

1 **Comparison of rapid QPCR-based and conventional culture-based methods for**
2 **enumeration of *Enterococcus* sp. and *Escherichia coli* in recreational waters**

3

4 *Rachel T. Noble¹, A. Denene Blackwood¹, John F. Griffith²,*
5 *Charles D. McGee³ and Stephen B. Weisberg²*

6

7 *1 University of North Carolina, Institute of Marine Sciences, Chapel Hill, NC*

8 *2 Southern California Coastal Water Research Project, Costa Mesa, CA*

9 *3 Orange County Sanitation District, Fountain Valley, CA*

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24 **Abstract**

25 Recreational water quality is currently monitored using EPA-approved culture-based methods
26 that require 18 to 96 hours for results, limiting protection of public health. Quantitative PCR
27 (QPCR) methods that can be completed in less than two hours have been developed, but could
28 yield different results than the slower methods they are intended to replace. Here, we present two
29 studies where samples were processed simultaneously using **proprietary** QPCR- and culture-
30 based methods of enumeration for *Enterococcus* sp. and *Escherichia coli* to compare these
31 methods. The first study involved processing 54 blind samples, where QPCR was conducted by
32 developers of the assays. The second study involved 163 samples processed by personnel from a
33 State-certified microbiology laboratory with little previous experience with QPCR. The
34 correlation between QPCR and culture-based methods was 0.79-0.86 ($R^2=0.62-0.74$) for
35 *Enterococcus* sp. and 0.83-0.86 ($R^2=0.69-0.74$) for *E. coli*, and comparable to, but less than the
36 0.91-0.94 ($R^2=0.83-0.88$) correlation observed between culture-based methods. There were no
37 false positives on blank samples and repeatability between replicates was as high for QPCR as it
38 was for the culture-based methods. The QPCR results would have led to the same beach
39 management decision as the culture-based methods for 88% of the samples: only slightly less
40 than the 94% agreement for the two culture-based methods. The samples showing disagreement
41 suggested a slight bias toward underestimation for QPCR, possibly due to PCR inhibition. While
42 there is still a need to understand these minor differences, the high level of agreement indicates
43 that QPCR could be a rapid future alternative.

44 **INTRODUCTION**

45 Presently, fecal indicator bacteria are measured to assess recreational water quality using one of
46 three EPA approved methods: membrane filtration, multiple tube fermentation, or **defined**

47 substrate technologies (DST, Weisberg *et al.* 2007). These methods are widely accepted because
48 of their relative ease of use, low cost, and demonstrated relationship to health risk. However, the
49 time required for sample processing ranges from 18 to 96 hours, with confirmation and
50 verification steps taking even longer. Beach bacterial indicator concentrations have been shown
51 to change substantially on time scales of less than a day (Boehm *et al.* 2002). Thus, contaminated
52 beaches remain open during the laboratory processing period, and often the contamination event
53 has passed by the time warnings are posted (Leecaster and Weisberg 2001).

54

55 Advances in water quality techniques provide new opportunities to measure bacteria more
56 rapidly (Haugland *et al.* 2005, Noble and Weisberg 2005, Layton *et al.* 2006, Bushon *et al.*
57 2009). While currently used methods rely on bacterial growth and metabolic activity, these new
58 methods directly measure cellular attributes, such as genetic material or surface immunological
59 properties. By eliminating the need for a lengthy incubation step, results are available in several
60 hours, enabling managers to take action to protect public health (i.e., post warnings or close
61 beaches) on the same day that water samples are collected. The most advanced of these new
62 methods is QPCR, which has been found to perform well in epidemiological studies as a
63 predictor of gastrointestinal illness risk in beachgoers (Wade *et al.* 2006, 2008).

64

65 While QPCR-based methods are promising, results may differ from those of the culture-based
66 methods that they are intended to replace. Some differences may be related to quantification of
67 nucleic acid endpoints, which could potentially result in detection of bacterial fragments that
68 would not have been measured by culture-based methods. Differences may also be related to
69 chemical inhibition of genetic amplification or challenges in training personnel without previous

70 experience using molecular methods. Acceptance of new methods by water quality professionals
71 with a long history of using culture-based methods will depend on understanding the frequency
72 and the underlying causes of these differences. Whereas a number of studies have assessed the
73 relative performance of the three most commonly used culture-based methods (Noble *et al.* 2003,
74 Griffith *et al.* 2006, United States Federal Register 2006), there have been few comparisons
75 between QPCR- and culture-based method performance. Here we compare performance of a
76 QPCR-based method with two culture-based methods for *Enterococcus* sp. and one culture-
77 based method for *E. coli*. We also quantify the effect of two different QPCR sample processing
78 approaches and assess the performance of each when implemented by personnel from a State-
79 certified water quality laboratory that would be expected to employ these approaches, if adopted.

80

81 **METHODS**

82 The study involved two **levels** of testing in which water samples were simultaneously processed
83 using QPCR-based and EPA approved culture-based methods. In the first test, the QPCR assays
84 were conducted by the scientist who developed the method. In the second test, the sample
85 processing and the QPCR analyses were conducted by personnel from a State certified water
86 quality microbiology laboratory without previous **QPCR** experience to assess whether a more
87 typical user could produce comparable results.

88

89 **Study Design**

90 The first study involved 54 blind samples consisting of triplicates of each of 18 different test
91 samples. Six samples were natural ambient samples collected at shoreline locations with
92 historically high concentrations of fecal indicator bacteria, including: Imperial Beach, San Diego,

93 CA; Doheny State Beach, Dana Point, CA; Cabrillo Beach, Los Angeles, CA; Surfrider State
94 Beach, Malibu, CA; Paradise Cove, Malibu, CA; and a freshwater sample from the Tijuana
95 River, San Diego, CA. Three samples were various types of blanks, consisting of sterile
96 phosphate-buffered saline (PBS, pH 7.2), uninoculated offshore seawater, and 0.2 µm filtered
97 offshore seawater. Three laboratory created samples were prepared using seawater collected
98 from 18 km offshore of Newport Beach, CA, at a depth of 10 m, in an area known to be free
99 from allochthonous fecal contamination. Three additional laboratory samples were inoculated
100 with differing concentrations of laboratory cultures (*Enterococcus faecium*, *Enterococcus*
101 *faecalis* and *E. coli*). Another additional set of three laboratory-created samples were inoculated
102 with differing concentrations of primary wastewater influent from Orange County Sanitation
103 District Plant #1 (OCSD; Fountain Valley, CA), or were inoculated with differing concentrations
104 of urban runoff collected from a Dominguez Channel storm sewer in Torrance, CA.

105

106 Sample processing for the culture-based methods was conducted by five local laboratories:
107 OCSD, Orange County Public Health Laboratory, City of Los Angeles, Los Angeles County
108 Sanitation District, and the City of San Diego, using methods employed in their routine water
109 quality monitoring programs. For *Enterococcus* sp., sample processing included the Enterolert™
110 (IDEXX Laboratories, Inc., Westbrook, ME) DST and the EPA Method 1600 membrane
111 filtration (Frahm and Obst 2003, Messer and Dufour 1998). For *E. coli*, only Colilert-18®
112 (IDEXX) DST was used.

113

114 Testing took place June 21-23, 2005. Samples were created or collected between 6 and 9 a.m.
115 each day and distributed to all laboratories no later than 11 a.m. Samples were all processed

116 starting at the same time in all laboratories and in numbered order to minimize any concentration
117 differences that might have developed from degradation during sample transport or laboratory
118 holding. Further details are available as to other results from this study in Griffith *et al.* 2007.

119

120 The second study was conducted from February through July 2006 and involved OCSD
121 microbiologists processing 163 samples using both culture-based and QPCR-based methods. Of
122 these samples, 137 were ambient samples collected from 41 locations that are part of the
123 microbiologists' typical weekly monitoring efforts. The remaining 26 samples were seawater
124 spiked with primary sewage influent (19 samples), or secondary effluent (6 samples; Table 1).
125 All samples were processed in duplicate using QPCR, EPA Method 1600, Enterolert, and
126 Colilert- 18. Forty-four samples were processed using bead beating followed by a commercial
127 DNA extraction kit; these samples were not included in the final data analysis. Therefore, the
128 comparison of the QPCR data was only with samples that were processed using bead beating.

129

130 Ambient water samples were collected from five location types: open ocean beaches distant from
131 creeks that drain land-based runoff (Open Ocean Beach); open ocean beaches near storm drains
132 (Open Beach Near Drain); enclosed embayment beaches (Enclosed Beaches); locations within
133 storm drains; and wet weather samples from open ocean beaches (Wet Weather; Table 1).

134 Sewage spiked samples were created by inoculating clean ocean water with varying
135 concentrations of either primary sewage influent or secondary sewage effluent (Table 1). Clean
136 ocean water was collected at a location 11 km offshore of Newport Beach, CA. Sewage was
137 obtained from OCSD wastewater stream. Following inoculation, sewage spiked samples were
138 stirred for a minimum of 15 minutes using a magnetic stirring plate.

139

140 **Sample Processing for QPCR**

141 The QPCR assays were a proprietary method developed for *Enterococcus* sp. targeting the
142 multiple copy 23S rRNA gene in an approach similar to that outlined by Ludwig and Schleifer
143 (2000). The *E. coli* assay targets the single copy *uidA* gene as discussed in Frahm and Obst
144 (2003). Scorpion™ primer/probe technology (DxS, Ltd., Manchester, UK) was utilized for assay
145 development. Primer and probe sequences for *Enterococcus* sp. and *E. coli* QPCR assays are
146 licensed and sold by Cepheid as proprietary Total *Enterococcus* and *E. coli* SmartBeads
147 (Cepheid, Inc., Sunnyvale, CA). A *Lactococcus* bead containing 100,000 cells, also marketed by
148 Cepheid, was used as a specimen processing control (SPC) to assess inhibition for each analysis.

