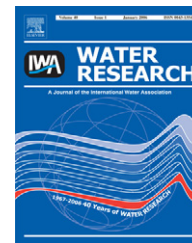


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Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution

Jorge W. Santo Domingo^{a,*}, Dustin G. Bambic^{b,1}, Thomas A. Edge^c, Stefan Wuertz^d

^aUS Environmental Protection Agency, NRMRL/WSWRD/MCCB, 26 W. Martin Luther King Dr., MS 387, Cincinnati, OH 45268, USA

^bLarry Walker Associates, 707 Fourth Street Suite 200, Davis, CA 95616, USA

^cNational Water Research Institute, Environment Canada, 867 Lakeshore Road, Burlington, Ont., Canada L7R 4A6

^dDepartment of Civil and Environmental Engineering, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

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ABSTRACT

Advances in microbial source tracking (MST) have largely been driven by the need to comply with water quality standards based on traditional indicator bacteria. Recently, a number of culture-independent, and library-independent methods based on polymerase chain reaction (PCR) have been gaining popularity among source trackers. However, only a limited number of these methods have been successfully used in field applications, primarily due to the fact that many of them are still being developed. In this critical outlook, we examine different viewpoints associated with the practical use of MST to identify critical research gaps, propose a priority-based timeline to address them, and outline emerging technologies that will likely impact the future of source tracking. We propose that it is necessary to consider each of these aspects in order to advance towards a unifying framework in source identification, so that fecal pollution monitoring can be reliably used for comprehensive environmental microbial monitoring, to develop risk assessment models, and to implement and validate adequate management practices.

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1. Introduction

Fecal microorganisms are one of the primary pollutants of concern in the world. For example, the 2000 National Water Quality Inventory reported approximately 93,000 river and stream miles in the United States as containing high levels of fecal bacteria (USEPA (United States Environmental Protection Agency), 2002). The most troublesome aspect of fecal pollution in the developed world is that uncharacterized sources seem to be mostly responsible for contaminating significant portions of our watersheds and coastal areas. Current regulations require state and local agencies to monitor fecal pollution levels based on methodology developed in the early 1900s that does not allow identification of the specific sources of microbial pollution. Microbial source tracking (MST) is a rapidly emerging area of applied environmental microbiology

that focuses on identifying the source(s) of fecal contamination impacting a water system (USEPA, 2005). Approaches used in MST are also relevant to other research fields like food safety and agricultural and veterinary microbiology.

In the US, the need for identifying fecal sources has recently become a priority to states, territories, and authorized tribes in light of the federal requirements to develop and implement total maximum daily loads (TMDLs). A TMDL determines the maximum amount of pollutant that an impaired waterbody is able to receive and still meet applicable water quality objectives (WQOs), and allocates that amount to existing waste loads from point sources and non-point sources including natural background and a margin of safety (USEPA, 2001). In most cases, non-point sources (e.g., faulty septic tanks, livestock, and wildlife) are believed to be responsible for WQO exceedances. Thus, it is necessary to determine

*Corresponding author. Tel.: +1 513 569 7085; fax: +1 513 569 7817.

E-mail address: santodomingo.jorge@epa.gov (J.W. Santo Domingo).

¹ Current address: AMEC Earth and Environmental, 3800 Ezell Road, Suite 100, Nashville, TN 37211, USA.

which non-point sources need to be controlled in order to establish adequate best management practices (BMPs), which are used to implement TMDLs and meet WQOs (Fig. 1). Without properly guided source control efforts, millions of dollars may be appropriated without much benefit to water quality or public health.

A multitude of source-identification methods have been developed in response to the need for controlling fecal loading into receiving waters (Fig. 2) (Simpson et al., 2002; USEPA, 2005). While requirements to develop and implement

TMDLs have been the primary driver for method development in the US, these methods can also be applied to help meet microbial quality standards of waters used for shellfishing and/or protected under the Beaches Environmental Assessment and Coastal Health (BEACH) Act. The number of beach closures and advisories in the US increased by 50% between 1997 and 1999, amounting to over 6000 days in 1999 (NRDC (Natural Resources Defense Council), 2000), and in 2005 more than 20,000 days of closings and advisories were reported (NRDC, 2006). While this apparent increase in posting days

