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# BETA TESTING OF RAPID METHODS FOR MEASURING BEACH WATER QUALITY


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# EXECUTIVE SUMMARY

Public health officials routinely monitor fecal indicator bacteria levels to assess beach water quality, but present laboratory processing methods generally require 24 hours for completion and swimmers can be exposed to poor water quality during this time. Several researchers have been developing new methods that produce results within two hours, but there is a need for independent testing to evaluate their performance. The Southern California Coastal Water Research Project (SCCWRP) has conducted two such evaluations to date, but they were based on evaluating methods that were implemented by the method developers. This report describes a third evaluation study in which local practitioners implemented two of the promising methods.

The study involved simultaneous processing of samples using rapid methods and existing methods to enumerate fecal indicator bacteria. All samples were processed in duplicate using both sets of methods. The rapid methods tested were Quantitative Polymerase Chain Reaction (QPCR) and Transcription-Mediated Amplification (TMA). The existing methods included EPA Method 1600 (mEI agar) and the IDEXX defined substrate method. QPCR was performed for both enterococci and *Escherichia coli* by the Orange County Sanitation District. TMA was performed for enterococci by the Orange County Public Health Agency.

One hundred sixty three samples were processed, 138 of which were ambient samples collected from 41 locations. The remaining samples consisted of clean seawater spiked with primary sewage influent or secondary effluent. A small set of additional samples was inoculated with secondary sewage effluent, treated with chlorine that was subsequently neutralized, and analyzed at several time points over the course of hours to assess how well the rapid methods enumerate enterococci in chlorine disinfected wastewater. Each method's performance was evaluated with respect to the State's water quality standards and variability among replicates. These evaluations were integrated into an overall assessment to determine if management decisions based on rapid method results would have been the same as decisions based on traditional methods.

Both of the rapid methods were more than 85% accurate with respect to the State standard for enterococci and had average coefficients of variation comparable to that for existing methods. Both methods produced results for enterococci that would have led a public health officer to make the same beach management decision that would have been made if the measurements had been obtained using EPA-approved methods approximately 70% of the time. This is nearly equal to the 75% decision agreement rate for Enterolert™ when compared to the decisions that would have been made using EPA Method 1600. However, the differences between Enterolert™ and EPA Method 1600 were random error associated with measurement precision, whereas the errors associated with the rapid methods were the result of underestimation bias. The underestimates were primarily associated with stormdrain samples and appeared to result from amplification inhibition.

In contrast to the underestimation for ambient samples, both rapid methods overestimated bacterial concentrations in the chlorinated effluent samples. Enterococci concentrations measured by traditional methods fell below detectable levels following chlorination, while enterococci measured by QPCR and TMA remained in the tens of thousands over the same period. The rapid methods target nucleic acids rather than viable cells, and they detected the residual genetic material of the non-viable cells, which would render these methods problematic for measuring samples in the vicinity of disinfected wastewater plumes.

While neither of the rapid methods performed well enough to be recommended for adoption, there is reason to be optimistic. Technology transfer to working laboratories was successful, as laboratory personnel with minimal previous experience in performing molecular methods were able to master the rapid techniques in a relatively short period of time. Although there was evidence of inhibition in some samples, the method developers are committed to future inclusion of new internal controls in the assays to detect inhibition and provide a correction factor if amplification inhibition occurs. However, further method evaluation tests will be required to assess whether these controls are effective.

# ACKNOWLEDGEMENTS

We wish to thank the staff at Orange County Sanitation District and Orange County Public Health Laboratory for their assistance in collecting and preparing test samples, and beta testing these methods. Thanks also to Cepheid, Inc. and Gen-Probe, Inc. for providing the necessary equipment and reagents to conduct this study. We also thank the California Beach Water Quality Work Group for their assistance in formulating our study plan.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# INTRODUCTION

California's beach water quality monitoring programs are the most comprehensive in the nation. State Health Services regulations require measurement of three indicator bacteria (total coliforms, fecal coliforms and enterococci) on at least a weekly basis at high use beaches, with many beaches even monitored on a daily basis. Regulations further require that the public be warned of possible health risk if any of these indicator bacteria exceed threshold values established through epidemiological studies.

While California's beach monitoring programs are extensive, there remains opportunity for improvement. Current laboratory measurement methods require up to 96 hours to enumerate indicator bacteria. Contaminated beaches remain open during this processing period, but indicator bacteria may already have returned to acceptable levels by the time laboratory results are available and warning signs are posted. 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 new 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, enabling managers to take action to protect public health (i.e., post warnings or close beaches) on the same day that water samples are collected.

While promising, these rapid methods require extensive independent testing before they can be adopted for use in beach water quality monitoring. The State of California has requested that the Southern California Coastal Water Research Project (SCCWRP) conduct such evaluation tests. SCCWRP has conducted two such evaluations to date, the first of which tested five methodologies in 2004 (Griffith *et al.* 2004). None of the rapid methods produced results equivalent to those of existing methods, but several performed well enough to be optimistic about their future development. In 2005, SCCWRP conducted a second evaluative test that included the two top performing methods from the 2004 evaluation. In this evaluation, three rapid methods were found to produce results that were nearly comparable to those of existing methods.

Following the 2005 evaluation study, California's Beach Water Quality Workgroup (BWQWG) was asked to evaluate the study's results to assess whether the new methods were ready for adoption within California. The BWQWG determined that while the testing was an appropriate first step for evaluating new rapid indicator measurement methods, the testing alone was insufficient to determine whether the new methods were ready for adoption. They identified two shortcomings in the testing. First, the test placed emphasis on laboratory-created samples, which were used to ensure that testing with a limited number of samples would cover a wide range of bacterial concentrations. The BWQWG felt that the tests needed to be expanded to include a greater number of ambient samples to address confounding factors less likely to be encountered in laboratory-created samples. Second, they were concerned that the method developers were the ones that had processed the test samples. Since molecular methods are not currently commonplace in water quality testing laboratories, the BWQWG identified the

need to establish that local water quality personnel could produce comparable results when running the assays themselves.

