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Evaluation of the Suitability of Individual Combinations of Indicators and Methods for Different Clean Water Act Programs

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List of Acronyms & Abbreviations

BEACH Act	Beaches Environmental Assessment and Coastal Health Act
CCE	calibrator cell equivalents
CE	cell equivalents
CFU	colony forming unit
CLAT	culture, latex agglutination, and typing
CSE	calibrator sequence equivalents
CT	Cycle Threshold
CWA	Clean Water Act
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
GI	gastrointestinal
Ml	milliliter
MST	Microbial Source Tracking
mTEC	modified membrane-Thermotolerant *Escherichia coli* agar
NEEAR	National Epidemiological and Environmental Assessment of Recreational
NPDES	National Pollutant Discharge Elimination System
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
POTW	Publicly-Owned Treatment Works
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RSD	Relative Standard Deviation
SCCWRP	Southern California Coastal Water Research Project
TMDL	Total Maximum Daily Load
WQS	Water Quality Standards

1.0 INTRODUCTION/ PROJECT DESCRIPTION

Project P18 as described in Environmental Protection Agency's (EPA's) Critical Path Science Plan is to "*identify and evaluate indicator/method combinations for strengths and limitations with respect to fecal source identification (human, animal); and performance in different waterbody types. The important features of each indicator/method will be described and the strengths and weaknesses of those features will be explained and evaluated. The ideal set of features will be proposed so that the indicator/methods can be compared to a hypothetical ideal indicator/method. The indicators/methods under consideration will be ranked for each feature with respect to ability to differentiate fecal sources, performance in different water body types and appropriateness for different CWA purposes. EPA plans to use the results of this evaluation to inform the decisions regarding which indicator/methods will be included in the new or revised criteria and under what conditions those indicator/methods will be recommended*." The purpose of this white paper is to report on the result of the evaluation of indicator/methods assessed by EPA in support of the development of new or revised recreational water quality criteria for bacteria.

Section 304(a)(1) of the CWA directs EPA to publish criteria for water quality accurately reflecting the latest scientific knowledge. The criteria published by EPA under section 304(a) are intended to provide guidance to States in setting water quality standards (WQS) to protect public health as well as to maintain and restore water quality and ecosystem integrity. CWA 304(a) criteria are typically expressed as numbers (i.e., concentrations of pollutants) or narratives that EPA recommends that states put into their WQS to protect waters for aquatic life, wildlife, consumption of aquatic organisms by humans, and primary contact recreation. CWA section 303 requires each state to adopt WQS for all waters of the state and to review, and revise them as necessary, every 3 years. Once adopted and approved, WQS are binding CWA regulatory standards and effective for the following CWA purposes:

- *Water Quality Assessments.* Sections 303(d) and 305(b) provide that states are required to assess their waters on a regular basis to determine if they are meeting WQS. The states' water quality criteria are an essential baseline against which states determine whether particular waters are "impaired."

- *Total Maximum Daily Loads (TMDL).* TMDL calculations are required for all waters that have been listed as "impaired" under section 303(d). A TMDL specifies the maximum amount of a pollutant that a waterbody can receive and still meet WQS, and "allocates" pollutant loadings among point and non-point pollutant sources. TMDL calculations for impaired waters must be written to implement the applicable State WQS.

- *National Pollutant Discharge Elimination System (NPDES).* NPDES permits are required under section 402 for point source discharges of pollutants to waters of the United States. NPDES permits must include effluent limitations more stringent than required by technology regulations, if necessary, to meet water quality standards, which include state water quality criteria.

- *Non-point Source Program.* Water quality standards (including criteria) play a similarly important role under the CWA section 319 non-point source program as part of the listing and TMDL processes to determine whether best management practices or other risk management control strategies are needed to address non-point source pollution.

- *Recreational Water Monitoring and Notification.* A State's recreational water quality criteria are used in beach monitoring and notification programs. States and beach managers typically make decisions about whether to issue advisories or closure notices by measuring the results of their monitoring against their WQS.

EPA's current recommended criteria for bacteria are based on bacterial indicator organisms – *Escherichia coli* (*E. coli*) and *Enterococcus* – which generally do not cause illness, but have characteristics that make them good indicators of fecal contamination, and thus by inference, of pathogens capable of causing human illness such as acute gastrointestinal illness. EPA's recommended bacteria criteria are intended to be adopted by states into state water quality standards to protect waters designated for recreational use activities such as swimming.

