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8 Are They Alive? Detection of Viable Organisms and Functional Gene Expression Using Molecular Techniques

Paul A. Rochelle, Anne K. Camper, Andreas Nocker and Mark Burr

Abstract

The ultimate measure of microbial viability and biological activity is growth in some form of culture system. Unfortunately, due to many limitations, growth is usually not the most sensitive or rapid detection method. This chapter describes many of the molecular-based tools for assessing viability and functional gene expression, and their applications to microbes in environmental samples. Methods include fluorescent nucleic acid binding dyes, enzymatic conversion of substrates to fluorescent compounds (often in conjunction with nucleic acid-based methods), various techniques based on amplification and detection of nucleic acids, nucleic acid amplification linked to biosensors and microarray detection platforms, detection and characterization of proteins, and molecular detection coupled with culturing. Principles supporting each of these techniques are discussed along with applications to bacteria, protozoa, and viruses, focusing primarily on microbes of concern to the drinking water and wastewater industries.

Introduction

Knowledge on the presence or absence of microorganisms in environmental samples is frequently sufficient information to address a particular problem or answer a specific question. However, it is often necessary to determine whether microbes in environmental samples are viable and active. Viability information is needed by the drinking water and wastewater industries to aid in assessing the public health risk represented by pathogens in environmental waters, and to assess the efficacy of disinfection and treatment techniques. Measurements of viability and activity are also required to ascertain whether the microorganisms are interacting with their environment and responsible for some of the biological, chemical, biogeological, and physical processes that occur within ecosystems. Gene expression, functionality and activity measurements are critical to establishing the link between microbial diversity and ecosystem functions.

Microbial culture methods have a variety of limitations when used for studying environmental populations. Many bacteria in environmental samples will not grow on laboratory culture media, those that can be cultured may grow at different rates so the faster growers dominate, and long incubation periods may be required. In addition, culturing methods greatly underestimate the functional capabilities of microbes in the environment. Nevertheless, culture-based methods provide a definitive measure of viability; if a bacterium grows on an agar plate and produces a visible colony, it is unquestionably alive and active. Although molecular detection of microbes in environmental samples can be more rapid, sensitive, and specific than conventional culture-based methods, in their most basic form, they are simply presence/absence tools. They do not provide information on the viability, activity, or infectivity of detected microbes. However, there is now a wide diversity of molecular tools that can be used to specifically differentiate between viable and dead microbes, and to assess and measure gene expression (potential activity) in environmental samples (Table 8.1). These methods encompass

Table 8.1 Methods for assessing microbial viability in environmental samples

Measured parameter	Method
Cellular integrity	Inclusion or exclusion of fluorescent dyes (e.g. propidium iodide, ethidium homodimer, SYTO-13, SYTOX)
Presence and integrity of nucleic acids:	
DNA	PCR (with propidium monoazide)
RNA	Long target region (LTR)-PCR
rRNA	Fluorescent *in situ* hybridization (FISH)
Gene expression: mRNA	Reverse transcriptase (RT)-PCR Nucleic acid sequence-based amplification (NASBA) Biosensors Functional microarrays
Cellular activity	Detection of proteins Metabolic dyes (e.g. Calcein-AM, Resazurin, CTC)
Cellular replication	Growth in complex media or growth in cell culture – both coupled with molecular detection for faster turnaround

inclusion or exclusion of fluorescent dyes by microbial cells, enzymatic conversion of substrates to fluorescent compounds (often in conjunction with nucleic acid-based methods), various techniques based on amplification and detection of nucleic acids, nucleic acid amplification linked to biosensors and microarray detection platforms, detection and characterization of proteins, and molecular detection coupled with culturing. These techniques were originally developed primarily for single species or relatively small groups of related microbes, but 'meta' approaches that allow community-wide investigations have been applied to all of the molecular targets (DNA, RNA, and proteins; Fig. 8.1). This chapter describes many of the technologies for assessing viability and functional gene expression, and discusses their application to bacteria, protozoa, and viruses in environmental samples, focusing primarily on microbes that are of concern to the drinking water and wastewater industries. The ultimate measure of viability and biological activity is growth of the microbe in some form of culture system or direct measure (see Chapter 10). However, this is often not practical because of the limitations described above. Therefore, the surrogate molecular methods described in this chapter allow inferences about microbial viability. However, as methods become further removed from a culture-based system, the confidence in viability or functional activity assessments may be reduced (Fig. 8.1).

Dye-based methods for assessing viability

Although fluorescent dye-based techniques are not usually regarded as 'molecular methods,' they usually involve nucleic acid-binding dyes [e.g. 4',6-diamidino-2-phenylindole (DAPI), propidium iodide, SYTO dyes, SYBR-Green, and SYTOX]. Cellular membrane integrity, as determined by inclusion and/or exclusion of membrane-permeable and impermeable dyes, is an accepted technique for distinguishing viable from dead cells in many areas of microbiology (Gregori *et al.*, 2001). For example, propidium iodide and ethidium homodimer dyes cannot cross intact membranes of healthy cells. However, they cross damaged cell membranes, bind to nucleic acids, and their red fluorescence can then be detected microscopically (528–536 nm excitation, 617 nm emission). A limitation of propidium iodide is that it actively stains dead cells but there is no direct staining of live cells, so a 'viable' count has to be obtained by subtraction from the dead count. Consequently, various combinations of stains are typically used. DAPI and SYTO-10 are used as counterstains to stain

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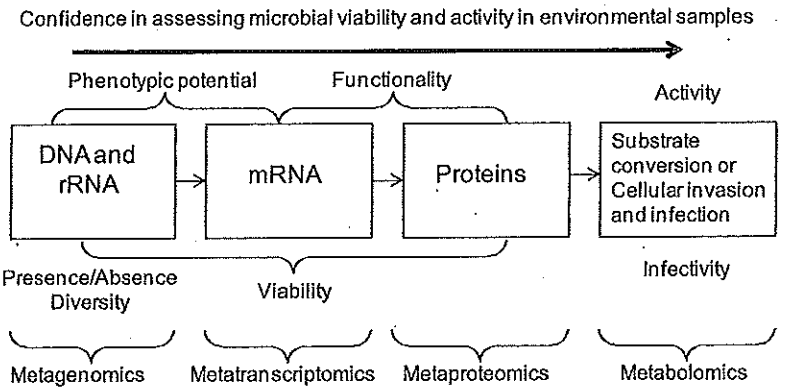

Figure 8.1 Confidence in assessing and measuring viability of microbes in environmental samples increases as assay methods get closer to culture-based approaches (figure adapted from Maron *et al.*, 2007).

the nucleic acids of all cells. Live stains are usually based on functional activity within a cell, such as the conversion of non-fluorescent calcein-AM to a fluorescent product by intracellular esterase. Viable cells with a functioning esterase enzyme will produce fluorescence, whereas dead cells will not stain because they have no cellular activity. Combining calcein-AM (or other intracellular enzyme substrates) with propidium iodide or ethidium homodimer provides direct staining of both live and dead cells. Methods based on dye exclusion/inclusion across intact or damaged membranes have been developed commercially (LIVE/DEAD™ staining kits; Invitrogen, Carlsbad, CA) and for analytical flow cytometry (Barbesti *et al.*, 2000; Gregori *et al.*, 2001). However, limitations of these viability dyes include non-specific binding and fluorescence, variable staining depending on the method of cellular inactivation (e.g. natural death versus chemical or physical inactivation methods), and cross-talk between dyes with different but overlapping excitation and emission spectra. Alternative dye combinations, such as SYTO-13 and SYTOX-Orange, were reported to improve live/dead discrimination due to reduced background fluorescence and non-specific binding (Biggerstaff *et al.*, 2006).

Flow cytometry of differentially stained cells is a useful method for sorting viable cells from dead or injured organisms. Viability has been detected on the basis of substrate-responsive and non-responsive cells, and actively respiring 5-cyano-2,3-ditolyltetrazolium chloride (CTC)-reducing cells from an aquatic ecosystem (Bernard *et al.*, 2001). Ben-Amor *et al.* (2005) separated a faecal microbial community by fluorescence activated cell sorting (FACS) after staining the sample with SYTO BC and propidium iodide. Three fractions were distinguished and defined as viable (SYTO BC-stained), injured (SYTO BC and propidium iodide-stained) and dead cells (just propidium iodide stained). Techniques using flow cytometry sorting depend on the ability to disperse a community into single cells. However, bacteria in many environmental samples form aggregates that are difficult to separate (Dunne, 2002; Bakker *et al.*, 2004), so flow cytometry has not been widely used to detect viable cells in environmental samples.