The BWQWG suggested the use of an additional type of testing, referred to as beta testing, in which local practitioners perform the new methods on typical ambient beach water samples in parallel with existing methods. They further recommended that this testing be conducted by at least two local laboratories, processing at least 100 samples. Developers of two of the most promising methods from the 2005 test agreed to collaborate with SCCWRP and two local water quality monitoring laboratories to conduct beta testing of these methods. This document details the results of that study.

# METHODS

This study involved simultaneous processing of samples using two new rapid methods and two existing methods. The rapid methods tested were Quantitative Polymerase Chain Reaction (QPCR) as developed by Dr. Rachel Noble of the University of North Carolina and Transcription-Mediated Amplification (TMA) as developed by Gen-Probe Incorporated (San Diego, CA). The existing methods included EPA Method 1600 (mEI agar) and the IDEXX defined substrate method (Westbrook, ME). QPCR was performed for both enterococci (ENT) and *Escherichia coli* (EC) by the Orange County Sanitation District (OCSD), Fountain Valley, CA. TMA was performed for ENT by the Orange County Public Health Agency Laboratory (OCPHL), Newport Beach, CA.

One hundred sixty three samples were processed, 138 of which were ambient samples collected from 41 locations. The remaining samples consisted of seawater spiked with primary sewage influent or secondary effluent. All samples were processed in duplicate using both sets of methods. Testing was conducted from February through July 2006.

Ambient water was collected from five categories of locations: open ocean beaches distant from storm drains that serve as outlets for land-based runoff; open ocean beaches near storm drains; enclosed embayment beaches; locations within flowing storm drains; and wet-weather samples from open ocean beaches (Table 1). Ambient samples were collected following Standard Methods 1060 protocol (APHA 1995) and transported on ice by the collecting agency to either OCSD or OCPHL.

Sewage spiked samples were created by inoculating clean ocean water with varying concentrations of either primary sewage influent or secondary sewage effluent (Table 1). Clean ocean water was collected outside of the influence of land-based sources of fecal contamination at a location seven miles offshore of Newport Harbor. Sewage was obtained from Orange County Sanitation District's Plant #2 wastewater stream. Following inoculation, sewage spiked samples were stirred for a minimum of 15 minutes using a magnetic stirring plate and a sterile stir bar to ensure even distribution of fecal indicator bacteria throughout.

All samples were split into thirds for processing. The first third was analyzed for ENT and EC using existing methods by the laboratory that collected the ambient sample or created the spiked sample. The second third was analyzed by the collecting agency using one of the new methods: OCSD used QPCR, and OCPHL used TMA. The last third was immediately transported on ice to the non-collecting agency for analysis using the other new method.

## QPCR Methods

The QPCR method was designed to detect and enumerate unique DNA target sequences found in *Enterococcus* spp. and *E. coli.* For this method, each 100ml sample was passed through a 0.4-µm polycarbonate membrane (Osmonics, Livermore, CA) to capture bacteria on the filter. The filter was then subjected to bead beating alone or in addition to chemical or thermal treatment to lyse the cells and release the target DNA. Two variants

of the QPCR method were used in this study. In the first, QPCR bead-beaten, the DNA was used directly in the QPCR reaction following the bead-beating step. In the second, QPCR extracted, the DNA was further purified and concentrated after bead beating using a commercially available DNA extraction kit (MoBio, Carlsbad, CA) before being added to the PCR reaction. In both cases, bead beating was followed by the quantification step, in which the DNA of the target organism was simultaneously amplified and measured using a system of primers and fluorescent probes, then analyzed using an advanced optics QPCR instrument. For a detailed description of the protocol for each variant of the QPCR method, see Griffith and Weisberg (2006).

QPCR was initially performed on 33 samples using the extracted method (Table 1). However, the multi-step purification and concentration portion of the method proved logistically difficult for personnel at the test laboratories to perform. As a result, a decision was made to switch sample preparation to the less labor-intensive bead-beaten approach. The balance of the samples, except for eleven samples lost to a laboratory accident, was analyzed for both EC and ENT using the bead-beaten approach. In addition, laboratory personnel began storing samples for batch analysis at –80ºC following the initial filtration. This increased processing efficiency, as the thermocycler was able to accommodate a week's worth of samples in a single run.

For absolute quantification of the ENT and EC targets using the QPCR assay, water quality personnel initially utilized a standard curve approach. However, because of workflow issues, this approach was changed to utilize a "calibrator" for each QPCR run. That is, known quantities of either *E. faecalis* or *E. coli* were added to blank filters, bead beaten, and analyzed in the same manner as the unknown water samples, thereby providing a basis for quantification. Results from the calibrators were then used 1) as positive controls for the *Enterococcus* target or the *E. coli* target in the QPCR assays, and 2) as the basis for target sequence quantification using the dCt method of Haugland *et al.* (2005). The calibrators provided a means for quantification of run-to-run variability, sequence recovery, and PCR efficiency.

