Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring *Enterococcus* in environmental waters

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Abstract

Draft criteria for the optional use of qPCR for recreational water quality monitoring have been published in the US. One concern is that inhibition of the qPCR assay can lead to false negative results and potentially inadequate public health protection. In this study we evaluated the effectiveness of strategies for minimizing the impact of inhibition. Five qPCR method permutations for measuring *Enterococcus* were challenged with 133 potentially inhibitory fresh and marine water samples. Serial dilutions were conducted to assess *Enterococcus* target assay inhibition, to which inhibition identified using four internal controls (IC) was compared. The frequency and magnitude of inhibition varied considerably among qPCR methods, with the permutation using a proprietary environmental master mix performing substantially better. Five-fold dilution was also effective at reducing inhibition in most samples (>78%). ICs were variable and often ineffective, with 54 to 85% agreement between ICs and serial dilution. The current IC methods appear not to accurately predict *Enterococcus* inhibition and should be used with caution; 5-fold dilution and the use of reagents designed for environmental sample analysis (more robust qPCR chemistry) may be preferable. Suitable approaches for defining, detecting, and reducing inhibition will improve implementation of qPCR for water monitoring.

Introduction

Quantitative polymerase chain reaction (qPCR) offers the possibility of providing results of beach water testing within a few hours, compared to the 18 to 96 hours required for culture-based methods (Haugland *et al*. 2005, Noble *et al*. 2010). This speed provides the opportunity for beach managers to issue swim advisories on the same day that water samples are collected, potentially reducing swimmer exposure to poor-quality water (Wade *et al*. 2008, Colford *et al.* 2011). As a result, these methods are already being used by health departments in demonstration projects (Griffith and Weisberg 2011) and EPA has recently published draft Recreational Water Quality Criteria incorporating this technology (USEPA 2011a).

A significant hurdle to be overcome before qPCR methods are widely implemented for water quality testing is the inhibition of the qPCR assay by interfering substances in water samples (Dorevitch *et al.*

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2010; Griffith and Weisberg 2011; USEPA 2011a,b). The recently published draft Recreational Water Quality Criteria recommends that "States evaluate qPCR performance with respect to sample interference prior to developing new or revised standards relying on this method for the purposes of beach monitoring." However, the EPA has not provided guidance about how to evaluate qPCR performance with respect to inhibition.

Inhibition occurs when substances present in a water sample interfere with PCR amplification, leading to target underestimation, which can, in turn, lead to false negative results when applied to a beach closure scenario (i.e., keeping a beach open that should be closed). Inhibition can be caused by a range of physical, biological, and chemical mechanisms. While inhibition is an issue for all qPCR methods in many application fields, there are a number of concerns particular to recreational water quality testing. First, ambient water is compositionally complex, containing many types of potential inhibitory substances, i.e., inhibitors. Second, DNA purification, which commonly uses commercial DNA extraction kits to remove inhibitors, is not a simple option in this application because the added processing time leads to health warnings being posted too late in the day to adequately protect swimmers (Griffith and Weisberg 2011) and the extra laboratory steps associated with commercial extraction kits can add to measurement error (Noble *et al*. 2010). Unpurified sample DNA is therefore used for the monitoring of bacteria concentrations in beach water (USEPA 2010).

Other options for minimizing the effect of inhibition include dilution of the inhibitory sample (Haugland *et al*. 2005) and use of internal controls (Haugland *et al*. 2005, Shanks *et al*. 2008, Noble *et al*. 2010). However each of these options has potential shortcomings. For instance, dilution alleviates inhibition by reducing concentrations of inhibitors, but also lowers the target concentration potentially below detection limit. Similarly, internal controls (IC) are utilized on the premise that the IC and target assays respond to inhibitors the same way, which may not be assumed for a given pair of reactions (Huggett *et al*. 2008). Alternatively, qPCR condition such as composition of reaction mixture may be varied to increase the robustness of an assay to inhibition.

The objective of this study was to examine the effectiveness of dilution, use of IC and varying qPCR conditions (composition of reaction mixtures including alternative primer/probes) in minimizing the impact of inhibition on applying qPCR methods for recreational water quality monitoring. We investigated the relative susceptibility to inhibition of five qPCR method permutations for enumerating *Enterococcus* for surface water quality applications, and we evaluated the effectiveness of dilution and IC as a means of resolving or identifying inhibition when it does occur.

