A review of technologies for rapid detection of bacteria in recreational waters

Rachel T. Noble and Stephen B. Weisberg

ABSTRACT

Monitoring of recreational beaches for fecal indicator bacteria is currently performed using culture-based technology that can require more than a day for laboratory analysis, during which time swimmers are at risk. Here we review new methods that have the potential to reduce the measurement period to less than an hour. These methods generally involve two steps. The first is target capture, in which the microbial group of interest (or some molecular/chemical/or biochemical signature of the group) is removed, tagged or amplified to differentiate it from the remaining material in the sample. We discuss three classes of capture methods: 1) Surface and whole-cell recognition methods, including immunoassay techniques and molecule-specific probes; 2) Nucleic acid methods, including polymerase chain reaction (PCR), quantitative PCR (Q-PCR), nucleic acid sequence based amplification (NASBA) and microarrays; and 3) Enzyme/substrate methods utilizing chromogenic or fluorogenic substrates. The second step is detection, in which optical, electrochemical or piezoelectric technologies are used to quantify the captured, tagged or amplified material. The biggest technological hurdle for all of these methods is sensitivity, as EPA's recommended bathing water standard is less than one cell per ml and most detection technologies measure sample volumes less than 1 ml. This challenge is being overcome through addition of preconcentration or enrichment steps, which have the potential to boost sensitivity without the need to develop new detector technology. The second hurdle is demonstrating a relationship to health risk, since most new methods are based on measuring cell structure without assessing viability and may not relate to current water quality standards that were developed in epidemiology studies using culture-based methods. Enzyme/substrate methods may be the first rapid methods adopted because they are based on the same capture technology as currently-approved EPA methods and their relationship to health risk can be established by demonstrating equivalency to existing procedures. Demonstration of equivalency may also be possible for some surface and whole-cell recognition methods that capture bacteria in a potentially viable state. Nucleic acid technologies are the most versatile, but measure nonviable structure and will require inclusion in epidemiological studies to link their measurement with health risk.

Key words | E. coli, enterococci, water quality, recreational, optical, electrochemical

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INTRODUCTION

Considerable resources are expended each year to measure indicator bacteria and assess whether recreational beaches are free from fecal contamination (Schiff *et al.* 2002).These monitoring programs are compromised, though, because current methods of enumerating bacteria are too slow to provide full protection from exposure to waterborne pathogens. The current United States Environmental Protection Agency (USEPA) approved methods to evaluate

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recreational waters require an 18 to 96 hour incubation period, while several studies have shown that temporal changes in indicator bacteria levels in beach water occur on much shorter time scales (Leecaster and Weisberg 2001; Boehm *et al.* 2002). Thus, contaminated beaches remain open during the laboratory incubation period and are often clean by the time warnings are posted.

This processing time lag can also negatively affect tracking of contamination sources. A frequently used tracking approach is to look for differential bacterial concentrations at the confluence of upstream tributaries. However, the fecal contamination signal can dissipate or disperse while the initial samples that would trigger such an investigation are being processed, making it difficult to subsequently track the sources of fecal contamination. Even when upstream tracking is successfully initiated, the slow laboratory processing time requires that many locations be examined simultaneously, rather than using spatially-sequential sampling that would be possible if a more rapid (and possibly field-based) method were available.

The limiting factor for present methods is that they rely on culturing techniques that either measure a metabolic endpoint or determine growth of a microorganism after an extended incubation period. New molecular methods that allow direct measurement of cellular properties without incubation are becoming available and have the potential to reduce the measurement period to less than an hour. These new technologies also allow expansion of the number and types of microbiological indicators that can be measured. Beach monitoring programs are presently based primarily on *E. coli* and *Enterococcus* spp. because they are easily and inexpensively cultured. Molecular methods do not require culturing and allow for pathogens such as viruses to be measured as easily as bacterial indicators, potentially providing a more direct link to public health risk.

While development of molecular methods has advanced considerably for use in several disciplines, such as the food service and hospital industries (Fung 2002), there has been less effort toward application of new methods for recreational water quality testing. Water testing can present challenges that are not frequently encountered in these other fields. Generally speaking, there are small numbers of the microorganisms of interest in a water sample, therefore large volumes of water must typically be processed, or some enrichment or concentration approach included in sample processing. In addition, presence/absence information is not adequate for assessing recreational waters. Methods must yield accurate quantitative information. Other problems with water samples include the presence of potential interferents to specific methodological approaches, such as salinity, humic acids, highly variable and complex sample matrices, and the presence of other confounding dominant native bacterial species. In this paper, we review rapid methodologies that are being developed for use in recreational waters and also identify the major impediments to adoption of these methods. For the purpose of this article, we treat “rapid” as methods that provide results in less than 4 hours (including sample preparation time), which is the longest time frame that reasonably allows managers to take action to protect public health (i.e. post or close a beach) on the same day that water samples are collected.

