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Comparison of *Enterococcus* quantitative polymerase chain reaction analysis results from Midwest U.S. river samples using EPA Method 1611 and Method 1609 PCR reagents

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ABSTRACT

Enterococci target sequence density estimates from analyses of diluted river water DNA extracts by EPA Methods 1611 and 1609 and estimates with lower detection limits from undiluted DNA extracts by Method 1609 were indistinguishable. These methods should be equally suitable for comparison with U.S. EPA 2012 Recreational Water Quality Criteria values.

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1. Introduction

The U.S. Environmental Protection Agency (EPA) is providing supplemental guidance in its 2012 Recreational Water Quality Criteria (RWQC) (U.S.EPA, 2012a) for states wishing to utilize beach action values (BAV) or to develop recreational water quality standards based upon EPA Method 1611. Method 1611 uses quantitative polymerase chain reaction (qPCR) technology for the detection of enterococci fecal indicator bacteria (FIB) in surface waters (U.S.EPA, 2012b). The guidance is based on analysis results of fresh and marine coastal beach water samples during the National Epidemiological and Environmental Assessment of Recreational (NEEAR) Water Study, which demonstrated that enterococci quantified by Method 1611 were predictive of swimming-related illness at beaches predominantly impacted by point source wastewater effluents (Wade et al., 2006; Wade et al., 2008; Wade et al., 2010). QPCR method results can provide increased public health protection by facilitating timely notification to swimmers of elevated levels of FIB (Ferretti et al., 2013; Griffith and Weisberg, 2011). The RWQC recommends qPCR-enumerated enterococci geometric means corresponding to NEEAR study-estimated gastrointestinal illness (NGI) rates of 32 NGI/1000 and 36 NGI/1000 (U.S.EPA, 2012a). BAV are values that correspond to the 75th percentile of the water quality distribution observed at the fresh and marine coastal NEEAR study beach sites, and can be used by states to make precautionary beach management decisions. The supplemental nature of the qPCR method guidance is largely due to the still limited experience with the performance of Method 1611 across a broad range of environmental conditions. The method has been extensively tested at only a limited number of sites to date. Therefore, EPA encourages a site-specific analysis of the method's performance prior to use in a beach notification program or for adoption of water quality standards based on the method.

Several studies (Cao et al., 2012a; Haugland et al., 2012; Strand et al., 2011) have indicated that a relatively new formulation of PCR reagent, Environmental Master Mix (EMM) (http://www3.appliedbiosystems.com/cms/groups/applied_markets_marketing/documents/generaldocuments/cms_049538.pdf), reduces the frequency of PCR inhibition by different types of surface waters compared to Universal Master Mix (UMM) which is used in Method 1611. EMM will be the reagent specified for use in EPA Method 1609 which is expected to be released in 2014. Method 1609 also will include a competitive internal amplification control (IAC) assay that is currently not part of Method 1611 (Haugland et al., 2012; Shanks et al., 2012). The inclusion of the IAC assay has been demonstrated to not significantly affect the Ct measurements or resultant slope and intercept values of standard curves obtained by the *Enterococcus* assay used in both methods (U.S. EPA, 2014). Otherwise, the reagents and sample analysis procedures in the two methods will be the same.

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The first objective of this study was to further investigate the frequencies of interference in qPCR analyses of water sample filters, in this instance from seven midwestern U.S. rivers including the Mississippi and several major tributaries of the Ohio River, using EMM and UMM. Previous studies have suggested that inland lake and river waters may be particularly good candidates for exhibiting interference in Method 1611 due to PCR inhibition, potentially at least in part due to greater impacts of runoff from land during precipitation events on these waters (Haugland et al., 2012; Kinzelman et al., 2011). The selected rivers are representative of watersheds that range from industrialized to predominantly agricultural land use and have varying levels of potential impact from treated and untreated fecal wastes (unpublished data and personal communication, Ohio River Valley Water Sanitation Commission). The performance of both qPCR reagents was evaluated based on results of the current salmon DNA sample processing control (SPC) assay, featured in both Methods 1611 and 1609, and also the more recently developed IAC assay specified in Method 1609. Recovery ratios of DNA from spiked target organisms in the presence and absence of the water sample matrices were also assessed as a performance metric for results from each reagent.

The second objective of the study was to determine whether enterococci density estimates obtained using analytical results from the two reagents are comparable. Both Methods 1611 using UMM and 1609 using EMM have independently gone through the EPA validation process (U.S. EPA, 2012c, 2013). However, this study was designed to provide the first assessment of whether a change from original Method 1611 to newer Method 1609 is likely to cause any significant differences in enterococci density estimates and thus in the potential relationship between results from the newer method with EPA's currently published BAV and water quality criteria that were based on the original method. The comparison of only the UMM and EMM reagents in this study should address this question due to the similarity of the two methods in most respects other than their difference in these two reagents.

