

Chapter 8

ANALYTICAL METHODS FOR MICROBIOLOGICAL WATER QUALITY TESTING

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8.1 Introduction

There is a wide range of microorganisms of interest in water quality testing. Here we describe the general suite of methods currently used for the major indicator organisms and many of the pathogens of concern.

A fundamental limiting factor in the assessment of microbial quality of waters, and especially drinking water, is often the very low number of each organism present. Therefore, it is important to note that most microbiological procedure consists of: concentration/enrichment, detection and quantification (Table 8.1). A consequence of this multi-step approach is that technological advancement of any one step (such as detection) while possibly revolutionary, may be of limited value if the target group can not be satisfactorily concentrated before being subjected to the detection system.

This chapter is organised around the logical sequence of these method steps and common approaches for different microbial groups are discussed in one section. Emerging technologies are also presented, including the possible automation of the complete method or part of it. Performance and validation of methods and the statistical considerations behind choosing sample numbers are examined. The chapter concludes with a summary tabulation of the major methods along with their advantages and disadvantages and a list of abbreviations.

Table 8.1. Example of the various method steps involved in the analysis of microorganisms

Common method components	Microbial groups		
	Viruses	Bacteria	Parasitic protozoa
Concentration	Adsorption-elution	Membrane filtration	Cartridge filtration/IMS separation
Detection/enumeration	Cell culture/cytopathic effect, count plaque forming units	Selective growth on agar, count colony forming units	Immunological staining/count fluorescent cysts

IMS: Immunomagnetic separation.

8.2 Recovery of target microorganisms

Traditional approaches to the isolation of microbial indicators have relied on various agar plate and liquid media methods. The basic pour plate technique has a maximum sample volume of about 1 ml whereas the spread plate technique uses 0.1 or 0.2 ml samples. For larger volume processing and rapid throughput, however, the membrane filtration technique is preferred if interfering particles are not concentrated simultaneously. Liquid cultivation techniques, either for the detection of the target organism (presence/absence test) or quantitatively, using multiple tube techniques and most probable number (MPN) calculations, allow flexible sample volume range and the handling of turbid samples. In liquid cultivation techniques, small volumes of sample dilutions or up to ten litre samples can be used. The detection of target microorganisms by non-cultivation methods is also presented for enteric viruses and parasitic protozoa.

8.2.1 Filtration methods

Bacteria are generally recovered on 47 mm diameter membrane filters with porosities of 0.22 to 0.45 μm . Membrane filters may be incubated on solid media, pads soaked in liquid media or as a MPN system in enrichment broth.

Cysts of protozoan parasites can be recovered on similar membranes but with larger surfaces (up to 293 mm diameter) and porosities as high as 2 μm (Ongerth and Stibbs, 1987). For convenience, however, various cartridge filters

are generally preferred to recover protozoan cysts from up to 100 l water samples even in the presence of some turbidity (USEPA, 1999). The co-concentration of non-target particulates can, in part, be removed by subsequent selective separation method(s) (such as immunomagnetic separation (IMS), gradient centrifugation or flow cytometry, outlined in sections 8.2.2, 8.2.4.2 and 8.2.5). In England and Wales treated water supplies (10 l samples) are, however, analysed using compressed foam filters. Such sampling and monitoring procedures have been specified in a number of documents published by the UK Drinking Water Inspectorate (DWI: <http://www.dwi.gov.uk/regs/crypto/index.htm>). A French Standard has also come into force in 2001 (NF T90-455, Publication date: 2001-07-01: Water quality Detection and enumeration of *Cryptosporidium* oocysts and of *Giardia* cysts- Concentration and Enumeration method) and an ISO is currently in preparation (ISO CD 15553 Water Quality-Isolation and Identification of *Cryptosporidium* Oocysts and *Giardia* Cysts from Water).

8.2.1.1 *Virus adsorption-elution methods*

A number of techniques have been described for the recovery of viruses by approaches based on the filtration of test water through filter media to which the phages/viruses adsorb. The phages/viruses are afterwards released from the filter media into a small volume suitable for quantitative plaque assays or presence/absence testing. The principle involved is that viruses/phages carry a particular electrostatic charge that is predominantly negative at or near neutral pH levels. This charge can be modified to predominantly positive by reducing the pH level to about 3.5. At this pH level viruses/phages will adsorb to negatively charged filter media. The balance involved is rather delicate because the lower the pH the better the adsorption, but low pH levels inactivate phages/viruses, and the sensitivity of different phages and viruses to low pH levels differs. Hydrophobic interactions also seem to play a role in the adsorption process (APHA, AWWA, WEF 1998). After adsorption, a small volume of an organic solution at pH 9.5 or higher is passed through the filter to reverse the charge on the viruses/phages to negative. This results in the release of the viruses/phages and they can be detected by conventional methods.