METHODOLOGICAL OVERVIEW

There are two general steps involved in the application of rapid technologies. The first is the capture, in which the microbial species or group of interest (or some molecular/chemical/or biochemical signature of the group) is removed, tagged or amplified to differentiate it from the remaining material in the sample. This step is typically responsible for the selectivity of the approach. The second step is the detection, in which the captured, tagged or amplified material is counted or measured quantitatively. The detector typically acts as a transducer, translating the biological, physical, or chemical alteration into a measurable signal.

These steps differ slightly among measurement approaches, but they provide a useful outline for organizing our discussion. In many cases, a third step, preconcentration, may be added prior to target capture because most recreational waters have relatively dilute levels of contaminants compared to other applications. Recreational water standards for bacterial indicators are roughly 100 cfu/100 ml, or 1 cell/ml. Since many detection technologies are based on measuring less than a single ml, preconcentration may be necessary to achieve acceptable precision.

Capture methods

There are three broad classes of capture methods used in rapid microbial detection technology. Firstly, molecular

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whole-cell and surface recognition methods capture and/or label the target microorganism by binding to molecular structures on the exterior surface or to structures within the interior of a bacterium, virus, or to genetic material of interest. These include immunoassay techniques, bacteriophage, and molecule-specific probes, such as lipid or protein attachment-based approaches. Secondly, nucleic acid detection methods target specific nucleic acid sequences of bacteria, viruses, or protozoa. These include polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), quantitative PCR (Q-PCR), nucleic acid sequence based amplification (NASBA), and microarrays. Thirdly, enzyme/substrate methods are based upon either existing chromogenic or fluorogenic substrate methods already in wide use, or new enzyme-substrate approaches.

Enzyme/substrate methods are enhancements of currently approved methods such as the defined substrate technology employed in the commercial kits, Colilert® and Enterolert® (IDEXX Laboratories, Inc). Several new technologies that are being developed use that technology in conjunction with high-sensitivity fluorescence detection instruments to reduce the time required for the assay. Capture is achieved through fluorophore-tagged growth substrates included in a proprietary powder media that are added to water samples. Upon growth, specific bacterial enzymatic activity cleaves the fluorophore from the substrates, causing fluorescence to increase (Edberg *et al.* 1989). This fluorescence can then be detected by a number of instruments.

Molecular recognition approaches have the potential for being more rapid, more sensitive and adaptable to a wider class of indicators and pathogens. Antibody (Ab)-based approaches, which take advantage of the specific binding affinities of Abs to specific antigens, can either be produced in the laboratory or purchased commercially. The Abs can be specific for a single strain (or serotype) of bacteria (e.g. *E. coli O157:H7*), or can potentially be produced for a single species (*E. coli*) or groups or families of organisms (enterococci), although the latter two approaches are generally more difficult. Once produced and tested for specificity, Abs are typically mounted onto a support system. Well-documented support systems include polystyrene waveguides, nylon supports, glass slides, and cantilevers (Kasempimolporn *et al.* 2000; DeMarco & Lim 2002; Dubitsky *et al.* 2002; Kooser *et al.* 2003). After antigen capture by the primary Ab, remaining excess material is typically washed away, and fluorescently labeled secondary Abs are used for detection. Immunomagnetic capture, in which organisms are captured using an Ab-antigen-magnet complex, can also be employed (e.g. Shelton *et al.* 2003). With use of an external magnet, the bound material can be effectively separated from the remainder of the sample. One advantage of Ab-based approaches is that captured bacteria are still viable and can be further studied, having the potential to yield more specific information about the sources of the bacteria through assessment of genotypic or phenotypic information (Scott *et al.* 2002).

The third type of capture technology is nucleic acid priming, which relies upon the affinity of specific nucleic acid sequences, or primers, to "fish" for a complementary sequence of interest. The biochemical basis for nucleic acid priming is the foundation for techniques such as polymerase chain reaction (PCR) based methods, microarrays and nucleic acid sequence based amplification (NASBA). Primers can be designed that are complementary to a single gene sequence, allowing these methods to be highly specific.