Two types of internal controls were also incorporated into the QPCR assay. The first of the internal controls was a Sample Processing Control (SPC) in the form of *Lactococcus lactis* cells. A known quantity of *L. lactis* cells were spiked onto every filter created from collected water samples, which allowed assessment of target recovery from the bead beating process. The second type of internal control was the Super Smart Internal Control (SSIC), developed by Cepheid (Sunnyvale, CA) as part of the SmartBead® system of reagents and included in all ENT and EC QPCR reactions. The SSIC is a proprietary combination of nucleic acid template, primers, and a fluorescent probe, that is incorporated into each lyophilized SmartBead. The SSIC contains a known copy of target DNA that, under ideal conditions in which no interfering substances are present in a sample, results in a consistent Ct value observed after the QPCR run is complete. However, when inhibition caused by various sample matrix constituents (e.g., humic or fulvic acids or colloidal substances) is present in the reaction, the SSIC amplifies at a higher Ct (>1.65 Ct difference from the normal condition) indicating that the reaction is inhibited. A Ct difference of greater than 1.65 was chosen as the threshold for designating an inhibited

sample based upon the ½ log variability typically seen with other EPA-approved methods (Noble *et al.* 2004, Griffith *et al.* 2006).

The initial calculation of QPCR results employed the $\Delta C_T$ (comparative cycle threshold) calculation method. This calculation method, derived by Applied Biosystems (Anonymous 1997) for calculating the ratios of target sequences in two DNA samples (e.g., a calibration and water filtrate sample), normalizes for differences in total DNA recovery from samples using QPCR analysis $C_T$ values for the SPC sequence. A second set of results adjusted for amplification efficiency was calculated by applying an amplification factor (AF) to the original results produced by the $\Delta C_T$ method. The AF, a measure of the average efficiency at which target DNA is amplified during QPCR cycling, was calculated by the laboratory staff at OCSD using the average of many standard curves.

### TMA Method

TMA is similar in concept to QPCR in that it amplifies a genetic target in the bacteria and uses a fluorescent probe for detection (Persimoni *et al.* 2002). TMA differs from QPCR in that it targets bacterial RNA, rather than DNA, and uses two enzymes (RNA polymerase and reverse transcriptase) to amplify the target sequence. In this application, the assay targeted Group II and Group III *Enterococcus* spp. As with the QPCR method, bacteria were first captured on a filter. Following this step, the filter was washed to remove interfering substances and chemical, and an enzymatic treatment was used to lyse the bacteria. A specific capture oligomer was then used to bind enterococcal rRNA which was subsequently captured by oligo dT sequences bound to magnetic beads. The bead/probe/RNA complex was then pulled out of solution using a powerful magnet, leaving cellular debris and other contaminants behind. The captured RNA template was then combined with amplification reagents and inserted into a real-time amplification engine.

The TMA method used in this study requires one priming oligonucleotide and one terminating oligonucleotide in the first step of the reaction. Both oligonucleotides hybridize to the target rRNA at defined sites. Reverse transcriptase creates a DNA copy of the target rRNA by extension from the end of the priming oligonucleotide to the terminating oligonucleotide. The RNA in the resulting RNA:DNA duplex is degraded by the RNAseH activities of the reverse transcriptase. A promoter oligonucleotide then binds to the DNA copy. This oligonucleotide contains a promoter sequence for RNA polymerase and a blocking group at the opposite end. A new strand of DNA is synthesized from the end of the promoter oligonucleotide by reverse transcriptase creating a double-stranded DNA molecule at the promoter site. The blocking group prevents DNA synthesis at the opposite end of the promoter oligonucleotide. RNA polymerase recognizes the promoter sequence in the DNA template and initiates transcription. Each newly synthesized RNA amplicon then re-enters the TMA process and serves as a template for a new round of replication, leading to an exponential expansion of the RNA amplicon. Since each of the DNA templates can make 100 to 1000 copies of RNA amplicon, this expansion can result in the production of up to 10 billion amplicons. The entire process is autocatalytic and performed at a single temperature.

Following the amplification process, a standard curve was used to calculate the number of ENT RNA copies in each sample. An equation was used to calculate the number of ENT cells per 100 ml based upon the expected number of rRNA copies per cell. No internal controls were utilized in the TMA assays during this study, although subsequently such controls have been developed for this assay.

TMA was performed on all 163 samples. Similar to QPCR, most samples were frozen following the initial purification step and processed in batches at the end of each week to maximize laboratory efficiency.

### Effect of Chlorination

In addition to the testing described above, a small set of laboratory-created samples containing secondary sewage effluent was treated with chlorine that was subsequently neutralized, and assayed over time. The purpose of these samples was to test the ability of the genetic methods to accurately enumerate ENT in chlorine disinfected wastewater. Sodium hypochlorite was added to 3.3L secondary sewage effluent from OCSD to achieve a final concentration of 8 mg/L. Samples were then treated with sodium thiosulfate to neutralize free chlorine, which was measured using EPA Method 330.5 (APHA 1995). Sub-samples were analyzed using both the new and existing methods prior to chlorination, immediately following chlorination and at 15 minutes, 30 minutes,1 hour, 2 hours, and 4 hours after chlorination.

### Data Analysis

The primary means of data analysis was to compare the results from the new rapid methods to those produced by EPA-approved methods. This was done in four ways. First, we assessed the number of individual samples from each new method that differed by half a log unit from the reference method median. One-half of a log unit was selected because previous laboratory intercalibration studies (Noble *et al.* 2004, Griffith *et al.* 2006) have demonstrated that this is the typical range of variability observed for EPA-approved methodologies, both within and among laboratories. For this analysis, samples that were identified as non-detect by the reference methods 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 ENT and 400 cells/100 ml for EC, as the State requires posting warning signs for any sample 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 methods.

The third analysis assessed precision of the measurements. Precision was quantified as the coefficient of variation (CV) and was compared between the new and existing methods. For this data analysis, the number 10 was substituted for nondetects in order to normalize the new methods with the detection limits of the EPA-approved methods.

