Real-time monitoring technologies for indicator bacteria and pathogens in shellfish and shellfish harvesting waters

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The counting of bacteria in water has been a critical element in protecting public health in the last century. In the early part of the century scientists were able to grow many species of bacteria and differentiate them from each other using biochemical tests. They also had observed that certain bacteria were always found in the faeces of humans and other warm-blooded animals and that significant disease was also associated with faecal wastes. This association was recognized early on by the scientists who were the fore-runners of our present-day public health scientists. A bacterium originally called *Bacillus coli*
was found in high numbers in the faeces of an infant and shortly thereafter was found in the faeces of healthy humans and warm-blooded animals (Escherich 1885). Pathogens, such as the microorganism that causes cholera, were difficult to grow with the nutrient media available at the time. \textit{B. coli}, on the other hand, grew on simple media and was easily detected in water. The specific identification of \textit{B. coli}, however, was not easy and microbiologists were soon isolating all of the bacteria that looked like and behaved like \textit{B. coli}. This resulted in the group name coliform, meaning having the form of a \textit{B. coli}. Soon, glucose was replaced in coliform media with lactose. The ability of coliforms to specifically metabolize lactose and produce acid and hydrogen gas as end-products allowed this group of organisms to be easily identified.

Dunham (1898) recognized the value of gas production for detecting coliforms and used this characteristic to identify the presence of coliforms in liquid culture. He simply inverted a small tube and placed it in the culture tube. This tube captured the gas produced by coliforms. Phelps (1907) developed a coliform index which used utilized gas production. The index was based on a dilution concept in which the reciprocal of the highest dilution where growth and gas production occurred was reported as the best estimate of the coliform density in a given volume of water.

McCready (1915) was the first to utilize statistical probability theory to estimate numbers of coliform bacteria using the fermentation tube method. The technique, based on the number of tubes showing growth and gas production, provided a most probable number (MPN) estimate of the number of coliform bacteria in a particular volume of sample (McCready 1915). However, solid media, to which water samples can be applied, were preferable to MPN methods because colonies growing on the surface of the medium could be counted rather than estimated. Initially the small volumes of sample that must be used with solid agar media precluded their use with samples that contain small numbers of the bacteria being counted. This problem was solved with the introduction of the membrane filter which allowed larger volumes of water to be analyzed. 100 ml or more of sample can be passed through the membrane filter, which can then be placed on a selective, solid medium and incubated for a selected time period, after which colonies on the filter can be identified and counted.

The common factor between these two quantitative approaches to counting bacteria is the requirement for cell multiplication to occur over a sufficient period of time, so they can be easily observed. In both cases the time to colony formation is between 20 and 24 hours. Compressing this time interval has been the goal of water microbiologists for many years. In one case this was accomplished using a membrane filter technique and a nutrient medium that
maximized the cell doubling time which allowed microscopic identification of the colonies in about seven hours (Reasomer et al. 1979). This is about the lowest time limit for obtaining results with culture methods for quantifying bacterial indicators in water samples. However, there is a new generation of instruments and techniques being developed to quantify indicator bacteria and pathogens that will provide monitoring results in a much shorter period of time.

Molecular methods are at the forefront of these new technologies. The advent of the polymerase chain reaction (PCR) technique (Mullis and Faloona 1987) holds great promise as a rapid method for measuring environmental water quality. The PCR was a significant step in the development of highly specific, rapid methods for identifying microbes associated with faeces. It did have drawbacks, however, in that the original post-amplification procedures were time consuming and did not lend themselves to quantification. In the mid-1990’s, the real-time quantitative PCR (qPCR) was introduced to the scientific community (Heid et al. 1996). This procedure allows both detection and quantification of PCR-amplified nucleic acid sequences without the need for post-amplification procedures. There are numerous thermal cycling instruments available commercially that are capable of rapidly detecting and quantifying microbes in environmental waters by this technique.

Other techniques are also gaining favour in the area of water quality monitoring. Chemical methods that measure adenosine triphosphate (ATP) and enzymatic reactions are also rapid and may be useful for measuring water quality. Antibody based methods that are used with flow cytometry and fibre optic technologies also have some potential, but problems with sensitivity and the small volumes used in these assays are limiting their use. Molecular methods however, are by far the most advanced of the technologies that have been used to quantify microbes used to measure faecal contamination in water.

