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Evaluation of Rapid Microbiological Methods for Measuring Recreational Water Quality

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EXECUTIVE SUMMARY

Public health officials routinely monitor fecal indicator bacteria levels to assess beach water quality. However, present laboratory processing methods require approximately 24 hours for completion and swimmers may be exposed to poor water quality during this time. New, more rapid assessment methods that would allow for same-day water quality warnings are under development, but require rigorous independent testing. This study evaluates new methods being developed by six research groups.

Three research groups utilized Quantitative Polymerase Chain Reaction (QPCR), a genetic method that quantifies a DNA target via a fluorescently tagged probe. A fourth research group used Transcription-mediated Amplification (TMA), another genetic method that relies on a fluorgenic probe for quantification, but targets RNA rather than DNA. The fifth group used Dual-Wavelength Fluorimetry (DWF), a defined substrate (DS) method that utilizes the same fluorogenic substrates as traditional IDEXX methods, but coupled with an advanced optical detection system to produce results after an incubation period of only a few hours. The sixth group used an immunological dipstick method that is designed to provide a binary answer about whether bacterial concentrations in a sample exceed California's single-sample standard. Although the study focused on methods for enumerating enterococci, two of the groups using a QPCR method and the group using DWF also measured *E. coli* in their test samples. The immunological dipstick method targeted only *E. coli*.

Testing involved processing 18 blind samples in triplicate and comparing these results to those obtained by 5 local laboratories that processed the same samples using traditional methods. Test samples included both natural and laboratory-created samples, ensuring method evaluation over a range of concentrations, matrices, and interferences. Each research group processed nine samples consisting of clean seawater inoculated with three different concentrations of laboratory culture, sewage, or urban runoff. Six were ambient water samples from locations throughout southern California and three were blanks. Each method's performance was evaluated with respect to individual sample and average concentration across replicates, the State's water quality standard, and variability among replicates by comparing new method results to results obtained through traditional methods. These evaluations were integrated into an overall assessment to determine if management decisions based on new method results would have been the same as decisions based on traditional methods.

Results from two of the QPCR methods and the TMA method were more than 80% accurate with respect to the State standard for enterococci. These methods also proved consistent in terms of effect on beach management decisions, concurring with decisions based on EPA approved methods results for more than 75% of the samples. Results for one of the *E. coli* QPCR methods were even more promising, with 90% agreement about beach management decisions. This is comparable to the rate of agreement between the two traditional methods used in this study.

The new methods measure different bacterial properties than EPA approved methods and are not expected to produce completely equivalent results. Readiness of the new methods for routine use is a subjective determination that should involve balancing equivalency between new and traditional water quality monitoring methods, with the desire to incorporate new rapid methods into a beach water quality warning system that is presently handicapped by extended sample

processing time. California's Beach Water Quality Workgroup is a collection of water quality specialists that was asked to define the necessary level of equivalency before recommending State certification of these methods. The workgroup identified six applications for rapid indicators, established desired levels of equivalency for each application, and determined that the QPCR and TMA methods appear ready for use in several, but not all, of the applications. However, the workgroup also identified the desirability for additional testing focused on ambient samples and conducted by personnel from local laboratories, rather than the method developers. This additional testing is now ongoing.

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INTRODUCTION

Public health officials monitor levels of fecal indicator bacteria (total coliforms, fecal coliforms and enterococci) in recreational water to protect swimmers from exposure to waterborne pathogens. In California, State Health Department regulations require a minimum of weekly measurements of indicator bacteria at high use beaches from April through October. These regulations further require that the public be advised of possible health risks when any of these bacterial indicators exceed threshold values that were established through epidemiological studies.

Despite California's commitment to protect the public from exposure to contaminated waters, current bacterial growth based methods for measuring microbiological water quality are too slow to prevent exposure to most contamination events. State and federally approved methods for testing recreational waters require an 18 to 96 hour incubation period once a water sample reaches the laboratory. Yet, several studies have shown that temporal changes in concentrations of fecal indicator bacteria occur much more rapidly (Leecaster and Weisberg 2001, Boehm et al. 2002). As a result, swimmers may be exposed to contamination during the sample processing period while warning signs informing the public of possible exposure to waterborne pathogens are often posted after waters are already clean. This time lapse also inhibits tracking of contamination sources, because the signal can dissipate before upstream tracking is initiated. Consequently, lacking a more rapid method, investigators are unable to follow the trail of contamination back to its origin.

Continued advances and improvements in molecular and immunological techniques provide new opportunities for measuring bacteria more rapidly. While current methods rely on bacterial growth and metabolic activity, these methods allow direct measurement of cellular attributes such as genetic material or surface immunological properties. By eliminating the necessity for a lengthy incubation step, some of these methods provide results in less than four hours, a short enough time for managers to take action to protect public health (i.e. post or close a beach) on the same day that water samples are collected.

Molecular and immunological methods have advanced considerably for use in fields such as the food service and hospital industries (Fung 2002, NRC 2004). Effort has recently begun to focus on application of these new methods for recreational water quality testing (Noble and Weisberg 2005). Water testing presents challenges not frequently encountered in other fields, such as complex sample matrices and the presence of other potentially confounding native bacterial species. As such, extensive testing of these methods is needed to ensure that they provide comparable reliability to the culture-based methods they are intended to replace.

In 2004, the Southern California Coastal Water Research Project (SCCWRP) conducted the first comparative testing of new methods for enumeration of fecal indicator bacteria against results produced by five reference labs using United States Environmental Protection Agency (USEPA) approved bacterial growth-based methods (Griffith et al. 2004). Five methods were evaluated. Although none of the new methods produced results equivalent to those of the reference laboratories, several performed well enough for researchers to be optimistic regarding possible implementation in the near future. Testing also revealed areas of concern, including the way

results are affected by constituents of urban runoff in samples or by the presence of high levels of suspended solids, requiring further method development and evaluation.

In response to a favorable showing in the first test, several participants in this study expressed interest in participating in further testing. Additionally, several other groups developing rapid detection technologies have approached SCCWRP to discuss inclusion of their methods in future tests. Toward that end, SCCWRP and the Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET) have developed a cooperative relationship and initiated a second evaluation test in June 2005. This document describes the results of this subsequent testing.

METHODS

The study involved assessing equivalency with traditional water quality monitoring methods through simultaneous processing of water samples using both new and EPA approved methods. Samples processed included both natural samples and laboratory-created samples, to ensure that a range of conditions was evaluated. Laboratory-created samples were included because they offer the ability to control the number of indicator organisms and potentially interfering contaminants present, but they do not completely mimic natural conditions. Environmental water samples were included because they contain complex combinations of interferences that cannot be duplicated in artificial samples, though they offer less control over specific variables that need to be evaluated.

Participants analyzed 54 blind samples consisting of triplicates of each of 18 different test samples. Sample processing occurred over three days, with triplicates of each of six samples processed on each day. Processing occurred over three days because participants identified that 18 samples were the most they could analyze within the four-hour time frame without duplicative equipment and personnel.

Nine of the eighteen samples consisted of laboratory-created samples in a seawater matrix inoculated with differing levels of fecal contamination. Seawater used to prepare these samples was collected from 18 kilometers offshore at a depth of 10 meters in an area known to be free from allochthonous fecal contamination. Three of these samples were inoculated with differing concentrations of laboratory cultures of *E. faecium, E. faecalis and E. coli* previously isolated from the environment. Another three samples were inoculated with differing concentrations of primary wastewater influent from the Orange County Sanitation District (OCSD) in Fountain Valley, CA, and three were inoculated with differing concentrations of urban runoff collected from the Dominguez Channel storm sewer in Torrance.

Six samples were natural samples, including shoreline samples collected at Imperial Beach, in San Diego, CA; Doheny State Beach, in Dana Point, CA; Cabrillo Beach, in Los Angeles, CA; Surfrider State Beach, in Malibu, CA; Paradise Cove, in Malibu, CA; and a freshwater sample from the Tijuana River in San Diego, CA.

The last three samples were various types of blanks. These consisted of sterile phosphate-buffered saline (PBS), uninoculated offshore seawater, and 0.2 μm filtered offshore seawater.

Testing took place June 21-23, 2005. Samples were created or collected between 6 and 9 AM each day and distributed no later than 11 AM. All participants began processing samples at the same time. Samples were processed in numbered order to minimize any concentration differences that might have developed from degradation during sample transport or laboratory holding.

All samples were also processed by five local laboratories: Orange County Sanitation District , Orange County Public Health Laboratory, City of Los Angeles, Los Angeles County Sanitation District, and the City of San Diego, using methods employed in their routine water quality monitoring programs. For enterococci, this included a defined substrate (DS) method, Enterolert™ (IDEXX, Westbrook, ME) and a membrane filtration (MF) method, USEPA

Method 1600 (Messer and Dufour 1998). A DS method, Colilert-18™ (IDEXX, Westbrook, ME), was the only method used to enumerate *E. coli*.

Methods Evaluated

Four types of methods, implemented by six investigators, were evaluated. Several investigators implemented multiple permutations of their method resulting in a total of 11 methods being tested.

The first method, quantitative polymerase chain reaction (QPCR), was implemented by three research groups using nine permutations of a similar approach. The basic steps included capturing bacteria on a filter and then using either bead beating, or bead beating coupled with chemical treatment to lyse the cells and release the target Deoxyribonucleic Acid (DNA). The DNA was then purified or used directly in a quantification step, where the DNA was simultaneously amplified and measured using a system of target specific primers and fluorescent probes. The differences among the methods generally involved the primers and probes used or the methods used to release and capture the bacterial DNA from the target cells. Table 1 reflects the time required for each method.

The University of North Carolina (UNC) performed three QPCR method permutations and applied them to both *E. coli* and enterococci. The first two methods differed only in the way the samples were prepared. In the UNC Extracted method, filters were prepared using a commercial DNA extraction kit that included bead beating of the filter and DNA capture and purification. In the UNC Bead Beaten method, the UNC team used bead beaten samples provided by the USEPA National Exposure Research Laboratory (NERL) team that were not subjected to the DNA concentration and purification step. The UNC Alternate Cycling Time method was identical to the UNC Bead Beaten method, except that it used a longer annealing time. The third method was performed on frozen DNA extracts the following week, but the results were submitted prior to unblinding of the test samples. Appendix A contains a detailed protocol for each of these permutations.

The USEPA NERL performed three QPCR method permutations and applied them to enterococci: The ABI-Taqman method (Haugland et al. 2005), the Omni-TaqMan method, and the Omni-TaqMan method conducted at an alternate temperature. The main difference between the ABI-TaqMan method and the Omni-TaqMan method was in the DNA polymerase used. The ABI-TaqMan method uses AmpliTaq™ DNA polymerase and reagents along with TaqMan™ probes (Applied Biosystems, Foster City, CA). The Omni-TaqMan method uses OmniMix™, a freeze-dried all-in-one polymerase and reagent system containing TaKaRa DNA polymerase (Cepheid, Sunnyvale, CA) and the same TaqMan probe described previously. Each method requires a separate set of thermocycler temperature settings and cycling times to achieve maximum efficiency. The Alternate Temperature Omni-TaqMan method was identical to the Omni-TaqMan method except for the temperature setting of the thermocycler was raised to 66°C. The Alternate Temperature Omni-TaqMan method was performed on frozen samples that were processed the following week, with results reported prior to the unblinding of the test samples. Details of these protocols have been included in Appendix A.

USEPA Region 1 (R1) performed three QPCR method permutations applied to both enterococci and *E. coli*. The R1 permutations differed only in the manner in which the result was quantified.

The first permutation was Absolute Quantitation, in which the gene copy number in each sample was interpolated to cell counts via a standard curve created using DNA standards. The second permutation was Adjusted Absolute Quantitation, which differed from Absolute Quantitation by assuming an altered number of gene copies per cell. The third permutation was Relative Quantitation, which used a standard curve generated from actual cell suspensions rather that a DNA standard to interpolate cell concentrations in samples. Appendix A provides a more detailed explanation of the different techniques used to calculate cell concentrations for esch permutation.

The second method type was transcription mediated Amplification (TMA), which was performed by Gen-Probe and applied to enterococci. TMA is similar to QPCR in that it amplifies a genetic target in the bacteria and uses a fluorescent probe for detection (Piersimoni et al. 2002), but differs in that it is a single-primer isothermal method that targets RNA rather than DNA. As with QPCR, bacteria are first captured on a filter. The cells then undergo enzymatic lysis to release the target 23SrRNA molecules, which are hybridized with species-specific probes and subsequently captured by magnetic beads. Following capture, a powerful magnet is used to separate the bead-bound RNA from the other constituents of the sample and a small portion of the captured target material is subjected to TMA, which is an isothermal reaction that incorporates reverse transcriptase and polymerase enzymes to amplify the 23SrRNA gene.

The third class of method was dual-wavelength fluorimetry (DWF), which was conducted by Rosewood Industries and applied to both enterococci and *E. coli*. DWF relies on the same sugar-fluorophore substrate as is used in the commercially available IDEXX™ Enterolert® and Colilert-18® assays, but advances the detection process. Through use of a novel fluorometer, the method simultaneously measures the rate at which bacteria take up the chromogenic substrate as well as the rate at which the fluorescent byproduct of substrate metabolism appears. This ratiometric measurement allows detection and enumeration of target bacteria in considerably less time than required for the visual color change detection used by IDEXX.

The fourth method type was an immunological dipstick manufactured by Silver Lake Research. In this method, sample water is combined with liquid growth media and incubated for 4 – 6 hours at constant temperature on an orbital shaker. Once incubation is complete, the dipstick, which contains antibodies specific to *E. coli* is immersed in the growth media. This method produces a binary answer. If the original concentration of *E. coli* was greater than 400 per 100 mL of water, then a black bar becomes visible on the dipstick, indicating a positive result.

Data Analysis

Results from the new methods were compared to those from the traditional methods employed by the reference laboratories in several ways. First, we assessed the number of individual samples from each new method that differed by half a log unit from the reference laboratory median. Half a log unit was selected because previous laboratory intercalibration studies have demonstrated that this is the typical range of variability observed for traditional methodologies,

both within and among laboratories (Noble et al. 2004; Griffith et al. in press). For this analysis, blank samples were counted as outside of range when values exceeded 50 cells/100 ml.

Second, results were evaluated for false positives and false negatives relative to the State of California standard of 104 cells/100 ml for enterococci and 400 cells/100 ml for *E. coli*, as the State requires posting warning signs for any sample that occurs above this level. The decision of whether a sign should have been posted that day, against which the new methods were being evaluated, was based on the median concentration for that sample as measured by the reference laboratories.

The third analysis assessed precision of the measurements, which we could accomplish because each sample was processed three times as blind replicates. Precision was quantified as coefficient of variation (CV) and was compared between the new methods and the reference laboratories. In all cases, the CV was calculated using the standard deviation and mean across all sample types for each method or reference laboratory. For purposes of data analysis, qualifiers (< or >) were ignored and only the numerical value was used.

The fourth analysis assessed the variability of the methods through linear regression across all samples. Each method was compared with the grand median of the reference laboratories and with each of the other methods. Samples were compared using both individual replicates and by averaging the results from the three corresponding replicates from each of the eighteen sample types. Linear regression was also used to compare each method across a range of concentrations within inoculant type (cultured-cells, sewage, and urban runoff), allowing us to assess the correlation when the percentage of cultivable and non-cultivable cells was held constant.

The final analysis was an integrated evaluation designed to discern how often the results from the new tests would result in a public health officer making the same or a different decision regarding issuing a public health warning. Here triplicate results from each sample processed by new methods were compared to those of the reference laboratories and categorized as "Equivalent", "Not Materially Different", or "Materially Different" than traditional water quality monitoring methods.