The fourth 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 duplicate results from each sample processed by new methods were compared to those of the reference labs and categorized as "Equivalent", "Materially Different", or "Not Materially Different" than existing methods.

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

- both replicates and the median were correct with respect to the AB411 standard
- both replicates were within ½ log unit of existing methods median
- replicates exhibited a variance smaller than 2x the variance of existing methods

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

- average of replicates incorrect with respect to standard
- average of replicates differed by >½ log unit from existing methods
- coefficient of variation is >4x that of existing methods

Samples that failed none of the materially different criteria, but did not meet all of the equivalency criteria, were classified as "Not Materially Different" than current methods.

# RESULTS

## QPCR

The QPCR bead-beaten method produced ENT results that were accurate with respect to the California AB411 standard 85% of the time, which is slightly less than the 93% correct classification rate when comparing between existing methods (Table 2). The correct classification rate for the QPCR extracted method was only 76%. For both QPCR methods, the number of false negative errors was much greater than the number of false positive errors (Table 3). The highest false positive rates were observed for storm drain samples, while open ocean beach samples (both methods) or ocean beaches near drains (bead beaten method only) accounted for the majority of false negative results (Table 4). Adjusting results for amplification efficiency caused a net reversal between false positive and false negative rates, with false positive results becoming dominant, but did not improve the accuracy of either method in relation to the AB411 standard for ENT (Table 5).

The bead-beaten method consistently underestimated ENT levels compared to existing methods (Table 6; Figure 1). Approximately one-tenth of samples from the bead-beaten method fell outside of the range of ½ log unit above or below the median value produced by the EPA-approved methods, with the majority falling below this range. The greatest percentage falling below the acceptable level was from storm drains, for which internal controls indicated some level of inhibition in nearly 30% of samples. Adjusting results for amplification efficiency led to overestimates of ENT concentrations (Table 7). The net effect of adjustment for controls was that the percentage of results below the acceptable range fell 10%, but the percentage of samples above the acceptable range increased ten-fold.

The pattern of underestimation was even more pronounced for the extracted method (Table 6; Figure 2). Over 40% of results were >½ log unit below the median of the reference methods, with no results >½ log unit above the reference methods. Wet-weather samples accounted for the majority of results outside of the acceptable range, although greater than 15% of the open ocean beach samples were also low. Adjusting results for amplification efficiency reduced the percentage of samples below the acceptable range, but caused an increase in results >½ log unit above the median (Table 7).

Precision of the QPCR bead-beaten method was comparable to that of Enterolert™ and almost equivalent to that of EPA method 1600 (Table 8). However, precision of the QPCR extracted method was almost three times greater than that of the existing methods. When results were adjusted for amplification efficiency, both QPCR methods exhibited precision three times that of existing methods.

The QPCR bead-beaten 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 obtained using existing EPA-approved methods 73% of the time (Table 9). Adjusting the bead-beaten results for amplification efficiency markedly

reduced the percentage of results that would have produced comparable management outcomes. For ENT, the extracted method would have resulted in the same management decision as that based on measurements obtained using existing EPA-approved methods only 50% of the time. Adjusting the extracted method results for amplification efficiency had little effect on management-decision outcomes.

QPCR results for EC were slightly better than those for ENT. Results were correct with respect to the California AB411 standard 92% of the time (Table 10). The false negative rate was 8%, with the majority occurring in seawater inoculated with sewage and ambient samples collected during wet weather. No false positive results were observed, but adjusting for amplification efficiency resulted in a false positive rate of 27%.

None of the EC results were >½ log unit above those from the median of the existing methods (Table 11). The percentage of samples below the acceptable range was also low, with storm drain and open ocean beach samples accounting for most of the underestimation. When results were adjusted for amplification efficiency, nearly half were >½ log unit above the reference lab median.

Precision between EC replicates for QPCR equaled that of the existing methods. However, as was the case with the QPCR methods for ENT, repeatability between replicates declined when results were adjusted for amplification efficiency (Table 12).

The QPCR method for EC produced results that would have resulted in a public health officer making the same management decision that would have been made if the measurements had been obtained using EPA-approved methods 70% of the time (Table 13). Results were equivalent to those of current methods for 54% of samples and not materially different for an additional 16%. In contrast, results adjusted for amplification efficiency were materially different than those of currently approved methods for almost 70% of samples.

### TMA

The TMA method produced results that were correct with respect to the California AB411 standard for ENT 86% of the time (Table 2). TMA had a false positive rate equal to that of the EPA-approved methods, but had a false negative rate of 33% (Table 3). Underestimation was especially pronounced for samples taken at enclosed beaches and from ocean beaches near storm drains, while the lowest false negative rates were for samples containing sewage influent and those collected during wet-weather (Table 4).

TMA performed best relative to existing methods for samples that contained moderate to high levels of ENT (Figure 3), but underestimated levels of ENT by >½ log unit for about one-fifth of all samples (Table 6). The percentage of results below the acceptable range was greatest for samples taken from storm drains, near ocean drains and in samples containing sewage effluent. Only 4% of results produced by TMA were >½ log unit above the median, but the majority of these occurred for samples collected from storm drains, which also accounted for the greatest percentage of TMA results that were >½ log unit below the median of the EPA-approved methods.

TMA exhibited precision between replicate samples that was in the same range as the QPCR bead-beaten and Enterolert™ methods, but was less precise than EPA Method 1600 (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 methods 69% of the time. Results measured by TMA were equivalent to current methods for 62% of samples and not materially different from existing methods for 7% of samples (Table 9). However, due to the tendency of the method to underestimate ENT in certain sample types, TMA produced results that were materially different than current methods almost one third of the time.