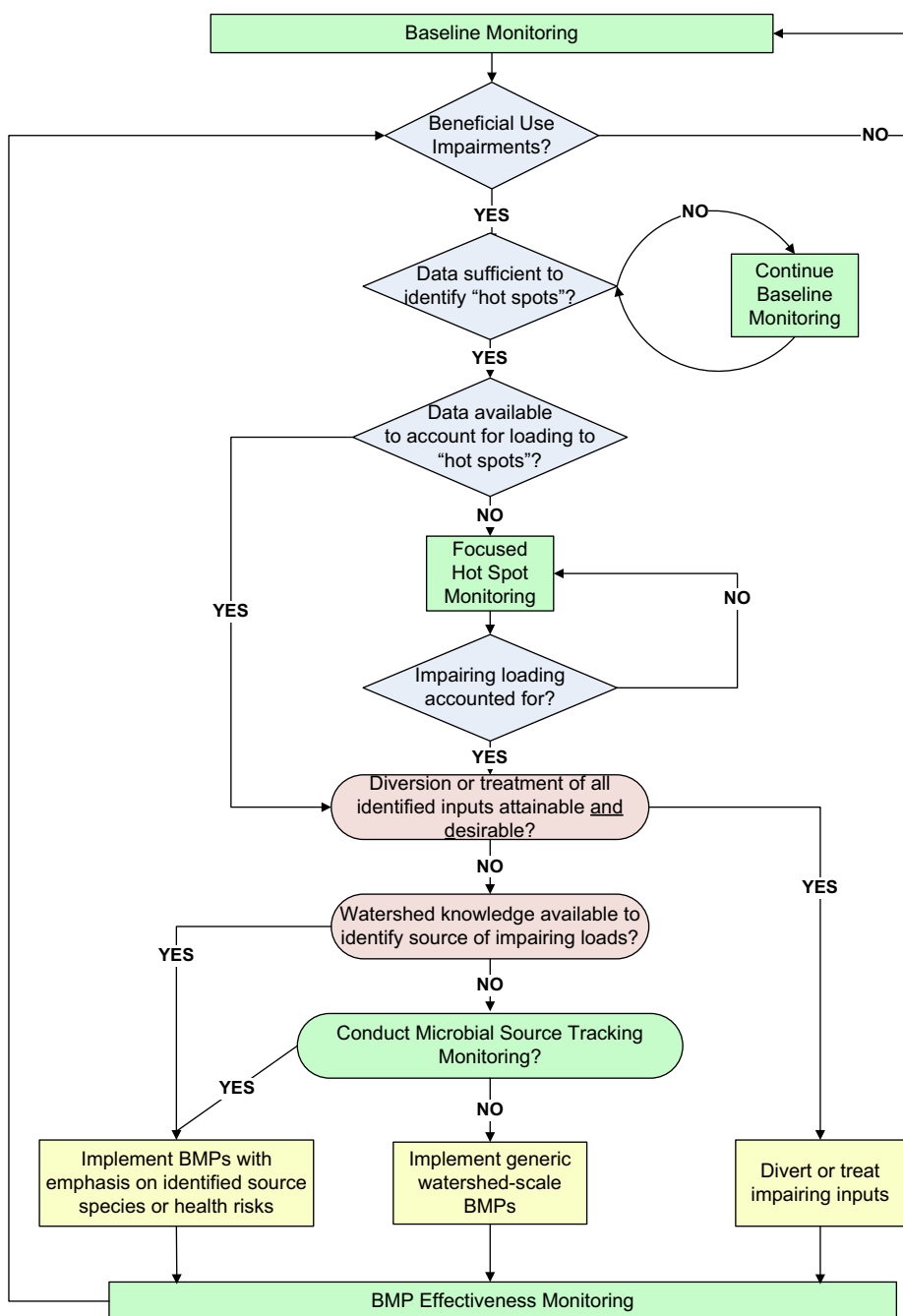


Fig. 1 – Example of how MST studies can assist with decision-making when attempting to meet water quality standards based on bacteria.

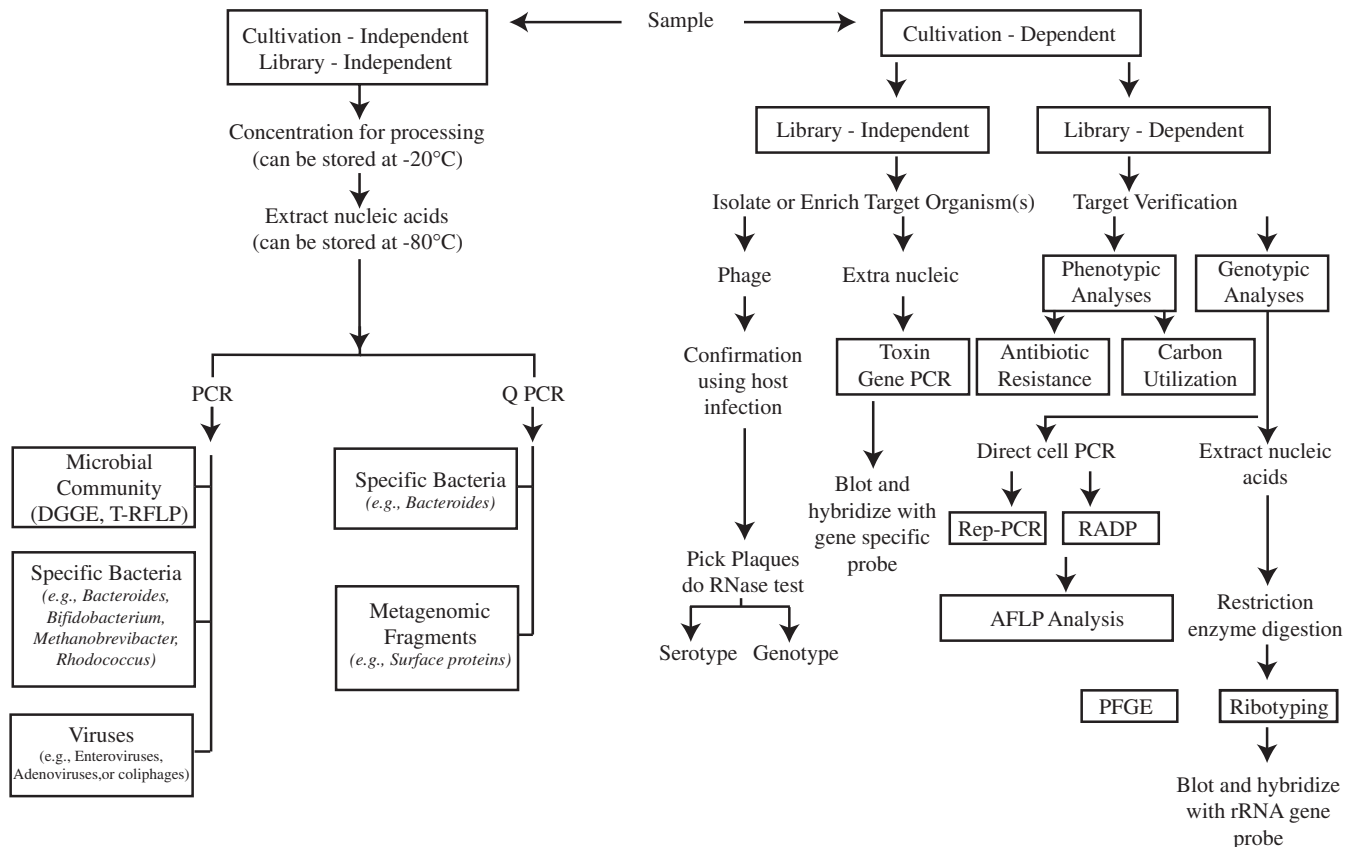


Fig. 2 – Schematic representation of some of the methods used for tracking sources of fecal pollution (modified from original provided by Cindy Nakatsu; USEPA, 2005).

could be in part due to an increase in monitoring frequency, these closures and advisories have a major economic impact considering that many coastal communities depend on recreational tourism for revenue. Similarly, temporary closures of shellfishing areas, in addition to the number of areas permanently excluded from commercial harvesting, translate into losses of hundreds of millions of dollars annually (Meschke and Boyle, 2007). The public health costs associated with exposure to fecal contamination may have huge economic impacts as well. Gastroenteritis caused by swimming in contaminated coastal waters has been estimated to result in an annual economic loss of \$21–51 million in Los Angeles and Orange Counties of southern California (Givens et al., 2006). While public advisories and closures associated with swimming and shellfishing are implemented regardless of the type of contamination, most postings are the result of unknown contamination (NRDC, 2006), and understanding the fecal sources impacting contaminated areas can be used to guide the implementation of remedial actions and to protect public health and minimize economic impacts.

Early source-tracking studies relied on bacterial DNA “fingerprints” of strains isolated from water samples and fecal sources to assign the contribution of different sources (Wiggins et al., 1999). However, recent developments in genomics and biotechnology are allowing microbiologists to use culture-independent techniques based on molecular markers to monitor the microbial quality of environmental waters. By avoiding culturing steps, such methods tend to be

more rapid and less expensive; they target a large number of organisms that otherwise would not be quantifiable, because they cannot easily be cultivated in the laboratory. However, source-tracking methodology seems to be lagging the real needs of field applications; that is, there are remaining research gaps that need to be addressed before the advantages of various established and emerging MST tools can be reliably used to their full potential. This is even more critical considering the federal requirements to meet bacteria-based WQOs in nearly all navigable water systems, the large number of systems that currently do not meet these objectives, and the complexity of many of these systems, which makes source identification and control a daunting task. Several reviews have been recently published summarizing the current status of source tracking and discussing the assumptions and limitations of existing methods and approaches (Scott et al., 2002; Simpson et al., 2002; Meays et al., 2004). In this critical outlook, we discuss the key research issues that need to be addressed in order to use source-tracking methods in field applications related to regulatory activities. Additionally, we propose a strategic framework based on timelines to guide researchers to develop MST methods within a time-frame compatible with current regulatory deadlines. Particular attention is given to culture-independent methods as biotechnological advances are providing the opportunity to develop rapid, cost-effective and reliable approaches to environmental monitoring without the requirement of culturing microbial source identifiers.