To be considered equivalent to current methods, a sample had to exhibit the following characteristics:

- 2 of 3 replicates and the median were correct with respect to the AB411 standard
- 2 of 3 replicates were within ½ log unit of reference lab median
- Replicate exhibited a smaller variance than worst reference lab

To be deemed materially different than current methods the criteria were:

- 2 of 3 replicates were incorrect with respect to standard
- Median value differed by >1/2 log unit from reference lab median
- Coefficient of variation is twice that of the worst reference lab

Samples deemed "Not materially different than current methods" failed none of the materially different criteria, but did not meet equivalency criteria.

Each of these evaluations were also performed for the traditional measurement methods by using DS as a reference for MF, and vice versa. This provided context for the certainty of EPA approved methods within which to interpret viability of the new methods. This analysis was only possible for enterococci, though, as the reference labs used only DS to measure *E. coli*. The Tijuana River samples were excluded from the comparative analysis for *Enterococci* Disagreement between the two growth-based methods made it impossible to determine whether the sample was above or below the AB411 standard.

The time elapsed between samples being provided and results submitted was also quantified. A target of four hours or less was established, as this turnaround time allows beach managers to collect samples in the morning and post warning signs by noon if bacterial concentrations are elevated.

RESULTS

Enterococcus Measurement Methods

QPCR: USEPA NERL

The NERL Omni-mix method required approximately 2.5 hours to complete and the ABI method took approximately four hours (Table 1). All of their methods produced similar results with respect to the AB411 Standard, producing the correct answer for 78-84% of the samples (Table 2). The Omni-TaqMan method exhibited the lowest rate of false positive results among the three, but had some false negative results, whereas the other two methods had none (Table 3). The percent of results that were > ½ log above the median value of traditional methods was also similar among the three NERL methods. The ABI-TaqMan method exhibited the lowest rate (29%) and the Alternate Temperature Omni-TaqMan method the highest (41%) (Table 4). This did not change significantly when the average of the replicates was used instead of the individual replicates (Table 6). When results were outside of the half-log unit range, they were typically higher than the results from traditional methods.

The precision among replicates varied among the three NERL QPCR methods. The Omni-TaqMan method were almost as precise as current methods. The other two NERL methods were not as precise, exhibiting similar precision across four of five categories of samples (Table 6).

The Alternate Temperature Omni-TaqMan method was best correlated with both traditional methods, explaining 60% and 58% of the variability for MF and DS, respectively (Table 7). Averaging results among the three replicates from each sample improved the r-squared values slightly for the ABI-TaqMan and Omni-TaqMan methods (Table 8).

The Alternate Temperature Omni-TaqMan method produced results that would have resulted in a public health officer making the same management decision that would have been made if the measurement had been derived using traditional methods for 71% of samples. The ABI-TaqMan and Omni-TaqMan methods would have resulted in the same public health decision for 65% of the samples (Table 9). Results measured by the Alternate Temperature Omni-TaqMan method were equivalent to current methods for 53% of samples, while the ABI-TaqMan and Omni-TaqMan results were equivalent for 47%.

QPCR: UNC

The UNC Bead Beaten method produced results in 2.5 hours, whereas the Extracted method required 3.5 hours (Table 1). All methods performed by UNC exhibited similar overall accuracy with respect to the AB411 Standard for *Enterococci* (Tables 2 and 3). The Extracted method recorded a low rate of false positive results, but a relatively high rate of false negative results on sewage and ambient water samples. The opposite was true for the two UNC methods that employed bead beating. For these methods, researchers experienced difficulty with false positives in both ambient water samples and laboratory created samples spiked with urban run off when levels of *Enterococci* were low. The tendency of the Extracted method to underestimate concentrations of *Enterococci* was also apparent in the number of results it produced that were > ½ log unit below the median value produced by current methods (Tables 4 and 5). The Bead Beaten methods produced similar results to one another, with approximately one-third of samples > ½ log unit above the median and approximately one-tenth > ½ log below.

Precision among replicates was very good for all three UNC methods (Table 6). Overall precision of the Bead Beaten method surpassed that of both traditional methods. The Extracted method was only slightly more variable, except in the case of ambient water samples.

All three UNC methods correlated well with current methods. When calculated using individual replicates, the Extracted method and the Alternate Cycling Time method were more highly correlated with the MF method than the DS method (Table 7). However, when average values from replicates were compared, r-squared all three UNC method were slightly more correlated with MF than DS with r-squared values approaching 0.90 for both the Extracted and Bead Beaten methods and around 0.80 for the Alternate Cycling Time method (Table 8).

The Bead Beaten method produced results that would have resulted in a public health officer making the same management decision as if the measurement had been made with current USEPA approved methods on 77% of samples (Table 9). The Alternate Cycling Time and Extracted methods would have resulted in the same management decision 71% and 53%, respectively. Results from the Bead Beaten method were equivalent to current methods on 53% of samples and not materially different from current methods an on additional 24% of samples. The Alternate Cycling Time method produced slightly lower equivalent results, but was otherwise the similar. The main difference between the Extracted method and the two Bead Beaten methods was its tendency to underestimate when concentrations of *Enterococci* were high.

QPCR: USEPA R1

The USEPA R1 QPCR methods required 11-21 hours before results were available (Table 1). The results overestimated *Enterococcus* concentrations by >1/2 log unit in most of the samples, regardless of which method for calculating the results was employed (Tables 4, 5 and 10). This overestimation resulted in a high percentage of false positive results with respect to the AB411 standard for *Enterococci* (Table 3).

Despite the overestimation bias, the USEPA R1 methods exhibited excellent precision among replicates (Table 6). Overall precision surpassed that of both reference methods. In contrast to the reference methods, variability was greatest for blanks and least for samples inoculated with urban runoff. Despite its repeatability, the method was poorly correlated to either the MF or DS methods across all test samples (Tables 7 and 8). However, when the methods were only compared across different concentrations of the same inoculum, precision increased and correlation improved dramatically (Tables 10 and 11).

Equivalency in terms of beach management decisions between current methods and the USEPA R1 QPCR method was between 10 and 20% (Table 9). This was true regardless of whether absolute or relative quantitation was used to obtain results. Relative quantitation produced 18% equivalency with current methods, adjusted absolute quantitation faired slightly less well, with 12% of results equivalent and 6% classified as "not materially different".

TMA: Gen-Probe

The TMA methods produced results in approximately five hours (Table 1). TMA exhibited a low rate of false positives and a similar rate of false negatives to traditional methods in regard to

the AB411 standard (Tables 2 and 3). This method performed best on samples that contained moderate to high levels of *Enterococci*. However, TMA underestimated levels of enterococci by >1/2 log unit in approximately half of the samples. This underestimation was especially apparent in ambient water samples and laboratory created samples where the proportion of inactive/dead cells to active/live cells might be expected to be higher (Table 4 and 5). This trend was also evident when comparing the different laboratory created samples across concentrations of the same inoculant, where underestimation increased successively in samples spiked with fresh lab cultures, urban runoff and sewage respectively (Figures 1 - 3).

TMA exhibited good precision between replicate samples (Table 6), but did not equal that of the reference methods. The greatest variability was observed for seawater samples inoculated with sewage. Variability was lowest for blanks (zero) and ambient water samples.

TMA was somewhat correlated with both traditional methods (Table 7). MF and DS methods explained 69 and 56% of the variability across samples respectively. However, when results from TMA replicates were averaged rather than taken individually, r-squared values approached 1.0 (Table 8).

TMA produced results that would have resulted in a public health officer making the same management decision as if the measurement had been made with current EPA approved methods approximately 77% of the time (Table 9). Results measured by TMA were equivalent to current methods on 59% of samples and not materially different from current methods on an additional 18% of samples. However, for 23% of samples, TMA produced an answer that was materially different than current methods due to its tendency to underestimate enterococci in certain sample types.

DWF: Rosewood

DWF generally took approximately 5 hours to produce results (Table 1). DWF exhibited a high number of false positive results and a moderate number of false negative results with respect to the AB411 Standard. Consequently, overall accuracy with respect to the standard was low (Tables 2 and 3). Approximately a third of the results produced by DWF were > ½ log unit above the median of the reference labs and approximately the same proportion were >½ log below (Tables 4 and 5). There was no discernable tendency toward overestimation or under estimation among a particular category of type of sample.

Precision of results among replicates was slightly greater than that of traditional methods (Table 6). However, precision varied greatly among sample types. Variability was greatest for seawater inoculated with sewage and least for seawater inoculated with urban runoff.

DWF results correlated poorly with traditional methods (Table 7), explaining only approximately 2% of the MF and DS results. When conducted using averages of replicates, the correlation improved slightly with DS, but not for MF (Table 8).

DWF produced results that would have led to public health officials making the same management decision as if samples had been measured with current methods only 30% of the

time (Table 9). Results measured by DWF were equivalent to current methods for 18% of samples.

E. coli Measurement Methods

QPCR: UNC

The two UNC methods for *E. coli* produced results in the same time frame as the UNC methods for enterococci (Table 1). Their accuracy with respect to the California AB411 Standard ranged from 74 – 85% (Table 12). The Extracted method exhibited a low rate of both false positives and false negatives (Table 13). False positives for this method were limited to seawater inoculated with sewage, while false negatives occurred only in ambient water samples. The false positive rate for the UNC Bead Beaten method was approximately four times that of the Extracted method, with false positive results present in three of five sample categories. False negative results for this method were very low and were limited to ambient water samples.

The proportion of samples > ½ log unit above and below the reference lab median followed the same pattern as the false positive and false negative rates for each method (Table 14). More than half of all results from the Bead Beaten method, or approximately ten times the percentage observed in the Extracted method, were > ½ log unit above the reference lab median. The percentage of samples > ½ log unit below the reference lab median was similar for both UNC *E. coli* methods. Substituting the average of the results from the three replicates had minimal effect on the number of samples above or below the target range (Table 15).

Precision among replicates for the Extracted method was relatively good, but still approximately twice approximately that of the DS (Table 16). The Extracted method was most precise on seawater inoculated with lab-cultured *E. coli* and least precise on seawater inoculated with urban runoff. The Bead Beaten method was less precise than the Extracted method on all sample types, except seawater inoculated with sewage.

Neither of the UNC QPCR methods for *E. coli* correlated well with traditional methods or with each other (Table 17). The DS method accounted for only 16% of the variability observed in the Extracted method and 51% of the variability in the Bead Beaten method. Averaging the results from the three replicate samples dramatically improved the correlation between the two UNC methods, but the correlation between the methods and DS improved only slightly (Table 18).

The UNC Extracted QPCR method for *E. coli* produced results that would have resulted in a public health officer making the same management decision as if the measurement had been made with traditional methods for almost 90% of the samples (Table 19). Results measured by the UNC Extracted method were equivalent to current methods 67% of the time and not materially different from current methods an additional 22%. In contrast, the UNC Bead Beaten method for *E. coli* produced results materially different from the reference method approximately 70% of the time.

QPCR: USEPA R1

The R1 QPCR methods for *E. coli* required the same time as the R1 methods for enterococci (Table 1). Accuracy of the 3 R1 QPCR methods with respect to the AB411 standard ranged

from 78 - 85% (Table 12). The false positive rate for the R1 Absolute Quantitation method was roughly four times that of the false negative rate across all samples (Table 13). False positives for this method occurred in all categories of sample except seawater inoculated with urban runoff, while false negatives were limited to ambient water samples. By comparison, the Adjusted Absolute Quantitation and Relative Quantitation false positive rates were near zero, but false negative rates increased by a factor of approximately three for these methods as compared to Absolute Quantitation.

More than 60% of results calculated by Absolute Quantitation were > ½ log unit above the median values produced by the reference labs (Table 14). This was consistent across all sample types, except seawater inoculated with laboratory-cultured cells. The other two R1 methods exhibited low rates of results > ½ log unit above the reference lab median, but also a dramatic increase in false negative results as compared to Absolute Quantitation. Averaging the values of the replicates did little to change the percentage of results > ½ log unit above the reference lab median for any of the three methods, but did lower the percentage > ½ log below the median for Relative Quantitation (Table 15).

The three R1 methods demonstrated excellent precision between replicates, meeting or exceeding that of the traditional method for two sample types (Table 16). Absolute Quantitation was the least variable, followed by Adjusted Absolute Quantitation, and Relative Quantitation respectively. Most of the variability between replicates followed the same trend for all three methods, with the highest variability occurring for replicates inoculated with urban runoff.

None of the R1 methods were well correlated with the reference laboratories (Table 17). DS explained 54% and 62% of the variability in Adjusted Absolute Quantitation and Relative Quantitation respectively, but only 19% in Absolute Quantitation. Substituting average values from the replicates slightly improved the relationship between the DS and the two more correlated methods, but did not change the relationship between DS and Absolute Quantitation (Table 18).

Equivalency in terms of beach management decisions varied greatly among the three R1 methods for *E. coli* (Table 19). Relative Quantitation produced results that would have led to the same management decision 72% of the time, while results produced by Absolute Quantitation were materially different than that from DS for more than 60% of the samples. Adjusted Absolute Quantitation would have produced the same outcome as DS for slightly more than half of samples.

DWF: Rosewood

The DWF method for *E. coli* produced results in approximately 5.5 hours (Table 1). DWF produced a correct answer with respect to the California AB411 Standard for 61% of the samples (Table 12). DWF exhibited a high rate of false negative results, especially on seawater inoculated with laboratory cultures and ambient water samples (Table 13). False positive results were limited to seawater inoculated with urban runoff.

Similarly, DWF exhibited results that were > ½ log below the median value produced by the traditional method for more than half of the samples (Table 14). The tendency to underestimate

concentrations of *E. coli* was especially apparent for seawater inoculated with laboratory-cultured cells or sewage, and to a lesser extent, ambient water samples. Averaging the results of the three replicates did not change this outcome (Table 15).

Overall precision of DWF among replicates was less than that for traditional methods (Table 16). DWF exhibited the best precision on seawater inoculated with sewage, while variation among replicates was highest for ambient water samples.

DWF was poorly correlated with the reference method for *E. coli* (Table 17), explaining only 36% of the variability seen in DS. Averaging results from the three replicate samples had little effect on this result (Table 18).

DWF produced results that would have resulted in a public health officer making a different management decision than if the measurement had been made traditional methods for approximately 60% of samples (Table 19). Results measured by DWF were equivalent to current methods 22% of the time and not materially different from current methods an additional 17%.

Immunological Dipstick: Silverlake Research

The Immunological Dipstick method produced results in 5.5 hours (Table 1). Overall accuracy of the Immunological Dipstick method with respect to the California AB411 Standard for *E. coli* was approximately 60% (Table 12). This was primarily due to false negative results for seawater inoculated with laboratory cultures or sewage.

At least two of three binary results from replicates agreed with the median value produced by the reference method approximately 60% of the time (data not shown). However, the binary nature of the results produced by this method made it impossible to perform numerical comparisons with traditional methods.

DISCUSSION

Several genetic methods exhibited dramatic improvement as compared with the previous evaluative testing of rapid indicator methods conducted in 2004 (Griffith et al. 2004). Of these, TMA and the QPCR methods developed by USEPA NERL and UNC in conjunction with Cepheid performed best in terms of agreement with routine EPA approved water quality monitoring methods. The QPCR methods were faster on average than was TMA. This was especially true of those QPCR methods that employed only bead beading prior to the amplification step.

The bead beating QPCR methods were among the most accurate with respect to the State's *Enterococcus* standard and scored well in terms of equivalency to traditional methods in regard to making beach management decisions. However, bead beating QPCR methods tended to overestimate enterococci compared to traditional growth-based methods. This overestimation is consistent with previous applications of QPCR methods to environmental water samples and probably results from QPCR being unable to differentiate between target DNA in cultivable and non-cultivable cells (Dupray et al. 1997, Brinkman et al. 2003, Frahm and Obst 2003, Griffith et al. 2004). This explanation is consistent with the greater observed overestimation for urban runoff inoculated samples than for those inoculated with sewage or cultured cells, which are fresher fecal sources and more likely to contain a higher percentage of cultivable cells. Though detection of non-culturable cells is likely the most important factor in the overestimation, another contributing factor may be variability in the number of copies of the target gene in different enterococcal species (Oana et al. (2002) demonstrated that enterococci may contain between 2 and six copies of 16S and 23S rRNA genes and that this number varies between enterococcal species. Thus, results from QPCR enumeration of enterococci may vary depending on the species composition of enterococci in a given sample.

