Advancements in Mitigating Interference in Quantitative Polymerase Chain Reaction (qPCR) for Microbial Water Quality Monitoring

Kaedra Jones, MPH (ICF) and Sharon Nappier, PhD, MSPH (U.S. EPA)

APHL Meeting

June 3, 2018

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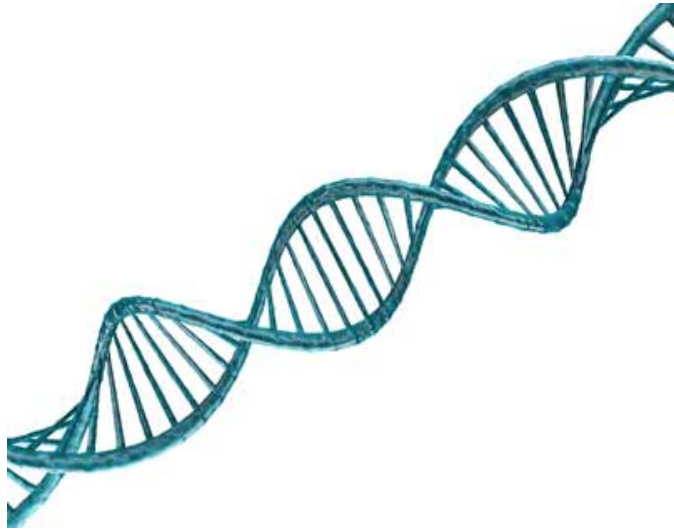
Agenda

- Background
- Literature Search Objectives
- Literature Search Screening and Review Approach
- Common qPCR Interference Controls
- Results
- Limitations
- Implications

Background

- **EPA Method 1611 proposed in the 2012 Recreational Water Quality Criteria (RWQC) as a supplemental indicator to detect and quantify *Enterococcus* spp. in ambient water on a site-specific basis.**
- **Advantages:**
  - Rapid detection results (2-6 hours) allows beach managers to make same-day decisions.
  - EPA's *Enterococcus* spp. qPCR (Method A) was significantly associated with gastrointestinal (GI) illness in the human-impacted EPA National Epidemiological and Environmental Assessment of Recreational Water (NEEAR) studies (Wade et al., 2006, 2008, 2010).
- **Disadvantages:**
  - Limited experience demonstrating method performance across range of environmental conditions.
  - Users cautioned of potential for qPCR interference, which can vary across waterbodies and on a site-specific basis.

What is Interference?

