# Appendix C. Review of Methods and Markers for Microbial Source Tracking

## Introduction

Fecal material containing pathogenic viruses, bacteria, and protozoa creates a public health risk in contaminated environmental water. Sources of fecal pollution in a watershed can be both point and non-point, from diverse human, agricultural and wildlife origins. The ability to track the fate and transport of fecal pollution and distinguish between sources is particularly important for mitigating and managing water quality and waterborne diseases. At present, monitoring for all waterborne pathogens is unrealistic due to the diversity present in sewage and the broad range of costly or challenging methods used to collect and identify pathogenic organisms in environmental samples.

To date, water quality monitoring and management practices have relied heavily on fecal indicator bacteria (FIB), including *Escherichia coli* and *Enterococcus,* which have low pathogenic potential but abundant presence in sewage and feces. FIB are therefore suggestive of pathogen presence. Studies have shown, however, that pathogen presence does not always co-vary strongly and consistently with FIB concentrations since the ecology and fate of FIB outside a host can vary widely (Anderson et al. 2005, Harwood et al. 2005, Colford et al. 2007, McQuaig et al. 2009). FIB can be native or adapted to stream, estuary, and bay habitats and some are shown to persist or even grow in association with aquatic sediments, aquatic vegetation, and terrestrial soils (Whitman et al. 2003, Ishii et al. 2006, Badgley et al. 2011). Furthermore, conventional indicators cannot discern between human and animal sources because FIB are present in the feces of most mammals and birds (Harwood et al., 1999; Souza et al., 1999; Leclerc et al., 2001). It is important to distinguish between human and animal derived fecal pollution because of the heightened health risks associated with human sewage and the different remediation strategies for mitigating contamination from sewage versus surface runoff carrying animal waste. As our knowledge of zoonotic disease potential increases, the need to identify specific sources of animal waste in contaminated water bodies also intensifies.

Despite the limitations of FIB methods, they continue to be broadly used because they offer fast, easy, inexpensive detection. Alternative indicators for waterborne fecal pollution also exist, including viruses, caffeine, and optical brighteners, and molecular markers (Noble et al. 2003, Buerge et al. 2003, Dixon et al. 2005; Glassmeyer et al. 2005, Hagedorn and Weisberg 2009). Using molecular markers to target DNA sequences from host-associated microorganisms or sequences derived directly from the

host offers an analytical approach with unprecedented specificity, sensitivity, and quantitative capacity. Differences in gut conditions such as temperature, diet, and type of digestive system shape the intestinal microbiota and select for microbial communities unique to their respective human or animal host (Sekelja et al. 2011, Shanks et al. 2011). Microbial source tracking (MST) relies on bacterial taxa or genetic markers that occur preferentially or exclusively in the intestinal system of a target host population and are excreted in high abundance through the host feces (Field and Samadpour, 2007). Ideally, MST markers are also directly correlated with public health risks and provide quantitative data for determining total daily maximum loads (TDML) of pollution in water bodies in accordance with state regulations and the federal Clean Water Act (US EPA CWA 303(d)). Advances in next-generation sequencing technologies and microbiome research have resulted in comprehensive inventories of microbial communities associated with a wide range of hosts and environments allowing rapid development and application of targeted genetic markers for microbial source tracking (MST) (Robinson et al. 2010, Lozupone et al. 2012, Quast et al. 2013, McLellan and Eren, 2014).

## Overview of Methods

Techniques for MST can be generally divided into two categories, library-dependent and library-independent, with a focus on genetic or phenotypic traits. Phenotypic analyses measure expressed traits of microorganisms whereas genotypic methods detect a specific gene sequence or evaluate genetic polymorphisms (differences) in DNA.