The bead beaten QPCR methods exhibited more variability between replicate samples than did growth-based or other QPCR methods. One explanation may be the manner in which samples were processed prior to amplification. Unlike the USEPA R1 and UNC Extracted methods, the Bead Beaten methods do not include DNA purification as part of sample preparation. Instead, once cells are broken open by bead beating, the crude cell lysate is used directly in the QPCR reaction. Omitting a DNA purification step can have both beneficial and deleterious effects. On the positive side, transferring the cell lysate directly to the QPCR reaction eliminates the possibility of sample loss during the extraction process and reduces the time needed to obtain results, as DNA purification techniques added approximately an hour to sample analysis. However, using unpurified sample DNA may introduce environmental contaminants such as humic acids that are can inhibit DNA amplification and detection, potentially increasing variability (Kreader 1996).

The UNC Extracted Method for enterococci was among the most accurate of all methods when concentrations of enterococci were low, but had a marked tendency toward underestimation when bacterial concentrations were $>10^3/100$ mL. One possible explanation for this is poor DNA recovery during purification, as underestimation increased with increasing concentration. In this method, DNA is captured on spin-columns which have a finite limit as to how much DNA can be bound to their membranes. DNA that is not bound to the capture membrane is lost. While further research will be necessary to confirm this possibility, this method was still able to

produce repeatable results at concentrations near the State standard, which is the range of most concern to beach managers.

While the UNC Extracted method performed reasonably well for enterococci, it outperformed all other methods in this study for measurement of *E. coli*. Results were deemed equivalent or not materially different than traditional methods for making beach management decisions for almost 90% of the samples. Despite this high performance, the UNC Extracted QPCR method for *E. coli* encountered some difficulty with underestimation for one set of ambient samples. This underestimation may result from interference with the QPCR reaction by environmental contaminants such as humic and fulvic acids, which are known to interfere with amplification of DNA by PCR (Kreader 1996). Although the DNA extraction steps in this method are designed to minimize or limit the presence of contaminants, they may not be fully effective.

The USEPA R1 methods were the most repeatable of the QPCR techniques, but they severely overestimated levels of both *E. coli* and enterococci. In the case of enterococci, this is unlikely to result from specificity of their primers, which were based on the same Ludwig and Schliefer assay used by the other QPCR method developers. A more likely explanation is systematic error in the calibration or calculation steps. The USEPA R1 methods were the most repeatable of any evaluated in the study. They also had the highest correlation with traditional methods when compared across several concentrations within an inoculant category (Table 6). High precision and correlation to traditional methods, yet with a significant overestimation bias, suggests a problem with calibration.

Over-estimation of enterococci levels in the samples was attributed by the USEPA R1 group to be due to possible differences between the actual number of genomic DNA molecules and target gene copies present in each enterococci cell isolated from the environmental samples and the *Enterococcus* DNA standards or calibrator cell samples used for the Absolute and Relative Quantitation Methods, respectively. The group discovered in experiments conducted subsequent to this study that the DNA recovery from the relatively "clean" calibrator samples was 50% lower than that of the "dirty" environmental samples. The resulting underestimation of the actual DNA recovery from environmental test samples was thought to be responsible for at least a two-fold over-estimation of concentrations of enterococci in the these samples.

While the USEPA R1 methods were designed to achieve a result in less than 4-h, several factors combined to prolong the time needed to complete analyses conducted during this study. The first limiting factor was sample filtration. Due to the design of the integrated 12-filter manifold (Millipore Corp., Bedford, MA) employed, filtration could proceed only as fast as the slowest filtering sample. AThis was dictated by the design of the manifold, which required that all filters be removed from the manifold as a batch. The use of individual filter units, would have improved filtration performance and disposable filter units may have reduced incidences of cross-contamination between samples and blanks. A second factor was the bead-beating and subsequent DNA extraction of 32 samples (18 samples plus extra optional extraction controls) in the MagNA Pure LC platform. Although this step produced very clean target DNA and likely enhaced the repeatability of the results, it required approximately two and a half hours. The third limiting factor was the availability of only one thermocycler to conduct the QPCR analysis. The LightCycler instrument upon which the QPCR analyses were run during this study could analyze

only 32 PCR reactions per run cycle, with each run taking about 3-h from set up to completion, but running assays for *E.* coli enterococci and associated controls required three runs. Access to a second LightCycler PCR instrument would have shortened the time from sample provision to result reporting by permitting the simultaneous running of the *E.coli* and *Enterococcus* QPCR assays in parallel rather than in series.

TMA was the most accurate of the genetic methods with respect to the State standard for enterococci. This method also performed well in the integrated evaluation, but tended to underestimate concentrations of enterococci, which is the opposite problem from most of the QPCR applications. There are several possible explanations for this, the most likely of which is that TMA targets cellular Ribosomal Ribonucleic Acid (rRNA) rather than Ribosomal Deoxyribonucleic Acid (rDNA). Levels of cellular rRNA are known to vary dramatically depending on the physiological state of the cell. In general, rapidly dividing bacteria harbor copious amounts of RNA used to produce the proteins needed for metabolic activities and to pass on to daughter cells (Brock et al. 1994). Fecal bacteria in a hostile environment, such as low temperature seawater, could be expected to divide very slowly, if at all, and would therefore be likely to contain less measurable RNA on average than the recently growing cultured bacteria used for calibration of the method. Thus, bacteria that are injured or in shock due to environmental factors such as temperature or salinity may contain less RNA than an average healthy cell, even though they may later be revived and enumerated in growth-based methods. Empirical evidence suggesting that the physiological state of the target bacteria played a role in underestimation of enterococci concentrations can be seen in the results from the laboratory created samples. TMA underestimated enterococci concentrations only slightly for samples inoculated with laboratory cultures or primary sewage influent, where the fecal material was only hours old, but underestimated by almost an order of magnitude for the urban runoff inoculum, in which the age of fecal material and physiological state of the bacteria was less certain (Figures 1-3). However, since this method is designed for use on true environmental samples, rather than the laboratory created samples from which these curves were created, the calibration of the method may partially explain these differences.

A second factor that would cause underestimation is that the TMA targets Group 2 and Group 3 enterococci, which include *E. faecalis* and *E. faecium* and are the dominant species of enterococci found in the human gut (Geldreich et al. 1978). This high level of specificity is designed to minimize cross-reactivity with non-enterococcal organisms. In contrast, traditional growth-based methods target a larger set of enterococcal species. However, speciation was conducted on isolates measured by traditional methods from samples used in the evaluation testing and the two species targeted by TMA consistently accounted for the majority of the enterococci in the test samples (Table 20). While this difference is real, in most cases it would only be a small contributor to the underestimation of TMA relative to traditional methods.

A third factor that may have affected the TMA results is contamination by laboratory dust. During the first day of testing, a large number of observers were present and there was extensive use of an outside door near where this method was being implemented. The TMA method is thought to be sensitive to airborne particles, which can inhibit the efficiency of nucleic acid amplification. While we cannot quantify this potential inhibition, the possible presence of airborne contaminants in the laboratory should be considered when contemplating use of this

method in future applications. This is especially important since many environmental microbiology laboratories may not meet the cleanliness standards found in the clinical laboratories where TMA is more routinely used.

DWF performed poorly compared to most other methods and to its own performance in testing conducted the previous year (Griffith et al. 2004). The performance of this method was hampered by both software and equipment problems. The software failed on the first day of testing. Repairs were made the following morning, but this required that the first day samples be held overnight before they could be run. As a result, we could only make a comparison to DS results from one laboratory that kindly volunteered to perform an additional analysis specifically for this purpose. The second day results were much improved, but on the third day, a hardware failure again compromised the results. A forensic investigation of the analytical instrument identified a voltage problem that produced spikes in the signal coming out of the detector. Further, testing will be necessary before an accurate evaluation of this method is possible.

The Immunological Dipstick method performed best on ambient samples, recording no false positives or false negatives. However, this method exhibited considerable difficulty with laboratory created samples, particularly those inoculated with cultured cells. On the surface, these samples should be simpler as they have less potential interference from the matrix or from other bacterial species. This method has great appeal because it could potentially be used to make water quality decisions in the field. However, to reach its potential, more research will be necessary to assess why there were false negatives for so many of the created samples.

In the overall assessment, methods performed well enough for researchers to be optimistic regarding possible implementation in the near future. In particular, results from the UNC QPCR, the NERL QPCR and the GenProbe TMA methods would have led to the same beach management decisions for more than 75% of the enterococci measurements. While this implies an error rate of approximately 25%, it must be considered in context of the error rate for traditional methods. Traditional methods are highly variable, with a typical confidence interval that is 50% or more of the measured value (Griffith et al. in press, Noble et al. 2004). In this study, we used both DS and MF as traditional methods for the enterococci samples and found that results from the two traditional methods would have resulted in a different management decision for 11% of the samples. Thus, the error rate for the new methods is only approximately twice that of traditional methods and is likely to improve as the developers learn where biases exist from evaluations such as this one.

For the UNC Extracted *E. coli* method, the results were even more promising, with almost 90% agreement in terms of beach management decisions, comparable to the rate of agreement between the two traditional methods.

The new methods measure different bacterial properties than traditional methods and are not expected to produce completely equivalent results. A preferred approach to method validation is to conduct epidemiological studies that establish a relationship between new method measurements and health risk, but this is a lengthy and expensive process. It potentially requires modification of the State's bacterial standards based on the results of such studies. Assessing equivalency is a more expeditious approach for incorporating rapid methods into a warning system that is presently hampered by extended processing time.

Thus, readiness of the new methods for routine use is a subjective determination that involves balancing equivalency between new and traditional methods with the desire to incorporate new rapid methods into a beach water quality warning system in need of improvement. To assist with this decision process, we consulted with Beach Water Quality Work Group (BWQWG) of the State Water Resources Control Board, which includes members of the regulatory, public health, scientific and environmental communities, and has historically been instrumental in providing the State of California recommendations regarding approaches to beach water quality monitoring. The BWQWG identified six applications for rapid indicators, including tracking spatial progress and dilution of inland sewage spills as they move toward the beach; re-opening beaches subsequent to a sewage spill; routine beach monitoring; tracking fecal contamination back to its source; National Pollutant Discharge Elimination System (NPDES) regulatory compliance; and tracking trends in beach condition. They established desired levels of equivalency for each application (Appendix B).

The BWQWG determined that the QPCR and TMA methods might presently be ready for use in several, but not all, of the applications (Appendix B). However, they identified the desirability for additional testing focused on ambient samples to ensure that the methods were evaluated under a full range of potential confounding factors. They also expressed preference that further testing be conducted by personnel from local laboratories, rather than the method developers, to ensure that the methods are readily transferable to personnel that would be responsible for their routine use once approved.

In response to these suggestions, supplemental testing for the TMA and QPCR methods has been arranged. Referred to as "Beta testing", this evaluation involves implementation by local practitioners performing traditional methods conducted in parallel with the new methods on 175 samples (Appendix C). This testing is now underway and the results will be returned to the BWQWG in summer, 2006 for their evaluation of whether these methods are ready for State of California approval.

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TABLES

Table 1. Average time needed to complete each method.

Method	Time (hours)
GenProbe	5.0
USEPA R1	15.4
USEPA NERL ABI-TaqMan	3.8
USEPA NERL Omni-TaqMan	2.5
UNC Extracted	3.6
UNC Bead Beaten	2.6
Rosewood	5.5
Silverlake Research	5.5

Table 2. Overall Accuracy in respect to the California single-sample standard for enterococci of 104/100 mL as a percentage of all samples.

Method	Overall Accuracy
Gen-Probe	88
USEPA R1: Absolute Quantitation	65
USEPA R1: Adjusted Absolute Quantitation	61
USEPA R1: Relative Quantitation	67
USEPA NERL: ABI-TaqMan	84
USEPA NERL: Omni-TaqMan	78
USEPA NERL: Omni-TaqMan Alternate Temperature	82
UNC: Extracted	80
UNC: Bead Beaten	82
UNC: Bead Beaten Alternate Cycling Time	82
Rosewood	65

Table 3. Percentage of *Enterococcus* samples for which there were false positive and false negative results relative to the California single-sample standard of 104 ENT/100 mL. The new methods were compared against the grand median of all reference labs. Membrane filtration was compared against the median of DS results and *visa versa.*

Method	All Samples		Blanks		Seawater Spiked with Culture		Seawater Spiked with Urban Runoff		Seawater Spiked with Sewage		Environmental Samples	
	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative
Gen-Probe	5	15	0	0	0	0	17	0	0	44	0	33
USEPA R1: Absolute Quantitation	67	0	67	0	0	0	100	0	0	0	100	0
USEPA R1: Adjusted Absolute Quantitation	100	0	100	0	0	0	100	0	0	0	100	0
USEPA R1: Relative Quantitation	94	0	89	0	0	0	100	0	0	0	100	0
USEPA NERL: ABI-TaqMan	61	0	0	0	0	0	83	0	0	0	100	0
USEPA NERL: Omni-TaqMan	61	0	33	0	0	0	100	0	0	0	66	0
USEPA NERL: Omni-TaqMan Alternate Temperature	50	3	0	0	0	11	100	0	0	0	100	0
UNC: Extracted	11	27	0	0	0	0	33	0	0	33	0	42
UNC: Bead Beaten	50	0	0	0	0	0	100	0	0	0	100	0
Alternate Cycling Time	50	3	0	0	0	0	100	0	0	0	100	25
Rosewood	50	27	0	0	0	56	100	0	0	44	100	0
MF	12	13	0	0	0	0	4	4	0	9	19	16
DS	13	12	0	0	14	0	9	0	0	17	10	14

Table 4. Percentage of *Enterococcus* samples >1/2 log above or below the median of all reference labs. Membrane filtration was compared against the grand median of DS results and *visa versa.*

Method	All Samples		Blanks		Seawater Spiked with Culture		Seawater Spiked with Urban Runoff		Seawater Spiked with Sewage		Environmental Samples	
	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median
Gen-Probe	2	51	0	0	0	44	0	33	0	100	7	67
USEPA R1: Absolute Quantitation	80	0	67	0	56	0	100	0	67	0	100	0
USEPA R1: Adjusted Absolute Quantitation	92	0	100	0	56	0	100	0	100	0	100	0
USEPA R1: Relative Quantitation	82	0	100	0	0	0	100	0	100	0	100	0
USEPA NERL: ABI-TaqMan	29	4	22	0	0	22	33	0	22	0	53	0
USEPA NERL: Omni-TaqMan	33	0	33	0	0	0	33	0	67	0	33	0
USEPA NERL: Omni-TaqMan Alternate Temperature	41	2	0	0	0	11	100	0	56	0	47	0
UNC: Extracted	6	41	0	0	0	100	22	0	0	33	0	60
UNC: Bead Beaten	33	10	0	0	0	22	100	0	22	0	40	20
Alternate Cycling Time	29	12	0	0	0	56	100	0	22	0	27	7
Rosewood	39	35	11	0	0	100	78	0	0	89	80	0
MF	2	1	0	0	0	0	4	4	0	2	8	3
DS	7	2	0	0	0	0	19	2	2	0	2	3

Table 5. Percentage of averaged *Enterococcus* repilcates >1/2 log above or below the median of all reference labs. Membrane filtration was compared against the grand median of DS results and *visa versa*.