2. MST status quo

2.1. From library dependent to library independent?

MST is based on the assumption that different intestinal systems will select for specific microbial populations primarily due to differences in the diet and digestive system of different hosts. As with any ecosystem, competition for space and nutrients are relevant evolutionary forces in the intestine (Savage, 1977). The host immune system and use of antimicrobials in the animal's diet add other layers of selection that can influence the success of a potential enteric colonizer. Early MST methods used bacterial indicators of fecal contamination (i.e., *Escherichia coli* and enterococci) as source identifiers. While these bacteria may be ubiquitous in urbanized watersheds (Surbeck et al., 2006), perhaps due to persistence in secondary habitats (Ishii et al., 2006), there are lines of evidence supporting their use in source tracking. First, analyses of phenotypic and genotypic fingerprint data have shown that there are subpopulations of indicator bacteria that show host-preferential distribution (Wiggins et al., 1999; Johnson et al., 2004). Second, a few studies have shown that virulence (Khatib et al., 2003; Scott et al., 2005) and other functional (Hamilton et al., 2006; Soule et al., 2006) genes from indicator bacteria could be potential targets for assays specific to cattle, pigs, humans, geese, ducks, and elk/deer.

In recent years, however, there have been a number of findings that undermine the utility of cultivation-dependent methods (Stewart et al., 2003). For example, library-dependent methods (LDMs) based on *E. coli* libraries can result in high source misclassification rates or the inability to classify many unknown source isolates (Stoekel et al., 2004). In studies using phenotypic and genotypic fingerprints, as the size of the libraries increases, the number of populations showing true host specificity decreases, due to transient and cosmopolitan isolates (Jenkins et al., 2003; Hamilton et al., 2006). Furthermore, the statistical methods used to classify environmental samples based on library isolates can have a large effect on MST results (Lasalde et al., 2005; Wilbur and Whitlock, 2007; Hassan et al., 2005). In the case of culture-based library-independent methods (LIMs) targeting pathogenic *E. coli* and enterococci strains, the densities of these subpopulations in the intestine tend to be 2–3 orders of magnitude less than those of their non-pathogenic counterparts (Scott et al., 2005), which may reduce our ability to reliably detect them in environmental samples. Due to these problems, and the time and expense associated with developing fecal isolate libraries, culture-independent LIMs are becoming increasingly popular among source trackers.

Currently, there is an apparent transition toward culture-independent LIMs using host-specific polymerase chain reaction (PCR) assays based on alternative targets. The most desirable aspect of host-specific PCR assays is that they generally do not require cultivation, which can save much time and expense. PCR assays can be performed in a matter of hours, and they have the potential of being sensitive, inexpensive, quantitative, and amenable to automation. Another advantage of PCR-based techniques is that multiple assays targeting multiple microbial targets and microbial

populations can be performed against the same DNA extract. This is particularly relevant when coupling microarrays or low-density real-time PCR arrays for screening of multiple potential host-specific markers. Furthermore, DNA extracts from environmental and fecal samples can be preserved for future analyses should more sensitive assays, or assays for other hosts of interest, become available. While archival of DNA is not indefinite, the addition of an internal DNA control of known concentration to the extracts can be used to estimate the rate of DNA degradation when samples are analyzed at a later stage.

While comparison studies and published field studies continue to suggest that LIM methods are very promising, there are still a number of critical limitations that need to be addressed for the field to move forward toward wide-scale application of LIMs, and before they can be used in source-tracking field studies to their highest potential. These limitations, and a prioritized timeline for the field-source tracking to address them, are detailed in Section 3.

2.2. MST goes global

In spite of regulations and guidelines regarding bathing and shellfishing water quality, there are no specific regulations in most countries comparable to the TMDL programs in the US that are encouraging MST studies. However, the need for identifying fecal pollution sources is apparent in countries around the world. While fecal contamination problems may be most pressing in developing countries, MST activity is predominantly occurring in developed countries, which have the most stringent water quality monitoring requirements. For example, the European Union Directive 2006/7/EC, COD/2002/0254 regulating bathing water quality has been an impetus for MST studies in Europe, and clearly established the importance of identifying sources of contamination as part of the modern management measures necessary to improve the microbial quality of bathing waters (www.europarl.europa.eu/oeil/file.jsp?id=226822). In Canada, the drivers for MST studies have been local concerns about beach and shellfish closures, protecting drinking water sources, and meeting federal and provincial water quality regulations, guidelines, and objectives (Edge and Schaefer, 2006).

Examples of recent MST activities outside the US include work performed in Canada (Cimenti et al., 2005; Martellini et al., 2005; Edge and Hill, 2007), Australia (Ahmed et al., 2005; Barnes and Gordon, 2004), New Zealand (Gregor et al., 2002; Gilpin et al., 2003), South-Eastern and Eastern Asia (Isobe et al., 2002; Savichtcheva and Okabe, 2006; Peng et al., 2005; Okabe et al., 2007), and Europe (Schonning et al., 2002; Reischer et al., 2006; Ebdon and Taylor, 2006; Blanch et al., 2006).

3. Key research gaps and timelines pertaining to LIMs

While the emergence of MST has been welcomed by environmental professionals, there are many research gaps that need to be addressed in order for the methods to be routinely used as part of environmental monitoring programs

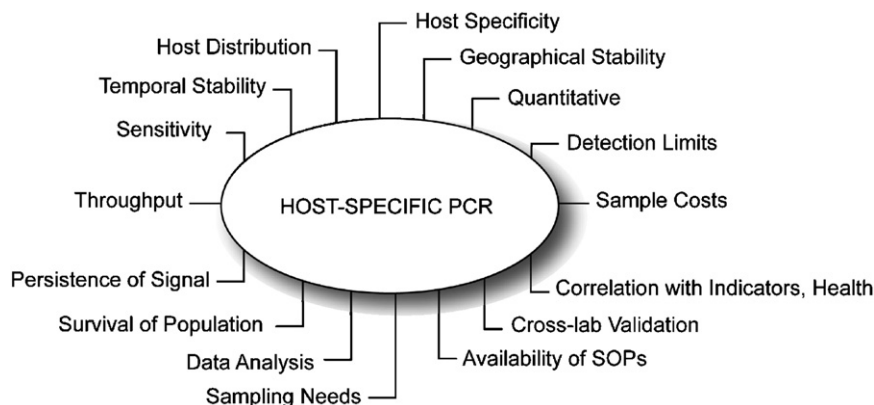


Fig. 3 – Research gaps associated with current host-specific PCR assays. These issues have been addressed at different levels by some of the method developers. The level of importance of these issues depends on the different perspectives associated with end users, regulators, and academic researchers.