Bacterial viruses can also be retained by membrane filters under acidic conditions in the presence of divalent or trivalent salts. Sobsey *et al.* (1990) developed a relatively simple, inexpensive and practical procedure for the recovery and detection of F-RNA coliphages using mixed cellulose nitrate and acetate membrane filters for analysis of 100 to 2 000 ml volumes of tap water and 100 to 300 ml volumes of surface water. The efficiency of recovery of seeded F-RNA phages from 100 ml samples of tap water was 49%, which

gradually decreased with increasing test volume to 12% for 2 000 ml. The efficiency of recovery from 100 ml and 300 ml samples of surface water was 34% and 18%, respectively. Although the procedure has attractive features, it should be weighed up against direct plaque assays on 100 ml samples, and presence/absence tests on 500 ml samples, both of which have theoretical efficiencies of 100% (Grabow *et al.*, 1998). Test volumes of the latter assays can be increased without loss of efficiency, as will be discussed later. Nonetheless, negatively or positively charged cartridge filters of various compositions remain the preferred approach for the concentration of viruses (enteric or bacteriophages) from large volumes of water.

Alternatively, filter media which carry a positive charge and hydrophobic binding sites at neutral pH levels, may be used to sorb negatively-charged viruses/phages at neutral pH levels (Sobsey and Glass, 1980). A variety of membranes and filter systems is available, among the well known ones are CUNO 1-MDS Virosorb and CUNO Zeta Plus 50-S or 60-S electropositive filters and glass wool. Application of these and related positively-charged filters in procedures with a wide variety of modifications and variations have been used to recover enteric viruses and phages (Singh and Gerba, 1983; Goyal *et al.* 1987). Efficiencies in the recovery of the coliphages (MS2, ØX-174, T2 and T4) from 17 litre volumes of tap water, sewage and lake water ranged between 34 - 100 % with positively charged Zeta Plus filters, however, MS2 appeared to be poorly recovered (range 0.3-1.8 %) with glass wool (Grabow *et al.*, 1998).

Therefore, although poliovirus and related viruses are recovered to some degree under certain conditions, evidence has been presented that phage recovery may be poor, probably because of poor adsorption as well as inactivation by exposure to the pH extremes required for adsorption and/or elution (Seeley and Primrose, 1982; Grabow *et al.*, 1998).

8.2.1.2 Ultrafiltration

Ultrafiltration is based on the filtration of water through membranes of polysulphonate or related material with a nominal molecular weight cut-off limit of about 10 000 Daltons. Particles with a diameter of 0.02 µm or more fail to pass through these membranes. Hence, dissolved organic molecules pass through the pores of these membranes but viruses and phages are too large to do so. It is, therefore, a process in which viruses are physically retained. Filter systems include spiral wound and sheet membranes (against which the water is kept in motion by means of a recirculating pump) or stirring apparatus (to enhance the filtration rate and avoid clogging) and yield close to 100% recovery (Grabow *et al.*, 1993). Other commercially available systems consist of units in

which filtration is enhanced by tangential flow through hollow fibres with a large total filtration surface area, with some as disposable modules (described for *Cryptosporidium* by Simmons *et al.*, 2001).

Advantages of ultrafiltration include high recovery efficiencies and viruses/phages are not exposed to pH extremes or other unfavourable conditions, which may affect their viability. Adsorption of viruses and phages to the membranes is minimal, and this can be reduced by pretreatment of the membranes with beef extract (Divizia *et al.*, 1989) or 1-2% Tween 80, which seems to block potential adsorption sites. The most important disadvantage is that the membranes clog rapidly which implies that the volumes of water that can be processed are restricted.

8.2.2 Immunocapture

Direct immunomagnetic separation (IMS) techniques involve incubation of magnetic beads that are coated with specific antibodies for a target organism (see Box 8.1), in a mixture of the cell suspension (*e.g.* a water sample). After incubation and efficient mixing of the particles with the sample, the target cells become bound to the magnetic beads. The particles are then separated from the rest of the suspension with the help of a magnetic particle separator and washed several times.

Box 8.1. Immunology techniques

A wide range of immunological methods, taking advantage of antibody-antigen interactions, is available, among them the enzyme immunoassays (EIA). EIA methods combine the specificity of antibody molecules with the amplification of antibody-antigen interactions by enzyme catalysis. Different EIA methods exist. Many assays are performed in the wells of microtitre plates in which the reactants are immobilised. Antigen in the sample may or may not be bound by a specific antibody immobilized on the surface (coating antibody). Direct assays employ specific antibody conjugated to enzyme (enzyme linked immunosorbent assay - ELISA), whereas in indirect assays (double antibody sandwich - DAS-ELISA) the antigen-specific detecting antibody is detected by an anti-immunoglobulin enzyme conjugate. A number of DAS-ELISA approaches take advantage of the strong interaction between biotin and avidin (or streptavidin). Biotinylated antibodies are easily detected by using a streptavidin-enzyme conjugate. The same conjugate may be used to detect a number of different antibodies.

