Multi-laboratory survey of qPCR enterococci analysis method performance in U.S. coastal and inland surface waters

Richard A. Haugland [a,*], Shawn Siefring [a], Manju Varma [a], Kevin H. Oshima [a], Mano Sivaganesan [b], Yiping Cao [c], Meredith Raith [c], John Griffith [c], Stephen B. Weisberg [c], Rachel T. Noble [d], A. Denene Blackwood [d], Julie Kinzelman [e], Tamara Anan'eva [e], Rebecca N. Bushon [f], Erin A. Stelzer [f], Valarie J. Harwood [g], Katrina V. Gordon [g], Christopher Sinigalliano [h]

[a] U.S. Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory, Cincinnati, USA
[b] U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, Cincinnati, OH, USA
[c] Southern California Coastal Water Research Project Authority, Costa Mesa, CA, USA
[d] Institute of Marine Sciences, University of North Carolina at Chapel Hill, Morehead City, NC, USA
[e] City of Racine Health Department, Racine, WI, USA
[f] U.S. Geological Survey, Columbus, OH, USA
[g] Department of Integrative Biology, University of South Florida, Tampa, FL, USA
[h] National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratories, Ocean Chemistry Division, Miami, FL, USA

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ABSTRACT

Quantitative polymerase chain reaction (qPCR) has become a frequently used technique for quantifying enterococci in recreational surface waters, but there are several methodological options. Here we evaluated how three method permutations, type of mastermix, sample extract dilution and use of controls in results calculation, affect method reliability among multiple laboratories with respect to sample interference. Multiple samples from each of 22 sites representing an array of habitat types were analyzed using EPA Method 1611 and 1609 reagents with full strength and five-fold diluted extracts. The presence of interference was assessed three ways: using sample processing and PCR amplifications controls; consistency of results across extract dilutions; and relative recovery of target genes from spiked enterococci in water sample compared to control matrices with acceptable recovery defined as 50 to 200%. Method 1609, which is based on an environmental mastermix, was found to be superior to Method 1611, which is based on a universal mastermix. Method 1611 had over a 40% control assay failure rate with undiluted extracts and a 6% failure rate with diluted extracts. Method 1609 failed in only 11% and 3% of undiluted and diluted extracts analyses. Use of sample processing control assay results in the delta–delta Ct method for calculating relative target gene recoveries increased the number of acceptable recovery results. Delta–delta tended to bias recoveries from apparent partially inhibitory samples on the high side which could help in avoiding potential underestimates of enterococci — an important consideration in a public health context. Control assay and delta–delta recovery results were largely consistent across the range of habitats sampled, and among laboratories. The methodological option that best balanced acceptable estimated target gene recoveries with method sensitivity and avoidance of underestimated enterococci densities was Method 1609 without extract dilution and using the delta–delta calculation method. The applicability of this method can be extended by the analysis of diluted extracts to sites where interference is indicated but, particularly in these instances, should be confirmed by augmenting the control assays with analyses for target gene recoveries from spiked target organisms.

1. Introduction

Quantitative polymerase chain reaction (qPCR) is becoming a frequently used method for quantifying enterococci in recreational surface waters (Boehm et al., 2009; Bourlat et al., 2013). The method's popularity stems from its speed advantage; whereas traditional culture methods require 18–72 h, qPCR methods can be conducted in as little as less than 2 h, creating the opportunity for same day health warnings (Griffith and Weisberg, 2011). QPCR has been adopted as an acceptable method by EPA (U.S. EPA, 2012a), supported by a number of studies demonstrating an association with health risk (Colford et al., 2012;

* Corresponding author.
E-mail address: haugland.rich@epa.gov (R.A. Haugland).

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Wade et al., 2008, 2010; Yau et al., 2014) and correlation of qPCR results with the traditional culture methods (Converse et al., 2012; Haugland et al., 2005; Noble et al., 2010; Raith et al., 2014).

QPCR methods continue to evolve and there are now multiple options for how they are performed. EPA has published two analysis methods for enterococci, EPA Method 1611 (U.S. EPA, 2012b) and EPA Method 1609 (U.S. EPA, 2013a), that differ primarily in the type of PCR mastermix reagents used. Method 1611 calls for the use of a long-available reagent, TaqMan® Universal Master Mix (UMM), whereas Method 1609 specifies the use of a more recently introduced reagent, TaqMan® Environmental Master Mix (EMM). Updated versions of both methods (U.S. EPA, 2015a, 2015b) have been recently published (http://www2.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological), primarily to improve the standardization of their results between laboratories and to facilitate comparisons with EPA recreational water quality criteria (RWQC) guideline values (Haugland et al., 2014). The updated Method 1611 also includes the recommended use of an internal amplification control (IAC) assay as described in Method 1609. Applying these or other related methods also requires decisions about whether to dilute the sample DNA extract, use cleanup procedures to purify the extract, and how to use the sample processing or other controls when calculating the final result (Cao et al., 2012; Haugland et al., 2012; Shanks et al., 2011; Sivaganesan et al., 2014). Additional consideration should be given to the nuanced differences in thermal cycler platform and reference materials among manufacturers that can lead to inter-laboratory variation (Cao et al., 2013).

The above and other reports have examined the effects of such permutations on method precision and repeatability, but only a few have focused on how they affect susceptibility to sample interference, particularly with different types of surface waters (Cao et al., 2012; Haugland et al., 2012). Interference, which can be caused by either PCR inhibition or poor recovery of amplifiable target gene sequences (Haugland et al., 2012), is of particular concern in beach water quality applications as it can lead to underestimates of fecal indicators, which is inconsistent with the public health goal of such monitoring. Here we conducted a multi-laboratory evaluation of how three method permutations: type of mastermix; sample extract dilution; and use of controls in results calculation, affect: 1) the frequency of samples from different water body types and from diverse individual sites that provide acceptable qPCR analysis results with respect to absence of significant sample matrix interference as determined by currently used controls and; 2) the reliability of the qPCR analysis results based on recoveries of target gene sequences from spiked enterococci in these sample matrices.

2. Materials and methods

2.1. Approach

Samples were collected by seven laboratories on a minimum of 12 separate days or visits from each of 22 sites representing a range of U.S. river, inland lake, Great Lake, and east and west coast marine sites (Tables 1 and S1). Six laboratories participated in analyses of the samples and replicate subsamples of each sample were processed and analyzed by at least two laboratories. Each laboratory employed the EMM and UMM mastermix reagents specified respectively in EPA Method 1609 and EPA Method 1611 (hereafter referred to as Methods 1609 and 1611 or simply the Methods) in analyses of both full strength and five-fold (5×) diluted DNA extracts of each sample to assess apparent interference. In analyses stemming from both Methods (i.e. with both types of mastermix reagents), a competitive IAC assay and an external sample processing control (SPC) assay were employed. Three approaches were used to assess interference by: 1) use of the control assays, 2) examination of linearity (i.e., Ct shift) between the full strength and diluted extract *Enterococcus* assay results as described by Cao et al. (2012), and 3) the recovery ratios of target gene sequences from laboratory spiked test matrix samples (STM samples) compared to spiked control matrix samples (SCM samples). The STM samples contained spiked enterococci cells that were extracted in the presence of filters and their retentates from each of the collected water samples, while SCM samples contained the same quantity of spiked organisms that were extracted in the presence of clean filters. The latter two approaches examine interference on the *Enterococcus* assay directly, while the control assay approach assumes that controls are affected by sample interference the same way as the *Enterococcus* assay.

Recovery ratio estimates for STM samples were determined using two alternative comparative Ct relative quantification models, commonly referred to as delta–delta Ct or ΔΔCt (Haugland et al.,

Table 1
Water sample collection sites.

