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REPORT OF THE EXPERTS SCIENTIFIC WORKSHOP ON CRITICAL RESEARCH NEEDS FOR THE DEVELOPMENT OF NEW OR REVISED RECREATIONAL WATER QUALITY CRITERIA

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CHAPTER 2
PATHOGENS, PATHOGEN INDICATORS, AND INDICATORS OF FECAL CONTAMINATION

Nicholas Ashbolt, Chair, USEPA
Roger Fujioka, University of Hawaii, Manoa
Toni Glymph, Wisconsin Department of Natural Resources
Charles McGee, Orange County Sanitation District, California
Stephen Schaub, USEPA
Mark Sobsey, University of North Carolina, Chapel Hill
Gary Toranzos, University of Puerto Rico, Rio Piedras

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2.1 Application of Microbial/Biomarker Parameters

The charge of the Pathogen, Pathogen Indicators, and Indicators of Fecal Contamination workgroup was to identify critical research and science needs in the development of new or revised criteria for recreational waters, including total maximum daily load (TMDL) implementation, and National Pollutant Discharge Elimination System (NPDES) implementation using microbial and chemical indicators. The discussions were limited to constituents for which methods are currently available or expected to be available within the next 3 years and focused around the following four issues:

1. Fecal matter indicators (as surrogates for gastrointestinal [GI] and non-GI illnesses);
2. Pathogens and their index organisms (GI and non-GI illnesses);
3. Application of fecal indicators, pathogen index organisms, and pathogens in combination for criteria development; and
4. Application of all the above for all categories of waters, climatology, and geographical considerations.

Currently, implementation of ambient water quality criteria (AWQC) for the four Clean Water Act (CWA) applications require monitoring fecal bacterial indicators to assess the degree to which the water is contaminated with sewage and sewage-borne pathogens with respect to the accepted risk for exposure. Development of the existing (US EPA, 1986) AWQC for recreational waters were based on epidemiological studies that related concentrations of fecal indicator bacteria at recreational waters impacted primarily by point sources of human sewage.

Since development of the currently used 1986 AWQC, research has shown that this narrow health effects-based standard (i.e., epidemiological studies at beaches with point sources of human sewage) is limited in that it does not take into account differences in geographical conditions, ecology of microorganisms, and varying sources of fecal indicator bacteria. In this regard, the expected relationship between illness and indicator organism densities would be high if the source of contamination is human sewage, moderate if the source was a mixture of human and animal feces, or lower if the source is the result of replication of the indicator bacteria in the environment, such as in soil, sediments, storm drains, or on plants or aquatic vegetative matter. Initially, replication of fecal indicator bacteria was reported in tropical areas (e.g., Hawaii, Guam, Puerto Rico) but has now been documented in subtropical areas such as south Florida and even temperate areas (Great Lakes States). A further but untested complication in interpreting fecal indicator bacteria results may arise due to different rates of pathogen inactivation in the environment relative to fecal indicators across different geographic and climatic regions.

It is for the above reasons that experts in the field of microbial water quality generally agree that the principles of microbial ecology must be considered in water quality assessment. Understanding and applying these principles requires an assessment of the sources of fecal contamination, selection of the appropriate methods used to assess these sources, a connection between the intended AWQC application, and the fecal and/or pathogen indicator or pathogen measured and an analysis of that indicator's fate and transport. Because of this understanding, workgroup members suggested a tiered assessment of a watershed, starting with traditional fecal indicators (conservative measures), and progressing to select a suite of indicators of

contamination (providing source specificity and contaminant load information). A characterization of contaminant inputs through a sanitary investigation of the watershed and the waterbody being assessed should be undertaken, specifically assessing hazardous events, such as rainfall-induced runoff or wastewater treatment failure. Key information would pertain to a cataloguing of point sources (e.g., sewage effluent) and non-point sources (e.g., animals, runoff, on-site septic systems, environmental regrowth) so that a comparative risk assessment can be made based on the concentrations of standard (traditional) monitoring fecal indicators (i.e., *E. coli*, enterococci) and the expected presence of human pathogens. This initial assessment should assist in understanding the relationship between the contamination and epidemiological studies (indicator levels and risks of illness) mentioned elsewhere in these proceedings. In order to select appropriate indicators, a tiered toolbox approach was preferred by workgroup members rather than promoting use of one particular indicator over another.

2.2 Tiered Toolbox Monitoring Approach

An initial cataloguing of fecal pollution sources should include a review of existing monitoring data and a sanitary investigation to assess contaminant levels and sources that impact a given recreational water site. Based on that information, the indicator used in monitoring or the predictive modeling tool most appropriate for each CWA AWQC application and contamination source would be selected for the situation. Water quality assessment for each recreational water site should begin with the simplest analyses and assessment and move on to the most appropriate (specific or targeted) indicator for that site or purpose. More refined tools to differentiate between human, domestic animal, or environmental sources of fecal contamination could subsequently be used if deemed necessary.

If a sanitary investigation determines that fecal pollution is human or animal origin, then *E. coli* or enterococci could be used in the tier one water quality assessment because many pathogens can be expected to multiply in human and animal intestines. If the source of the "fecal" indicator organisms is determined to be from the environment (i.e., from growth in soil/sand, sediment, or water), then *E. coli* and enterococci may be inappropriate because most pathogens are not capable of environmental multiplication. As a result, the monitoring for this tier would need to be a fecal organism/chemical that does not amplify in the environment, such as spores of *Clostridium perfringens* or male-specific (F+) coliphages measured by culture- or molecular-based methods, specific members of the *Bacteriodes* bacteria measured by a molecular method, or use of a chemical indicator of fecal material.