(USEPA, 2005). There are different paths one could take to minimize the existing gaps. The approach for this review is to tackle them by ranking priorities and suggesting timetables. Using this rationale, we propose short-, mid-, and long-term goals to assist method developers with setting research priorities relevant to TMDL deadlines. While US TMDL deadlines are seemingly not relevant to priorities for government agencies in other countries, the outcomes of the proposed research activities are indeed relevant to recreational water quality programs and public health in general. The length for each term was arbitrarily set considering the current state of the science and the different water quality programs that will benefit from source-identification studies. It is also recognized that these timetables will largely depend on government agency priorities and availability of research funds. In the following sections, with particular attention to LIMs, we will identify some of the critical variables in MST (Fig. 3), briefly discuss the research needs associated with them, and provide examples of how some of the research gaps fit within the proposed scheme.

3.1. Short-term goals (1–3 years)

In order to use MST methods in field applications and to comply with approaching TMDL deadlines, the following research gaps will need to be addressed in the short term. Thus far, none of the currently available methods have been fully developed and validated with respect to the criteria described herein. Consequently, results from the following short-term research goals will establish which method(s) should be emphasized (or scrutinized) as part of the mid- and long-term goals.

3.1.1. Performance criteria

Perhaps the most critical issue in MST is the lack of performance standards to evaluate the accuracy of any of the existing and emerging methods (Stoeckel and Harwood, 2007). Those methods that do not pass a certain performance “score” (e.g., sensitivity or accuracy) should be discouraged for use in source-tracking field studies even though they might still be useful tools in academic research. Different sets

of criteria will be needed depending on the type of method, but some criteria can be universally applied (e.g., host distribution, sensitivity). Results from studies using performance criteria will ultimately determine whether federal agencies recommend source-identification activities to be part of funding initiatives and whether state agencies will adopt any of the current MST methods as part of their environmental monitoring schemes. Results can also be used to ensure that stakeholder needs are met without sacrificing scientific validity of applied projects, as long as agencies share similar goals and set reasonable expectations. Funding initiatives to conduct these studies should be aggressively pursued by federal agencies and should be compatible with timetables and with a balanced portfolio of methods.

It must be acknowledged that the minimal criteria that any given method should comply with depend on the objective of the study. For example, for presence/absence studies, quantification is not a requirement, while for TMDL purposes, quantification is an important criterion. The level of host specificity is important in complex watersheds as a myriad of temporally variable sources might impact water quality, while small rural watersheds might be impacted by only a few sources. In the latter cases, methods that might also produce signals with sources that are not present in that given environment could still provide useful information to the end users. Criteria needed to assess the performance of each method are discussed in Sections 3.1.2–3.1.6.

3.1.2. Host specificity

Ideally, MST assays should not cross react with non-specific targets as it is difficult to determine the true rate of false-positive signals in environmental samples, even when positive and negative controls are part of the experimental design. Several studies have shown high levels of host specificity (Shanks et al., 2006; Carson et al., 2005). However, in most cases, the frequencies have been determined with a limited number of target and non-target samples. No studies have been performed to determine the minimal number of individual target and non-target samples that are needed in order to establish acceptable levels of correct classification for quantitative PCR-based methods. It should be acknowledged

that this number might depend on the targeted population, targeted genetic marker, and availability of genetic databases. This is particularly critical when evaluating human-specific assays as humans may share flora with animals with which they cohabitate.

There has been a conspicuous absence of probability analysis in the development and application of genetic markers given that no assay can be expected to be 100% sensitive and specific. Calculated sensitivities and specificities are likely to be adjusted as assays are applied to more species, individuals and watersheds. Hence, it is important to be able to assign conditional probabilities to host-specific MST markers. This approach has been demonstrated in markers using Bayes' theorem in the case of host-specific *Bacteroidales* assays (Kildare et al., 2007) and it can be applied to any MST method, be it LDM or LIM based. Assays are assumed to be independent discrete random variables. As an example, Eq. (1) illustrates the use of Bayes' theorem to calculate the conditional probability of the human-specific assay to detect a marker sequence originating from humans in a water sample (true positive), and not fecal *Bacteroidales* sequences originating from another known source (false positive). In other words, Eq. (1) estimates $P(H/T)$, the probability of a human source of contamination (H) in an analyzed water sample given a positive test result (T) with the human-specific assay

$$P(H/T) = \frac{P(T/H)P(H)}{P(T/H)P(H) + P(T/H')P(H')}, \quad (1)$$

where $P(T/H)$ is the probability of a positive signal with the human-specific assay in a fecal sample that is human derived, $P(T/H')$ is the probability of a positive signal with the human-specific assay in a fecal sample that is not human derived, and $P(H)$ is the background probability of detecting the human-specific marker in a given watershed. It is important to consider that calculated Bayesian probabilities are not fixed but are subject to changes based on background probabilities in other watersheds and further testing of animal stool samples. However, a Bayesian approach provides a simple and universally applicable means to validate the utility of MST assays in a monitored watershed.

3.1.3. Host distribution

Genetic targets used to develop host-specific markers should be present in the vast majority of fecal samples from the individuals of the same host type, and ideally at approximately the same abundance level (Harwood, 2007). If the bacterial populations linked to the host-specific markers are present in few individuals belonging to a host species, the probability of detecting them in environmental samples is also relatively low, unless they are present in high numbers in those individuals serving as hosts. The former is often the case with markers targeting *Cryptosporidium* and other pathogens, and, therefore, their detection in environmental waters requires methods with extremely low method detection limits (MDLs) and/or concentration of large volumes of water. Also, if the target populations fluctuate significantly between different individuals it will be difficult to establish a correlation between signals and fecal loads. The studies that have addressed this issue generally used a limited number of

individuals (Layton et al., 2006; Okabe et al., 2007), and, therefore, the true distribution of the markers is unknown.

3.1.4. Temporal and geographical stability

An assay targeting a specific fecal source must be capable of producing signals in different seasons as contamination may persist throughout the year (Harwood, 2007). Assays targeting populations that exhibit significant seasonal fluctuations due to the host's diet, health, or diel patterns might not be sensitive enough to pick up a signal of that fecal source downstream from the fecal input. Moreover, PCR-based assays must target genetic regions that are not subject to frequent mutations; otherwise they will interfere with target detection. Only a handful of studies have applied MST assays for more than a year, and, therefore, additional information is needed to understand the temporal stability of most methods.