Berney *et al.* (2008) proposed using SYBR Green I, propidium iodide, bis (1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC) and carboxyfluorescein diacetate (CFDA) in combination with flow cytometry and total adenosine triphosphate (ATP) measurement as a cultivation-independent approach to assess viability of bacteria in drinking water. More details on methods based on ATP measurement can be found in Chapter 1. SYBR Green stains nucleic acids in all bacterial cells regardless of their physiological state, but the intensity of staining varies depending on the amount of nucleic acids in the cell. DiBAC is a green fluorescent stain that only enters a cell if its membrane potential is lost, and so stains dead cells but not live healthy cells. CFDA is a non-fluorescent enzyme substrate that

is hydrolysed by intracellular esterase to produce fluorescent 6-carboxyfluorescein. The esterase-positive cell fraction (CFDA-positive), total ATP values, and high nucleic acid (HNA) bacterial fraction (most intensely stained with SYBR Green) were most representative of the active or viable cell population. The strongest correlations ($R^2 > 0.75$) between individual measures of viability were: esterase-positive vs. total ATP; intact cells vs. DiBAC-negative; HNA vs. total ATP; total counts vs. DiBAC-negative; and HNA vs. esterase.

Staining nucleic acids has also been combined with a short culture period. Microcolonies (approximately 10–50 μm in diameter) that developed after 18 h incubation on membrane filters were stained with SYBR Green II (Wang *et al.*, 2007), which was quicker than waiting the 5–7 days required for regular size colonies to develop. Bacterial numbers in compost based on macroscopic colonies on LB plates, or SYBR-Green stained microcolonies, were 3% and 52% of the esterase-positive fraction, respectively.

Detecting viable microbes using the polymerase chain reaction (PCR)

Although cultivation is still frequently regarded as the 'gold standard', it is not necessarily the most appropriate method for detecting target microbes in environmental samples. Testing and comparison of methods other than culturing, such as PCR, would make a significant contribution towards improved pathogen detection by the water industry, and will ultimately increase public health protection. However, there are remarkably few published studies showing how well PCR detection methods correlate with culturing, with the majority of work being undertaken in the food industry. A review by Maciorowski *et al.* (2005) stated that some commercial PCR diagnostic kits generated mixed results when compared to cultural methods but, overall, agreement was good. In a variety of food matrices, there was 96% agreement between the BAX™ method (DuPont Qualicon, Wilmington, DE) and cultural methods for detecting *Salmonella* (Bennett *et al.*, 1998). In another study with *Salmonella*, there was agreement in 99% of meat samples analysed with conventional methods and PCR (n=100; Kimura *et al.*, 1999). A similar correlation (98%) was seen when Probelia™-based PCR and culturing were used to test minced beef, egg, fish, milk, and water for *Salmonella* (Coquard *et al.*, 1999; Fach *et al.*, 1999). Similar studies should be undertaken with matrices and microbes relevant to the water industry to verify the applicability of specific PCR-based methods because the methods that correlate well with culturing are most likely to gain acceptance and widespread use.

DNA intercalating dyes and PCR: ethidium and propidium monoazide

Conventional PCR with no modifications can detect the DNA of specific microbes but is largely blind to the viability status of the target organism, unless the DNA is so degraded in dead cells that it cannot be amplified. In fact, DNA in dead organisms and naked DNA in environmental samples can be detected by PCR (Masters *et al.*, 1994; England *et al.*, 1997; Dupray *et al.*, 2003). Although a single instance of detecting DNA using a molecular method indicates the presence of that organism at some time prior to sampling, it provides no information about the viability of the organism. On the other hand, consistent detection of DNA from a particular organism over an extended period of time implies the presence of a viable population of that organism. Nevertheless, unmodified PCR is generally regarded as inappropriate for assessing viability (see Chapter 7 for other *in situ* methods). Combining PCR of DNA targets with membrane impermeable dyes provides a robust and relatively simple method for assessing viability. Ethidium monoazide (EMA) penetrates cells with compromised membranes (dead or dying cells), but not healthy cells with intact membranes (Nogva *et al.*, 2003; Rudi *et al.*, 2005). This fluorescent dye contains an azide group that intercalates with the DNA. Upon exposure to a strong light source, the dye becomes covalently linked to DNA and remains linked through DNA extraction procedures. The modified DNA strongly inhibits PCR amplification so that DNA from dead cells is not amplified. However, EMA is not entirely specific to a particular viability status because it does not penetrate damaged membranes in some bacterial species,

and can enter live cells of other species (Nocker *et al.*, 2006; Flekna *et al.*, 2007). Because of this limitation, it is prudent to perform preliminary analyses to test the appropriateness of the dye for the target organism(s).

Propidium monoazide (PMA) is structurally similar to EMA and propidium iodide. Compared to EMA, PMA is less able to cross the cell membranes of a variety of live Gram-negative and Gram-positive bacteria, probably due to the presence of two positive charges compared to the single positive charge on EMA (Nocker *et al.*, 2006). PMA has been used to monitor the disinfection efficacy of chlorine, benzalkonium chloride, heat, and alcohol exposed bacterial cultures by quantitative-PCR (qPCR; Nocker *et al.*, 2007). The method was rapid and simple to perform. However, PMA-PCR cannot be used to measure disinfection by ultraviolet light (UV) because membrane integrity is not affected by UV. PMA treatment followed by qPCR was also used to quantify fungal viability in air and water samples (Vesper *et al.*, 2008). Heat treatment of different fungal species, and subsequent PMA treatment, showed a 100- to1000-fold difference in cell viability. This result was consistent with viability loss based on culturing. PMA-PCR has also been used to assess viability in Bacteroidales bacteria in wastewater influents and effluents, *Bacillus subtilis* spores, *Enterobacter* spp., and *Listeria monocytogenes* (Pan and Breidt, 2007; Cawthorn and Witthuhn, 2008; Bae and Wuertz, 2009; Rawsthorne *et al.*, 2009). PMA has also been coupled with microarray analysis. In an analysis of four different pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Serratia marcescens*) damaged cells did not produce amplicons for *cpn60* genes (coding for *GroEl*). The PCR products were hybridized to microarrays containing strain-specific *cpn60*-based 35-mer oligonucleotide probes. The results were not strictly quantitative, but there was a significant signal reduction when damaged cells were PMA-treated, and the results correlated well with PMA-qPCR (Nocker *et al.*, 2009). In addition, PMA-PCR targeting the 18S ribosomal RNA (rRNA), and heat shock protein (*hsp70*) genes were used to simultaneously assess viability and genotype *Cryptosporidium* oocysts (Brescia *et al.*, 2009). The assay was demonstrated with raw waste water and surface water spiked with 1×10^5 oocysts, which were recovered and purified by immunomagnetic separation (IMS) prior to PMA-PCR. PMA effectively prevented amplification of DNA from oocysts that were killed by heating or 14 months' storage at room temperature.

Detecting RNA

Compared to DNA, RNA is intrinsically unstable and prone to degradation by ubiquitous RNases. Purified *Salmonella* DNA persisted for 2–8 days in seawater, whereas the DNA of dead *Salmonella* cells could be detected by PCR after 10–55 days storage in seawater (Dupray *et al.*, 2003). Although the half-life of purified DNA added to soil was 9–28 h (Lorenz and Wackernagel, 1996), bacterial DNA persisted for weeks in soil (England *et al.*, 1997) and has been detected for up to 3 weeks after cell death (Josephson *et al.*, 1993; Masters *et al.*, 1994). In addition, DNA added to soil and water retains its ability to transform recipient bacteria (Nielsen *et al.*, 2000), indicating both functional and physical stability. Consequently, DNA of a specific microbe may be detected long after the organism has died (due to disinfection or other environmental stresses), or ceased to contribute to the overall activity of an ecosystem. In contrast, RNA is degraded reasonably quickly, and is generally considered to be more labile than DNA. However, poliovirus RNA was stable, based on detection by reverse transcriptase (RT)-PCR, for up to 21 days in filter sterilized seawater, but could not be detected after 2 days in unfiltered seawater (Tsai *et al.*, 1995).

