A critical evaluation of a flow cytometer used for detecting enterococci in recreational waters

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ABSTRACT

The current U. S. Environmental Protection Agency-approved method for enterococci (Method 1600) in recreational water is a membrane filter (MF) method that takes 24 hours to obtain results. If the recreational water is not in compliance with the standard, the risk of exposure to enteric pathogens may occur before the water is identified as hazardous. Because flow cytometry combined with specific fluorescent antibodies has the potential to be used as a rapid detection method for microorganisms, this technology was evaluated as a rapid, same-day method to detect enterococci in bathing beach waters. The flow cytometer chosen for this study was a laser microbial detection system designed to detect labeled antibodies. A comparison of MF counts with flow cytometry counts of enterococci in phosphate buffer and sterile-filtered recreational water showed good agreement between the two methods. However, when flow cytometry was used, the counts were several orders of magnitude higher than the MF counts with no correlation to *Enterococcus* spike concentrations. The unspiked sample controls frequently had higher counts than the samples spiked with enterococci. Particles within the spiked water samples were probably counted as target cells by the flow cytometer because of autofluorescence or non-specific adsorption of antibody and carryover to subsequent samples. For these reasons, this technology may not be suitable for enterococci detection in recreational waters. Improvements in research and instrument design that will eliminate high background and carryover may make this a viable technology in the future.

Key words | autofluorescence, enterococci, flow cytometry, fluorescence, membrane filtration, recreational water

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INTRODUCTION

Recreational water is an important potential transmission vehicle for fecal contamination containing human infectious disease agents. Since 1993, recreational waters have been implicated as the cause of an increasing number of outbreaks of human gastrointestinal and other types of illness, such as dermatitis, respiratory disease, meningitis, and chemical exposure (Yoder *et al.* 2004). Potential sources of fecal contamination for recreational water are sewage releases or effluents (point sources), storm water runoff from agricultural, forest, and residential areas, and floods (Yoder *et al.* 2004).

In 1986, the U. S. Environmental Protection Agency (EPA) recommended that enterococci concentration limits (geometric mean of 5 samples/month: 33 per 100 ml for fresh water and 35 per 100 ml for marine water) (U. S. Environmental Protection Agency 1986) be used to determine whether recreational water was safe for swimming (U. S. Environmental Protection Agency 1986). These levels

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were based on epidemiological studies that showed that illness rates in swimmers were directly related to enterococci levels in both fresh and marine water, while *Escherichia coli* concentrations were only correlated with illness rates in fresh water, and total and fecal coliforms showed no correlation with illness at all (U. S. Environmental Protection Agency 1986).

The current EPA-approved membrane filter method (Method 1600) (U. S. Environmental Protection Agency 1997; Messer & Dufour 1998; U. S. Environmental Protection Agency 2000) for monitoring enterococci in recreational waters is based on the growth of the indicator bacteria and requires at least 24 hours to obtain results. Due to the dynamic nature of beaches, with the constant input and mixing of potentially contaminated waters, and the normal variability from sample to sample, 24-hour measurements offer little insight for beach managers because exposure can occur before results are obtained indicating the water is unsafe for swimming. The quality of recreational waters changes day to day, even minute to minute with the input of contaminants. For this reason, a new rapid, same-day (preferably 2-hour) method for monitoring recreational water is needed to protect the public (U.S. Environmental Protection Agency 1999).

Flow cytometry has the potential for use as a rapid detection method for fecal bacteria from environmental samples, such as recreational water. This technology, coupled with the use of fluorescent biomarkers, has previously been used to detect bacteria in environmental samples. For example, flow cytometry and fluorescent nucleic acid dyes that identified target cells based on their G-C/A-T content were used to detect *Escherichia coli* in well water (Stopa & Mastromanolis 2001), and similar methods were used to detect bacteria in water distribution systems (Hoefel *et al.* 2003). Flow cytometry analysis has also been used in conjunction with fluorescein-bound specific antibodies and propidium iodide to monitor cooling water towers for *Legionella* spp. (Tyndall *et al.* 1985). This method was reported to be much quicker and less labor intensive than fluorescent microscopy.

The goal of this project was to evaluate the use of flow cytometry as a same-day analytical method, preferably two hours, to detect enterococci in recreational waters.