This chapter will describe some of the available technology for the rapid measurement of water quality and shellfish. Although many high technology methods are described in the literature, very few have been used to test natural samples, such as water samples or shellfish tissue samples. Only methods that have been used to measure natural water samples, whether marine, estuarine or freshwater, or shellfish tissues for faecal indicator bacteria or pathogens will be described in this chapter. The approach will be to describe the procedure in some detail and then briefly review one or two papers from the literature describing how the method has been used to measure indicators or pathogens in samples taken from natural environmental waters or from harvested shellfish. No attempt has been made to provide a comprehensive literature review.
7.1 MOLECULAR APPROACH

The most studied of the new methods for quantifying microbes in water is the qPCR. This technology has many attributes which make it attractive for measuring microbes in water. First, the qPCR method is very specific to the target microbes being detected. Contemporary culture techniques depend on phenotypic characteristics whose presence may be governed by several enzymes that frequently are affected by the physiological state of the microbes. A variable physiological state will result in variable phenotypic characteristics which can at times make identification of the microbe difficult. This variability does not occur with qPCR which detects cells on the basis of specific nucleotide sequences that are unique to the microbes under study. In addition, the qPCR technology is very rapid. Detecting and identifying microbes with cultural methods usually require about 24 hours, the amount of time it takes microbes to grow to the point where growth can be visualized. qPCR results, on the other hand, can be observed in two to three hours, because of the logarithmic amplification of the sequences of interest.

The qPCR process consists of two steps that occur at different temperatures. At a high temperature, double-stranded DNA is denatured to two single strands, completing the first step. At the lower temperature, a number of reactions take place. The first is the hybridization of short pieces of DNA (oligonucleotides) called primers to specific locations on the single strand of DNA. These primers provide a starting point for the synthesis of new double-stranded DNA. A second hybridization involving a highly specific oligonucleotide called a probe, takes place at a point on one of the single-strands of DNA which is between the two primer sites. This probe is unique to the microbe being detected. One of the most commonly used types of probes is called a hydrolysis of Taqman® probe. These probes have a fluorescent reporter dye attached to one end and a quencher dye attached to the other end. When these two dyes remain in close proximity to each other on the probe the reporter dye cannot fluoresce. After the probe attaches to the target sequence, a polymerase begins extending the primer toward the probe, forming new double-stranded DNA. As the extended DNA meets the probe, the probe is cleaved, freeing the reporter dye so that it is no longer in proximity to the quencher dye and can now fluoresce. The formation of double-stranded DNA completely removes the probe from the target sequence allowing the primer extension to continue until a new double-stranded DNA is formed, ending the second step.

These cycles are programmed into a spectrofluorometric thermocycler, which continuously proceeds through the two steps, measuring the amount of fluorescent dye freed in each annealing step. The fluorescent signal intensity is proportional
to the amount of DNA produced. Quantification of the PCR process is measured in terms of the number of two-step cycles and the accumulation of the fluorescent signal to that point where it crosses a baseline and is first detected. The magnitude of the signal generated under a given set of PCR conditions is determined from standard samples of known concentrations used to establish a standard curve.

Quantitative molecular methods for measuring microbes in shellfish have been used to detect both viruses and bacteria. A quantitative reverse transcriptase qPCR method (RT-qPCR) was used by Jothikumar et al. (2005) to determine the norovirus density in shellfish meat. The viral RNA from purified shellfish concentrates was recovered by binding to size-fractionated silica after lysis of the viral particles with guanidine isothiocyanates. After elution from the silica particles, the RNA was precipitated in ethanol and sodium acetate. Reverse transcription was then performed using a Geneamp RNA PCR corekit in a Geneamp 9700 PCR system (Applied Biosystems). Finally, Taqman PCR was performed with a QuantiTect probe PCR kit. Compared to other conventional multiplex RT-PCR assays, the Taqman RT-qPCR results were much faster because they do not require additional nested amplification steps.