*Library-Dependent*

Library-dependent techniques require a cultivation step to generate the library of known bacterial isolates from water sources and fecal samples, to which unknown bacterial isolates from environmental samples can be compared. The library of isolate bacteria is characterized by an identifying attribute such as genetic signature, antibiotic resistance or carbon source utilization (Hagedorn et al. 1999, Moore et al. 2005). Phenotypic analyses like antibiotic resistance and carbon source utilization assume that selective pressure alters the antibiotic resistance or metabolic profile of fecal bacteria from different animals and humans because they are likely exposed to different types of antibiotics or organic substrates. Therefore, antibiotic resistance or carbon utilization profiles of easily cultured FIB bacteria from known fecal samples can be used to classify unidentified environmental isolates based on profile similarity. Genotypic library-dependent analyses generally discriminate between *E. coli* or *Enterococcus spp*. based on the assumption that these organisms are uniquely adapted to their known host environment therefore differ genetically from other strains found in other host species.

Ribotyping and Pulsed Field Gel Electrophoresis (PFGE) are commonly used library-dependent genetic techniques (Carson et al. 2001, Stoeckel et al. 2004). Both methods use restriction enzymes to cut bacterial DNA into fragments that are separated by size and visualized as unique banding patterns, or fingerprints, that can be compared to a library of characterized DNA from known fecal bacteria. PFGE digests whole genomic DNA of bacterial isolates and visualizes large DNA fragments on a specialized gel. Ribotyping is based on differences in the genomic sequences within 16S ribosomal ribonucleic acid (rRNA) gene, a gene that is universally present in bacterial genomes and contains hyper-variable regions that are widely used for taxonomic classification of bacterial communities (Chakravorty et al. 2007).

In a comparison of phenotypic and genotypic library-dependent techniques using blind samples containing one to three of five possible fecal sources (human, dog, cattle, seagull, or sewage) all methods could correctly identify the dominant source in most samples (Harwood et al. 2003, Myoda et al. 2003). Overall, the genotypic library-based techniques performed better than the phenotypic techniques (Stoeckel and Harwood, 2007, Sargeant et al. 2011). While the phenotypic methods had high false positive rates (i.e. a source was identified when it was not actually present) the genotypic analyses showed variable sensitivity (Myoda et al. 2003). Issues with all methods were attributed to the statistical tests used to match patterns from blind sample isolates with the host library database and the limited representativeness of libraries (Stoeckel and Harwood, 2007, Sargeant et al. 2011). In order to establish a comprehensive library, observational knowledge of potential sources of fecal contamination is required and many representative fecal samples from target organisms across all geographic sites of interest must be collected. In general, the accuracy of with which environmental samples are classified into fecal source categories varies widely with library size and representativeness (Stoeckel and Harwood, 2007). The need to develop large site-specific libraries (>1000 isolates), that are both time and labor intensive, has decreased interest in using library-dependent approaches (Johnson et al. 2004, Santo Domingo et al. 2011).

*Library-Independent*

In contrast, library-independent techniques do not require the development of a source library database. These techniques rely on a species-specific genotype or characteristic detected within a mixed environmental sample. Nucleic acid replication via polymerase chain reaction (PCR) is an important genetic tool in library-independent approaches that can be applied to both laboratory-cultivated bacteria and DNA sequences obtained directly from environmental samples. PCR facilitates rapid, preferential amplification of specific nucleotide sequences from a mixture of non-target sequences. As a result, PCR allows detection and examination of gene targets that are strong indicators of fecal source DNA and only requires a small amount of starting

material from cultured bacterial cells or environmental DNA. PCR protocols that detect the presence or absence of a gene sequence are referred to as end-point PCR.