In order for any MST method to be considered useful by regulators they must be applicable across different geographic regions (USEPA, 2005). Due to the different type of matrices, it is ideal for an MST assay to have broad application and thus it should be able to deal with peculiarities of various sample types whether freshwater, estuarine, marine, or coming from temperate or tropical sites. The validation of methods using a large number of samples collected from different geographic locations over time is not a common practice in source-tracking studies. Archival of fecal samples is also an important issue when dealing with both geographical and temporal stability of markers; however, the impact of fecal storage on marker/assay stability has not been studied in great detail.

3.1.5. Sensitivity and accuracy

Oftentimes confused with method detection limits (MDLs) or sample limits of detection (SLODs), sensitivity is defined as the percentage of instances an assay determines the presence of a host-specific signal in samples containing the source in question (Deep, 2006). Sensitivity relates to the robustness of the method providing that the markers are present at established detectable levels. Most studies have relied on relatively small sample volumes and as such the method's suitability needs to be determined prior to field applications. The rate of false negatives is determined with direct testing of fecal samples, matrix spikes, or host distribution data, which can also provide information regarding how sensitive a method is relative to another. Most applied studies have used a limited number of controls to estimate method sensitivity (Lamendella et al., 2007). However, before markers can be applied in a variety of watersheds, their sensitivity will need to be tested using a very large set of fecal and spiked-matrix samples from a variety of locations. If such validation studies were performed for each method, then users could compare the sensitivities of different host-specific markers and make decisions regarding which assays provide the greatest cost-benefit.

The accuracy of a given method is defined as the percentage of instances that an assay correctly determines the presence of a host-specific signal in all samples tested, which is affected by both the number of false positives and false negatives. The number of false positives can be addressed by

challenging the tests against non-targeted fecal samples. Estimating false-positive PCR signals from environmental samples involves confirming the identity of the PCR product using sequence analysis or DNA hybridization. This is seldom performed as most researchers rely on PCR size product data as a line of evidence.

3.1.6. Sample and method limits of detection

In general, it is understood that different assays have different MDLs (Bernhard and Field, 2000), and individual samples have varying SLODs (Rajal et al., 2007a, b). Note that MDLs are the theoretical detection limit under ideal (laboratory water) conditions, while SLODs are the estimated detection limit for individual samples, based on the level of inhibition of PCR and other factors. In theory, PCR assays are capable of detecting a few copies of a gene per reaction. MDLs can be estimated using known quantities of the target, normally using a plasmid containing the marker in question. However, in practice, DNA extracts from environmental samples contain inhibitors and a mixture of competing targets that increases the SLOD of host-PCR assays. As a result, to understand the MDL of an assay, experiments should be performed using DNA extracts from different individual fecal samples and different environmental samples. Calculations of SLODs should account for all sources of error during analysis, including nucleic acid extraction efficiency, inhibition of PCR amplification, and filtration losses (Fig. 4) (Rajal et al., 2007a, b).

3.1.7. Sampling requirements

The minimal number of sampling points to accurately represent the dynamics of a watershed and to statistically defend the interpretation of field observations must be established, independent from the available methods, by the

scientists/engineers planning to apply the MST technique. This step is seldom performed in source-tracking studies. It should be noted that methods that require filtration of large volumes are generally not compatible with sampling plans that collect a large number of samples during each monitoring event (i.e., laboratory throughput is limited by the number of filtration systems, which are relatively expensive), and therefore the conclusions will likely be reached with datasets based on a smaller number of samples per day. In contrast, methods that require small sample volumes (which normally correspond to relatively high SLODs) will be compatible with studies that collect a large number of samples per day. For quantitative methods, once information is available regarding appropriate values to use for estimates of coefficients of variation, then conventional study design techniques (e.g., power analyses) can be used to evaluate the minimum number of samples to be collected at each sampling site. But estimating sample requirements is not a simple task as the spatial and temporal scales impacting the concentrations of fecal indicators and pathogens are influenced by a number of factors including fluctuating environmental conditions (e.g., water temperature) and by the genetic attributes of the organisms in question.

3.1.8. Standard operating procedures (SOPs)

To set performance standards for an individual method, it will be necessary to establish standard operating procedures (SOPs). SOPs will be needed for all the different steps of the experimental design (i.e., from sample collection to data analysis). This is ultimately necessary in order for an MST method to be used in regulatory activities, including the development of water quality standards based on LIMs. Procedures must be validated by independent laboratories using the same performance standard criteria. Additionally, there is a need to establish the type of reagents and equipment that should be used in MST in order to obtain reproducible results. To facilitate standardization, studies should be performed to address issues like the efficiency of DNA extraction procedures/kits, the stability of DNA polymerases, performance of thermal cyclers, and possibility of sample contamination via laboratory equipment (e.g., filters), among others. Standards like fecal samples or DNA extracts to be used by source trackers need to be established and protocols for archival of such standards need to be developed and validated. Tests that quantify the efficiency of DNA extractions for both fecal and water samples are not often performed by researchers, but clearly are necessary in order to determine the accuracy of any PCR-based method. This is not a simple task as the type and quantity of sample spikes have not yet been standardized and it is necessary to apply performance standards to individual fecal samples. Similarly, there are issues that need research involving DNA extracts, including PCR efficiency and PCR artifacts, particularly when using quantitative PCR (Q-PCR) (Rajal et al., 2007b). Addressing these issues is complicated by the fact that the number of samples required to estimate host distribution of the markers and to accurately represent pollution dynamics is seldom determined. Eventually, a laboratory certification program will need to be developed, just as laboratories that analyze drinking water samples that are used for compliance

MST and Pathogen Quantification

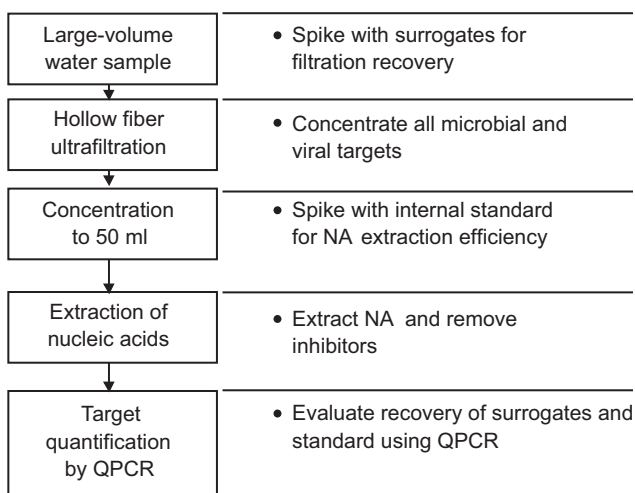


Fig. 4 – Flow chart depicting QA/QC protocols to ensure quantitative determination of target MST markers or pathogens based on spiking with appropriate surrogates and internal standards.

testing in the US must be certified (www.epa.gov/safewater/labcert/index.html).