149

150 Samples were processed on a six-place filtration manifold and vacuum pump assembly with Pall
151 disposable filter funnels (Pall Corp., East Hills, NY). The mixed-ester cellulose filters that had
152 been provided with the funnels from the manufacturer were replaced with 47-mm diameter, 0.45-
153 µm pore size polycarbonate filters (HTTP; Millipore Corp., Bedford, MA). Each 100-ml sample
154 (measured using a sterile 50-ml conical tube) was filtered within 30 minutes of receipt. Sample
155 filtration was conducted until no further moisture appeared on the filter. Each filter was
156 subsequently rinsed with a small volume (~20 ml) of PBS, which was also filtered to visible
157 dryness.

158

159 For the first study, replicate filters were processed twice, using either bead beating only or bead
160 beating followed by a full DNA extraction. Filters were immediately removed from the vacuum
161 manifold using sterile disposable forceps, gently folded in half and placed into a pre-labeled 2.0-

162 ml screw-cap microcentrifuge tube. For bead beating, the 2.0-ml tube contained 0.3 g of 1-mm
163 zirconium silica beads (Biospec Corp., Bartlesville, OK). DNA was recovered from the
164 organisms retained on the filters by addition of 600 µl of Buffer AE (QIAGEN, Valencia, CA).
165 Approximately 10^5 *Lactococcus lactis* cells were added as SPC to each tube. Tubes were placed
166 in a 48-position mini bead beater (BioSpec Corp.) and shaken for 2 minutes at the highest speed
167 setting. The tubes were then centrifuged at 12,000 x g for 1 minute to pellet the beads and debris.
168 Resulting supernatants were transferred to sterile 1.6-ml microcentrifuge tubes. Sample
169 processing using this method took less than 30 minutes for each sample. Full DNA extraction
170 was conducted using the Mo Bio Fecal DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad,
171 CA) according to the manufacturer's instructions for maximum yield. Filters were processed
172 using Mo Bio bead tubes to bead-beat for two minutes as described above.

173

174 **Standards and Standard Curves**

175 Standards for use in the QPCR assays were created based on **cultures** obtained from the
176 American Type Culture Collection (ATCC) and cultured overnight at 37°C. *E. coli* (ATCC
177 25922) was grown in Tryptic Soy Broth, while *E. faecalis* (ATCC 29212) and *L. lactis* (ATCC
178 11454) were grown in Brain Heart Infusion Broth. Overnight cell suspensions were either
179 counted using epifluorescence microscopy following the method of Noble and Fuhrman (1998)
180 or by DST. Cell suspensions were diluted in PBS to concentrations listed below and frozen at -
181 80°C in single-use aliquots. Aliquots were thawed and extracted in the same manner as described
182 previously for samples.

183

184 Standard curves, produced by diluting the cell suspensions listed above, were processed in
185 duplicate four-log dilutions for each reaction. Standard curve cell counts ranged from 1.63×10^4
186 to 1.63 per reaction for *E. Faecalis*, and from 4.6×10^4 to 4.6 for *E. coli*. Inhibition was judged
187 via the variation of $1/2 \log$ (1.5 cycle threshold (Ct)) from the expected Ct of 26.58 for a spike of
188 1.0×10^5 SPC *L. lactis* cells that were co-extracted with the samples (i.e., if the Ct value for the
189 sample was greater than 28.23 the sample was treated as inhibited). Inhibited samples were
190 diluted 10-fold with sterile water and re-analyzed. Quantification during the first study (for
191 which QPCR analysis was conducted by the method developers) relied on interpolation of cell
192 numbers from the standard curve generated during each analysis. Results for the second study
193 (for which QPCR analysis was conducted by OCSD microbiologists) were generated using the
194 delta Ct approach (Haugland *et al.* 2005). Given the similarities observed between results
195 calculated using standard curve and delta Ct approaches and results calculated by the EPA using
196 the delta Ct approach, the delta Ct approach was adopted for the second part of this study. A
197 calibrator curve was run in duplicate during each run using the calibrator (at a concentration of 1
198 $\times 10^5$ *E. faecalis* cells) and three serial 10- fold dilutions. In this way, the amplification
199 efficiency (*E*) was calculated using the given slope from the SmartCycler™ software (Cepheid):
200 $E = 10^{(-\text{slope})}$. The ratio of target DNA in the samples to that in the calibrator was calculated
201 following Pfaffl (2001). The ratio was then multiplied by the amount of target DNA in the
202 calibrator to get the sample quantities in number of cells.

203

204 **QPCR Reactions**

205 Lyophilized OmniMix® (Cepheid) and Total *Enterococcus*, *E. coli*, or *Lactococcus* SmartBeads
206 were dissolved in RNase and DNase-free sterile water to create a master mix. For each master

207 mix, 20- μ l aliquots were pipetted into reaction tubes, followed by 5 μ l of sample processed using
208 bead beating only, bead beating followed by a commercial DNA extraction kit, or PBS for no
209 template controls. The Omnimix and SmartBeads contained all required QPCR reagents, and
210 primer/probe sets for total *Enterococcus*, *E. coli*, or *Lactococcus*. All SmartBeads contained a
211 propriety PCR positive internal control template (IC; Cepheid) and a primer/probe set (Cepheid)
212 for this template. All probes incorporated Scorpion chemistry. All reactions were prepared in 25-
213 μ l optical tubes (Cepheid). The reactions were monitored in a Smart Cycler IITM sequence
214 detection instrument (Cepheid). Thermal cycling conditions for all reactions (*Enterococcus*/IC,
215 *E. coli*/IC, and *Lactococcus*/IC) were the same, consisting of 1 cycle at 94°C for 2 minutes (hot
216 start), then 45 cycles at 94°C for 5 seconds, and 62°C for 43 seconds (optics on). Determinations
217 of Ct were performed automatically by the instrument after manually adjusting the threshold
218 fluorescence value to 8 units. The Smartcycler II detected fluorescence emissions at three
219 wavelengths that were specific to the fluorophores associated with each of the three probes. Total
220 *Enterococcus* and *E. coli* probes were tagged with the fluorophore FAM (emission maximum at
221 515 nm), *Lactococcus* probes were tagged with the fluorophore Cy 5 (emission maximum at 554
222 nm), and the IC probes were tagged with the fluorophore Cal Fluor Red® (emission maximum at
223 610 nm; Biosearch Technologies, Inc., Novato, CA). Results of unknowns were calculated using
224 fluorescence signals emitted at the correct wavelength and appropriate SmartCycler software
225 associated with the respective standard curve generated or delta Ct.

226

227 During the second study conducted by OCSD personnel, samples were processed as described
228 above, but results are only presented for the bead beating approach. At the beginning of the
229 second study, samples were processed using both bead beating only and bead beating plus a

230 commercial DNA extraction kit. After processing of roughly 40 samples in the second study, we
231 determined that the use of a full commercial DNA extraction kit proved to be too difficult and
232 time consuming for OCSD microbiologists, and that bead beating only fit better into the work
233 flow of their daily routine monitoring. Therefore, for the remainder of the second study the
234 samples were processed using only bead beating. Through examination of the first and second
235 study data, we also observed that the standard curve and delta Ct quantification approaches
236 yielded highly similar results. Therefore, during the second study, to reduce resource expenditure
237 the delta Ct quantification approach was employed as by Haugland *et al.* (2005). The delta Ct
238 method uses an abridged standard curve of the respective calibrator, *Enterococcus* sp. or *E. coli*,
239 to derive the QPCR amplification efficiency (E_{QPCR}). The ratio of change between the calibrator
240 Ct for a known cell amount and the unknown Ct was multiplied by E_{QPCR} to arrive at the cell
241 number. Even though the delta Ct approach was used for enumeration, standard curves were run
242 with every sample batch to assess E , which exceeded 90% for every run.

243

244 **Data Handling and Statistical Calculations**

245 All QPCR- and culture-based datasets were tested for normality and failed. Data was log-
246 transformed and subsequent *Enterococcus* or *E. coli* QPCR results were compared to the culture-
247 based method results using **Pearson product-moment analysis**. For *E. coli*, we compared QPCR
248 cell equivalent results to mean Colilert-18 analyses only. Nondetect culture-based values were
249 reported as one-half of the detection limit (i.e., <10 became 5) and greater-than values were
250 deleted from the data pool. Samples that yielded a non-detect QPCR result were assigned a
251 concentration of 5 cells per 100 ml. **To assess inhibition, the results for the SPC (*Lactococcus*)**
252 **and the IC for each reaction were examined. Inhibition was defined as a delay in amplification**

253 by 1.5 Ct of an unknown as compared to either a filter processed only with the SPC, or the IC
254 with no added sample. Although samples were identified as inhibited, they were diluted and
255 reanalyzed and the values were still included in the overall analyses for *E. coli* and *Enterococcus*
256 sp. to provide a true comparison of a range of sample types.

257

258 RESULTS

259 Testing Conducted by Method Developer

260 Concentrations of *Enterococcus* sp. measured using QPCR were significantly correlated with
261 those as measured using Enterolert and EPA Method 1600 (Table 2; Figure 1). However, the
262 correlation with Enterolert was stronger for samples processed using bead beating alone. These
263 correlation coefficients compared favorably with, but were not quite as strong as, the relationship
264 between the two culture-based methods, EPA 1600 and Enterolert (Table 2).

265

266 The slope of the regression for QPCR-based vs. culture-based methods was less than 1.0,
267 indicating relative underestimation by QPCR. The slope was higher for samples processed using
268 bead beating only, suggesting some loss of target cells in the processing conducted by bead
269 beating plus an additional DNA extraction step. The correlation between *E. coli* QPCR and
270 Colilert-18 was also significant and very similar to that for *Enterococcus* QPCR vs. culture-
271 based methods (Table 2; Figure 2). However, unlike *Enterococcus* QPCR, the slope of the
272 relationship between culture-based methods and *E. coli* QPCR was nearly unity for both bead
273 beating and bead beating plus the commercial DNA extraction kit: $y = 1.0346x + 0.0324$ and $y =$
274 $0.9635x + 0.0991$, respectively.