2.0 BACKGROUND

Beginning with the freshwater National Epidemiological and Environmental Assessment of Recreational (NEEAR) Water Study in 2003, EPA has been evaluating recreational water quality using traditional culture methods and rapid genetic methods based on qPCR. EPA's research goal is to determine the indicator/method combination with the best correlation to illness in fresh and marine recreational waters. EPA was also responding to a provision in the Beaches Assessment and Coastal Health Act (BEACH Act) of 2000 which required the Agency to provide additional information for use in developing "appropriate, accurate, and expeditious and cost-effective methods (including predictive models) for detecting in a timely manner in coastal recreation waters the presence of pathogens that are harmful to human health."

A culture method for bacteria is one that allows direct propagation of the bacteria, and for the visible (to the naked eye) increase in the number of cells, which can allow for enumeration via solid (*i.e*., membrane filtration technique) or liquid media (i.e., multiple tube fermentation technique). The currently approved EPA culture methods involve culturing and enumerating fecal indicator bacteria (*E. coli* or *Enterococcus spp.*). Current culture-based methods require 24 to 48 hours to obtain results (Wade *et al*., 2008).

Rapid methods allow for the direct measurement of genetic material such as DNA without the need for incubation and can provide results between 2 to 6 hours from time of receipt by the laboratory to time results are available. Rapid methods are being evaluated for the development of new or revised criteria because they provide more timely results than traditional culture methods, allowing beach managers to make beach notification decisions on the same day water samples are collected.

2.1 Indicator/Methods Evaluated in the 2003-2005 NEEAR Study

The rapid indicator/methods evaluated in the 2003-2004 freshwater and 2005 (Biloxi, MS) marine NEEAR studies were commercially-available test methods or validated or experimental research protocols. The methods included in the NEEAR study met the following criteria:

(1) results could be obtained within a few hours;
(2) *Enterococcus* or the new potential indicator, Bacteroidales, was detected by the method;
(3) the detection limit of the method was sufficiently low to allow detection of indicators in the majority of samples from recreational water environments;
(4) the method was sufficiently resistant to water sample inhibitory effects to allow detection of indicators in the majority of samples from recreational water environments.

The four tests included in the 2003-2005 freshwater and marine NEEAR Water Studies are described below. The following descriptions are based on the methods available at that time and may not reflect recent changes to these methods.

1. Quantitative Polymerase Chain Reaction (qPCR) Method, two new rapid gene probe methods developed by Dr. Richard Haugland of the EPA (Haugland *et al*., 2005) (hereafter referred to as the EPA qPCR Assay), were used to detect *Enterococcus* and Bacteroidales in water samples based on the collection of these organisms on membrane filters, extraction of their total DNA, and qPCR amplification (*i.e.*, a process whereby target DNA strands are doubled in each cycle of amplification) of a genus-specific (*Enterococcus*) or order–specific (Bacteroidales) rDNA sequence using the TaqMan™ system.

2. RAPTOR Fiber optic Biosensor, a portable, automated fiber optic biosensor developed by Research International, Woodinville, Washington that can be used to detect microbiological and chemical analytes in water samples. The RAPTOR Biosensor, used in the summer of 2003, was removed from the study in 2004 because the capture and detection antibodies were not sensitive enough.

3. Luminex 100 System, a compact flow cytometer, developed by the Luminex Corporation, Austin, Texas and MiraiBio, Alameda, California, that analyzes immunoassays, complex genetic analyses, and/or enzymatic assays through the use of optics, fluidics, and advanced signal processing. The Luminex method was removed from further study in 2004 because it lacked adequate sensitivity.

4. EPA Method 1600 is the EPA-approved membrane filter method using mEI Agar for the detection of *Enterococcus* in recreational water. This method was included in the study as a reference method for *Enterococcus*.

Of the new methods described above, only the qPCR method produced results that were sufficient to be included for further evaluation in the 2007 marine epidemiological studies in Fairhope, Alabama and Goddard, Rhode Island. The antibodies used in the RAPTOR and

Luminex 100 methods lacked sensitivity for *Enterococcus* and Bacteroidales but these methods may be useful if new antibodies can be produced to increase the sensitivity and specificity of the methods. Both the RAPTOR and Luminex methods were dropped from further evaluation.

In the 2003-2005 freshwater and marine studies *E. coli* methods were not tested. EPA did not include an *E. coli* qPCR method because at that time, a method was not available. Since no qPCR method was available for comparison with the culture method, the culture method was not included.