Methods

Approach

Inhibition was assessed for 133 samples from diverse waters in two ways. The first was based on examining linearity of response in *Enterococcus* cycle threshold (Ct) values across four 5-fold serial dilutions of the unpurified sample DNA. A 5-fold dilution is expected to delay the detection of the target by 2.32 cycles, as $2^{2.32} = 5$ assuming 100% amplification efficiency (i.e., perfect doubling of target concentration with each qPCR cycle). The sample was deemed inhibited if the *Enterococcus* Ct difference between this dilution and the next serial 5-fold dilution was one cycle less than expected without inhibition (i.e., 1 cycle less than 2.32 = $\log_2 5$). More details on this assessment of inhibition are available in Supporting Information. The second was inhibition detected by the IC assays. For each IC assay in each qPCR method, a sample was deemed inhibited if the IC Ct in the sample increased by a threshold (3.0, 1.7, or 1.0 cycles) from an uninhibited reference analyzed on the same plate. The thresholds 3.0 and 1.7 were selected based on literature (as used in USEPA draft Recreational Water Quality Criteria (USEPA 2011a) and/or previous studies (Haugland *et al*. 2005, Griffith and Weisberg 2011)). The threshold 1.0 was selected because one may not expect Ct difference between an uninhibited sample and a reference to be more than 1 cycle if assuming 0.5 cycle variability between qPCR replicates (1.0 cycle = 2 x 0.5 cycle). For samples in which an IC was not amplified, the maximum number of cycles was assigned as the Ct value. All qPCR reactions (for samples, standard curves, uninhibited references, and no-template controls) were run in triplicates. Data from the whole plate was discarded and analysis redone if any no-template control was amplified. Figure 1 shows the overall experimental design and sample processing procedure.

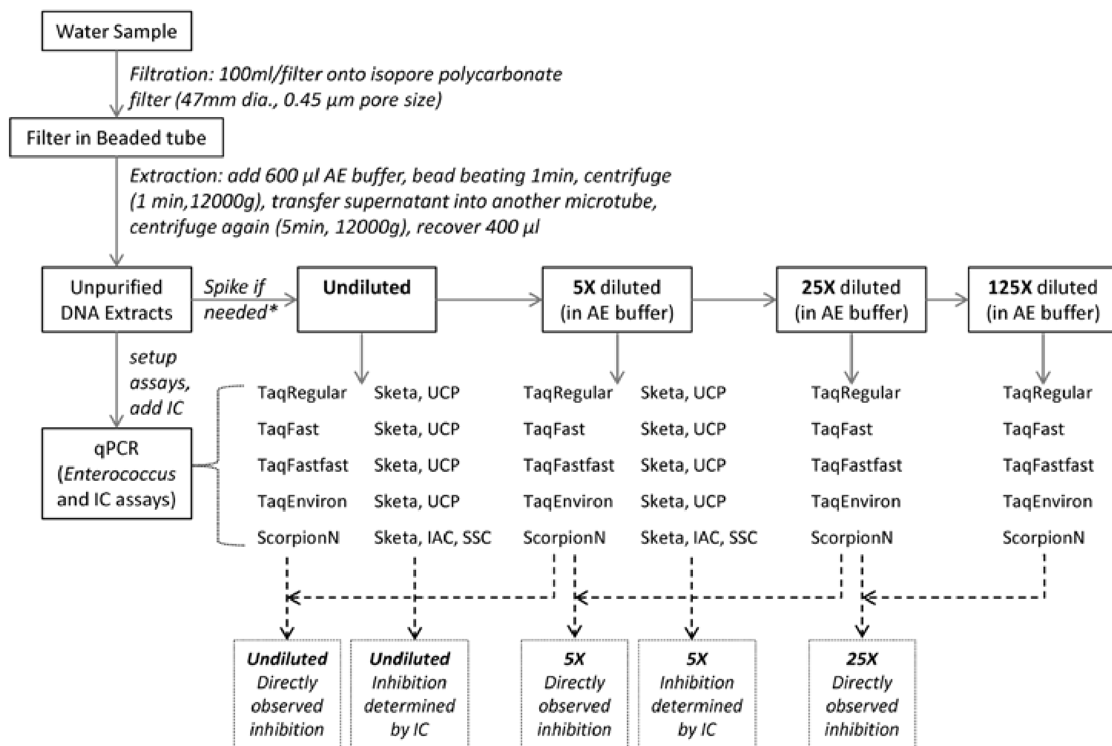

Figure 1. Diagram depicting sample processing and experimental design. Abbreviations for qPCR (*Enterococcus* and IC assays) are as specified in Materials and Methods. * The samples with insufficient *Enterococcus* concentrations (<1500 cells per 100 ml) to allow precise Ct results at higher dilutions (25x or 125x) were spiked with genomic DNA equivalent to 1500 *Enterococcus* cells prior to inhibition analysis.