Site name	Site ID	Water body type (abbreviation)	Location	GPS coordinates (N,W)	
Virginia Key Wetlands	A	Brackish Stream (BS)	Miami, Florida	25.7384	−80.1501
Horlick Dam, Root River	B	River or Stream (RS)	Racine, Wisconsin	42.7519	−87.8241
Liberty Street, Root River	C	River or Stream (RS)	Racine, Wisconsin	42.7303	−87.8018
Oak Creek	D	River or Stream (RS)	At mouth, South Milwaukee, Wisconsin	42.9071	−87.8434
Pike River	E	River or Stream (RS)	Near mouth, Kenosha, Wisconsin	42.6082	−87.8213
Little Miami River	F	River or Stream (RS)	Near mouth, Cincinnati, Ohio	39.0855	−84.4201
Hillsborough River	G	River or Stream (RS)	Riverfront Park, Tampa, Florida	27.9527	−82.4659
Brooks Beach	H	Inland Lake (IL)	Buckeye Lake, Ohio	39.9014	−82.5167
Crystal Beach	I	Inland Lake (IL)	Buckeye Lake, Ohio	39.9325	−82.4772
Fairfield Beach	J	Inland Lake (IL)	Buckeye Lake, Ohio	39.9222	−82.4708
White Sands Beach	K	Inland Lake (IL)	Lake Carroll, Florida	28.0543	−82.4887
Fischer Park Beach	L	Inland Lake (IL)	Browns Lake, Wisconsin	42.6814	−88.2375
Quarry Lake Park Beach	M	Inland Lake (IL)	Racine, Wisconsin	42.7396[a]	−87.8160[a]
North Beach	N	Great Lakes (GL)	Racine, Wisconsin	42.7400[b]	−87.7800[b]
Cabrillo Beach	O	Pacific Ocean (PO)	San Pedro, California	33.7090	−118.2830
Doheny Beach	P	Pacific Ocean (PO)	Dana Point, California	33.4614	−117.6893
Long Beach	Q	Pacific Ocean (PO)	Long Beach, California	33.7633	−118.1694
Newport Dunes Beach	R	Pacific Ocean (PO)	Newport, California	33.6159	−117.8922
Jockey's Ridge Beach	S	Atlantic Ocean (AO)	Outer Banks, North Carolina	35.9511	−75.6322
South Nags Head Beach	T	Atlantic Ocean (AO)	Outer Banks, North Carolina	35.8470	−75.5627
Iula, Wrightsville Beach	U	Atlantic Ocean (AO)	Wilmington North Carolina	34.1967	−77.8053
Snyder, Wrightsville Beach	V	Atlantic Ocean (AO)	Wilmington North Carolina	34.2042	−77.7992

[a] Composited samples from: 42.746953, −87.822427; 42.747274, −87.821256.
[b] composited samples from: 42.739861, −87.778833; 42.740917, −87.779361; 42.742417, −87.779972; 42.743667, −87.780444.

2005) and delta Ct or ΔCt (Converse et al., 2012). The main difference between these two models is that the former attempts to quantitatively adjust the enterococci target sequence density estimates in the samples for minor variations in amplification efficiency due to PCR inhibition and/or in total DNA recovery by analysis of an unrelated reference target gene that is added to the samples prior to extraction, whereas the latter does not. While this adjustment is employed in the Methods, there is not yet a consensus on which of these models is more appropriate for beach water monitoring applications (Cao et al., 2012).

2.2. Water samples, sample collection and distribution

The water sampling sites in this study and their locations are described in Table 1 and are shown in supplemental Fig. S1. Selection of sampling sites emphasized waters anticipated to challenge qPCR analyses based upon the local laboratory's previous analytical experience at those sites and/or other subjective factors such as historically observed high turbidity levels due to suspended sediments or coloration due to humic, tannic, algae or other materials (see Table S1). Approximately 2-L bulk water samples were collected from each site on a minimum of 12 separate days or site visits following general guidelines for water sample collection provided in the Methods. A list of the laboratories and an overview of the study design are shown in Fig. 1. Water samples were transported on ice and processed by filtration within 6 h of collection. A minimum of 12 replicate 30 to 100 mL aliquots (subsamples — see Table S1) of each of the bulk water samples were filtered through 0.45 μm pore size polycarbonate filters (Millipore # HTTP04700) according to the Methods. While 100 mL is recommended in the Methods, lesser filtration volumes within this range, i.e. 50 mL, are currently considered acceptable depending on the time required for filtration (U.S. EPA, 2015a, 2015b).

Filters containing retentates of each of the water subsamples were transferred to 2 mL, semi-conical microcentrifuge tubes containing 0.3 g of 212–300 μm, acid-washed glass beads (GeneRite #S0205-50) as indicated in the Methods and stored at <— 70 °C by each of the laboratories until they were extracted within that laboratory or shipped to another laboratory. Replicate subsample filters were shipped to other laboratories on dry ice and stored at <— 70 °C by the receiving laboratories until they were extracted. The distribution of replicate subsample filters among the different laboratories is shown in Fig. 1. Holding times of the subsample filters at <— 70 °C prior to DNA extraction and qPCR analysis ranged from about 1 to 18 months.

2.3. Reference, spiking and control materials

Cells used by all laboratories for spiking control and test matrix samples originated from *Enterococcus faecalis* strain ATCC 29212 (ATCC™, Manassas VA). Cultured *E. faecalis* cells were prepared for this purpose by Laboratory 1 and were enumerated from plate counts as described in the Methods. An aliquot of the cell preparation was diluted with AE buffer to a concentration of about 10^6 cells/mL. The diluted cell suspension was divided into 0.2–0.25 mL aliquots in sterile micro-centrifuge tubes and frozen at <— 70 °C. Tubes containing the cell aliquots were either held by the source laboratory or shipped on dry ice to the other laboratories in quantities determined to meet their needs for the study. Tubes were stored by each of the laboratories at <— 70 °C until used for preparing SCM and STM samples as described in Section 2.4.

Cells used to estimate absolute ambient enterococci quantities in the water samples by Laboratory 1 only (Table S1) originated from single Multishot 550 Bioballs® (catalog #56015, BioMerieux Inc., Lombard, IL), batch# B-1664 (mean of 501 CFU/Bioball based on manufacturer estimates). *E. faecalis* genomic DNA standards used by Laboratory 1 only to estimate a reference number of average target gene sequences recovered from the Bioballs and also from the SCM samples (see Section 2.7) were prepared and quantified by a most probable number (MPN) qPCR estimation method as previously described (Sivaganesan et al., 2011).

The internal amplification control (IAC) assay DNA template and extraction buffer containing the salmon DNA sample processing control (SPC) also originated from Laboratory 1. The IAC5 plasmid DNA template (IDT, Inc., Coralville, IA) is described in Method 1609 and in the updated Methods and was linearized and diluted to a concentration of about 50 copies/μL as described in these Methods. Extraction buffer containing 0.2 μg/mL salmon DNA (Sigma #1626) in AE buffer (Qiagen #19077) was also prepared as described in the Methods. Aliquots of the IAC template and bulk volumes of the extraction buffer were either retained by the source laboratory or shipped to each of the other laboratories on dry ice in quantities determined to meet their needs for the study and were stored at −20 °C and 4 °C, respectively until used as described below.

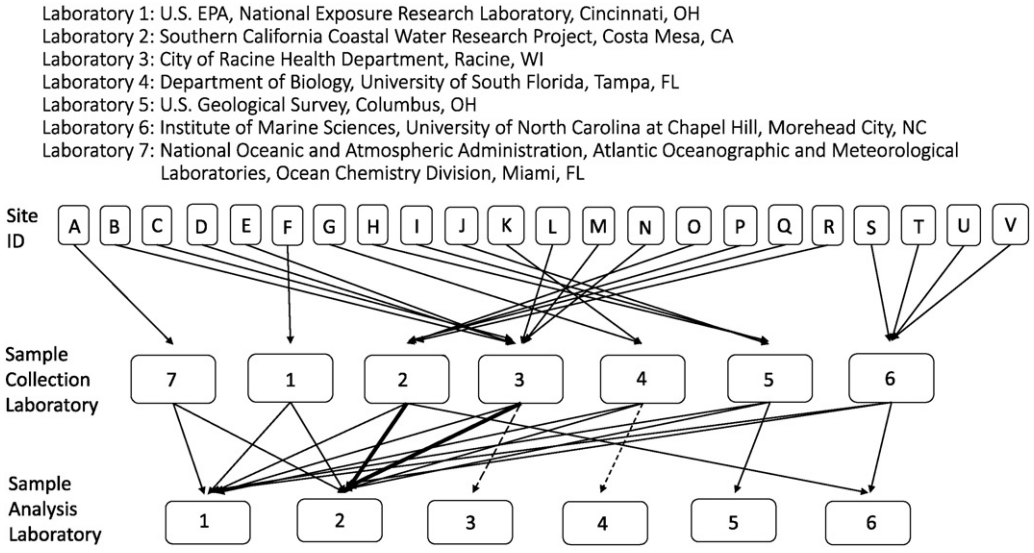

Fig. 1. Study design for water sample collection and analyses. Site IDs are described in Table 1. Arrows originating from each site point to the laboratory that collected samples from that site. Arrows originating from each sample collection laboratory point to the laboratories that received filter samples from that laboratory for analysis. Dashed arrows indicate that not all samples originating from the collection laboratory were analyzed by the receiving laboratory. Laboratory 3 analyzed samples only from sites B, M and N. Laboratory 4 analyzed samples only from site I. Bold arrows indicate that duplicate filter samples were received by Laboratory 2. Selected duplicate samples from sites B, C, E, N, O, P, Q and R were analyzed by this laboratory (N = 21 samples from fresh water sites B, C, E, N and 21 samples from marine sites O, P, Q and R).