Method	All Samples		Blanks		Seawater Spiked with Culture		Seawater Spiked with Urban Runoff		Seawater Spiked with Sewage		Environmental Samples	
	>1/2 log Above Median	>1/2 log Below Median	>1/2 log Above Median	>1/2 log Below Median	>1/2 log Above Median	>1/2 log Below Median	>1/2 log Above Median	>1/2 log Below Median	>1/2 log Above Median	>1/2 log Below Median	>1/2 log Above Median	>1/2 log Below Median
Gen-Probe	6	47	0	0	0	33	0	0	0	100	20	80
USEPA R1: Absolute Quantitation	82	0	67	0	67	0	100	0	67	0	100	0
USEPA R1: Adjusted Absolute Quantitation	94	0	100	0	67	0	100	0	100	0	100	0
USEPA R1: Relative Quantitation	82	0	100	0	0	0	100	0	100	0	100	0
USEPA NERL: ABI-TaqMan	24	0	0	0	0	0	33	0	0	0	60	0
USEPA NERL: Omni-TaqMan	35	0	33	0	0	0	33	0	67	0	40	0
USEPA NERL: Omni-TaqMan Alternate Temperature	47	6	0	0	0	33	100	0	67	0	60	0
UNC: Extracted	6	35	0	0	0	100	33	0	0	33	0	40
UNC: Bead Beaten	29	6	0	0	0	0	100	0	0	0	40	20
Alternate Cycling Time	35	12	0	0	0	67	100	0	33	0	40	0
Rosewood	35	35	0	0	0	100	67	0	0	100	80	0
MF	6	0	0	0	0	0	33	0	0	0	0	0
DS	18	0	0	0	0	0	33	0	0	0	0	0

Table 6. Average coefficient of variation for *Enterococcus sp.*

Method	All Samples	Blanks	Seawater Spiked with Culture	Seawater Spiked with Urban Runoff	Seawater Spiked with Sewage	Environmental Samples
Gen-Probe	44	0	79	66	42	39
USEPA R1: Absolute Quantitation	19	31	29	12	18	13
USEPA R1: Adjusted Absolute Quantitation	22	46	29	12	18	13
USEPA R1: Relative Quantitation	20	47	14	12	19	14
USEPA NERL: ABI-TaqMan	62	116	45	60	43	50
USEPA NERL: Omni-TaqMan	38	65	35	20	39	36
USEPA NERL: Omni-TaqMan Alternate Temperature	58	116	44	53	29	44
UNC: Extracted	36	0	23	40	30	52
UNC: Bead Beaten	27	0	49	29	29	27
UNC: Bead Beaten Alternate Cycling Time	26	0	27	14	17	41
Rosewood	38	58	39	13	94	40
MF	32	0	16	43	18	26
DS	31	0	20	28	28	38

Table 7. Correlation (r^2)-of *Enterococcus sp.* estimates among methods across all samples.

Method	MF	DS	Gen-Probe	USEPA R1: Absolute Quantitation	USEPA R1: Adjusted Absolute Quantitation	USEPA R1: Relative Quantitation	USEPA NERL: ABI-TaqMan	USEPA NERL: Omni-TaqMan	USEPA NERL: Omni-TaqMan Alternate Temperature	UNC: Extracted	UNC: Bead Beaten	UNC: Bead Beaten Alternate Cycling Time
DS	0.83											
Gen-Probe	0.69	0.56										
USEPA R1: Absolute Quantitation	<0.01	<0.01	0.02									
USEPA R1: Adjusted Absolute Quantitation	<0.01	<0.01	0.02	1.00								
USEPA R1: Relative Quantitation	<0.01	<0.01	<0.01	0.99	0.99							
USEPA NERL: ABI-TaqMan	0.19	0.21	0.21	0.65	0.65	0.61						
USEPA NERL: Omni-TaqMan	0.41	0.38	0.41	0.99	0.47	0.42	0.74					
USEPA NERL: Omni-TaqMan Alternate Temperature	0.60	0.58	0.41	0.33	0.33	0.30	0.79	0.80				
UNC: Extracted	0.92	0.81	0.77	0.02	0.02	0.01	0.23	0.47	0.60			
UNC: Bead Beaten	0.74	0.95	0.42	0.03	0.03	0.02	0.33	0.45	0.71	0.72		
UNC: Bead Beaten Alternate Cycling Time	0.90	0.79	0.70	0.05	0.05	0.03	0.25	0.43	0.61	0.92	0.73	
Rosewood	0.02	0.02	0.01	0.91	0.91	0.94	0.54	0.24	0.19	0.01	0.00	<0.01

Table 8. Correlation (r^2)-of *Enterococcus sp.* among methods across all samples.

Method	MF	DS	Gen-Probe	USEPA R1: Absolute Quantitation	USEPA R1: Adjusted Absolute Quantitation	USEPA R1: Relative Quantitation	USEPA NERL: ABI-TaqMan	USEPA NERL: Omni-TaqMan	USEPA NERL: Omni-TaqMan Alternate Temperature	UNC: Extracted	UNC: Bead Beaten	UNC: Bead Beaten Alternate Cycling Time
DS	0.99											
Gen-Probe	0.98	0.99										
USEPA R1: Absolute Quantitation	0.01	0.01	0.01									
USEPA R1: Adjusted Absolute Quantitation	0.01	0.01	0.01	1.00								
USEPA R1: Relative Quantitation	<0.01	<0.01	<0.01	0.99	0.99							
USEPA NERL: ABI-TaqMan	0.26	0.26	0.26	0.81	0.81	0.76						
USEPA NERL: Omni-TaqMan	0.52	0.50	0.51	0.53	0.53	0.48	0.89					
USEPA NERL: Omni-TaqMan Alternate Temperature	0.58	0.55	0.53	0.40	0.40	0.33	0.80	0.91				
UNC: Extracted	0.89	0.85	0.83	0.03	0.03	0.02	0.34	0.63	0.73			
UNC:Bead Beaten	0.89	0.87	0.84	0.09	0.09	0.06	0.46	0.71	0.84	0.95		
UNC: Bead Beaten Alternate Cycling Time	0.83	0.79	0.75	0.08	0.08	0.04	0.43	0.67	0.84	0.98	0.98	
Rosewood	<0.01	0.25	0.02	0.20	0.20	0.22	0.05	0.01	<0.01	0.01	<0.01	<0.01

Table 9. Integrated evaluation of equivalency between new and EPA approved methods for *Enterococcus sp.*

Method	Equivalent to Current Methods	Not Materially Different from Current Methods	Materially Different from Current Methods
Gen-Probe	59	18	23
USEPA R1: Absolute Quantitation	6	6	88
USEPA R1: Adjusted Absolute Quantitation	6	12	82
USEPA R1: Relative Quantitation	18	0	82
USEPA NERL: ABI-TaqMan	47	18	35
USEPA NERL: Omni-TaqMan	47	18	35
USEPA NERL: Omni-TaqMan Alternate Temperature	53	18	29
UNC: Extracted	35	18	47
UNC: Bead Beaten	53	24	23
UNC: Bead Beaten Alternate Cycling Time	47	24	29
Rosewood	18	12	70

Table 10. Logarithmic offset of USEPA R1 methods for *Enterococcus sp.* from grand median values produced by reference labs.

Sample Type and Relative Concentration	Absolute Quantitaion	Adjusted Absolute Quantitation	Relative Quantitation
Cultured Cells - low	0.4	0.4	0.4
Cultured Cells - medium	0.5	0.5	0.5
Cultured Cells - high	0.5	0.5	0.5
Primary Sewage influent - low	1.7	1.7	1.7
Primary Sewage influent - medium	1.7	1.7	1.7
Primary Sewage influent – high	1.7	1.7	1.7
Urban Runoff - low	2.7	2.7	2.7
Urban Runoff - medium	2.9	2.9	2.9
Urban Runoff - high	2.8	2.8	2.8

Table 11. Regression analysis of laboratory created samples containing three concentrations of inoculum between new and EPA approved methods for enterococci.

Method	Cultured Cells		Primary Sewage Influent		Urban Runoff	
	r^2	slope	r^2	slope	r^2	slope
Gen-Probe	0.64	1.09	0.93	1.01	0.54	0.76
USEPA R1: Absolute Quantitation	0.96	1.18	0.98	0.97	0.97	1.06
USEPA R1: Adjusted Absolute Quantitation	0.97	1.19	0.99	0.98	0.97	1.06
USEPA R1: Relative Quantitation	0.99	1.12	0.99	0.99	0.97	1.08
USEPA NERL: ABI-TaqMan	0.76	1.22	0.91	0.94	0.69	1.09
USEPA NERL: Omni-TaqMan	0.83	0.62	0.85	0..67	0.94	0.80
USEPA NERL: Omni-TaqMan Alternate Temerature	0.98	1.06	0.96	0.91	0.72	0.81
UNC: Extracted	0.91	0.78	0.91	0.79	0.27	0.31
UNC:Bead Beaten	0.84	1.20	0.97	1.00	0.88	0.77
UNC Bead Beaten Alternate Cycling Time	0.91	1.17	0.98	0.83	0.73	0.64

Table 12. Overall Accuracy in respect to the California single-sample standard for *E. coli* of 400/100 mL as a percentage of all samples.

Method	Overall Accuracy
USEPA R1: Absolute Quantitation	78
USEPA R1: Adjusted Absolute Quantitation	80
USEPA R1: Relative Quantitation	85
UNC: Extracted	85
UNC: Bead Beaten	74
Rosewood	61
Silverlake	58

Table 13. Percentage of *E. coli* samples for which there were false positive and false negative results relative to the California single-sample standard of 400 *E. coli*/100 mL. The new methods were compared against the grand median of all reference labs.

Method	All Samples		Blanks		Seawater Spiked with Culture		Seawater Spiked with Urban Runoff		Seawater Spiked with Sewage		Environmental Samples	
	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative	False Positive	False Negative
USEPA R1: Absolute Quantitation	38	10	22	0	0	0	11	0	100	0	100	0
USEPA R1: Adjusted Absolute Quantitation	0	37	0	0	0	33	0	0	0	17	0	47
USEPA R1: Relative Quantitation	4	27	0	0	0	0	0	0	33	17	0	47
UNC: Extracted	13	17	0	0	0	0	0	0	33	0	0	33
UNC: Bead Beaten	50	7	33	0	0	0	100	0	0	0	22	11
Rosewood	13	60	0	0	0	100	33	0	0	0	0	50
Silverlake	25	43	0	0	0	100	33	0	33	44	0	0

Table 14. Percentage of *E. coli* samples >1/2 log above or below the median of all reference labs.

Method	All Samples		Blanks		Seawater Spiked with Culture		Seawater Spiked with Urban Runoff		Seawater Spiked with Sewage		Environmental Samples	
	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median
USEPA R1: Absolute Quantitation	63	2	67	0	0	0	78	0	100	0	67	6
USEPA R1: Adjusted Absolute Quantitation	3	37	0	0	0	100	56	0	0	11	0	56
USEPA R1: Relative Quantitation	9	17	0	0	0	33	44	11	11	22	0	60
UNC: Extracted	4	11	0	0	0	0	11	0	11	0	0	33
UNC: Bead Beaten	52	15	33	0	67	0	100	0	100	0	6	39
Rosewood	14	52	11	0	0	100	67	0	0	100	0	56

Table 15. Percentage of average of *E.coli* replicates >1/2 log above or below the median of all reference labs.

Method	All Samples		Blanks		Seawater Spiked with Culture		Seawater Spiked with Urban Runoff		Seawater Spiked with Sewage		Environmental Samples	
	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median	>1/2 log **Above** Median	>1/2 log **Below** Median
USEPA R1: Absolute Quantitation	56	0	67	0	0	0	100	0	100	0	67	0
USEPA R1: Adjusted Absolute Quantitation	11	33	0	0	0	100	67	0	0	0	0	50
USEPA R1: Relative Quantitation	11	11	0	0	0	33	67	0	0	0	0	17
UNC: Extracted	6	11	0	0	0	0	0	0	33	0	0	33
UNC: Bead Beaten	56	6	33	0	67	0	100	0	100	0	17	6
Rosewood	11	50	0	0	0	100	67	0	0	100	0	50

Table 16. Average coefficient of variation for *E. coli*.

Method	All Samples	Blanks	Seawater Spiked with Culture	Seawater Spiked with Urban Runoff	Seawater Spiked with Sewage	Environmental Samples
USEPA R1: Absolute Quantitation	28	15	24	65	18	22
USEPA R1: Adjusted Absolute Quantitation	38	0	12	77	66	38
USEPA R1: Relative Quantitation	51	72	11	90	65	34
UNC: Extracted	42	0	20	99	51	40
UNC: Bead Beaten	67	0	63	104	38	98
Rosewood	60	111	58	42	21	63
DS	19	0	17	30	25	22

Table 17. Correlation (r^2)-of *E.coli* estimates among methods across all samples.

Method	DS	USEPA R1: Absolute Quantitation	USEPA R1: Adjusted Absolute Quantitation	USEPA R1: Relative Quantitation	UNC: Extracted	UNC: Bead Beaten
USEPA R1:Absolute Quantitation	0.19					
USEPA R1: Adjusted Absolute Quantitation	0.54	0.45				
USEPA R1: Relative Quantitation	0.62	0.25	0.90			
UNC: Extracted	0.14	0.44	0.26	0.11		
UNC: Bead Beaten	0.51	0.41	0.45	0.30	0.46	
Rosewood	0.36	0.09	0.42	0.54	0.00	0.00

Table 18. Correlation (r^2)-of *E.coli* among methods using average of replicates across all samples.

Method	DS	USEPA R1: Absolute Quantitation	USEPA R1: Adjusted Absolute Quantitation	USEPA R1: Relative Quantitation	UNC: Extracted	UNC: Bead Beaten
USEPA R1: Absolute Quantitation	0.19					
USEPA R1: Adjusted Absolute Quantitation	0.62	0.52				
USEPA R1: Relative Quantitation	0.66	0.26	0.93			
UNC: Extracted	0.27	0.86	0.37	0.16		
UNC: Bead Beaten	0.69	0.63	0.55	0.37	0.81	
Rosewood	0.36	0.10	0.51	0.59	0.00	0.01

Table 19. Integrated evaluation of equivalency between new and DS (IDEXX) methods for *E.coli*.

Method	Equivalent to Current Methods	Not Materially Different from Current Methods	Materially Different from Current Methods
USEPA R1: Absolute Quantitation	39	0	61
USEPA R1: Adjusted Absolute Quantitation	33	22	45
USEPA R1: Relative Quantitation	55	17	28
UNC: Extracted	67	22	11
UNC: Bead Beaten	17	11	72
Rosewood	22	17	61

Table 20. Species composition of *Enterococcus sp.* isolated from growth-based methods.