Immunoaffinity methods in combination with antibody coated magnetic beads have been used to isolate a number of different organisms from water samples, including hepatitis A virus (HAV), group A rotaviruses, pseudomonads, *E. coli* O157:H7 and *Cryptosporidium parvum*. The isolation of

bacteria from water can be improved using enrichment followed by IMS and plating on selective agar. Moreover, magnetic beads coated with antibodies that specifically recognise various surface exposed epitopes of a variety of target organisms are already commercially available.

A basic laboratory infrastructure would be an advantage but is not absolutely necessary. The assays are easy to perform in a few hours. In addition, purification “kits” based on the immunocapture principle exist for several organisms. Although the technique is simple and fast, the efficiency of the reaction relies on the specificity and affinity of the commercially available monoclonal antibody and on the turbidity of the water sample. Immunocapture-based methods can be used as sound basis for other detection techniques (such as polymerase chain reaction (PCR), reverse transcriptase-polymerase chain reaction (RT-PCR), flow cytometry and fluorescent in-situ hybridisation (FISH), covered in Sections 8.2.5, 8.3.2.1 and 8.3.2.2).

8.2.3 Flocculation

Relatively successful techniques are on record for the recovery of enteric viruses from water by adsorption of viruses to flocculants such as aluminium hydroxide (APHA, AWWA, WEF 1998). The process probably involves both electrostatic interactions between the negatively charged virus surface and the positively charged aluminium hydroxide surfaces and coordination of the virus surface by hydroxo-aluminium complexes. Flocs are generally recovered by centrifugation or filtration. The flocs are then disintegrated by vigorous shaking and the viruses recovered by centrifugation (APHA, AWWA, WEF, 1998). The procedure is suitable for the recovery of viruses from relatively small volumes (several litres of water). This has been confirmed in tests using ammonium sulphate supplemented with beef extract for flocculation which yielded efficiencies of recovery of up to 85% for phages MS2, ØX174 and T3 (Shields and Farrah, 1986). Modifications of the procedure include magnetic organic flocculation, in which casein flocs are formed in the presence of magnetite for subsequent collection of the flocs by means of a magnet. The recovery of coliphages from waste- and lake water by this procedure has been described (Kennedy *et al.*, 1985).

A method for the concentration of particles in the *Cryptosporidium* oocyst size range from water has been developed based on the calcium carbonate flocculation (crystallisation) (Vesey *et al.*, 1993). An aliquot volume of water sample is treated by adding solutions of calcium chloride and sodium bicarbonate and raising the pH value to ten with sodium hydroxide, resulting in the formation of crystals of calcium carbonate, which enmesh particles. The

crystals are allowed to settle, the supernatant fluid is discarded and the calcium carbonate precipitate is dissolved in sulphamic acid. This process yields reproducibly high recovery rates. It has, however, been suggested that the oocysts may not be used for the viability test because solution of the calcium carbonate with sulphamic acid has been reported to affect viability measured by fluorescent dye exclusion (Campbell *et al.*, 1994).

8.2.4 Centrifugation

8.2.4.1 Continuous flow centrifuge

The most common separation method is that of differential centrifugation (pelleting) using either a swinging bucket or a fixed angle rotor. However, this conventional method is limited to small volumes of water. For harvesting microbes to be tested from source and drinking waters, continuous flow rotors are preferred as they allow efficient processing of large volumes of water in a single run regardless of turbidity of the sample water.

The basic instrument is a continuous flow rotor in combination with a refrigerated centrifuge and a simple peristaltic pump. Continuous flow experiments are normally carried out in the cold in order to avoid heating the particle concentrate. In practice, sample water is pumped in continuously through the centreline of the seal assembly of the rotor while it is spinning at operating speed. The sample flows along the bottom of the core and moves over the centripetal surface of a solution. The centrifugal separation therefore accounts for two fractions:

- A sedimenting particle that moves out into the rotor cavity.
- A supernatant fraction that continues to flow along the core and over the centripetal surface of the water, then out of the rotor via the outlet lines.

The sample particles are allowed to pellet on the rotor wall.

The continuous flow centrifuges currently commercially available are large and stationary, and are not suited to concentrating water samples on site. Recently, a compact, continuous flow centrifuge with disposable plastic bowls (a modified blood component separation system) has been applied to the concentration of *Cryptosporidium* oocysts and *Giardia* cysts from large volumes of water. The robustness and accuracy of this system has not yet been fully examined and further experiments are also needed to examine the

reproducibility and ease of recovery of the microbes from the disposable plastic bowl.