2.4. DNA extractions

Clean and test matrix filters were spiked with 0.6 mL of a 50-fold dilution of the *E. faecalis* cell suspensions distributed to each of the laboratories (about 10^4 cells) to prepare SCM and STM samples, respectively. Dilutions of the cells were made with the 0.2 μg/mL salmon DNA extraction buffer that was also distributed to each of the laboratories. Replicate test matrix filters for each water sample and clean filters serving as negative controls were amended with 0.6 mL of only the salmon DNA extraction buffer. Each sample tube containing a filter, glass beads, salmon DNA extraction buffer and with or without spiked *E. faecalis* cells was sealed, bead milled at 5000 reciprocations/min for 60 s, and centrifuged at 12 000 $\times g$ for 1 min as described in the Methods. Continuing with the protocol described in the Methods, about 400 μL of the supernatants from each tube were transferred to clean, low-retention microfuge tubes, re-centrifuged at 12 000 $\times$ *g* for 5 min and then about 350 μL of the clarified supernatants were transferred to clean tubes to serve as undiluted DNA extracts. A 30 μL aliquot of each undiluted DNA extract was diluted by each of the laboratories with 120 μL of AE buffer to prepare 5-fold diluted DNA extracts. Laboratory 2 similarly prepared 25-fold diluted extracts from the 5-fold dilutions of their DNA extracts. All DNA extracts were stored at 4 °C and were analyzed within 24 h of their preparation. Spiked and unspiked replicate filters of each water sample were extracted in parallel with the SCM samples by each of the laboratories except Laboratory 1. In that laboratory the unspiked water sample filters were extracted and analyzed in parallel with similarly prepared Bioball® calibrator samples about three months prior to the STM and SCM samples.

2.5. QPCR analyses

Amplifications were performed in either an ABI StepOnePlus™ sequence detector (ThermoFisher/Life Technologies/Applied Biosystems, Foster City CA) or by Laboratory 2 only in a CFX96 real-time PCR detection system (BioRad, Hercules, CA). Multiplex *Enterococcus* assay reaction mixtures contained either 1X TaqMan® Universal PCR Master Mix or 1X TaqMan® Environmental PCR Master Mix (ThermoFisher/Life Technologies), plus 0.2 mg/mL bovine serum albumin (Sigma #A-5611), 1 μM of each primer, 80 nM each of FAM™ labeled *Enterococcus* TaqMan® probe and VIC™ labeled UC1P1 (IAC) TaqMan® probe (both labeled with TAMRA™ as the quencher dye (ThermoFisher/Life Technologies), about 100 copies of IAC5 and 5 μL of undiluted or diluted DNA extracts in a total reaction volume of 25 μL as previously described (Sivaganesan et al., 2014). The use of this multiplex assay reaction mixture is recommended in Method 1609 and in the updated Methods (U.S. EPA, 2013a, 2015a, 2015b). Primers and probe of the Sketa22 assay described in the Methods were used in simplex reactions with the other reaction components mentioned above for the detection of salmon DNA SPC target sequences in all sample extracts. Thermal cycling conditions for all reactions included an initial incubation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Data were analyzed at a threshold value of 0.03 ΔRn on the StepOnePlus™ sequence detectors and 100 RFU on the CFX96 sequence detectors. Threshold cycle (Ct) values were exported to Microsoft Excel and subsequently to SAS 9.2 for Windows for further analysis.

2.6. QPCR data analyses for relative target gene sequence quantification

Prior to the analysis of the STM samples, all of the laboratories performed preliminary extractions of six SCM samples and a series of three, 5-fold serial dilutions were prepared from each DNA extract. The undiluted and serially diluted extracts were analyzed in duplicate by the *Enterococcus*/IAC multiplex assay in three separate plate runs by each laboratory. The mean slope values determined from the *Enterococcus* assay Ct measurements and arbitrarily chosen log10 target sequence copy numbers per reaction corresponding to the relative dilution factors of the extracts were used to calculate an amplification factor for this assay with each Method and for each laboratory as described in Eq. (1):

$$AF = 10^{(1/-\text{slope})} \tag{1}$$

where AF is equivalent to the amplification efficiency of the target sequence (E) + 1 (Applied Biosystems, 1997). IAC assay Ct values were used to determine the range of quantification (ROQ) threshold of this assay with each reagent and for each laboratory (Shanks et al., 2011). In this study, the threshold was defined as the mean *Enterococcus* assay Ct value at the lowest dilution of the SCM sample extracts where the corresponding mean IAC assay Ct measurements were within two standard deviations of the mean measurements from analyses of the negative control samples. The negative control samples consisted of no template control samples and DNA extracts from clean filters that were each analyzed by each laboratory in quadruplicate in each plate run.

Recovery ratios of total target gene sequence copies from the STM samples compared to the SCM samples were determined for all of the laboratories by the following simple formulas that are also expressions of the ΔCt and ΔΔCt comparative Ct relative quantification models:

$$\Delta\text{Ct ratio} = AF^{(-(a-c))} \tag{2}$$

$$\Delta\Delta\text{Ct ratio} = AF^{(-((a-b)-(c-d)))} \tag{3}$$

where AF = amplification factor, a = mean STM sample *Enterococcus* assay Ct, b = mean STM sample SPC assay Ct, c = mean SCM sample *Enterococcus* assay Ct, d = mean SCM sample SPC assay Ct. Mean Ct values were from duplicate analyses of three SCM samples prepared with each batch of STM samples and duplicate analyses of each STM sample. Recovery ratios determined from Eqs. (2) and (3) were converted to percent recovery values by multiplying by 100. Corresponding unspiked water sample filter DNA extracts were analyzed in duplicate at the same time as the STM and SCM samples by all of the laboratories except Laboratory 1. Mean *Enterococcus* and SPC assay Ct values from the unspiked samples were used as "a" and "b" in Eqs. (2) and (3) to calculate percent recovery values for all of these samples. Analyses giving undetermined Ct values were assigned a value of 40 (the total number of thermal cycles run) for the calculations. Percent recovery values from the unspiked samples were subtracted from the percent recovery values from the corresponding STM sample analyses to obtain net percent recoveries from the STM samples. Negative net recoveries were set to zero.