3.2. Mid-term (3–5 years)

3.2.1. Quantification

The TMDL as well as other water quality programs need quantitative approaches to determine fecal loads of specific sources and to develop accurate risk assessment models. While this is a high-priority issue, particularly due to approaching TMDL deadlines in the US, we see this as a mid-term goal in light of the lack of host-specific PCR methods that have been fully validated and can therefore provide accurate quantitative information in the next few years. Researchers should not be discouraged to develop Q-PCR assays with the currently available markers. However, the host distribution and relative abundance of such markers will need to be determined in non-target hosts and incorporated in algorithms used to calculate source-specific loads. The Q-PCR host-specific assays available in the literature have only been tested by a handful of laboratories (Dick and Field, 2004; Seurinck et al., 2005; Layton et al., 2006; Reischer et al., 2007; Okabe et al., 2007; Kildare et al., 2007). Admittedly, estimating the relative abundance of a particular source using these Q-PCR assays will be useful to stakeholders if there is a correlation between the signal and indicator bacteria counts, which has been the case in at least one instance (Dick and Field, 2004), or if there is a desire to eliminate inputs that are most impacted by a specific host of concern (e.g., humans). Just as enzyme inhibition during PCR increases SLODs, it also skews calculated marker concentrations. Mass balance losses due to filtration and extraction as well as PCR inhibition should be noted on a per-sample basis, which can be performed using surrogate markers that are spiked into the environmental sample prior to filtration and DNA extraction (Fig. 4). Extensive sampling of fecal sources and receiving waters will be needed to develop accurate estimates of target densities and corresponding rates of fecal loading.

3.2.2. Persistence of genetic target in nature

A microbial population that persists in the environment for prolonged periods of time is not a good target for monitoring recent contamination events. In most cases, while the use of anaerobic targets is often encouraged due to assumed limited persistence, the survival of targeted populations has been investigated in a limited manner (Walters and Field, 2006; Kreader, 1998). There has been some application of ratios of host-specific to universal *Bacteroidales* markers in field studies and, ultimately, the use of such ratios to estimate the “percentage” of fecal inputs due to dominant sources may be an overarching goal for LIMs.

3.2.3. Sediments

The potential contribution of sediments as secondary habitats of source identifiers and their influence on source-tracking studies is poorly understood. Indicator bacteria can survive for prolonged periods of time in sediments, especially in high organic carbon content environments (e.g., Davies et al., 1995; Anderson et al., 2005). Resuspension of sediments can introduce source identifiers from previous contamination

events into the water table, possibly skewing source identification analyses. Also, sediments might act as a repository of subpopulations that cannot be classified during validation efforts, decreasing the predictive value of some MST methods. Consequently, LIMs should not target populations that can survive for prolonged periods of time in sediments as they might not be useful at identifying sources implicated in recent pollution events. Analysis of sediment-spiked water samples or laboratory studies of the persistence of targets in sediment mesocosms are needed to evaluate the potential impacts of sediment on collected data (Anderson et al., 2005).

3.3. Long-term (5–10 years)

3.3.1. Epidemiological studies

Perhaps the most notable goal of MST is to determine the correlation between a source-identifier signal and the occurrence of illness associated with a particular water use. Epidemiological studies, which are the basis of existing water quality standards, should incorporate MST data in order to develop risk assessment models and/or new standards (Wade et al., 2006). Ideally, quantitative methods for both source identifiers and pathogens will be available by then. While the risks associated with human fecal pollution are generally accepted to be higher, the correlation between non-human sources and health risks is poorly understood (Colford et al., 2007). MST offers a number of tools to develop risk assessment models that can be used to develop scientifically sound regulations, including perhaps different WQOs for water systems with different dominant fecal sources. Therefore, future epidemiological studies should target surface waters impacted by a variety of pollution sources, including non-human sources, and incorporate source-tracking efforts to the maximum extent possible.

3.3.2. Long-term field studies

To fully validate the potential of MST, long-term, large-scale field studies need to be conducted with the methods that meet standardized performance criteria. Long-term studies are needed to evaluate if a given method can be used to understand the pollution dynamics in a watershed. For instance, if indicator bacteria spatial patterns remain relatively uniform from year to year, while marker concentrations vary greatly, then source-tracking results might not be very useful unless they correlate better with epidemiological data. As more TMDLs move into the implementation phase, multi-year studies should be conducted to evaluate the effectiveness of BMPs in different geographic locations, including tropical waters.

3.3.3. Microbial ecology

MST studies have not looked at the ecology of the so-called “host-specific” populations. To further understand the ecology of host-specific populations there is a need to use a microbial community approach to study the biotic interactions that might play a role in the survival of fecal bacteria (Sadowsky et al., 2007). Fecal pollution also has an impact on watershed nutrient levels and, therefore, it is important to determine the spatial and temporal scales where the occurrence of source identifiers correlates with the structure and function of water and

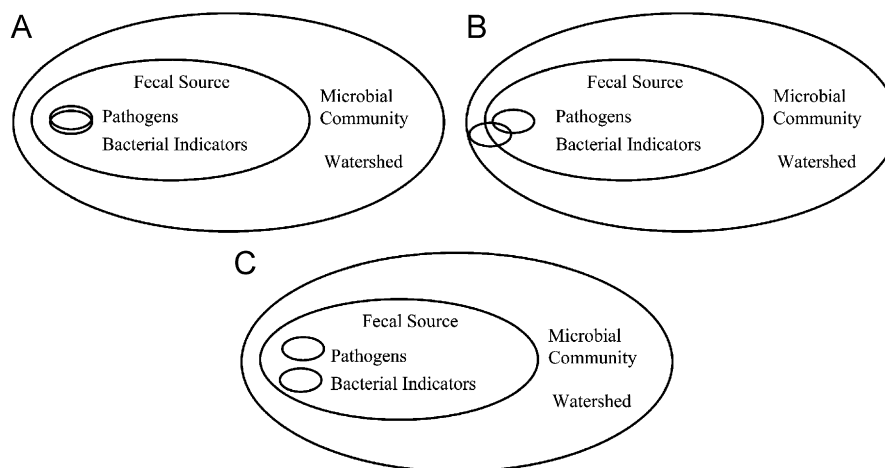


Fig. 5 – Potential scenarios associated with the use of indicator bacteria to predict the presence of pathogens and their sources. Overall, once fecal sources impact a water system, discharged fecal organisms become part of the aquatic ecosystem (in this case a watershed). The fecal pollutants come from a particular source (or multiple sources), and assuming that there is a good correlation between pathogens and indicator organism (A) it is then possible to use the surrogate indicator to predict health risks. However, when this association is tenuous like in (B), the correlation is relatively poor as well as the predictive power of the indicator in question. In some cases (C), there is no correlation between the indicator and the pathogen(s) due to different survival rates, due to adapted populations, or they are associated with another source. While some subpopulations of indicator organisms can be used to identify primary sources (i.e., used as source identifiers), in some cases they might represent a small fraction of the source indicators.

sediment communities. Data on how these populations interact with biotic and abiotic factors are also needed to confirm host specificity and to understand the dynamics of pollution sources, indicators, and pathogens once fecal waste is introduced into the aqueous environment (Fig. 5).