8.2.4.2 *Gradient density separation/isolation*

A centrifugation technique is also commonly used for separation/isolation of microbes, such as *Cryptosporidium* oocysts and *Giardia* cysts, from particle concentrates. In this case, a density gradient within a medium is centrifuged, separating microbes/particles from a thick mixture based on their specific density. The density gradient method involves a supporting column of fluid (such as sucrose or Percoll) where the density increases either zonally or linearly toward the bottom of the tube. If the density gradient column encompasses the whole range of densities of the sample particles, each particle will settle only to the position in the centrifuge tube at which the gradient density is equal to its own density. Thus, resulting in the separation of particles into zones solely on the basis of their density differences, although with environmental samples, the density gradient centrifugation step may lead to more than 30% losses with oocysts or cysts.

It is sometimes easier to start with a uniform solution of the sample and the gradient material such as a self-generating caesium chloride gradient for virus purification. Under the influence of centrifugal force, the material redistributes in the tube so as to form the required density gradient. Meanwhile, sample particles, which are initially distributed throughout the tube, sediment or float to their isopycnic positions. The target microbes can be recovered by removing the required density zone from the centrifuge tube. Development of density markers, which can be mixed in a particle concentrate prior to centrifugation should easily differentiate the zone to be collected.

Biohazard

Concentration or separation of pathogenic materials by preparative centrifugation is deemed a biohazard. Extreme precautions must be taken when such samples are used because of the possibility of seal leakage or rotor mishaps. There is no standard method for decontaminating rotors exposed to pathogenic materials. Rotors should be cleaned with appropriate detergents and/or disinfectants according to the manufacturer's instructions. The widely used method is autoclaving and most commercially available rotors can be autoclaved, although the instruction manuals should always be consulted to ascertain any specific handling requirements.

8.2.5 *Flow cytometry*

Flow cytometry is a technology in which a variety of measurements can be made on particles, cells, bacteria and other objects suspended in a liquid. In a flow cytometer, particles are made to flow one at a time through a light beam (laser beam) in a sensing region of a flow chamber. They are characterised by light scattering based on their size, shape and density and also on the dyes that are used either independently or bound to specific antibodies or oligonucleotides that endow a fluorescent phenotype onto components of interest. As a particle flows through the beam, both light scattered by the particle and fluorescence light from the labelled particle is collected either by a photomultiplier or photodiode in combination with light splitters (dicroic mirrors) and filters. This makes it possible to make multiple simultaneous measurements (up to six parameters) on a particle. A solid phase laser scanning analyser might be an alternative for the flow cytometry technology, though it is still in its infancy. In the latter system, the fluorescent dye-stained samples loaded on a membrane filter are scanned by a laser beam, and fluorescence emitted from the dye attached to the target particle is similarly measured.

For the concentration of target organisms, a flow cytometer with the additional capacity to selectively sort (such as fluorescently activated cell sorting [FACS]) any selected particle from the suspension can be used. The ability to sort particles is an important feature for environmental microbiology since it makes it possible to collect presumptive organisms and to confirm results by, for example, visual examination. However, incorporation of a sorting unit into the system not only doubles the cost of the basic instruments but is also problematic for the development of the automatic monitoring system. Alternatively, additional detection parameters, such as dual staining with a second monoclonal antibody can be used to determine that both antibodies are binding an authentic target organism. This results in an increase in sensitivity of the detection method to such a degree that nonsorting detection (analyser only mode) is possible, although this has yet to be applied in routine practice (Vesey *et al.*, 1994).

A particularly valuable aspect of flow cytometry is its capability of rapid analysis: the assay itself can be completed within three to five minutes. This is likely to be one of the key devices for the routine multiple monitoring of microbes of interest (including a variety of indicator or pathogenic microbes and even viable but non-culturable bacteria). Although the applicability of this system is very broad, the current application of flow cytometry for monitoring of drinking water is limited (Deere *et al.*, 2002).

The basic instrument is the flow cytometer, which requires a skilled operator. The main consumables (in cost terms) are primarily (monoclonal) antibodies. The first and foremost problem, affecting the use of flow cytometry technology in this field is the high capital cost.

The technological limitation of this system is the number of dye combinations that can be used, where the combinations of excitation and emission spectra must be significantly different. The number and variety of specific labelling reagents will be another limitation of the system. Availability of commercial kits is expected to increase the use of this technique in many research fields including safe drinking water. In addition, most of the pathogenic microbes to be measured occur in drinking water at very low concentrations. When a negative sample is analysed no particles should be detected and a sample seeded with an aliquot of organisms should have an exact number of particles added. However, at present, it is difficult to obtain this level of sensitivity. Often a negative sample will contain some particles due to non-specific binding of antibodies to some interfering particles found in water samples, no matter how specific the antibody is.

Biohazard

Handling of the particle concentrate to be measured and the effluent from the flow cytometer is deemed to be a biohazard. Effluents must be autoclaved before discarding. The cytometer can be decontaminated (disinfected) between samples and at the end of the run by running 10% sodium hypochlorite (bleach solution) for 30 seconds and detergent solution for two minutes followed by a distilled water flush.