For a subsequent tier of monitoring, infectivity and/or molecular methods could be used for specific groups of pathogens such as, for bacterial pathogens (shiga-toxin producing *E. coli* [STEC] or *Salmonella*), for protozoa (*Cryptosporidium* or *Giardia*), and for representative human sewage-borne viruses (enteroviruses, adenoviruses, polyomaviruses, or noroviruses).

Location-specific data should be archived for potential use in future predictive modeling that might allow for management of site-specific fecal contaminants. Finally, if possible, archiving samples for further characterization and national comparison of new indicators, and/or pathogens and their respective methods would be advantageous assuming a national repository database and sample archive facility could be established.

Several non-GI illnesses have been associated with recreational uses of water but these are not addressed by monitoring for fecal indicator microorganisms or chemicals because the etiological agents for these waterborne diseases come from non-sewage sources. Examples include animal urine (*Leptospira* spp.), shedding from human skin (*Staphylococcus aureus*), or microorganisms that are naturally present in freshwater environments (*Aeromonas hydrophila, Naegleri fowlerii, Legionella pneumophila*). Further, several human pathogenic *Vibrio* species (*V. cholerae, V. vulnificus, V. parahaemolyticus, V. alginolyticus*) are indigenous to marine and brackish waters. Because reliable indicators have not been developed for non-GI etiological agents, the best approach to address aquatic non-enteric pathogens is to characterize the aquatic conditions that increase the risk for these pathogens. For pathogenic *Vibrio* spp., this includes saline waters of warmer temperature and waters that contain high levels of nutrients.

2.3 Parameters for Hazardous Event Pollution Monitoring

The first approach to investigate a hazardous event (sewage discharge, rainfall impact, etc.) would be to assay for fecal indicators (appropriate for a climatic/geographic area of concern, see Section 2.4). The primary indicators of fecal contamination are *E. coli* or enterococci; however, based on the classification from an initial sanitary investigation, alternatives may include *Clostridium perfringens* or F+ coliphages (dependent on a robust method being confirmed). These must be demonstrated to relate to a possible health outcome (see Section 2.4). When information is required on source characterization, then additional microbial indicators (see Section 2.5) are generally preferred over chemical biomarkers (see Section 2.7).

Focused sampling during and after higher risk periods is important when information from the sanitary investigation (which may include system models) is used to predict such risk. For these applications, the context of the likely pathogen group(s) should dictate the type of indicator to assay. For example, for rainfall in an area possibly impacted by on-site septic systems, viruses are considered the most mobile pathogen group so use of virus model organisms, such as the F+ coliphages, would be informative. For sites contaminated by concentrated animal feeding operations (CAFOs), reasonable pathogen index tests include shiga-toxin producing *E. coli* or *Cryptosporidium*. This approach assumes that some background level of the targeted group is known for the area of concern (see Section 2.8, research needs).

2.3.1 Microbiological Parameters

The Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 requires States with coastal or Great Lakes recreational waters to adopt the current (US EPA, 1986) criteria for *E. coli* and enterococci. In November 2004, EPA promulgated a final rule that put federal standards in place for the 21 coastal states that had not adopted the 1986 criteria or established criteria as protective of human health as EPA's 1986 criteria. However, these federal criteria apply only to coastal states and Great Lakes waters. In many cases, a fecal coliform-based standard still applies to many states having only inland waters. It is important to note that the results of the epidemiological studies used to generate the 1986 criteria for coastal and Great Lakes waters may not be directly applicable to all inland waters.

2.4 Traditional Fecal Indicators (Coliforms and Enterococci)

Consideration of the environmental context for which these traditional indicators are used is critical to the interpretation of the results. For example, wastewater treatment/disinfection may be effective in reducing the number of these traditional fecal indicators but ineffective in reducing/inactivating some pathogens of concern (Blatchley et al., 2007). Some industrial treatment systems may contain/enable replication of high number of "fecal indicators," which are not necessarily associated with fecal sources (Degnan, 2007; Gauthier and Archibald, 2001). Ambient water and soils in tropical environments may be conducive to the growth of environmental strains of *E. coli* and enterococci (Fujioka and Byappanahalli, 2001). A similar situation was found in temperate Australian waters (Ashbolt et al., 1997; Barnes and Gordon, 2004; Davies et al., 1995) and these indicators have also been found to persist in U.S. beach sand/sediments (Whitman et al., 2006).

The range of strains of fecal indicators identified by traditional culture-based methods may differ from those identified by enzyme-based and quantitative polymerase chain reaction (qPCR [molecular])-based methods. Further, the strains more associated with fecal matter are not differentiated from the environmental strains by all of these methods. There are commercially available systems that can aid in the discrimination of strains; however, less expensive typing kits do not accurately provide such discrimination for environmental strains. It is important to note then when quantifying fecal indicator organisms, different methods target different strains. For example, cells stressed by wastewater disinfection processes may be enumerated using MPN (Most Probable Number) methods but excluded by methods enumerated by colony forming unit (cfu) methods. When current qPCR methods are used, both viable and non-viable cells are detected. In addition, the number of gene targets may vary per cell and therefore do not provide comparable information to culture-based results.