### Effect of Chlorination

Immediately following chlorination, concentrations of ENT measured by EPA Method 1600 and Enterolert™ fell to and remained at the lower limit of detection for each method. In contrast, chlorine produced no discernable effect on ENT measurements obtained using QPCR or TMA methods, which remained constant at about 10,000 ENT/100 ml for the duration of the experiment (Figure 4). Chlorine demand in the secondary effluent remained relatively constant throughout the course of the experiment (Table 14).

# DISCUSSION

Technology transfer of rapid measurement methods to working water quality laboratories was largely successful. Except for the QPCR extracted method, which was abandoned because OCSD staff found it difficult and time-consuming, personnel from both beta testing laboratories were able to competently execute the rapid methods. While both laboratories identified numerous opportunities for automating several steps in the process, they found the methods workable and were able to achieve results that were comparable to those produced by expert operators in the previous evaluation studies (Griffth and Weisberg 2006).

Both methods produced ENT results that would have led to a public health officer making an equivalent decision as would have been made if the measurement had been obtained using an existing EPA-approved method about 70% of the time. This is only slightly less than the 75% agreement rate between Enterolert™ and EPA Method 1600 for the same samples. However, the differences between the two existing methods resulted from random measurement precision error, whereas the rapid methods were biased low relative to existing methods. This led to a false negative rate almost three times higher than that observed when the two current methods were compared to each other.

A false negative, in which a sample that actually exceeds standards is measured as below standards, is problematic in a public warning system. Beach managers place a high priority on ensuring that the public is not swimming in contaminated water. When a false negative occurs, there is no other mechanism for capturing the fact that a problem exists. In contrast, a false positive would lead to an inappropriate posting, which could be remedied by additional sampling with alternative methods or alternative indicators that would be triggered by the positive measurement.

The most likely explanation for underestimation is inhibition of nucleic acid amplification reactions associated with both methods. Inhibition typically occurs when there are compounds in the source water, often high molecular weight compounds, such as humic acids and other complex carbohydrates, that combine with metal ions to sequester nucleic acids from polymerases and prevent amplification (De Boer *et al.* 1995, Kreader 1996). The TMA method possesses a target capture step which has been shown to be effective at removing inhibitors prior to the transcription mediated amplification reaction. Despite this, this study's finding of greater underestimation for the storm drain samples is consistent with inhibition, as compounds that inhibit nucleic acid amplification are likely to occur in storm drains. These drains discharge a complex mixture of inputs from an urbanized landscape, including humic and fulvic acids, as well as other organic compounds from the breakdown of plant material, and a wide variety of metal ions from automobiles.

The addition of the extra cleanup steps in the QPCR extracted method was intended to reduce inhibition, but the method was neither repeatable nor simple enough for the laboratory personnel to master. The extracted method had a coefficient of variation

between replicates that was three times higher than that for the bead-beaten or existing methods. Inclusion of the extra steps also had little effect on underestimation, though this may have had more to do with implementation than with the underlying efficacy of the method. The implementation difficulty resulted from the many pipetting steps of the extracted method that introduced opportunities for imprecision, but part of the difficulty with implementation likely stemmed from the manner in which the laboratory attempted to integrate the method into their regular workflow. Their approach was to break the protocol into segments that could be performed by a succession of technicians. This likely introduced error due to varying levels of familiarity with the purification method and opportunities for miscommunication between technicians performing successive steps of the protocol. Perhaps the precision, convenience, and suitability of this method for reducing inhibition will improve over time as method developers establish a means for automating steps in the process.

Developers of the QPCR methods incorporated two internal controls into their assays that were intended to provide a means to measure and compensate for inhibition or recovery issues. One of these controls, the SSIC, was used in calculating the "adjusted" ENT values. While the adjustment for controls reduced the false negative error rate, it led to an overall increase in the level of error. For example, the percentage of QPCR bead-beaten samples for which a materially different decision would have been made compared to a decision based on existing methods increased from 27% to 77% after adjustment (Table 9). It is unclear why the adjustment failed to improve comparability with existing methods. One possibility is that the target primers and probe that comprise the SSIC have a different amplification efficiency than those used to detect ENT or EC. For example, the SSIC may be more sensitive to inhibitory compounds than are the ENT and EC assays because it is designed to be particularly sensitive toward inhibition. If this is the case, then extrapolating inhibition rates from the SSIC might lead to the type of overestimation that was observed. Regardless of the cause, developers of both methods have indicated that they are placing priority on developing improved internal controls for implementation in future evaluation studies.

It is also possible that freezing the samples led to some of the underestimation. Samples were frozen in both beta testing laboratories because the number of samples collected daily for this effort was too small to cost-effectively quantify them on a daily basis. Freezing would not be part of normal practice if these methods were adopted for routine use. However, it is unlikely that freezing after extraction was a major contributor to the underestimation. Previous studies have shown that genetic material remains intact when frozen at –80°C as were the samples used in this study (Smith 2005). Moreover, an improvement in classification accuracy was not observed for those samples that were processed without freezing.

Previous evaluations of genetically based methods have suggested that overestimation relative to existing methods is a greater concern than underestimation (Noble and Weisberg 2005). Overestimation typically results because nucleic acid methods are based on measuring the presence of specific genetic fragments without assessing the presence of live, viable cells. This concern was certainly manifested in the chlorination

portion of the study, in which no viable cells were measured by existing methods, but bacterial concentrations, as measured by the genetic methods, were relatively unchanged following chlorination. This indicates that the new methods should not be used for measurement near a chlorinated effluent; however, evidence that overestimation due to viability was a concern at other locations was not observed. Many of this study's sampling sites were located in or near storm drain effluents, which are likely to contain several day old fecal sources originating upstream. Perhaps the lack of overestimation relative to existing methods reflects moderation by inhibition, but an alternative explanation is that genetic material breaks down at rates comparable enough to inactivation of live cells that the ratio of live:dead cells is not large enough to be of concern. Certainly this is an area that requires further investigation.