*Vibrio parahaemolyticus* is a halophilic, gram-negative bacterium that has frequently been associated with shellfish-associated illness (Rippey 1994). These shellfish-associated outbreaks have stimulated a great interest in the availability of rapid methods for detecting and identifying this microorganism. Ward and Bej (2006) examined multiplex real-time PCR to detect specific genes related to the virulence and species of *V. parahaemolyticus*. They developed the assay using four sets of gene-specific oligonucleotide primers and four corresponding Taqman probes labeled with four different fluorogenic dyes. Ward and Bej used Gulf of Mexico oysters to evaluate their multiplex system. Oyster homogenates were enriched for 24 hours. Following enrichment, DNA was extracted and a small sample of extract was amplified in a thermocycler. Shellfish sample homogenates were seeded with purified genomic DNA of the four genes being amplified as a positive control. Their results showed that 17 of the 34 shellfish were positive for *V. parahaemolyticus* and that four of the positive samples contained a gene indicating that the strain was pathogenic. The other two genes coding for pathogenicity were negative in the 17 samples. This approach will lend itself to delivering timely results in the examination of suspected contaminated oysters or other shellfish meats in outbreak situations.

The use of qPCR to monitor the quality of shellfish harvesting waters has not been considered up to this point in time. However, qPCR has been used to monitor the quality of recreational waters (Haugland et al. 2005). Enterococci in beach waters were measured with qPCR and the results, which were obtained in about three to four hours, were shown to have a direct relationship to
gastrointestinal illness in swimmers (Wade et al. 2006). The assay is straightforward. A 100 ml water sample is filtered through a polycarbonate filter which is placed in a centrifuge tube containing glass beads. Violent shaking of the beads breaks the cells open, freeing the DNA. The cell debris is sedimented by centrifugation and the supernatant is analyzed by qPCR. This method has also been applied to Bacteroides species and it may also have utility in identifying sources of faecal contamination using mammalian species-specific Bacteroides spp. strains.

7.2 CHEMILUMINESCENCE APPROACH

The term bioluminescence refers to chemical reactions that occur in vivo and which result in the emission of light. If the chemical reaction takes place in vitro, the emission of light is termed chemiluminescence. The best known chemiluminescence reaction is the luciferin–luciferase reaction, which has been used for many years to measure ATP from living microbes. ATP can be extracted from living cells and assayed in vitro with the luciferin–luciferase system. The resulting emission of light is detected by a luminometer photomultiplier tube. ATP can be measured quantitatively with this system. The amount of light reaching the photomultiplier tube is proportional to the amount the ATP in the sample. Furthermore, the ATP measured should also be proportional to the number of viable cells in the sample.

Chemiluminescence tests that measure the presence of ATP have been used in the food industry, the pharmaceutical industry and the cosmetics industry for many years. Even though this technology has been available for years it has not gained favour for measuring the quality of drinking or surface waters. The reason for this is that the ATP measured in the above industries is from the total microbial populations rather than from specific faecal indicator microorganisms used to measure the quality of water. This shortcoming has been overcome by the availability of magnetic beads coated with antibodies specific for the indicator bacteria used to measure water quality. The antibody captures the specific indicator bacterium and the captured cells are separated from the remainder of the sample with magnets. The separated cells are then assayed for ATP. The perceived need for more rapid methods for measuring water quality, especially in the areas of recreational waters and drinking water security, has established new interest in the use of luminescence. Measuring ATP has several advantages with regard to measuring water quality. This technology is relatively inexpensive, the results can be obtained in a very short time, the test is very sensitive and the technique can be used to measure analytes other than ATP. Furthermore, ATP can be used to estimate the number of viable cells in
a water sample. There is a paucity of research information on the use of the measurement of ATP for monitoring water quality. There are, in fact, no references to the use of ATP for measuring faecal indicators in marine waters or shellfish meats.

Lee and Deininger (2004) did show the use of this technology for measuring *E. coli* in fresh surface waters. Their method consisted of the following steps. Between 100 and 500 ml of water sample was first passed through a nylon pre-filter with a pore size of 20 μm to separate large particles from the sample. Following the pre-filter step, the sample was passed through a 0.45 μm filter. The retained cells were washed from the filter with phosphate buffered saline (PBS) containing Tween 20. The bacterial suspension was then mixed with anti-*E. coli* antibody adsorbed to the surface of magnetic beads. The solution was mixed for 15 minutes at 60 RPM. The *E. coli* captured by the beads were removed and concentrated from the buffer solution with a magnet applied to the side of the tube. The buffer solution was discarded and the captured cells were then washed twice in PBS. After washing, the retentate was suspended in 1 ml PBS and pipetted to a centrifuge tube. The magnet was applied again to separate the cells from the PBS, followed by the addition of 50 μl of somatic cell releasing agent. This step removed the non-bacterial ATP from the mixture. After further magnetic separation, the buffer was removed and the retentate was washed with PBS. After a fourth magnetic separation, the PBS was discarded and the ATP was extracted from the cells by a solution that dissolved the *E. coli* cell wall. This solution was transferred to a cuvette and 50 μl of luciferin/luciferase solution was added for light development. The light emission was measured in relative light units (RLU) with a microluminometer.