	Culture	Primary Wastewater Influent	Urban Runnoff	Tijuana River	Imperial Beach	Cabrillo Beach	Doheny Beach	Surfrider Beach	Paradise Cove
Enterococcus faecalis	88	34	1	11	8	23	42	12	1
Enterococcus faecium	11	30	58	45	18	38	22	12	78
Enterococcus avium	0	0	0	0	0	0	3	0	0
Enterococcus casseliflavus	0	8	16	12	17	23	8	54	9
Enterococcus gallinarum	0	5	2	1	0	3	11	7	4
Enterococcus hirae	0	4	5	5	7	5	0	2	0
Enterococcus mundtii	0	3	9	3	2	3	2	5	8
Ambiguous *Enterococcus*	2	4	3	8	5	2	6	2	0
non-*Enterococcus*	0	11	7	15	43	3	8	7	1

FIGURES

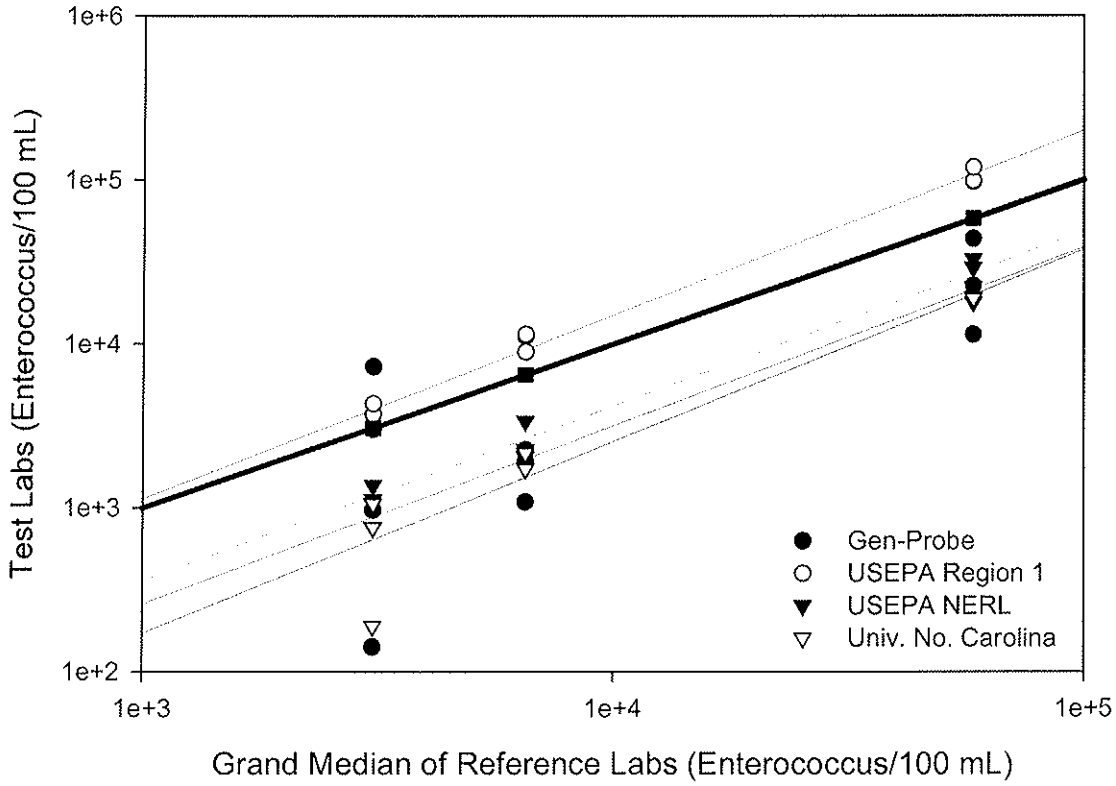

Figure 1. r^2 plots across three concentrations of laboratory cultured *Enterococcus sp.*.

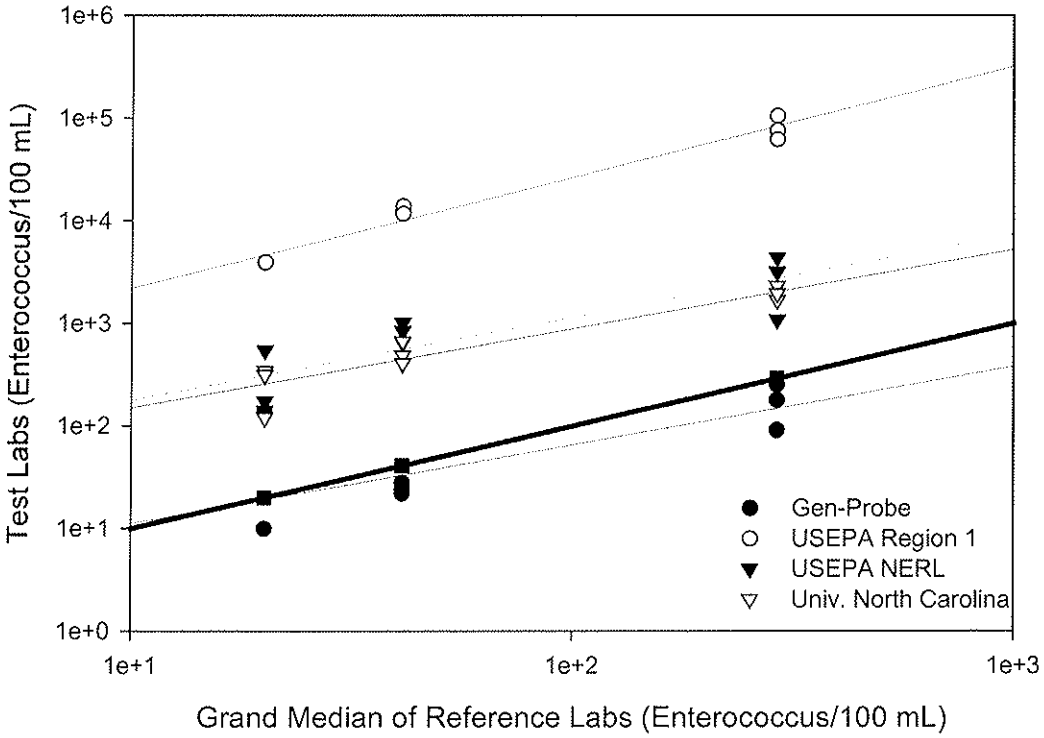

Figure 2. r^2 plots across three concentrations of primary wastewater influent inoculum.

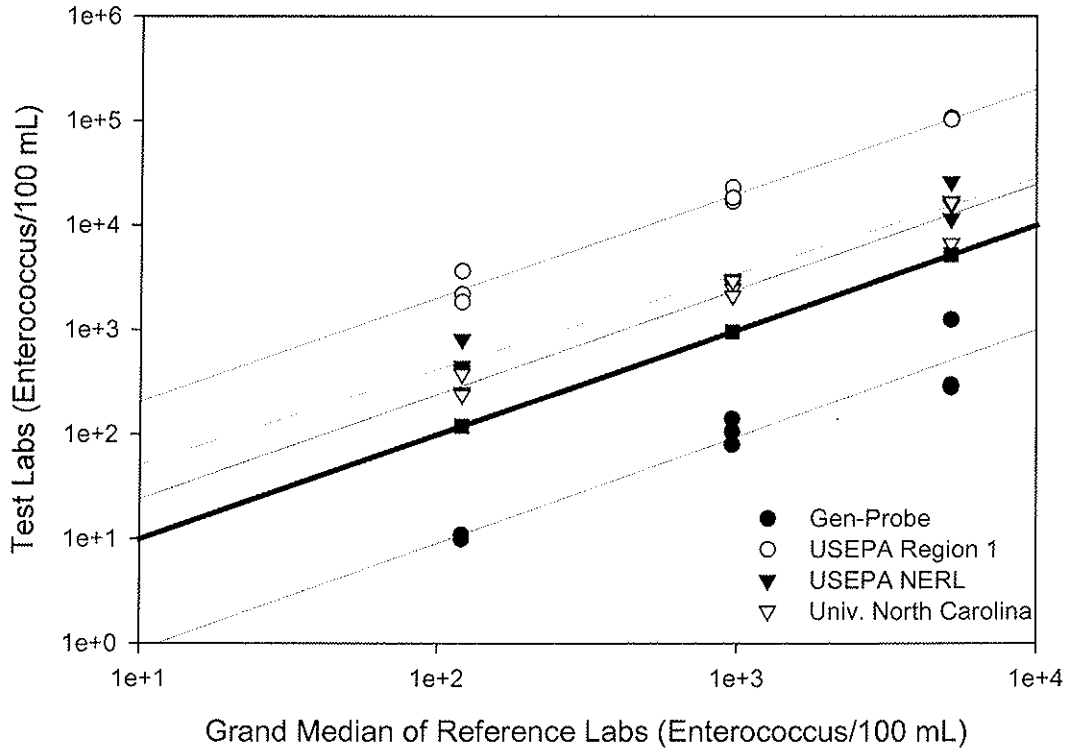

Figure 3. r^2 plots across three concentrations of urban runoff inoculum.

APPENDIX A

Comparison of QPCR Based Methods for Rapid Quantification of Enterococcus sp. and E. coli from Environmental and Laboratory-manipulated Samples

COMPARISON OF QPCR BASED METHODS FOR RAPID QUANTIFICATION OF ENTEROCOCCUS SP. FROM ENVIRONMENTAL AND LABORATORY-MANIPULATED SAMPLES

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INTRODUCTION

Current U.S. EPA approved methods for measuring concentrations of indicator bacteria such as *Enterococcus sp.* (ENT) and E. coli (EC) in recreational waters include membrane filtration (MF), multiple tube fermentation (MTF), and directed substrate technologies (DST, such as those produced by IDEXX Laboratories, Inc.). These well established methods have been discussed individually in relation to their accuracy and precision, and their results have been extensively compared to one another in the analyses of a variety of recreational water samples (Noble et al. 2003, Griffith et al. 2006). Major advantages to the use of these methods by water quality agencies worldwide are relative ease of use, low cost, and the acceptance among professionals of indicator bacteria as adequate proxies of potential public health risk. The major disadvantage of these methods is the time required from sample collection to results. This time ranges from 18 to 96 hours, with confirmation and verification steps taking even longer. Given the period of time required to results, accurate management of recreational waters is impossible. When beaches are contaminated, they remain open to swimming for at least one full day while they should have been closed. Conversely, upon closure of areas it is likely that the sources of contamination have long disappeared, as most contamination events tend to be episodic rather than chronic in nature (Leecaster and Weisberg, 2003). There is a vital need for rapid methods to quantify indicator bacteria in recreational waters to solve this problem.

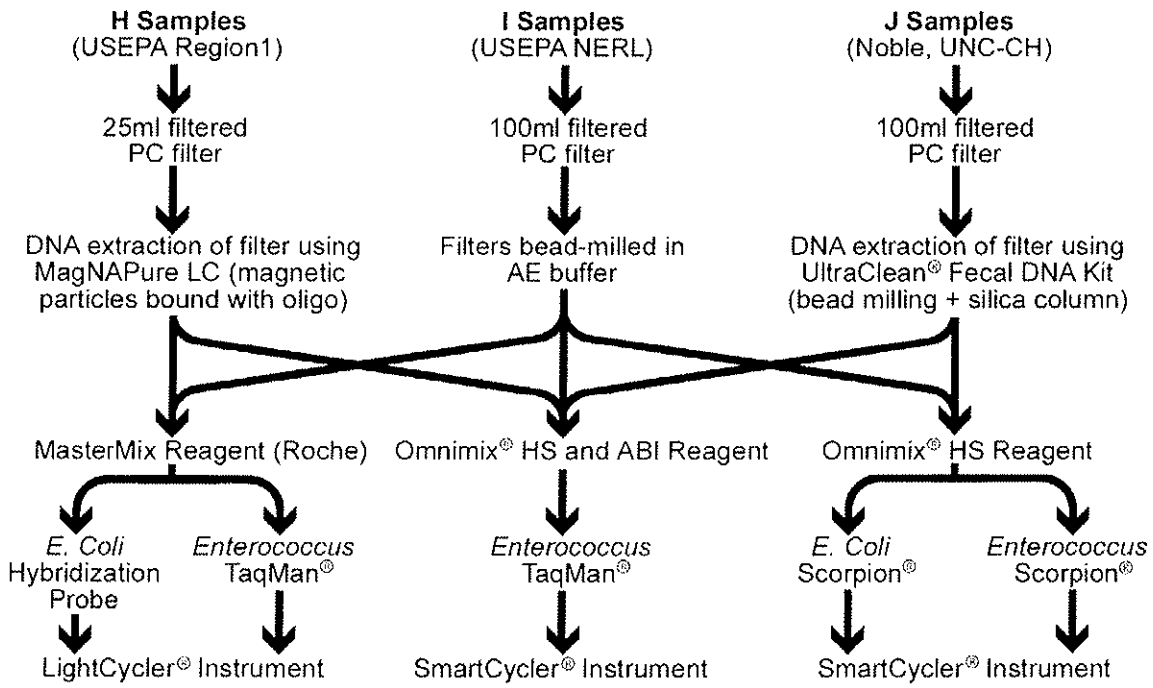
In recent years, molecular assays have gained attention for their successful application to the field of recreational water quality (Noble and Weisberg, 2005). More specifically, the quantitative polymerase chain reaction (QPCR) has been successfully used in epidemiology studies to measure concentrations of *Enterococcus* sp. at freshwater recreational beaches in the Great Lakes (Haugland et al. 2005, Wade et al., 2005) and in methods comparison studies. There are now several groups that are developing and validating the use of rapid QPCR methods for enumerating both ENT and EC in recreational waters (e.g. Griffith et al. 2004, Blackwood and Noble 2005, Haugland et al. 2005). These methods are capable of quantifying indicator bacteria below the existing single sample thresholds, and can be completed from sample to results in several hours.

The QPCR methodologies currently in development are generally similar to one another, with basic steps including sample filtration, cell lysis, and QPCR cycling. There are, however, variations in these protocols that warrant investigation. For example, most protocols include sample filtration similar to membrane filtration protocols, but different volumes of sample may be filtered and characteristics of available filter matrices may result in dissimilar recovery of indicator bacteria cells. Further differences may arise from post filtration processing. One of the current protocols features only a crude bead milling approach for cell lysis and DNA release. The filters are rapidly shaken for a short time in a tube with buffer and glass beads, followed by a brief centrifugation step and the supernatants are then directly analyzed by QPCR. This approach is simple and fast, but might not purify the DNA enough for successful QPCR analysis of water samples with high levels of inhibitory compounds. Other protocols include bead milling with an additional DNA extraction and purification step using commercial kits. This approach is more complex and time consuming, but yields higher quality DNA for QPCR analysis. Finally, the QPCR analyses themselves can be performed with different types of instruments (cycling

platforms) and with different thermal cycling parameters and probe chemistries. With all of the possible permutations in the developing QPCR assays, there is a need to determine whether the variations have an impact on results.

This paper is the result of three research groups coming together to conduct rapid QPCR analysis for ENT on a wide range of laboratory-manipulated and natural environmental samples. To the fullest extent possible, the three groups shared filters, extracts, and results in order to create comparisons of specific components of each QPCR assay. For example, one group used the crude bead milling approach, while the others performed additional DNA extraction and purification protocols on the samples using different methods. The groups shared extracts and analyzed them with all other parts of the analyses being the same. The goal of this report is to compare various components of the methods, and to discuss the results. A flow chart comparing sample processing and assay permutations is presented in Figure 1.

Figure 1.



Material and Methods:

Experimental Design:

The three teams conducting the QPCR analyses were 1) University of North Carolina at Chapel Hill research group headed by Rachel Noble (UNC), 2) U.S. EPA National Exposure Research Laboratory research group headed by Richard Haugland (USEPA NERL), and U.S. EPA Region 1 (USEPA R1) Genomics laboratory, headed by the contractor, Mark M. Dolittle of Lockheed Martin Environmental Support and Assistance Team. The study, conducted at the Orange County Sanitation District (OCSD) Environmental Laboratory in Fountain Valley, CA, included analysis of 18 blind samples run in triplicate (54 samples total). Samples were generally laboratory generated (spiking buffer or seawater with *Enterococcus* sp. or sewage) or were collected directly from beach and storm drain areas with known chronic contamination. Samples were provided to each group (USEPA R1 received samples marked H1-54, UNC received samples marked J1-54, USEPA NERL received samples marked I1-54), and were simultaneously filtered by each group's existing filtration approach (Figure 1).