Methods that can discriminate between recent and non-recent pollution events will also benefit from a community approach. For this reason, methods that target viable organisms will be of great benefit as they can discriminate between background noise (e.g., cells not surviving environmental pressures or killed during wastewater disinfection, or free DNA bound to soil or sediment particles) and signals generated from active microorganisms. It is hypothesized that alternate populations (i.e., other than those consisting of fecal bacteria) may be useful temporal indicators of pollution age, along with other biogeochemical processes that are stimulated as a result of nutrients (i.e., nitrogen) associated with fecal loading. Such pollution “dating” methods could be used to determine whether risks diminish with the age of fecal pollution, and to evaluate the impact of fecal pollution in ecosystem stability.

4. Emerging approaches and technologies

The future of MST is somewhat difficult to predict as scientists still have to deal with the research gaps mentioned in the previous sections. However, it is clear that recent biotechnological advances and the emergence of genomics will allow environmental microbiologists to address questions that seemed unanswerable a few years ago (Devereux et al., 2006). New methods are constantly emerging in the field of source tracking, a trend that promises to continue as methods based on alternate targets (Martellini et al., 2005; Fong et al., 2005; McQuaig et al.,

2006; Ufnar et al., 2006) become part of the MST “tool box”. On the other hand, some methods are regaining popular acceptance among source trackers, like the detection of chemicals associated with anthropogenic activities (e.g., detergents, pharmaceuticals) as they can signal human impacts (Glassmeyer et al., 2005) or can discriminate between different types of feces (e.g., ratios of coprostanol:24-ethylcoprostanol; Gilpin et al., 2003). Of course, each new (or re-emerging) method has its own drawbacks and limitations. This section highlights many of the emerging LIMs and MST approaches, in order to encourage the use of multiple methods and facilitate a unifying framework for source tracking.

4.1. Polyphasic/multi-tier approaches

The main focus in source tracking in recent years has been on the development of new methods, primarily because many of the currently available markers do not meet critical criteria like host specificity, sensitivity, and temporal and geographical stability. Perhaps one way of interpreting this trend is that it is difficult to develop a perfectly host-specific MST assay with the currently available information and, therefore, there is a need to use several approaches to fully characterize the array of fecal sources impacting water systems. Such a polyphasic approach could be conducted simultaneously. For example, McQuaig et al. (2006) recently combined the results from three different methods targeting the *esp* gene of *E. faecium*, *Bacteroides* 16S rDNA, and human polyomaviruses to infer human fecal contamination in surface waters. Similarly, Blanch et al. (2006) used microbial and chemical methods to develop predictive models that could distinguish human fecal sources from non-human fecal sources. In each of these studies, although most of the methods alone were not capable of providing 100% correct

classification rates, combining methods increased the predictive power of the MST tool box.

Alternatively, it would be possible to use a multi-tier approach, that is, the use of additional methods to further confirm the presence or absence of a particular source. This is necessary if only the most expensive methods are sensitive enough to track the sources, for example, in some of the less contaminated sites in a watershed. However, a multi-tier approach offers the opportunity to decide which methods might be useful for a particular scenario, potentially resulting in a significant reduction of costs. An example of a multi-tier approach is the recent study by McDonald et al. (2006) using targeted sampling, fluorimetry, and enterococci markers. In addition, Boehm et al. (2003) also applied a tiered approach to track human fecal pollution using human-specific *Bacteroidetes* and enterovirus assays.

4.2. Targeted sampling

In general, many MST methods are cumbersome, and require large sample volumes, unconventional equipment, and significant technical expertise. More importantly, most methods are relatively expensive when considering the number of samples that need to be processed to represent watershed-scale pollution dynamics. The price tag for source-tracking studies is critical as most agencies have a finite amount of funds to develop and implement bacterial TMDLs and identify pollution sources. Targeted sampling has been suggested as necessary prior to engaging in intensive source-tracking studies (Kuntz et al., 2003). This approach consists of performing a general sanitary survey to identify hot spots, using local community knowledge of potential sources, and studying hydrological flow data to identify areas that should be scrutinized more rigorously with an MST method. Using this approach and a combination of *esp* gene detection and fluorimetry, McDonald et al. (2006) were able to quickly and inexpensively identify leaking sewer lines as in part responsible for pollution on an island off the coast of southern Georgia.

4.3. Multiple host-specific PCR assays

Another emerging approach is to develop multi-marker conventional PCR assays for each specific source. This approach can take advantage of the availability of several assays for each host. These assays could be used in microarrays to analyze DNA extracts from each water sample. In fact, this approach has been used to identify appropriate markers (Soule et al., 2006), so it could easily be extended to instead apply an array of validated markers on collected water samples. One concern is that the relatively low DNA yields from environmental samples might restrict the total number of assays per water sample. However, if quantification is not necessary, a whole-genome amplification (WGA) step could be performed (Yokouchi et al., 2006), increasing the number of presence/absence tests that the sample could be challenged against and increasing the detection capability of less MST-sensitive assays.

4.4. Pathogen source tracking

Often, there is no detectable relationship between fecal indicator bacterial densities and enteric pathogens

(e.g., *Cryptosporidium* or enteroviruses) in environmental samples. In this case, a fecal source with high pathogen titers might be presumed non-important for meeting bacterial WQOs if using an indicator-based MST method. Consequently, management practices targeting the primary indicator bacterial sources might not be able to adequately reduce the loads of the pathogen(s) and corresponding health risks. For this reason, pathogen source tracking (PST) has been suggested when the goal is to reduce a specific pathogen or health risks. There are some examples of PST in the literature, particularly with protozoa and enteric viruses (Xiao et al., 2000; Fong et al., 2005). Due to the host-specific nature of these organisms, it is likely that their use in field applications will increase in the near future if epidemiological data are available, as suggested in a recent review (Field and Samadpour, 2007). Improvements in water concentration methods have been made, eventually reducing the sample limit of detection and sampling costs (Rajal et al., 2007a,b). Additionally, the importance of non-specific hosts as carriers of protozoan pathogens should be examined carefully in light of recent findings by Slodkowitz-Kowalska et al. (2006), who detected human microsporidian species in aquatic birds. Targeting enteric viruses can potentially be used as an indication of human health risk, although the presence of infectious viral particles must be assessed in order to use the data in risk assessment models (Choi and Jiang, 2005).

4.5. Multilocus sequence typing (MLST)

While PST could be performed with protozoan and viral pathogens using conventional PCR approaches, tracking waterborne bacterial pathogens like *Salmonella* poses a more difficult challenge as these organisms can inhabit multiple hosts. Although their host ranges are likely much narrower than those of commensal bacteria like *E. coli*, it has been necessary to use MLST to discriminate between human and bovine clinical *Salmonella* isolates (Alcaine et al., 2006). *Salmonella* MLST has been performed with three to seven genes, primarily in epidemiological studies of food-borne outbreaks. MLST can potentially identify host-specific alleles and therefore could be used for MST method development.