In one of the first library-independent studies, Bernhard and Field demonstrated the use of end-point PCR of the 16S rRNA gene of human-associated *Bacteroidales* to detect human fecal contamination (Bernhard and Field, 2000). This method served as a precursor for many other library-independent gene-specific PCR analyses (reviewed in Harwood et al. 2013). A common end-point PCR method for identifying human fecal pollution not based on the 16S rRNA gene, uses a culture step to enrich for target *Enterococcus faecium* cells and then amplifies and detects the enterococcal surface protein (esp) gene (Scott et al. 2005, Ahmed et al. 2008). Both methods have been shown to be highly sensitive and specific (>90%) (Ahmed et al. 2009, Boehm et al. 2013, Harwood et al. 2013) although additional studies have detected some level of *E. faecium* and human-associated *Bacteroidales* in the feces of animals (Kildare et al. 2007, Whitman et al. 2007; Layton et al., 2009; Boehm et al. 2013). In addition to human-associated microbial gene targets, many PCR methods have been developed to detect common animal sources including dogs, pigs, cows, poultry, gulls and other wild birds. These, and other gene-specific PCR targets discussed below, are adequate to determine the source of fecal microbial pollution in the environment, however, they cannot be used to quantify the amount the fecal pollution and evaluate associated public health risks.

Recently, quantitative real-time PCR (qPCR) assays which allow for more rapid detection of markers, as well as determination of their relative concentrations, have been developed (Dick and Field 2004, Seurinck et al. 2005). qPCR works much like end-point PCR but the accumulation of PCR products is quantified with each reaction cycle using a fluorescence detector. The strength of the fluorescent signal indicates the relative amount of a specific target DNA sequence in a sample (Walker, 2002) and thus can be used in TMDL analysis and subsequent management decisions. In many studies of human and animal-associated gene targets, qPCR methods have been found to more precisely correlate with pathogen presence compared to end-point PCR or other MST methods (Savichtcheva et al. 2007; Walters et al. 2009; Harwood et al. 2013). It should be noted that correlations between MST markers and pathogens have not been found in all studies yet the general conclusion in the field is that *Bacteroidales* markers have a comparable or better ability to predict pathogens compared with conventional FIB methods (Fremaux et al. 2009; Schriewer, et al. 2010).

Oligotyping is a recently introduced computational method that allows the identification of closely related but distinct bacterial strains that would normally be classified as one taxonomic unit. Variations within a single bacterial taxa can result in differential distribution patterns between geographically distinct host populations that can then be used to identify a source population. Eren and colleagues (2015) identified host-specific oligotypes of the bacterial taxon *Blautia* that occurred exclusively in fecal

samples of humans, swine, cows, deer or chickens. Oligotyping has also been used to distinguish between members of the taxon *Helicobacter* found in the gut and feces of wild and domestic animals including seabirds, marine mammals, and dogs (Oxley and McKay 2005).

Whole-community analysis based on bacterial 16S rRNA gene sequencing of fecal and environmental microbial communities demonstrate evidence of host patterns in entire bacterial assemblages. Early studies using whole bacterial communities demonstrated that the native microbial communities in water are changed by the addition of fecal contamination from bovine or equine sources (Cho and Kim 2000, Simpson et al., 2004). More recently, Newton and colleagues (2013) used community sequencing of bacterial 16S rRNA gene to describe three sewer infrastructure-associated bacterial genera and five fecal-associated bacterial families that served as signatures of sewer and fecal contamination in urban rivers and lakes. Other studies have found that microbial communities from the same fecal origin were highly similar and could be used to determine the dominant sources of fecal contamination in water samples (Lee et al. 2011, Cao et al. 2013).

Microarray technology provides high-throughput comprehensive screening of whole microbial communities or targeted MST markers. Microarray platforms contain thousands of short gene sequences for classes of markers specific to indicator organisms, pathogens, and source identifiers that hybridize with PCR products or whole genomic DNA in samples. Multiple microarrays have been designed and used to specifically detect waterborne bacterial pathogens (Miller et al. 2008, Gomes et al. 2015). Specifically for MST applications, the Phylochip microarray for 16S rRNA bacterial community analysis was modified by Dubinsky and colleagues (2016) to detect and distinguish fecal bacteria from humans, birds, ruminants, horses, pigs and dogs. Also, Li and colleagues (2015) developed a custom microarray targeting waterborne viral, bacterial, and protozoan pathogens, well-studied fecal indicator bacteria and markers, antibiotic resistance genes, as well as universal bacterial probes for whole community characterization. While microarray tests can be used to rapidly screen for multiple sources of fecal contamination and identify human health risks, they do not provide quantitative information about the identified sources that may be critical for environmental monitoring applications.