8.2.6 *Pre-enrichment and enrichment techniques*

As outlined in Chapter 2, detection and enumeration of index and indicator parameters rather than the search for specific pathogenic bacteria is used in routine bacteriological analysis of water. Nevertheless, under special circumstances the search for pathogenic bacteria may be necessary, *e.g.* during an epidemic (see Chapter 7) or when evaluating new water resources (WHO, 1984). Typically the number of pathogenic microorganisms is low (Emde *et al.*, 1992) and their recovery is low because they are in a stressed conditions. Therefore, the chances of detecting pathogenic bacteria will be greater by using a pre-enrichment step prior to enrichment and selective plating. This allows environmentally stressed organisms to recover and grow before selective pressures are applied. Generally, pre-enrichment media contain no antibiotics or

other selective agents and this allows the growth of most microorganisms in the sample. Subsequent inoculation into enrichment media selects for the pathogen of interest, which can be detected by plating onto solid selective media. It should also be pointed out that this limits the ability to later quantify the pathogens in the sample (Ericksen and Dufour, 1986). Table 8.2 is based on the principles described above. Note that cell culture enrichment of viruses and phages is also used prior to detection by plaque assay (Grabow *et al.*, 1998) or PCR (as cell culture-PCR).

Table 8.2. Procedures for the pre-enrichment and enrichment of bacterial pathogens using liquid media

Organisms	Enrichment conditions	Reference
<i>Yersinia enterocolitica</i>	"Cold enrichment" at 15°C in peptone-yeast extract broth. Selective medium, alkaline bile-oxalate-sorbose broth, pH 7.6.	Schiemann (1990) Schiemann (1990)
<i>Salmonella</i> spp.	Pre-enrichment in buffered peptone water, then enrichment in, e.g. selenite-containing broth.	WHO (1984)
<i>Shigella</i> spp.	Enrichment medium, e.g. alkaline nutrient broth pH 8.0.	WHO (1984)
Cholera and non-cholera <i>Vibrios</i>	Enrichment medium, alkaline peptone water, or taurocholate tellurite peptone water.	WHO (1984)
<i>Legionella</i> spp.	Selective medium, charcoal yeast extract base amended with selected antibiotics.	States <i>et al.</i> (1990)

8.2.7 Other techniques

8.2.7.1 Hydro-extraction

This procedure is based on placing a water sample into a cellulose dialysis bag, which is exposed to hygroscopic material such as polyethylene glycol (PEG). The PEG extracts water and micro-solutes through the semipermeable membrane while viruses and other macrosolutes remain inside. The procedure is recommended as an option for the recovery of viruses from small volumes of water, not more than a few hundred millilitres (APHA, AWWA, WEF 1998). The method may also be suitable for phages and has been used to recover cyanophages from ponds (Padan *et al.*, 1967).

8.2.7.2 Solvent-extraction

Solvent extraction is often applied as the initial step in separating viruses from solids, prior to polyethylene glycol precipitation, chromatography and guanidinium isothiocyanate (GIT) extraction (Shieh *et al.*, 1997).

8.3 Detection, identification and quantification of microorganisms

This section describes the more “classical” methods, which depend largely on cultivation techniques, as well as molecular methods. A number of them, particularly most of the recent techniques require standardisation and validation. Nonetheless, the majority of the methods presented here have already proven to be useful in drinking water microbiology and/or medical diagnostics, or display great potential.

In the detection, identification and quantification of target organisms some approaches are solely based on a single technique whereas other strategies take advantage of a combination of different methods. For example, to identify *Escherichia coli* reliance can be placed on a one-day-cultivation on chromogenic media. Alternatively, in a much faster approach, short pre-cultivation on an artificial medium can be combined with labelling using fluorescent probes, microscopy, and laser scanning techniques (section 8.4.1).

In the following sub-sections, alternative approaches are offered for a number of target organisms. The traditional cultivation techniques are usually sensitive but the identification is often not as reliable as might be desired. Methods based on molecular biology tend to be sensitive and yield reliable identification, but cultivation techniques always show viable organisms whereas molecular methods often reveal dead or inactivated target organisms/nucleic acid. This is of relevance in disinfected waters and should be considered in the interpretation of results.

8.3.1 Cultivation techniques

8.3.1.1 Cultivation of bacteria

It has long been recognised that culture media lead to only a very small fraction (0.01 – 1 %) of the viable bacteria present being detected (Watkins and Xiangrong, 1997). Since MacConkey's development of selective media for *E. coli* and coliforms at the beginning of the 20th century, various workers have shown these selective agents inhibit environmentally or oxidatively stressed

coliforms (McFeters *et al.*, 1986). Specially developed media without selective detergent agents (*e.g.* the m-T7 medium of LeChevallier *et al.*, 1982) permit a significant improvement in the recovery of stressed target bacteria. In addition, peroxides and superoxides are generated through auto-oxidation and photochemical reactions during the process of preparing, sterilising and storage of selective media (Lee and Hartman, 1989). Stressed cells have reduced catalase activity (Calabrese and Bissonnette, 1990) and are subject to additional stress once placed on selective media. Coupled with this is the accumulation of toxic hydrogen peroxide generated by aerobic respiration. Media without harsh selective agents have, therefore, taken over from the traditional approach (Hurst *et al.*, 2001).