Fluorescence *in situ* hybridization (FISH) targeting 18S rRNA was useful for identifying *Cryptosporidium parvum* oocysts (Alagappan *et al.*, 2008), and was proposed as a species-specific viability assay for *C. parvum* (Vesey *et al.*, 1998). However, 18S rRNA was detectable by PCR for at least 11 weeks following inactivation of *C. parvum* oocysts at 65°C for 15 min (Widmer *et al.*, 1999), demonstrating the unsuitability of rRNA as a target for viability in this organism. More recently, it was concluded that the extended half-life of *C. parvum* 18S rRNA results in over-estimation of oocyst viability (Smith *et al.*, 2004). The authors recommended treating oocysts with RNase prior

to permeabilization to eliminate rRNA from recently killed and damaged oocysts, thereby reducing the occurrence of FISH false-positives; the FISH signal of intact oocysts was not affected by RNase treatment. However, the utility of rRNA in general as a marker of viability has been questioned because it may persist for long periods in dead cells. Although FISH allows direct visual observation and enumeration of target organisms, its use for determining microbial viability is limited by (i) relatively complicated and time-consuming sample processing procedures, (ii) lack of sensitivity with single-copy messenger RNA (mRNA) targets limiting target selection to multi-copy rRNA genes, (iii) signal amplification techniques to increase sensitivity with mRNA targets, adding to the cost and complexity of analyses, (iv) stability and persistence of rRNA in dead cells, and (v) additional procedures to circumvent rRNA stability in dead cells that do not perform consistently and add further complexity to the method. Consequently, RT-PCR targeting mRNA has become the preferred culture-independent method for assessing microbial viability.

Messenger RNA generally has a shorter half-life than rRNA, and consequently mRNA is perceived to be a better indicator of viability (King and Schlessinger, 1987). The average half-life of *E. coli* mRNA was one minute based on decay of isotopically labelled RNA (Baracchini and Bremer, 1987; Conway and Schoolnik, 2003). However, there are differences in reported half-lives for mRNA. Bernstein *et al.* (2002) reported that 80% of *E. coli* transcripts have half-lives of 3–8 min. Different parts of the transcripts are degraded at different rates; sometimes the 5′ end of a mRNA transcript (the end which is made first) is degraded faster than internal sequences. Furthermore, the persistence of mRNA in dead cells depends on how the cells were inactivated and stored following inactivation (Sheridan *et al.*, 1998). Therefore, the choice of target sequences and primers is important when using mRNA as a viability indicator.

Reverse transcriptase (RT)-PCR

RT-PCR assays for mRNA targets have been developed to assess viability in a wide variety of microbes, including pathogens of interest to the water industry. Morin *et al.* (2004) reported the simultaneous detection of viable *E. coli* O157:H7, *Vibrio cholerae* O1, and *Salmonella enterica* Typhi cells using a multiplex mRNA-based assay with PCR amplification of cDNA from specific lipopolysaccharide genes and a flagellin gene. As few as 30 cells of *E. coli* O157:H7 and *Salmonella* Typhi were detected in clinical samples without interference from other bacteria. For ethanol inactivated cells, mRNA of the *Salmonella rpo*D gene was detected 1 and 48 h post treatment when cells were stored at room temperature or 4°C, respectively, but it was undetectable almost immediately in heat-killed cells (Rijpens *et al.*, 2002). While recognizing that RT-PCR of mRNA is useful for live/dead discrimination, the variability between species of mRNA persistence in dead cells and differences between individual genes made it unreliable for assessing viability of *Campylobacter* spp. in food (Sung *et al.*, 2005). An assay targeting *hsp*70 mRNA in *Pneumocystis carinii* detected 100 viable trophozoites, but generated no signal from 10^6 trophozoites that had been inactivated by UV light, heat, or desiccation (Maher *et al.*, 2000) and a quantitative viability assay was developed for *Cryptococcus neoformans* using real-time RT-PCR targeting a capsular gene transcript (Amjad *et al.*, 2004).

Detection of a single viable *C. parvum* oocyst by RT-PCR targeting *hsp*70 mRNA was reported (Stinear *et al.*, 1996), and a similar assay coupled with immunomagnetic separation (IMS) capture detected 10 viable oocysts in 100 l of drinking water (Hallier-Soulier and Guillot, 2003). Rapid decay of β-tubulin mRNA in heat inactivated *C. parvum* oocysts indicated its suitability as a sensitive viability marker with complete reduction in amplification signal within one hour of inactivation (Widmer *et al.*, 1999). In addition, an RT-PCR method targeting mRNA from the *hsp*70, structural giardin, and alcohol dehydrogenase genes of *Giardia* was developed as a viability assay (Maux *et al.*, 2002). Detection of penicillin-binding protein mRNA in *Enterococcus faecalis* correlated with metabolic activity and resuscitation capability, demonstrating the continuing but declining viability of bacterial cells in the viable but non-culturable (VBNC) state (Mar Lleo *et al.*, 2000). Bacteria in the VBNC state were also

studied by RT-PCR targeting house-keeping (rRNA and *rpoS*) and virulence gene transcripts in *Vibrio parahaemolyticus* (Coutard *et al.*, 2005). Only house-keeping genes were expressed by VBNC bacteria, indicating that virulence genes are only expressed by actively growing bacteria.

Approaching the ultimate 'promise' of molecular detection methods, a fully automated RT-PCR viability assay was developed for *E. coli* (Rauter *et al.*, 2003). The system included cell concentration, nucleic acid extraction, and robotic real-time RT-PCR targeting the heat inducible *degP* gene. While the principle has been demonstrated, this and similar systems have not yet reached the level of maturity, reproducibility, and robustness for routine use. Further extending method capabilities, RT-PCR of mRNA from four genes in *E. coli* O157:H7, *Vibrio cholerae* O1, and *Salmonella* Typhi was integrated with a microarray detection platform to demonstrate the potential for high throughput viability screening of multiple pathogens (Liu *et al.*, 2006).

Nucleic acid sequence-based amplification (NASBA)

Although the reverse transcription (RT) step of RT-PCR is specific to RNA, small amounts of DNA can carry through the extraction and RT processes, leading to amplification signal in the absence of target mRNA. Nucleic acid sequence-based amplification (NASBA) is an amplification process that exclusively targets RNA; DNA is not amplified so there is no risk of false positives from extraneous DNA when targeting mRNA. It is an isothermal technique so there is no requirement for thermal cyclers, and it uses three enzymes – reverse transcriptase, RNaseH, and T7 RNA polymerase – and two sequence specific primers, one of which carries a bacteriophage T7 promoter at its 5′ end. The four steps of the technique are: (i) Primer elongation of the attached T7 promoter sequence (primer 1) by the reverse transcriptase, (ii) RNA template degradation by RNase H, (iii) Synthesis of the second strand by elongating primer 2, resulting in a double-stranded cDNA copy of the original RNA sequence, and (iv) Synthesis of RNA by T7 RNA polymerase, which binds to the T7 recognition site of primer 1 (see Chapter 1 for a diagrammatic representation). Repetition of these steps, as with multiple PCR cycles, increases the amount of product, with greater than 10^8-fold amplification reported (Compton, 1991).

Because the process is isothermal, and normally performed at 40 ± 1°C, genomic DNA from the target microorganism remains double-stranded and cannot serve as a template for amplification (Cook, 2003). It also eliminates the necessity for DNase treatment (which is essential for RT-PCR-based detection of RNA targets). Another advantage is that because NASBA yields single-stranded products, they are directly available for hybridization-based detection without denaturation.