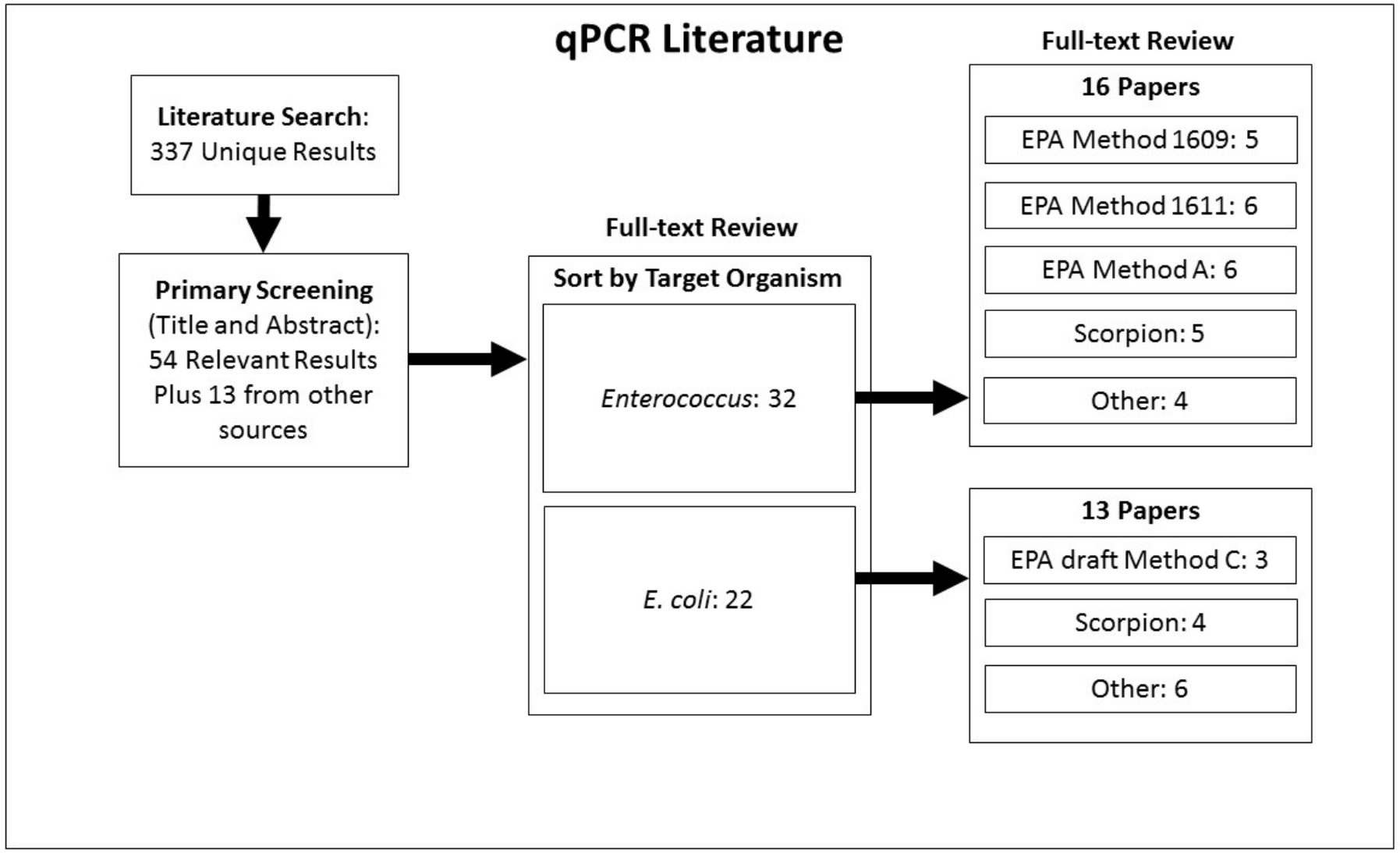


- **Process that results in lower quantitative estimates than actual or expected values.**
  - False negatives
- **Occurs when substances in the test sample inhibit polymerase function or cause DNA to be lost or unavailable for amplification.**
- **Potential interference-causing substances: humic acids, coral sands, calcium, specific types of clay particles, and other substances.**
- **Adaptations to qPCR methods improve estimation and control of sample interference.**

Literature Search Objectives

1) **Identify where *Enterococcus* spp. and *E. coli* qPCR methods been applied since 2010.**
2) **Identify the rate of interference when using molecular methods in those waterbodies.**
3) **Identify method improvements that have reduced interference.**
4) **Identify method or water matrix attributes (e.g., turbidity) and dynamics of fecal contamination that may continue to contribute to poor performance or increased interference.**

Literature Search, Screening, and Review Approach



Note: Some studies included in the full-text review reported multiple organisms. Some studies reported multiple qPCR methods.