MATERIALS AND METHODS

Reagents and media

Type I distilled water (DW) (American Public Health Association 1998), produced by a Millipore Milli Q Synthesis A-10 System, was used to make all media and reagents. Syto 62 nucleic acid stain was purchased from Molecular Probes (Eugene, OR), and Bovine serum albumin (BSA), Fraction V powder, 99% was purchased from Sigma-Aldrich (St. Louis, MO). The fluorochrome (Alexa Fluor 647) - labeled rabbit anti-*Enterococcus faecium* and anti-*Entercococcus faecalis* IgG antibodies [525 μg/ml solution in phosphate-buffered saline (PBS), pH 7.2], BRAG 3 (a proprietary fluorescence quencher used to reduce background counts), and the instrument calibration beads were purchased from Advanced Analytical Technologies Inc. (AATI) (Ames, IA). The mEI agar was made using mE agar base from BD Biosciences (San Jose, CA), as described in the USEPA Recreational Water Methods Manual (U.S. Environmental Protection Agency 2000). Indoxyl-β-D-Glucoside was obtained from Sigma-Aldrich (St. Louis, MO).

Cultures

Enterococcus faecalis (ATCC 19433) and *Enterococcus faecium* (ATCC 19434) cultures were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Stock cultures of each strain were grown in Tryptic soy broth (TSB) and stored on Tryptic soy agar (TSA) slants at 4°C. For use in this study, stock cultures of *E. faecalis* and *E. faecium* were inoculated into tubes of Tryptic soy broth (TSB), incubated for 24–48 hours at 35°C, transferred to new tubes of TSB, and incubated for 24 hours at 35°C. The cells were concentrated by centrifugation at 3000 rpm for 30 minutes in an IEC model HN-S centrifuge, washed once in *Standard Methods* phosphate-buffered dilution water (American Public Health Association 1998) (PB), and resuspended in an equal volume of the PB. Ten-fold dilutions of each culture made in PB were used to spike filter-sterilized natural water samples. Culture dilutions in PB were analyzed in the following order using the RBD 2100: 10^{-2}, 10^{-8}, 10^{-4}, 10^{-6}.

Natural water samples

Natural recreational water samples were obtained from the Great Miami River (Dayton, OH), the Ohio River at the public landing (Cincinnati, OH), Burnett Woods Lake (Cincinnati, OH), and Acton Lake (Hueston Woods State Park, near Cincinnati, OH). Samples were placed on ice during the transport to the laboratory, and stored at 4°C until analyzed. The natural samples were analyzed using the flow cytometer manufacturer's protocol (Advanced Analytical Technologies Inc. 2003), and filter-sterilized samples (0.22-μm Nalgene units; Nalge Nunc International, Rochester, NY) were spiked with enterococci cultures in a 1:1 ratio. Tenfold dilutions of spiked and non-spiked natural water samples were analyzed in the following order using the RBD 2100: 10^{-2}, 10^{-8}, 10^{-4}, 10^{-6}.

Membrane filtration assay

Each natural water sample and ten-fold dilutions of the pure enterococci cultures in PB were analyzed using Method 1600 (U.S. Environmental Protection Agency 1997; Messer & Dufour 1998; U.S. Environmental Protection Agency 2000) in order to compare the viable counts with the flow cytometer data. Natural water samples were filtered through a 3-μm pore size Millipore (Billerica, MA) polycarbonate membrane filter and spiked with equal amounts of centrifuged and washed cultures of *E. faecalis* and *E. faecium*. The spiked sample was then filtered through a 0.45-μm pore size Sartorius (Edgewood, NY) cellulose nitrate membrane filter, and the filter was placed on mEI agar plate and incubated in a 41°C water bath (U. S. Environmental Protection Agency 1997; Messer & Dufour 1998; U. S. Environmental Protection Agency 2000). Blue colonies or those with blue halos were counted as enterococci, and counts were converted to colony-forming-units per 100 ml.

Flow cytometer

The evaluated flow cytometer uses a red 35 mW, 635 nm diode laser for detection of the target organisms. This model is considered portable (approximately 125 lbs) with an on-board touch screen computer. The computer runs on Microsoft® Windows NT 4.0 with a 133 MHz processor and 4GB hard drive (enough memory to hold approximately 25,000 samples). Proprietary RBD2100 software is used (21 CFR Part 11 compliant). A pressure source of He, air or N_2 regulated at 45 to 55 psig is required. All water and other fluids must be 0.2 micron filtered. The operating temperature of the machine is between zero and 50°C.