2.7. QPCR data analyses for absolute and relative target gene sequence quantification

Absolute enterococci calibrator target sequence equivalent (CSE) and corresponding calibrator cell equivalent (CCE) estimates, adjusted to the CSE/CCE ratio used to determine the RWQC guideline values for CCE (Haugland et al., 2014), were determined for the unspiked ambient and STM water samples by Laboratory 1 only. The estimates for the ambient samples came from duplicate analyses of three Bioball® calibrator sample DNA extracts per batch of samples and duplicate analyses of each of the unspiked water sample filter DNA extracts, as described in the updated Methods, using a prototype of an Excel calculation spreadsheet that is now available at http://www2.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological. The formula used for CSE estimation in this spreadsheet can be represented as follows:

$$\text{CSE} = AF^{(-\Delta\Delta\text{Ct})} * R \tag{4}$$

where AF = amplification factor and R = reference number (defined below) and ΔΔCt is calculated from the mean Ct values of the

Enterococcus and SPC assays for both the calibrator and test samples as previously described (Haugland et al., 2005; see also Eq. (3)). Log_{10} target sequences per reaction of the *E. faecalis* genomic DNA standards were approximately 3.71, 3.21, 2.71, 2.21, 1.70 and 1.20. The mean slope value of a composite standard curve, generated from ten independent analyses of the DNA standards (duplicate analyses of each standard for each analysis), was used in the spreadsheet to calculate the amplification factor (AF) for the *Enterococcus* assay using the formula in Eq. (1). The average of the Ct measurements from extracts of thirty *E. faecalis* Bioball® calibrator samples (each analyzed in duplicate) was used in the spreadsheet to determine the reference number (R) of average *E. faecalis* target sequence copies recovered from the calibrator samples by the formula:

$$R = 10^{((y-c)/s)}/(v_a/v_t) \quad (5)$$

where y = y-intercept from standard curve, c = average calibrator sample reaction Ct, s = slope from standard curve, v_a = volume of calibrator sample extracts analyzed and v_t = total volume of the calibrator sample extracts. Ambient water sample CSE and CCE estimates were obtained using results from the Methods (Table S1). Below method reporting limit (BMRL) estimates shown in this table were based on previously determined estimates of the lower limits of quantification for Method 1611 (U.S. EPA, 2013b) that are also specified in the calculation spreadsheet (568 target gene sequence copies per filter for undiluted extracts and 2841 copies for five-fold diluted extracts — equal to 37.9 and 189.4 CCE, respectively), however, mean Ct values for all of the samples (including values of 40 that were assigned to undetermined analysis results as well as values from samples that failed the control assay acceptance criteria) were used to obtain CSE estimates for purposes of determining net recoveries from the STM samples as described below.

Total CSE estimates also were determined for the STM samples in the same manner as described for the ambient unspiked water sample filters but substituting the SCM samples for the Bioball samples as the calibrators. CSE estimates of the unspiked samples were subtracted from those of the corresponding STM sample analyses to determine net CSE estimates. These values were divided by the reference number of average target sequences recovered from all SCM (calibrator) samples to determine net relative recovery ratios from the STM samples for Laboratory 1 and then converted to percent recovery values as indicated in Section 2.6.

2.8. Control criteria for determining qPCR interference

The acceptance criterion for mean IAC assay Ct values from duplicate analyses of each of the unspiked water sample filter extracts was ≤ the mean of the Ct values from duplicate analyses of the negative control filter extracts (that were analyzed in parallel in each plate run) plus 1.5 Ct units as indicated in Method 1609 and the updated Methods (U.S. EPA, 2013a, 2015a, 2015b). For the STM samples, the negative control filter Ct values were replaced with the mean of the SCM sample Ct values from the corresponding plate runs for making these determinations. The maximum acceptable mean IAC assay Ct value from the negative control or SCM samples was 35.0. The acceptance criterion for mean SPC assay Ct values from duplicate analyses of the unspiked water sample filter and STM sample extracts was ≤ the means of the Bioball (Laboratory 1 only) or SCM sample Ct values plus 3.0 Ct units as indicated in the Methods. The acceptance criterion for effects of DNA extract dilutions on *Enterococcus* assay Ct measurements was based on an expected shift of 2.32 Ct units resulting from analyses of five-fold dilutions of the DNA extracts when the amplification factor is 2 (100% efficiency), i.e. $2^{2.32} = 5$. The Ct shift acceptance range was 2.32 ± 1 Ct units. Shift values below this range are assumed to be associated with sample matrix inhibition of the qPCR and values above this range are assumed to result from technical error (Cao et al., 2012). A summary of these control analyses and acceptance criteria as they relate to different, currently available *Enterococcus* qPCR methods is provided in Table 2. As further discussed in Section 4.7, all of these current acceptance criteria were empirically determined and are not based upon statistical analyses.

3. Results

3.1. Enterococcus and IAC assay parameters

Table 3 shows the *Enterococcus* assay amplification factors that were calculated for each laboratory from preliminary analysis results of their serially diluted SCM samples with the mastermix reagents from both Methods, i.e. EMM and UMM. This table also shows the IAC assay ROQ thresholds determined for each of the laboratories with both of the reagents. In greater than half of the laboratory/reagent combinations, the IAC assay gave Ct values that exceeded this threshold in analyses of the undiluted SCM sample DNA extracts. As a consequence, IAC assay Ct values from the SCM samples were used to compare with the IAC assay results from the STM samples while the negative control samples were used to compare with the unspiked water sample filter IAC assay results for determining unacceptable sample matrix interferences for all laboratories.

The mean slope and intercept values from the *Enterococcus* assay standard curves used by Laboratory 1 to calculate target gene copy reference numbers for the Bioball and SCM calibrator samples were −3.28 and 36.80 from analyses with EMM and −3.33 and 37.0 for UMM. Bioball and SCM calibrator sample reference numbers were 4929 and 168,030 target gene copies, respectively, from analyses with EMM and 4719 and 159,333 copies for UMM in this laboratory.

3.2. Acceptable STM sample analyses determined from control results

Table 4 summarizes the percentages of all STM sample analyses that met the acceptance criteria currently specified in the Methods for SPC and IAC control assay results as well as the percentages of samples that met the acceptance criterion range for shifts in *Enterococcus* assay Ct values from undiluted to 5×-diluted extracts specified by Cao et al. (2012). Table 5 provides a summary of the results for sub-groups of similar water body types and Table S2 provides the results for each of the individual sampling sites. Results are not shown for analyses of undiluted extracts by Method 1611 in these tables since only analyses of 5×-diluted extracts are recommended in this method and, consistent with this recommendation, over 40% of the total STM sample analyses in this study failed the EPA control assays acceptance criteria by this approach. The unequal numbers of undiluted and 5×-diluted extract analyses shown in these tables resulted from failures to follow the dilution protocols specified for this study by one of the laboratories with some of their samples and from the analysis of only 5×-diluted extracts by another laboratory. Further analyses of *Enterococcus* assay Ct shifts resulting from additional 5×-dilutions of the 5×-diluted extracts were only performed by Laboratory 2 and so these results also are not shown.

3.3. Estimated target sequence recoveries from STM samples

Tables 4, 5 and S2 also summarize the percentages of the analyses giving net percent recoveries of target gene sequence copies from the STM samples compared to the SCM samples within a selected benchmark range of 50–200%. Fig. 2 summarizes the distributions of total percent recoveries of target gene sequence copies from all STM samples compared to the SCM samples calculated for each of the laboratories and the composites of these distributions for all laboratories. Results are presented for groupings of similar water body types as in Table 5. Since these total recovery results were obtained from the same sample analysis protocol and by the same calculation methods

Table 2
Summary of important analytical permutations and control analysis acceptance criteria for addressing sample matrix interference in *Enterococcus* qPCR methods.

Method	Analytical permutations		Control analyses			
	PCR Reagent	Sample Extract Dilution	*Enterococcus* assay Ct shift across 5× sample extract dilutions	Salmon DNA sample processing control (SPC) assay	Competitive Internal Amplification Control (IAC) assay	STM/SCM sample target gene sequence recovery ratio
Cao et al. (2012)	UMM, EMM and others[a]	Undiluted, 5×-diluted, Optional: 25×-diluted	Acceptance criterion: test sample or STM sample Ct shift within 2.32 ± 1 units Recommendation: recommended in methods	Acceptance criterion: test sample Ct within 3, 1.7 or 1 units of uninhibited reference samples Recommendation: not recommended in methods for detecting PCR inhibition	Acceptance criterion: test sample Ct within 3, 1.7 or 1 units of uninhibited reference samples Recommendation: not recommended in methods	Not evaluated
EPA Method 1611	UMM	5×-diluted	Not evaluated	Acceptance criterion: test sample Ct within 3 units of calibrator samples Recommendation: mandatory in method	Acceptance criterion: test sample Ct within 1.5 units of negative control samples Recommendation: recommended in updated method[b]	Acceptance criterion: 50–200% recovery (this study) Recommendation: recommended for initial site evaluation and periodically thereafter
EPA Method 1609	EMM	Undiluted, Optional: 5×-diluted	Not evaluated	Acceptance criterion: test sample Ct within 3 units of calibrator samples Recommendation: mandatory in method	Acceptance criterion: test sample Ct within 1.5 units of negative control samples Recommendation: recommended in method[b]	Acceptance criterion: 50–200% recovery (this study) Recommendation: recommended for initial site evaluation and periodically thereafter

[a] UMM: TaqMan® Universal master mix, EMM: TaqMan® Environmental master mix. Other reagents evaluated: TaqMan® Fast Environmental Master Mix, OmniMix™ HS.
[b] See Section 4.1 for further discussion.

for each laboratory's data, as described in Section 2.6, they provide a direct comparison of the distributions obtained by each laboratory. Distributions of net percent recoveries calculated for each laboratory are not shown in this figure due to the difference in the sample analysis and calculation approach used by Laboratory 1 as described in Section 2.7. However, the composites of these net percent recovery distributions for all laboratories are shown for comparisons with the corresponding total percent recovery distributions. Fig. S2 further illustrates the net percent recoveries obtained for all sample analyses by all laboratories and from all sites in relation to the aforementioned selected benchmark range of 50–200%. Net percent recoveries from analyses that met and failed the acceptance criteria threshold values for control assay results and shifts in *Enterococcus* assay Ct values are also shown in different panels of this figure.