PCR utilizes a combination of reagents and temperature change schemes to anneal and denature nucleic acid sequences for exponential amplification of the gene of interest (Saiki *et al.* 1985). Quantitative PCR (Q-PCR) is a primer-based molecular technique that combines the specificity of conventional PCR with the quantitative measurement of fluorescence for determining the presence of specific types of nucleic acid in environmental samples. One type is the molecular beacon approach, which employs the use of dual-labeled oligonucleotide probes, that typically bear a 5′-fluorescent reporter dye and a "dark" quencher group in the 3′-position (most commonly Dabcyl). The probe has a unique structure designed to specifically hybridize to a target sequence. When the probe hybridizes to its target sequence, the structure is disrupted and the 5′-reporter is physically separated from the 3′-quencher, allowing fluorescence emission to be detected and measured quantitatively (Heid *et al.* 1996). Lyon (2001) has used this approach for detection of *Vibrio cholerae* in both oysters and seeded water samples. Other Q-PCR approaches are also possible, including the use of

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Taqman® and Scorpion® primer-probe chemistries which can be used with various flourescent dyes for sensitive, high-fidelity Q-PCR.

Microarrays (or 'microchips') involve the attachment of a sequence specific probe on a slide, or array, where specific hybridization of the sequence of interest occurs after a series of linking and wash steps, and subsequent color change indicates positive detection. Microarrays involve the use of cDNA probes, often greater than 200 nucleotides in length, or smaller oligonucleotides (20–100 nucleotides in length) that are fashioned to glass supports, nylon strips, or silica wafers (Richmond *et al.* 1999; Bavykin *et al.* 2001; Wu *et al.* 2003). With a single microarray, thousands of microorganisms can be analyzed at a single time. Upon hybridization of a target to the probe of interest, fluorescence is generated as data on the surface of each slide or "chip". This fluorescence can be read in a number of ways. A planar waveguide can be used for successful imaging of surface confined fluorescence, in conjunction with the use of a cooled charge coupled device (CCD) camera (Rowe-Taitt *et al.* 2000). Laser scanning can also be used to "read" the fluorescent signals, the approach that the well-known microarray manufacturing company Affymetrix recommends (www.Affymetrix.com). Microarrays can also be analyzed with wide-field-high aperture fluorescence microscopes equipped with cooled CCD cameras.

NASBA is similar to PCR technology, but is an isothermal based method of RNA amplification that was applied originally to HIV-1 detection (Kievits *et al.* 1991). Instead of utilizing a thermostable DNA polymerase, as PCR does, RNA is amplified using an enzyme mixture at a fixed temperature. NASBA detection systems are currently commercially available from BioMerieux, Inc (http://www.biomerieux.com) and have shown tremendous potential for use in environmental samples (Collins *et al.* 2003; Paul *et al.* 2003). One advantage of NASBA over PCR-based rapid detection methods is it does not require a thermal cycler, improving portability. However, some Q-PCR manufacturers have already addressed portability of thermal cyclers. For example, Cepheid, Inc. manufactures a portable, car battery powered Q-PCR instrument system, already in use for real-time sampling for a variety of applications.

Detection technology

There are many instruments which can be used to detect a range of chemical, optical, and biological signals generated by the aforementioned capture methods. Most detection technologies revolve around measurement of optical, electrochemical, or piezoelectric properties. The technical aspects of these approaches have been reviewed by Deisingh (2003).

Optical methods are the most frequently used detection approach. The simplest detection units are spectrometers and fluorometers, which can be used for spectroscopic or fluorescence detection of indicator bacteria, respectively, and are field portable. Another widely used option is flow cytometry (FCM), in which cells are physically analyzed based upon characteristics such as natural fluorescence or light scattering (Collier and Campbell 1999; Veal *et al.* 2000). FCM is often paired with immunomagnetic capture to concentrate cells which are passed single file in a fluid stream with the light scatter from a laser defining cell count. Advanced flow cytometers can even sort target cells away from waste materials onto membranes or slides, for further verification methods. FCM systems have been deployed in the field, but they are generally not portable or robust and require advanced training to operate.

Other options for optical detection systems include fiber optics and laser-based interferometry. Both of these are evanescent wave-based technologies, allowing measurement of binding (e.g. of fluorescently labeled antibodies to antigens) at the fiber surface. This greatly reduces the number of separation steps required to separate target from non-target organisms in environmental samples.

Fiber optics is the most advanced of these technologies. Fiber optic devices appear to be promising for environmental applications for two reasons: the ability to make remote *in situ* measurements and the inherent sensitivity of optical approaches. Most currently used fiber optics biosensors involve the use of a combination of immuno-based capture approaches, depending upon a series of non-labeled and fluorescently labeled Abs designed specifically for the organism(s) of interest.