Several sets of comparisons were made via the sharing of samples as outlined in Figure 1. First, the results generated using different sets of QPCR reagents were compared. Samples as bead milled by USEPA NERL were analyzed using both the Applied Biosystems (ABI) Mastermix and Cepheid Omnimix. Samples processed by USEPA NERL were also analyzed by UNC using OmniMix reagent but substituting a Scorpion probe for the TaqMan® probe used by USEPA NERL. Second, cell lysis/DNA extracts created by each of the three research groups were analyzed by USEPA NERL on a single Smart Cycler instrument. Additionally, filters that were crude bead milled by USEPA NERL were run using the QPCR assays of both USEPA NERL and UNC, and similarly the purified DNA extracts generated by UNC were shared and analyzed by USEPA NERL. Finally, results were generated by running the same extracts on different cycling platforms (LightCycler vs. SmartCycler) to compare the results from these instruments. Samples prepared by USEPA R1 using bead milling and MagNA Pure DNA extraction/purification were analyzed by QPCR hydrolysis (TaqMan®) and hybridization (Roche) probes using the Light Cycler Instrument with Roche Fast Start reagents and were also analyzed by USEPA NERL using the Smart Cycler instrument with ABI Mastermix.

Sample processing approaches follow the format A) Sample filtration B) Sample Extraction, and C) QPCR, for each of the three groups.

UNC Group: The assays developed for ENT targeted the multiple copy 23S rDNA gene (Ludwig and Schleifer, 2000). Scorpions® primer probe technology was also utilized for this assay.

A) Sample Filtration: Water samples were collected from beach, storm drain, and natural environments, or created in the laboratory by Griffith et al. (2006). The samples were processed on a six-place filtration manifold and vacuum pump assembly with Pall disposable filter funnels. The glass fiber filters provided with the funnels were replaced with 47 mm diameter, 0.45μm

pore-size polycarbonate filters (HTTP, Millipore, Bedford Mass or Osmonics). One hundred ml samples (measured using sterile 50 ml conical tubes) were filtered within 30 minutes of receipt. Filtration of samples was conducted until no further moisture appeared on the filter. Each filter were subsequently rinsed with a small volume (~20 ml) of Phosphate-Buffered Saline (PBS) which was also removed by vacuum filtration.

B) Sample Processing: Filters were immediately removed from the vacuum manifold using sterile disposable forceps, gently folding in half and placed in a microcentrifuge tube (2 ml screw cap tube) containing not more than 300 μg of 0.1 mm zirconia beads (BioSpec Corp) and 600 μl of AE buffer (QIAGEN). The tubes were placed in an 8-place bead beater (BioSpec) and shaken at maximum speed for 2 minutes. Following bead milling the extracts were briefly spun (30 sec, 10,000 x g) in a microcentrifuge to pellet filter debris and beads. Supernatants were carefully removed and placed in microcentrifuge tubes and then processed using the Mo Bio Fecal DNA extraction kit, according to the manufacturer's instructions for maximum yield. (Fecal DNA Extraction Kit, Mo Bio.). While waiting for QPCR, DNA was stored on ice or placed in a -20 freezer.

C) QPCR Approach:
Standards and Standard Curves: For *Enterococcus sp.* enumeration, a cell suspension was used as given by the USEPA NERL Group to the UNC Group. The initial cell suspension concentration was 1.0×10^9 ml^{-1}, which was diluted to a concentration of 1.0×10^5 per 10 ul for analysis. The crude cell suspension was first lysed by boiling for 1 minute and then run directly in the QPCR reaction (no extraction).

Standard curves (cell suspensions created as listed above) were run in duplicate, 5 log dilutions for each reaction. Standard curve cell counts ranged from 8.3×10^4 to 0.83 per reaction for Enterococcus. Inhibition was judged via the variation of 1 log from the expected Ct of 34.5 for a spike of 1.0×10^5 positive control *Lactococcus* cells that were co-extracted with the samples, i.e. if the Ct value for the sample was greater than 37.9 the sample was considered to be inhibited. Cell numbers in unknown samples that were not inhibited were calculated straight from the standard curves, and the values were multiplied by the factor of concentration (i.e. for the DNA extraction approach, 50 ul of extracted material represents the entire 100 ml of filtered sample, therefore, a 5 ul PCR reaction unknown value was multiplied by a factor of 10 to calculate the cells/100 ml volume.

QPCR Reactions: Generally, an appropriate number of lyophilized OmniMix beads were dissolved in RNase and DNase-free sterile water prior to the analyses to create a master mix. Twenty μl aliquots of each master mix were pipetted into each reaction tube, followed by 5 μl of DNA extracts or buffer for no-template controls. The OmniMix beads, contained all required QPCR reagents including probe and primer sets for either *Enterococcus* or *Lactococcus*. The Lactococcus beads also contained a propriety internal positive control template (IC, Cepheid) and a primer and probe set for this template. All probes incorporated Scorpion® chemistry. All reactions were prepared in 25 μl optical tubes (Cepheid). The reactions were monitored in a Smart Cycler II™ sequence detection instrument (Cepheid). Thermal cycling conditions for all reactions (ENT, and *Lactococcus*/IC) were the same, consisting of 1 cycle at 95°C for 2 minutes (hot start), then 45 cycles at 95°C for 5 sec, and 62°C for 43 sec (optics on). Determinations of

cycle threshold (C_T) were performed automatically by the instrument after manually adjusting the threshold fluorescence value to 8 units. The SmartCycler detected fluorescence emissions at three wavelengths that were specific for the different fluorophore dyes associated with each of the three probes. ENT probes were tagged with the fluorophore FAM (emission maximum at 515 nm), *Lactococcus* probes were tagged with the fluorophore Cy 5 (emission maximum at 554 nm), and the IC probes were tagged with the fluorophore Texas Red (emission maximum at 601 nm). Results of unknowns were calculated using fluorescence signal emitted at the correct wavelength, and using the SmartCycler software given the respective standard curve generated as described previously.

NERL Haugland Group:

The USEPA NERL Group conducted ENT analysis using 1) two different reagent systems (Cepheid, OmniMix, as described above and TaqMan Universal MasterMix from Applied Biochemical Inc. 2) simple bead milling and direct analyses of the samples, 3) TaqMan hydrolysis probe chemistry, and 4) the delta, delta Ct comparative cycle threshold calculation method featured in Haugland et al. 2005.

A) Sample Filtration: Sample filtration was performed as described for the UNC Group above.

B) Sample Processing: Following filtration, each filter was folded into a cylinder with the sample side facing inward, and inserted (using sterile forceps) into a 2 ml semiconical screw-cap microcentrifuge tube (extraction tube) containing 0.3 g of acid-washed glass beads. DNA was recovered from the organisms retained on the filters by addition of 600 µl of AE buffer (Qiagen, Valencia, CA) containing 0.2 µg/ml salmon testes DNA and ~10^5 *Lactococcus lactis* cells (added as positive controls) to each extraction tube and shaking in an eight-position mini bead beater for 60 sec. at maximum rate. The tubes were then centrifuged at 12,000 *x g* for 1 min to pellet the glass beads and debris. Resulting supernatants were transferred to sterile 1.6 ml microcentrifuge tubes. Sample processing using this method took less than 30 min for the 18 unknown samples plus two calibration standard samples analyzed each day in the study.

C) QPCR Approach:
Calibration standards:
Each day a 1.0 x 10^9 ml^{-1} cell suspension of *Enterococcus faecalis* was diluted in phosphate buffered saline to a concentration of 1.0 x 10^7/ml. Ten ul aliquots of this diluted suspension (10^5 cells) were added to 3 extraction tubes containing glass beads and AE buffer with salmon testes DNA, as described above, in addition to a clean filter of the same type as used for water sample filtrations. A *Lactococcus lactis* cell stock suspension was similarly diluted and ~10^5 cells were added to each of these extraction tubes as well. The tubes were processed by bead milling and centrifugation as described above. Five ul aliquots of the recovered supernatants were analyzed at 1:5, 1:50 and 1:500 dilutions to generate data for comparative cycle threshold calculations as previously described (Haugland et. al. 2005)

QPCR Reactions:
QPCR analyses were performed using two different commercially available reagent systems with different *Enterococcus* and positive control-specific primer and probe sets customized for each

system. QPCR analysis for EC was not conducted by the USEPA NERL Group. The USEPA NERL Group featured the use of TaqMan-based QPCR probe chemistry, as opposed to the Scorpions chemistry utilized by the UNC Group. Reagent mixes with the first system were prepared by dissolving OmniMix™ reagent beads (Cepheid, Sunnyvale, CA) in 35 µl distilled water and adding 5 µl of a mixture of forward and reverse primers (5 µM each) and 400 nM TaqMan™ probe. Each of these mixes was sufficient for two reactions. Reagent mixes with the second system were prepared by combining 12.5 µl of TaqMan™ Universal Master Mix, (Applied Biosystems, Foster City, CA); 5 µl of primer and probe mix as described above, and 2.5 µl of 2 mg/ml bovine serum albumin (fraction V, GibcoBRL, Gaithersburg, MD). All reactions were prepared in 25 µl optical tubes (Cepheid) by addition of 20 µl of reagent mixes and 5 µl of DNA extracts from the samples, diluted from 5-fold in AE buffer. The reactions were monitored in a Smart Cycler II™ sequence detection instrument (Cepheid). Thermal cycling conditions for the first reagent system (OmniMix™) consisted of 2 min at 95°C, followed by 40 cycles of 5 s at 95°C and 30 sec at either 64 or 66°C. Thermal cycling conditions for the second reagent system (TaqMan™) consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 2 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument after manually adjusting the threshold fluorescence value to 8 units.

USEPA Region 1 Group

A) Sample Filtration: Twenty-five ml volumes of each water sample were filtered through 25-mm diameter, 0.4 µm pore-size polycarbonate filters (Whatman Nucleopore or Millipore Isopore) inserted into 12-position vacuum filter manifolds (Millipore Corporation, Bedford, MA). A second (duplicate) 25-mL volume of one the 18 samples was also filtered along with two blanks (sterile buffer) and two positive control "calibrator" samples that were spiked with 1 x 10^5 cells of *Enterococcus faecalis*. After two rinses with Phosphate-Buffered Saline (PBS), the filters were suctioned dry by the vacuum pump.

B) Sample Processing: DNA was extracted from the filters by aseptically folding the filters into individual tubes containing siliconized ceramic "Green Beads" (Roche Applied Sciences). Roche MagNA Pure LC DNA Isolation Kit III (Bacteria; Fungi) reagents were used in subsequent steps to isolate purified bacterial DNA from the cells retained on the filters. After adding a volume of Roche Lysis Buffer the tubes were bead beaten using a Roche MagNA Lyser for 1-minute at 5,000 rpm to disrupt and lyse the cells on the filter. Proteinase K was added to hydrolyze protein and held at 65 °C for 10 min.. Cell debris was then collected at the bottom of the tube by centrifugation. Half of the clarified lysate volume was transferred from each bead-tube to a 32-well sample processing cartridge. Additional positive and negative control samples were subjected to bead-beating or added directly to the sample cartridge to monitor the efficiency of extraction and cross-contamination. The cartridge was loaded into a Roche MagNA Pure LC automated nucleic acid extraction platform. DNA was extracted and purified from a batch of 32 control and "unknown" sample lysates by the robotic MagNA Pure LC platform using standardized reagent volumes (e.g. Lysis Binding Buffer; Magnetic Glass Particles, Wash Buffers and Elution Buffer) according to the computer software program for the MagNA Pure LC DNA Isolation Kit III Protocol.

Before starting the DNA extraction and purification process, the Lysis Binding Buffer (LBB) loaded in the reagent tray was spiked with surrogate extraction control DNA (Salmon

testes DNA, Sigma Chemical Company, St. Louis, MO). This was necessary to ensure that each lysate would be spiked with the same specific number of surrogate DNA molecules during extraction so that differences in DNA extraction efficiency and recovery could be quatified . At the end of the DNA extraction and purification process, nucleic acids (DNA and RNA) were desorbed from the Magnetic Glass Particles into 100-µL of Elution Buffer.

C) QPCR Approach: Ten µl volumes of each DNA extract were initially analyzed by Salmon DNA PCR assays in order to quantify surrogate DNA recovery and to detect PCR inhibition or fluorescence quenching. When inhibition or quenching was observed, 5 µl volumes of DNA extracts were analyzed for the ENT and EC assays.
The number of ENT cells present in the water samples was reported in two ways 1) the number of gene copies or genomic equivalents (GEQs) in each sample were derived from comparison of fluorescence data with those of a generated standard curve (absolute quantitation), and 2) QPCR results (Ct values) of a sample "unknown" were compared with the PCR results of "calibrator" samples that contained a known number of ENT cells (relative quantitation, Haugland et al. 2005).

i) Salmon DNA PCR Analysis of Sample DNA Extracts
The DNA extracts were analyzed for Salmon DNA GEQs recovered by the filter concentration and DNA extraction protocols using the Sketa-2 Primer Probe Set (designed by R. Haugland and provided by TIB Mol Biol, Inc.) added to Roche FastStart (Taq) Hybridization Probe Master Kit reagents. The Salmon DNA (SS-DNA) PCR assay used the same PCR thermal cycling parameters as the Ludwig and Schliefer (2000) PCR assay for quantitation of *Enterococcus*: 10 min denaturation and FastStart Taq Activation at 95 °C, 45 cycles of 15-sec denaturation at 95 °C and 2-min annealing / extension at 60 °C. Fluorescence was detected by the LightCycler (LC) during each cycle of PCR amplification of target gene sequences (amplicons), and the LC software determined the PCR cycle number at which amplicon numbers caused the fluorescence to exceed the background threshold level, or cycle threshold (Ct).

ii) ENT QPCR analysis
PCR thermal cycling conditions were as described above for the SS-DNA analyses. An external standard curve that was previously generated was imported into the "analysis window" of each *Enterococcus* PCR assay and was used to determine the GEQ value from each PCR reaction's Ct value.

Data Handling and Statistical Calculations:

All three groups collected PCR data, including assay name, assay date, sample number, sample label, Ct value, target copy or calibrator cell numbers, and standard curve name and parameters that were exported from the respective software into Microsoft EXCEL. For the UNC and USEPA R1 Groups, a standard curve was used for some of the calculations. That is, the Ct value for each PCR reaction was used by the software to extrapolate the starting copy number of ENT genomic DNA copies in each PCR reaction based on standard curves, which were derived from regression plots (Log DNA concentration vs. Ct). For USEPA R1, an external standard curve that was previously generated using the LightCycler PCR Quantitation Software

for the ENT assay, and was imported into the "analysis window" to determine the GEQ value from each PCR reaction's Ct value. The Delta Ct approach (relative quantitation) for ENT cell concentrations was also performed by each group by comparing the results of PCR analyses of the SCCWRP Study water samples with those of "Calibrator" samples and computing results in EXCEL software. Each sample's quantified target copy number was corrected for PCR sample volume, concentration factors, and dilution factors to standardize results in units of genomic equivalents (GEQs) or calibrator cell equivalents (CCE) per 100mL. The mean, standard deviation, and coefficient of variation values of replicate (intra- and inter-batch) PCR reactions (same and different sample volumes) and sample duplicates were calculated.

Raw Ct data was compiled for each group for all samples. Ct values for blanks were removed from the analysis. Average Ct values for each triplicate set of samples were generated, along with their respective coefficient of variation. Most statistical analyses were regression analyses of all compared samples, and ANOVA using Microsoft Excel statistical packages.