## Microbial Targets

Recent MST research has focused on fecal anaerobe markers because of the unlikelihood that these organisms will successfully grow and reproduce outside their host. They are either specifically adapted to or selected for by the host gut, and consequently will be more tightly associated with fecal pathogen presence in the environment. Fecal anaerobes of the taxonomic order *Bacteroidales* have received the

majority of MST research effort (Bernhard and Field, 2000, and reviewed in Harwood et al. 2013); other potential indicators include members of *Clostridiales* and direct pathogen detection.

*Bacteriodes*

Selected for its high concentrations in feces and tendency to coevolve with its host, the Bacteroides-Prevotella taxon was one of the first targets of library-independent detection based on the HF183 end-point PCR of the 16S rRNA gene (Bernhard and Field, 2000). *Bacteroidales* are gram-negative, obligate anaerobes that occur in human and animal feces at concentrations from $10^9$ to $10^{11}$ cells · $g^{-1}$ and at concentrations of $10^9$ cells · $100ml^{-1}$ in sewage (Holdeman et al. 1976, Wexler 2007) compared to traditional FIB that exist at orders of magnitude lower concentrations ($10^6$ to $10^7$ CFU · $100ml^{-1}$ in sewage) (Harwood et al. 2005, Converse et al. 2009). Many studies have confirmed the high sensitivity and general specificity of HF183 and related *Bacteroides* markers for human and animal targets (Kildare et al., 2007, Harwood et al. 2013, Boehm et al. 2013).

*Clostridiales*

Obligate anaerobes of the phylum Firmicutes, members of the *Clostridiales* are commonly found in the gut of humans and animals. Within this group of organisms, MST focus has been on *Lachnospiraceae*, one of the most abundant groups of faecal bacteria in sewage (McLellan et al. 2013). A strong correlation was observed between between *Lachnospiraceae* and adenovirus, indicating a link between these markers and human pathogen presence (Newton et al. 2011). Members of *Clostridiales* have also been found in high abundance in avian and marine mammal hosts and feces and subsequently been developed as MST markers for these organisms (Oxley and McKay 2005, Green et al. 2012, Koskey et al. 2014).

*Pathogens*

Direct detection of pathogens in watersheds is beneficial for assessing public health risk. The Centers for Disease Control and Prevention (CDC) has found fecal pathogens shiga-toxin producing *E. coli, Shigella, Salmonella,* and *Campylobacter* as the dominant sources of fecal-associated waterborne disease (Lee et al. 2002). Among the fecal coliform bacteria strains of shiga-toxin *producing E. coli* O157:H7 and the pathogen *Shigella sonnei* both cause a range of intestinal illnesses. The *E. coli* O157 serotype and other pathogenic *E. coli* can be identified by the PCR detection of specific shiga-toxin genes and surface proteins (Maurer 1999, Osek 2003, Duris et al. 2009). Certain *E. coli* toxin genes can also distinguish between cattle and swine fecal pollution presence (Duris et al. 2011). Campylobacter is another leading cause of bacterial gastroenteritis in developed regions. Wild birds and poultry are recognized as sources

of the Campylobacter taxa, *C. jejuni, C. coli* and *C. lari*, frequently implicated in human illness (Butzler 2004). Campylobacter qPCR markers can discern between pathogenic and non-pathogenic strains and have been used to inform public health risk assessment from gull fecal pollution (Lu et al 2011). The issue with direct detection is pathogen strains are normally found in low densities in environmental water, and a cultivation step is required to increase the sensitivity of the assays (Duris et al. 2011).