Laser-based interferometry is based upon the fact that planar waveguides have evanescent fields that are responsive to changes in index of refraction. By optically combining guided and reference beams in an interferometric

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configuration, this response can be measured with high sensitivity. Schneider *et al.* (1997) discuss the Hartman Interferometer, which allows a microsensor fitted with the proper chemical/biological coatings to detect multiple contaminants in soil, groundwater, and air. Interferometric technology can easily be combined with Ab-antigen binding mechanisms. Detection is based upon small shifts in optical properties of the organism (upon Ab binding), making this approach highly sensitive. Hartman *et al.* (1995) originally applied this technology to detect proteins specific to *Salmonella* spp in food, and have more recently applied their technology to detect 12 different pathogens. Interferometric approaches have been demonstrated to be highly sensitive, able to detect down to 1 cell. However, the availability and specificity of the Abs used must be predetermined and empirically demonstrated. This approach has not been fully developed for recreational water testing, but with an appropriate preconcentration method, this technology holds promise for development.

Electrochemical signal detectors measure an electrochemical response, of which there are three basic types: 1) conductometric, which is a change in conductance of a bacterial cell between a pair of electrodes due to cell metabolism; 2) potentiometric, which is the difference in electrical potential between a sample and a reference electrode; and 3) amperometric, which is a response due to oxidation or reduction of a specific chemical at a constant applied potential. Electrochemical detection approaches are typically fast and the equipment necessary to measure the signal can be low-cost. Electrochemical approaches are not as susceptible to turbidity interference as optical-based detection and typically have very low detection limits. However, seawater is highly buffered, which can interfere with some electrochemical-based measurements.

Several groups have been working to develop novel electrochemical applications to measure bacteria. Perez *et al.* (2001) has developed an *E. coli* method based on hydrolysis of 4-APGal to 4-aminophenyl (4AP), which is a highly electro-active process that can be detected by small shifts in amperes. This approach is paired with the measurement of cellobiose oxidation, which is indicative of viability, but has been limited by the time necessary for bacterial growth to produce the 4AP molecule. Nistor *et al.* (2002) have used amplification to reduce the time required for *E. coli* detection in natural samples. Even though they succeeded in reducing detection time, their currently reported detection limit was still $>10^4$ cfu/100 ml, too high for use in recreational waters.

Piezoelectric-based biosensors are based on quartz crystals that oscillate at a defined frequency when an oscillating voltage is applied, allowing high sensitivity. Binding of an analyte to the quartz crystal surface changes the mass of the crystal and causes a measurable change in the oscillation frequency. Piezoelectric detection approaches are currently most commonly paired with Ab-antigen capture modes. Microorganisms captured by specific antibodies are immobilized onto the surface of the quartz crystal, which is then subjected to an electrical field. Once the electrical field is applied, the quartz begins to oscillate with increasing amplitude. At a specific oscillation, the antigen (virus or bacteria) suddenly is removed from the surface of the crystal. The noise created during this disruption is proportional to the number of antigens that were originally attached to the surface of the crystal. Piezoelectric biosensors have been used to detect *Salmonella typhimurium* in food (Babacan *et al.* 2002), and for the detection of *Listeria monocytogenes* (Vaughan *et al.* 2001). Sensitivity levels have not been demonstrated at 1 cell/ml to date for piezoelectric-based detection, but flow-through systems as presented by Babacan *et al.* (2002) have the potential to be combined with preconcentration systems.

SAMPLE PREPROCESSING

The biggest technical impediments to the implementation of these methods are the detection sensitivity and the volume assayed. Most detection technologies are based on measuring sample volumes less than 1 ml. EPA's recommended marine bathing water standard is 35 enterococci per 100 ml, which equates to less than one cell per ml. Thus, detectors measuring only a 1 ml volume, even if they are capable of detection of one cell per ml, will necessarily produce unacceptable sensitivity and poor precision at concentrations near the standard.

There are two possible approaches to overcoming inadequate sensitivity. The first is to improve detector technology to allow measurement of larger volume samples, but this is a longer term option. Most researchers already

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use the most advanced detectors available and more sensitive detector technology is not likely to be available in the near future because of the cost and time necessary for development. The preferred option at the present time is preconcentration, which can enhance sensitivity several fold by increasing the number of target organisms per unit volume at a relatively modest cost.