4. Discussion

4.1. IAC assay results

Previous reports have discussed the need for establishing a ROQ for competitive IAC assays that are multiplexed with target organism gene sequence assays such as the *Enterococcus* assay used in Methods 1609 and 1611 (Haugland et al., 2010; Shanks et al., 2011). The upper limit or threshold of the IAC assay ROQ normally results from the competitive effects of increasing concentrations of the target gene sequences on the amplification of typically lower and fixed concentrations of the IAC templates that are added to the reactions. While an upper limit to the IAC assay ROQ was identified in more than half of the laboratory/reagent combinations in this study, this limit was associated with a decrease in IAC assay Ct values with increasing *Enterococcus* target sequences in all instances except one involving analyses with UMM. Competitive effects from increasing concentrations of target gene sequences are normally associated with increasing IAC assay Ct values and so the effects of the *Enterococcus* DNA on the IAC assay seen most often in this study can be more accurately described as facilitation rather than competition. While not evaluated further in this study, unpublished results from Laboratory 1 have indicated that this facilitation effect is transitory in analyses with UMM reagent, i.e. further increases in *Enterococcus* target sequences in multiplex analyses with a fixed IAC template concentration will lead to the expected competition-associated increases in IAC Ct values that were observed by only one laboratory in this study. However, such a competitive effect has not been observed in analyses with EMM reagent. It is presently unclear whether this facilitation has an effect on the sensitivity of the IAC assay in detecting

Table 3
Enterococcus and IAC assay parameters for spiked control matrix (SCM) samples.

Lab	EPA Method (PCR Reagent)	Enterococcus assay amplification factor (mean slope)	Enterococcus assay mean Ct for undiluted SCM samples (std deviation)	IAC assay mean Ct for undiluted SCM samples (std deviation)	IAC assay mean Ct for negative control samples (std deviation)	Enterococcus assay mean Ct at IAC assay ROQ threshold
1	1609 (EMM)	1.98 (−3.37)	26.48 (0.20)	30.77 (0.31)	31.45 (0.40)	ND[a]
	1611 (UMM)	1.87 (−3.69)	26.76 (0.12)	31.15 (0.21)	31.57 (0.31)	ND[a]
2	1609 (EMM)	2.04 (−3.23)	28.04 (0.13)	32.59 (0.31)	32.83 (0.35)	ND[a]
	1611 (UMM)	1.95 (−3.44)	28.30 (0.13)	34.72 (0.48)	32.95 (0.22)	30.65
3	1609 (EMM)	1.74 (−4.14)	26.02 (0.09)	30.47 (0.13)	31.23 (0.16)	28.98
	1611 (UMM)	1.67 (−4.51)	26.33 (0.08)	31.02 (0.24)	31.72 (0.28)	29.58
4	1609 (EMM)	2.05 (−3.20)	25.79 (0.18)	29.48 (0.21)	30.77 (0.33)	28.07
	1611 (UMM)	1.93 (−3.51)	26.13 (0.18)	29.75 (0.22)	31.19 (0.28)	28.53
5	1609 (EMM)	1.96 (−3.42)	26.53 (0.08)	31.05 (0.26)	31.52 (0.17)	28.93
	1611 (UMM)	1.9 (−3.57)	26.82 (0.12)	31.38 (0.44)	31.57 (0.21)	ND[a]
6	1609 (EMM)	1.95 (−3.45)	26.23 (0.21)	31.18 (0.22)	31.52 (0.47)	ND[a]
	1611 (UMM)	1.91 (−3.56)	25.82 (0.11)	30.86 (0.32)	31.31 (0.22)	28.34

[a] Not determined, < mean Ct for undiluted SCM samples.

Table 4
Effects of methodological choices on number of acceptable results across all sites.

Method (sample extract dilution[a])	Total analyses (N)	Percent of analyses passing SPC & IAC control assay criteria	Percent of analyses passing Enterococcus assay Ct shift criterion	Percent of ΔCt net recovery analyses within 50–200% recovery range STM:SCM[c]	Percent of ΔΔCt net recovery analyses within 50–200% recovery range STM:SCM[d]
1609 (1×)	732	89%	81%	71%	91%
1609 (5×)	775	97%	NS[b]	85%	93%
1611 (5×)	778	94%	NS	87%	84%

[a] Results from 1611(1×) not shown, see Section 3.2.
[b] Results not shown, see Section 3.2.
[c] ΔCt analyses are not used for estimating enterococci densities in EPA Methods 1609 and 1611.
[d] ΔΔCt analyses are used for estimating enterococci densities in EPA Methods 1609 and 1611.

inhibition by surface water test samples. Partly due to this uncertainty about the sensitivity of the IAC assay and partly due to the recognized additional technical and cost requirements associated with implementing it, this assay is currently recommended but not mandatory in updated Methods 1609 and 1611 (U.S. EPA, 2015a, 2015b). Overall frequencies of IAC assay failures in this study were 4.4%, 0.8% and 3.7%, respectively, for full strength and 5×-diluted extract analyses by Method 1609 and for 5×-diluted extract analyses by Method 1611, compared to 7.7%, 2.6% and 3.5% for the SPC assay. Frequencies of analyses failing only the IAC assay were 3%, 0.6% and 3%, suggesting that inclusion of the IAC assay along with the SPC assay may have a nominal effect on overall sample analysis failure rates, particularly in Method 1609 analyses of diluted extracts.

4.2. Acceptability of sites for qPCR monitoring based on control analyses

The EPA has produced a document containing current guidelines (available at http://www2.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological) to help state or local beach management authorities determine whether a qPCR test would be acceptable for use in public notification programs at their sites (U.S. EPA, 2013c). The principal recommendation in this document is that at least 90% of either full strength or 5×-diluted extract analyses of at least 10 water samples collected on different days and under representative conditions from a site should meet the control assay criteria as specified in the Methods and in Section 2.8 of this report. All of the water body types shown in Table 4 and all but two of the 22 individual sites shown in Table S2 (>90%) would be considered acceptable for use of Method 1609 based on all laboratory results obtained in this study. While the Pacific Ocean samples as a group, narrowly missed this cutoff, all but six of the individual sites (73%) would be considered acceptable for the use of Method 1611. Using results from Method 1609 with 5×-diluted DNA extracts which provided the most complete set of at least 10 usable analysis results per site from all laboratories, only 28 of 37 pairwise comparisons between laboratories (76%) were in agreement in terms of whether the sites passed or failed this acceptability guideline (results not shown). The overall percentages of sample analyses by this method permutation that met the control assay criteria differed among the laboratories (88–100%). Part of this difference may be attributable to the different samples (e.g. from different sites) that the laboratories analyzed in some cases. Part of this difference also may be attributable to differences in the efficiency of the assays in the hands of the different laboratories as suggested by an empirically observed correlation between lower estimated amplification factors (see Table 2) and higher frequencies of control assay criteria failures among the laboratories (results not shown). Based on analyses by this method permutation performed only by Laboratory 2 (see Fig. 1), the overall frequency of differences in the acceptability of control assay results between duplicate filters of the same water samples within that laboratory was 7%. Thus while variations in method performance between and within laboratories cannot be ruled out, the overall results also suggest that the minimum number of 10 sample analysis results per site from the individual laboratories in this study may not have been sufficient in some instances to adequately assess the acceptability of a site for qPCR analysis based on the current EPA guideline recommendations. A number in the range of the combined analyses obtained from all laboratories per site in this study, e.g. at least 20 analyses and potentially more if any criteria failures occur, may be more suitable for this purpose. As

Table 5
Effects of methodological choices on number of acceptable results as a function of water body type.