Samples

Sixty-one of the samples were collected from 24 marine beach sites in Southern California between 2008 and 2010. Fifty-nine samples were collected from 15 freshwater sites in the Greater Chicago area from April through July, 2009. Another 13 samples (storm drain water, sewage, elutriate from washing algae and sediment, or combined sewage overflow; 1 from Chicago area and 12 from California) were also included as potential sources for beach contamination and/or inhibitory substances. A flow chart for sample processing is presented in Figure 1. Briefly, all samples were filtered within 6 hours of collection and stored at -80°C until extraction. Unpurified DNA extracts were obtained via bead beating and centrifugation from the filters as described in EPA Method A (USEPA 2010), with the exception of the step at which a particular internal control was added (described below, Figure 1). Extracts were stored at 4°C and analyzed within 8 hours. About 31% of the samples had insufficient *Enterococcus* concentrations (<1500 cells per 100 ml, screened by culture or qPCR methods prior to inhibition analysis) to allow precise Ct measurements at higher dilutions (25x and 125x). In an effort not to exclude these samples, the unpurified DNA extracts (400 µl) from samples with insufficient *Enterococcus* concentrations were spiked with genomic DNA equivalent to 1500 *Enterococcus* cells prior to inhibition analysis (5 µl extract used in each 25 µl qPCR reaction). Mixed genomic DNA from three common *Enterococcus* species (E. faecalis ATCC 29212, E. faecium ATCC 35667, and E. casseliflavus ATCC 700327) was used for spiking.

qPCR Methods

Five *Enterococcus* qPCR methods (Table 1) were included in this study. The TaqRegular (i.e., EPA Method A (Haugland *et al*. 2005, USEPA 2010) and ScorpionN (Noble *et al*. 2010) methods were as described previously. Three variations of the regular TaqMan® method (TaqFast, TaqFastfast, and TaqEnviron with either faster cycling time or potentially more robust reaction mixture) were also included to evaluate how differing master mixes and thermal cycling times affected inhibition. Briefly, the qPCR reaction mixture (25 µl including 5µl DNA template) for the four TaqMan® methods contained: 1X corresponding master mix (Applied Biosystems), 0.2 mg/ml bovine serum albumin (Sigma), 1 µM

Table 1. Description of the five qPCR methods.

qPCR Method	Master Mix*	Ramping Speed**	Average Running Time	qPCR Platform***
TaqRegular	TaqMan® Universal PCR Master Mix	Regular	1 hr 41 min	Applied Biosystems 7500 fast
TaqFast	TaqMan® Universal PCR Master Mix	Fast	1 hr 26 min	
TaqFastfast	TaqMan® Fast Universal PCR Master Mix	Fast	52 min	
TaqEnviron	TaqMan® Environmental Master Mix 2.0	Fast	1hr 33 min	BioRad CFX96
ScorpionN	OmniMix™ HS	Fast	1 hr 13 min	

* According to the manufacturer, the TaqMan® Environmental Master Mix 2.0 is specially formulated for qPCR in the presence of high levels of inhibitors.

** Ramping speed is determined by the qPCR platforms, and refers to how fast the temperature of the thermal block is raised and lowered.

*** The threshold crossing method was used to determine Ct on both platforms. Thresholds of 0.03 ΔRn and 100RFU were empirically determined and used throughout the study for AB7500fast and BioRad CFX96, respectively.

of each primer and 0.08 µM of TaqMan® probe (Applied Biosystems); for the ScorpionN method, 1X OmniMix™ HS (Cepheid), 0.25 µM primer and 0.25 µM Scorpion probe (Biosearch). Thermal protocols (Supplemental Information (SI) Table SI-1; Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_20SI.pdf) and primer/probe sequences (Table SI-2) for each qPCR method are provided in Supporting Information.

Standard curves (eight points with 5-fold serial dilution from 1.57 x 10^6 cells per filter to 20 cells per filter in triplicate at each point) of reference material were performed to confirm performance of each qPCR method before analyses of samples. Reference materials were also analyzed in triplicate on each sample plate for quality control. The reference material was purified genomic DNA from frozen filters with *Enterococcus* faecalis (ATCC29212, 1.57 x 10^6 cells per filter) as described previously (Griffith and Weisberg 2011). For environmental water samples, unpurified DNA extracts were analyzed by each of the *Enterococcus* qPCR method at four 5-fold serial dilutions (undiluted, 5x, 25x, 125x; Figure 1).