NASBA-based methods have been used to detect bacterial pathogens including *Campylobacter* spp., *Listeria monocytogenes, Salmonella enterica,* and *C. parvum* (Esch *et al.*, 2001; Simpkins *et al.*, 2000). For example, a NASBA assay for detection of viable *Salmonella enterica* was based on amplification of mRNA transcribed from the *dnaK* gene, which encodes the bacterial chaperone hsp70 protein (Simpkins *et al.*, 2000). Heat exposure (65°C for 5 min) resulted in an 86% reduction in NASBA signal. Signal from viable cells was eliminated by RNase treatment, while PCR amplification of RNase-treated and untreated samples was not affected, indicating that the assay was blind to background DNA. A review by Cook (2003) noted that a NASBA signal was sometimes obtained from pasteurized *Salmonella* spp. if the RNA was extracted directly after heat treatment. This was remedied if the cultures were held for 15 min before RNA was extracted. NASBA, coupled with an electrochemiluminescence (ECL) detection assay, was used for detecting viable *E. coli* cells in drinking water. Incubation for 5–40 min at 41–50°C induced mRNA synthesis from the heat shock gene *clpB*, which was detected by NASBA-ECL (Min and Baeumner, 2002). The assay was specific to only cells that could respond to elevated temperatures so were deemed to be viable. The method detected 40 viable *E. coli* cells/ml in drinking water in 4 h, and there were no false-positive signals from dead cells. This approach was modified to replace ECL detection with a liposome-based, dipstick format, biosensor that relied on a membrane-bound

oligonucleotide capture probe (Baeumner *et al.*, 2003; see Chapter 1 for more details). The assay was again specific for viable *E. coli* cells, although only extreme heat (98°C for 15 min) was used for the killed controls. Other inactivation methods such as chlorine disinfection, ultraviolet light, and more moderate heat regimes need to be evaluated to ensure the method consistently differentiates between live and dead cells. This kind of isothermal mRNA amplification coupled with biosensor technology offers the potential of rapid detection of viable organisms with a field-portable instrument (Baeumner *et al.*, 2003). The same approach was used for detection of *Bacillus anthracis* with a reported sensitivity of a single viable spore (Hartley and Baeumner, 2003). Liposome-based reporting was also used for a NASBA method that detected viable cells while increasing sensitivity (Esch *et al.*, 2001). The method used a microfluidic chip to detect NASBA-amplified mRNA produced by heat shocked or viable *C. parvum* oocysts. The lower limit of detection was 10 oocysts. Each liposome contained up to 10^5 molecules of a fluorescent dye, which increased sensitivity 1000-fold compared to a single fluorescent molecule.

These promising studies illustrate that NASBA-based methods have the potential for detecting viable cells. However, as with all methods, there are limitations and cautions. The persistence of mRNA may vary significantly based on environmental conditions, the cause of cell death, and the cell's physiological state before death (Birch *et al.*, 2001). NASBA signals from heat-killed *E. coli* O157:H7 cultures could be detected for up to 30 h after exposing cells to 65°C for 30 min. In another study, the persistence of the *fliC* transcript (30 h) was significantly greater than for the *rfbE* transcript (7 h), with an explanation that different transcripts (or regions of transcripts) have different susceptibilities to degradation (Alifano *et al.*, 1994; Norton and Batt, 1999). This highlights the importance of selecting appropriate NASBA primers. It is also feasible that RNA expression in viable cells may depend on their physiological status (Barer and Harwood, 1999). Organisms that are stressed or VBNC may have RNA levels below the detection threshold. Therefore, NASBA primers that target mRNA transcripts of constitutively expressed genes in all physiological states are recommended (Deiman *et al.*, 2002).

The NASBA method can be made quantitative using a method similar to the Ct approach used in qPCR. With accumulating RNA amplicons detected by molecular beacons, a mathematical model was developed based on initial template concentrations of HIV-1 virus (Weusten *et al.*, 2002). The model described the fluorescence curve as a function of amplification time and relevant kinetic parameters. The curves have a linear component that can be used to quantify the target RNA by comparing the amplicon formation rate relative to a RNA calibration curve. El Galil *et al.* (2005) used a quantitative real-time NASBA assay using molecular beacons to detect hepatitis A virus with a detection limit of 1 PFU. The assay detected as few as 10 PFU from spiked lake water samples when combined with immunomagnetic separation.

Comparing NASBA and RT-PCR

In some respects, NASBA is simpler than RT-PCR for detection of specific RNA targets. It exclusively amplifies RNA so there is no concern of false-positives from extraneous DNA, and it is isothermal. However, developing and optimizing a NASBA assay for detection of expressed mRNA in environmental samples can be just as challenging as working with RT-PCR. There are at least four published studies comparing NASBA with RT-PCR for viruses or bacteria. NASBA was 10–25 times more sensitive than RT-PCR and detected hepatitis A virus in inoculated samples of wastewater, lettuce, and blueberries (Jean *et al.*, 2001). The specificity and detection limits of the NASBA depended on the primers and assay conditions. In a comparison of a real-time NASBA assay with conventional RT-PCR for detecting noroviruses in large volume river water samples, the inhibitors that reduced the efficiency of RT-PCR were not a factor in NASBA detection (Rutjes *et al.*, 2006). NASBA was also more sensitive than PCR and RT-PCR in an assay detecting *E. coli* O157 RNA (Birch *et al.*, 2001). El Galil *et al.* (2005) showed that NASBA had a much higher inherent amplification capability, and decreased detection time compared to RT-PCR (~ 100 min for NASBA vs. ~ 180 min for TaqMan® RT-PCR).

It is possible that the specificity of NASBA and other isothermal amplification methods may be reduced compared to RT-PCR because of the lower amplification temperature of ~40°C (Notomi *et al.*, 2000). NASBA has not been widely applied to environmental samples so it is possible that method limitations and problems have not yet been identified. Although some reports indicate that NASBA is less sensitive than RT-PCR to inhibition, further research into potential inhibitors is warranted since the technique involves three nucleic acid processing enzymes. In addition, methods for enhancing amplification would be useful.

The studies cited above demonstrate that, although targeting mRNA is a useful tool for monitoring microbial viability, suitable target selection is critical to its successful application. Even with an appropriate target, the method may not provide absolute discrimination between live and dead cells because mRNA persistence may depend on the type of stress to which an organism is exposed and various physical and biological properties of different species. Although NASBA does not suffer from false-positives due to DNA carryover, these same limitations apply regardless of the amplification method because they are biology-dependent rather than assay-dependent. Viability interpretations are further complicated by cells that are in the gray zone of dying or barely surviving, rather than the absolutes of (i) alive and healthy or (ii) dead.

Assessing potential infectivity of viruses

A variety of methods have been developed to assess or infer the infectivity of viruses, both with and without the use of cell culture. Receptor-mediated attachment of virus capsid binding sites to specific host cell receptors is the first step in the virus infection cycle, so structural integrity and biological function of virus capsids is critical to virus infectivity. Therefore, assuming that an intact and functional capsid is a prerequisite for successful virus infection, methods have been developed to capture viruses by targeting capsid components prior to release of nucleic acids and detection by PCR. Viruses with damaged capsid components will not be infectious, will not be captured, and so will not be detected by PCR. This approach was developed for coxsackievirus B3 using magnetic beads coated with soluble coxsackievirus-adenovirus cellular receptor (CAR) to capture virus particles (Cromeans *et al.*, 2004). Following release from the magnetic beads and RNA extraction, specific virus RNA was detected by RT-PCR. The method did not detect coxsackievirus B3 that had been exposed to chlorine disinfection at a CT of 10 mg · min/l. Similar approaches relied on pre-PCR capture of viruses by attachment to cell monolayers, or magnetic beads coated with anti-virus antibody targeting intact capsid antigens (Nuanualsuwan and Cliver, 2003). However, there are conflicting reports on the ability of immunocapture to differentiate between live and inactivated viruses, particularly for UV disinfection. Because its mode of action is primarily focused on nucleic acids, UV may not change the antigenic properties of a virus (Wang *et al.*, 1995).

Protease pretreatment of viruses degrades capsids that have been damaged by disinfection or environmental stresses, but does not affect intact capsids. Subsequent incubation with nucleases degrades the now exposed nucleic acids so that they cannot be amplified by PCR. The premise of the method is that non-infectious viruses often have damaged protein capsids, which are more susceptible to protease-mediated degradation than the intact capsids of infectious viruses. This approach was used to differentiate between intact viruses and those that had been inactivated and damaged by disinfection (Nuanualsuwan and Cliver, 2002). For RNA viruses, RNase is added to the pretreated samples. If the capsid is compromised, RNase will diffuse into the virus particle, degrade the viral RNA, and exclude these particles from subsequent molecular analysis using RT-PCR. The method has been tested with several viruses including coxsackievirus, echovirus, norovirus, and poliovirus.