E. coli

Of the traditional fecal indicators (see also Appendix E, Text Box E-1), only *E. coli* has been shown in epidemiological studies to consistently relate to health outcomes for freshwater recreational water users (Cabelli et al., 1982; Wade et al., 2003; Wiedenmann et al., 2006). In marine/estuarine waters, *E. coli* is more readily inactivated than enterococci and appears to correlate less well to health risk than enterococci for saline water environments.

Subtyping of different strains of *E. coli* (library-dependent microbial source tracking methods) appears to be very site-specific if useful at all. Thus, it is not generally suggested as an effective way forward to separate environmental sources of *E. coli* from fecal sources across the United States (see Section 2.5).

Enterococci

The enterococci are the major group of fecal indicators that have a clear link to GI illness and upper respiratory disease in bathers in marine and fresh recreational waters (Kay et al., 2004).

There are, however, several shortcomings in the use of current methods for enterococci. Most importantly, there is a range of different *Enterococcus* spp. detected by current methods. Based on unpublished Californian studies (Stephen Weisberg, SCCWRP, personal communication, 2007), greater fecal specificity may result from specific identification and enumeration of *E. faecalis* or *E. faecium* or molecular-based methods targeting specific genes within these species (e.g., ribosomal RNA or enterococcal cell surface-associated protein and its gene *Esp*) (Lehner et al., 2005; Liu et al., 2006). However, no robust method is currently available that readily provides such information, nor has this concept been verified at other U.S. recreational water sites (Anderson et al., 1997).

2.5 Alternative Fecal Indicators

2.5.1 Bacteria

Clostridium perfringens

C. perfringens is a member of the sulphite-reducing clostridia (SRC), which are spore-forming anaerobic bacteria excreted in human and animal fecal matter, but unlike other SRC, do not appear to grow in the aquatic/soil environment. These bacteria have been used as fecal indicator organisms for decades. Australian and North Carolina studies show *C. perfringens* levels in humans comparable to levels found in dog and feral pig feces, but low levels in cattle, sheep, horses, and birds (Leeming et al., 1998; Mark Sobsey, University of North Carolina, Chapel Hill, personal communication, 2007). Importantly, because *C. perfringens* does not appear to grow in aquatic/soil environments, it has potential to be useful as a fecal indicator for tropical environments such as in Hawaii where growth of *E. coli* and enterococci in soil/sand, sediment, and water make those indicator organisms less useful (Byappanahalli and Fujioka, 1998; Hardina and Fujioka, 1991; Roll and Fujioka, 1997). For example, in ambient streams in Hawaii, concentrations of fecal coliforms, *E. coli*, and enterococci consistently exceed recreational Water Quality Standards due to contribution by extra enteric sources (Hardina and Fujioka, 1991, Luther and Fujioka, 2004). Thus, monitoring inland and coastal waters for *C. perfringens* provides reliable data for sewage contamination and is used by the Hawaii State Department of Health to confirm a sewage contamination event (Fujioka and Byappanahalli, 2001).

The presence of *C. perfringens* (spores) in water, therefore provides evidence of existing human/urban fecal contamination, which may reflect either recent or historical fecal contamination from humans or animals. Although methods have been available for some time, confirmation of a robust and consistent method approach should be developed. For example, the advantages of heat-treating samples (or not) to remove background vegetative cells and induce spore germination remains unclear.

The environmental resistance of *C. perfringens* spores has both advantages and disadvantages in their application as a fecal indicator, pathogen indicator, and as an indicator of wastewater treatment efficacy. Collectively, these make *C. perfringens* spores better indicators of persistent and treatment-resistant pathogens, such as *Cryptosporidium* oocysts (resistance to chlorine) and adenoviruses (resistant to UV radiation). However, they can be so persistent in the environment

that they may not indicate the presence of pathogens coming from recent (contemporary) fecal contamination.

Recent studies on the partitioning of *C. perfringens* and other fecal indicator microbes in environmental waters, such as *E. coli* and coliphages, indicate differences in the extent of their association with settleable particulate matter (Characklis et al., 2005; Krometis et al., 2007). To date, limited data have been collected on any potential relationship between *C. perfringens* counts and recreator health outcomes (see Section 2.8).

Bacteroides

Bacteroides spp. are members of the normal microbiota of warm blooded animals and studies have shown them to be among the most prevalent genera in feces (Holdeman et al., 1976). Because they are strict anaerobes that grow in the GI tract of humans and animals, they do not survive for long periods of time under aerobic conditions (Kreader et al., 1998). However, their survival under different redox potential conditions (e.g., sediments) has not been thoroughly studied. Recent research based on molecular methods has demonstrated that some isolates may be strictly associated with human feces (Walters et al., 2007). If this is the case, these microorganisms also have the potential to be used for microbial source tracking (MST) applications.

Studies have indicated human versus bovine specificity in certain 16S rRNA genes therefore, 16S rRNA *Bacteroides* genes have been used as an index of human or animal contamination in Europe and the United States. The ability to differentiate sources of fecal contamination is very attractive when it comes to determining risk as a result of exposure via recreational waters. The molecular methodology has been shown to be robust and applicable in the United States and Europe, though it remains to be seen if this robustness holds across temperate versus tropical or subtropical zones of the world. Some results from Hawaii and Europe indicate that these methods may be useful under those climatic conditions (Betancourt and Fujioka, 2006; Seurinck et al., 2006). Either way, it is unclear whether quantification of human/animal fecal loads will be consistent or indeed possible using these molecular-based methods.