Internal Controls

Four ICs were tested in this study: Salmon testes DNA (USEPA 2010), a plasmid DNA composite construct (Shanks *et al*. 2008), and two commercially available ICs: BioGx IAC (Cat # 700-003, BioGX Inc., Birmingham, AL) and Super Smart Control SSC (lyophilized beads with premixed primers/probe, Cat # QC-DNA-SSC, Cepheid, Sunnyvale, CA). For simplicity, these ICs were referred to, in the order above, by abbreviations of their qPCR probes or of their commercial product names, as Sketa (for salmon testes DNA), UCP (for the plasmid DNA composite construct), IAC, and SSC, respectively. Based on common usage in practice, Sketa, IAC, and SSC were evaluated in the ScorpionN method; Sketa and UCP were tested in all four TaqMan® qPCR methods (Figure 1). Note that, under the current protocol (USEPA 2010), salmon testes DNA is added prior to DNA extraction and the Sketa result is used as a combined sample processing and inhibition control to control for both DNA recovery and presence of inhibition (USEPA 2010). In this study, salmon testes DNA were added to the final reaction mixture (described below), rather than before DNA extraction, such that its efficacy as an inhibition control alone could be assessed. Additionally, although SSC is a TaqMan®-based chemistry, it was included for evaluation in ScorpionN, which uses a master mix in lyophilized bead form (produced by Cepheid; the same company that produces SSC), in anticipation of complete dry chemistry for the duplex qPCR method in the future. All IC assays were duplex with the *Enterococcus* target assay, except for Sketa, which were simplex assays for all five qPCR methods.

Reaction conditions were as described previously (Shanks *et al*. 2008, USEPA 2010) or per manufacturer's instructions. Briefly, for the simplex Sketa assay in all four TaqMan® methods, reaction mixtures (25 µl) contained 1X corresponding master mix (Applied Biosystems), 0.2 mg/ml bovine serum albumin (Sigma), 1 µM of each primer, 0.08 µM of TaqMan® probe (Applied Biosystems), and 0.04 ng/µl salmon testes DNA (Sigma). For the duplex UCP assay in all four TaqMan® methods, additional reagents included 0.08 µM of probe for the IC and

50 copies of the IC. For the simplex Sketa assay in the ScorpionN method, reaction mixtures (25 µl) contained 1X OmniMix™ HS (Cepheid), 0.25 µM primer and 0.25 µM Scorpion probe (Biosearch), and 0.04 ng/µl salmon testes DNA (Sigma). For the duplex IAC assay, additional reagents included 0.08 µM Scoprion primer, 0.8 µM Scorpion probe, and 100 copies of the IAC as specified by the manufacturer. For the duplex SSC assay, lyophilized beads containing premixed (10X) primer, probe and IC were used in reaction setup in order to achieve a 1X concentration in the final duplex reaction mixture.

Standard curves using reference materials (as described above) were also performed to confirm performance of ICs and the concentration ranges where quantification of *Enterococcus* and IC does not interfere with each other. For environmental water samples, unpurified DNA extracts were analyzed by the IC assays undiluted and at 5x dilution.

Data Analysis

We compared 1) directly observed inhibition (by serial dilution) among five *Enterococcus* qPCR methods to assess their robustness against inhibition; 2) directly observed inhibition against inhibition detected by IC within each qPCR method to assess reliability of ICs; and 3) directly observed inhibition between undiluted and diluted sample DNA, as well as inhibition detected by ICs between undiluted and diluted sample DNA, within each qPCR method to assess how well dilution resolved these two assessments of inhibition. The EPA method A was used as the benchmark and the other four qPCR methods were referred as the variant qPCR methods. An IC was considered to have missed inhibition (i.e., false negative) if it failed to identify inhibition that had been identified using the serial dilution approach. An IC was considered to have given a false alarm (i.e., false positive) if it indicated inhibition while the serial dilution approach did not.

ANCOVA was used to compare standard curves generated by different qPCR methods and between simplex and duplex *Enterococcus* assays within a qPCR method. To define the range where competition from an *Enterococcus* assay did not interfere with quantification of ICs, ANOVA with Tukey's HSD test was used to compare Ct values from a fixed amount of IC in the presence of different concentrations of *Enterococcus*. Because a balanced data set (i.e., the same number of uninhibited and inhibited samples for the *Enterococcus* target assay) is required (Kubat *et al*. 1998) for adequately assessing the reliability of IC to reflect inhibition in the *Enterococcus* target assay, random sampling without replacement of the observed results (from all analyzed water samples) was performed 100 times, and the results were averaged. All statistical analyses were performed in R v2.11.1 (R Core Development Team 2008).