This approach to measuring water quality shows some promise for *E. coli*. The detection limit is about 20 cfu/100 ml. The ATP measurement method results in an underestimate of the *E. coli* densities as measured by a membrane filter method. This ATP method was evaluated by a second group (Bushon *et al.* 2004) who compared it to a membrane filter method at three sites along a freshwater river. The correlation of the ATP method with the membrane filter method was reasonably good at two of the sites, but showed no relationship to the membrane filter method at the third sampling site. It was recognized that further research would be required to optimize the effectiveness of this method.

### 7.3 ENZYMATIC APPROACH

Specific enzymes in indicator bacteria have been used for many years to quantify the microbes associated with faeces. Enzymes, such as galactosidase, glucosidase and glucuronidase, are detected through the use of specific fluorogenic
or chromogenic substrates, such as 4-methylumbelliferyl-beta-D-galactoside, 4-methylumbelliferyl-beta-D-glucoside and indoxyl-beta-D-glucuronide. The substrates, which are colourless in the conjugated state, either fluoresce or present a colour after they have been hydrolyzed to form a fluorogenic or chromogenic compound and a sugar or an acid. In the culture approach, the substrates are incorporated into culture media for estimating the number of microbes in a water sample by the MPN or membrane filter procedures. These chromogens and fluorogens are used to specifically differentiate the target organisms from other bacteria that might grow on the selective media. These media generally require 24 hours or more for growth of colonies for an estimate of their density.

Enzymatic methods followed two approaches for quantifying coliforms and faecal coliforms. The first approach for measuring faecal coliforms is to pipette a fluorogenic substrate directly into a mixture of water sample and buffer. The total volume of the solution is 12 ml. The mixture is incubated for one hour at 44.5°C. After the incubation period the medium is cooled very rapidly to stop the enzyme reaction and the solution is adjusted to pH 10 to optimize the fluorescent intensity. A fluorescent calibration curve is produced relating standard concentrations of the fluorogen to fluorescent intensity. The fluorescent intensity of an unknown sample is compared to the standard curve to estimate the number of cells in the sample.

A second approach involves the incubation of a membrane filter in a flask with 12 ml of buffer, fluorogen and a surfactant. The water sample (100 ml) is passed through the membrane filter before the membrane is placed in a 200 ml flask. The mixture is incubated in a water bath at 44°C. Every 5 minutes for 30 minutes a 2 ml aliquot is removed and placed in a cuvette containing sodium hydroxide. The fluorescent intensity is measured with a spectrofluorometer and is expressed as the amount of fluorescence liberated per minute for 100 ml of sample. The time interval at which the fluorescence is first detected is related to the number of bacteria in the sample.

Davies and Apte (1999) examined 254 water samples for faecal coliforms. The enzymatic test measured the hydrolysis of 4-methyl-umbelliferyl-beta-D-galactoside. The results showed a linear increase between fluorescent intensity and colony forming units (cfu) above 300 cfus. Below 300 faecal coliforms there was no relationship between fluorescent intensity and cfus. It was suggested that this enzymatic test could be used in the presence/absence mode. Lebaron et al. (2005) examined the rate of hydrolysis of a fluorogenic substrate, 4-methylumbelliferyl-beta-D-glucuronide, to detect the presence at E. coli in seawater. Twenty-six beach water samples were assayed using the multi-well MPN cultural procedure and a method using enzymatic hydrolysis of a substrate
over time to measure the *E. coli* density in the water sample. The results for both assays were compared and the findings showed that the results were similar, however the enzymatic results were somewhat higher than those from the cultural procedure. The authors attributed this to multiple *E. coli* cells that may have attached to particles and were counted as one cell in the MPN method. They also found the limit of sensitivity of the enzymatic method was about 5 cells/per 100 ml.