Several available modes of preconcentration are being used, including filtration, size-fractionation, centrifugation and immunomagnetic separation or combinations of these methods. Preconcentration needs vary according to the amount of indicator bacteria present in the sample, the detection limit of the technology being used and the presence of abiotic and biotic confounding factors that are commonly found in recreational water samples. The biggest drawback to preconcentration is the additional time it requires, potentially pushing some methods past the 4-hour criterion. Depending on the method employed, preconcentration could also result in partial loss of target organisms or the unintended concentration of environmental contaminants, both of which could have unpredictable effects on results. Still, these potential shortcomings can be overcome and preconcentration developments will play an important role in advancing the field.

Preprocessing may also be necessary to remove potential biotic and abiotic interferences in the sample, particularly if the samples are preconcentrated. Organic matter (e.g., humic acids), cellular debris and heavy metals can inhibit the reactions necessary for measurement of nucleic acids (Reynolds *et al.* 1997). Preprocessing to separate microorganisms or molecular targets in microorganisms from matrix constituents may involve chemical precipitation, solvent extraction, adsorption to charged surfaces, chelation, or binding through immunomagnetic separation.

SPECIFIC TECHNOLOGIES IN DEVELOPMENT

A number of researchers are presently working to develop technologies for recreational water quality assessments based on combinations of the above-mentioned capture and detection concepts. Below, we highlight a few of the technologies that show promise for accurately measuring indicator bacteria and which are likely to be available for widespread use in the next few years. Other researchers are working to adapt similar techniques toward measurement of specific pathogens, but we have chosen to focus on methods being developed for *E. coli* or enterococci because the management of recreational waters is presently based upon comparison to these bacterial standards.

Dual wavelength fluorometry

Rapid methods based on the enzyme-substrate capture approach are likely to be available commercially in the shortest time-frame, because they are enhancements of pre-existing technology. Whereas commercial applications of this technology, such as those produced by IDEXX Laboratories, Inc., rely on technicians looking for a visible color change after an 18-hour incubation, advanced fluorometry techniques enhance the time to results by quantifying bacterial concentration based on the rate of color change early in the incubation process. Researchers at the University of Connecticut have further refined the method of Jadamec *et al.* (1999), through the use of dual wavelength fluorometry to simultaneously assess both enzymatic hydrolysis and the loss of substrate. A Farrand dual wavelength fluorometer is used at excitation and emission wavelengths of 340 and 450 nm, respectively, with the ratio between the two readings used to infer bacterial concentrations without lengthy incubations. Dual wavelength fluorometry is less susceptible to interference from environmental contaminants because detection of both substrate and product would be affected equally leaving the ratio unchanged regardless of turbidity or the presence of colored substances. To date, the researchers have been able to detect *Enterococcus* spp., *Escherichia coli* and total coliforms at EPA's recommended bathing water standards in less than 6 hours. Given the relationship of the change in fluorescence signal intensity of the product and substrate ratio with increasing cell numbers, along with an algorithm for determining the starting concentrations of the cells of interest, a measurement of original bacterial indicator concentration can be made (Figures 1 and 2). Further optimization of the growth conditions for several of the bacterial indicators, and optimization of dual wavelength instrumental detection parameters has recently reduced the time to detection by 25%, resulting in a current

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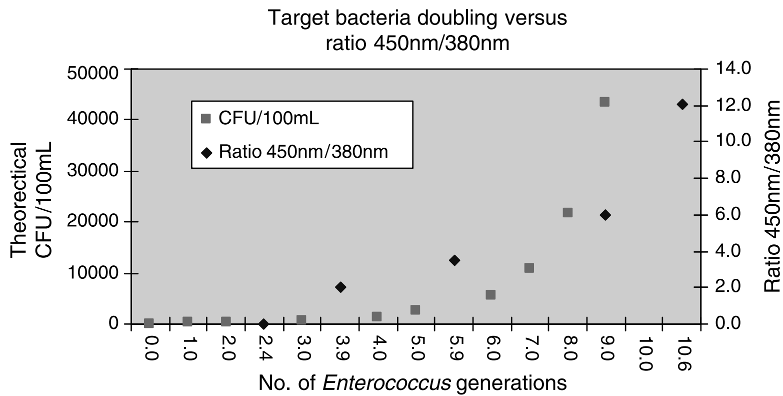

Figure 1 | The relationship of *Enterococcus* growth rate with the ratio of change in fluorescence signal intensity of the product and available substrate using dual-wavelength fluorometry.