Method (sample extract dilution[a])	Water body type	# of Sites	Total analyses (N)	Analyses passing SPC & IAC control assay criteria	Analyses passing Enterococcus assay Ct shift criterion	ΔCt net recovery analyses within 50–200% recovery range	ΔΔCt net recovery analyses within 50–200% recovery range
1609 (1×)	Atlantic Ocean	4	83	93%	95%	80%	86%
	Brackish Stream	1	27	93%	93%	59%	70%
	Great Lakes	1	59	93%	97%	90%	95%
	Inland Lake	6	214	86%	53%	40%	94%
	Pacific Ocean	4	131	88%	85%	81%	86%
	River or Stream	6	218	91%	94%	88%	94%
1609 (5×)	Atlantic Ocean	4	116	100%	NS[b]	94%	97%
	Brackish Stream	1	30	100%	NS	87%	100%
	Great Lakes	1	44	93%	NS	89%	98%
	Inland Lake	6	196	97%	NS	68%	89%
	Pacific Ocean	4	210	94%	NS	90%	93%
	River or Stream	6	179	98%	NS	92%	93%
1611 (5×)	Atlantic Ocean	4	116	100%	NS	93%	98%
	Brackish Stream	1	30	100%	NS	87%	93%
	Great Lakes	1	44	93%	NS	86%	98%
	Inland Lake	6	196	96%	NS	84%	94%
	Pacific Ocean	4	213	89%	NS	85%	69%
	River or Stream	6	179	92%	NS	88%	75%

[a] Results from 1611(1×) not shown, see Section 3.2.
[b] Results not shown, see Section 3.2.

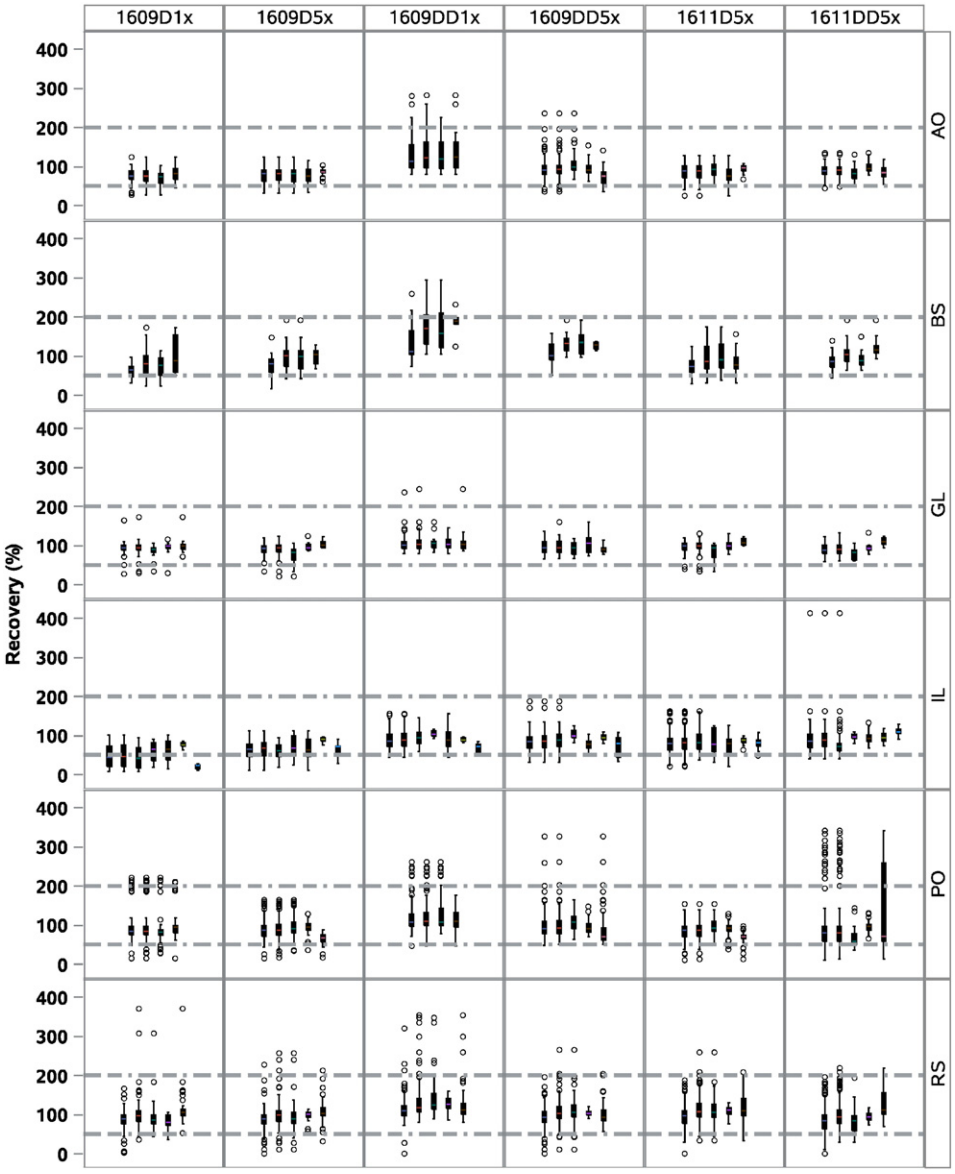

Fig. 2. Box and whisker plots showing distributions of estimated net and total enterococci target gene sequence recoveries from STM filter samples as percentages of corresponding SCM sample recoveries from different analysis method permutations and water body types. The bottom and top edges of the boxes indicate the intra-quartile range (IQR) — that is, the range of values between the first and third quartiles (the 25th and 75th percentiles). The lines inside the boxes indicates the median value. The whiskers that extend from each box indicate the range of values that are outside of the intra-quartile range, however, the values are close enough not to be considered outliers (a distance less than or equal to 1.5*IQR). Any points that are a distance of more than 1.5*IQR from the box are considered to be outliers. Recovery estimates are only from sample analyses that met the control assay acceptance criteria of the EPA methods. Analysis method permutations included different combinations of EPA method (PCR reagent), comparative Ct quantification model and analyzed DNA extract dilution that are indicated at the top of each column of panels: 1609 = EPA Method 1609 (EMM); 1611 = EPA Method 1611 (UMM); D = ΔCt model; DD = ΔΔCt model; 1× = undiluted extracts; 5× = 5-fold diluted extracts. Water body types (see Table 1) are indicated to the right of each row of panels: AO = Atlantic Ocean; BS = Brackish Stream; GL = Great Lakes; IL = Inland Lakes; PO = Pacific Ocean; RS = Rivers and Streams (see Table 1). The left-most plot in each panel is of net enterococci target gene sequence recoveries from all laboratories and the next adjacent plot to the right in each panel is of total enterococci target gene sequence recoveries from all laboratories. Additional plots in each panel are of total enterococci target gene sequence recoveries from individual laboratories that analyzed samples of the indicated water body type and by the indicated method permutation. Dashed horizontal lines denote 50% and 200% enterococci target gene sequence recoveries. Negative net percent recoveries are set to zero.

indicated in the EPA guidelines, site managers also may wish to perform these evaluations over an entire bathing season to fully capture variations in the characteristics of their waters.

The overall percentages of acceptable results obtained using the *Enterococcus* assay Ct shift criterion did not differ substantially from those obtained from the control assay criteria when evaluating the directly comparable analysis results from the undiluted DNA extracts with the control assay criteria. A notable exception occurred, however, at the three inland lake sites in Ohio (sites H, I and J) where much lower percentages of samples met the Ct shift criterion.

4.3. Estimation of target sequence recoveries from STM samples and effects of the ΔΔCt model adjustment with Method 1609 analysis results

The 50–200% range of net target sequence recoveries from the STM samples compared to the SCM samples was selected as a benchmark to evaluate the performance of the Methods and the effects of the different sites on this performance. This acceptance range is more stringent than the ranges of recoveries obtained in previous multi-laboratory investigations of the Methods (U.S. EPA, 2012b, 2013a) but is fairly consistent with previous single laboratory relative recovery

estimates by Method 1611 (Haugland et al., 2005) and by other similar qPCR methods (Brinkman et al., 2003; Haugland et al., 1999).