2.2 Indicator/Methods Evaluated in the 2007/2009 Marine Epidemiological Studies

In 2007, EPA conducted two marine epidemiological studies in Fairhope, Alabama and Goddard, Rhode Island at beaches predominantly impacted by treated Publicly-Owned Treatment Works (POTW) effluent. In 2009, EPA initiated two additional epidemiological studies at a tropical beach impacted predominantly by human sources of fecal pollution, and a beach impacted by urban runoff using several rapid and cultural indicator/method combinations. The tropical epidemiological study was conducted at Boquerón Beach in Puerto Rico; and the urban runoff study at Surfside Beach in South Carolina. Additional information on the site selection process for the urban runoff epidemiological study can be found at http://www.epa.gov/nheerl/neear/.

Table 1 presents a comprehensive list of the methods tested in the EPA epidemiological studies conducted to support the development of new or revised recreational water quality criteria. Several methods were tested at multiple epidemiological sites dating back to the freshwater Great Lakes studies that were initiated in 2003 as part of the NEEAR Water Study. *E. coli* culture was not included in the methods tested in marine waters because *E. coli* is not stable and cells lyse easily in saltwater environments.

The Fecal *Bacteroides* assay was evaluated because of its potential as a human associated marker. However, the evaluation of this indicator/method combination in the 2007 epidemiological studies resulted in its elimination due to its lack of human source specificity and the relatively high proportion of sample where target organisms were not detectable. A similar problem was encountered with the *E. coli* qPCR assay. However, evaluation of this assay was continued in 2009 in conjunction with an added DNA concentration and purification procedure. The male-specific F+ Coliphage methods were eliminated due to the low density of the indicator in surface water.

Table 1 – Indicators/ Methods Tested by EPA in NEEAR Epidemiological Studies	
Methods	**EPA Epidemiological Studies Where Methods Were Used**
Enterococcus spp. qPCR	Freshwater: 2003, 2004[1] Marine: 2005, 2007, 2009[1]
Enterococcus culture (EPA Method 1600)	Freshwater: 2003, 2004[2] Marine: 2005, 2007, 2009[2]
Total Bacteroidales *spp.* qPCR	Freshwater: 2003, 2004[3] Marine: 2005, 2007, 2009[4]
Fecal *Bacteroides spp.* qPCR	Marine: 2007[5]
Human-specific Bacteroidales markers	Marine: 2009[6,7]
E. coli qPCR	Marine: 2007[8], 2009[8,9]
Clostridium *spp.* qPCR	Marine: 2007, 2009[8]
F+ RNA Coliphage CLAT assay	Marine: 2007[10]
F+ DNA Coliphage CLAT assay	Marine: 2007[10]
F+ Coliphage 24-hr SPOT assay	Marine: 2007[11]

[1]Haughland *et al.*, 2005
[2]USEPA 2002
[3]Dick and Field, 2004
[4]Siefring *et al*., 2008
[5]Converse *et al*., 2009
[6] Shanks *et al.*, 2009
[7]Haugland *et al.*, 2010.
[8]Chern *et al*., 2009
[9]Chern *et al.*, nd
[10] Love and Sobsey, 2007
[11]USEPA 2001

2.3 Other Indicator Methods

2.3.1 Microbial Source Tracking Methods

There is uncertainty about the risk to human health associated with non-human sources of fecal pollution. The ability to differentiate sources of fecal contamination site-specifically may be important for an accurate assessment of the risks to human health from domestic, agricultural animals and wildlife. Source differentiation is also important with regard to CWA monitoring and assessment (§303[d] and §305[b]) and TMDL programs. Some states have expressed a desire to be able to adjust the applicable criteria based on data that the indicator levels present in the waterbody are not due to human sources of fecal pollution. The stated concern is that once waters are listed as impaired, states must then expend resources to develop a TMDL to restore waters that pose less of a risk to human health. In this context, microbial source tracking (MST) methods are useful to supplement sanitary survey investigations (i.e., to identify sources of contaminants or TMDL sources) and for risk analysis (human vs. non-human vs. domestic animals). Most MST methods attempt to identify specific fecal sources to assist with prioritizing polluted areas for restoration.