Though data from molecular techniques have shown that there is specificity in the human versus animal strains, the fact that both human and animal feces contain a diverse population of *Bacteroides* spp. may limit the usefulness of some detection methods. Methods that focus on one target may have reduced sensitivity as a result of the lower concentrations of a specific *Bacteroides* strain. Data have shown that *Bacteroides* spp. does not survive for long periods of time in the environment; thus, *Bacteroides* detected by qPCR in ambient waters includes a high percentage of inactivated microorganisms. The fact that qPCR detects both live and dead organisms needs to be considered when data are applied in different contexts (e.g., different AWQC applications). That is, qPCR detection is linked to the time the nucleic acid remains within the cell without being degraded. EPA data have demonstrated that the DNA remains undegraded for up to 20 days (Kevin Oshima, USEPA, Office of Research and Development, personal communication, 2007) in the inactivated unlysed cells. This may be equivalent to the survival of some enteric pathogens under environmental conditions. Thus, the presence of *Bacteroides* may have possible use as an indicator of health effects. Because the concentration

of *Bacteroides* spp. in feces is much higher than other fecal bacteria, once the persistence of PCR-detected types is better understood, it may also be useful for TMDL applications, although this possibility needs further evaluation.

The molecular methodology for the detection of general and human-specific *Bacteroides* spp. is already being tested and has proven to be robust (Gawler et al., 2007; Walters et al., 2007). Thus, if detection methods are validated in the United States there is an excellent opportunity for short-term advances in quickly adapting the use of this alternate indicator for the rapid analyses of recreational waters and fecal source identification.

2.5.2 Bacteriophages

Coliphages

Bacteriophages (viruses) that infect *E. coli* and possibly other closely related coliform bacteria are called coliphages. There is a long history of research documenting the possible uses of phages as indicators of fecal contamination (Grabow et al., 1998). Coliphages were first proposed as indicators of the presence of *E. coli* bacteria and are taxonomically very diverse, covering the following six virus families: three families of double-stranded DNA viruses (*Myoviridae*, *Styloviriae*, *Podoviridae*), two families of single-stranded DNA phages (*Microviridae* and *Inoviridae*), and one family of single-stranded RNA viruses (*Leviviridae*).

Coliphages that infect via the host cell wall of *E. coli* are called somatic coliphages (including families *Myoviridae*, *Styloviriae*, *Podoviridae*, and *Microviridae*). Male-specific (also called F+) coliphages (*Inoviridae* and *Leviviridae*) infect by attaching to hair-like appendages called F-pili protruding from the host bacterium surface.

Somatic phages have been explored as fecal, treatment efficacy, and health effects indicators. However, little is known about the specificity of their occurrence in human or animal feces. Furthermore, their considerable taxonomic diversity and the lack of readily available and convenient methods to distinguish or specifically detect the different groups has made it difficult to determine which, if any, are effective fecal, treatment efficacy, or health effects indicators. In a recent study by Colford et al. (2007), somatic coliphages were not predictive of human health risks from bathing in marine recreational water largely impacted by non-point sources of fecal contamination. Furthermore, there is very little information on the sources and ecology of the somatic coliphages, especially for the different taxonomic groups. With rare exceptions, they are detected as a broad group with no effort to identify specific taxonomic groups or relate or attribute these different taxonomic groups to specific sources of human or animal fecal contamination or possibly non-fecal environmental sources.

Male-specific coliphages have been studied extensively as fecal indicators and for water/wastewater treatment/disinfection efficacy. Furthermore, F+ RNA coliphages can be distinguished genetically (via nucleic acid detection methods) or antigenically (via immunological methods), into four distinct subgroups: I, II, III, and IV. There is reasonably good evidence that Groups II and III are associated primarily with human fecal waste and that Groups I and IV are associated primarily with animal fecal waste (Furuse et al., 1975; Hsu et al.,

1995; Osawa et al., 1981) in the United States. Male-specific coliphages have been included in some epidemiological studies of recreational water. In the recent study by Colford et al. (2007) at a marine recreational water site impacted primarily by non-point source fecal contamination, F+ coliphages were the only microbial indicator whose levels were associated with risks of swimming-associated illness.

Strengths of Coliphages as Indicators

Advantages of both somatic and F+ coliphages as fecal indicators include their (1) presence in relatively high concentrations in sewage; (2) relatively high persistence through wastewater treatment plants, compared to typical bacterial indicators like *E. coli* and fecal coliforms (coliphages may behave similarly to human viruses during wastewater treatment); and (3) ability to be detected in relatively small (100 mL) to medium (1,000 mL) volumes of fecally contaminated water.

Coliphages can be detected by relatively simple, affordable, and robust culture methods—several of which have been standardized and collaboratively tested as EPA, EU, and ISO (International Organization for Standardization) water methods. However, the EPA methods for somatic and F+ coliphages have been fully validated only for groundwater and not for ambient surface waters or wastewaters. Recent research also describes a rapid, simple, and affordable method to detect and group infectious F+ coliphages by short-term (3-hour) enrichment culture, followed by quick (<1 minute) detection of positive cultures by a simple immunological (particle agglutination) method scored by simple visual examination (Love and Sobsey, 2007). The method can be conducted in an MPN format to quantify concentrations of the different F+ coliphage groups (F+ DNA and F+ RNA Groups I, II, III, and IV).