Based on the assumption that viruses with degraded nucleic acids probably have damaged capsids, and are therefore not infectious, direct PCR approaches have been developed that take advantage of the easily degraded 5′ and 3′

non-coding regions of viral genomes or target long fragments of the genome (Bhattacharya *et al.*, 2004; Allain *et al.*, 2006). The 5′ non-translated region (NTR) of the hepatitis A virus genome is the first and most easily degraded region upon exposure to chorine disinfection (Bhattacharya *et al.*, 2004). Thus, reduced or lack of amplification signal in a 5′ NTR-specific assay can be inferred to result from genome degradation, translating to loss of infectivity. Reduced amplification of the 5′ NTR correlated with the loss of infectivity in cell culture (Li *et al.*, 2002). Various approaches to amplification of long target regions (LTR-PCR) have also been tried assuming that an intact viral genome is a requirement for infectivity. Nucleic acid damage is more readily detected as the size of the analysed region increases. When a poly(T) primer is used for reverse transcription of the poliovirus genome, priming off the 3′ poly(A) region of the single-stranded RNA genome, followed by PCR targeting the 5′ NTR, most of the genome must be transcribed to cDNA to provide the target sequence for successful PCR (Fig. 8.2). This indicates that the genome is complete and intact. Using this approach to analyse 93% of the poliovirus genome, there was a 3-log reduction in the level of amplifiable genome after chlorine dioxide disinfection (Simonet and Gantzer, 2002). However, this approach has not been widely used, and its correlation with infectivity in cell culture has not been evaluated thoroughly.

The replication of viruses in their host cells varies with each type of virus, and the methods described above for inferring virus infectivity are not appropriate for all viruses. Consequently, it is unlikely that direct PCR approaches such as 5′-NTR, LTR-PCR, or PCR following capsid digestion will be completely reliable or satisfactory for assessing infectivity and inactivation of all relevant viruses (Rodriguez *et al.*, 2009). Combining *in vitro* cell culture with PCR, or alternative amplification techniques, is currently the best approach for assessing the infectivity of viruses. However, norovirus is very important from a public health perspective, but cannot be grown using traditional cell culture techniques.

Combining culture and molecular methods

Cultural enrichment of bacteria in selective media is typically performed to increase the amount of 'signal' by the growth of viable target cells. The

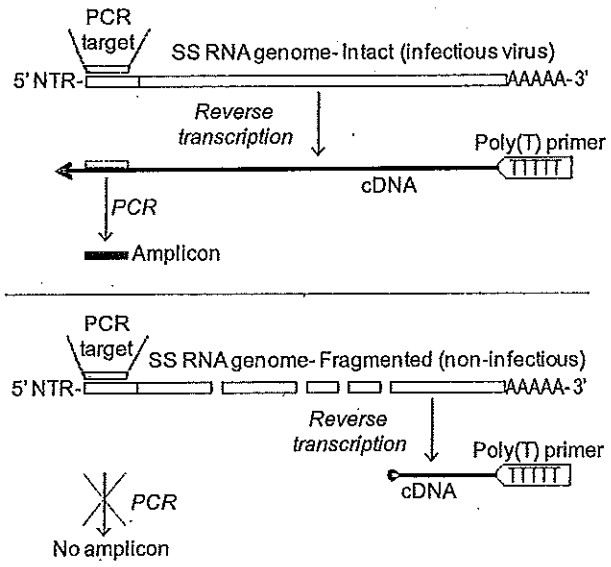

Figure 8.2 Schematic of long target region (LTR)-reverse transcriptase (RT)-PCR for detecting polioviruses with intact single-stranded (SS) RNA genomes, which are presumed to be infectious.

appropriate choice of enrichment conditions may resuscitate injured or stressed bacteria (Clark and Ordal, 1969; Jameson, 2008) or, if desired, suppress their growth (Clark and Ordal, 1969; Chen *et al.*, 1993). If the 'signal' is metabolic function detected with phenotypic/biochemical tests, incubation times are often several days. This time can be significantly reduced if molecular methods are used as the downstream analytical tool because the only requirement to prove viability is that the molecular signal has increased during the incubation period.

There are several instances where molecular methods have been combined with enrichments. PCR-hybridization analysis after enrichment worked as well as, or better than, traditional bacteriological methods for detecting *Salmonella* serotypes in porcine faecal and water samples (Feder *et al.*, 2001). In addition to increasing sensitivity by cell growth, there was the added benefit of dilution of PCR inhibitors. The agreement between *Salmonella* serotype detection using cultivation with pre-enrichment/PCR detection (samples taken after an overnight enrichment) was 76%. In another study, Mozina *et al.* (2005) showed that enrichment combined with PCR shortened the detection time for *L. monocytogenes* from 96–120 h for cultivation alone to 30–72 h for enrichment-PCR. This study also illustrated that the choice of the enrichment medium was critical because only one of four media was reliable. Enrichment coupled with multiplex PCR and microarray detection of pathogenic vibrios in oyster homogenates had a sensitivity of 1 CFU per gram (Panicker *et al.*, 2004), while culture combined with real-time PCR detected *E. coli* O157: H7 at a concentration of 50 CFU in 40 l of surface water (Mull and Hill, 2009).

These studies show the usefulness of coupling molecular methods with traditional enrichment culturing. Although the approach is not quantitative, it has the advantages of detecting viable target organisms from an environmental sample, improving sensitivity, and removing inhibitory substances. A disadvantage is that culturing conditions for the target organism must be available. Another limitation is that enrichment may not resuscitate injured or VBNC cells that could be outcompeted during enrichment and, therefore, excluded from detection (Maciorowski *et al.*, 2005). It is also problematic to enrich for more than one target organism in a single culture.

Cell culture combined with molecular detection of infectious viruses

Determining the infectivity of difficult to culture or non-culturable viruses is necessary for assessing their survival in environmental waters, their susceptibility to disinfection, and their risk to public health. Viruses in environmental samples have traditionally been detected using *in vitro* cell culture. However, many viruses are difficult to culture, very slow growing, do not produce cytopathic effects (CPE), or do not replicate in the cell lines typically used (e.g. norovirus). In addition, it can take weeks to generate a cell culture result. Therefore, PCR and other molecular methods have been used to decrease analysis time and widen the variety of viruses that can be detected. However, traditional PCR is limited by its inability to differentiate between infectious and non-infectious viruses. Cell culture combined with a PCR-based detection step (integrated cell culture-PCR, ICC-PCR) can reduce detection time from weeks to a few days, but is still limited to those viruses that can replicate in the particular cell line.

In ICC-PCR, detection of infectious viruses depends on an initial biological amplification (replication) of viral nucleic acids followed by PCR-mediated *in vitro* amplification. This approach can detect viruses that replicate but do not produce CPE, but may also detect high titres of a virus that remain on the cells without initiating an infection. It has an added advantage of often overcoming PCR inhibition because the interfering substances present in the environment are diluted to subinhibitory concentrations by the cell culture procedures and media. Sensitivity is increased because the pathogen replicates in the host cell line.

ICC-PCR has been used to detect infectious enteroviruses, hepatitis A virus, adenoviruses, reovirus, and astroviruses. Reynolds *et al.* (1996) detected infectious polioviruses in distilled water and enteroviruses in primary sewage effluent within 24 h of culturing. Lee *et al.* (2004) detected infectious adenoviruses and enteroviruses

from river water using two different cell lines and reverse transcription/multiplex PCR. Detection of infectious adenoviruses in environmental samples increased from 29% when tested with conventional cell culture to 50% using ICC-PCR (Greening *et al.*, 2002). A potential drawback of ICC-PCR targeting viral DNA is false-positive detection of nucleic acid from inactive viruses that adhere to the cell monolayer but do not initiate an infection. This can be circumvented by ICC-RT-PCR targeting viral mRNA, which is only produced by infectious viruses that are actively transcribed during replication (Ko *et al.*, 2003).

Definitive evidence of virus infection in cell culture is provided by detecting nucleic acids that are only produced once the virus starts to replicate. Single-stranded positive-sense RNA viruses such as enteroviruses, coxsackieviruses, norovirus, and hepatitis A virus produce a double-stranded RNA replicative form (RF) in the early stages of replication. This RF is only produced during infection of suitable host cells, so its presence indicates an active infection. Following RNA extraction and DNase and RNase digestion to remove DNA and single-stranded RNA, respectively, RF-RNA is detected by RT-PCR with primers selected to be specific for positive- and negative-strand RF detection. This method was applied to coxsackievirus B3 and hepatitis A virus inoculated into BGM and FRhK-4 cells, respectively, with a reported sensitivity of four infectious units detected within 24 h of cell inoculation (Cromeans *et al.*, 2004). The method was specific for infectious viruses, and did not detect RF from viruses inoculated onto cell monolayers following chlorine disinfection. Replicative forms of wild-type viruses recovered from sewage were detected long before CPE developed, further demonstrating the utility of the method.