EPA has been conducting research on rapid MST methods and has evaluated qPCR and PCR genetic markers of human and bovine pollution. This research includes a performance evaluation of (1) seven PCR and qPCR assays targeting Bacteroidales genes reported to be associated with either ruminant (goat, sheep, deer and others) or bovine feces (Shanks *et al*., 2010a); and (2) five PCR and ten qPCR assays targeting Bacteroidales genes reported to be associated with human feces (Shanks *et al*., 2010b). The bovine assay study found large discrepancies in the performance of qPCR assays across different bovine populations; and recommended that the use of bovine-associated MST applications require an a priori characterization of each watershed due to variability in genetic marker abundance and prevalence between populations. Study results also suggest that some assays are more suitable for the characterization of fecal contamination than others, and that the assay of choice can vary from one bovine population to another.

The human assay performance study included the evaluation of two qPCR assays for quantification of recently developed human-associated genetic markers targeting putative Bacteroidales-like cell surface-associated genes (Shanks 2009), seven qPCR assays for quantification of 16s rRNA gene markers from human-associated Bacteroidales species (Haugland *et al*., 2010), and several other published assays. Some assays showed human source specificity levels exceeding 97% when tested against a panel of reference fecal samples originating from cattle, poultry, swine, and various wildlife animal sources. Based on assay performance and the prevalence of DNA targets in a collection of reference untreated sewage samples collected from 54 different waste water treatment facilities in the United States, this research suggests a potential application for human-associated quantitative methods for monitoring fecal pollution in ambient waters.

EPA is also conducting research to identify genetic sequences that could form the basis of chicken and seagull-associated MST methods as specific fecal source assays. EPA has selected PCR-based assays that could uniquely identify avian sources (primarily chicken and seagull) of fecal pollution. Four assays are currently being evaluated and additional molecular data are being collected to further validate existing assays and determine if additional assays can be developed. If EPA determines that chicken and seagull specific assays can be developed, EPA will evaluate chicken and seagull-associated MST PCR-based assays for sensitivity and specificity using reference fecal samples and environmental water samples with known sources of fecal contamination.

2.3.2 Chemical Methods

In addition to the microbial indicators discussed in sections 2.2 and 2.3, EPA also tested water samples for chemical constituents. The chemical constituents tested included coprostanol, a product of cholesterol metabolism in feces; and urobilin, a bile pigment found in human feces and urine. Additionally, 48 different chemicals distinctly associated with humans that could be markers of human sewage or human fecal contamination (e.g., caffeine, cotininine, Acetaminophen, and codeine) were tested. Preliminary data suggest that chemical detections were not very frequent at freshwaters beaches, but were more frequent and occurred at higher concentrations at marine beaches tested in 2005 and 2007. Preliminary analyses did not show a consistent relationship with health outcomes, and the chemical measurements were discontinued in the 2009 epidemiological studies due to a lack of funding.

2.3.3 Southern California Coastal Water Research Project Research

Southern California Coastal Water Research Project (SCCWRP) has been conducting research on indicator/methods with the goal of developing rapid methods that can augment or replace existing traditional culture methods for one or more types of indicator bacteria. SCCWRP is assessing water quality by measuring both traditional and non-traditional indicators. Traditional indicator methods include total coliform, fecal coliform, *Enterococcus* using membrane filtration and Enterolert chromogenic substrate method, *E. coli* using Colilert chromogenic substrate method and membrane filtration, and Coliphage. Nontraditional measurements include rapid methods for quantifying *Enterococcus* and *E. coli*, *Bacteroides*, *Bacteroides thetaiotamicron*, adenovirus, norovirus, enterovirus and Coliphage (somatic and F+), among others. These indicator methods are being tested by SCCWRP as part of a 4-year project on Rapid Bacterial Indicator Development and then validated in three large-scale epidemiological studies conducted at Avalon Bay Beach, Malibu Surfrider Beach and Doheny State Beach in southern California. As part of the epidemiological studies, SCCWRP has analyzed more than 4,000 water samples using 36 different analytical methods (SCCWRP, 2010).

As part of its 2010/2011 Research Plan, SCCWRP is conducting a 3-year study to assess which source identification methods are optimal for differentiating fecal sources with the goal of bringing together a team of water quality experts experienced in source identification methods to create a source identification manual, implement selected protocols at several beaches of high interest to California, and then transition source identification capabilities to local laboratories.