These findings indicate that robust, simple, rapid, and low-cost F+ coliphage methods could be implemented within the 2 to 3 year time frame if correlations to health targets are observed in epidemiological studies. It would be valuable if water samples from upcoming EPA and SCCWRP (Southern California Coastal Water Research Project; see also Appendix F) marine recreational water epidemiological studies are collected and archived for analysis by these emerging qPCR methods once they are fully developed and validated. In addition, research is suggested to compare the performance of methods for rapid coliphage detection by short-term enrichment-particle agglutination and qPCR and to consider the advantages and disadvantages of these two methods for application to recreational water quality monitoring.

Limitations of Coliphages as Indictors

Although effective methods are available to recover, detect, and quantify coliphages, limitations and unsolved problems with these methods remain. The single agar layer method (EPA Method 1601) for enumeration of coliphages by counting plaques is limited to sample volumes of about 100 mL. Analyzing larger volumes is cumbersome and consumes considerable materials, such as Petri plates. Although the enrichment culture-spot plate method can be used to conveniently analyze sample volumes of up to 1 L, the method makes it more difficult to resolve coliphage mixtures when more than one type of coliphage is present in the enriched sample volume. In some cases, one coliphage will grow faster and to a higher concentration. This makes it difficult

to detect and isolate minority coliphages that grow more slowly and to lower concentrations. However, detection of all of the different coliphages present as a mixture in enriched sample is possible by either nucleic acid or immunological (particle immunoagglutination) methods.

The ecology of both somatic and F+ coliphages remains poorly documented and inadequately understood. Information is lacking on bacterial host range, sources, occurrence, and behavior (survival, transport, and fate) in different geographical regions having different climates (temperate, subtropical, and tropical) and in waters and wastewaters of different microbial quality.

F+ coliphages can also be detected by molecular-based methods, including conventional and qPCR methods, according to recent studies. Careful review of these studies suggests that there may be deficiencies in the ability of these qPCR methods to detect the broad range of F+ DNA and F+ RNA coliphages and their subgroups. Nevertheless, research is now in progress to further improve F+ RNA qPCR by developing and performance-validating primer sets for all four genogroups of F+ RNA coliphages (Stephanie Friedman, EPA Environmental Effects Research Laboratory Laboratory, personal communication, 2007). Reliable methods have not been developed for genetic analysis and characterization of different somatic coliphage taxonomic groups.

Very few studies have been conducted to evaluate F+ coliphages as predictive indicators of human health risks from recreational use of water. The most extensive study was conducted by Colford et al. (2007). That study showed no health relationship for somatic coliphages, but a weak relationship for F+ coliphages examined by two different assay methods—an MPN version of EPA Method 1601 (enrichment-spot plate method) and EPA Method 1602 (saline agar layer plaque assay). However, these methods have not been performance characterized and fully validated for use in fresh and marine recreational waters according to EPA collaborative study protocols. Additional studies of this type are needed to clarify their potential criteria uses.

Bacteroides phages

Bacteroides phages, viruses that specifically infect *Bacteroides* spp., have been tested as indicators of fecal material in Spain and more recently in the U.K. The former used a method (bacterial host) that was tested in some labs in the United States but further efforts were not made as a result of the perceived difficulty in dealing with anaerobic methodology. Attempts to use the *B. fragilis* strain VPI 3625 showed low occurrence of these phages in the United States (Chung and Sobsey, 1993). Spanish data initially supported the use of *B. fragilis* HSP40, which is specific to phages that only occur in human feces. More recent British work indicated human specificity and high phage counts for a newer Spanish host *Bacteroides* (GB-124), thus providing the opportunity for determining human fecal contamination and virus transport using a rapid and inexpensive phage method (Ebdon et al., 2007).

Strengths of Bacteroides Phages as Indicators

The methods for the detection of *Bacteroides* spp. phages are inexpensive and their presence indicates human fecal contamination. In addition, there is research that indicates specific

Bacteroides hosts are susceptible to phages that are possibly useful for MST, which would be beneficial for its use for CWA §304(a) criteria (Chung and Sobsey, 1993; Ebdon et al., 2007).

Limitations of Bacteroides Phages as Indicators

The diversity of phages including their specificity for human host strains is not yet well characterized over a range of locations. This type of data could be easily obtained in 2 to 3 years, but if it is discovered that there is wide variability in their validity for MST, then their attractiveness for use in national AWQC would be reduced. Many laboratory personnel may not have the experience required to work with anaerobic microorganisms; however, little additional laboratory equipment would be required. Because detection methods have not been standardized in the United States, it would likely take several years to develop standardized methods for enumeration of *Bacteroides* phages in water samples.

2.5.3 EU Project Summary of Tracers

Several microbes and chemicals have been considered as potential tracers to identify fecal sources in the environment. However, to date, no single approach has been shown to accurately identify the origins of fecal pollution in all aquatic environments. In a European multi-laboratory study, different microbial and chemical indicators were analyzed in order to distinguish human fecal sources from nonhuman fecal sources using wastewaters and slurries from diverse geographical areas across Europe. Twenty-six parameters, which were later combined to form derived variables for statistical analyses, were obtained by performing methods that were achievable in all the participant laboratories and include the following: enumeration of fecal coliform bacteria, enterococci, clostridia, somatic coliphages, F+ RNA phages, bacteriophages infecting *Bacteroides fragilis* RYC2056 and *Bacteroides thetaiotaomicron* GA17, and total and sorbitol-fermenting bifidobacteria; genotyping of F+ RNA phages; biochemical phenotyping of fecal coliform bacteria and enterococci using miniaturized tests; specific detection of *Bifidobacterium adolescentis* and *Bifidobacterium dentium*; and measurement of four fecal sterols. A number of potentially useful source indicators were detected (bacteriophages infecting *B. thetaiotaomicron*, certain genotypes of F+ bacteriophages, sorbitol-fermenting bifidobacteria, 24-ethylcoprostanol, and epicoprostanol), although no one source identifier alone provided 100% correct classification of the fecal source. Subsequently, 38 variables (both single and derived) were defined from the measured microbial and chemical parameters in order to find the best subset of variables to develop predictive models using the lowest possible number of measured parameters. To this end, several statistical or machine learning methods were evaluated and provided two successful predictive models based on just two variables that provided 100% correct classification—(1) the ratio of the densities of somatic coliphages, and phages infecting *Bacteroides thetaiotaomicron* to the density of somatic coliphages and (2) the ratio of the densities of fecal coliform bacteria and phages infecting *B. thetaiotaomicron* to the density of fecal coliform bacteria. Other models with high rates of correct classification were developed but they required higher numbers of variables (Blanch et al., 2006).