Detecting infectious *Cryptosporidium*

Cell culture combined with molecular methods for detecting infection has also been applied to *Cryptosporidium* spp. These techniques are highly specific and sensitive and, because they have been adapted to 96-well formats, can be used to screen a large number of samples. There have been many outbreaks of cryptosporidiosis associated with either drinking water or recreational use of water; the largest waterborne outbreak to date occurred in 1993 in Milwaukee infecting up to 400,000 individuals (MacKenzie *et al.*, 1994). The continued detection of *Cryptosporidium* oocysts in source waters, and occasionally in treated drinking water, makes the organism a cause of significant concern for the water industry, and federally-mandated monitoring under the Long Term 2 Enhanced Surface Water Treatment Rule will determine whether water utilities need to install additional treatment based on the level of *Cryptosporidium* in their source water (U.S. EPA, 2006). An assay using RT-PCR to amplify *C. parvum*-specific mRNA from a region of the *hsp*70 gene was used to detect infection in Caco-2 and HCT-8 cells (Rochelle *et al.*, 1997). The assay was also used to measure the efficiency of UV inactivation of *C. parvum*, and generated inactivation results that showed very close agreement to published mouse-derived data (Mofidi *et al.*, 2001; Rochelle *et al.*, 2004). In extensive evaluations with five isolates of *C. parvum*, the cell culture-RT-PCR assay was equivalent to the widely used CD-1/ICR mouse assay for measuring the infectivity of untreated *C. parvum* oocysts (Rochelle *et al.* 2002). In addition, the assay was used to demonstrate that HCT-8 cells supported infection by the human-specific pathogen *C. hominis* (Rochelle *et al.*, 2002). Using HCT-8 cells and PCR detection targeting *C. parvum hsp*70 DNA, 4.9% of raw water samples and 7.4% of filter backwash samples were found to contain infectious *C. parvum* (Di Giovanni *et al.*, 1999). The sensitivity of this assay was less than five infectious oocysts. The same method also detected infectious oocysts in 3.9% of untreated source waters (n=560, LeChevallier *et al.*, 2003) and 1.4% of treated drinking water samples (Aboytes *et al.*, 2004). Cell culture combined with real-time qPCR was used to evaluate the disinfection efficacy of *C. parvum* oocysts (Keegan *et al.*, 2003).

Although RT-PCR and PCR-based infection detection methods can be used to quantify the level of infection, either by qPCR (Keegan *et al.*, 2003; Di Giovanni and LeChevallier, 2005) or per cent infectivity converted to a logit response (Rochelle *et al.*, 2002), absolute quantification requires visualization of the infection. Therefore, a

colorimetric *in situ* hybridization (CISH) method was developed that provides the specificity and sensitivity of molecular assays with direct microscopic enumeration of infections (Rochelle *et al.*, 2001). The oligonucleotide probe conjugated to alkaline phosphatase was specific to *C. parvum* rRNA, and converted substrate to a coloured precipitate at the sites of hybridization to each life-cycle stage of the parasite within the cell monolayer (Fig. 8.3). This allowed for quantification of infections by enumerating the number of infectious foci (clusters of individual life stages that develop from a single oocyst) and the number of life stages per foci.

Assessing functional gene expression and microbial activity

Although DNA-based methods, including microarrays, have been used to assess the occurrence of functional genes in environmental samples, and their relative abundance in contaminated and uncontaminated environments (Wu *et al.*, 2001; He *et al.*, 2007; Yergeau *et al.*, 2007), interrogation of whole community DNA assesses only the phenotypic potential of a community. In some cases, significant correlations between enzymatic activity in soil (laccase and cellulase) and microarray data indicated that the detected genes were also expressed in soil (Yergeau *et al.*, 2007). However, to determine the genes that are actually being expressed by viable microbes in natural microbial communities, and therefore contributing to biological activity within that particular ecosystem, it is necessary to detect mRNA of functional genes. By probing community mRNAs, community level gene expression can be measured. Gene expression, functionality and activity measurements are critical to establishing the link between microbial diversity, viability, and ecosystem functions. Increased knowledge and understanding of gene expression patterns in microbial communities, and the role of environmental factors in the up- or down-regulation of metabolic activity, could benefit the water and wastewater industries by improving processes that depend on microbial activity or providing enhanced control measures. These include biofiltration in drinking water treatment to remove biodegradable organic matter (a source of nutrients that can support bacterial growth in the distribution system), activated sludge processes, biodegradation of pollutants such as perchlorate, and prevention of nitrification in treated water reservoirs.

Functional gene microarrays can potentially assess the expression of the majority of functional genes and, hence, the viability of organisms in a particular microbial community. Dennis *et al.* (2003) demonstrated the feasibility of a bacterial catabolic/metabolic gene microarray for detecting and comparing gene expression levels in waste waters. However, the concentration of RNA extracted from many environmental samples is typically too low to be useful for direct microarray probing. Therefore, mRNA amplification is

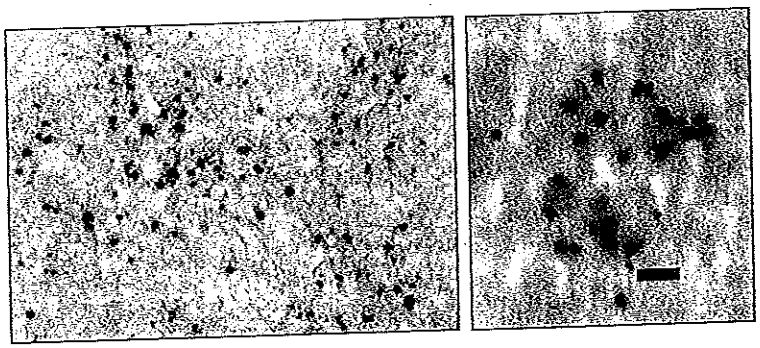

Figure 8.3 Detection of *Cryptosporidium parvum* life cycle stages (black dots) in HCT-8 cell culture by colorimetric *in situ* hybridization using the method described by Rochelle *et al.* (2001). Scale bar in right panel is 10 µm.

required. Whole community RNA amplification (WCRA) preserves the relative abundance of each mRNA in a community so that they can be quantified by hybridization to functional gene microarrays (Moreno-Paz and Parro, 2006; Gao *et al.*, 2007). In WCRA, a T7 promoter sequence is attached to a short random sequence oligonucleotide primer, which is then used for reverse transcription of RNAs extracted from an environmental sample. The resulting double-stranded cDNAs are used as templates for linear RNA amplification with T7 RNA polymerase to achieve up to 1800-fold amplification of community mRNAs, followed by synthesis of fluorescently labelled cDNA, and finally hybridization to a microarray containing probes specific for functional genes (Fig. 8.4). Although the various cDNA synthesis and transcription steps use random primers, the length of the random primers used for first strand cDNA synthesis (indicated with an asterisk in Fig. 8.4) is critical in limiting representative bias in the amplified RNA. Primers with 6–9 nucleotides have been used (Moreno-Paz and Parro, 2006; Gao *et al.*, 2007), but 6-mer