3.0 QUANTITATIVE EVALUATION OF INDICATOR/METHOD COMBINATIONS BASED ON PERFORMANCE CRITERIA

EPA's approach to evaluate the suitability of individual combinations of indicators and methods for different CWA programs (P18) was to conduct two separate analyses – a quantitative analysis of indicator/method combinations based on a set of performance criteria; and a qualitative evaluation of the appropriateness of the qPCR and culture methods for each CWA program. *Enterococcus* qPCR, Bacteroidales qPCR, Method 1600 (membrane filtration method for Enterococci,) and Method 1603 (membrane filtration method for *E. coli* using modified mTEC agar) were evaluated because they have an association with illness in swimmers (Fleisher *et al*., 2010; Wade *et al*., 2008; Wade *et al*., 2006; and USEPA 1986) (Table 2). The performance criteria used to evaluate these methods are as follows: (1) established health relationship, (2) limit of detection, (3) sensitivity, (4) specificity, (5) precision, (6) percent false positive and (7) percent false negative. Table 2 shows the results of the quantitative evaluation of the qPCR and culture methods. The methods were also evaluated on their ability to differentiate fecal sources and performance in different waterbody types.

3.1 Established Health Relationship

3.1.1 Enterococcus Culture and qPCR

A demonstrated relationship between indicator concentration and gastrointestinal (GI) illness is the most important criterion for selection of the method for new or revised ambient water quality criteria. The method must demonstrate a positive relationship between illness in humans and with indicator levels (*i.e*., as indicator levels increase, risk of illness increases) in recreational waters.

Enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens. Results from EPA's recent epidemiological studies showed a correlation between qPCR measured *Enterococcus* levels to GI illness at freshwater beaches impacted by POTW sources (Wade *et al*., 2008). Additionally, the recent studies point to significantly increased illness rates among swimmers 10 years and younger exposed above 35 CFU of *Enterococcus* using the culture method compared to non-swimmers in the Great Lakes study, but the trend is not as strong (or evident) at low exposures. QPCR cell equivalent (CE) levels were a stronger predictor of GI illness than the CFU measure in the NEEAR study (Wade *et al*., 2008). Other studies have shown enterococci levels measured by culture methods correlated with GI illness levels in marine and fresh waters (Au-Yeung *et al*., unpub; Zmirou *et al*., 2003). The meta-analysis of the Epibathe studies found an increased risk of gastroenteritis in both marine and freshwater water sites when bathers were exposed to enterococci concentrations higher than 100 enterococci/100 ml in marine water and 200 enterococci/100 ml in fresh waters (Au-Yeung *et al*., unpub).

3.1.2 E. coli Culture and qPCR

Epidemiological studies conducted by EPA and SCCWRP separately found that there was no relationship between *E. coli* qPCR and GI illness in marine waters. There are no recent EPA data on the health relationship between *E. coli* culture and swimming-related illness. *E. coli* culture was not included in the list of methods tested by EPA in the NEEAR epidemiological studies. However, in epidemiological studies conducted by EPA in the 1970s, there was an association between *E. coli* culture and GI illness in fresh water but not marine waters.

More recent data from randomized controlled trial epidemiological studies conducted in Europe found a weak relationship between *E. coli* culture and GI illness in freshwater only. A meta-analysis of the (Epibathe) studies found an increased risk of gastroenteritis in both marine and fresh water sites in bathers exposed to *E. coli* concentrations higher than the level that represents the 2006/7/EC[1] "excellent quality" criteria (< 500 *E.coli*/100 ml) (Au-Yeung *et al*., unpub).

[1] Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. The purpose of this Directive is to preserve, protect and improve the quality of the environment and to protect human health by complementing Directive 2000/60/EC. This Directive lays down provisions for: (a) the monitoring and classification of bathing water quality; (b) the management of bathing water quality; and (c) the provision of information to the public on bathing water quality.

Table 2 –Evaluation of Rapid and Culture Methods				
Performance Evaluation Criteria	**Rapid Methods**		**Culture Methods**	
	Enterococcus 23S qPCR Diluted crude extract	Bacteroidales 16S qPCR Diluted crude extract	Method 1600 (*Enterococcus* by MF)	Method 1603 (*E. coli* by MF)
50% Limit of detection (CCE)	65[1]	99[1]	NR	NR
50% Limit of detection (CSE)	904[1]	1,380[1]	–	–
Sensitivity	100%[2]	NR	NR	NR
Specificity	100%[3]	100%[4]	NR	NR
Precision (log 10 standard deviation)	0.24[4]	0.20[4]	2.2% - 18.9%[5]	25.9%[6]
False positives	0%[1], 19%[7]	0%[1]	6%[5]	6%[6]
False negatives	1.1-4.5%[1], 2.0%[7]	1.1-4.5%[1], 2.0%[7]	6.5%[5]	5%[6]
Health relationship established	Yes[8,9]	Yes (Marine)[9]	Yes	Yes (Freshwater)

Notes:

Results presented in this table may be modified as additional information is collected and peer reviewed.