The dynamic range of this instrument is 100 to 10,000,000 colony forming units/ml with a cell size of 0.1 micron and larger. Samples are loaded into the injection port and analyzed one at a time. Following sample preparation to label microbes of interest with fluorescent markers, a continuous fine stream of the microbial suspension sends the microbes single-file past the laser beam. A syringe pump delivers the sample at a controlled rate to the center of a fast-flowing sheath flow. Hydrodynamic forces focus the sample into a small flow path to be excited by the laser. The detectors consist of two photomultiplier tubes that collect the emitted signals for fluorescence and side scatter. Microbes that have been labeled with Alexa Fluor 647 antibodies emit laser-induced fluorescence at wavelengths longer than 635 nm, and individual particles and microbes within the sample will scatter a portion of light, which is collected and represented as bitmaps by the instrument. Bitmaps separate target organisms based on their unique size and fluorescence.

Flow cytometer assay preparation

A working solution (20 μg/ml final concentration) of each of the fluorochrome (Alexa Fluor 647) - labeled rabbit anti-*Enterococcus faecium* and anti-*Entercococcus faecalis* IgG antibodies was made in PBS (U.S. Environmental Protection Agency 2000), and a final concentration of 0.05% BSA was added. The two antibody solutions were sterilized by filtering through 0.22-μm Nalgene filter units. Forty μl of each antibody working solution was added to each 1 ml water sample or control volume to be analyzed on the RBD 2100 flow cytometer.

Carryover verification test

A verification test to determine if there was carryover from sample to sample was conducted between each water sample analysis to see if the instrument rinsing procedure was adequate for the production of valid data. The test was prepared by incubating 1 ml PB with 1 μl Syto 62 for 5 minutes in the dark at room temperature. One μl of BRAG 3 was then added, and the solution was incubated in the dark at room temperature for an additional 2 minutes. The PB/Syto/BRAG was analyzed in the RBD 2100 following each water sample analysis. Carryover from the previous sample was assumed to have occurred whenever >10 flow cytometer counts of the target organisms were observed with the verification test. To remove any additional sample from the instrument, a filter-sterilized solution of undiluted bleach (5.5% sodium hypochlorite) was run through the instrument. At least 2 complete runs using filter-sterilized distilled water were needed to flush the bleach out of the system. This was followed by another verification test to determine if the counts were reduced to <10, indicating that any residual microorganisms had been removed.

Flow cytometer assay procedure for spiked natural water samples

Each water sample was filtered through a 3-μm polycarbonate membrane filter, according to the manufacturer's procedure (Advanced Analytical Technologies Inc. 2003), to remove larger particles that could plug the sample intake openings to the instrument. The cultures of *E. faecalis* and *E. faecium* were prepared as described above, and appropriate dilutions containing equal amounts of the two organisms were added to the processed water samples. BSA was added to a final concentration of 0.05%. The samples were concentrated to 1 ml by centrifugation in an Eppendorf 5804 R Centrifuge (Westbury, NY) for 45 minutes at 3100 *x g* at 4°C. Forty μl each of anti-*E. faecium* and anti-*E. faecalis* were added to the sample, and the solution was incubated in the dark at room temperature for 30 minutes. Two μl of BRAG 3 was added to the sample solution, which was incubated then in the dark at room temperature for an additional 2 minutes. Controls of unspiked water samples and verification tests (PB/Syto/BRAG), described above, were analyzed with each processed sample to determine if sample carryover occurred in the instrument.

Filter-sterilized natural water samples

In order to determine the effect of dissolved minerals on the analysis results, natural water samples were filtered through 0.22-μm Nalgene filter units, spiked as before, and analyzed using the same procedure as the manufacturer's protocol, described in a previous section. Sterile PB was spiked with enterococci as a positive control to show the results that would be obtained without the effects of dissolved minerals and particulates that are usually present in natural samples.

Quantitative PCR of unspiked samples

Quantitative PCR (QPCR) assays for *E. faecalis* and *E. faecium* were run on each natural water sample prior to being spiked with organisms (Haugland *et al.* 2005). The purpose of this was to determine if any enterococci were present. Water samples were prepared by filtering 100 ml of each sample through a 0.4-μm pore size polycarbonate filter. Filters were placed in a screwcap tube containing salmon DNA extraction AE buffer (Qiagen, Valencia, CA), and any cells from the samples were lysed by bead beating. Cellular debris was pelleted by centrifugation, and the supernatant was removed and diluted 1:10 with AE buffer. The diluted samples were analyzed using a Cepheid Model SC1000-1 Smart Cycler (Sunnyvale, CA) for 40 cycles (Haugland *et al.* 2005). All assays were performed in duplicate.