Results

All *Enterococcus* and IC assays had amplification efficiency >1.90, except for UCP in some qPCR methods. For the simplex *Enterococcus* assay, all qPCR methods had standard curves with comparable slopes ($p > 0.05$) but different intercepts ($p < 0.001$). Raw Ct values from ScorpionN and TaqEnviron were approximately 2 and 1.5 cycles higher, respectively, than raw Ct values from the other three qPCR methods. Amplification efficiency of ICs was also checked using serial dilutions of the ICs in simplex assays. All ICs had high amplification efficiency (>1.95) except for UCP, the efficiency of which varied with qPCR method: TaqRegular (1.81 ±0.03), TaqFast (1.80 ±0.04), TaqFastfast (1.65 ±0.005), TaqEnviron (1.99 ±0.01). Note that in this paper amplification efficiency is reported as amplification factor (1 - 2) instead of percentage efficiency (0 - 100%). All standard curve equations (and R^2) are provided in Table SI-3).

In addition to amplification efficiency, the potential for substrate competition (Hoorfar *et al*. 2004) between *Enterococcus* and IC in duplex reactions was determined using non-inhibitory reference materials. Duplexing did not affect amplification efficiency of the *Enterococcus* assays ($p > 0.05$). Duplexing also did not affect quantification of low *Enterococcus* concentrations with any of the ICs, except that duplexing with SSC in the ScorpionN method led to *Enterococcus* becoming undetectable at the lowest dilution of 20 cells per filter. The *Enterococcus* concentration ranges where IC quantification was unaffected varied with IC and sometimes with qPCR method. For most ICs, the ranges were similar among qPCR methods, ranking from the full range tested (up to 1.57×10^6 cells per filter) to the narrowest ($<1.26 \times 10^4$ cells per filter) as follows: SSC>UCP (excluding TaqEnviron) >IAC. Interestingly, UCP quantification was unaffected throughout the full range of *Enterococcus* concentrations tested when using the TaqEnviron qPCR

method. More details on the ICs' application ranges are provided in Figure SI-1.

Susceptibility to inhibition differed substantially among the *Enterococcus* qPCR methods. TaqEnviron was the most robust, with only four samples inhibited (Table 2). TaqEnviron, TaqFast, ScorpionN, and TaqFastfast were 0.2, 1.3, 2.0, and 3.0 times as likely to be inhibited, respectively, compared to TaqRegular. Susceptibility to inhibition was similar between freshwater and marine samples for all five qPCR methods.

None of the ICs accurately reflected the presence or absence of *Enterococcus* target assay inhibition for more than 85% of the samples, and this overall accuracy varied with the different qPCR methods and IC thresholds for the same IC (Table 3). While ICs overall averaged 70% agreement with inhibition detected by serial dilution, the errors by ICs were generally unbalanced with respect to missed inhibition (i.e., false negative) and false alarm (i.e., false positive). Sketa and SSC appeared insensitive with high specificity: they largely had very few false alarms, but failed to identify about half to two-third of the inhibited samples, except for Sketa in ScorpionN. In contrast, UCP and IAC appeared sensitive with low specificity: they usually gave false alarms more frequently than missed inhibition, except for with IC threshold of 3.0.

In absence of inhibition (i.e., with uninhibited reference materials), most ICs produced Ct values of 30 to 34 with low variability (average within-plate standard deviation ranged from 0.27 to 0.5 cycles). However, Sketa assays produced Ct of approximately 21 (in the four TaqMan® qPCR methods) or 27 (in the ScorpionN method) with minimal variability (average within-plate standard deviation ranged from 0.06 to 0.09 cycles). This perhaps reflected the relative high concentration (hence smaller Ct with low variability) of salmon testes DNA used for the Sketa assay as this assay was typically used as a combined sample processing and inhibition control (USEPA 2010). Note that Sketa in the four TaqMan® methods vs. that in the ScorpionN method were two different assays (based on TaqMan® vs. Scorpion probe chemistry, respectively) of the same internal control (i.e., salmon testes DNA), which may explain the Sketa Ct difference above (21 vs. 27). Additionally, little difference in Ct variability was observed for a given IC across the five (Sketa) or four (UCP) qPCR methods. Nevertheless, UCP appeared to have more stable Ct values in TaqEnviron than in others (Table SI-4).

Table 2. Percentage of *Enterococcus* target assay inhibition in undiluted sample DNA.

Variant qPCR Method*	Sample Size	% Inhibited in TaqRegular Method	% Inhibited by Variant qPCR Method
TaqFast	92	33%	41%
TaqFastfast	110	27%	82%
TaqEnviron	73	29%	5%
ScorpionN	100	28%	57%

* TaqRegular was used as the benchmark.