275

276 Repeatability was similar between QPCR and culture-based methods for *Enterococcus* sp. (Table
277 3). The QPCR methods using bead beating yielded a slightly smaller coefficient of variation
278 (CoV) than that for EPA Method 1600 and Enterolert, and even smaller CoV for samples
279 processed using bead beating followed by DNA extraction. In contrast, culture-based methods
280 had substantially lower CoV than that observed for *E. coli* QPCR (Table 3).

281

282 **Testing Conducted by Water Quality**

283 **Microbiologists**

284 The correlation between QPCR- and culture-based methods for *Enterococcus* sp. were nearly the
285 same when employed by the OCSD microbiology laboratory personnel as when employed by the
286 method developers (Table 2; Figures 1 through 4). Moreover, the correlation between QPCR and
287 Enterolert and EPA Method 1600 was nearly as high as that between the two culture-based
288 methods, and the culture-based methods comparison was noticeably weaker (Table 2). When the
289 results were examined with respect to whether the measurement exceeded 104 colony forming
290 units (CFU) per 100 ml, the concentration at which beach water quality warnings are issued, the
291 *Enterococcus* QPCR agreed with EPA Method 1600 and Enterolert for 88% and 87% of the
292 samples, respectively. This was close to the 94% agreement rate between the two culture-based
293 methods. There were 12 and 13% error rates in method agreement, therefore, as compared to the
294 6% disagreement between culture-based methods. Culture-based methods were not perfect with
295 Enterolert and EPA 1600 showing 83% successful prediction of the EPA 1600 concentration as
296 by Enterolert-based measurements, indicating that 17% of the variability between methods is not
297 explained. With the QPCR-based measurement for *Enterococcus*, that percentage of successful
298 prediction was reduced to 74%, or 26% of the variability between methods not explained.

299

300 The relationship between QPCR and Colilert-18 was similar for *E. coli* to that observed during
301 the testing conducted by the method developers (Table 2; Figures 2 and 4). When assessed
302 relative to the beach warning decision criterion, the agreement rate between QPCR and Colilert-
303 18 was 94%.

304

305 Repeatability of the *Enterococcus* QPCR assay was nearly the same as that for the culture-based
306 methods when testing was conducted by water quality agency personnel (Table 3). Unlike the
307 testing conducted by the method developers, for which we observed a large difference in CoV
308 between the *E. coli* QPCR assay and Colilert-18, the CoV for the two methods during the testing
309 conducted by the water quality microbiologists was identical.

310

311 Identified as delay in the SPC and IC amplification of more than 1.5 Ct values, inhibition was
312 observed in 8 of the 163 samples. Three of these were enclosed beach samples, and five were
313 storm drain samples. The three enclosed beach samples were collected from Newport Dunes, in
314 Newport Beach CA. Four of the inhibited storm drain samples were collected from the same
315 location: Back Bay Storm Drain in Newport Beach, CA.

316

317 **DISCUSSION**

318 Results based on QPCR-based methods were significantly correlated with, and as repeatable as,
319 EPA-approved culture-based methods. However, there was a 9% reduction in the explanation of
320 variability moving from the comparison in culture-based methods ($R^2=0.83$ or 83%) to the
321 comparison between EPA 1600 and *Enterococcus* QPCR ($R^2=0.74$ or 74%). The disagreement

322 rates between the culture-based methods and the *Enterococcus* QPCR-based methods for a
323 management decision were higher than that for the culture-based methods compared to one
324 another (88% compared to 94%). Interestingly the *E. coli* comparison for management action
325 was very high, showing 94% agreement between *E. coli* QPCR and Colilert-18. Similar findings
326 were recently reported in Lavendar and Kinzelman (2009) for Great Lakes waters. Given the
327 fact that the QPCR methods have been shown to be significantly related to human health in a
328 range of epidemiological studies (Wade et al. 2006, Wade et al. 2008), this may not be a major
329 hurdle to successful implementation. Clearly, there are differences in the method agreement
330 regarding beach posting or closure based upon the existing *Enterococcus* single sample standard.
331 However, given the benefit of the rapidity of the methods, and provided a newly developed set of
332 QPCR-based criteria, there may be a significant potential public risk tradeoff. The slope of the
333 correlation for both the first and second study consistently indicated a bias toward
334 underestimation by QPCR relative to culture-based methods. This contrasts with concerns that
335 have been expressed about potential QPCR overestimation relative to culture-based methods
336 because it does not differentiate DNA fragments from culturable cells. Notably, we did not
337 observe such overestimation in samples analyzed during this study, even when examining the
338 sewage influent or effluent samples alone (data not shown).

339
340 There are several possible explanations for the observed underestimation; one of which is
341 inhibition of DNA amplification during QPCR. Inhibition typically occurs when high molecular
342 weight compounds, and heavy metals in the source water (e.g., humic acids and other complex
343 carbohydrates) combine with metal ions to sequester nucleic acids from polymerases and prevent
344 amplification (Thurman *et al.* 1988, Tsai and Olson 1992, De Boer *et al.* 1995, Kreader 1996,

345 Watson and Blackwell 2000). We observed a lower slope for storm drain samples than for the
346 beach samples, which is consistent with inhibition because storm drain samples contain a
347 complex mixture of organic inputs running off of the urbanized landscape. Notably, we did not
348 observe the same extent of inhibition for the *E. coli* QPCR analysis. Inhibition should be
349 detected by the SPC and IC which were incorporated into the analysis. Although inhibition was
350 observed in five of the storm drain samples analyzed for *Enterococcus* sp., it is possible that the
351 criteria used for the SPC and IC may have been too lenient resulting in missed identification of
352 inhibited samples. Also, even though a SPC (*Lactococcus*) was used to identify inhibition, it is
353 possible that *Lactococcus* cells were present in the natural environment, thereby causing an
354 underestimation in the true amount of inhibition. This has been observed previously for the
355 beaches of Avalon, CA, and eastern NC, and caused a switch from the use of *Lactococcus* as a
356 SPC to the use of salmon sperm DNA (e.g. Converse et al. 2009). Inhibited samples were
357 included in the *E. coli* and *Enterococcus* sp. comparisons when the QPCR analysis produced Ct
358 values; removing inhibited samples would have resulted in even closer agreement between
359 QPCR- and culture-based results.

360

361 Another possible explanation for underestimation is that the molecular primers may be more
362 specific to the target species, as compared to the wide range of *Enterococcus* species that are
363 enumerated using EPA Method 1600. We used a pan-*Enterococcus* primer-probe set for
364 *Enterococcus* QPCR in an attempt to detect a wide range of members of the *Enterococcus* genus.
365 However, this approach may have been too specific to include all *Enterococcus* species that
366 grow on the mEI agar used for EPA Method 1600. For example, EPA Method 1600 has been

367 reported to grow a range of non-target species, with false positives rates as high as 17 - 40%
368 reported in some instances (Moore *et al.* 2008).

369

370 Regardless of the reason, underestimation is a substantial management concern because beach
371 managers place high priority on ensuring that the public is not swimming in contaminated water.

372 A false negative, in which a sample that actually exceeds standards is measured as below
373 standards, is problematic because there is no subsequent mechanism for determining that a
374 problem exists. In contrast, a false positive would lead to an inappropriate warning, but one
375 which could be remedied by additional sampling with alternative methods that would be
376 triggered by the positive measurement.

377

378 The second study permitted assessment of whether QPCR technology could be successfully
379 transferred to a local laboratory, which we generally found to be the case. The OCSD
380 microbiology personnel were able to produce results in about two hours, even though their
381 testing included a wider array of sample types than in the first study. The relationships with EPA
382 approved methods and repeatability between replicates were as strong as those observed for
383 QPCR assays conducted by the method developers. However, not all aspects of technology
384 transfer were successful. We abandoned the DNA purification step using the commercial DNA
385 extraction kit prior to QPCR, which was intended to reduce inhibition, because OCSD staff
386 found it complex and time consuming. In particular, they found that the many pipeting steps
387 introduced opportunities for imprecision, which was confirmed by the observed CoV between
388 replicates being three times higher than that for samples processed using bead beating. In
389 contrast, the lyophilized bead technology associated with sample processing that used beat

390 beating only reduced sample manipulation to only two pipeting steps. Additional method
391 automation is desirable, particularly if more complex procedures to minimize inhibition are to be
392 adopted by similar water quality monitoring laboratories across the country. In conclusion, these
393 studies demonstrate the comparability of *Enterococcus* and *E. coli* QPCR to currently used
394 culture-based methods. As further improvements, such as automation and user-friendliness, are
395 made to QPCR-based methods, they should become a possibility for future use.

396

397 LITERATURE CITED

398 Boehm, A.B., S.B. Grant, J.H. Kim, S.L. Mowbray, C.D. McGee, C.D. Clark, D.M. Foley and
399 D.E. Wellman. 2002. Decadal and shorter period variability of surf zone water quality at
400 Huntington Beach, California. *Environmental Science & Technology* 36:3885-3892.

401

402 Bushon, R.N., A.M. Brady, C.A. Likirdopulos and J.V. Cireddu. 2009. Rapid detection of
403 *Escherichia coli* and *enterococci* in recreational water using an immunomagnetic
404 separation/adenosine triphosphate technique. *Journal of Applied Microbiology* 106: 432-441.

405

406 Converse, R. R., Griffith, J. F. and **R. T. Noble**. 2009. Rapid QPCR-Based Assays for Fecal
407 *Bacteroides* and *Enterococcus* Speciation as Tools for Assessing Fecal Contamination in
408 Recreational Waters. *Journal of Water Research*. 43(19):4828-37.

409

410 De Boer, S.H., L.J. Ward, X. Li and S. Chittaranjan. 1995. Attenuation of PCR inhibition in the
411 presence of plant compounds by addition of BLOTTO. *Nucleic Acids Research* 23:2567-2568.