Common qPCR Interference Controls

Interference Controls	Abbreviation	Application	Common Types
Sample Processing Control (EPA Method A, 1611, and 1609)	SPC	Non-target DNA sequence used to estimate recovery efficiency. Control involves spiking known quantity of non-target DNA into sample prior to processing.	Salmon testes DNA (i.e., Sketa 22)
Internal Amplification Control (EPA Method 1611 and 1609)	IAC	Non-target DNA sequence added to reaction mix prior to qPCR reaction. If non-target DNA does not amplify as expected, a problem with the reaction is indicated (e.g., DNA polymerase inhibition).	IAC5
Dilution (Cao et al., 2012)	Dilution	Dilution of sample can result in dilution of other compounds that interfere with DNA amplification. Different dilutions can be compared (i.e., serial dilutions).	5x 25x
Ratio spiked test matrix/spiked control matrix (Haugland et al., 2016)	STM/SCM	The recovery of target DNA sequences from target organisms spiked into water samples (STM) can be compared to recovery of DNA from spiked target organisms in control samples (SCM). STM/SCM ratio can provide additional measure of interference caused by inhibitors in water matrix.	Not applicable
Addition of higher salmon DNA concentrations to samples during extraction (Haugland et al., 2012)	Not applicable	Demonstrated at one tropical site to reduce interference due to DNA loss during sample extraction.	25x increase in salmon DNA concentration

qPCR Methods: *Enterococcus* spp.

Method	Analytical Permutations		Performance/Interference Evaluation Analyses		
	Master Mix	Recommended Sample Extract Dilution	SPC: Acceptance range	IAC: Acceptance Range	TSC or CE Spike Recovery: Acceptance Range
EPA Method 1609 (U.S. EPA, 2013b; Haugland et al., 2016)	EMM	Undiluted (5x diluted optional)	Sketa 22: Test sample Ct within 3 units of calibrator samples (mandatory in method)	IAC5: Test sample Ct within 1.5 units of negative control samples (recommended in method)	TSC: 50 – 200%[b]
EPA Method 1611 (U.S. EPA, 2012b; Haugland et al., 2012)	UMM	5x diluted	Sketa 22: Test sample Ct within 3 units of calibrator samples (mandatory in method)	IAC5: Test sample Ct within 1.5 units of negative control samples (recommended in method)	TSC: 50 – 200%[b]
EPA Method A (U.S. EPA, 2010a)	UMM	5x or 25x diluted	Sketa 2[a]: Test sample Ct within 3 units of uninhibited reference samples	Not evaluated	CE: Acceptance ranges defined by study results[c]; Fresh Water: detect – 333% Marine Water: detect – 1,123%
Scorpion method (Noble et al., 2010)	OmniMix	10x diluted, if needed	*Lactococcus* (SmartBeads): 1.5 Ct shift	*Enterococcus* IC, *Lactococcus* IC (SmartBeads): 1.5 Ct shift	Not evaluated

Note: Adapted from Haugland et al. (2016)

[a] Sketa 22 was also evaluated

[b] Recovery ratio of spiked test matrix (filters and retentates from collected water samples spiked with *Enterococcus* spp. cells) to spiked control matrix (clean filters spiked with *Enterococcus* spp. cells).

[c] Recovery ratio of estimated qPCR cell equivalents in spiked test matrix to estimated colony forming units (CFU) in the spikes. Spiking done with 550 CFU Bioballs™.

Acronyms and Abbreviations: CE = cell equivalent; EMM = Environmental MasterMix; IAC = Internal Amplification Control; IC = propriety PCR positive internal control template; UMM = Universal MasterMix; SPC = Sample processing control; TSC = target sequence copy

Summary of Interference Rates for *Enterococcus* spp. qPCR Methods (2010–2017)