Five-fold dilution of the unpurified and undiluted DNA extracts resolved inhibition for 78 to 100% of the samples, depending on the qPCR method (Table 4). Effectiveness of further dilutions was not evaluated, as the 1:5 dilution was already effective. Additionally, assessment of dilution effectiveness for TaqEnviron was limited because only four samples were inhibited when undiluted. However, inhibition detected by ICs responded to the 5-fold dilution differently than did the *Enterococcus* target assay inhibition. For example, the 5-fold dilution resolved 95% of the true inhibition for TaqRegular (Table 4), but only relieved <84% of the inhibition detected by UCP (Table 5). In contrast, for TaqFastfast, the 5-fold dilution was more effective on resolving inhibition detected by Sketa (>87%) than resolving inhibition of the *Enterococcus* assay (79%).

Discussion

This is the first study to compare IC and directly observed inhibition across qPCR permutations. Directly observed inhibition was defined by qPCR detection occurring earlier than expected following sample dilution; qPCR permutations included assay reagents and cycling time. We found considerable difference in susceptibility to inhibition among the five qPCR method permutations we tested. Reagent mixtures were important. The TaqEnviron method was the least susceptible to inhibition, with the improved performance likely resulting from the polymerase used (in the TaqMan® Environmental Master Mix 2.0, Table 1), as polymerases have been found to be differentially susceptible to various inhibitors (Abu Al-Soud and Radstrom 1998, Eilert and Foran 2009). The bovine serum albumin added in the four TaqMan® methods has also been considered a common PCR facilitator in presence of

Table 3. Reliability of IC for detecting inhibition on the *Enterococcus* target assays.

IC	IC Threshold (cycles)	qPCR Method*	n†	False Negative‡	False Positive§	Overall Accuracy**
Sketa	3	TaqRegular	78	74%	0%	63%
		TaqFast	82	73%	0%	63%
		TaqFastfast	42	72%	5%	62%
		ScorpionN	104	26%	4%	85%
	1.7	TaqRegular	78	64%	0%	68%
		TaqFast	82	66%	0%	67%
		TaqFastfast	42	65%	5%	65%
		ScorpionN	104	11%	40%	74%
	1	TaqRegular	78	59%	0%	71%
		TaqFast	82	51%	0%	74%
		TaqFastfast	42	52%	5%	71%
		ScorpionN	104	4%	67%	64%
UCP	3	TaqRegular	78	44%	15%	71%
		TaqFast	80	20%	40%	70%
		TaqFastfast	42	8%	38%	77%
	1.7	TaqRegular	78	26%	29%	72%
		TaqFast	80	10%	55%	67%
		TaqFastfast	42	2%	67%	66%
	1	TaqRegular	78	23%	39%	69%
		TaqFast	80	10%	72%	59%
		TaqFastfast	42	1%	90%	54%
IAC	3	ScorpionN	100	28%	24%	74%
	1.7	ScorpionN	100	22%	44%	67%
	1	ScorpionN	100	8%	60%	66%
SSC	3	ScorpionN	108	61%	6%	67%
	1.7	ScorpionN	108	54%	7%	69%
	1	ScorpionN	108	45%	9%	73%

* TaqEnviron was not included due to small number of undiluted samples (n=4) exhibiting Enterococcus target assay inhibition.

† As described in the Materials and Methods, because a balanced data set is required for adequately assessing reliability of IC in reflecting Enterococcus target assay inhibition, random sampling without replacement (n out of results from all samples) was performed to ensure n/2 samples were inhibited and n/2 samples were uninhibited for the Enterococcus qPCR assays.

‡ False negative: percentages of samples (out of n/2) that were inhibited for Enterococcus qPCR but the inhibition was not detected by IC.

§ False positive: percentages of samples (out of n/2) that were not inhibited for Enterococcus qPCR but were flagged as inhibited by IC.

** Overall accuracy: percentage of samples (out of n) for which IC reliably reflected either presence or absence of inhibition on the Enterococcus assay, i.e., % true positives + % true negatives.

inhibitors, presumably due to its stabilizing effect on polymerase and/or the DNA template (Kreader 1996, Wilson 1997). The TaqMan® and ScorpionN methods also differed in their primers and probes, and primer-probe properties were suggested to affect inhibition through altered annealing and extension efficiency in presence of inhibitors (Chung 2004).