412

413 Frahm, E. and U. Obst. 2003. Application of the fluorogenic probe technique (TaqMan PCR) to
414 the detection of *Enterococcus* and *Escherichia coli* in water samples. *Journal of Microbiological*
415 *Methods* 52:123-131.

416

417 Griffith, J.F., L.A. Aumand, I.M. Lee, C.D. McGee, L.L. Othman, K.J. Ritter, K.O. Walker and
418 S.B. Weisberg. 2006. Comparison and verification of bacterial water quality indicator
419 measurement methods using ambient coastal water samples. *Environmental Monitoring and*
420 *Assessment* 116:335-344.

421

422 Griffith, J.F., D. Moore, C. McGee and S.B. Weisberg. 2007. Beta testing of rapid methods for
423 measuring beach water quality. Technical Report 506. Southern California Coastal Water
424 Research Project. Costa Mesa, CA.

425

426 Haugland, R.A., S.C. Sieftring, L.J. Wymer, K.P. Brenner and A.P. Dufour. 2005. Comparison of
427 *Enterococcus* measurements in freshwater at two recreational beaches by quantitative
428 polymerase chain reaction and membrane filter culture analysis. *Water Research* 39:559-568.

429

430 Kreader, C.A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4
431 gene 32 protein. *Applied Environmental Microbiology* 62:1102-1106.

432

433 Layton, A., L. McKay, D. Williams, V. Garrett, R. Gentry and G. Sayler. 2006. Development of
434 *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human,
435 and bovine fecal pollution in water. *Applied Environmental Microbiology* 72:4214-4224.

436 Lavender, J. S., and J. L. Kinzelman. 2009. A cross comparison of QPCR to agar-based or
437 defined substrate test methods for the determination of *Escherichia coli* and enterococci in
438 municipal water quality monitoring programs. *Water Research*. 43 (19):4967-4979.
439

440 Leecaster, M.K. and S.B. Weisberg. 2001. Effect of sampling frequency on shoreline
441 microbiology assessments. *Marine Pollution Bulletin* 42:1150-1154.
442

443 Ludwig, W. and K.H. Schleifer. 2000. How quantitative is quantitative PCR with respect to cell
444 counts? *Systematic and Applied Microbiology* 23:556-562.
445

446 Messer, J.W. and A.P. Dufour. 1998. A rapid, specific membrane filtration procedure for
447 enumeration of enterococci in recreational water. *Applied Environmental Microbiology* 64:678-
448 680.
449

450 Moore, D.F., J.A. Guzman and C. McGee. 2008. Species distribution and antimicrobial
451 resistance of enterococci isolated from surface and ocean water. *Journal of Applied*
452 *Microbiology* 105:1017-1025.
453

454 Noble, R.T. and J.A. Fuhrman. 1998. Use of SYBR Green I for rapid epifluorescence counts of
455 marine viruses and bacteria. *Aquatic Microbiology and Ecology* 14:113-118.
456

457 Noble, R.T. and S.B. Weisberg. 2005. A review of technologies for rapid detection of bacteria in
458 recreational waters. *Journal of Water Health* 3:381-392.

459

460 Noble, R.T., S.B. Weisberg, M.K. Leecaster, C.D. McGee, K. Ritter, K.O. Walker and P.M.

461 Vainik. 2003. Comparison of beach bacterial water quality indicator measurement methods.

462 *Environmental Monitoring and Assessment* 81:301-312.

463

464 Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time PCR.

465 *Nucleic Acids Research* 29:2002-2007.

466

467 Sivaganesan, M., S. Seifring, M. Varma, R.A. Haugland and O.C. Shanks. 2008. A Bayesian

468 method for calculating real-time quantitative PCR calibration curves using absolute plasmid

469 DNA standards. *BMC Bioinformatics* 9:120-132.

470

471 Thurman, E.M., G.R. Aiken, M. Ewald, W.R. Fischer, U. Forstner, A.H. Hack, R.F. C.

472 Mantoura, J.W. Parsons, R. Pocklington, F.J. Stevenson, R.S. Swift and B. Szpakowska. 1988.

473 Isolation of soil and aquatic humic substances. pp. 31-43 *in*: F.H. Frimmel and R.F. Christman

474 (ed.), *Humic Substances and Their Role in the Environment*. John Wiley and Sons, Ltd. New

475 York, NY.

476

477 Tsai, Y.L. and B.H. Olson. 1992. Rapid method for separation of bacterial DNA from humic

478 substances in sediments for polymerase chain reaction. *Applied Environmental Microbiology*

479 58:2292-2295.

480

481 United States Federal Register. 40 CFR Parts 9, 141 and 142, January 5, 2006.

482

483 Wade, T.J., R.L. Calderon, E. Sams, M. Beach, K.P. Brenner, A.H. Williams and A.P. Dufour.
484 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-
485 associated gastrointestinal illness. *Environmental Health Perspectives* 114:24-28.

486

487 Wade, T.J., R.L. Calderon, K.P. Brenner, E. Sams, M. Beach, R. Haugland, L. Wymer and A.P.
488 Dufour. 2008. High sensitivity of children to swimming-associated gastrointestinal illness: results
489 using a rapid assay of recreational water quality. *Epidemiology* 19:375-383.

490

491 Watson, R.J. and B. Blackwell. 2000. Purification and characterization of a common soil
492 component which inhibits polymerase chain reaction. *Canadian Journal of Microbiology*
493 46:633-642.

494

495 Weisberg, S.B., R.T. Noble and J.F. Griffith. 2007. Microbial Indicators of Marine Recreational
496 Water Quality. pp. 280-289 in: C.J. Hurst, R.L. Crawford, J.L. Garland, D.A. Lipson, A.L. Miles
497 and L.D. Stetzenbach (eds.), *Manual of Environmental*
498 *Microbiology*, 3rd edition. ASM Press.

499 Washington, DC.

500

501 **ACKNOWLEDGEMENTS**

502 The authors would like to thank Seth Yu, Richard Haugland and Shawn Siefring for sample
503 processing and QPCR assistance during the first study. The authors also thank Cepheid, Inc.,
504 Sunnyvale, CA, for donating reagents and equipment.

505

506

507 CAPTIONS

508

509 **Table 1. Type and number of samples analyzed for *Enterococcus* sp. and *E. coli* analyses**
510 **during the second study.**

511

512 **Table 2. Regression analysis results for the first and second testing of rapid QPCR methods**
513 **for *Enterococcus* and *E. coli* as compared to culture-based methods. First study testing was**
514 **conducted by method developers; second study testing was conducted by water quality**
515 **microbiologists. BB = bead beating; BB+DNA = bead beating plus DNA purification using**
516 **a commercial extraction kit.**

517

518 **Table 3. Average coefficient of variation for testing methods for enumeration of**
519 ***Enterococcus* sp. and *E. coli* during the two studies. BB = bead beating.**

520

521

522 **Figure 1. Comparison among multiple measures of *Enterococcus* sp. concentration, EPA**
523 **Method 1600, and Enterolert (black diamonds), or *Enterococcus* QPCR for a range of**
524 **water samples. QPCR testing was conducted by the method developers. Log-transformed**
525 **EPA Method 1600 versus log-transformed rapid QPCR for *Enterococcus* results (gray**
526 **squares) represent samples processed using bead beating only; gray triangles represent**
527 **samples processed using bead beating followed by a commercial DNA extraction kit. CE =**
528 **cell equivalents; CFU = colony forming unit. Best linear fit equations are reported in Table**
529 **2.**

530

531 **Figure 2. Comparison between Colilert-18 and QPCR for a range of water samples. The *E.***
532 ***coli* QPCR testing was conducted by the method developers. Log-transformed Colilert-18**
533 **results in open squares represent samples processed using bead beating only; log-**
534 **transformed QPCR results in black diamonds represent samples processed using bead**
535 **beating followed by a commercial DNA extraction kit. CE = cell equivalents; MPN = most**
536 **probable number.**

537

538 **Figure 3. Comparison among multiple measures of *Enterococcus* sp. concentration; EPA**
539 **Method 1600 versus Enterolert (black diamonds) or *Enterococcus* QPCR (open squares)**
540 **for a range of ambient southern California marine water samples. The QPCR testing was**
541 **conducted by water quality personnel at Orange County Sanitation District. All QPCR**
542 **analyses were conducted on samples processed using bead beating only. CFU = colony**
543 **forming units; CE = cell equivalents; MPN = most probable number. Best linear fit**
544 **equations are reported in Table 2.**

545

546 **Figure 4. Comparison between concentrations of *E. coli* measured using Colilert-18 and *E.***
547 ***coli* QPCR for a range of ambient southern California marine water samples. The QPCR**
548 **testing was conducted by water quality personnel at Orange County Sanitation District. All**
549 **QPCR analyses were conducted on samples processed using bead beating only. CE = cell**
550 **equivalents; MPN = most probable number. Best linear fit equations are reported in Table**
551 **2.**

552

553
554

TABLE 1. Type and number of samples analyzed during the second study.