Though the rapid methods beta tested in this study are not yet ready to adopt for beach water quality monitoring, they are approaching the accuracy of some existing EPA-approved methods. Further, this study showed that the technology needed to perform these methods can be successfully transferred to working water quality laboratories and performed by technicians with little or no prior experience with molecular methods. Despite these encouraging findings, there is still work to be done. It is apparent that the internal controls developed for the QPCR method to guard against false negative results have not been perfected at this time, and that given the tendency of TMA toward underestimation, an internal control is needed for this method as well. Finally, labor requirements are an impediment to implementing these methods in their current iterations. The challenge will be for method developers to introduce a level of automation to their methods that minimizes errors and reduces labor costs while keeping instrumentation affordable.

**Table 1. Type and number of samples analyzed for ENT. A dash indicates no analyzed samples for that method.**

| Sample Type | Number of Samples | QPCR Extracted | QPCR Bead-Beaten | Hydrus | EPA-Approved |
|---|---|---|---|---|---|
| Ocean Beach Near Drain | 18 | - | 18 | 18 | 18 |
| Open Ocean Beach | 44 | 12 | 24 | 44 | 44 |
| Enclosed Beach | 22 | - | 19 | 22 | 22 |
| Storm Drain | 29 | - | 28 | 29 | 29 |
| Wet Weather | 25 | 21 | 4 | 25 | 25 |
| Sewage Influent | 19 | - | 20 | 19 | 19 |
| Sewage Effluent | 6 | - | 6 | 6 | 6 |
| Total | 163 | 33 | 119 | 163 | 163 |

**Table 2. Percent accuracy with respect to California AB411 standard of 104 ENT/100 ml.**

| Method | Correct |
|---|---|
| EPA Method 1600 | 94 |
| Enterolert™ | 93 |
| QPCR Extracted | 76 |
| QPCR Bead Beaten | 85 |
| QPCR Extracted (Adjusted) | 66 |
| QPCR Bead Beaten (Adjusted) | 70 |
| TMA (Hydrus) | 86 |

**Table 3. Percent false positive or false negative with respect to the AB411 standard of 104 ENT/100 ml.**

| Method | False Positive | False Negative |
|---|---|---|
| EPA Method 1600 | 4 | 10 |
| Enterolert™ | 4 | 12 |
| QPCR Extracted | 3 | 58 |
| QPCR Bead Beaten | 9 | 29 |
| QPCR Extracted (Adjusted) | 24 | 6 |
| QPCR Bead Beaten (Adjusted) | 31 | 3 |
| TMA (Hydrus) | 4 | 33 |

**Table 4. Percent false positive and false negative results with respect to the AB411 standard of 104 ENT/100 ml by sample type.**

| Sample Type | TMA (Hydrus) | | QPCR Extracted | | QCPR Bead Beaten | |
|---|---|---|---|---|---|---|
| | False Positive | False Negative | False Positive | False Negative | False Positive | False Negative |
| Open Ocean Beach | 2 | 57 | 0 | 100 | 0 | 83 |
| Ocean Beach Near Drain | 0 | 100 | - | - | 0 | 50 |
| Enclosed Beach | 0 | 100 | - | - | 0 | 0 |
| Storm Drain | 2 | 42 | - | - | 44 | 42 |
| Wet Weather | 1 | 13 | 13 | 62 | 0 | 0 |
| Influent | 0 | 10 | - | - | 10 | 10 |
| Effluent | 0 | 25 | - | - | 0 | 0 |

**Table 5. Percent false positive and false negative results with respect to the AB411 standard of 104 ENT/100 ml after adjustment for amplification efficiency.**

| Sample Type | QPCR Extracted | | QCPR Bead Beaten | |
|---|---|---|---|---|
| | False Positive | False Negative | False Positive | False Negative |
| Open Ocean Beach | 13 | 8 | 10 | 0 |
| Ocean Beach Near Drain | - | - | 25 | 0 |
| Enclosed Beach | - | - | 42 | 0 |
| Storm Drain | - | - | 52 | 11 |
| Wet Weather | 7 | 0 | 0 | 0 |
| Influent | - | - | 10 | 0 |
| Effluent | - | - | 20 | 0 |

**Table 6. Percent samples >½ log unit above or below median values of EPA-approved methods for ENT.**

| Sample Type | TMA (Hydrus) | | QPCR Extracted | | QCPR Bead Beaten | |
|---|---|---|---|---|---|---|
| | >1/2 log Above | >1/2 log Below | >1/2 log Above | % >1/2 log Below | >1/2 log Above | >1/2 log Below |
| Open Ocean Beach | 3 | 16 | 0 | 17 | 2 | 27 |
| Ocean Beach Near Drain | 0 | 26 | - | - | 3 | 8 |
| Enclosed Beach | 0 | 14 | - | - | 0 | 3 |
| Storm Drain | 14 | 31 | - | - | 13 | 29 |
| Wet Weather | 2 | 8 | 0 | 50 | 0 | 0 |
| Influent | 3 | 15 | - | - | 0 | 5 |
| Effluent | 0 | 30 | - | - | 0 | 20 |
| All | 4 | 19 | 0 | 41 | 4 | 16 |