In terms of cycling time, there appeared to be a tradeoff between speed and sensitivity to inhibitory compounds; this tradeoff may need to be seriously considered to make implementation practical. Methods using a shorter running time (TaqFast, TaqFastfast, ScorpionN; Table 1) had higher susceptibility to inhibition, possibly because more time allows the reaction to achieve full replication at each cycle even in the presence of inhibitors. Longer extension time has been found to improve PCR efficiency (Wei *et al*. 2008). While timing is crucial for rapid recreational water monitoring, slightly slower but more robust qPCR methods might still allow same day water quality warnings (Griffith and Weisberg 2011) with lower probability of qPCR results being affected by inhibition. Additionally, inhibition can be chronic (i.e., inhibition consistently occurs over time at certain sites), so it maybe that

Table 4. Effectiveness of a 5-fold dilution on resolving inhibition on *Enterococcus* qPCR in undiluted sample DNA.

qPCR method	# Sample Inhibited Undiluted	% Inhibition Resolved by 1:5 Dilution
TaqRegular	38	95%
TaqFast	41	95%
TaqFastfast	89	79%
TaqEnviron	4	100%
ScorpionN	68	78%

the tradeoff between time and relieving inhibition need only be made at a subset of sites. Further work is being conducted to assess robustness (against inhibition) of an even faster version (possibly <1 hour) of the TaqEnviron method (currently 1 hour 33 minutes).

A frequent approach to mitigating inhibition is to adjust the results based on the performance of an assay for exogenous DNA that serves as a control. The foundation of this approach is the assumption that the exogenous DNA assay is inhibited the same way as the target assay (Huggett *et al*. 2008). Our results suggest that this is an imperfect approach with the ICs we tested, as they all detected

Table 5. Effectiveness of a 5-fold dilution on resolving inhibition detected by IC in undiluted sample DNA.

qPCR Method	IC	IC Threshold (cycles)	# Sample Inhibited Undiluted	% Inhibition Resolved by 1:5 Dilution
TaqRegular	Sketa	3	10	90%
		1.7	14	93%
		1	16	94%
	UCP	3	35	83%
		1.7	56	84%
		1	66	79%
TaqFast	Sketa	3	12	92%
		1.7	15	93%
		1	21	95%
	UCP	3	57	86%
		1.7	70	84%
		1	82	83%
TaqFastfast	Sketa	3	31	90%
		1.7	38	89%
		1	52	87%
	UCP	3	107	67%
		1.7	120	58%
		1	126	46%
TaqEnviron	Sketa	3	0	n/a
		1.7	1	0%
		1	3	33%
	UCP	3	1	100%
		1.7	1	100%
		1	1	0%
ScorpionN*	Sketa	3	56	89%
		1.7	88	69%
		1	108	58%
	IAC	3	61	79%
		1.7	75	68%
		1	92	61%

* SSC in ScorpionN was not included due to limited data at the 1:5 dilution.

inhibition inconsistently with that identified through dilution on the *Enterococcus* target assays (Table 3). Additionally, disagreement between inhibition detected by ICs and inhibition on *Enterococcus* qPCR was not resolved by simple linear offsets of IC threshold (from 3.0 to 1.0 cycles), nor did the two assessments of inhibition respond to 5-fold dilution to the same extents (Tables 4 and 5), further indicating inhibition on ICs and *Enterococcus* were mechanistically different in the ambient samples. While the ICs may reflect target assay inhibition if inhibitors are affecting the polymerase alone, exogenous DNA would likely respond to inhibitors differently than target DNA if the inhibition effect is through inhibitor interacting with DNA template or DNA-polymerase complex (Opel *et al*. 2010). In the latter case, a competitive IC designed to share more sequence similarity with the target DNA would be more reliable than ICs that share little sequence similarity with the target DNA (Hoorfar *et al*. 2004). This may explain why UCP, which is a competitive IC, performed slightly better than the other IC in the TaqMan® methods, though only <80% of target assay inhibition was reliably reflected even by UCP. Thus, use of any of the four ICs we tested to numerically correct for inhibition for the *Enterococcus* assay may not be appropriate. Further studies with more samples from wider geographic regions will shed additional light on this subject.

Nevertheless, the decision to use any of the four ICs we tested for detecting inhibition for the *Enterococcus* assay may be one for end users, who must decide if the frequencies of missing and/or falsely identifying inhibition by ICs are acceptable for their research or monitoring applications. Simulation assessing the impact of inhibition can assist such decision making (An example of such a simulation is provided as Appendix A in the Supplemental Information). Consideration of application ranges (Hoorfar *et al*. 2004) and preference of duplex versus simplex assays (Shanks *et al*. 2008) is also needed in choosing ICs for detecting inhibition. Although it is important to note that different concentrations of the same IC can affect performance of the IC, the IC assays in this study were used as optimized by the corresponding assay developer or manufacturer. Regardless, it is important that an IC is specifically evaluated for its reliability in mimicking inhibition of the target assays.