Sample Type	Number of Samples Collected	Samples included in data analysis	Samples analyzed using Culture-Based Methods
Ocean Beach Near Drain	18	18	18
Open Ocean Beach	44	33	36
Enclosed Beach	22	22	22
Storm Drain	29	29	29
Wet Weather	25	25	25
Open Beach Spiked with Sewage Influent	19	19	19
Open Beach Spiked with Sewage Effluent	6	6	6
Total	163	152	155

555
556
557

558 **TABLE 2. Regression analysis results for the first and second testing of rapid QPCR**
 559 **methods for *Enterococcus* and *E. coli* as compared to culture-based methods. In the first**
 560 **study, samples were processing using two approaches 1) BB= bead beating, and**
 561 **2)BB+DNA= bead beating plus DNA purification using a commercial extraction kit. In the**
 562 **second study, the QPCR data comparison was only conducted on samples that were**
 563 **processed using bead beating only.**
 564

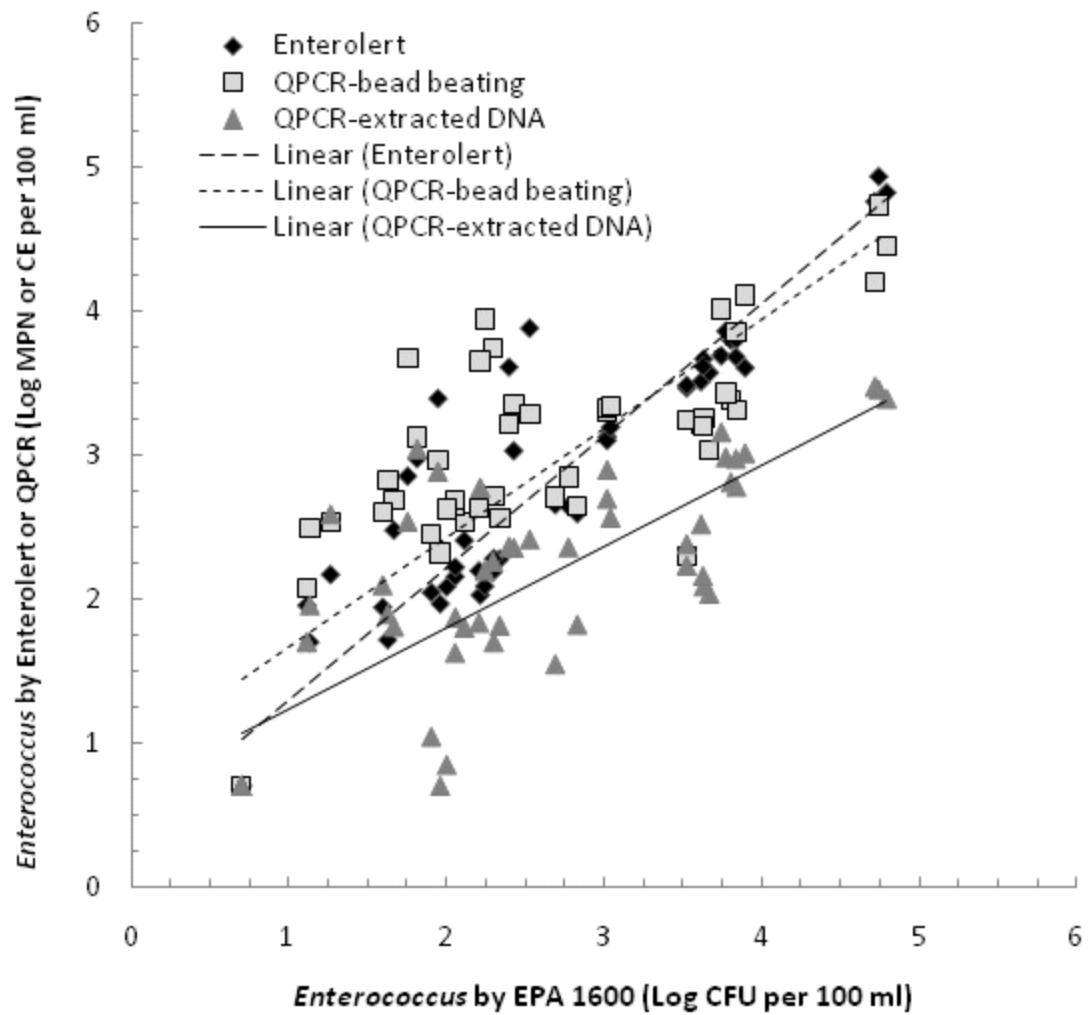
First study: Testing conducted by method developers		Best linear fit	n	R ²	P-value
<i>Enterococcus</i>	EPA 1600 vs. QPCR BB	$y=0.7571x +0.9206$	54	0.69	<0.0001
	EPA 1600 vs. QPCR BB+DNA	$y=0.5635x +0.6800$	54	0.62	<0.0001
	Enterolert vs. QPCR BB	$y=0.8155x +0.6290$	54	0.77	<0.0001
	Enterolert vs. QPCR BB+DNA	$y=0.6370x +0.3857$	54	0.76	<0.0001
	EPA 1600 vs. Enterolert	$y=0.9171x +0.3878$	54	0.87	<0.0001
<i>E. coli</i>	Colilert-18 vs. QPCR BB	$y=1.0346x+0.0324$	53	0.74	<0.001
<i>E. coli</i>	Colilert-18 vs. QPCR BB+DNA	$y= 0.9635x +0.0991$	54	0.69	<0.0001
Second study: Testing conducted by water quality microbiologists					
<i>Enterococcus</i>	EPA 1600 vs. QPCR	$y=0.7509x + 0.1559$	119	0.74	<0.0001
	Enterolert vs. QPCR	$y =0.7516x +0.2549$	119	0.69	<0.0001
	EPA 1600 vs. Enterolert	$y=0.8869x + 0.0942$	152	0.83	<0.0001
<i>E. coli</i>	Colilert-18 vs. QPCR	$y=0.7058x + 0.0596$	119	0.71	<0.001