RESULTS

The current study began as an evaluation of flow cytometry technology to detect enterococci in recreational waters. A comparison of the flow cytometer counts to the membrane filter counts, on mEI agar, indicated a fairly good agreement between these two methods in extremely clean matrices (phosphate buffer or sterile-filtered environmental water) (Tables 1 and 2). The exceptions included very high ($\geq 10^6$/100 ml) and very low (<100 organisms/100 ml) spikes.

Table 1 | Comparison[a] of EPA Method 1600[b] colony counts to flow cytometer results for phosphate buffer spiked with *E. faecium* and *E. faecalis*

Date of analysis	Dilution of spike[c]	Flow cytometery (counts/ml)	Method 1600[b] (cfu/ml)	Carryover[d] (counts/ml)
12/16/2003	10^{-2}	2.15E + 06	1.21E + 07	**352**
	10^{-4}	3.39E + 05	1.21E + 05	**36**
	10^{-6}	4.40E + 03	1.21E + 03	**52**
	10^{-8}	**9.00E + 02**	**12**	4
2/3/2004	10^{-2}	4.08E + 06	8.90E + 06	NA[e]
	10^{-4}	9.31E + 04	8.90E + 04	0
	10^{-6}	1.62E + 03	8.90E + 02	8
	10^{-8}	**3.08E + 02**	**9**	8
	Control[f]	**9.04E + 02**	**0**	NA[e]
2/26/2004	10^{-2}	4.22E + 06	1.32E + 07	**4892**
	10^{-4}	9.69E + 04	1.32E + 05	**20**
	10^{-6}	1.36E + 03	1.32E + 03	**12**
	10^{-8}	**2.12E + 02**	**13**	136
	Control[f]	**8.60E + 02**	**0**	8
3/17/2004	10^{-2}	3.00E + 06	1.56E + 07	**72**
	10^{-4}	1.30E + 05	1.56E + 05	**32**
	10^{-6}	1.65E + 03	1.56E + 03	**20**
	10^{-8}	**2.12E + 02**	**16**	**16**
	Control[f]	**3.16E + 02**	**0**	8

[a]The comparison counts that lack agreement by one log or more are shown in bold.
[b]EPA Method 1600, using mEI agar, is the approved cultural method used for monitoring enterococci in recreational water (U. S. Environmental Protection Agency 1997; Messer & Dufour 1998; U. S. Environmental Protection Agency 2000).
[c]PB spiked with a 1:1 concentation of *E. faecalis* and *E. faecium*. Tenfold dilutions of spiked samples in PB were analyzed in the following order using the RBD 2100: 10^{-2}, 10^{-8}, 10^{-4}, 10^{-6}.
[d]The carryover counts that exceed the acceptable limit of 0 to 10 counts per milliliter are also shown in bold.
[e]NA, Not applicable (Test not completed).
[f]The control consisted of sterile non-spiked PB (American Public Health Association 1998).

Lack of agreement at very high levels of target organisms may not represent a problem in the application of this methodology in analyzing environmental samples, as these levels are generally not seen in actual recreational water samples. However, the lack of agreement at low levels is problematic. The analysis of PB or sterile-filtered natural water samples (used as negative controls), which included the addition of fluorescent antibody and BRAG 3, yielded a background of 100–1000 counts (Tables 1 and 2). These counts were generally confined to the far left edge of the

Table 2 | Comparison[a] of EPA Method 1600[b] colony counts to flow cytometer results with filtered natural recreational water samples[c] spiked with *E. faecium* and *E. faecalis*.