**Table 7. Percent samples >½ log unit above or below median value of EPA-approved for ENT after adjustment for amplification efficiency.**

| | QPCR Extracted | | QCPR Bead Beaten | |
|---|---|---|---|---|
| Sample Type | >1/2 log Above | >1/2 log Below | >1/2 log Above | >1/2 log Below |
| Open Ocean Beach | 13 | 17 | 12 | 5 |
| Ocean Beach Near Drain | - | - | 47 | 0 |
| Enclosed Beach | - | - | 53 | 0 |
| Storm Drain | - | - | 70 | 11 |
| Wet Weather | 29 | 21 | - | - |
| Influent | - | - | 31 | 13 |
| Effluent | - | - | 90 | 0 |
| All | 38 | 24 | 47 | 6 |

**Table 8. Average coefficient of variation (CV) among ENT replicates.**

| Method | Average CV |
|---|---|
| EPA Method 1600 | 0.18 |
| Enterolert™ | 0.21 |
| QPCR Extracted | 0.59 |
| QPCR Bead Beaten | 0.21 |
| QPCR Extracted (adjusted) | 0.59 |
| QPCR Bead Beaten (adjusted) | 0.60 |
| TMA (Hydrus) | 0.25 |

**Table 9. Percent agreement in beach management decisions using existing EPA-approved methods compared to agreement in decisions based on new rapid measurement methods for ENT. EPA-approved methods were compared to one another.**

| Method | Equivalent | Not Materially Different | Materially Different |
|---|---|---|---|
| EPA Method 1600 | 72 | 10 | 18 |
| Enterolert™ | 59 | 16 | 25 |
| QPCR Extracted | 47 | 3 | 50 |
| QPCR Bead Beaten | 67 | 6 | 27 |
| QPCR Extracted (adjusted) | 30 | 15 | 55 |
| QPCR Bead Beaten (adjusted) | 17 | 6 | 77 |
| TMA (Hydrus) | 62 | 7 | 31 |

**Table 10. Percent false positive and false negative results with respect to the AB411 standard of 400 EC/100 ml before and after adjustment for amplification efficiency.**

| | QCPR | | QCPR (Adjusted) | |
|---|---|---|---|---|
| Sample Type | False Positive | False Negative | False Positive | False Negative |
| Open Ocean Beach | 0 | 0 | 31 | 13 |
| Ocean Beach Near Drain | 0 | 6 | 12 | 8 |
| Enclosed Beach | 0 | 5 | 27 | 0 |
| Storm Drain | 0 | 8 | 45 | 8 |
| Wet Weather | 0 | 50 | 0 | 25 |
| Influent | 0 | 19 | 30 | 0 |
| Effluent | 0 | 20 | 0 | 0 |
| All | 0 | 8 | 27 | 7 |

**Table 11. Percent samples >½ log unit above or below median value of EPA-approved methods for EC.**

| | QCPR | | QCPR (Adjusted) | |
|---|---|---|---|---|
| Sample Type | >1/2 log Above | >1/2 log Below | >1/2 log Above | >1/2 log Below |
| Open Ocean Beach | 0 | 17 | 44 | 38 |
| Ocean Beach Near Drain | 0 | 6 | 28 | 20 |
| Enclosed Beach | 0 | 5 | 67 | 7 |
| Storm Drain | 0 | 23 | 55 | 23 |
| Wet Weather | 0 | 0 | 0 | 13 |
| Influent | 0 | 0 | 65 | 9 |
| Effluent | 0 | 0 | 0 | 20 |
| All | 0 | 10 | 45 | 19 |

**Table 12. Average coefficient of variation (CV) between replicates for EC.**

| Method | Average CV |
|---|---|
| EPA-Approved | 0.25 |
| QPCR | 0.25 |
| QPCR (Adjusted) | 0.50 |

**Table 13. Percent agreement in beach management decisions using existing EPA-approved methods compared to agreement in decisions based on QPCR methods for EC.**

| Method | Equivalent to Current Methods | Not Materially Different | Materially Different |
|---|---|---|---|
| QPCR | 54 | 16 | 30 |
| QPCR Adjusted for Efficiency | 20 | 12 | 68 |

**Table 14. Chlorine demand in secondary effluent.**

| Time (h) | Total Chlorine (mg/L) | Chlorine Demand (mg/L) |
|---|---|---|
| 0 | 8 | 0 |
| 0.25 | 7.5 | 0.5 |
| 0.5 | 7.3 | 0.7 |
| 1.0 | 7.3 | 0.7 |
| 2.0 | 6.3* | 1.7 |
| 4.0 | 7.2 | 0.8 |