Citation	Water Type	Location (# of sites)	Fecal Source	# of Samples Undiluted (% interference)	# of Samples Diluted 5X (% interference)	Strategies to Test Interference[d]
EPA Method 1609 (EMM)						
Dorevitch et al. (2017)	FW	IL (9)	WW, NPS	1256 (1.1)	540 (0.37)	SPC (Sketa 22) (Ct 3)
Haugland et al. (2016)	M	FL, CA, NC (9)	Not reported	241 (10)	356 (4)	SPC (Sketa 22) (Ct 3)[a] IAC (IAC5) (Ct 1.5)
Haugland et al. (2016)	FW	WI, OH, FL (13)	Not reported	491 (11)	419 (3)	SPC (Sketa 22) (Ct 3)[a] IAC (IAC5) (Ct 1.5)
Sivaganesan et al. (2014)	FW	OH, KY, IN, PA, IA (7)	NPS, SS, WW, AW, HW	221 (5)	221 (3)	SPC (Sketa 22) (Ct 3) IAC (IAC5) (Ct 1.5)
Haugland et al. (2012)	FW	OH, KY (5)	NPS, SS, WW, AW, HW	268[b] (0)	268[b] (0.7)	SPC (Sketa 22) (Ct 3)[a] IAC (IAC5) (Ct 1.5)
Cao et al. (2012)	M, FW	CA, IL (52)	NPS, HW	133 (0)	Not reported	SPC (Sketa 2)[c] (Ct 3)[a]
Cao et al. (2012)	M, FW	CA, IL (52)	NPS, HW	133 (11)	133 (0)	IAC (IAC5) (Ct 1.7)[a]

[a] Other interference controls evaluated (dilution and/or STM/SCM).
[b] Interference rates shown are based on SPC assay only, IAC assay results were generally in agreement when available.
[c] Deviated from Method 1609 by using Sketa2 rather than Sketa22 SPC assay
[d] Composite of undiluted and 5X dilution results. 5X dilutions analyzed only for undiluted samples that failed Sketa2 assay acceptance criterion.
Abbreviations and Acronyms: Master Mixes: EMM = environmental master mix; UMM = universal master mix; Waterbody Types: M = marine; FW = Freshwater; Fecal Sources: NPS = non-point source/urban runoff; HW = human waste; AW = animal waste; SS = spiked samples; WW = waste water; Interference Controls: SPC = sample processing control; IAC = internal amplification control; Ct shift = Difference in Cycle Threshold values for between control and environmental samples; STM/SCM = recovery ratio of spiked test matrix (STM) (filters and retentates from collected water samples spiked with *Enterococcus* cells) to spiked control matrix (SCM) (clean filters and buffers spiked with *Enterococcus* cells); Sketa 22 = primers for salmon sperm DNA; Sketa 2 = primers for salmon sperm DNA

Results: *Enterococcus* spp. qPCR Methods

- **Identified 16 papers with relevant information on selected methods.**
- **Only 1 of the 16 papers addressed potential reason for interference in tested samples (Haugland et al., 2012).**
  - *Polymerase inhibitory compounds affecting amplification (would affect both IAC and SPC assay results) present in source water, in addition to DNA binding compounds that would affect the SPC assay results in Boquerón Bay, could explain the discrepancy in failure rates observed for these two control assays among samples from both locations.*
- **Some authors did speculate causes of interference.**
  - Kinzelman et al. (2011): Runoff from land during precipitation events could have been a factor.
  - Wang et al. (2016): Spiked qPCR reactions with organic (humic acid) and inorganic (calcium) matter to test their inhibitory effects on PCR reactions; small concentrations of both caused significant inhibition.

Results: *Enterococcus* spp. qPCR Methods

EPA Method A

- Interference rate observed to be significantly higher when using Sketa 2, as compared to using Sketa 22 in EPA Method 1611 for analyses of Ohio River water samples (Haugland et al., 2012).

EPA Method 1611

- Much higher average interference rate in undiluted samples (18 – 53%) in studies of temperate marine and freshwater sites (Sivaganesan et al., 2014; Haugland et al., 2012, 2016).
- Five-fold dilution of sample extracts significantly reduced the interference rate in both marine and freshwaters to acceptable levels of interference (<10%) at most sites studied.

