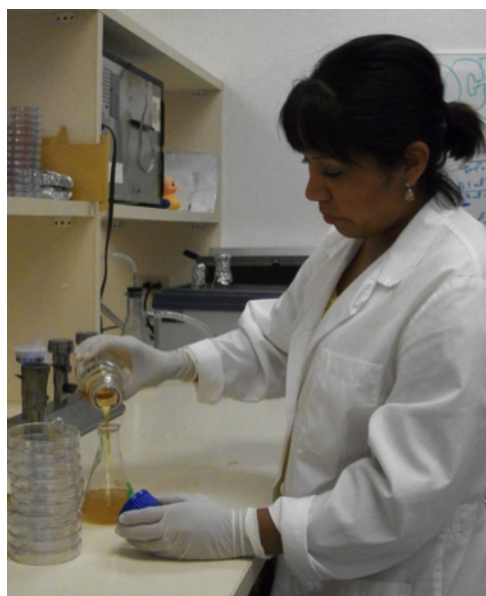
While this can be useful for a specific location, they are generally not as applicable on a broader watershed scale or on statewide issues.

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 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 enables researchers 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 relatively short amount of time (Figure 5). 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 (US EPA 2005).

What MST methods are currently being used?

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 US 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 (US EPA 2005). Comparison studies have shown that no single method is clearly superior
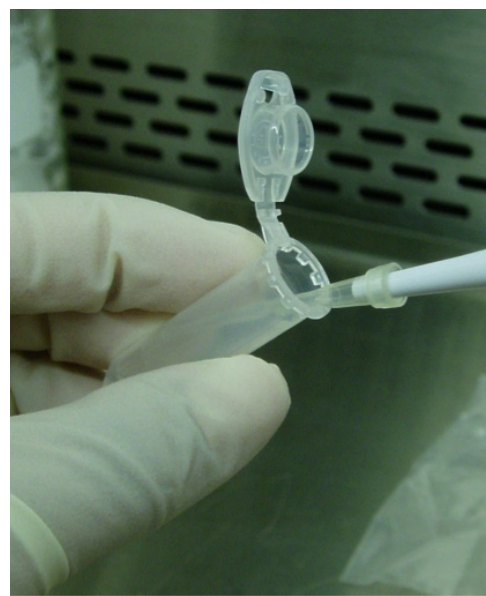

Figure 4. PhD student, Berenise Rivera, demonstrates sterile technique while assaying water samples for fecal bacteria.

Figure 5. DNA Extraction/Concentration.

Table 1. Common Types of MST Methods (ref: US EPA 2011)

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

to the others (US 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 to 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. The following table describes existing MST methods that are currently being used and the general purposes for each (Table 1).

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 seem to have led recent method development. 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.*

What is Bacteroides?

The genus *Bacteroides* contains Gram negative, nonspore-forming, non-motile, 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. These conditions are gastrointestinal surgery, perforated or gangrenous appendicitis, perforated ulcer, diverticulitis, trauma and inflammatory bowel disease. Another important aspect of *Bacteroides* biology is their lack in ability to proliferate in the environment as well as their potential to survive in the environment at a rate directly proportional to the pathogens of concern. *Bacteroides* depend primarily on temperature and presence of predators, and have been found to survive for up to six days under oxygen stressed conditions similar to other pathogens. (Field and Dick 2004)

Due to the abundance of this bacterium in human and animal feces, it has allowed for host-related analysis targeting genes present in the *Bacteroides* genome. Layton *et al.* (2006) states, bacteria belonging to the genus *Bacteroides* have been suggested as 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 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. Katherine Field and colleagues, in particular, have performed extensive research into the use of *Bacteroides* 16S rRNA-based PCR assays for MST. Field and Bernard (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 differentiating between human and ruminant associated *Bacteroides*, therefore identifying the possible source of contamination. As Scott *et al.* (2002) mentions, this approach offers the advantage of circumventing the need for a culturing step, which allows a more rapid identification of target organism.

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. PCR was found to be a reliable indicator of fecal contamination. *Bacteroides* was observed in 95% of fecal samples in all sewage treatment plant 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, Weisberg and McGee (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.

MST Supporting Watershed Characterization and Source Identification in Arizona.

The Arizona Department of Environmental Quality (ADEQ) was established by the Arizona Legislature in 1986. ADEQ's goal is to protect and enhance public health, welfare, and the environment in Arizona. Today, ADEQ manages a variety of programs to bring awareness of the water issues Arizona is currently facing. Also, ADEQ uses programs to improve the welfare and health of Arizona's citizens through ensuring that water resources meet regulatory standards. This regulatory agency maintains a 303d list of locations that do not meet clean water regulatory standards across the State of Arizona (ADEQ 2010). Section 303d requires total maximum daily loads (TMDL) be established for the impaired waters by states, territories, and authorized tribes with oversight by the US EPA (Simpson, Santo Domingo and Reasoner 2002). A TMDL is defined as the maximum amount of a pollutant the water body can receive and still meet regulated limits for that pollutant. As of 2008, ADEQ listed 17 impaired watersheds throughout the state of Arizona on the 303d list due to *E. coli* levels higher than the set standards (US EPA 2008). It is anticipated that the number of impaired watersheds will increase by the year 2012. ADEQ works diligently to bring those impaired watersheds to standard.

Recently, an approach used by the ADEQ section tasked with TMDL implementation has involved intensive water quality monitoring by trained volunteers coupled with the use of innovative MST methods. This approach aims to better understand and outline the courses of action necessary to restore impaired waters and to protect and maintain unimpaired waters across the State of Arizona. As part of this approach, local stakeholder driven watershed groups and the State agency have begun to collaborate with research institutions, the University of Arizona and Northern Arizona University, to utilize MST techniques to identify sources of fecal bacteria and microbial contamination within impaired watersheds. The objective of this approach is to identify and appropriately characterize the pollutant sources causing the impairments. In watersheds where sources are not known or understood, MST techniques can help to identify and also eliminate potential sources of fecal bacteria.

To date, over 181 surface water samples have been collected by volunteers trained by faculty, staff and students from the University of Arizona Cooperative Extension in three watersheds currently classified as impaired for

Figure 6. Volunteer water quality monitoring team receives training from UA Cooperative Extension.

Figure 7.Volunteer water quality monitoring in the Santa Cruz River, AZ.

E.coli bacteria by ADEQ (Figures 6 and 7). Research at the University of Arizona is currently evaluating published MST methods that produce reliable data from these watersheds for TMDL development and implementation. MST methods were specifically chosen within these select regions in the State due to the anticipated source(s) of bacteria not visibly obvious in these watersheds. More specifically, methods were selected to differentiate between Human and Bovine sources of *Bacteroides* present in water samples collected by volunteers. Each of the different watersheds included in this study has different land-use characterization (urban vs. rural) and potential inputs of pollution within their area. Using the methods mentioned above to identify the sources of fecal pollution will empower ADEQ to work with stakeholders within the community to monitor and remediate locations contributing to contamination with the ultimate intent to de-list impaired waters of Arizona.

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Table 2. Commonly Used Terms (ref. US EPA 2011)

Commonly Used Terms:

Biochemical (aka 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 antibiotic or utilization of carbon or nutrient source.

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

Colony Forming Units (CFU) refers to the unit of measure or the concentration of cultured bacteria.

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 of 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 for a library.

Microbial Source Tracking (MST) refers to a group of methods intended to discriminate between human and non-human 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 (aka genotypic) methods utilize variations in the genetic makeup or the DNA of each individual organism or bacteria. This is often referred to as "DNA fingerprinting".

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