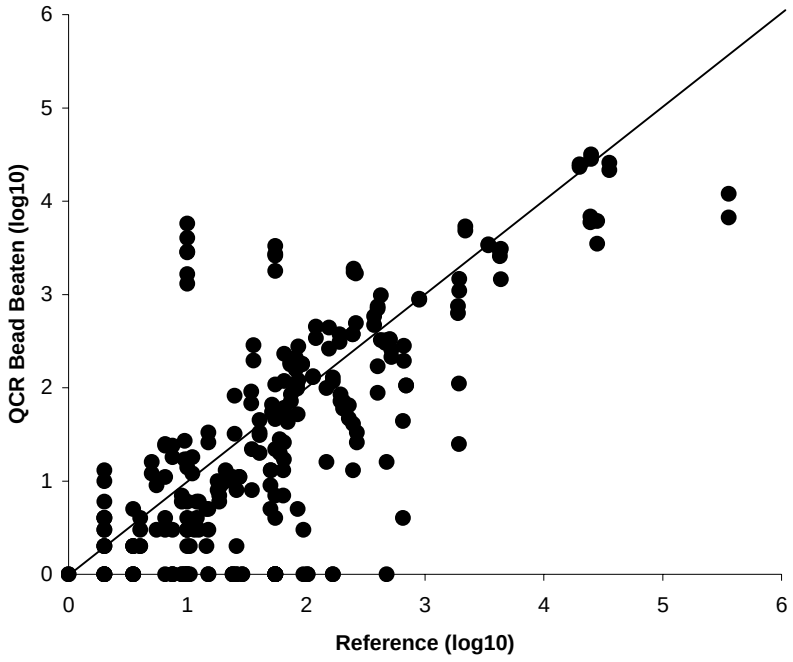


**Figure 1. QPCR Bead-beaten method vs. median value for ENT produced by EPA-approved methods.**

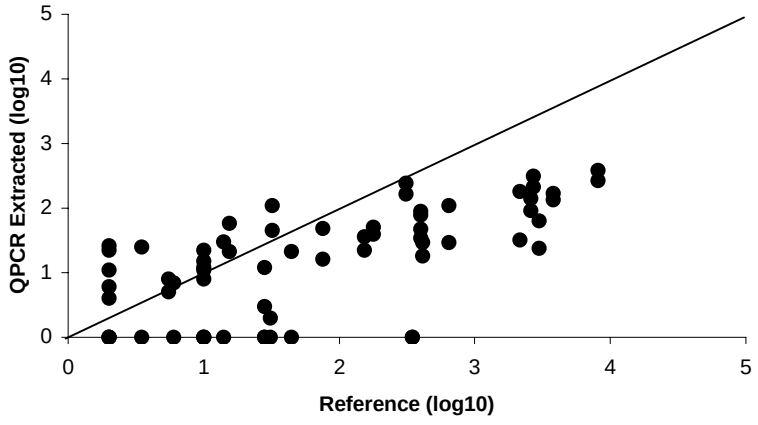


**Figure 2. QPCR Extracted method vs. median value for ENT produced by EPA-approved methods.**

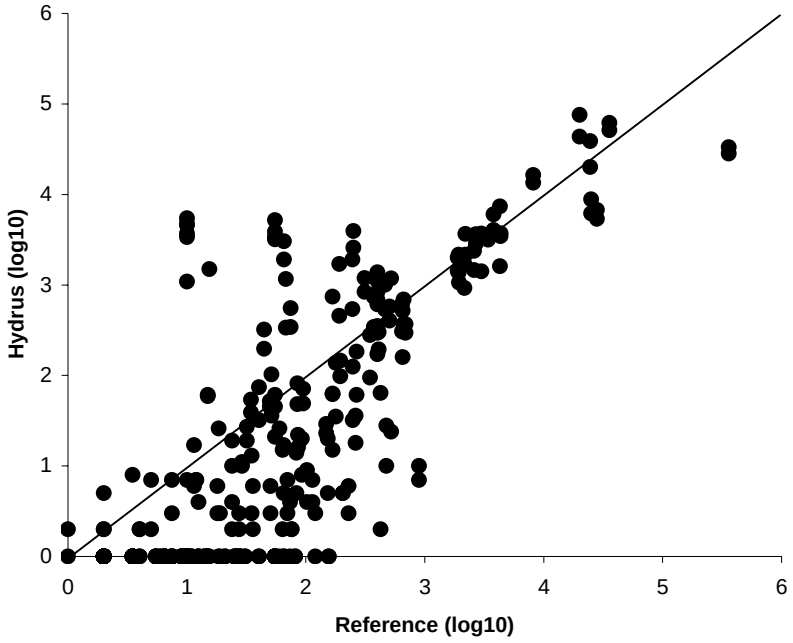


**Figure 3. TMA method vs. median value for ENT produced by EPA-approved methods.**

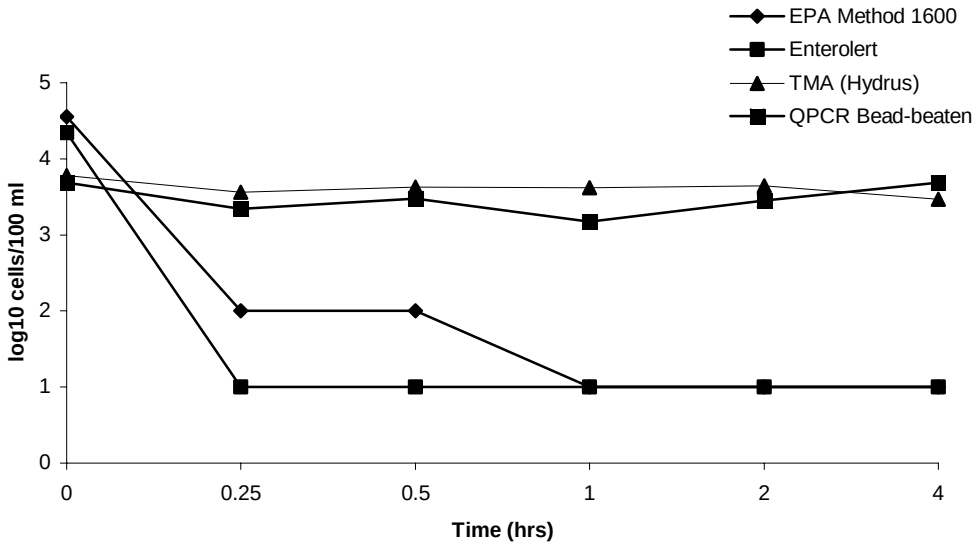


**Figure 4. Effect of chlorination of levels of enterococci in sterile water spiked with secondary sewage effluent as measured by EPA-approved and rapid methods.**

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