The overall percentages of sample analyses using the ΔΔCt model with Method 1609 analysis results that gave net target sequence recoveries within the 50–200% acceptance range were fairly similar to the percentages that met the control assay acceptance criteria (Table 3). Potential discrepancies were observed, however, for some of the individual sites and, as illustrated in Figs. 2 and S2, conclusions from the control assay results were not always correct in predicting net recoveries that fell within or outside of this range. Considerably lower percentages of the overall analyses (20% lower for analyses of undiluted extracts and 8% lower for 5×-diluted extract analyses by this method) fell within the recovery acceptance range using the ΔCt model. The distributions of target sequence recovery percentages illustrated in Figs. 2 and S2 provide further insight into these differences. In the case of the ΔCt analyses, nearly all of the net percent recovery estimates that were not within the acceptance range were below the 50% lower limit. These observations were largely consistent with *Enterococcus* assay Ct shift results which ideally should provide the most direct indication of inhibition specifically to the *Enterococcus* qPCR assay. The lower percentages of sample analyses giving acceptable target sequence recovery estimates by the ΔCt method and meeting the acceptance criterion of the *Enterococcus* assay Ct shift approach, particularly from sites H, I and J, suggests that many of these sample extracts contained PCR inhibitory substances. Analyses of the 5×-diluted extracts diminished but did not eliminate the trend of low percent recovery estimates by the ΔCt method suggesting that this commonly used dilution approach for eliminating PCR inhibition may have been only partially effective.

The difference between the ΔCt and ΔΔCt models in the proportions of acceptable target sequence recovery estimates was more pronounced in analyses of the undiluted extracts. This observation suggests that parallel inhibition of the SPC assay may have been partially responsible for the upward adjustments of recovery estimates into the acceptable range by the ΔΔCt model. It further suggests that the SPC assay may be slightly more sensitive to inhibitory effects than the *Enterococcus* assay. Previous analyses of Ohio River water samples indicated that an earlier version of the SPC assay (Sketa2) had this characteristic of greater sensitivity to PCR inhibition than the *Enterococcus* assay but did not show this characteristic in the current Sketa22 version of the assay (Haugland et al., 2012). As mentioned in that report, however, the principal role of the SPC assay in the ΔΔCt model is to adjust for specific interferences to the recovery of total amplifiable target sequences that were either demonstrated or suggested to be caused by some waters. This function of the SPC assay results in the ΔΔCt model provides an alternative or additional explanation for the higher percent target sequence recovery estimates observed using this model, compared to the ΔCt model, from a number of the STM samples in this study.

4.4. Analyses of undiluted versus diluted DNA extracts by Method 1609

Method 1609 recommends the use of undiluted calibrator and water sample DNA extracts if only one dilution can be analyzed. Additional analyses of 5-fold diluted calibrator and water sample extracts can be performed if practical and necessary to mitigate and/or further assess PCR inhibition. These recommendations are based upon several factors including the increased simplicity and added analytical sensitivity afforded by analysis of undiluted extracts as well as previous analysis results indicating that enterococci CSE density estimates obtained by this approach were statistically indistinguishable from those obtained from 5× diluted extracts in analyses of river water samples (Sivaganesan et al., 2014). Results from the present study indicate that undiluted extract analyses alone may not be sufficient for some sites to meet the current EPA guideline recommendations for site acceptability based on control assay results. Part of the higher overall control assay failure rate for undiluted versus diluted extracts was associated with samples that gave acceptable target sequence recoveries (5.5% for undiluted versus 1.8% for diluted). In addition, acceptable control assay results more often correctly predicted when net recovery estimates from the ΔΔCt model fell within the 50–200% acceptance range for the undiluted extracts (96%) than for the diluted extracts (94.5%, see also Fig. S2). It is also noteworthy that the majority of the undiluted sample extract analyses that passed the EPA control assay criteria or the *Enterococcus* assay Ct shift criterion and that failed to give acceptable recoveries gave recovery estimates from the ΔΔCt model that were above the upper 200% limit of this range (67% and 77%, respectively). In contrast, a large majority (91%) of 5×-diluted extract analysis results in this category (i.e. passing the EPA control assay criteria and outside the acceptable recovery range) gave recovery estimates that were below the 50% lower limit. Thus, while fewer samples analyses may be considered as acceptable based on the current criteria for control assay results, these results suggest that there is a greater likelihood that conclusions of sample acceptability drawn from the control assays will be supported by acceptable target sequence recovery estimates in analyses of undiluted sample extracts than in analyses of the diluted sample extracts by Method 1609 using the ΔΔCt model. They also suggest that analyses of undiluted sample extracts by Method 1609 using this model are more likely to provide overestimates of enterococci densities in water samples where some degree of interference is encountered. As such, this recommended analysis permutation in Method 1609 is expected to err on the side of being more protective of public health at sites with problematic water samples of this nature.

4.5. Comparison of Methods 1611 and 1609

Analysis of undiluted extracts is not recommended in Method 1611 due to the commonly high percentages of water sample analyses that do not meet the control assay acceptance criteria. This limitation of Method 1611 was corroborated by the sample analyses in this study. Overall and for the majority of the water body types and individual sites, however, updated Method 1611 using both control assays performed nearly as well as Method 1609 using the ΔΔCt model in analyses of 5×-diluted extracts, both in terms of the percentages of samples that met the control assay criteria and also in terms of the frequencies of samples that fell within the 50–200% acceptable net target sequence recovery range. Notable exceptions occurred in the analyses of samples from two Pacific Ocean sites (sites O and P — see Table 1) where a number of net target sequence recovery estimates were above 200% with Method 1611 results but not with Method 1609 using the ΔΔCt model. This discrepancy between the Methods was not reflected by ΔCt model estimates and, as illustrated in Fig. 2, could be largely attributed to results from just one of the three laboratories that analyzed these samples. The atypical target sequence recovery estimates obtained from the ΔΔCt model in this instance illustrate the need for additional control measures in the analyses of calibrator samples in both Methods 1609 and 1611 that are discussed in the following section.

4.6. Study limitations

Despite the recent revisions in Methods 1609 and 1611 (U.S. EPA, 2015a, 2015b) that place greater emphasis on quantifying target sequences as opposed to cell equivalents in samples, comparison of *Enterococcus* and SPC assay Ct measurements of DNA extracts from whole cell calibrator samples to those from test samples remains an integral part of the ΔΔCt calibration model used in the Methods (Haugland et al., 2014). Maintaining consistency in calibrator or SCM sample Ct measurements is the best means available for ensuring batch to batch consistency in the extraction and analyses of test samples. The current version of the EPA calculation spreadsheet contains guidance and automatic calculations for users of the updated Methods to determine the normal variability, expressed as standard deviation values, of calibrator sample Ct measurements in their

laboratory. The spreadsheet then automatically applies these standard deviation values in assessing whether Ct measurements from subsequent calibrator samples that are extracted and analyzed with each batch of test samples fall within an acceptable range of variability, currently defined as ± three times the laboratory's initial standard deviations. This new quality control feature of the Methods had not been introduced at the time of this study. Thus while a potentially sufficient number of *Enterococcus* assay analyses of preliminary SCM sample extracts were performed by each of the laboratories, as described in Section 2.6, to pre-determine the normal variability of this assay in their hands, only one initial run of Sketa22 SPC assay analyses were performed by each laboratory. This limited preliminary data from the SPC assay prevented the pre-determination of normal variability in this assay and of ΔCt (*Enterococcus* Ct − SPC Ct) values from the SCM samples and thus precluded the use of the new quality control measures in the ΔΔCt model. Several instances of potentially atypical SCM sample results were observed among the different batch analyses performed by different laboratories in this study. One of the more pronounced examples occurred in Method 1611 analyses of the Pacific Ocean STM samples by one of the laboratories, as indicated in Section 4.5. Fig. S3 shows the distribution of *Enterococcus* and SPC assay Ct measurements from the six batches of SCM samples that were analyzed by this laboratory. While the mean *Enterococcus* assay Ct values varied over a range of <0.25 units for each of these batches, the mean Ct value of the SPC assay for batch 2 was >2 Ct units lower than those of the other five batches. This batch of SCM samples was analyzed together with most of the west coast STM samples from sites O and P by that laboratory which accounts for the high percent target sequence recovery estimates that were determined for these samples.