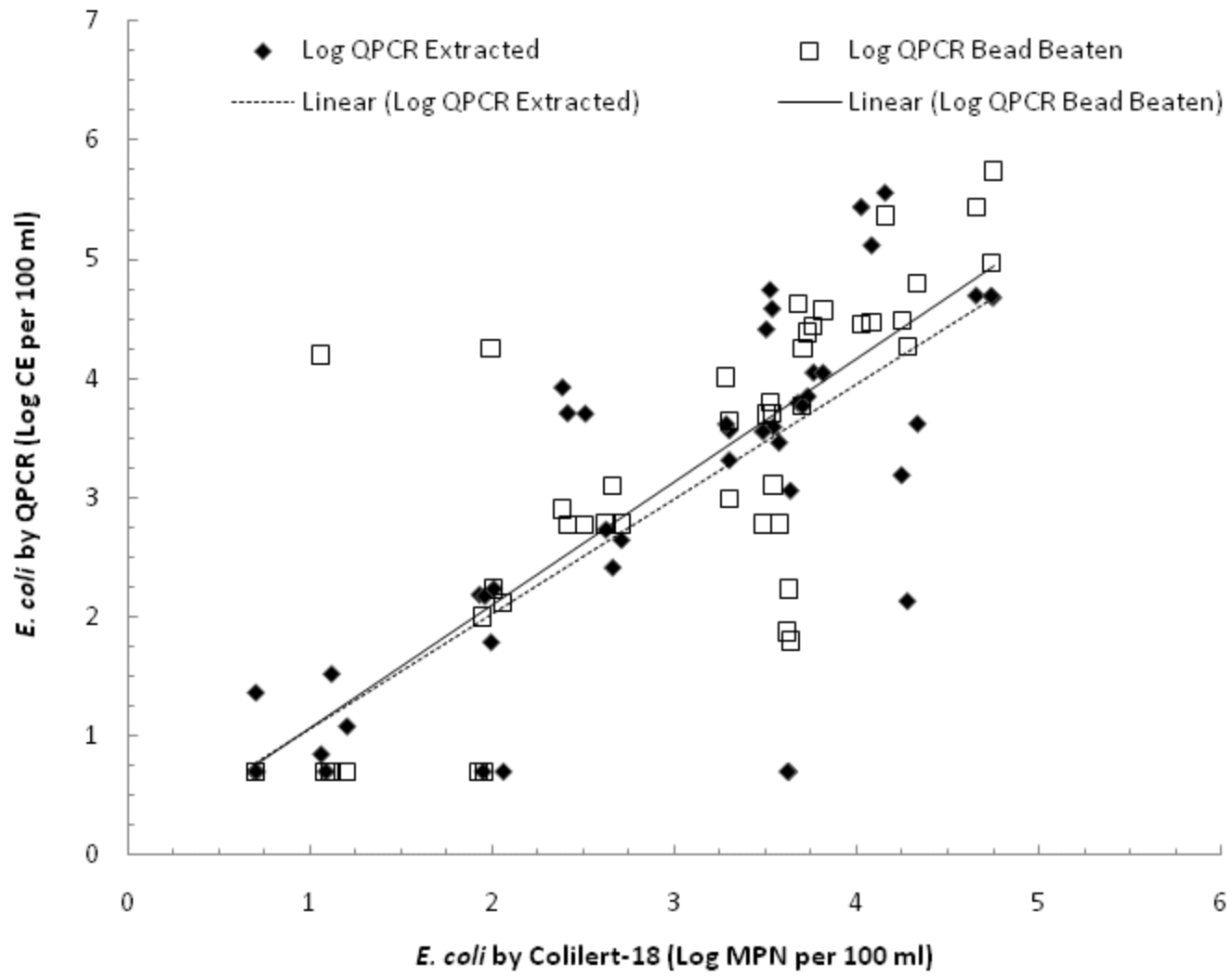
565
566

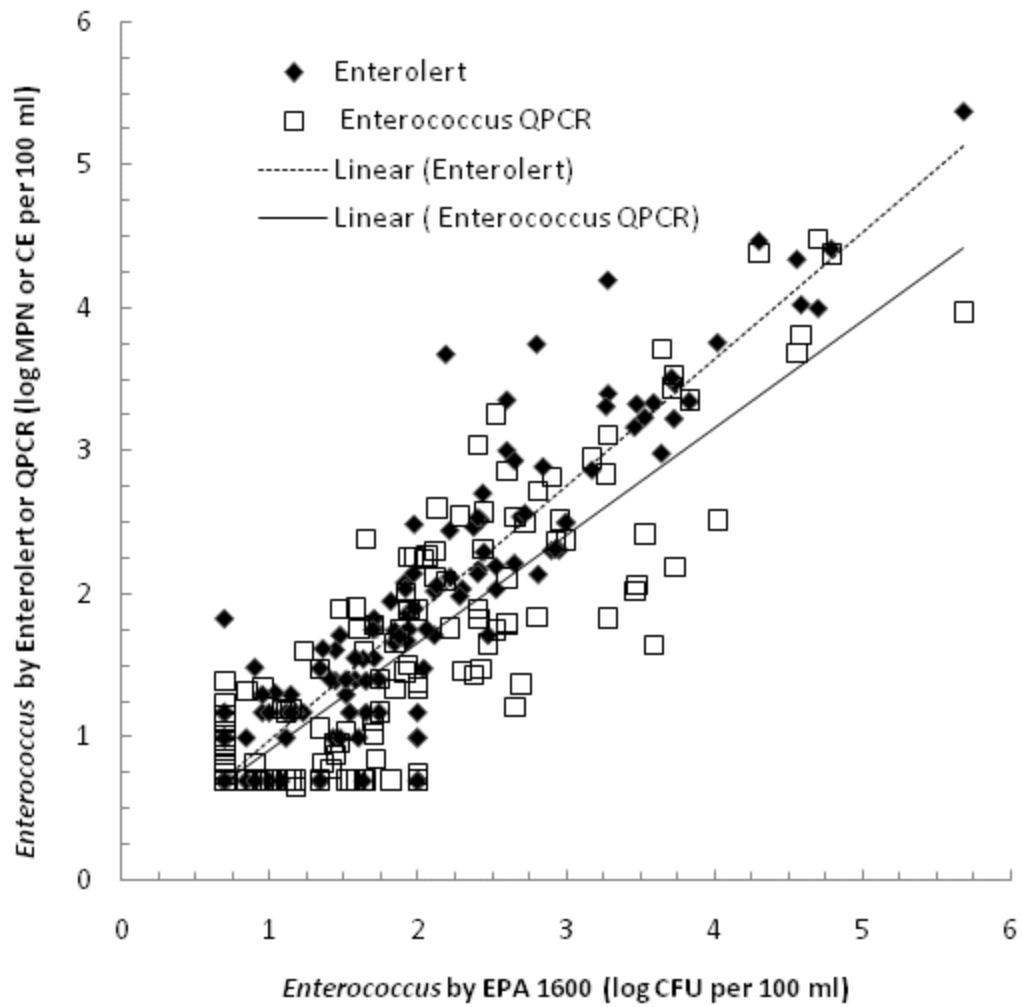
567 **TABLE 3. Average coefficient of variation (CoV) for testing methods for enumeration of**
 568 ***Enterococcus* and *E. coli* during the two studies. The average CoV was determined for each**
 569 **entire study by calculating the CoV for each sample based upon replicate analyses, and**
 570 **then calculating an average of those determined values. BB=bead beating, BB+DNA=bead**
 571 **beating plus DNA extraction kit.**
 572

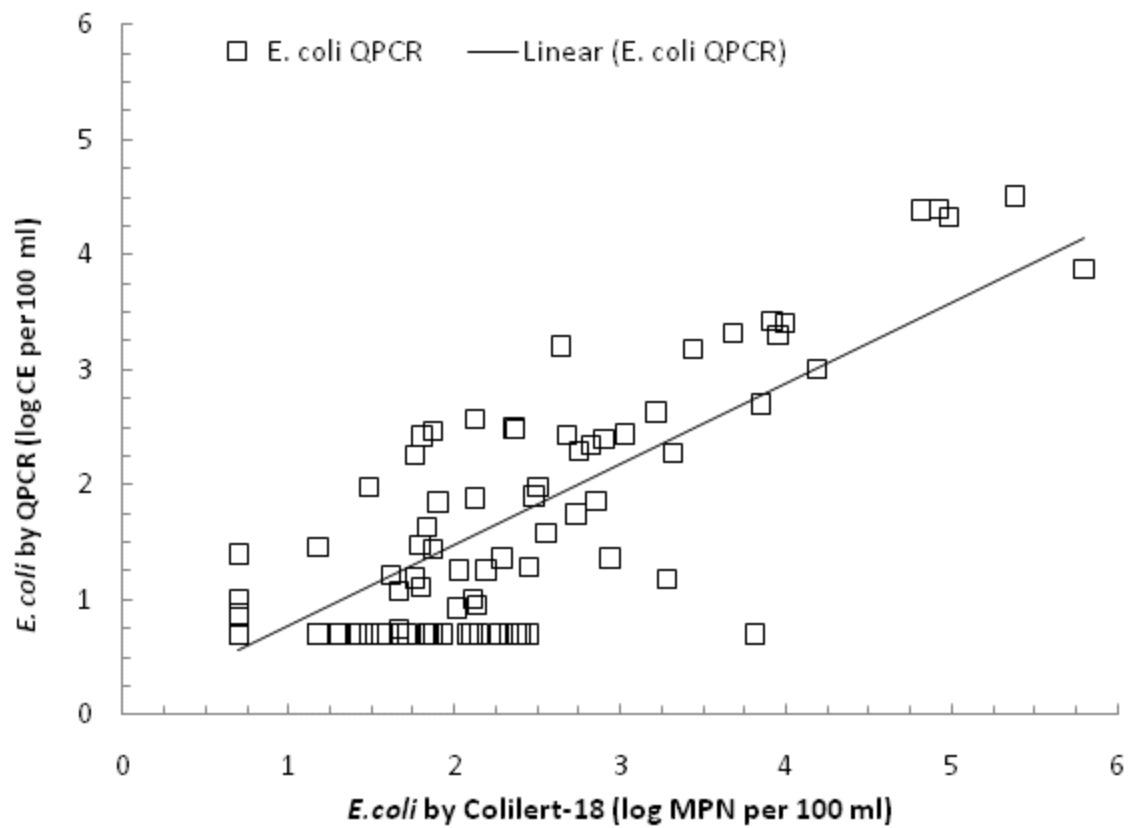
Testing conducted by method developers		Coefficient of variation
<i>Enterococcus</i>	QPCR: BB	0.27
	QPCR: BB + DNA extraction kit	0.36
	Enterolert	0.31
	EPA 1600	0.32
<i>E. coli</i>	QPCR BB only	0.67
	Colilert-18	0.19
Testing conducted by water quality microbiologists		
<i>Enterococcus</i>	QPCR: BB	0.21
	Enterolert	0.21
	EPA 1600	0.18
<i>E. coli</i>	QPCR: BB	0.25
	Colilert-18	0.25

573
 574
 575









This document details the reviews and comments from the editor for the first submission of the manuscript entitled "Comparison of Rapid qPCR-Based and Culture-Based Methods for Enumeration of Enterococcus spp. and Escherichia coli in Recreational Waters (AEM00408-09 Version 1)". Where a longer description/discussion is warranted the response includes all necessary information. All requested modifications to the previously submitted manuscript have been made.

Dear Dr. Noble:

Review comments on your manuscript have been received from three members of the editorial board or ad hoc reviewers. The reviewers felt that this method has the potential to be an important contribution to the field of microbial indicators of water quality. However, it was found that the method need further testing to demonstrate its widespread applicability in order to justify your conclusions. For these reasons, and the reasons in the attached reviews, I am unable to accept your manuscript for publication. The reviewer comments are attached and I believe that they will be helpful to you. Your interest to publish in Applied and Environmental Microbiology is very much appreciated.

REVIEWER 1:

Comments:

This work is a much-needed exploration of the extent to which rapid assessment methods for fecal indicator bacteria (e.g. qPCR) correspond to the agency-approved, culture-based methods currently used for monitoring. The use of the proprietary Scorpion technology and beads has pros and cons that should be made more transparent in the paper; while one must use the company's products to reproduce these results (con), the bead technology undoubtedly yields greater reproducibility and allows laboratory workers with minimal previous training in molecular biology to successfully employ the methodology.

Abstract

The use of the proprietary qPCR method needs to be mentioned in the abstract.
RESPONSE: DONE. We have added "proprietary" to line 29.

Introduction

The first sentence needs references. RESPONSE: Done.

L 57 "DST" stands for defined substrate technology (not directed) RESPONSE: Change made. Line 47.

L 65 to 66 A sampling of the original studies should be cited here rather than just a review. RESPONSE: AGREED, DONE. Lines 56-57.

L 70 Two hours is really overly optimistic (in the best of all possible worlds) unless one has one sample to process and the distance from the beach to the lab is a block or two. I would suggest giving a realistic time frame (e.g. several hours). RESPONSE; DONE. Line 59.

L 74-75 I would suggest replacing "traditional" here with culture-based, which is more specific terminology. RESPONSE: DONE FOR ALL INSTANCES IN THE ENTIRE MANUSCRIPT. Line 65.

L 87 Rather than "two tests" I suggest two levels of testing RESPONSE: DONE.
Line 82.

L92 Add biology to "molecular" (molecular biology) RESPONSE: DONE CHANGED
WORDING TO QPCR INSTEAD.Line 86.

L142 Change rDNA to rRNA; also after "gene" add IN AN APPROACH RESPONSE:
DONE Line 142.

L 143 Check format of "uid a" (should be uidA ?) RESPONSE: DONE. Line 143.

L 157 pH and reference (or formula) for phosphate buffered saline? The
written-out term should be lower case. RESPONSE: DONE at line 96.

L173 Replace 'cell lines" with cultures RESPONSE: DONE
RESULTS. Line 175.

I could not find any data for inhibition - this should be shown. RESPONSE:
The information on inhibition is now reported on LINES 251-256, AND LINES
311-315.

Not all of the graphs are necessary. I would suggest showing an example
graph or two for Figs 4-9, particularly since equations are given in Table 2.
RESPONSE: DONE. There are now 4 figures instead of nine and the best fit
equations are only reported in Table 2 as requested.

DISCUSSION

L 332 "The QPCR extracted approach..." is awkward; suggest replacing with
something like "The approach of extracting DNA prior to QPCR..." RESPONSE:DONE
THE WORDING HAS BEEN CHANGED COMPLETELY SO THAT THIS NO LONGER APPEARS.