For Method 1600 (Enterococcus by MF) and Method 1603(E. coli by MF), performance criteria are based on the results of the inter-laboratory validation in disinfected wastewater matrices.

NR = Not reported.

[1] Chern *et al.*, 2009
[2] Ludwig and Schleifer, 2000
[3] Frahm and Obst, 2003
[4] Siefring *et al.,* 2008
[5] USEPA, 2006a
[6] USEPA 2006b
[7] Haughland, *et al.,* 2005
[8] Wade *et al.*, 2008
[9] Wade *et al.,* 2010

3.1.3 Bacteroidales qPCR

Bacteroidales is a group of anaerobic bacteria commonly found in the gut of humans. Bacteroidales densities, as measured by qPCR, are orders of magnitude higher in raw waste streams than enterococci or *E. coli* densities due in part to its greater abundance in feces. There are qPCR primers available that target the general Bacteriodales population and other primers that are thought to target human-specific strains. A positive relationship has been observed between general Bacteroidales and GI illness in marine waters in the NEEAR epidemiological studies (Wade *et al*., 2010).

3.2 Limit of detection

3.2.1 qPCR

The limits of detection of the *Enterococcus* and Bacteroidales qPCR methods have been reported as the estimated number of target organism calibrator cell equivalents (CCE) or target organism calibrator sequence equivalents (CSE) that need to be present per total water sample filter extract in order to allow detection in 50% of analyses of that extract by the specified method (Chern *et al*., 2009). Chern *et al.* (2009) reported mean limits of detection of 65 CCE (904 CSE) for Enterococcus qPCR and 99 CCE (1,380 CSE) for Bacteroidales qPCR.

3.2.2 Culture

The limits of detection of the *Enterococcus* and *E. coli* culture methods (Method 1600 and Method 1603, respectively) have not been reported.

3.3 Precision

3.3.1 qPCR

Precision of a qPCR method is the estimate of total variability of Cycle Threshold (CT) measurements obtained in analyses of common water sample filter extracts by single or multiple laboratories. It can be expressed in terms of standard deviation, incorporating between lab, between run and random error estimates.

At this time, EPA has completed a Single Laboratory Validation Study for *Enterococcus* qPCR and Bacteroidales qPCR. A Multi-Laboratory Validation Study is on-going; however, the results will not be available until 2012 and are therefore, not presented or discussed in this report. Estimates of method precision (expressed as $\log_{10}$ standard deviations of CCE) from analyses of 10,000 *Enterococcus* and *Bacteroides* cells spiked into 51 different fresh and marine water samples have been reported as 0.24 and 0.20, respectively (Siefring *et al*., 2008).

3.3.2 Culture

Precision for Method 1603 has been characterized by laboratory-specific relative standard deviations (RSDs) from disinfected wastewater samples spiked with laboratory-prepared. For Method 1603, the within-laboratory pooled RSD was 25.9% (USEPA 2006b). Precision estimates in the range of 2.2% - 18.9% has been reported for Method 1600 (USEPA 2006a).

3.4 Sensitivity

Sensitivity can be expressed as a percentage of targeted species that are detected by a method.

3.4.1 qPCR

It has been reported that the *Enterococcus* qPCR method detects all validly described species of *Enterococcus (*Ludwig *et al*., 2000). For the Bacteroidales qPCR method, there are no publish data available on the sensitivity of the method.

3.4.2 Culture

For the *Enterococcus* and *E. coli* culture methods there are no published reports available on method sensitivity. However, as part of the formal QA process for each laboratory that uses Methods 1600 and 1603, the methods recommend that laboratories develop a statement of accuracy for method by calculating the average percent recovery and the standard deviation of the percent recovery (USEPA 2006a, b).

3.5 Specificity

Specificity is the ability of a method to select and or distinguish the target bacteria under test from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. In the following sections, data are provided on the specificity, false positive and false negative rates for *Enterococcus* qPCR, Bacteroidales qPCR, Method 1600, and Method 1603.