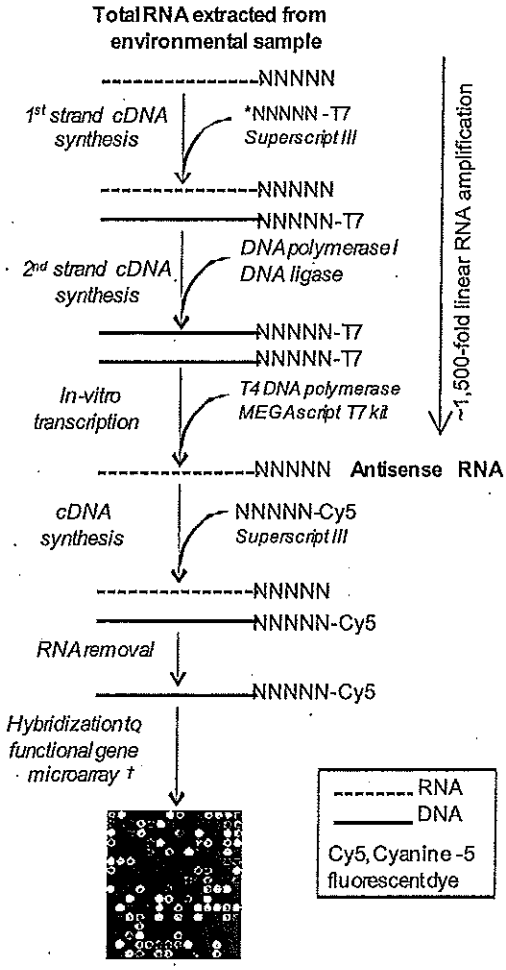

Figure 8.4 Whole community RNA amplification to ascertain the functional genes that are expressed by microbial communities in the environment (adapted from procedures described by Gao *et al.*, 2007). *NNNNN-T7: Random oligonucleotide primer linked to a T7 promoter (T7 promoter sequence: AATTGTAATACGACTCACTATAGGG). †Functional gene microarrays contain probes for genes involved in carbon, nitrogen, and sulfur cycling, organic contaminant degradation, metal resistance and reduction.

oligonucleotides amplified mRNAs with the closest relative abundance to the original community mRNA (Gao *et al.*, 2007). The WCRA method was applied to the active microbial populations in contaminated groundwaters. A total of 39 positive hybridization signals were obtained on the microarray (out of 2006 probes in total), most of them corresponding to genes involved in nitrate and sulfate reduction, and degradation of various organic compounds (Gao *et al.*, 2007).

Gene expression in the bacterioplankton community of two aquatic saline environments was assessed by extracting total RNA, enriching for mRNA by subtractive hybridization of rRNA, reverse transcribing mRNA using random primers, and amplifying cDNA to produce a clone library (Poretsky *et al.*, 2005). The authors calculated that 2.4×10^{13} bacterial mRNA were present in 1l of water, of which 80,000 were unique, representing 200 bacterial species. Although the majority of recovered sequences were not full length transcripts (most were ~200–500 bp), they represented a relatively diverse range of expressed genes for various housekeeping functions, energy metabolism, transport systems, sulfur oxidation, assimilation of C1 compounds, and nitrogen acquisition. Expression of these genes indicated that organisms with these capabilities were viable and metabolically active either at or shortly before the time of analysis.

Another approach to assessing the functionality of microbial communities involves stable isotope probing (SIP) and microautoradiography. When coupled with FISH targeting rRNA genes, the technique reveals the phylogenetic diversity and spatial organisation of microbes that actively incorporate the radioactive label and are, therefore, actively metabolizing (Lee *et al.*, 1999). The sensitivity of FISH is limited by the low copy numbers of most functional genes and their associated mRNA molecules. Consequently, techniques such as tyramide signal amplification (TSA) and primed *in situ* amplification (PRINS) have been incorporated into FISH protocols to increase sensitivity (Kenzaka *et al.*, 2005; Kubota *et al.*, 2006).

The GeoChip is a microarray containing 24,243 oligonucleotide probes, covering approximately 10,000 genes, and representing over 150 functional gene families involved in nutrient cycling, metal reduction and resistance, and degradation of organic contaminants (He *et al.*, 2007; described in detail in Chapter 11). Linking such microarrays with SIP may allow the active functional groups in a community to be identified.

Although they are potentially very powerful tools for assessing microbial activity in the environment, transcriptomic studies in environmental samples have so far been limited (Maron *et al.*, 2007). This is due to the short half-life of many RNAs, co-extraction of PCR-inhibiting humic acids from environmental samples, differential transcription kinetics of similar genes in different populations, and relatively low correlation between RNA levels and synthesis of corresponding proteins.

Beyond nucleic acids: detecting and analysing proteins

Detecting specific protein-encoding genes within an environment's metagenome provides occurrence and diversity data, and defines the phenotypic potential of that community, or at least the phenotypic potential of individuals within the community. Detecting mRNA reveals which genes are being expressed and which functional proteins are probably being produced by viable organisms – the expressed phenotypic potential. However, different levels of functional activity may depend on post-translational modification of proteins rather than changes in abundance of gene transcripts. Therefore, detecting the products of these expressed genes (their corresponding proteins) provides the greatest confidence that characterization of a community's metabolic activity is accurate. Production of proteins encoded by functional genes provides insights into the viability status of the community, and can be considered as fulfilling the phenotypic potential of the community.

Methods have been developed for detecting and characterizing proteins in environmental samples, although they have not yet been widely adopted. While protein-based methods have been

developed to study individual functions (e.g. mercury resistance; Ogunseitan, 1998), greater value in terms of assessing functional activity within ecosystems is derived from a metaproteomics approach. The large-scale study of the entire protein complement of microbial communities in the environment is an important component in developing a full understanding of functional activity in ecosystems. Following extraction from environmental samples, proteins are analysed and identified by 1-D and 2-D gel electrophoresis, liquid chromatography, chromatographic-capillary electrophoresis, mass spectrometry, and protein microarrays or protein-chips (Maron *et al.*, 2007). As with nucleic acid-based methods, extraction is the most critical step for successful subsequent analyses. Proteomic studies have been conducted on microbial biofilms, fresh and ocean waters, contaminated groundwater, soil, wastewater treatment reactors, and activated sludge (Ogunseitan, 1996; Wilmes and Bond, 2004; Ram *et al.*, 2005;Benndorf *et al.*, 2007; Lacerda *et al.*, 2007).

However, as with all of the '-omics' approaches when applied to environmental samples, protein extracts are often contaminated with interfering compounds, making protein characterization difficult (Maron *et al.*, 2007). Direct lysis of environmental samples recovers proteins from bacteria, fungi, protozoa, and multicellular organisms, which can complicate interpretation of protein analyses.

Sample processing considerations

One of the most critical issues with detecting microbes in water is their low concentrations, which necessitates various concentration methods. These include filtration, size fractionation, centrifugation, immunomagnetic separation, or combinations of these methods. A detailed discussion of sample processing methods is provided in Chapter 2. The method selected depends on the type of organism, their relative abundance, and the sensitivity of the downstream detection method. Typically, concentration must reduce samples from many litres down to millilitre or even microlitre volumes. One of the most significant effects is the concomitant concentration of compounds that interfere with molecular detection methods. Common inhibitors are humic substances, polysaccharides, heavy metals and urea. Humic substances are a significant problem because they have physical and chemical properties similar to nucleic acids, co-extract with DNA (Lakay *et al.*, 2007), and interfere with PCR even at trace amounts (Tsai and Olson, 1992). Humic substances also reduce adsorption efficiency of viruses (Sobsey and Hickey, 1985; Tsai and Olson, 1992) and compete with DNA on purification columns (Harry *et al.*, 1999).

The goals of efficiently removing all inhibitors, while at the same time avoiding loss of nucleic acids, may seem to be mutually exclusive. Additional purification steps often reduce the nucleic acid yield and increase shearing. Some purification methods result in substantial loss of target and may lead to significant underestimation of target quantities (Moreira, 1998). Difficulties can arise in any of the sample processing steps, which include cell lysis, nucleic acid extraction, and purification of the released nucleic acids. Published protocols may be appropriate for specific applications, but a universal technique for preparing high-quality DNA or RNA from a range of environmental samples is not yet available.

To avoid the bias of differential response to lysis by microbial cells, lysis techniques should be adequate to disrupt all target organisms without degrading the nucleic acids. Issues associated with cell lysis have been summarized by Schneegurt *et al.* (2003). An additional source of bias is nucleic acid extraction techniques. Ogram *et al.* (1995) used three methods for RNA extraction from oligotrophic environments. Two methods had extraction efficiencies of approximately 6%, and the third was 26.4%, demonstrating a significant impact of method on recovery. This study also demonstrated that even the most effective method left approximately 75% of the signal undetected.