Recreational water sample[c]	Date of analysis	Dilution of spike[d]	Flow cytometer (counts/ml)	Method 1600[b] (cfu/ml)	Carryover[e] (counts/ml)
Burnet Woods Lake, Cincinnati Ohio	12/18/2003	10^{-2}	**6.05E + 05**	**1.31E + 07**	NA[f]
		10^{-4}	1.08E + 05	1.31E + 05	4
		10^{-6}	1.15E + 03	1.31E + 03	**24**
		10^{-8}	84	13	0
		0.22 μm Control[g]	196	0	NA[f]
Ohio River, Public Landing	2/10/2004	10^{-2}	3.43E + 06	1.09E + 07	**4444**
		10^{-4}	9.18E + 04	1.09E + 05	**44**
		10^{-6}	1.36E + 03	1.09E + 03	4
		10^{-8}	**1.72E + 02**	**11**	**20**
		0.22 μm Control[g]	**5.16E + 02**	**0**	NA[f]
Great Miami River, Dayton Ohio	3/2/2004	10^{-2}	3.74E + 06	1.32E + 07	**1992**
		10^{-4}	9.10E + 04	1.32E + 05	**280**
		10^{-6}	1.15E + 03	1.32E + 03	**28**
		10^{-8}	**2.64E + 02**	**13**	**72**
		0.22 μm Control[g]	**2.12E + 02**	**0**	NA[f]
Acton Lake, Hueston Woods State Park, Ohio	3/18/2004	10^{-2}	2.14E + 06	1.52E + 07	**196**
		10^{-4}	1.26E + 05	1.52E + 05	**28**
		10^{-6}	2.16E + 03	1.52E + 03	**28**
		10^{-8}	**6.48E + 02**	**15**	8
		0.22 μm Control[g]	**1.17E + 03**	**0**	NA[f]

[a]The comparison counts that lack agreement by one log or more are shown in bold.
[b]EPA Method 1600, using mEI agar, is the approved cultural method used for monitoring enterococci in recreational water (U. S. Environmental Protection Agency 1997; Messer & Dufour 1998; U. S. Environmental Protection Agency 2000).
[c]Natural water samples were filtered through a 0.22-μm filter; then spiked with a 1:1 concentration of *E. faecalis* and *E. faecium*.
[d]Tenfold dilutions of spiked samples in natural water were analyzed in the following order using the RBD 2100: 10^{-2}, 10^{-8}, 10^{-4}, 10^{-6}.
[e]The carryover counts that exceed the acceptable limit of 0 to 10 counts per milliliter are also shown in bold.
[f]NA, Not applicable (Test not completed).
[g]The control consisted of sterile non-spiked natural water samples that were filtered through a 0.22-μm filter.

counting box (gate). In other applications, these counts may be omitted from the data collection by altering the size of the gate. This narrowing of the gate is recommended by the instrument manufacturer and is based on the range of anticipated data. However, in this study, spiking experiments with different sterile-filtered natural water samples showed that the majority of the counts for the target cells could be found in different locations within the gated area on the bitmap. Figure 1A-1B illustrates this observation for two different water samples. Because this

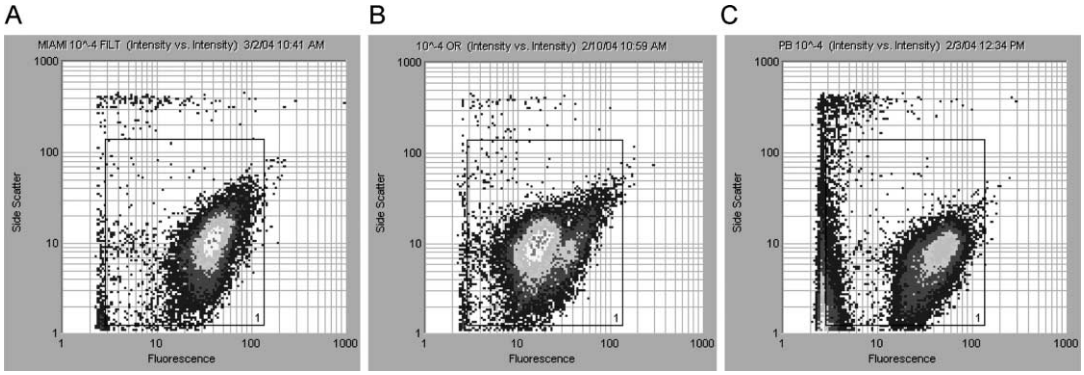

Figure 1 | Each sample was spiked with a 10^{-4} dilution of 1:1 *E. faecalis* and *E. faecium* after being filter-sterilized. The Great Miami River sample (Bitmap A) shows a population of target organisms further to the right within the gated area than the Ohio River sample (Bitmap B). Both river water samples show populations located higher in the counting box than the spiked PB sample (Bitmap C).

shift is unpredictable, it is difficult to know how narrow the gate should be, so that the target cells are counted while eliminating the background counts (on the far left side of the box).