3.5.1 qPCR

The specificity of the *Enterococcus* qPCR method was estimated from experimental analyses of five closely related non-*Enterococcus* species showing at least 10,000 times higher limit of detection when compared to *Enterococcus* species (Frahm and Obst, 2003). For the Bacteroidales qPCR method specificity was experimentally assessed using representative species of the related bacterial classes, *Flavobacteria* and *Sphingobacteria* (Siefring *et al*., 2008).

The percent false negatives for the *Enterococcus* and Bacteroidales qPCR methods have been reported as the percentage of water sample filter extracts that fail salmon DNA sample processing control assay quality control criterion of > 3 CT units higher than the mean of associated calibrator samples in the analysis of NEEAR study samples (Haugland *et al*., 2005; Chern *et al*., 2009). Additionally, Griffith *et al.* (2007) reported false negative results of 58% for enteroccocci qPCR extracted using a multi-step purification and concentration process after bead beating and 29% for enterococci qPCR bead beaten method. The analysis for false negative rates was conducted relative to the State of California's (AB411) standard of 104 cells/100 ml for enterococci (Griffith *et al.*, 2007).

Percent false positives for the qPCR methods have been reported as the percentages of negative control samples, consisting of clean filters that were subjected to the entire method including DNA extraction and qPCR analysis, that gave positive detection of target sequences *(i.e.*, a true logarithmic amplification trace) during analyses of NEEAR study samples (Haugland *et al*.,

2005; Chern *et al*., 2009). It should be noted that the mean CT value from the false positive analyses shown for the study cited in footnote 7 in Table 2 was 43.65 which would not be detected as positive in a more recent version of the method where only 40 thermal cycles are run.

Additionally, Griffith *et al.* (2007) reported false positive results of 3% for enteroccocci qPCR extracted using a multi-step purification and concentration process after bead beating and 9% for enterococci qPCR bead beaten method. For *E. coli* qPCR, the false positive results were 0% before and 27% after adjustment for amplification efficiency. The analysis for false positive rates was conducted relative to the State of California's (AB411) standard of 104 cells/100 ml for enterococci and 400 cells/100 ml for *E. coli* (Griffith *et al.*, 2007).

3.5.2 Culture

For the culture methods, the percent false negative is the percentage of the samples that had a negative result but were actually positive. For Method 1600 and 1603, the percent false negatives are reported from analyses of various environmental water samples (USEPA 2000). Five percent of the *E. coli* colonies observed gave a false negative reaction (USEPA, 2006). In unspiked CSO samples, false negative rates of 37.5% and 1.7% have been reported for methods 1600 and 1603, respectively (USEPA, 2008). Additionally, Francy *et al.* (2000) reported false negative results of 11% for the modified membrane-Thermotolerant *Escherichia coli* agar (mTEC) (Method 1603) when compared to the mTEC (EPA Method 1103.1). Griffith *et al.* (2007) reported false negative results of 10% for Method 1600 with respect to the AB411 standard of 104 enterococci/100 ml.

For Method 1603, the percent false positives reported from analyses of various environmental water samples were <1% (USEPA, 2000) and averaged 6% for marine and fresh water samples (USEPA, 2006). False positive confirmation rates were 0% and 6.7% for Method 1600 and 1603, respectively (USEPA, 2008) were reported in unspiked CSO samples. Francy *et al*. (2000) reported false positive results of 0% for the modified mTEC (Method 1603) when compared to the mTEC (Method 1103.1). Griffith *et al.* (2007) reported false positive results of 4% for Method 1600 with respect to the AB411 standard of 104 enterococci/100 ml.

3.6 Ability to Differentiate Fecal Sources

This criterion evaluates the ability of the indicator/method combination to identify the sources contributing to fecal pollution in a waterbody. None of the indicator/methods evaluated are able to differentiate between fecal contamination sources. However, current research by EPA on microbial source tracking assays for use in detecting bovine and human fecal pollution suggests that these assays maybe useful in monitoring fecal contamination in ambient waters (see Section 2.4.1).

3.7 Performance in Different Waterbody Types

This criterion evaluates how well the method performs (*i.e.,* does it work or provide usable results) in different types of recreational waters (*e.g.*, freshwater, marine water, temperate, tropical, and subtropical waters). Recent epidemiological studies conducted by EPA have shown that *Enterococcus* qPCR and Bacteroidales qPCR performed well in both marine and freshwaters.

Results from earlier EPA epidemiological studies found that *Enterococcus* culture works well in both marine and freshwater, whereas *E. coli* only had good correlation ($R^2 \geq 0.5$) with GI illness in freshwater (Cabelli, 1983; Dufour, 1984).