2. Materials and methods

2.1. Water samples

Ohio River tributary water samples were collected approximately bimonthly from the Beaver, Great Miami, Licking, Little Miami, Tennessee and Wabash Rivers at mile points 1.2, 5.2, 0.9, 1.4, 5.1 and 32.5 respectively, from their confluences with the Ohio River from August through November, 2011 and from March through July, 2012. Mississippi River water samples were collected daily from the Buffalo Shores Park recreational beach near Davenport, Iowa from June 29 to June 30 and then twice weekly from July 2 to August 7, 2009. Ohio River tributary water samples (100 mL) were collected in 110 mL Corning™ Coliform Containers by collaborators at the Ohio River Valley Water Sanitation Commission by their standard operating procedure for grab sampling which is adopted from a standard EPA method (U.S. EPA, 1978). Mississippi River water samples were collected as previously described (Haugland et al., 2005). All water samples were stored on ice during transport to a laboratory (a mobile laboratory in the case of the tributary samples) and were processed as described below within 6 h of collection. Each water sample was shaken thoroughly and two 50 mL (Ohio River tributaries) or 100 mL (Mississippi River) subsamples were filtered through 47-mm, 0.4-μm pore size polycarbonate filters (Millipore #HTTP04700) followed by a rinse of the sides of the funnels with 20 mL of sterile, phosphate buffered saline and continued filtration to remove all visible liquid. The filters were transferred to 2 mL semiconical screw-cap microcentrifuge tubes (extraction tubes) containing 0.3 g of acid-washed glass beads as previously described (Haugland et al., 2005) and stored on dry ice and then at −80 °C until extraction (tributary water sample filters were held at −20 °C in the mobile laboratory for up to 1 week prior to transferring to −80 °C).

2.2. Calibrator, spiked matrix and negative control samples

Calibrator sample filters were prepared for extraction and analysis in advance of the study and again in triplicate for extraction and analysis with each batch of water sample filters. Spiked water sample filters were prepared for extraction and analysis in parallel with each water sample filter. For the preparation of calibrator samples, approximately 10^4 cells of laboratory grown *Enterococcus faecalis*, strain ATCC 29212 (ATCC™, Manassas VA), were suspended in 600 μl AE buffer (Qiagen, Valencia CA) containing 0.2 μg/mL salmon DNA (#D-1626, Sigma, St. Louis MO) and transferred to extraction tubes containing glass beads and clean polycarbonate filters of the same type as used for water sample filtration. Spiked samples were prepared by adding these cells in the same manner to one of the replicate filters from each water sample. Laboratory grown *E. faecalis* cells (ATCC® 29212™) were cultured, harvested, washed and enumerated as previously described (Siefring et al., 2008). Negative control samples were prepared in the same manner by additions of salmon DNA extraction buffer with no cells to the filters. The calibrator, spiked matrix and negative control samples were extracted and analyzed immediately after preparation as described in Sections 2.3–2.4.

2.3. DNA extraction

DNA was released from the cells and filters by bead milling in a mini bead beater (Biospec Corp., Bartlesville, OK) for 60 s at maximum rate in the presence of 600 μL of AE buffer, containing 0.2 μg/mL salmon testes DNA as an SPC. The tubes were centrifuged at 12,000 ×*g* for 1 min to pellet the glass beads and debris. Resulting supernatants were transferred to sterile low-retention 1.7 mL microcentrifuge tubes (GeneMate, #C-3228-1, BioExpress, Kaysville UT) and centrifuged a second time as above for 5 min. These supernatants (DNA extracts) were routinely analyzed by qPCR both undiluted and after 5-fold dilution in AE buffer.

2.4. QPCR analysis

Amplifications were performed in an ABI StepOnePlus sequence detector (Life Technologies/Applied Biosystems, Foster City CA). Multiplex *Enterococcus* assay reaction mixtures contained either 1× TaqMan® Universal PCR Master Mix or 1× TaqMan® Environmental PCR Master Mix (Life Technologies/Applied Biosystems), plus 0.2 mg/mL bovine serum albumin (Sigma), 1 μM of each primer, 80 nM each of FAM™ labeled *Enterococcus* TaqMan® probe and 80 nM VIC™ labeled UC1P1 (IAC) TaqMan® probe (both labeled with TAMRA™ as the quencher dye), ~100 copies of the IAC assay plasmid DNA template IAC5 and 5 μl of DNA extracts in a total reaction volume of 25 μl as previously described (Haugland et al., 2012; Shanks et al., 2012). Primers and probe of the previously reported Sketa22 assay (Haugland et al., 2012; U.S.EPA, 2012b) 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 were 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C, after an initial incubation at 50 °C for 2 min and 95 °C for 10 min. Data were analyzed at a threshold ΔRn value of 0.03 on the sequence detector. Unless otherwise specified, all sample analyses were performed in duplicate. Cycle threshold (Ct) values were exported to Microsoft Excel for further analysis.

2.5. Data collection for estimation of calibration model parameter values

Data collection for most probable number (MPN) estimation of target sequence recoveries from calibrator samples was performed using

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