Carryover from the previous sample was observed. The standard procedure to detect carryover was to analyze a negative control of PB/Syto/Brag 3 solution after each sample. The counts should be ≤ 10 if carryover was absent. The data in Tables 1 and 2 indicate notable carryover occurred when the counts were $10^3 - 10^7$ in spiked PB or sterile-filtered environmental water. Carryover could be removed using a time-consuming washing procedure, which consisted of cycling bleach through the instrument, followed by several distilled water washes (Figures 2–3 bitmaps show carryover after a highly concentrated sample) and another PB/Syto/BRAG 3 control to document that the carryover was reduced to ≤ 10 counts.

The method recommended by the manufacturer for the analysis of natural water samples required filtration through a 3-μm filter, which removed large particles that could plug up the instrument. However, following this method consistently yielded high background counts with natural water samples (Table 3). The information in Figure 3 depicts an unspiked river sample that was prepared for analysis according to the manufacturer's method with the addition of the fluorescent antibodies and BRAG 3. Regardless of the sample source, prominent levels of background counts were always observed. Evidence that these counts were not entirely due to enterococci is provided by viable MF counts and QPCR analyses on each of these samples. The MF analysis indicated

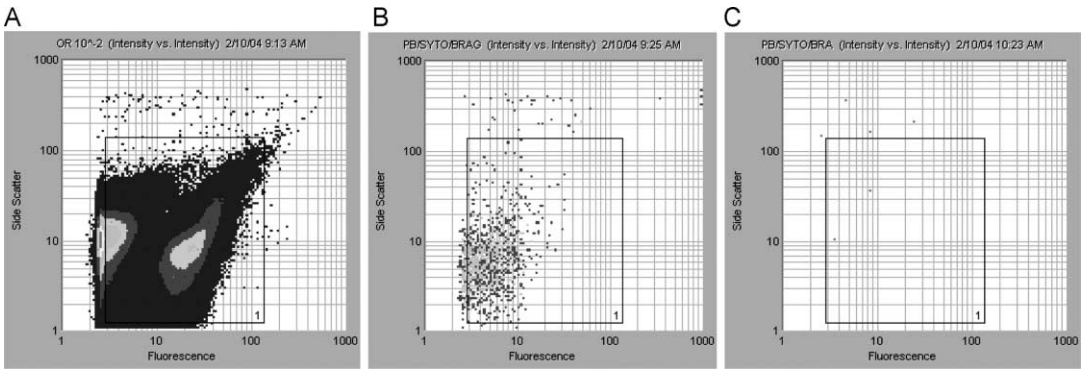

Figure 2 | These three bitmaps show carryover after analysis of a highly concentrated sample of enterococci in PB. Bitmap A is the result of testing a 10^{-2} dilution of a 1:1 ratio of *E. faecalis* and *E. faecium*. The negative control (Bitmap B) contains a nucleic acid stain revealing the amount of carryover from sample A. After a 30 minute bleach cleaning, the machine is ready to analyze another sample which is indicated by the negative control (Bitmap C).

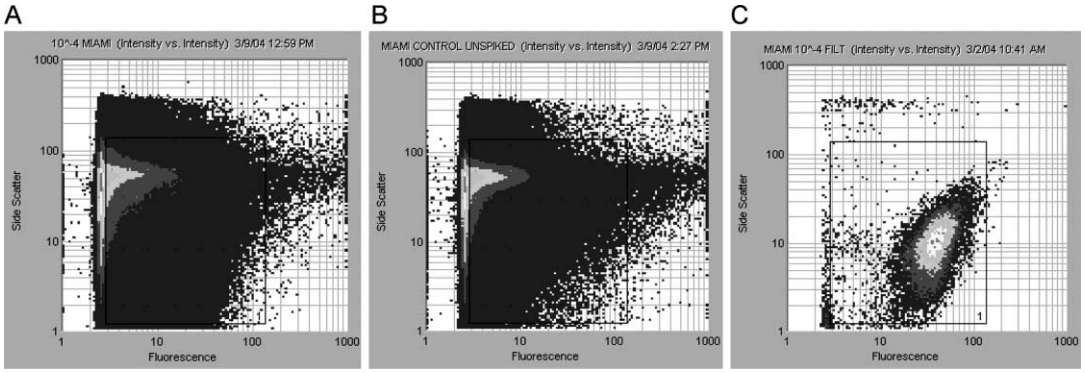

Figure 3 | Bitmap A is the result of running unspiked Great Miami River water through the instrument according to the manufacturer's protocol. Bitmap B is a portion of the same water sample as A spiked with a 10^{-4} dilution of a 1:1 ratio of *E. faecalis* and *E. faecium*. Bitmap C is a portion of the same sample as A that has been sterile filtered, then spiked with a 10^{-4} dilution of a 1:1 ratio of *E. faecalis* and *E. faecium*.