One alternative to ICs would be to assess inhibition on the target assay itself using a spiking-followed-by-dilution approach. If a DNA sample is inhibited, then the difference between Ct values of this DNA sample and its further dilution will be smaller than expected for samples not inhibited. However, to ensure that the diluted DNA (if not inhibited) has enough targets to produce a reliable Ct (low target concentration lead to high variability in Ct, see below), a fixed amount of reference material (i.e., that used as standard for quantification in the given qPCR method) is spiked into the DNA sample prior to dilution. A full protocol for conducting such an approach is provided as Appendix B in Supporting Information. Because spiking and dilution add analysis time and supply cost, this approach may not be appropriate for routine ambient water monitoring. Yet, it may be useful for applications in microbial source tracking (Silkie and Nelson 2009) and clinical diagnosis (Paiva-Cavalcanti *et al*. 2010) where timing and cost may be of lesser concern and it is not feasible to design and conduct comprehensive evaluation of ICs for every given qPCR assay.

We also found that a 5-fold dilution of the unpurified DNA extract was effective in resolving inhibition in the samples we tested, but this approach has drawbacks because dilution reduces method sensitivity. The theoretical detection limit for TaqRegular is 27 cells per 100 ml following the standard filtration and extraction protocol (Haugland *et al*. 2005). Implementing a 5-fold dilution would raise the detection limit to 135 cells per 100 ml, above the current recreational water quality criteria of 104 *Enterococcus* per 100 ml. Additionally, qPCR variability greatly increases when the target concentrations are low (Whitman *et al*. 2010, Griffith and Weisberg 2011). DNA amplification relies on the target coming into contact with the reactive agents (primer, polymerase etc.). When target concentration is low, amplification becomes stochastic resulting in higher variability in Ct values (Sivaganesan *et al*. 2010). Although dilution can be an effective mechanism for reducing inhibition, the resulting low precision may be undesirable for beach management decisions unless the measured concentrations far exceed the present concentration for public health notification. Careful characterization of study sites would help determine strategies that minimize the impact of inhibition. In situations where a 5-fold dilution can be effective, it is suggested to perform qPCR with both undiluted and diluted sample DNA if one suspects high probability of inhibition at certain monitoring sites. This will avoid delaying production

of usable qPCR results due to the need to dilute and reanalyze inhibited samples.

Inhibition can result from several potentially interactive mechanisms (Opel *et al*. 2010), including: a) reducing polymerase activity; b) reducing availability of DNA template for amplification through interaction of inhibitors with the DNA; and c) interfering with primer extension of the polymerase-DNA complex. While most inhibition occurs during qPCR, which is the focus of our and most other investigations, mechanism b could also have an effect during DNA extraction, prior to qPCR. It is possible that inhibitors such as humic substances may bind to double stranded DNA (dsDNA) during the extraction step when dsDNA is released by cell lysis, making it unavailable for subsequent qPCR. The inhibition assessment by dilution (this study) may account for this type of pre-qPCR inhibition if dilution shifts the binding equilibrium (between inhibitor and dsDNA) and frees some dsDNA. However, as the thermodynamics for interaction between humic substances and dsDNA remain unclear (Sutlovic *et al*. 2008), we consider this type of pre-qPCR inhibition beyond the scope of our study.

Although this study focused on *Enterococcus* qPCR, our findings provide useful guidance in broader applications. We demonstrated that inhibition compatibility between IC and target assays cannot be assumed. While a more susceptible IC can be employed to screen samples for inhibition for less susceptible target assays, using one IC for identifying inhibition in multiple target qPCR assays, or using an IC to numerically adjust qPCR results without comprehensive validation, should be discouraged. Additionally, the spiking-followed-by-inhibition approach (Appendix B in Supplemental Information) can be a useful tool to detect inhibition for a variety of qPCR applications (e.g., microbial source tracking or clinic diagnosis) where turn-around time and cost are of relatively lesser concern as compared to recreational water quality monitoring.

As US regulators move toward recommendation of qPCR-based methods for *Enterococcus* quantification in ambient waters, it is imperative to establish a uniform definition of inhibition and to recommend specific approaches to mitigating inhibition. Our work suggests that the salmon testes DNA (i.e., Sketa), which is specified in the EPA Method A as a sample processing and inhibition internal control, does not reliably reflect *Enterococcus* inhibition. The potential impacts of using Sketa for adjusting *Enterococcus* qPCR results via the delta-delta-Ct approach need to be comprehensively evaluated prior to routine usage. The use of the dilution method described here, along with the use of reagents designed for analysis of environmental samples may offer a more consistent and accurate approach for identifying and mitigating qPCR assay interference.

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Acknowledgements

The authors would like to thank Richard Haugland for helpful review comments, Denene Blackwood, Wayne Litaker, King-Teh Lin, Rachel Noble, and Orin Shanks for insightful discussion, Martin Getrich, Jian Peng, Charles McGee, and Theodore von Bitner for providing samples, and Meredith Raith, Darcy Ebentier, and Elizabeth Scott for technical assistance.

Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_20SI.pdf