Each of the cultivation techniques has a particular detection range depending on the sample volume. Whereas the lower detection limit depends on the maximum sample volume that can be processed, the upper limit can be freely chosen by selection of the dilution of the sample assayed. The measurement uncertainty related to each cultivation technique and statistical considerations based on Poisson distribution of target organisms in the sample have been described in documents produced by the Technical Committee on Water Quality of the International Organization for Standardization (ISO/TC 147/SC 4/WG 12).

The presence/absence test is sometimes used to monitor high quality samples where the presence of the target organism is improbable. It yields no information on the contamination level if a positive result is observed. The sensitivity of this technique depends on the sample volume analysed and the precision on the number of samples analysed in parallel at each dilution step. When using enough replicates good precision can be achieved. Computer programs now available for the calculation of MPN, give freedom to optimise the design without the restrictions of fixed MPN tables (Gonzales, 1996). In the techniques based on colony counting, the precision increases with increasing total number of colonies counted from replicate plates and from different dilutions. High densities of colonies on plates can cause overlap error and the interference of non-target colonies also limits the number of colonies to be reliably counted from one plate. Therefore, the upper working limit for a plate in colony counting techniques depends on the method (selectivity and distinction of the target), the target organism (size of target colonies), and the sample (background growth). In all of the enumeration techniques, the cultivation conditions are selected to promote the multiplication of the target organisms while simultaneously inhibiting the growth of other organisms. The balance between sensitivity and selectivity is the reason for different methods or sample processing for drinking water and highly contaminated waters.

Table 8.3 summarises the advantages and disadvantages of the commonly used cultivation techniques.

Table 8.3. Established cultivation techniques

Technique	Advantages	Disadvantages
Most probable number (MPN) using liquid media	• Flexible sample volume range • Applicable to all kinds of samples • Allows resuscitation and growth of injured organisms • Usually easy interpretation of test results and no special skills required • Minimal time and effort needed to start the test • The precision and sensitivity can be chosen by selection of volumes analysed, number of dilution levels and number of replicate tubes • Media often inexpensive	• In routine application, when few replicates are used, the precision is often low • Confirmation steps involving new cultivations are usually needed, which increase costs and time • When the selectivity of the medium is not adequate, the target organisms can be masked due to the growth of other microorganisms • Sample may contain inhibitors affecting the growth of the target organisms • For the isolation of pure cultures, further cultivation on solid media is necessary • If big sample volumes are studied costs of media increase and large space for incubation is needed
Presence/absence test using liquid media	• As above	• As above • No information on level of concentration of target organisms
Pour plate	• Simple and inexpensive method	• The sample volume analysed routinely is a maximum of 1 ml • Thermal shock, caused when melted agar is poured on the sample, inhibits sensitive organisms • Scoring of typical colonies not easy

Table 8.3. Established cultivation techniques (*continued*)

Technique	Advantages	Disadvantages
Spread plate	• Strictly aerobic organism are favoured because colonies grow on the agar surface (unless anaerobic conditions are applied) • Differentiation of the colonies is easier than from pour plates	• The sample volume analysed routinely is a maximum of 0.1 ml • Scoring of typical colonies not always easy
Membrane filtration	• Flexible sample volume range enabling the use of large sample volume and therefore increased sensitivity • Water soluble impurities interfering with the growth of target organisms separated from the sample in the filtration step • Quantitative result and good precision if the number of colonies grown adequate • Further cultivation steps not always needed, which lowers the costs and time needed for the analysis • When confirmation is needed, isolation from well separated colonies on membrane is easy	• Quality of membranes varies • Solid particles and chemicals adsorbed from sample to the membrane during filtration may interfere with the growth of the target organism • Not applicable to turbid samples • Scoring of typical colonies not always easy
Liquid enrichment + confirmation and/or isolation on solid media	• Liquid enrichment in favourable media and incubation temperature allows resuscitation of injured or stressed cells • Streaking of a portion of enrichment culture on an agar medium allows isolation of separate colonies • Differentiation and preliminary identification is possible on selective solid media • Detection and identification of organisms occurring in low numbers possible (e.g. <i>Salmonella</i>)	• Many cultivation steps increase costs of media, labour, skills needed and duration of the test

Chromogenic media-based detection methods

Media without harsh selective agents, but specific enzyme substrates allow significant improvements in recoveries and identification of target bacteria. In the case of coliforms and *E. coli*, such so-called ‘defined substrate methods’ were introduced by Edberg *et al.* (1991). This has evolved into the Colilert® technique and has been shown to correlate very well with the traditional membrane filter and MPN methods when used to test freshwater (Fricker *et al.*, 1997; Eckner, 1998). A number of such enzyme-based methods, allowing quantification within 24 hours is now available, including:

- Enterolert®, manufactured by IDEXX.
- Colisure® manufactured by IDEXX.
- Colilert®, manufactured by IDEXX.
- m-ColiBlue®, manufactured by Hach.
- ColiComplete®, manufactured by BioControl.
- Chromocult®, manufactured by Merck.
- MicroSure®, manufactured by Gelman.