L 343 I think the sentence would read better if it were made into two
sentences at the semi-colon RESPONSE:DONE

L 353 Adoption of QPCR methods for what and by whom? I would also suggest an
adjective other than "viable" - maybe useful. RESPONSE: DONE. THE WORDING
HAS BEEN COMPLETELY CHANGED AND APPEARS LINES 390-395.

Table 2. Lower case "n" might be more easily recognizable as sample number,
and "P" could be italicized (or represented as P-value) for ease of
interpretation. RESPONSE:ALL DONE

Table 3. The CoV applies to the methodology, not the organism, therefore the
legend should be changed to reflect that (e.g. Average CoV for testing
methods used to quantify E. coli...) RESPONSE:DONE

REVIEWER 2:

Comments:

This is an interesting paper comparing results of traditional cultivation
versus a non-cultivation based Q-PCR approach to estimate numbers of
Escherichia coli and Enterococcus spp. in recreational waters. Although many
groups have been proposing alternative microbial targets as indicators of
water quality these investigators have recognized that considerable more
research is still necessary before alternative standards can be implemented.
Because of the rich history of using these two indicator species they may
continue to be sufficient to assess water quality. Whereas the other more
specialized targets being developed may be useful to identify sources of
contamination and estimate pathogenic potential. The testing of the method
by a traditional lab is of interest to users of these approaches but my
greatest concern is that alpha testing was insufficient and was prematurely
sent to beta testing. RESPONSE: We have now changed all wording in the
manuscript to reflect that we conducted two studies without the terminology
of Alpha and Beta testing. One study is described as being "conducted by the
method developers" and the second study "conducted by water quality
personnel" to alleviate these concerns.

This was evident in the complexity of the extraction protocol that was luckily discovered early and modifications could be made prior to project completion. At times reading this manuscript there seems to be an eclectic data set that was put together to try to tell a story that does not have an ending. RESPONSE: DONE. The manuscript has been reworded throughout and we feel it is much improved.

Additionally samples are being amplified using a commercial kit that needs to be checked for accuracy and precision by independent researchers and not taken at face value based on the distributor. RESPONSE: These kits are widely cited in water quality studies and were the standard for use at the time (e.g. Noble et al. 2006, Ufnar et al. 2007, Layton et al. 2006). The fact that the specimen processing control did not exhibit greater than 95% loss over the extraction procedure as compared to bead beating provides confirmation of their use. Secondly, the fact that the loss observed for bead beating as examined by the best fit equation by comparing the culture-based methods with the QPCR based method was similar to the loss observed when using the DNA extraction kit, shows, that the kits are doing a commendable job in retaining the target DNA.

Additional comments

Is the term "bead beaten" as opposed to "bead beating" being used correctly throughout the manuscript? The method is typically called "bead beating" and I have not seen the alternative terminology used by any others. RESPONSE: DONE. Thanks for catching this, we have changed this throughout the entire manuscript.

Also the use of QPCR extracted is a little confusing since DNA has been extracted in all methods. If I understand correctly the difference is in using or not using bead beating. RESPONSE: CHANGED. We apologize for the confusion, the difference is whether the DNA extraction kit was used to purify the DNA beyond the bead beating procedure. We now are more specific about this throughout the manuscript.

L98. Include method used to create different levels of fecal contamination. Or are these the samples described L104-112? I would not call this creation of different levels of fecal contamination. RESPONSE: DONE. This has been deleted and the information described more clearly in a completely different way.

Otherwise: What was the fecal source? Were different levels based on fecal mass or indicator organism numbers? How were fecal samples homogenized prior to inoculation to ensure that differences due to fecal sample heterogeneity were minimized? RESPONSE: We used EPA-approved methods the day before to assess the indicator organisms at each location, or from each sample type, and then used this as a guide for our inoculation. This information is completely available in the Griffith et al. 2007 technical report. We have added some pertinent information and have reworded this section.

L113. Does this mean that in alpha testing the research lab did not do any of the cultivation to determine differences between them and the traditional lab?

RESPONSE: NO. The researchers that developed the QPCR methods did only the QPCR methods and the state certified WQ personnel did the cultivation based methods so that they could be done simultaneously.

Since this was a test between a research lab and traditional water quality assessment lab there should have been inclusion of potential error cultivation results. The research lab should have determined (at least in a subset of samples) the incorrect identification of E. coli and Enterococcus by the traditional labs used in this study.

RESPONSE: The test was not to see if the researchers, who are not state certified to do the culture based methods, could do the culture based methods. The test was to compare the results of the culture based methods and the QPCR based methods, when correctly conducted by their respective users.

The investigators are unable to draw conclusions because of the lack of this information. This is very important for this paper since its objective is to assess the value of using QPCR. RESPONSE: This comparison that the reviewer is suggesting is precisely what was compared during the "second study" and why we feel it is so important to report on both versions of the study. There were verification analyses conducted during the first study. These are presented in Griffith et al. 2006. Since we are comparing methods across types of samples, we did not describe the verification analyses. Certainly false positives of the membrane filtration and DST methods played a role in the imperfect relationships among the methods. The urban runoff had very high levels of non-Enterococcus, so that might explain some of the variability seen. During the second study, no verification analyses were done.

L117. Why three methods for Enterococcus and only one for E. coli? Was the comparison of Enterococcus cultivation methods another objective?

RESPONSE: The authors would love to have a dataset that includes all of the culture based methods that are approved by EPA. The laboratories that were involved in this study only wanted to do the methods that they are state certified for and that they do regularly. These are the methods that are in use also in southern California, so it was of interest to them to compare the QPCR only to what they currently use.

L126. Is my interpretation correct that only one traditional lab actually tested QPCR and all the other labs listed previously were only involved in cultivation? This is a little misleading and takes away from the stated objectives of this manuscript.

RESPONSE: Not completely correct. For the first study (where the METHOD developers conducted the QPCR), multiple laboratories were used to do the culture-based methods, because many groups wanted to be involved and because qualified (less than and greater than data) made it important to use multiple data sets. For the second study (where the water quality agency personnel were trained to do the QPCR), the agency personnel did both the QPCR and the culture-based analyses all themselves (i.e. there were not multiple laboratories involved).

L159. The folding and likely rolling of filters required to fit them into 2 ml microcentrifuge tubes would limit contact of beads to much of the cells on the filters. During alpha testing were any measures made to test the efficiency/completeness of this method for cell lysis?

RESPONSE: This is the currently accepted approach for the QPCR analysis, including the analyses conducted by EPA for epidemiology studies, etc. For every sample analyzed we run a specimen processing control (this was Lactococcus cells in this study) by spiking the cells onto the filter that is

in the buffer with the silica/zirconium beads. Our standards are processed the exact same way as the unknown samples. Both the standards and specimen processing controls are amplified to within 0.1 Ct (+/-) over hundreds of standards, controls, and calibrator runs. Therefore, we feel the cell lysis, and DNA removal from the filters is highly reproducible. With the use of the silica/zirconium beads, the PC filter is actually almost completely shredded by the bead beating. Therefore even if the filters are rolled, the shredding of the filter by the beads ensures that all surfaces of the filter were contacted and scrubbed by the bead.

L163. Addition of suspended cultures to the tubes is not good controls to assess filter bound cell lysis. Under these conditions the greatest challenge is lysing cells adhered to filters. It was necessary to include the Lactococcus from the filtering step to use it as a control for cell processing and as a measure of DNA extraction efficiency.

RESPONSE: We did add the standards/control cells to the filter, it was just inside the tube and not on the filtration manifold so as to prevent any cross contamination. We observe highly repeatable recovery from our standards and specimen processing controls, so we are confident in our approach.

We are confident that all cells are lysed during the bead beating step since the filter is shredded during the processing, and since both bead beating and heating via the hotstart step of the QPCR are used to make sure all cells are fully lysed. We use the same cell standard as our target, which is filtered and processed in the same manner as the samples. From historical averages, we have not seen any significant differences between standard filters for the bead beating. The ct values between 10 replicate filters vary by less than 0.3 Ct, indicating that all of the cells are efficiently lysed by the method and the DNA is available for amplification.

L136. The spiked samples are not evident in Table 1 as indicated in this sentence.

RESPONSE: DONE. Sorry for the confusions here, the first column of the table is clearly marked (spiked with sewage effluent) at the bottom.

L212. Should be "...though..." not "thought" RESPONSE: DONE

L227. Is my interpretation correct, that cultivation results were based on averages from 10 different independent measures whereas QPCR was based on just the values calculated by the research lab? Basically one measure was highly replicated and the other was not. Is this a fair comparison?

RESPONSE: There were 5 laboratories that conducted the culture based methods, and these laboratories conducted most of their analyses in singlicate. The QPCR analyses were conducted in duplicate. By the time qualified values were thrown out from the laboratories conducting the culture based methods, we had triplicate usable results for each sample. We have repeated the comparison using only a single laboratory (the laboratory where the QPCR was conducted) versus the QPCR based results and the results are the same, but since the qualified values from this lab have to be thrown out of the analysis, the power of the correlation analysis is reduced due to the remaining sample size. Since the results are not different, we prefer to use the analysis that has more statistical power.

L242. The terms "strong" and "highly" correlated are a matter of opinion and should not be used extensively in description of results. In my opinion highly correlated is greater than 0.95 but that is also just an opinion.

RESPONSE: We agree and have softened the use of these adjectives for statistical description.

L255-259. Include n for the number of samples used. If you did replicates for each sample then how do you get just one COV? Is this an average of average or based on just a single sample?

RESPONSE: The number of samples used was 54, the samples were run in triplicate, so each set of triplicate values produced a COV, and those COV were averaged to generate one COV estimate for the entire dataset. This is now clearly stated in Table 3.

L312. Should be "...specific to..." not "too" RESPONSE: DONE

L332. Should be "...extraction..." not "extracted" RESPONSE: DONE

Discussion. With proper experimental design many of the unanswered questions in the discussion could have been answered. To truly compare QPCR to standard methods there is insufficient data to draw conclusions at this time and more research is needed.