2.6 Pathogens and Pathogen Indicators

Many beach regulators and scientists believe that there are significant opportunities to utilize specific pathogens or pathogen indices to better understand or characterize potential health risks from recreational exposures. Some reasons for not doing so, however, remain—especially that pathogen numbers are generally significantly lower and more variable than fecal indicator organisms. Nonetheless, pathogens could be utilized to accurately determine risks as there have been a number of studies that define actual human dose-response from oral exposures such as may be encountered during swimming. Enteric pathogens are found in raw and even treated sewage so there is merit in using them in water quality monitoring to assess the risks from exposure. Also, it is possible that an entire "class" of pathogen risks can be determined by the presence of an "index pathogen" representing that group. The current capabilities of molecular methods to detect, identify, and enumerate pathogens has increased regulators' and stakeholders' interest in seeing these applied to ambient water quality monitoring to better protect public health.

There are a number of criteria related capabilities that may be provided by use of specific pathogen or index pathogen monitoring, such as the following: (1) determination of specific pathogen residuals from sewage discharges, the data from which could then be used to conduct quantitative microbial risk assessment (QMRA) studies to assess relative levels of public health concern at a beach; (2) establishment of "model" pathogens and index pathogens that could be used to assess risks from new or reemerging pathogens (an example would be the use of a virus model to assess the recreational risks from avian influenza [H5N1] because this virus can be released from infected human and animal feces [especially waterfowl] and can directly or indirectly contaminate recreational waters); and (3) determination of levels of pathogens that can subsequently be used in QMRA studies to inform decision making relative to whether or not a beach should be closed or reopened after a closure.

There are currently two approaches to pathogen detection, identification, and enumeration, (1) the traditional culture-based techniques that are especially useful in determining viability of the sampled materials; and (2) the molecular-based methods (PCR, antibody-based, and metabolic-based) that generally cannot distinguish between viable and non-viable pathogens, but which may be quite useful in further differentiating or speciating pathogens in water samples. The culture-based methods are useful for recreational waters in that they can determine if there is a viable disease risk from exposure while the molecular methods may not be capable of discerning viability.

Moreover, the culturable isolate can be further characterized for the presence of human virulence genes and compared to clinical isolates in waterborne disease outbreaks. In contrast, molecular-based methods may not be capable of discerning viability although the presence of virulence genes can also be assayed by molecular methods. Because molecular methods do not recover the entire microorganism, further characterization of that microorganism is limited.

Specific tracking of host sources using molecular techniques for pathogens can be very useful in setting TMDLs, as it can help identify the source of the pathogen and its magnitude. Recent improvements in molecular science applications have brought about a capability to

simultaneously sample and evaluate large numbers of pathogens (e.g., microarray technology). Microarray technology still requires high concentrations of pathogens for detection. However, ambient waters generally contain pathogen levels below the limits of detection and are unevenly distributed in the water matrix. Thus, research is needed to determine how to best apply these advanced technologies for characterizing enteric and non-enteric disease contaminants, their levels, and potential risks associated with their presence in recreational waters.

Workgroup members expressed some concerns about using either specific pathogens or pathogen class indices as a first tier monitoring requirement for infectious disease risks in a recreational water setting. First, pathogens are typically present in low concentrations in treated sewage, receiving waters, and also in recreational waters; therefore, high volumes of water need to be sampled, which is time consuming, costly, and contributes to analytical variability. Second, pathogen presence is typically sporadic in a community as many waterborne diseases may not be endemic, but are rather transient/episodic so they do not represent a constant contaminant source of fecal pathogens to monitor. Third, there is a variable component in terms of fecal contributions from humans and various animal sources in ambient waters that may have an impact on determining recreational exposure risks. Typically, a number of the bacterial pathogens (e.g., toxigenic *E. coli*, *Campylobacter*) are found in both humans and animals, but there may be differences in strain virulence or infectivity potential from different sources. Likewise, there are a number of protozoan pathogens that cross-infect animal species and humans (*Giardia* spp. and *Cryptosporidium parvum*). On the other hand, human enteric viruses have a much more limited host range and except for a potential few (e.g., hepatitis E virus [HEV]), animal sources of enteric viruses are not a major public health concern in recreational waters. Lastly, it is important to note that at any given time only a small portion of the human population may be infected and excreting any specific pathogen or index pathogen. Thus, large wastewater treatment systems may always contribute a small level of pathogens of concern while septic systems or small treatment systems may not have enough contribution from the infected population to ensure that those effluents would contain specific pathogens of concern to use as a routine measure of contamination—even if the disease organisms are endemic in the population. Also, many types of pathogens are associated with a seasonality or periodicity to their occurrence in a given population.