The Colilert® method is based upon the sample turning yellow, indicating coliforms with β -galactosidase activity on the substrate ONPG (O-nitrophenyl- β -D-galactopyranoside), and fluorescence under long-wavelength UV light when the substrate MUG (5-methylumbelliferyl- β -D-glucuronide) is metabolised by *E. coli* containing β -glucuronidase. The analytical method involves adding commercial dried indicator nutrients containing the two defined substrates to a 100 ml volume of water and incubation at 35-37°C (APHA, AWWA, WEF 1998). The result is either presence/absence testing in the 100 ml volume or quantification in a proprietary tray (QuantiTray™) which separates the sample into a series of test wells and provides a most probable number per 100 ml of water.

Table 8.4 shows some regularly used chromogenic substances available for the detection of indicator bacteria.

Table 8.4. Examples of chromogenic substrates for the detection of indicator bacteria

(Adapted from Manafi, 1996)

Bacteria	Chromogenic substance	Enzyme tested
Coliform bacteria	o-nitrophenyl- β -D-galactopyranoside (ONPG) 6-bromo-2-naphtyl- β -D-galactopyranoside 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (XGAL)	β -D-galactosidase (E.C.3.2.1.23)
<i>E. coli</i>	5-bromo-4-chloro-3-indolyl- β -D-glucuronide (XGLUC) 4-methylumbelliferyl- β -D-glucuronide (MUG) p-nitrophenol- β -D-glucuronide (PNPG)	β -D-glucuronidase (GUD) (E.C.3.2.1.31)
Enterococci	4-methylumbelliferyl- β -D-glucoside (MUD) indoxyl- β -D-glucoside	β -D-glucosidase (β -GLU) (E.C.3.2.21)

A major concern with any assay based on enzyme activity, is the interference that can be caused by the presence of other bacteria. In addition, the use of β -galactosidase in coliform detection has other disadvantages, as the enzyme can be found in numerous organisms (including *Enterobacteriaceae*, *Vibrionaceae*, *Pseudomonadaceae* and *Neisseriaceae*, several Gram-positives, yeasts, protozoa and fungi).

β -glucuronidase activity although produced by most *E. coli* strains is also produced by other *Enterobacteriaceae* including some *Shigella*, *Salmonella*, *Yersinia*, *Citrobacter*, *Edwardia* and *Hafnia* strains. The presence of this enzyme in *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp., *Streptococcus* spp., anaerobic corynebacteria and *Clostridium* has also been reported. This could lead to the detection of a number of false positive organisms. On the other hand, some strains of *E. coli* (among them pathogenic strains) cannot be detected with this technique since they are (phenotypically) β -glucuronidase negative. Nonetheless, the above problems generally result in fewer errors than traditional cultivation-based methods.

8.3.1.2 *Cultivation in host cells*

Cultivation of phages

Numbers of phages are generally determined by direct quantitative plaque assays, the principles of which were designed by Adams (1959). Basically, soft agar is mixed with a suitable volume of the water under investigation and a culture of the host bacterium of choice at a temperature just above the solidification temperature of the agar. This mixture is poured on top of a bottom agar in a conventional 90 mm diameter Petri dish, yielding what is called a double agar layer (DAL) assay. The plates are incubated and plaques scored the following day. In tests on waters containing high numbers of bacteria (which may interfere with the host strain and the visibility of plaques) antibiotics such as nalidixic acid may be added to the agar medium and a resistant host strain is used.

A significant limitation of DAL methods is that only approximately 1 ml of test water can be used per 9 cm Petri dish. The quantitative detection of phages in numbers below the detection limit of direct plaque assays is, therefore, carried out by direct plaque assays using large Petri dishes, or the recovery of phages from large volumes of water followed by conventional plaque assays on the concentrates. Small numbers of phages in large volumes of water may also be detected by qualitative enrichment procedures.

Cultivation of viruses

The detection of viruses following the concentration step is performed in flat-bottom stationary flasks/wells or in rotating test tubes (roll-tubes) containing specific cell lines. Viruses are thus counted as plaques (clearings) in solid monolayers of cells, as tissue culture for 50% infective dose (TCID₅₀) or most probable number (MPN) in liquid suspensions (Payment, 2001).