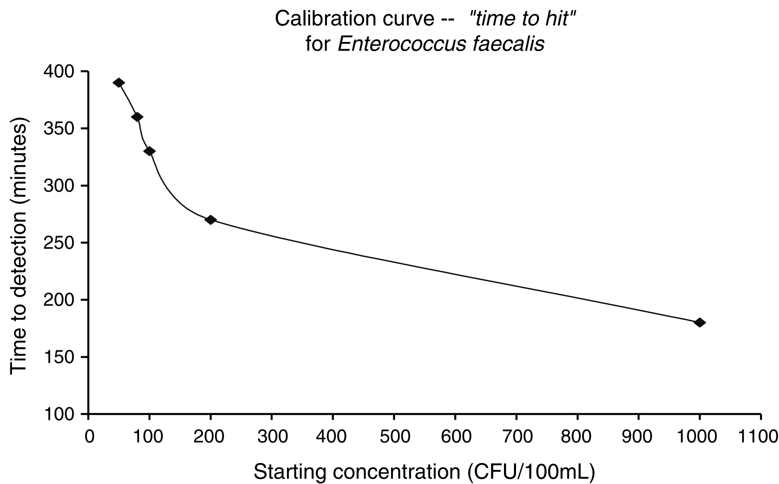

Figure 2 | A preliminary algorithm demonstrating the time to detection for a given starting concentration of *Enterococcus faecalis* in an environmental sample using dual wavelength fluorometry.

detection time of about 4 hours for a starting population of 1 CFU/ml.

Immunoassay approaches

There are several promising immunoassay approaches in development that have been adapted from technologies applied for rapid detection of food borne pathogens (Lim 2000; Lim 2001). An example is the evanescent wave fiber optic biosensor (Tims *et al.* 2001; DeMarco and Lim 2001; Lim 2003; Kramer and Lim 2004). This approach is based on a biosensor sandwich immunoassay that utilizes antibodies on a fiber optic waveguide to detect the target pathogen (Figure 3). The captured target is illuminated by a fluorophore-labeled (Cy5 or Alexa Fluor 647) antibody within an evanescent wave and is detected with a narrow-band laser light from the biosensor. The data are expressed as increases in fluorescence proportional in magnitude to target pathogen concentrations. Lim and colleagues have developed an innovative system to detect pathogens such as *E. coli* O157:H7 directly from ground beef, apple juice, and raw sewage (DeMarco *et al.* 1999; Demarco and Lim, 2001, DeMarco and Lim 2002; Kramer *et al.* 2002). Biotin-streptavidin interactions were used to attach polyclonal anti-*E. coli* O157:H7 antibodies to the surface of the fiber optic probe. These researchers are experimenting with hollow fiber filters and incubation in selective enrichment broth for 3 to 6 hours prior to biosensor assay to detect low bacterial concentrations for application to recreational waters. If the preconcentration

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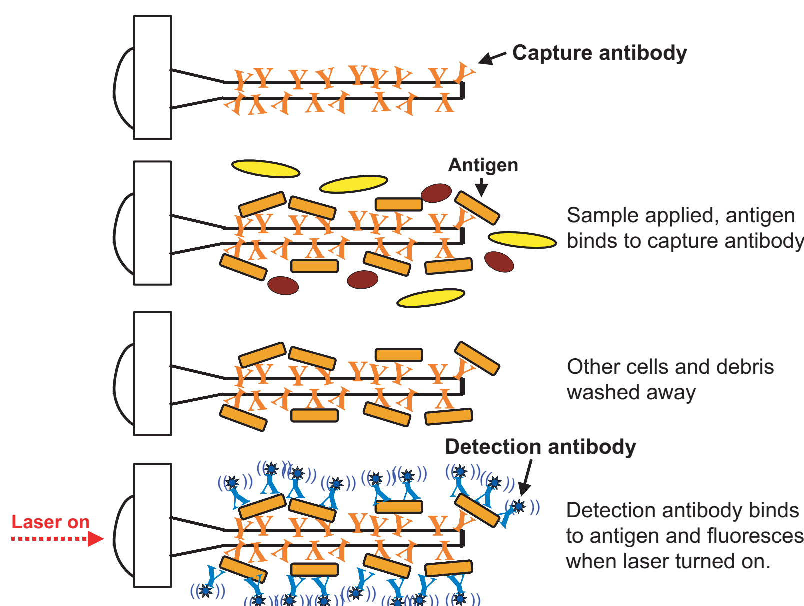

Figure 3 | Biosensor sandwich assay. Target antigen is bound by a capture antibody on the fiber optic waveguide. A fluorophore (Cy5)-labeled detection antibody is then attached to form a sandwich assay. The fluorophore is excited by a laser to generate a detectable signal.

needs can be addressed, this assay is advantageous because live bacteria can be recovered from fiber optic waveguides after the assay to confirm viability or other analyses (Kramer *et al.* 2002; Tims and Lim 2003). In addition, the fiber optic biosensor platform is portable and can be used by minimally-trained personnel in the field.