## Non-Bacterial Targets

*Viruses*

Monitoring for human viruses has been suggested as an alternate approach to assess human health risks in environmental waters. Viruses are generally highly host-specific and do not multiply in the environment or readily degrade under environmental stressors, such as UV irradiance and water treatment processes, unlike traditional FIBs. However, pathogenic viruses usually infect a small percentage of any given population, making them relatively rare targets (and thus more difficult to detect) (Pina et al. 1998). Certain non-pathogenic human viruses have a wider distribution in human populations than pathogenic viruses and their stable nature makes them ideal indicators of other viral pathogens, such as noroviruses and hepatitis A viruses, persistent in the environment (McQuaig et al. 2009). The human adenovirus (HAdV) and human polyomavirus (HPyV) are promising as human fecal indicators, as they are frequently excreted in the feces or urine of humans both with and without clinical symptoms and they are commonly detected in urban wastewater (Bofill-Mas et al., 2001). Certain adenoviruses exist that are specific to livestock as well providing distinction between human or animal-derived fecal pollution (Rusinol et al. 2014). Studies have demonstrated HPyV targets to be 100% specific, showing no cross-reactivity to animal fecal samples (Harwood et al. 2009, McQuaig et al. 2009, Ballesete et. al. 2010)

*Archaea*

Archaeal methanogens are commonly associated with the oral, vaginal, and intestinal mucosa of mammals (Belay et al. 1998, Belay et al. 1990, Miller et al. 1982, Miller et al. 1986). *Methanobrevibacter ruminantium* and *M. smithii* have been tested for possibilities as ruminant and human markers, respectively (Ufnar et al. 2006, Ufnar et al. 2007). *M. smithii* a methanogenic archaeon found exclusively and abundantly in the human gut and human fecal samples (Lin and Miller 1998, Dridi et al., 2009). Likewise, *M. ruminantium* is specific to the rumen of domesticated animals (Smith and Hungate 1958). The nifH gene is targeted in archaeal indicators because it is a predominantly methanogen-specific gene with sequence differences that can be used to discriminate between methanogen groups. The *M. ruminantium* nifH assay is shown to be successful at detecting cattle, sheep, and goat feces and contamination by agricultural lagoon

waste in environmental water samples (Ufnar et al. 2007). The *M. smithii* marker has high sensitivity against human sewage pollution especially in coastal waters but did show some cross-reactivity with bird feces (Ufnar et al. 2006, Johnston et al. 2010).

*Direct source detection*

The first fecal source tracking method based on a eukaryotic genetic marker was the end-point PCR assay targeting the human mitochondrial DNA (mtDNA) NADH dehydrogenase subunit (Martellini et al., 2005). mtDNA was proposed as a marker based upon the premise that it should be abundant in feces and especially host-specific. Other studies have used qPCR probes to target human, bovine, ovine and swine mtDNA for use as indicators in source tracking studies of shellfish harvesting areas (Baker-Austin et al. 2010). Developments in biodiversity monitoring using environmental DNA (eDNA), genetic material obtained directly from environmental samples from any organism, have also found application in fecal source tracking. Utilization and contamination of waterbodies by various wildlife, human, and domesticated animals can be detected through eDNA markers (Thomsen and Willerslev 2015).

## Evaluation of Source Tracking Methods

Any satisfactory MST method must comply with a set of performance criteria (Stoeckel and Harwood, 2007). Some performance criteria are universally applicable while others depend on the objectives of a particular study (Santo Domingo et al. 2007). The key universal criteria are described here.

*Sensitivity*

The sensitivity of a MST method is defined by the percentage of true positive results detected. Sensitivity indicates the robustness of an assay provided that targets are present at or above detection levels. Samples spiked with fecal material or other known contaminated samples are used to directly test the number of positive controls correctly identified as positive by the assay. Physical or chemical properties of the water matrix or sample type may impair the sensitivity of certain methods (Siefring et al. 2008).