One of the most useful techniques for separating specific microbes from environmental sample debris prior to DNA or RNA extraction is IMS. Target organisms are captured on the surface of antibody coated magnetic beads, which are retained by magnets during wash steps. IMS is compatible with a wide spectrum of downstream diagnostic methods including bacterial and cell culture, and nucleic acid and protein-based

detection techniques. IMS normally preserves the physiological status of the captured organisms, so that viability can be assayed, and because IMS requires a specific recognition event, it adds a layer of specificity to detection. Numerous studies show increased assay reliability, greater sensitivity, and improved inhibitor removal using IMS for sample purification prior to nucleic acid extraction (Myrmel *et al.*, 2000; Hwang *et al.*, 2007). For example, in a study using PCR to detect *Giardia muris* cysts, DNA extracted from seeded river water concentrates with high turbidities (~2x 10^5 Jackson turbidity units) failed to amplify. Inhibition was overcome when IMS was used to isolate the cysts from these same turbid samples (Mahbubani *et al.*, 1998). The method has already been incorporated into microscopic methods for detection of *Cryptosporidium* oocysts and *Giardia* cysts in water (U.S. EPA, 2005). However, IMS is limited to studies of single or relatively few organisms due to the relatively narrow specificity of antibodies attached to the magnetic beads. Typical IMS products and procedures target whole individual organisms and, consequently, do not lend themselves to meta-approaches. However, following lysis of cells in environmental samples, IMS can be used to purify nucleic acids using non-specific DNA- or RNA-targeted antibodies, or random primers conjugated to magnetic beads.

Future directions

Methods to assess the viability of microbes in the environment continue to evolve as detection techniques are advanced. However, the application of all molecular methods to environmental samples, whether they focus on DNA, RNA, or proteins, requires high quality target molecules. While this is currently achieved with many types of environmental sample, it is not universal and many investigations stall due to poor recovery, inefficient amplification, sample biases, or difficult interpretation of results. Therefore, there is still a need for improved sample processing and extraction techniques. Microfluidics and lab-on-a-chip type technologies may provide some of these improvements, but the sample volumes that can be handled by these systems are even smaller than those that current technology uses, so existing problems may be compounded as the drive towards miniaturization continues. As interest in determining viability continues, driven by the need to improve risk assessments and evaluating the efficacy of disinfectants, there is likely to be increased use of real-time PCR and other amplification techniques to quantify mRNA in environmental samples. This will necessitate improved correlations between the detected markers of viability and actual viability as determined by culturing.

Microarray screening has become an accepted technology for monitoring changes in gene expression in many areas of the biological sciences. However, microarray technology for pathogen detection is still in a stage of dynamic development (Bodrossy and Sessitch, 2004). Early technical difficulties such as printing defects, high background and low signals have mostly been overcome. Lemarchand *et al.* (2004) and Bodrossy and Sessitch (2004) comprehensively reviewed microarrays in terms of pathogen detection, immobilization chemistries, technical problems, and requirements for sample preparation, and Chapters 5 and 11 in this book are dedicated to the application of microarrays. Addressing viability with microarrays adds another level of complexity; combining PMA and PCR ahead of hybridization, or targeting mRNA, provide options for high throughput array-based viability assays. The enormous multiplexing capability of microarrays offers the potential of simultaneous monitoring of multiple microorganisms, including non-culturable ones, and viability assessment.

Considering some of the difficulties in analysing relevant volumes of sample in single tube assays, automated platforms that can analyse large volume samples split over many assay 'tubes' may be adventitious. The Biodetection Enabling Analyte Delivery System (BEADS) automates the concentration and purification of target analytes and reduces interference (Straub *et al.*, 2005). It can use IMS or an RNA-based method. The IMS-DNA-Luminex® version uses IMS to selectively concentrate target cells. DNA from captured cells is PCR-amplified, using labelled primers and amplicons, are bound to colour-coded Luminex®-microspheres with distinct capture probes on their surfaces. Beads are passed through a flow cytometer, which both recognizes

the bead colour code and detects and quantifies the PCR amplicons bound to the bead surfaces. This BEADS version successfully purified and amplified ten *E. coli* O157:H7 cells from river water samples. Multiplexing capability was tested for simultaneous detection of *E.coli* O157:H7, *Salmonella*, and *Shigella*. The detection limit was 100 cells for each organism. The RNA version uses purified and directly labelled sample RNA. The RNA is hybridized to a planar microarray for detection. The RNA approach limits detection to viable cells, but the lack of an amplification step limits sensitivity. The detection limit was 5–40 ng of mRNA (or 10 μg of total RNA). Transcripts from inducible genes (e.g. heat- or cold-shock genes) were thought to be good candidates for improving detection limits. Complete automation of the RNA version may be achieved by switching from a planar (surface) to a suspension array.

Cepheid, Inc. (Sunnyvale, CA) has commercialized a fully automated system utilizing qPCR to amplify and detect target DNA (GeneXpert®). The platform integrates all the steps required for PCR-based DNA testing (sample preparation, DNA amplification, and detection), and provides PCR test results in approximately 30 min. A test cartridge for *Bacillus anthracis* is available, and cartridges for other targets are in development (Lim *et al.*, 2005). The United States Postal Service in Baltimore, MD is using a pilot system designed for sorting centres. Roche Applied Science (Indianapolis, IN) provides a system that combines nucleic acid purification and PCR in one instrument (MagNA Pure LC). The instrument has been used to purify nucleic acids from serum, whole blood, stool samples, urine and swab specimen, tissue, sputum, cerebrospinal fluid, synovial fluid, ticks and powders (Lim *et al.*, 2005). Both of these systems could be used for detecting viable cells if upstream sample processing methods were modified to include PMA or other approaches that limit PCR detection to intact viable cells.

Advanced Analytical Technologies, Inc. (Ames, IA) developed a high throughput rapid microbial detection device (RBD 3000) capable of monitoring total waterborne microbes, total biomass, and specific pathogens. Viability assessment is based on inclusion/exclusion of fluorescent dyes. The detection device is fully automated, processes up to 42 samples at a time, and results can be obtained within 18 min. A membrane-permeable nucleic acid dye is added so that the fluorochrome intercalates with the DNA of all cells. A non-membrane permeable counterstain is added which can only enter membrane-compromised cells. Once inside these damaged cells, the counterstain reduces the fluorescence intensity of the nucleic acid dye. As a result, only cells with intact membranes, which did not take up the counterstain, fluoresce. The stained cells are counted in a flow cytometer. Numbers of events correlate with bacteria with intact cell membranes. A detection limit of 10 CFU/ml for total bacterial counts was reported. Noble and Weisberg (2007) reported that the RBD 3000 detected 100 CFU/100 ml within 2 h.

Most molecular characterization of microbes in natural environments has focused on DNA, using selected genes, high-throughput genomics or metagenomic approaches. However, tools have now been developed that allow other cellular components and functions to be interrogated. Transcriptomics targets mRNA as an indicator of gene expression, proteomics identifies, quantifies, and characterizes proteins (primarily enzymes), and metabolomics studies the metabolic products and intermediates of cellular function. It is important to be able to assess viability and functionality of individual species and cells. It is also important to assess viability and activity of an entire microbial community within a particular habitat. Consequently, a variety of 'meta' approaches have been developed for studying microbial diversity, gene expression, translation, and activity on a community-wide scale. A combination of molecular and biochemical tools, targeting functional genes, their transcription products, and corresponding proteins will address the link between genetic and functional diversity, and lead to a better understanding of the functions, viability, and dynamics of complex microbial communities in the environment. For example, a combination of genomic and mass spectrometry-based proteomic methods was used to evaluate gene expression, identify important activities, and assess partitioning of metabolic functions in an acid mine drainage, low-complexity microbial

biofilm (Ram *et al.*, 2005). Proteins involved in protein refolding and response to oxidative stress were highly expressed, but the biofilm was dominated by novel proteins with limited homology to proteins with identified functions. Future studies of more complex environments, using a wider variety of tools, will increase our knowledge of the diversity and types of activity of microbes in the environment, which will lead to a better understanding of bio/geological and bio/limnological processes.

Microbial culturing, molecular detection, and automation may all come together for studying microbial viability, activity, and function with the development of high-density micro-Petri chips. A recent report described a microbial culture device containing 340,000 culture chambers per cm^2 (Ingham *et al.*, 2007). Bacterial microcolonies grown on the micro-Petri chip were exposed to a fluorogenic substrate without altering the spatial arrangement of the colonies. The few microcolonies that converted the substrate and fluoresced were then retrieved using a micromanipulator. Future applications of this technology could see high throughput screening of microbial communities using many of the viability and activity assessment tools described in this chapter.

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Orin C. S
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