Results and Discussion:

The USEPA NERL approach was arguably the simplest of the three methods. This method involves filtration of the sample, crude bead milling of the filter to generate a crude extract of the sample, and then direct QPCR analysis of the extract. The method can take as little as ~1 hr from sample to result. The UNC Group conducts a similar sample filtration, DNA extraction and purification using a commercially available kit, and QPCR analysis. This method can be completed from sample to result in less than 2 h. The USEPA R1 method is more complex than the other two, utilizing a similar sample filtration approach, but incorporating bead milling, DNA extraction and target capture into sample processing. The complexity of this method is offset, however, by its availability in a semi-automated format. The USEPA R1 method requires roughly 4-h from sample to result, depending on the target and chemistry of the primer and probe combination used in the QPCR step. In this study, the Ludwig and Schliefer (XXXX) based primers for enterococci and TaqMan probe used by USEPA R1 required about 2-h. Each method has a feature that separates it from the others as far as feasibility and results. The USEPA NERL method is simple to perform, and is the fastest. The UNC method features the use of patented lyophilized bead technology (OmniMix reagent beads that also contain primers and probes, Cepheid, Sunnyvale, CA), which reduces sample manipulation by the end-user (reduces QPCR setup to only two pipetting steps, sample and water). Additionally the UNC Group has applied the use of Scorpions probe technology, with the assistance of David Whitcombe (DxS Limited, Manchester, UK), instead of the TaqMan probe technology (Haugland et al. 2005). Finally, the USEPA R1 method features automation and associated relatively low variability/ high precision in the analysis results.

A flow chart is provided in Figure 1 to depict the general ways that the three groups processed and shared samples. We focused our initial comparative analysis on raw Cycle threshold (Ct) data, rather than on the final calculated results (generally presented as QPCR cell equivalents/100 ml) because of differences in the types of standards and calculation methods used by the different groups. These differences in standards and calculation methods likely contributed to variations in the final reported results from the different laboratories and point to a need for standardization in future studies of these methods. By limiting our comparisons to the raw Ct data, however, we were able to more accurately compare 1) reagent systems, 2) extraction

methods, and 3) QPCR platforms.

Comparison of different reagent systems using the Smart Cycler platform:

DNA samples, prepared by USEPA NERL using the simple bead milling extraction method, were analyzed using both the ABI Universal Mastermix and Cepheid OmniMix reagent systems as described in Materials and Methods. Comparisons of mean raw cycle threshold (Ct) values results for the different sets of replicate samples (excluding PBS and filtered seawater controls), together with linear regression analyses, are shown in Figure 2. The relationship between results using the two reagents was strongly affected by the choice of annealing/extension temperature with the OmniMix reagent. At 66°C, a strong correlation (R^2 = 0.97) was observed, while at 64°C, a weaker relationship was observed (R^2 = 0.76). The slope and intercept values were not significantly different from 1 and 0, respectively ($p > 0.05$) in the regression at 66°C. The discrepancy between ABI reagent results and OmniMix reagent results at 64°C appeared to be associated primarily with a leveling off of the OmniMix results at ~32-33 Ct. A mean Ct value of 32.34 was obtained for the unfiltered seawater samples using the OmniMix reagent at 64°C, suggesting that under these conditions the reagent may contribute to detection of a background of non-target DNA sequences from unknown seawater organisms. Since unfiltered seawater was a component of the environmental samples as well as a diluent for a number of the other samples, this background effect presumably also contributed to relatively low Ct values for some of these samples depending on the levels of ENT that were added as spikes. In contrast, the unfiltered seawater samples gave virtually no signal when using the OmniMix reagent at 66°C or ABI reagent indicating the elimination of this background effect. Beyond sensitivities to annealing temperature changes, our comparison demonstrates that the reagents utilized in this study did not cause a significant difference in results.

Samples processed by USEPA NERL were also analyzed by the UNC Group using OmniMix reagent but substituting the TaqMan probe used by USEPA NERL with a Scorpion probe. The results of these analyses also were statistically indistinguishable with the ABI reagent analysis results (R^2 = 0.98, slope and intercept were not significantly different from 1 and 0, $p > 0.05$, Figure 2). Analyses of the unfiltered seawater samples with this system also gave no background signal despite the fact that a lower annealing/extension temperature of 62°C was used. These results suggest that the reagents used were inconsequential in the results generated. However, the specificity of the Scorpion probe appears to be less sensitive to annealing temperature differences than the TaqMan probe.

Comparison of different sample extraction methods on the Smart Cycler.

DNA samples prepared by each of the three research groups using their individual protocols were analyzed by USEPA NERL on a single Smart Cycler instrument to determine the influence of the different extraction/purification methods on Ct values with minimal variability introduced by different reagents, instruments and analysts. Because the results of these analyses were also intended to be compared with results of the other groups, reagent systems were used that were as similar as possible to those employed by the other groups. Identical sample volumes of 5 μl at the typical dilution/concentration factors associated with each method were analyzed in each instance.

Figure 2 shows a linear regression comparison of mean Ct results for the different sets of replicate samples obtained using OmniMix reagent analyses at the 64°C annealing/extension

temperature of simple bead milled samples (USEPA NERL) versus bead milled samples that were DNA extracted (UNC Group, excluding PBS and filtered seawater controls). Fig. 3 shows a similar comparison of results obtained using ABI reagent analyses of simple bead milled samples (USEPA NERL) versus bead milled samples that were DNA extracted using the MagNA Pure system (USEPA R1). In general, these data demonstrate that the different sample preparation approaches (extraction vs. no extraction) yield more variable QPCR results (lower correlation between approaches) than that seen during a comparison of the same sample preparation approach with different reagents. This variability reduced the ability of the regression analyses to identify significant differences between sample preparation methods (no significant differences from 1 and 0 were observed in the slope and intercept in either of the comparisons, $p > 0.05$). However both of the methods that used DNA extraction and sample concentration showed trends toward higher sensitivity than the simple bead milling method as evidenced by lower Ct values for analyses of corresponding samples. In the case of the samples processed by the UNC Group, this difference appeared most pronounced at lower target DNA concentrations in the samples. Extrapolation of the regression line from this comparison to the analytical method end point of 40 thermal cycles of QPCR for the simple bead milled samples (USEPA NERL) gives an intercept of ~35 cycles for the DNA extracted samples of UNC. This roughly translates into an approximately 40-fold lower theoretical target sequence detection limit for the sample processing approach used by the UNC Group. At higher target DNA concentrations the relative recovery efficiency of the approach used by the UNC Group appears to decrease, meaning that at higher copy numbers the two approaches give more similar results. This is probably due to the fact that at high DNA concentrations, some commercially available kits can become saturated with DNA and recovery efficiencies decrease. The same extrapolation of the regression line from comparisons of the DNA extraction approach employed by the USEPA R1 Group and the simple bead milling method (USEPA NERL) suggests a fairly similar target sequence detection limit for these two approaches. However, the relative recovery efficiency of the MagNA Pure extraction approach (USEPA R1) appeared to increase with higher target DNA concentrations.

Comparison of SmartCycler and Light Cycler platforms:

Samples prepared by USEPA R1 using bead milling and MagnPure DNA extraction/purification were analyzed using the Light Cycler Instrument with Roche Fast Start reagent and were also analyzed by USEPA NERL using the Smart Cycler instrument with ABI Mastermix. Comparisons of Ct values for the different sets of replicate samples (excluding PBS and filtered seawater controls), together with linear regression analyses, are shown in Fig 4. A fairly high level of variability was observed between results generated by the two systems, although a major difference was observed in just one set of samples (from Cabrillo Beach) that explains much of the variability ($R^2 = 0.82$ with Cabrillo Beach results and $R^2 = 0.90$ without them). The slope and intercept values for this regression (1.04 and -1.4, respectively), did not significantly differ from 1 and 0 ($p > 0.05$), indicating that there is no significant difference between QPCR results generated by the two platforms.

Summary:

In summary, we have conducted comparisons of a variety of different manipulations of

rapid QPCR approaches for measuring *Enterococcus* sp. concentrations in recreational waters and laboratory manipulated samples. Our findings indicate that:

1. A variety of different QPCR reagents are available commercially. Reagents in this study included ABI Mastermix, Cepheid OmniMix and Roche FastStart Mix. Our comparison of the use of these reagents for analysis of samples showed that the reagents produced statistically indistinguishable results from one another although additional studies involving larger sample numbers are needed to confirm this. Each of these reagent systems appears appropriate for use in QPCR analysis of environmental samples. However, some benefit may be associated with the use of Cepheid OmniMix given the fact that the reagents are sold in a freeze-dried format to which water and sample DNA are the only added components.

2. The cell lysis/DNA extraction approaches compared as part of this study generally yielded statistically indistinguishable results although, as mentioned above, further confirmation of the comparability of the different methods through the analyses of larger numbers of samples is advisable. The MagNA Pure DNA extraction approach of the USEPA R1 Group yielded results with greater precision than the other two methods. The DNA extraction approach of the UNC Group offered approximately a 50-fold relative concentration factor that permitted a lower detection limit as compared to the crude bead milling approach of USEPA NERL.

3. The QPCR methods tested yielded statistically indistinguishable results regardless of the platforms (Roche LightCycler vs. Cepheid SmartCycler). The assays tested are fully optimized however, and any application on other platforms would likely have to be optimized for specific use.

4. Crude bead milling approaches require less time, training, and effort than bead milling plus DNA extraction for processing of environmental samples. However, the current DNA extraction approaches appear to offer the benefits in terms of a) sample concentration, which permits a lower limit of detection, and b) purity of sample, which translates to fewer interfering substances to alter QPCR efficiency. At this time, it appears that crude bead milling and bead milling plus DNA extraction approaches need to be tested across additional beach environments to assess the need for DNA extraction on all samples. Currently, some concentration and/or purification step beyond the glass bead milling approach appears necessary to effectively enumerate ENT in samples below the equivalent of 30 MPN or cfu/100 ml.

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Figure 1: Comparison of NERL Crude Bead Milled Samples run using the ABI assay plus Taqman probe versus 1) UNC assay using Omnimix and Scorpion Probe, regression y = 0.9636x + 1.4871, r2 = 0.9816, 2) NERL Omnimix assay run at 64 C, regression y = 0.5523x + 12.889, r2 = 0.7618 and 3) NERL Omnimix assay run at 66 C, regression y = 0.928x + 2.5457, r2 = 0.967.

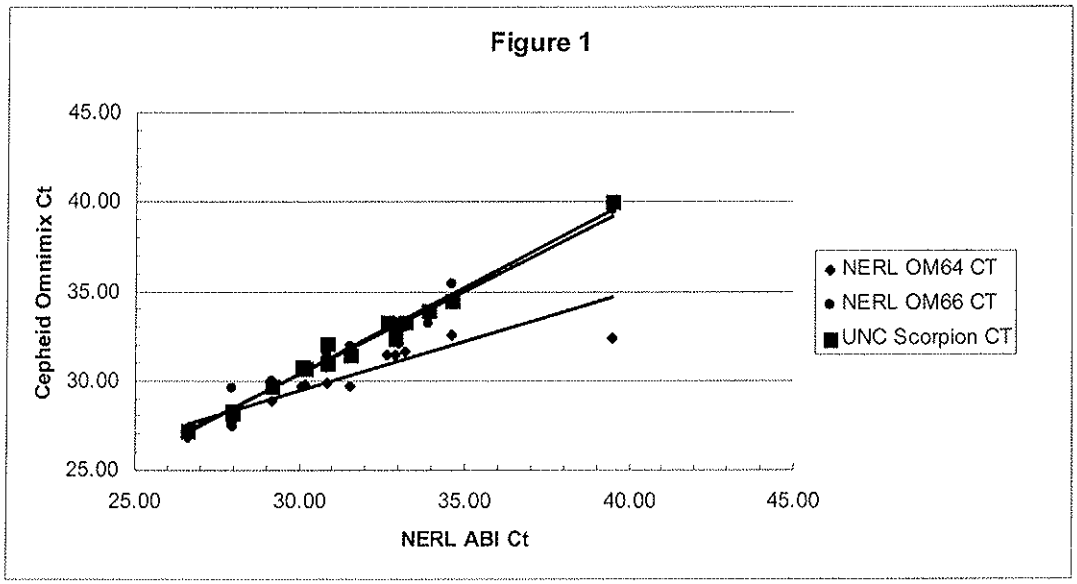


Figure 2: Direct comparison of the difference between the crude bead milling approach of NERL and the full DNA extraction approach of UNC. Regresssion analysis y = 0.7596x + 4.5342, r2 = 0.6955.

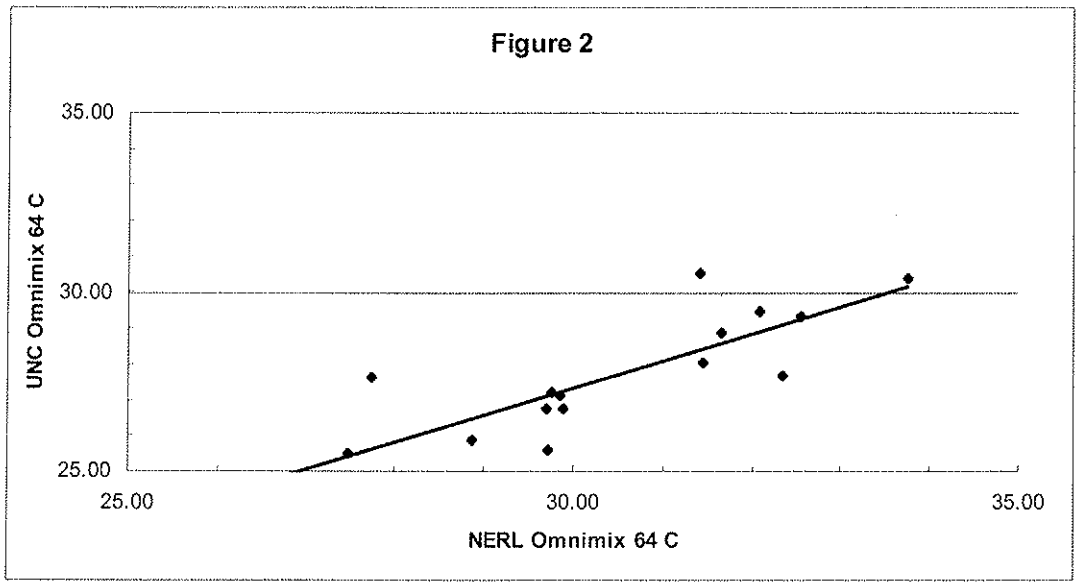


Figure 3: Comparison of ABI reagent analyses of simple bead milled samples (NERL Haugland Group) versus bead milled samples that were DNA extracted using the MagnaPure system (EPA Region 1 Doolittle Group). Regression analysis y = 1.1564x – 5.6323, r2 = 0.8578.