RESPONSE: We respectfully disagree with this comment. There are multiple questions raised in the discussion that are beyond the scope of an "equivalency test". These two studies were designed to determine whether QPCR for Enterococcus and E. coli yielded quantitative results that were equivalent to existing culture-based methods. In the absence of epidemiology studies, a main question of QPCR methods is whether they are equivalent to existing methods. The questions raised about inhibition, and implementation hurdles are extremely important in the development of rapid methods, but we argue that assessing equivalency is a first step in this process. To appease the reviewer, we have softened the tone of our final conclusions (lines 390-395) and we have altered the wording of "alpha" and "beta" testing so as to convey that our work is simply a step in the development and implementation of rapid methods and not the final word on rapid methods.

Table 1. The numbers do not always add up for Q-PCR. If the samples were not used for Q-PCR analyses were they relevant to this study? It is also a little confusing that there are a number of samples listed as QPCR extracted for beta testing but the QPCR results presented in subsequent tables only list results from bead beating. Is the error in Table 1 or Tables 2 & 3.

RESPONSE: We have several errors in this that have now been fixed, so the numbers are consistent. We thought that it was important to convey that an honest stab was given by the water quality agency personnel to conduct the DNA extraction. But because they were always uncomfortable with, and inaccurate with their pipetting during this processing, the standard curves generated were not of the quality that we required to conduct the quantification. We now describe this more fully in the discussion.

Table 2. Can actual numbers be listed in a supplement? This would give others interested in these approaches an idea of the magnitude they can expect in samples.

RESPONSE: Most of the raw data is available in the Griffith et al. 2006 and 2007 paper. The authors would have to gain approval of the individual laboratories participating in order to do this.

Table 4. This table is not readily understood. Is there a better way to present this information?

RESPONSE: This table has been deleted.

Figure 2-9 (L469-495) descriptions should begin with more descriptive title. Example, Comparison between Q-PCR gene copy numbers and EPA 1600 CFU.

RESPONSE: DONE

Figure 1. Not necessary; should not be difficult to describe in text.

RESPONSE: We have deleted this figure as requested.

Figure 2 & 3 labels on figure look incorrect. Additionally, these two figures should be combined into a single figure for easy comparison.

RESPONSE: DONE

Figure 4-9. Should be combined into logical groups for readers to easily compare results. RESPONSE; DONE there are now only 4 figures.

REVIEWER 3:

Comments:

Noble et al describe a study in which they have compared QPCR methods with traditional EPA-approved methods for the quantitative detection of *E. coli* and *Enterococcus* spp. in recreational waters. The results indicate that 60 to 70% of the variability observed in QPCR data was also observed in the data from the traditional methods and that in 88% (*Enterococcus*) and 94% (*E. coli*) of the cases the decision made by the beach manager would be the same when QPCR results would have been used. Up till now, not many studies have compared traditional methods with QPCR methods for the detection of *E. coli* and *Enterococcus*, and, therefore, are of interest to the readers to AEM. However, the conclusions made on basis of these results are not always justified, the papers contains redundant information and experiments and the statistical analysis can be improved. Overall, I suggest to rewrite the paper in a short-note format.

Major comments

1. The beta testing part should be omitted from the paper, because:

- one laboratory with little previous experience with molecular methods was tested. No general conclusions can be made on basis of the results from only one less-experienced lab. A round robin test, involving both experienced and non-experienced molecular labs, must be conducted to conclude if these molecular methods can be easily implemented.
- Moreover, as long as training and coaching of personnel that have no experience with molecular methods is adequate, they can implement the methods. Consequently, the authors have studied if they can adequately train non-experienced personnel instead of proven that these methods can be implemented in a non-experienced laboratory.
- The results from the beta testing experiments were similar to results from the alpha testing and as such they do not provide any new information.

RESPONSE: We respectfully disagree with these thoughts and feel that the testing done by the water quality agency personnel is an important part of our study. In the implementation of rapid methods, there will have to be training sessions for all water quality personnel involved. The training that we conducted for this group was rapid (less than 3 days), and they conducted all of the analyses without our assistance. To help find a compromise for the thoughts presented by this reviewer, we have changed the title of this study to " QPCR comparison study conducted by water quality

agency personnel", or similar words throughout the manuscript and we no longer refer to it as "Beta testing". Therefore, we are now conveying that we are now doing an assessment of only one laboratory's ability. Round robin testing was not our objective, and no general conclusions about technology transfer to all water quality laboratories is suggested.

2. The Materials and Methods section contains a lot of redundant information and can be reduced by 50%. RESPONSE; DONE

3. Page 11 line 232-239

- line 232 - 235 gives redundant information and can be omitted.

RESPONSE: DONE

- The authors use both correlation and regression, but these are two different statistical methods. Because the relationship between QPCR results and results from traditional methods were not yet known and thus are two independent parameters, the authors have performed a correlation analysis and not a regression analysis. This should be corrected in the manuscript.

RESPONSE: We conducted Pearson Product Moment analysis and this is now reported on line 247 in the Methods section. The mention of regression analysis has been replaced with best-fit linear analysis. This kind of comparative analysis is useful to assess relative under- or over-estimation of one method versus another, an important concern for rapid methods.

- What kind of correlation analysis was performed: Pearson or Spearman Rho? This information must be included in the Materials and Methods section.

RESPONSE: DONE, see line 247.

- Why were the data log transformed? It is tested with the correlation analysis if the numbers obtained by QPCR are identical to the numbers obtained by the traditional methods, not if log transformed numbers are identical. Therefore, the authors must explain why numbers were log transformed, because log transformation results in loss of data variability.

RESPONSE: The data were tested for normality and all failed, therefore, the data were log transformed and retested for normality and they passed. This is appropriate treatment of microbiological data.

- When were correlations considered to be significant. RESPONSE: This has been added to the statistical calculations section, and to the tables. DONE.

- The r-values of the correlations are given, but the R^2 values hold more information on the amount of variability that is explained by both parameters. Therefore, not the r-value but the R^2 value should be given.

RESPONSE: DONE. We now report R^2 values and discuss their meaning for the reader.

4. Table 4 and 5 can be omitted from the manuscript, because the most important information from these tables is provided in the text.

RESPONSE: DONE, We have deleted these.

5. Page 12 line 271 and in other places in the manuscript

The authors should provide whether differences in r-values are significantly different. In the case on page 12 line 271 the r-value of the beta-testing was 0.84 and for alpha testing 0.83. These differences are probably not significant. In addition, the r-value of 0.83 was obtained with DNA-extraction method, whereas the r-value of 0.84 was obtained by bead-beating. These differences should be considered in the statements made by the authors.

RESPONSE: We have considered these statements and have modified some of the language regarding these values.

6. Page 13 line 283

Although r-values are relatively high, still 30 to 40% of the variability in Q-PCR results were not seen by culturing methods. At least, the authors must state this percentage somewhere in their paper and explain to the reader what it means.

RESPONSE: AGREED, DONE

7. Page 13 line 287

The equivalent decision percentage of 88% means that in 12% the beach manager would have made another decision, resulting in another action (closure vs non-closure of the beach). To my opinion that is a major difference and, therefore, in contrast to the conclusion of the authors, one should be very careful in changing methods. I also would like to read in the paper what the effect is of a different decision by the beach manager on the health risk for swimmers.

RESPONSE: The currently used methods can have similar disagreement rates in many different environments as reported in the literature previously. We have modified the strength of some of our statements regarding this agreement, but the truth is that all of these methods measure slightly different things, so an agreement of 100% is unrealistic and not seen in the natural recreational water environment. We now convey the converse of our statements so that the reader can think about the implications.

8. Page 14 line 314-316

This conclusion is only justified when the authors can exclude that PCR-inhibition occurred. From the previous paragraph I understand that they cannot exclude that inhibition during PCR has occurred. Consequently, they should be much more careful in making this statement.

RESPONSE: WE HAVE REMOVED THIS STATEMENT.

9. Page 15 line 331

If the authors want to stress that some relationships are better, although I doubt if these differences are significant, they should also indicate that some relationships were weaker (Enterolert vs QPCR results)

RESPONSE: Agreed, modifications have been made to subjective text such as this.

10. Page 16 line 350-353.

The authors should be careful in making this statement. 30 to 40% of the variability in the Q-PCR dataset was not observed in the dataset from the traditional methods and in 12% of the cases the beach manager makes another decision when QPCR results are used. Based on these percentages it is too early to conclude that QPCR results can be adopted to replace traditional methods and the authors should weaken their conclusions.

RESPONSE: DONE, we have made strong modifications to all suggestive text such as this throughout the entire manuscript.

11. Figures

The correlation data is presented in both Table and Figure format. This makes the paper unnecessary long. I suggest to omit Figure 2 to 9, because Table 2 provides the necessary results from the correlation analysis.

RESPONSE: DONE. We have remove the best-fit and correlation information from the graphs. We now have 4 figures, making a good compromise between your suggestions and other reviewers. We suggest that it is important for the reader to be able to simultaneously view a comparison of the culture-based and QPCR-based methods of the real data figures for a few instances, and have not created such representation in single figures for the reader.

Minor comments

Page 3 line 55 omit '(E. coli)' RESPONSE: DONE

Page 3 line 57 Please provide references for the directed substrate technologies (RESPONSE: DONE)

Page 3 line 64 replace 'are often clean' with 'the fecal contamination is often over' RESPONSE: DONE

Page 3 line 65-66 replace 'for measuring' with 'to measure' RESPONSE: DONE

Page 3 line 77 some scientist would not consider QPCR one of the most promising methods. It is better to be neutral and state and state 'a promising new method' RESPONSE: DONE

Page 7 line 156 Omit phosphate buffered saline because abbreviation is already explained before RESPONSE: DONE

Page 12 line 270 E. coli in italics RESPONSE: DONE

Page 12 line 275 delete 'the' from was the nearly RESPONSE: DONE

Page 14 line 309 Enterococcus in italics RESPONSE: DONE