that these unspiked samples had ≤ 10 enterococci per 100 ml of sample. The QPCR data showed that *E. faecalis* was not detected and that seven out of eight samples (each sample was analyzed in duplicate) were negative for *E. faecium*. One of the two replicates of Burnet Woods Lake did detect *E. faecium* cells (Haugland *et al.* 2005).

DISCUSSION

The manufacturer of the flow cytometer evaluated in this study claims their method can be completed in approximately ten minutes after sample preparation is complete (sample preparation took approximately 1 hour). While this may be true in a technical sense, unspiked ambient water samples in this study required a sodium hypochlorite cleaning procedure after every sample in order to insure the prevention of carryover. The amount of carryover between samples without sodium hypochlorite addition was unacceptable, since these carryover counts would be added on to the counts for the subsequent samples. Since the acceptable limit for enterococci in recreation waters is less than 33 organisms per 100 ml of freshwater and 35 for marine water, even a small amount of carryover could affect the decision of whether to close a beach. The additional step of sodium hypochlorite cleaning adds approximately an hour to each sample analysis and greatly reduces the number of samples that can be processed in one day. For example, if carryover occurred in every sample, only six samples could be analyzed in an eight hour time period. This makes the instrument unsatisfactory for compliance monitoring of recreational water.

Although the carryover problem can be solved, other problems were encountered in the instrument evaluation. Abnormally high flow cytometer counts were observed in unspiked recreational water samples. In addition, the data showed little correlation between the flow cytometry counts analyzed according to the manufacturer's method and those of the MF assay. The flow cytometry counts tend to be much higher than the MF counts (up to 6 logs of magnitude higher), and the unspiked samples sometimes had higher counts than the samples spiked with enterococci. These data suggest that some component of the water samples, perhaps organic debris, was autofluorescent or binding the antibody, causing false-positive counts. The source of the false-positive counts can sometimes be removed by sterile-filtering the samples through a 0.2 μm filter. However, the counts from both the sterile-filtered samples and the control samples (PB) that were spiked indicate that, even in the absence of non-target particles, the counts from the RBD 2100 still did not correlate well with the MF counts at very high and very low concentrations of spiked enterococci.

The high level of background noise in the flow cytometery data from the two lake and river samples made it difficult to determine how many actual target bacteria were present. Although the addition of antibodies was associated with an increase in these background counts, the water samples themselves appeared to account for most of the problem. Sample matrix interference has been an issue with flow cytometry in other published works.

Table 3 | Comparison[a] of Method 1600[b] colony counts to flow cytometer results with non-filtered natural recreational water samples[c] spiked with *E. faecium* and *E. faecalis*.

Recreational water sample[c]	Date of analysis	Dilution of spike[d]	Flow cytometer (counts/ml)	Method 1600[b] (cfu/ml)	Carryover[e] (counts/ml)
Burnet Woods Lake, Cincinnati Ohio	1/7/2004	10^{-2}	2.43E + 06	1.14E + 07	NA[f]
		10^{-4}	1.55E + 06	1.14E + 05	**3296**
		10^{-6}	**1.55E + 05**	**1.14E + 03**	**1916**
		10^{-8}	**4.64E + 05**	**11**	**640**
		3 μm Control[g]	**2.97E + 05**	**0**	**16**
Ohio River, Public Landing	2/24/2004	10^{-2}	**3.75E + 05**	**1.22E + 07**	**2375**
		10^{-4}	7.91E + 05	1.22E + 05	**44**
		10^{-6}	**2.30E + 05**	**1.22E + 03**	**92**
		10^{-8}	**1.07E + 06**	**12**	**56**
		3 μm Control[g]	**6.37E + 04**	**0**	8
Great Miami River, Dayton Ohio	3/9/2004	10^{-2}	**1.28E + 06**	**1.03E + 07**	**2524**
		10^{-4}	2.02E + 06	1.03E + 05	**108**
		10^{-6}	**9.22E + 05**	**1.03E + 03**	**12**
		10^{-8}	**1.41E + 06**	**10**	**24**
		3 μm Control[g]	**1.73E + 06**	**0**	**160**
Acton Lake, Hueston Woods State Park, Ohio	3/23/2004	10^{-2}	**1.59E + 06**	**1.80E + 07**	**2468**
		10^{-4}	8.58E + 05	1.80E + 05	**76**
		10^{-6}	**1.13E + 05**	**1.80E + 03**	**24**
		10^{-8}	**1.18E + 05**	**18**	**28**
		3 μm Control[g]	**1.85E + 05**	**0**	**32**