Another source of potential error in the STM sample net percent target sequence recovery estimates arose from samples with high ambient densities of enterococci target sequences. Enterococci CCE estimates in a small number of unspiked ambient samples approached or even exceeded the estimated cell quantities added to the SCM and STM samples (Table S1). Net target sequence recovery estimates from the STM samples corresponding to these ambient samples were subject to a higher level of potential error due to the combined uncertainty of the total target sequence recovery estimates from both the STM and unspiked ambient samples. Several negative net percent target sequence recovery estimates obtained from the river and stream samples (set to zero as shown in Fig. 2) were each associated with samples having high estimated ambient target sequence densities. While all filter samples were stored at <−70 °C, differences of as much as >1 year between the laboratories in their sample holding times before analysis also could have affected their respective net recovery estimates.

Methods 1609 and 1611 both require the analysis of representative spiked water samples for the purpose of site characterization and recommend the use of the same cell suspensions for spiking test samples and calibrator or SCM samples. This protocol is similar to the protocol performed in this study, with the only difference being that the spikes normally would be added directly to the calibrator or SCM and water samples prior to, rather than after, filtration. The recommended spiking quantity of ~10^4 cells in the updated Methods is similar to the quantities used in this study and represents a compromise between approximating the EPA RWQC STV guideline levels of either 1280 or 2000 CCE/100mL (U.S. EPA, 2012a) and trying to ensure that the spiked cell quantities are sufficiently greater than ambient quantities to minimize excessive errors in net percent recovery estimates. To maintain simplicity in the calculations, the amplification factors of the *Enterococcus* and SPC assays are assumed to be the same for analyses of all test samples and calibrator or SCM samples in the ΔΔCt model. If in fact the results of the SPC assay are compensating for minor PCR inhibitory effects by certain water samples, the extent of compensation should decrease as the quantities of enterococci target sequences from these test samples decrease in relation to the calibrator or SCM samples. As further discussed by Chern et al. (2009), this is one of the reasons why it is desirable to determine target sequence recovery estimates from test sample spikes that approximate the RWQC guideline levels as closely as possible.

4.7. Methods limitations

Results from this study revealed an imperfect relationship between conclusions of sample analysis acceptability drawn from the control assay results and criteria currently recommended in the Methods and net enterococci target sequence recovery estimates from the ΔΔCt model that were within the benchmark 50–200% acceptance range. A similar frequency of discrepancies (5%) was noted using the alternative control measures and criteria recommended by Cao et al. (2012). Some of these discrepancies may have originated from variability associated with the SCM sample analyses or from other sources of error and variability in the recovery estimates in this study as discussed in Section 4.6. Variability in the control measurements could also contribute to such discrepancies. Another question that can be asked, therefore, is whether the pre-established, one value fits all acceptance criteria for controls that are currently used with these and other closely related methods (Cao et al., 2012; Kinzelman et al., 2011; Noble et al., 2010) are always the most appropriate for detecting the presence, or absence, of interference by the samples. Efforts are currently in progress to develop a more statistically supported approach for identifying unacceptable sample analysis results in other EPA qPCR methods (O. Shanks and M. Sivaganesan, personal communication). A potential limitation of this type of an approach is its requirement for more replicate analyses of both control and test samples in each batch of analyses. At least for routine, same-day beach notification programs, this requirement for more analyses per sample could challenge the sample analysis throughput capabilities of some laboratories that would be responsible for analyses of large numbers of samples on a daily basis. As illustrated in Figs. 2 and S2, the analyses of undiluted DNA extracts by Method 1609 using the specified control assays and ΔΔCt model were found in this multi-laboratory study of 22 diverse and potentially problematic sampling sites to provide a very low number (<2%) of net target sequence recovery estimates below 50%. This observation also applied to the alternative *Enterococcus* assay Ct shift approach of Cao and coworkers. These results suggest that either of these controls may be effective in minimizing the acceptance of sample analysis results that would falsely indicate that enterococci densities are below the EPA RWQC guidelines for beach notifications. In this context, when combined with the recommended analysis of undiluted DNA extracts, the control assays and relatively simple acceptance criteria presently indicated in Method 1609 appear to be adequate for implementing health protective beach notification programs when the control assay acceptance criteria can be met. Particularly at sites where preliminary results from the control assays may indicate that EPA site acceptability guideline recommendations are not being met and/or when analyses of diluted extracts are needed to meet these guidelines, analyses of spike recoveries from representative water samples, as indicated in both Methods 1609 and 1611, are warranted to help substantiate and interpret the control assay assessments of the Methods' performances.

4.8. Conclusions

Results from this study indicate that the analytical permutations and control assays currently recommended in EPA Methods 1609 and 1611 (U.S. EPA, 2015a, 2015b) can provide reliable estimates of enterococci densities by different laboratories in a variety of different, and potentially challenging, U.S. water bodies. Additional findings, such as results from the *Enterococcus* assay Ct shift control approach described by Cao et al. (2012), indicated that these methods did not eliminate matrix interferences from all samples. However, inferences drawn from matrix spike recoveries indicated that these methods, and particularly Method

1609 in analyses of undiluted extracts, adequately adjusted enterococci density estimates (adequate estimates defined here as ≥ 50% of expected true density) for any such interferences in up to >98% of all sample analyses passing control assay acceptance criteria by the use of the SPC control assay results in the ΔΔCt calculation method. The use of matrix spike recovery analyses is described in the EPA Methods and, together with the SPC and IAC control assays, should be considered as an important component in assessing the performance of these methods.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.01.017.

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Glossary of acronyms and terms

AF: amplification factor (see Eq. (1)).

calibrator samples: Spiked control matrix samples with associated reference numbers (cells in EPA method 1611 and recoverable target gene sequence copies in EPA Method 1609 and updated Methods1609.1 and 1611.1). Used in the EPA methods to estimate absolute quantities of CSE and/or CCE in test samples. (See Eq. (1)).

CCE: calibrator cell equivalents (from RWQC and EPA Method 1611. Redefined based on a standardized ratio of 15 CSE/CCE in EPA Methods 1609, 1609.1 and 1611.1).

CFU: colony forming unit.

CSE: calibrator target gene sequence equivalents (from EPA Methods 1609, 1609.1 and 1611.1, see Eq. (1)).

Ct: cycle threshold (also commonly referred to as Cq or quantification cycle). Instrument-measured (fractional) thermal cycle number at which the fluorescence generated within a real time polymerase chain reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence (defined for the instrument in the Method). At the threshold cycle, a detectable amount of amplicon product has been generated during the early exponential phase of the reaction. The threshold cycle is inversely proportional to the original $\log_{10}$ quantity of target sequences in the reaction. Definition paraphrased from: , http://www.experts123.com/q/what-is-the-threshold-cycle-or-ct-value.html.

ΔCt: delta Ct comparative Ct model for relative target gene sequence quantification (from Pfaffl (2001), see Eq. (2)).

ΔΔCt: delta Ct comparative Ct model for relative target gene sequence quantification (from Applied Biosystems (1997), see Eqs. (3) and (4)).

EMM: Environmental Master Mix.

EPA: U.S. Environmental Protection Agency.

IAC: internal amplification control.

MPN: most probable number.

qPCR: quantitative polymerase chain reaction.
R: reference number. Average absolute quantity of *E. faecalis* cells contained in (EPA Method 1611) or target gene sequence copies recovered from (EPA Methods 1609, 1609.1 and 1611.1) a series of calibrator samples (see Eq. (5)).
ROQ: range of quantification.
RWQC: EPA 2012 Recreational Water Quality Criteria.
Sketa22 assay: qPCR primers and TaqMan probe for detection of the salmon DNA used in the SPC.
SCM: spiked control matrix (samples). Similar to calibrator samples but not necessarily requiring an associated reference number. Used for relative target gene sequence quantification of equivalently prepared STM samples. (See Eqs. (2) and (3)).
SPC: sample processing control (Sketa22).
STM: spiked test matrix (samples).
STV: statistical threshold values (from RWQC).
UMM: Universal Master Mix.