4.0 EVALUATION OF APPROPRIATENESS OF INDICATOR/METHODS FOR CWA PURPOSES

The appropriateness of the method for the various CWA programs is important for state adoption and implementation of EPA's new or revised criteria recommendations. To assess the potential use and applicability of new fecal indicators and methods to meet CWA program needs, EPA's Monitoring and Assessment, TMDLs, and NPDES programs evaluated the impact of new indicators/methods on their programs. Each program identified attributes necessary for indicator-methods to meet their programs' CWA needs. These attributes are shown in Table 4 and ranked on whether they are important (Yes) or not important (No) for each CWA program.

Table 3 –Evaluation of Rapid and Culture Methods for Clean Water Act Programs Implementation Purposes

Method Attributes	Beach Program	NPDES	TMDL	Assessment
Low Cost	Yes	Yes	Yes	Yes
Ease of Use	Yes	Yes	Yes	Yes
Time to results (rapid/results within 4 hours of sample process/analysis)	Yes	No	No	No
Allows for use of historical data for model development, etc.	Yes	Yes	Yes	Yes
Demonstrates effectiveness of treatment from source to beach	No	Yes	No	No
Count/signal associated with human health risk pathogens	Yes	Yes	Yes	Yes
Precise/accurate	Yes	Yes	Yes	Yes

While beach advisory decisions and closures require same day results to protect public health, for the Assessment, TMDLs and the NPDES programs same day results are not necessary. Therefore, rapid methods are suggested as a key component of the criteria for the Beach Program only. Other important attributes for indicator/methods for the Beach Program include ease of use of the method and cost. Ease of use refers to how easily the method can be applied by those skilled in analysis. The cost associated with new indicator/method combination in criteria could include capital cost, training cost, per sample cost and additional sampling requirements. Capital costs include the upfront cost such as equipment purchase and space required to conduct the test. For example, when performing genetic testing, aside from the equipment needed (e.g., platform [i.e., specific machine]), laminar flow hoods, dedicated pipettes), space is needed, ideally in separate rooms, for reagent preparation (material not containing any genetic materials). Space is also needed for the two types of sample preparation, those containing high target sequence DNA concentrations such as DNA standards and calibrator samples, and those containing expected low target sequence DNA concentrations (e.g., filter blanks and water samples) – the latter of which should also be in separate laminar flow hoods (USEPA, 2007).

Training costs are incurred prior to routine testing so that the user can perform the test within the performance criteria of the test (USEPA, 2007). Training cost would be dependent on the types of training available, i.e., whether workshop-type training with hands-on experience or completing a training module.

Sample cost can vary and becomes an important cost consideration with a new method depending on the volume of tests needed to be completed on a routine basis. Additional sampling is generally an effort that results from rapid testing. For example, if an early morning sample yields a positive result resulting in beach closures, it may then lead to additional sampling to determine if the beach still needs to be closed in the mid-afternoon (USEPA, 2007). Therefore, depending on the number of designated bathing beaches that need to be monitored, the monitoring frequency, number of samples, and the need for additional sample analysis per beach, the sample costs associated with the use of rapid method could become cost prohibitive or result in beaches being sampled less frequently or not re-sampled to revise advisory or closure decision during the day.

Ease of use and cost are also important factors for Assessment, TMDL and NPDES programs. For the TMDL and Assessment programs the ability to use prior historical data for modeling loadings is also important. Current historical data for *E. coli*, enterococci, and fecal coliform are derived from culture methods data. Criteria based on a rapid method without the necessary linkage between qPCR and the culture method would not allow for the use of historical data.

For the NPDES Program, the ability of the indicator/method to reflect the treatment efficacy (e.g., chlorination) is crucial. Traditional culture methods for fecal indicator bacteria detect only a subset of the total viable population within any given water sample. The qPCR method will theoretically detect all intact cells in a water sample whether they are viable or not.

Precision and accuracy of methods are also important to each program as well as the ability to associate indicator counts/concentrations to health risk.

5.0 STUDY LIMITATIONS

There are several weaknesses in this evaluation with regard to how the project was defined in the Critical Path Science Plan. Due to limited data on various water body types, EPA is unable to evaluate method performance in different water body types based on the performance criteria in Table 2. Additionally, method performance with respect to fecal source identification (*i.e.*, human vs. animal) could not be evaluated because none of the current indicator/method combinations can distinguish between sources.

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