[a]The comparison counts that lack agreement by one log or more are shown in bold.
[b]EPA Method 1600, using mEI agar, is the approved cultural method used for monitoring enterococci in recreational water (U. S. Environmental Protection Agency 1997; Messer & Dufour 1998; U. S. Environmental Protection Agency 2000).
[c]Natural water samples filtered through a 3-μm filter, as recommended by the manufacturer(Advanced Analytical Technologies Inc. 2003), then spiked with a 1:1 concentration of *E. faecalis* and *E. faecium*.
[d]Tenfold dilutions of spiked samples in natural water were analyzed in the following order using the RBD 2100: 10^{-2}, 10^{-8}, 10^{-4}, 10^{-6}.
[e]The carryover counts that exceed the acceptable limit of 0 to 10 counts per milliliter are also shown in bold.
[f]NA, Not applicable (Test not completed).
[g]The control consisted of non-spiked natural water samples that were filtered through a 3-μm filter.

Variable and low recoveries, as well as high false-positive counts, have been associated with sample turbidity (Felini & Pirovano 1998; Lindquist *et al.* 2001). Further, a study relying on flow cytometry to detect bacteria in foods showed that non-target components within the sample matrix can affect counts (Patchett *et al.* 1991). Samples with high levels of particulate material could only be analyzed if the bacteria were separated from the background light

scattering material within the food matrix. The studies described above and the present study indicates that substances within the sample matrix can have a considerable effect on flow cytometer target counts.

Although it was not possible to determine the exact source(s) of the background counts observed in this study, the possibility of dead or dying enterococci cells being present was eliminated by negative QPCR assays. Autoflourescent particles in the water samples may be a cause of background counts in this study. Autoflourescence has been suggested as a problem in a study that used flow cytometry to identify *E. coli* in well water samples (Stopa & Mastromanolis 2001), although the actual source of the autoflourescence was not determined.

Some of the false-positive counts observed in this study could also have been caused by antibody cross-reactivity. It is generally recognized that different species or genera of bacteria may share similar antigenic components, allowing for non-specific binding of fluorescent-labeled antibodies (Laplace-Builhe *et al.* 1993). A study using *Cryptosporidium*-specific and *Giardia*-specific monoclonal antibodies in a flow cytometry-based detection assay for these protozoa in water samples has shown that the antibody chosen can greatly affect the accuracy of the results (Ferrari *et al.* 1999).

Other published studies using flow cytometry have shown that discriminatory gating (resizing and repositioning of the gated area of the bitmaps) is necessary to obtain accurate counts of the target (Patchett *et al.* 1991; Prakash *et al.* 2001). This strategy works well in theory, with less complex sample matrices, but the problem of population shifts encountered in this study made it difficult to construct a discriminatory gate on the bitmap that would detect all *E. faecalis* and *E. faecium* counts while excluding most of the background. The manufacturer recommends drawing a box around a pure culture of the bacterium of interest that is spiked into phosphate buffer at a level of 0.00001 colony forming units. The differences seen in this study between the populations from the spiked cultures in phosphate buffer and the 0.2-μm filtered water samples indicate that shrinking the gate would not be possible because counts of target bacteria from some water (or matrix) sources would be excluded. Therefore, it would be difficult to reduce background interference by changing the size or shape of the gate.

CONCLUSION

Evaluation of this flow cytometer indicates carryover between samples and population shifts on the bitmaps interfered with accurate data collection. The methods used in this study also permitted a high number of background counts to occur when analyzing natural water sources. Although this flow cytometer has the potential to produce same-day results, this study demonstrated that the instrument used for this analysis would not be suitable for enterococci detection in recreational waters without significant modifications. It is important to note that the manufacturer of this instrument has developed a new flow cytometer that may minimize or eliminate the problems encountered in this study. While not currently available, advances in antibody development and research on the reduction of high background fluorescence could also make this a more viable technology.

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DISCLAIMER

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