Several other technologies under development rely upon the Ab-antigen binding mechanism. Lee and Deininger (2004) have developed a system for trapping bacteria on a filter, resuspending it in a small amount of buffer and washing it with a Fluor-Ab-magnetic bead mixture specific to the bacterial species of interest. A magnetic field is used to separate the tagged bacteria from the remainder of the sample. Bacterial cell counts are then assessed by determining the concentration of ATP in the sample using a luciferase assay. ATP provides a measure of cell viability, as ATP degrades rapidly upon cell death (Deininger and Lee 2001). In a California laboratory intercalibration study, enterococcus concentrations for six of eight ambient water samples estimated using this method were within 10% of the median concentration from 26 laboratories that used conventional culture-based methods.

The Advanced Analytical Technologies, Inc. (AATI) commercially available Rapid Bacteria Detection (RBD) system is based on laser-based flow-through technology that identifies bacteria cells which have been labeled with fluorochrome-conjugated antibodies. The RBD provides graphical output with fluorescence intensity (x-axis), 90 degree scattered laser light (y-axis) and count (z-axis) as a dot density display (Figure 4). The operator defines an analysis box to encompass the labeled bacterial cells and output is provided in counts per unit volume within the box at the end of an eight-minute analysis time. Using controlled speed centrifugation for preconcentration, AATI has been able to achieve detection in the 10^2/100 ml range within two hours. AATI is also exploring use of the RBD with fluorochrome labeled rRNA specific peptide nucleic acid (PNA) probes.

PCR-Based technologies

Q-PCR methods are sufficiently advanced that EPA has already incorporated them into two epidemiology studies. The methods that they have used are outlined in Haugland *et al.* (2005) and modifications of approaches used by Bernhard *et al.* (2003), with techniques for application in water analysis described by Brinkman *et al.* (2003). Ambient water samples are collected on a polycarbonate filter, followed by disruption of cells on the filter with glass bead milling in buffer and brief centrifugation. Aliquots of the supernatants are diluted, if necessary, to overcome PCR

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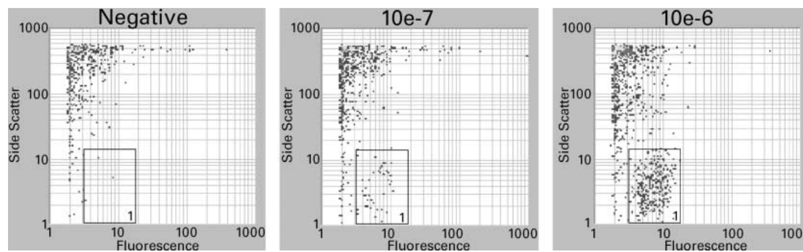

Figure 4 | Rapid Bacterial Detection (RBD) analysis and enumeration of antibody labeled *E. faecalis* and *E. faecium* in concentrated ocean water. The left panel represents a negative water sample (in box count of 6/0.25 ml), while the middle and the right panels represent artificially contaminated water samples from a dilution series with in box counts of 44/0.25 ml (middle panel) and in box counts of 333/0.25 ml (right panel).

inhibition. Detection is accomplished using TaqMan™ chemistry in a real time PCR instrument (e.g. Cepheid Inc., Sunnyvale, CA). Specialized primer/probe sets have thus far been used in assays for two groups of indicator organisms, enterococci and *Bacteroides-Prevotella* spp. Quantitative measurements of these organisms are obtained either by comparing test sample cycle threshold values to a standard curve of values from similarly prepared DNA extracts of known quantities of the target organisms, or by the comparative cycle threshold (C_T) method. The comparative C_T method employs an arithmetic formula to determine target sequence quantities in DNA extracts from test samples relative to those in similarly-prepared DNA extracts from calibration samples containing a known quantity of the target organism cells (Brinkman *et al.* 2003). The method takes approximately 2–3 hr to perform. An advantage of this method is that it is an extension of one that has been used successfully for source identification (Field *et al.* 2003a,b; Bernhard *et al.* 2000, 2003), potentially providing additional information which can assist in health risk management decisions.