It is reasonable to use specific pathogens or their index organisms (or model organisms) in a toolbox or tiered approach to monitoring if considered as other than as a first tier measure of fecal contamination. In a toolbox approach, the determination of the presence and concentration of specific pathogens or their index organisms could be useful to characterize risks once it has been determined that there is a trigger level of fecal contamination at a given recreational water site. Dose-response data for a number of the primary pathogens from oral exposures is available and these data would help more narrowly define exposure risks for a detected pathogen. Because of the costs, time for analysis results, and expertise needed to test specific pathogens or index organisms, these measurements would be the last set of measurements applied to monitoring of recreational sites for determining potential sources. The specific pathogen monitoring tools for other AWQC applications (e.g., TMDLs) could allow States to determine sources and concentrations of the pathogens for particular upstream contamination events. Also, pathogens could be incorporated into future NPDES permit limits and be used in the future to assess

wastewater treatment plant discharges for specific pathogens of concern downstream and to provide a better understanding of the efficacy of treatment and disinfection processes.

2.7 Chemical Biomarkers of Fecal Contamination

Various shortcomings have been identified in relying solely on indicator bacteria or pathogen/index microorganisms for CWA criteria uses. Methods for MST in aquatic environments have been developed and discussed above that distinguish animal from human sources in the United States and in Europe (Blanch et al., 2006). However, for some specific tiered approaches in sanitary investigations, certain chemical biomarkers of sources may provide timely or higher resolution information in fecal source tracking. Some of the most promising are discussed below.

2.7.1 Fecal Sterols

The most commonly known fecal sterol, coprostanol (5β-cholestan-3β-ol), is largely produced in the digestive tract of humans and dogs by microbial hydrogenation of cholesterol (Leeming et al., 1996). The term "sterols" is generally used for all sterols and stanols (i.e., "fecal sterols") and is also a more specific term denoting a steroidal alcohol with at least some degree of unsaturation.

Two pathways have been proposed for the biotransformation of cholesterol to coprostanol, one in the gut and the other in natural sediments. The α-configured form (cholestanol) is the most thermodynamically stable of the reduction products and is found ubiquitously in the environment; whereas coprostanol is largely of fecal origin, but some reisomerization can yield low levels in natural sediments. Both forms are easily resolved by gas chromatography-mass spectrometry (GC/MS) analysis.

An important advance in using these fecal sterols has been the realization that it is critical to measure both the ratios and absolute concentration of at least four of these related compounds to attribute fecal source contributions between humans, herbivores, and birds (Ashbolt and Roser, 2003). Coprostanol alone has never really been embraced as an indicator for sewage pollution because its presence is not considered as indicative of a health risk due to multiple sources and low level environmental production in sediments.

The fecal sterol biomarker technique offers many diagnostic and quantitative advantages when used in conjunction with traditional techniques for detecting sewage pollution. When careful data interpretation is undertaken, fecal sterol analysis, although expensive and complex, has resolved problems of source attribution in urban and rural environments not possible with use of traditional fecal indicator bacteria and coliphage assays (Roser and Ashbolt, 2007).

2.7.2 Caffeine

Caffeine has been extensively examined as a tool for assessing human influence on aquatic systems. Although caffeine is metabolized when consumed, a small amount (<10%) of ingested caffeine remains intact when excreted (Peeler et al., 2006). Most work in the past decade has

focused on heavily polluted systems and efficiency of caffeine removal in sewage treatment plants, although with improvements in techniques and the lowered detection limits, the scope of application has broadened to include stream, wetland, estuarine, and groundwater systems.

A major disadvantage is that caffeine is often present in the urban environment from numerous plant species debris as well as from human "dumping" of coffee wastes. Further, the current methods used (specific extraction and GC/MS analysis) are relatively complex and expensive. Nonetheless, based on the recent work of Peeler et al. (2006) in southwest Georgia, caffeine appears immediately below wastewater discharge sites and within towns, but not in rural watersheds. Overall, aquatic concentrations of caffeine are typically less than for fecal sterols, but caffeine tends to stay in solution, whereas the sterols associate with fine particulates.

2.7.3 Optical Brighteners and Other Sewage Markers

Recent sewage contamination may be readily identified in waters by the presence of ammonium, turbidity/particle counts, phosphate, odor, and a range of organics present. Depending on the sensitivity and AWQC applications, some of these analytes may provide value in fecal source identification.

One relatively inexpensive and sensitive fecal source identification method is fluorometry (Hartel et al., 2007). Fluorometry identifies human fecal contamination by detecting optical brighteners (also called fluorescent whitening agents) in water. Optical brighteners are compounds added primarily to laundry detergents, and because these brighteners emit light in the blue range (415 to 445 nm), they compensate for undesirable yellowing in clothes (Kaschig, 2003). In the United States, 97% of laundry detergents contain optical brighteners (Hagedorn et al., 2005). Because household plumbing systems mix effluent from washing machines and toilets together, optical brighteners are associated with human sewage in septic systems and wastewater treatment plants. However, in order to use optical brighteners to detect human fecal contamination properly, they must be combined with use (counts) of fecal indicator bacteria. For example, effluent from a wastewater treatment plant contains optical brighteners, regardless of how effective the treatment processes have been at removing or inactivating pathogens. Thus, data on the presence of optical brighteners without accompanying data on viable fecal indicators does not provide information on the potential health risk from pathogens.