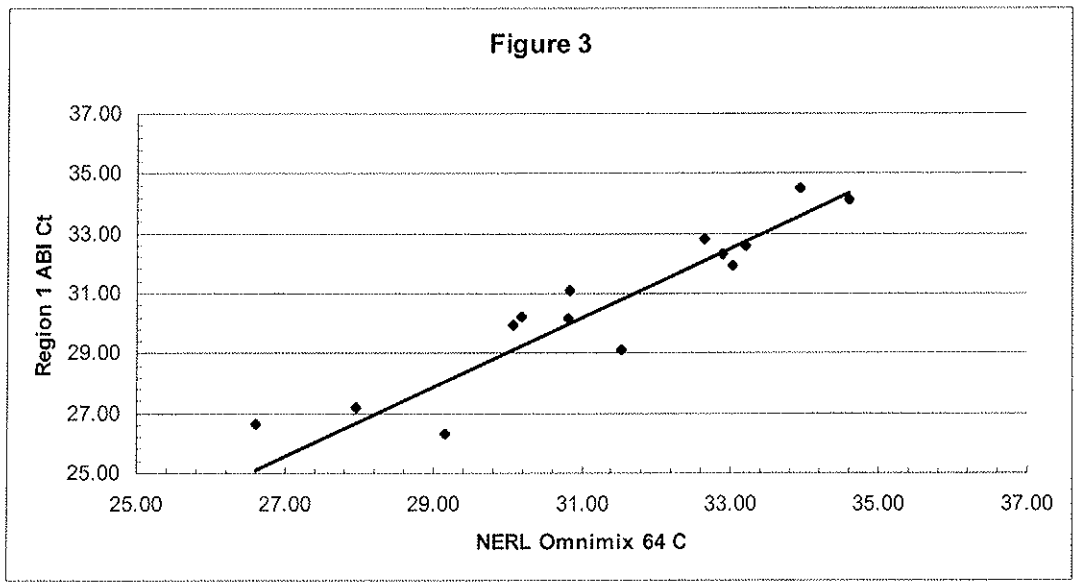
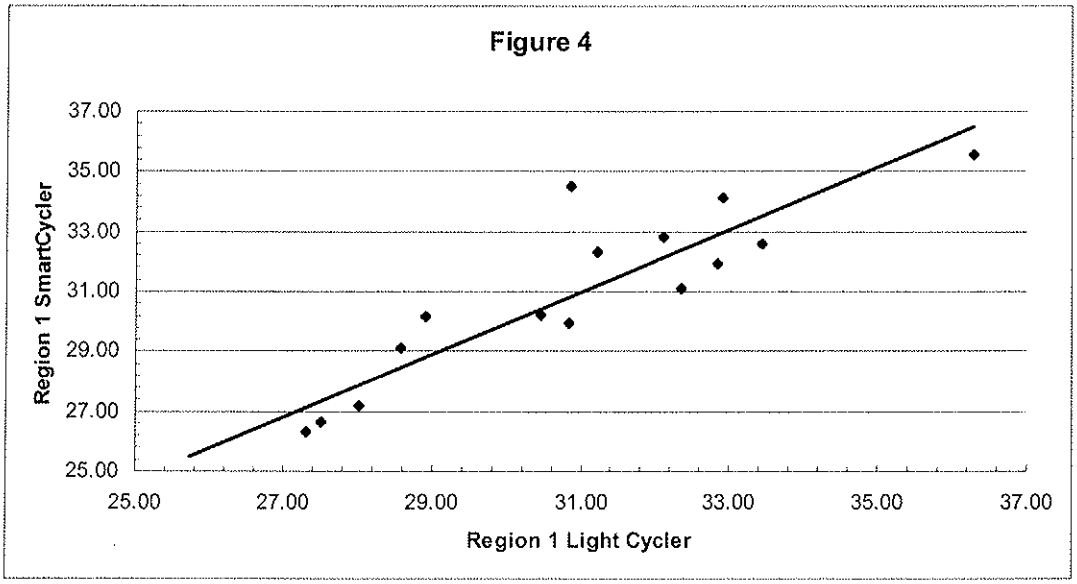


Figure 4: Comparison of QPCR results using two different QPCR platforms, the Roche Light Cycler (Region 1 ABI Ct) and the Cepheid SmartCycler. Regression analysis y = 1.0453x – 1.3955, r2 = 0.8248.



APPENDIX B

Recommendations from the California Beach Water Quality Workgroup

Recommendations from the California Beach Water Quality Workgroup

On December 15, 2005, and January 12, 2006, the Monitoring and Reporting Subcommittee of the California Beach Water Quality Workgroup (BWQWG) met to discuss results from the June 2005 Rapid Microbiology Method Evaluation study and to determine:

- Criteria for deciding whether a new, rapid evaluation method is ready for adoption by the State of California
- Which, if any, of the methods tested in the June 2005 study met these criteria
- Whether the June 2005 tests were adequate to assess criteria compliance
- The need for any additional evaluation testing

The Subcommittee identified two general approaches for determining if a new method is ready for adoption. The first approach involves demonstrating a level of consistency in quantification between the new and EPA approved methods; this approach is similar to methodology used to develop the BWQWG's recommendation for State adoption of the chromogenic substrate technique. The second involves conducting epidemiological studies that establish a relationship between new method measurements and relevant health risks. The Subcommittee preferred the second approach because the new rapid methods measure different endpoints than EPA approved methods, and thus the methods are not anticipated to be fully equivalent. However, the Subcommittee also recognized that the epidemiological approach is a lengthy and expensive process, potentially requiring modification of the State's bacterial standards based on the results of such studies. The Subcommittee recommended the pursuit of epidemiologic studies, but agreed that establishing equivalency criteria is a more expeditious approach for incorporating rapid methods into a warning system that is already hampered by extended processing time for EPA approved methods.

The Subcommittee went on to identify the following four criteria for determining whether there is sufficient equivalency for State acceptance of the new methods:

Accuracy – the new method should produce results that are equivalent to those of traditional water quality monitoring methods, without significant bias and within the bounds or variability observed for EPA approved methods (approximately one half of a log unit above and below the median value).

Repeatability – the new method should reproduce values for replicate samples with variability similar to that seen in traditional water quality monitoring methods, typically one half of a log unit (all of the methods tested were found to be more repeatable than EPA approved methods).

False Positives – the new method should not produce incorrect values indicating that a sample is above the AB411 standards when traditional water quality monitoring methods indicate the sample is below standards (false positives could lead to unnecessary beach closures).

False Negatives – the new method should not produce incorrect values indicating that a sample is below AB411 standards when traditional water quality monitoring methods indicate the sample is above standards (false negatives could result in swimmers being exposed to unsafe water quality).

Furthermore, the Subcommittee acknowledged the importance of the type of beach management application in determining how these evaluation criteria should be applied. Six types of management applications for which the new methods might be used were identified. These applications, along with potential advantages of having a rapid method for each application, and relevant evaluation criteria for acceptability of a new method with respect to each application, are described below.

APPLICATION #1: Tracking Spatial Progress and Dilution of a Sewage Spill from an Inland Source to a Beach

Potential Advantages– When a spill occurs, public health managers need to determine which beaches to close and when those closures need to occur. Those decisions depend on the rate at which contamination is moving down the watershed and the degree of dilution occurring along the way. With delayed information inherent in EPA approved methods, managers typically make conservative assumptions that tend toward closure of more beach area than may actually be necessary. New methods could allow for hourly progress reports on plume location and concentration so that managers could make better-informed decisions regarding appropriate beach closures.

Evaluation Criteria– The principal criterion for this Application is repeatability. Extreme precision or sensitivity is not essential to this Application because data interpretation is primarily limited to differences between high concentration plumes and background concentrations.

APPLICATION #2: Decision Support Relevant to Re-opening of Closed Beaches

Potential Advantages – Currently, California's public health managers reopen a closed beach after two consecutive samples indicate bacterial indicator levels below State standards. Rapid methods will provide this information to managers a full day sooner than EPA approved methods, allowing for avoidance of unnecessarily prolonged closures.

Evaluation Criteria– The principal criterion for this Application is that the rate of false negatives needs to be no greater than that for EPA approved methods, to prevent beach managers from incorrectly reopening a contaminated beach. If the rapid method is used alone, the Subcommittee determined that the rate of false positives should not be more than twice that of EPA approved methods. A higher allowable rate of false positives, which could lead to beaches remaining closed longer than necessary, would be more than offset by the rapidity with which beaches could be reopened because of the faster method.

APPLICATION #3: Routine Beach Monitoring

Potential Advantages – Presently, warning signs are posted in the afternoon of the day following the collection of a sample that exceeded standards. Rapid methods could allow warnings to be posted in the afternoon of the day the sample is collected.

Evaluation Criteria– Three criteria were identified for this Application:

1) A false negative rate that is no higher than that of EPA approved methods (a high priority being placed on ensuring that bathers aren't swimming in contaminated water).

2) Variability that is no greater than that for EPA approved methods.

3) A false positive rate that:

   a) Is no higher than 20%,

   b) Is no more than twice the false positive rate of EPA approved methods, and

   c) Does not lead to a net increase in beach signage, with the idea that the false positives could be more than offset by more timely removal of warning signs due to the speed of the new methods.

The Subcommittee was particularly concerned about this Application's potential for false positives because of the potential economic and aesthetic hardships for beachfront property owners when unnecessary warnings are issued. However, the Subcommittee anticipated the possibility that increased warnings generated from false positives could be balanced by the shorter duration of warning events due to the rapidity of the new methods. Criterion 3C is intended to quantify that balance between false positives and more timely removal of signage.

The Subcommittee was also concerned that this Application might require that new methods be available for at least two indicators, as the practices advocated for open coastal beaches by the BWQWG can lead to same-day posting in the event of two indicators exceed standards. Some members of the Subcommittee maintained that a single-indicator method would still be useful as EPA approved methods could be used to quantify the other indicators, but the method developers suggested that developing analogous methods for a second indicator (*E. coli*) would not be a difficult advance.

The Subcommittee also expressed concern that while the State may have the authority to allow the use of new methods for some purposes, it is important to maintain a level of sampling effort with EPA approved methods to remain responsive to the federal BEACH Act and to AB411 requirements. The Subcommittee felt that once per week sampling using EPA approved methods would be appropriate for this purpose and that these samples could also be used to address the geomean criteria of AB411. Thus, the new methods might not be used at beaches that are presently sampled only once per week; however, individual counties could augment present sampling practices with the use of new methods at the beaches where early warning is considered most important. The rapid methods might also be used for a faster confirmation sampling at such beaches, still speeding the warning system by a day.

APPLICATION #4: Tracking Fecal Contamination Sources to Their Origins

Potential Advantages – Identifying the source of high concentration contaminants is important to effective site remediation. At present, determining the source of high concentrations of fecal contamination can be done through sanitary surveys, in which upstream samples are collected to establish a spatial concentration gradient. However, traditional water quality monitoring methods are slow and such surveys proceed so slowly that the source has often dissipated by the time the survey is underway. Moreover, EPA approved methods require that sampling proceed in all directions simultaneously, which is expensive. Rapid methods would allow surveys to be initiated more quickly and to proceed sequentially from confluence to confluence, with the rapidity of the answer allowing a choice of which upstream direction to proceed with at each confluence.

Evaluation Criteria – Similar to the criteria for Application #1, the criteria for this Application does not require extreme precision as analysis involves only gross spatial patterns.

APPLICATION #5: NPDES Regulatory Compliance Assessment

Potential Advantages – Various facilities, such as wastewater treatment plants, conduct daily (or more frequent) sampling of their effluent for both compliance and plant process assessment. A rapid method would allow these facilities to respond more quickly to atypical results and contamination events.

Evaluation Criteria – New methods need to be nearly 100% equivalent to EPA approved methods for this Application, as false negatives are unacceptable for public health purposes and false positives are unacceptable because of the potential regulatory actions associated with recording elevated effluent concentrations.

APPLICATION #6: Tracking Trends in Beach Condition

Potential Advantages – None. Although managers are often interested in assessing changing beach conditions over time, typically there is no urgency in the reporting of such information.

Evaluation Criteria – A high degree of equivalency to EPA approved methods is important to the integrity of tracking trends. The level of consistency must be high enough to ensure that there is no confounding between potential trends and method bias.

The Subcommittee determined that two of the new methods (TMA and QPCR) had results during the June testing that were close to achieving the criteria for several applications, but concern was expressed that those tests were insufficient on their own for a complete evaluation. The Subcommittee was satisfied that the 2005 testing was a good initial step, but was concerned about the tests' emphasis on created samples to ensure a known concentration range, at the cost of including only six ambient samples in the assessment. The Subcommittee felt that ambient samples may contain potential confounding factors that were not encountered in the testing. The Subcommittee also raised a second concern that the evaluation of new methods in the June test

was based on implementation by the method developers. The Subcommittee recognized a need to establish that local practitioners are capable of producing comparable results.

The Subcommittee suggested that an additional type of testing, beta testing, be implemented by local practitioners employing the new methods in parallel with EPA approved methods on typical ambient samples. The Subcommittee recommended the use of at least two independent laboratories, processing at least 100 samples. In response, two local laboratories, the Orange County Sanitation District and County of Orange Public Health Laboratories, agreed to participate as beta testing facilities.

The Subcommittee further suggested that beta testing be a supplement, not a replacement for the type of testing conducted last summer. The testing conducted in June 2005 is an excellent selection process for determining which new methods are worthy of beta testing, which is a valuable process that should be repeated in the future as new methodology arises. Because beta testing requires considerable effort on the part of the local laboratories, it is an impractical means for initial evaluation of all methods.

APPENDIX C

Study Plan For Beta Testing Of New Rapid Bacterial Measurement Methods For Recreational Beach Applications

Study Plan For Beta Testing Of New Rapid Bacterial Measurement Methods For Recreational Beach Applications

The Monitoring and Reporting Subcommittee of California's Beach Water Quality Workgroup (BWQWG) was asked to examine results from the June 2005 study to evaluate new, rapid beach bacterial measurement methods. The BWQWG determined that the testing was an appropriate first step for evaluating new methods, but was insufficient in itself for making recommendations as to whether the new methods were ready for adoption by the State of California. The Subcommittee identified two shortcomings in the testing. First, the test placed emphasis on created samples, which were used to ensure that testing with a limited number of samples covered a wide range in bacterial concentration. This led to the inclusion of only six ambient samples, a number that the Subcommittee felt needed to be expanded because ambient samples can contain confounding factors that are less likely to be encountered in created samples. Second, the Subcommittee were concerned that the method developers processed the test samples and recommended that local practitioners produce comparable results.

The Subcommittee suggested that there should be an additional type of testing, referred to as beta testing, in which local practitioners perform the new methods on typical ambient samples in parallel with EPA approved methods. It was further recommended that this type of testing be conducted by at least two local laboratories, processing at least 100 samples. This document contains the study plan that was created in response to the Subcommittee's request.

The study will involve simultaneous processing of samples using both the new rapid methods and two EPA approved methods. Traditional water quality monitoring methods will include both EPA Method 1600 (mEI agar) and DS (IDEXX) methods. All samples will be processed in duplicate using both the new and EPA approved methods, to allow distinction of whether any observed differences in outcome resulted from natural processing variability or method bias. Samples of bacterial lysates will be stored so they can be used for confirmatory testing. Approximately 10% of the colonies will be isolated from mEI plates and IDEXX wells and stored on slants for later speciation by Vitek, a similar automated method, or phenotypic analysis.

Simultaneous processing using both new and EPA approved methods will be conducted for 175 samples. Those samples will consist of 25 samples from each of seven types:

- Open beach
- Near a drain
- Within a drain
- Embayment beaches
- Wet weather from areas that are influenced by a drain
- Sewage influent inoculated into urban runoff
- Sewage effluent inoculated into seawater

The sampling locations and sample types were selected to provide a variety of concentrations, matrices, and possible matrix interferences.

Two new methods did well enough in the June testing to advance to the beta testing. The QPCR method was most successfully implemented by both USEPA and UNC; the TMA implemented by Gen-Probe west the second most successful. Both UNC, in partnership with Cepheid, and Gen-Probe have agreed to participate in the second-phase testing by providing training and necessary equipment/supplies to implement their methods.

The Orange County Sanitation District (OCSD) and Orange County Public Health Laboratory (OCPHL), will serve as local practitioners. OCPHL will employ TMA and OCSD will employ QPCR. Samples from within drains and from embayment beaches will be collected by OCPHL, with the rest of the samples being collected by OCSD. Both laboratories will have flexibility to modify this during the course of the study). The laboratory that collects the sample will be responsible for processing them using EPA approved methods for that sample.

The sampling plan will remain flexible during the study. Data will be shared among OCSD, OCPHL, SCCWRP and the method developers on a daily basis. A conference call will be conducted among these participants after the first two weeks to discuss modification of sampling plans based on initial results.

Sampling will be initiated by the end of February to enhance the opportunity for collecting the desired number of wet-weather samples. Initially, each group will run four samples per day, though this number may be increased if logistically feasible and if the increase does not interfere with the laboratories' other responsibilities.

Both method developers will provide training to the local laboratories and be permitted to observe at the testing labs during the study to ensure that any inconsistency in results between new and EPA approved methods is due to the method itself and not method implementation. Gen-Probe will provide training during the week of February 6. The UNC-Cepheid team will provide training Feb 13-14.