*Specificity*

The specificity of a MST marker is represented by the rate of false positive results or the percentage of negative results correctly ascribed to samples known to lack the host target in question (Stoeckel and Harwood, 2007). A highly-specific MST marker should not cross-react with unintended targets and accurately identify only target source species. It is desirable that a marker is tested against as many nontarget

fecal samples as possible to better constrain limitations of method specificity (Harwood and Stoeckel, 2011)

*Stability*

The stability criteria dictates that changes in environmental or biological conditions due to seasonal or regional differences should not affect the presence of MST targets in host feces. A stable marker does not vary in frequency or concentration over time at the population level, has consistent detection across all geographic regions of the host range, and exhibits predictable rates decay in all habitats and water matrices (Sargeant et al. 2011).

*Challenges for stream, river, and estuarine systems*

Understanding eDNA detection rates in lotic systems is critical for inclusion of eDNA analysis as a reliable survey method in fecal source tracking. The concentration of DNA in rivers and streams depends on dynamics between eDNA released into the water, downstream transport and losses to the system through physical, chemical and biological processes. The contribution and rate of production of eDNA by various organisms has been the focus of only a few studies (Pilliod et al. 2014, Thomsen et al. 2012, Klymus et al. 2015) and is likely influenced by the size, sex, health, and density of members in a population. Difficulty measuring the transport and residence time of eDNA in riverine systems also poses challenges to describing the geographic origin of eDNA and making spatial interference about the source organism(s). A study by Deiner and Altermatt (2014) observed movement of eDNA five to ten kilometers downstream of the source population within a 24hr sampling period, indicating that eDNA can persist over relatively large distances in a river system. It has been shown, however, that eDNA concentrations are generally localized and do not appear to accumulate downstream (Deiner and Altermatt 2014, Pilliod et al. 2013, Laramie et al. 2015). Dilution and removal processes such as settling and degradation, likely reduce the amount of detectable eDNA over time and as it travels downstream thereby limiting accumulation (Dejean et al. 2011, Jane et al. 2014).

## *Recommendations*

In a review and critique of MST methods, the Washington State Department of Ecology highlighted the lack of standardized, validated, promulgated, and U.S. Environmental Protection Agency approved molecular MST methods. Sargeant and colleagues (2011) proposed the following quality assurance sampling for substantiation of results: 1) Field samples duplicated for reproducibility information; 2) Preliminary testing of source feces from the study area to confirm the source-specific MST indicator or marker is present; 3) Samples spiked with fecal material from each potential source per study as positive controls; 4) Samples from presumably uncontaminated sites as

field negative controls. The use of multiple MST techniques in parallel, was also recommended to overcome the experimental nature of fecal source tracking methods and to produce acceptable levels of accuracy, reproducibility, and investigation of numerous potential source types. Furthermore, library-independent methods are recommended over library-dependent methods because they typically have a lower cost and provide much faster results (Sargeant et al. 2011).

## The Toolbox Approach

Because rivers, streams and estuaries can have considerable temporal and spatial variability in microbial water quality from a multitude of human and animal-derived sources, a monitoring strategy that captures data about all potential sources is optimal. No one marker has all the requisite performance qualities for identifying and quantifying the source and magnitude of fecal pollution in water. Thus, a toolbox approach using a suite of techniques and molecular markers, producing multiple lines of evidence, is considered important to effective microbial source tracking (Harwood et al. 2013).

Monitoring, mitigation, and management of fecal pollution can be costly to coastal communities, which depend on uncontaminated water bodies for tourism, recreation, and fisheries (Rabinovici et al. 2004). Most public advisories and closures in recreation areas and shellfisheries are posted without specific knowledge of the type and source of fecal contamination (NRDC, 2006). A better understanding and implementation of MST will facilitate targeted remediation, enhance protection of public health, and minimize economic costs associated with fecal pollution in water systems.

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