However, results of studies that have combined fluorometry with counts of fecal bacteria have been contradictory. Although various reports have documented a strong fluorescent signal and high numbers of fecal enterococci, cases of no correlation between fluorometry and counts of fecal bacteria have also been reported (Hartel et al., 2007). One key confounder has been the presence of organic matter that fluoresces and interferes with fluorometry. Yet, this interference can be reduced by adding a 436-nm emission filter to the fluorometer, which may reduce background fluorescence by over 50%. As long as the fluorometer used is equipped with a 436-nm filter, it appears that targeted fecal indicator sampling combined with fluorometry can be a relatively inexpensive method for identifying human fecal contamination in water.

In summary, chemical biomarkers appear to have niche applicability for those with the resources and expertise to use them and where such biomarkers are advantageous, such as where other less expensive MST options have shown to be unsatisfactory or provide ambiguous results.

2.8 Research Needs

2.8.1 Near-term (1 to 3 Years)

1. Validate the range and species or sub-species diversity qPCR assays identify, and how they may relate to health outcomes for recreational exposures (also using archived epidemiological study material) (**high priority**).
    a. Example priority list of organisms: enterococci, *Bacteroides*, *C. perfringens*, *E. coli*, F+ RNA coliphages, and somatic coliphages
2. Investigate the potential for speciation of enterococci to identify fecal-specific (preferably human) from environmental strains, then apply results to future MST and epidemiological studies (**high/medium priority**).
3. Ensure that archived samples (collected from epidemiological/specific studies) are suitably sorted and stored (to maintain their integrity) for future viability as well as molecular-based method comparison or validation studies for candidate indicators/methods (**high priority**).
4. Validate *C. perfringens* (SRC) assay's robustness over a range of water and sediment sample characteristics and correlate health effects relationships to this indicator (**high priority**).
5. Determine if there are *Bacteroides* analytical targets that are human-specific and validate their use over a range of geographic areas, diverse populations, climates, and water quality conditions to correlate levels to health targets (**high priority**).
6. Conduct health and epidemiological studies with as wide a range of microorganisms (indicators/MST organisms) as possible to identify risk correlations for a range of pathogens/indicators (including bacteriophages) from various nonhuman sources; at a minimum would include *E. coli*, enterococci, enterococci-qPCR, coliphages, *Bacteroides*-PCR, *C. perfringens*; where possible, *Bacteroides* phage GB-124, enterohemorrhagic *E. coli* (EHEC); and check for absence of human Norovirus-qPCR, adenovirus-qPCR, Pan-enterovirus-qPCR, polyoma viruses (**high priority**).
7. Conduct health and epidemiological studies with microorganisms from nonhuman sources such as *Leptospira* spp. in fresh and *S. aureus* and pathogenic *Vibrio* spp. in marine recreational waters and determine appropriate indicators for these pathogens (**medium priority**).
8. Conduct epidemiological studies incorporating the measurement of pathogens of interest (along with indicators) as monitoring tools in sewage in order to determine the correlations of the occurrence of these pathogens to indicators, and to better understand their association with diseases at downstream recreational locations. For instance, while it is strongly suggested that enteric viruses are major contributors to illness from swimming, there have not been prospective epidemiological studies to actually support this association. Use serology (also consider collecting saliva and possibly fecal

samples) to help identify the etiological agents from sewage that are impacting on recreational water sites (**high priority**).

   a. Conduct similar studies in recreational waters (above refers to studies in sewage) (**medium priority**).

9. Systematically identify and evaluate more reproducible, accurate, and cost effective methods to sample and identify priority pathogens or their index organisms (including the total adenoviruses, [e.g., Groups A-F and adenovirus 40/41], but also JC virus, and Norovirus) in ambient waters (**medium priority**).
10. Determine if there are any appropriate sewage associated bacterial pathogens that can adequately serve as an index of any of the currently known sewage-borne bacterial organisms to use on a more routine basis in recreational water criteria. For example, determine if monitoring recreational waters for *Salmonella* spp. bacteria and phages of *Salmonella* can fulfill the criteria of a pathogen index for sewage-borne bacterial pathogen can be developed (**medium/low priority**).
11. Conduct microbial fate and transport studies to determine relationships between traditional and new fecal indicators, index pathogens, and priority pathogens in treated effluents and in downstream recreational waters to compare and validate their applicability for specific criteria uses (**high/medium priority**).

2.8.2 Longer-term Research Goals

The research below may take longer than 2 to 3 years of research to complete. These are *not* presented in order of priority.

1. Review archived samples to look for trends in evolution of viruses (new or cyclic re-emergence of viruses) and the efficacy of current indicator targets used by molecular methods for health based correlations.
   a. Develop predictive models to understand the conditions that promote the emergence or re-emergence of new pathogens.
2. Continue to conduct additional epidemiological studies on non-point sources of fecal contamination and assess illness relationships to pathogen/indicators.
3. Continue to conduct sewage surveillance for pathogens as a means of public health surveillance and informing pathogen monitoring programs for CWA purposes.
4. Develop robust method for speciation of enterococci with a view to identify fecal-specific (preferably human) from environmental strains; then apply to future MST and epidemiological studies (assuming initial studies suggest that this should be explored further).
5. Conduct studies on beaches to characterize the usefulness of total adenoviruses (Groups A-F), adenovirus 40/41, JC virus, and Norovirus to meet recreational water quality criteria purposes.
6. Conduct health/epidemiological studies to identify a range of pathogens/indicators from various nonhuman sources of fecal contamination.

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