EPA Method 1609

- Lower frequencies of interference in analyzed samples, as compared to EPA Method A, EPA Method 1611, and the Scorpion-based method.
- Use of EMM and, when necessary, sample dilution addressed interference at the 9 marine and 23 of the 25 freshwater sites in 10 states investigated in EPA studies (Haugland et al., 2012, 2016; Sivaganesan et al., 2014).

qPCR Methods: *E. coli*

- **Commonly used methods include EPA draft Method C (U.S. EPA, 2014) and the Scorpion method (Noble et al., 2010).**
- **More recently, researchers have developed other methods for use with ambient water samples.**
  - Examples: Bergeron et al., 2011; Sauer et al., 2011; Converse et al., 2012b; Zhang et al., 2012; Krometis et al., 2013; Painter et al., 2013; Walker et al., 2013; Byappanahalli et al., 2015; Cloutier and McLellan, 2017.
- **These methods have not been directly compared to EPA draft Method C in ambient waters. Differences in performance between the methods are unclear.**

Results: *E. coli* qPCR Methods

- **We identified 13 studies.**
- **Three of these studies used the *E. coli* primers specified in EPA draft Method C.**
  - In two of these studies, Sketa 22 used for SPC, CowM2 plasmid used as IAC, and EMM used to minimize interference (Peed et al., 2011; Molina et al., 2014).
- **Four of these studies used Scorpion method (Noble et al., 2010).**
- **Six of these studies used other qPCR-based methods.**
- **Number of reported sites and samples is significantly smaller than for *Enterococcus* spp. qPCR.**
- **Currently no peer-reviewed demonstrations of use for routine monitoring.**

Results: *E. coli* qPCR Methods

EPA draft Method C

- Low rates of interference (<10%).
- Shows promise to have similar performance characteristics as EPA Method 1609 based on current use of the same reagents (EMM), controls (Sketa 22, SPC and IAC5, IAC assays), and target genes (23S rRNA) for use on a site-specific basis.

Scorpion (Noble et al., 2010)

- Two studies with low rates of interference (<5%) and one study (Krometis et al., 2013) with 31% rate of interference.
- Sites impacted by human and animal waste and urban runoff.

Limitations

- **Search limited to peer-reviewed literature published 2010 – 2017.**
  - Literature search included years in which published studies were more likely to include consideration of sample interference and the utilization of advanced technology to reduce sample interference.
  - Prior to 2010, products to address sample interference in qPCR (e.g., EMM, UMM) were limited as technology was still emerging.
- **We acknowledge there are previous published analyses and monitoring efforts that utilized qPCR to assess water quality.**
  - These analyses and assessments generally failed to address or remediate sample interference or interference rate.

Implications

- ***Enterococcus* spp. qPCR:**
  - Use of interference controls and effects of sample interference more frequently reported than for *E. coli* qPCR.
  - Results support use of *Enterococcus* spp. qPCR for routine monitoring on a site-specific basis.
  - Of the available *Enterococcus* spp. qPCR methods, EPA Method 1609 is best suited for site-specific use, as the fewest number of samples with interference (as compared to EPA Method A, Method 1611, and Scorpion-based method) were reported when the proper controls were in place (Haugland et al., 2014, 2016).
- ***E. coli* qPCR:**
  - Based on available literature, more work is needed to demonstrate that *E. coli* qPCR is also ready for use for routine monitoring.
  - Studies with larger sample sizes and more sampling sites are needed to determine the characteristics of a sampling site where the use of *E. coli* qPCR is suitable for monitoring purposes.

Implications

- **Research needs:**
  - A peer-reviewed demonstration of the use of EPA draft Method C to determine if this method is suitable for use for routine water quality monitoring on a site-specific basis.
  - A direct comparison between EPA draft Method C and other *E. coli* qPCR methods that use various primers and probes to determine advantages and disadvantages of each method in ambient waters.
    - Consider efficacy of available strategies on reducing the rate of interference, such as dilution.
  - Additional evaluation of digital PCR in ambient waters to determine its utility for routine water quality monitoring applications.
- **Results of our literature review to be published (manuscript is under development).**

Questions?

Kaedra Jones: kaedra.jones@icf.com

Sharon Nappier: nappier.sharon@epa.gov

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