These methods may provide more rapid measurements of water quality. The enzymatic endpoint approach described by Davis and Apte (1999) would have limited application to shellfish harvesting waters because of the limit of sensitivity, which is rather high. The limit of sensitivity of the enzymatic hydrolysis of substrate approach (LeBaron 2004), on the other hand, is low, well within the range of shellfish harvesting water coliform limits.

### 7.4 CONCLUSIONS

The methods described above have the potential to measure the quality of both types of samples in a timely manner. Situations where rapid monitoring methods might be used include shellfish harvesting waters contaminated by an accidental sewage spill when it is important to know as soon as possible that the waters have returned to ambient conditions. There may also be instances where it is critical to know if harvested shellfish are contaminated and this information must be obtained in a timely manner. The methods discussed here are reported to furnish results in approximately one (Davies and Apte 1999; Lee and Deininger 2004; LeBaron et al. 2005) to four hours (Haugland et al. 2005).

It is frequently important to know if the pathogens being measured are viable and therefore able to cause infections. The available molecular methods are not able to identify and quantify viable microbes. Quantitative PCR measures both viable and non-viable bacteria and, therefore, this technique gives results that are greater than those obtained with cultural methods. Chemiluminescence and enzymatic methods, on the other hand, do measure viable microbes. Therefore, the estimates of bacterial densities in water samples by those methods are more likely to be comparable to those obtained with cultural methods.

The sensitivity of these methods is very good. All of these methods should, in theory, be able to measure one cell. In practice, samples for the qPCR technique and the chemiluminescence technique must be concentrated using membrane filtration. The former method requires that 100 ml of the sample is filtered for the assay and the latter method requires filtration of 100–500 ml of sample for the assay. The enzymatic test described by Davis and Apte (1999) had a limit of sensitivity of about 300 cells. They did not discuss the use of a concentration
step as a means of increasing the sensitivity of the test. The procedure that measured the rate of hydrolysis of a substrate (LeBaron et al. 2005) had a limit of sensitivity of five microbial cells using a 100 ml water sample, which is well within the range needed to measure currently-used indicator bacteria without further concentration of the sample.

The specificity of the new technologies for measuring microbes associated with faeces is frequently much better than that observed with cultural methods. This is especially true for molecular methods that detect unique portions of the genome of the indicator bacterium or pathogen. Methods that use antibody capture may also be highly specific, depending on the quality of the antibodies attached to the magnetic beads. Enzymatic methods may at times show some non-specificity with regard to target analytes. If non-target microorganisms with enzyme systems similar to the microorganisms of interest are contained in the water sample, it is possible that they might hydrolyze the substrate and cause a false positive result to occur. This situation would probably not occur more frequently than it would using a cultural approach.

There are several excellent reviews in the literature which address new methods and techniques for detecting and quantifying microbes in water and other media. Two of the reviews (Sidorowicz and Whitmore 1995; Rompre 2002) specifically address the rapid monitoring of coliforms in drinking water. The two papers describe the better-known of the rapid technologies such as flow cytometry, in situ hybridization, the PCR and enzyme-based approaches, as well as their advantages and disadvantages. A review of biosensors describes some of the more esoteric methods and techniques such as optical transducers, bioluminescence sensors, piezoelectric biosensors systems and electrical impedance biosensors. Other biosensors based on fluorescence labelled antibodies or electrochemical immunodetection and flow immunosensors are also discussed (Ivnitski et al. 1999). Although many of the detection and enumeration systems described in this review are not available to measure microbes in water and food, they do provide heuristic examples of some of the technologies that may become available in the future. Mandrell and Wachtel (1999) reviewed novel detection techniques for human pathogens that contaminate poultry. They addressed mainly the use of immunomagnetic separation and molecular techniques for a couple of the more common pathogens found in poultry, such as a Salmonella and Campylobacter, two bacterial pathogens that are sometimes associated with shellfish.

The rapid advances being made in the development of new methods and technologies for detecting and quantifying microbes in water and food, coupled with the great interest in maintaining the quality of shellfish harvesting waters, will undoubtedly lead to greater use of high technology, rapid methods in
the future. The many advantages of these new and emerging technologies are already evident and they provide a sound basis for transition from the methods of the last century to new means for protecting public health through better monitoring of the quality of our foods and water.

7.5 REFERENCES


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