Q-PCR methods specific for enterococci and other pathogens have been developed by Blackwood *et al.* (2004), using multiplex reactions that allow for simultaneous quantification of three targets, such as total enterococci, *E. faecalis* and *Salmonella* spp., or total enterococci, *Bacteroides* spp., and adenovirus, yielding information that could potentially indicate source of fecal contamination and a link to public health risk. For example a multiplex assay has been developed for quantification of *B. thetaiotaomicron*, *Salmonella sp*., and total enterococcus. Preliminary results demonstrate efficiencies in the multiplex reactions ranging from 95–122% with no significant differences in the slopes within a reaction (r-squared >0.995) with detection over a wide dynamic range from greater than 10^5 to less than 2 bacteria per reaction (Blackwood *et al.* 2004).

IMPEDIMENTS TO IMPLEMENTATION

While the largest technical impediment to implementation of rapid methods is sensitivity, another factor that will limit use of new methods is regulatory acceptance. Most recreational water quality monitoring is required by, or conducted with funding from, EPA, whose approval will be necessary before most practitioners will transition to a new method. Some of the testing necessary for approval, such as demonstration of method accuracy, specificity, and precision, is relatively easy to accomplish. However, the most important criterion for evaluating acceptability of a new method is a demonstrated relationship to human health risk.

A relationship to health risk is critical because current water quality standards are based on epidemiology studies in which exposure was assessed using culture-based methods which measure some aspect of metabolic activity. In contrast, most new methods, particularly nucleic acid methods, are based on measuring the presence of specific genes without assessing cell viability. As such, the new methods have the potential to overestimate health risk relative to present standards. Unfortunately, the epidemiological studies necessary to establish the health risk relationship are expensive and time consuming. However, many of the rapid techniques described above are equally applicable to a wide array of microbes, perhaps allowing adoption of indicators that have an even better relationship to pathogens or health risk than occurs for existing methods

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measuring current indicator bacteria (Jiang *et al.* 2001; Noble and Fuhrman 2001).

For this reason, the enzyme substrate methods are most likely to be the first rapid methods adopted for recreational water quality. Enzymatic substrate methods are based on the same capture technology as currently-approved EPA methods, with greater speed attained through enhanced detection technology. As such, the relationship to health risk can be established by demonstrating that the new detection capability produces equivalent results to existing procedures.

Demonstration of equivalency may also be possible for some surface recognition capture methods, such as antibody-based systems, which capture bacteria in a potentially viable state. For instance, Deininger and Lee (2001) combined immunomagnetic capture with a luciferase measure of cell viability. However, such measures of viability do not measure the same growth properties used in the current approved methods, so it is unclear whether they will provide comparable results. Epidemiological studies would not be required, though, if equivalency with existing methods could be demonstrated.

Epidemiology studies may also be required because of improvements in capture approaches. New antibodies for surface recognition, or new primers for nucleic acid approaches, have the potential to increase capture specificity. Whereas present culture-based methods measure a broad class of indicator organisms, such as enterococci, new molecular methods provide the opportunity to capture individual species, such as *Enterococcus faecalis*. This will improve monitoring systems by allowing measurement of indicators, or even the pathogens themselves most closely associated with swimmer health. However, new epidemiology studies will be required to establish water quality standards associated with the measurement of more specific target organisms.

Cost is another potential impediment to the adoption of new technologies. Many new methods require sophisticated detection instrumentation costing in excess of $30,000. There are also additional training costs, as the technicians at most public health agencies are unfamiliar with molecular techniques. However, disposable material costs and personnel time required for analysis are generally less for the new methods and the initial capital and training costs may be recouped over time if enough analyses are run, although these costs may remain an impediment for smaller laboratories.

CONCLUSIONS

The development of rapid microbial indicator methods is moving quickly and they will likely become available for use within the next few years, allowing managers to take action toward protecting swimmers from exposure to waterborne pathogens on the same day that samples are collected. Sensitivity is the major technological hurdle facing all three classes of rapid methods, but this may be overcome through a preconcentration step, which has the potential to boost sensitivity without the need to develop new detector technology.

In the short term, available methods are likely to be based on technological improvements to existing enzyme/-substrate-based methods that reduce incubation times from overnight to a few hours and can be approved for use based on demonstrated equivalency with current EPA approved methods. Molecular surface recognition-based methods will also become available soon, but may require inclusion in an epidemiological study to gain EPA approval. Eventually, rapid methods will gravitate toward newer nucleic acid technologies, which are more versatile and sensitive, but are still early in their development and will also require inclusion in an epidemiological study for approval.

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