4.6. Emerging paths to genetic marker discovery

Recent work by Lamendella et al. (2007) further supported the host-specific fecal *Bacteroidetes* 16S rDNA clusters identified by Bernhard and Field (2000). However, in the same study rarefaction analysis of over 1200 fecal and water clones indicated that *Bacteroidetes* is a widely diverse group and that additional sequencing is needed in order to resolve the level of specificity of 16S rDNA-based assays. Functional genes involved in host-microbial interactions represent a potential pool of host-specific genetic markers for MST method development. Genes associated with microbial surface proteins, cellular processes, metabolism, and host immunity have been shown to be present in gut bacterial symbionts (Ley et al., 2006; Bäckhed et al., 2005). However, the genetic identity and sequence information for such genes are not

available. Using competitive hybridization of fecal metagenomes, Shanks et al. (2006) were able to enrich for cow metagenomic fragments. The enriched fragments were used to develop PCR assays that showed high host specificity when challenged against 279 fecal samples from 27 different animal species. The assays generated positive signals in 72–91% of DNA extracts from cow feces collected in five different states. The same approach has been used to develop human-specific (Shanks et al., 2007) and chicken-specific assays (Lu et al., 2007). DNA-enriched fragments appear to be similar to surface proteins, suggesting their involvement in host–microbial interactions. Coupling the detection of function-specific markers to 16S rDNA-based assays would further verify that a specific fecal source is present in the sample.

4.7. Microbial water quality biochip and low-density PCR array

Given the ever-increasing number of genetic markers being developed, perhaps the grail of LIM source tracking is to combine all of these methods into one comprehensive array. Such a multi-level approach would enable source-tracking studies to address goals from an array of fields including environmental microbial monitoring, risk assessment, and risk management. Comprehensive screening could be achieved by developing a microbial water quality “biochip” or microarray capable of simultaneously targeting different classes of markers specific to indicator organisms, pathogens, and source identifiers (Stewart et al., 2007). Microarrays have been used to study pathogens, including waterborne bacteria (Maynard et al., 2005; Hamelin et al., 2006, 2007). Currently, there are several platforms as well as different hybridization strategies involving oligonucleotide-, PCR product-, and genome-based microarrays (Call et al., 2003). While conventional microarrays may not provide the quantitative information that is needed for environmental monitoring applications, they have been very useful at screening for MST markers (Soule et al., 2006). Alternatively, end-point Q-PCR platforms might be a potentially powerful alternative to conventional microarray platforms. Such an approach would increase the throughput of multi-marker PCR assays. End-point Q-PCR is more sensitive and less time consuming than conventional PCR, plus the data are easier to archive as they are stored in spreadsheet files generated by the detection system. Regardless of the platform, the use of comprehensive arrays and/or biochips will necessitate development of statistical methods with input from computational biologists in order to analyze collected datasets.

5. Conclusions

- Problems associated with the poor correlation between pathogen levels and bacterial indicator densities have been the subject of criticism for several decades. Nonetheless, regulatory statutes require compliance with WQOs based on these indicators, which has pressured stakeholders to identify types of pollution sources using methods that might not be ready for field applications, and

without understanding the differences in health risks associated with each potential source. Without such information, the implementation of pollution control and remediation strategies can only be labeled a temporary yet expensive “band-aid” approach. But even if improved indicators of human health risk are developed, in the US, identifying the sources impacting environmental waters will continue to be critical due to legal requirements to meet existing WQOs.

- While the impetus for the development of methods for fecal source identification has been driven by regulations in developed countries, the need for methods and approaches that might also be applicable in developing countries should be recognized. To this purpose, MST end users would benefit from particularly affordable and reliable MST methods.
- The field of source tracking is currently evolving as methodological innovations in genomics, biotechnology, and engineering are beginning to impact genetic marker discovery and sample processing technology. However, before any of the current and future assays can supply all the information needed to implement successful management practices, several research gaps must be addressed. In this regard, the field is struggling as research priorities can differ depending on the viewpoint. From an academic standpoint, some of the most critical issues relate to the characteristics per se of the markers, while assay cost, ease of analysis, quantitiveness, and correlation with current fecal indicators are some of the top priorities to stakeholders dealing with TMDLs. We have proposed that these priorities should be addressed considering the existing timetables and the complexity of the issues in hand. While there might be a difference of opinion regarding which issues should be considered the top research priorities, setting such priorities will stimulate communication between developers, end users, and regulators and promote a structured path toward applied environmental monitoring.
- LDMS have been used to develop and/or implement a significant number of TMDLs in the US. As methods continue to be improved upon, state and regional managers could face the possibility of having to revisit some of the TMDL studies performed with techniques that over time have been determined to be inaccurate. Consequently, there will be the need to compare data obtained with newer methods against the results obtained with older ones to verify the very large number of TMDLs that will have been completed by the time newer, more accurate methods emerge.
- Development of performance criteria is considered the highest priority research gap, as criteria are needed to determine which methods are indeed acceptable for regulatory activities. Without performance criteria and established SOPs, the results of MST studies will not be reliable and in most instances the interpretation of case studies might be erroneous. At the same time, stakeholders must understand the assumptions and limitations of MST and set specific goals and expectations for source-tracking studies. Regulators must decide if the current standards are indeed the best predictors of risks, or if there is a need to change to new

standards in light of evidence showing poor correlation between fecal indicators and source identifiers. All parties involved need to commit to spending an appropriate amount of budget for source-tracking studies to incorporate quality assurance efforts including fecal sample challenge tests, field and laboratory duplicates, and field blanks.

- Ultimately, quantitative assays will be needed in order for the TMDL process to establish fecal allocations and to predict the levels of reduction that can be achieved by targeting particular sources. Such assays are also needed to further evaluate the efficacy of management practices at temporal and spatial scales. While different quality criteria are used for recreational waters, shellfishing waters, and source waters, the development of rapid, sensitive, and quantitative assays will transform environmental monitoring in the years to come.
- Due to the complexity of watersheds, methods compatible with high-throughput technologies should be a high priority to method developers. Methods that need large sample volumes to attain sufficient SLODs and that can only target one marker might not have the throughput needed to accurately represent temporal and spatial dynamics. Methods requiring steps that cannot be automated are time consuming and should be a lower priority, and/or be used to supplement data collected using more rapid methods.
- The future of microbial water quality studies will also benefit from studying ecological principles impacting the different microbial populations relevant to health, and by using novel computational methods to better understand the risks associated with different pollution sources. Epidemiological studies will need source-tracking data in order to improve the predictive power of current risk models, which in turn might result in the development of new set of standards and/or novel environmental monitoring schemes.

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