Monolayer Plaque Assay: The cultivable enteroviruses produce a characteristic cytopathic effect and some can also produce visible plaques under a solid nutrient overlay. For the detection of plaque-forming enteroviruses, the plaque assay has been widely used. It has the advantage of providing results for rapidly growing viruses and can provide an isolated plaque (the equivalent of a bacterial colony), which can be picked up and contains a single virus type useful for virus identification and propagation. Disadvantages include under estimating the number of slow-growing viruses and not being able to detect those that are not plaque-producing.

A draft European Committee for Standardisation document (CEN/TC230/WG3/TG4) describes the monolayer plaque assay for enterovirus as follows: Confluent monolayer of Buffalo Green Monkey (BGM) cells in flasks or cell culture grade dishes are inoculated with the sample and incubated for one hour at 37°C (+/- 1.5°C). Excess sample is removed and an overlay medium containing agar and neutral red is added and allowed to set. After incubation pale areas of cell death (plaques) develop and are counted up to seven days. The cytopathic effect is localised because the agar will only allow spread of virus from cell to cell and neutral red is only taken up by living cells. It is assumed that a plaque is the progeny of a single virus infectious unit and they are referred to as plaque forming units (pfu). The number of pfu in the original sample can be calculated, utilising whole or part of the sample concentrate in multiple assays.

Liquid overlay assays: Slow-growing and non-plaque producing enteroviruses as well as viruses from the other groups (adenoviruses, reoviruses, hepatitis A, rotaviruses, etc.) replicate in cells but do not always produce any microscopic changes. To increase the probability of finding the viruses, one, two or even three passages incubated for seven to 14 days increase the probability of virus detection by allowing several cycles of replication. These techniques, under liquid nutrient medium can be performed in a macro-technique (tubes or flasks) or, more commonly, in a micro-technique (multiwell plates: 96, 24, 12, six or four wells). The number of inoculated tubes or wells determines the precision of the assay. When testing samples with a probable low number of viruses, a small number of flasks with a large surface area is preferable in order to maximise isolation and reduce the required labour time. The assay relies on various detection methods to enumerate the viruses in the original samples, including:

- Cytopathic effect (microscopy).
- Immunofluorescence (with specific or group antisera).
- Immunoperoxidase (with specific or group antisera).
- Molecular methods (PCR, hybridisation, etc.).
- Detection of virions in the supernatant by electron microscopy.
- ELISA methods (specific for one or more viruses).

Examples of frequently used cell lines are: MA 104, BGM-F1, BGM-H, RD, Frhk 4, HFS, HEP, Vero, CaCo-2.

Cultivation of protozoa in cell culture

In contrast to most bacteriological and virological assays, parasitological (protozoological) samples do not incorporate an enrichment step based on *in vitro* cultivation of the captured organisms in general. Improved *in vitro* assays for *Cryptosporidium parvum* have been developed to demonstrate the infectivity of the parasite. The majority of the life cycle can be completed in tissue culture but the production of new oocyst numbers is low and usually less than that used for the inoculum.

The methods for the cultivation of *C. parvum* may serve as an example for other protozoa (such as *Toxoplasma gondii*, *Isospora belli*, *Cyclospora cayetanensis* and various genera of Microsporidia). A variety of cell lines (e.g. CaCo-2 cells, bovine fallopian tube epithelial cells, Mardin Darby Bovine Kidney cells, HCT-8 cells) are currently in use for the cultivation of *C. parvum* (Slifko *et al.*, 1997; Gasser and Donaghue, 1999). One typical cultivation method is outlined below. *C. parvum* oocysts are treated with 10% bleach (5.2% sodium hypochlorite, or the sporozoites freshly recovered by the process of the excystation) and plated onto HCT-8 cells grown to approximately 60 to 80% confluency in a 5% CO₂ atmosphere at 37°C. Oocyst formation can be detected three days after inoculation. Propagation in cell cultures may be used in combination with polymerase chain reaction (see Box 8.3) for the detection of infectious oocysts, however, it requires specific staff training and experience and special equipment.

Cultivation of protozoa on artificial media

Artificial culture media for both *Entamoeba histolytica* and *Giardia lamblia* have been developed and used for diagnosis in the medical field. Historically, these lumen-dwelling protozoa have been grown in culture media with and without one or more of the microorganisms with which they are associated in their normal habitat within the hosts (xenic culture). Cultivation techniques so far developed are not quantitative and have never been successfully applied to environmental samples.

8.3.1.3 Standardisation of methods

Established standard methods are available, e.g. those of the International Organization for Standardization (ISO), the European Committee for Standardisation (CEN) and the American Public Health Association (APHA). Methods for the detection and enumeration of indicator bacteria and some

pathogenic or opportunistic bacteria are so widely needed that international standardisation is well underway. Table 8.5 outlines the state of the art of international standardisation of microbiological methods relevant for drinking water analysis.

Table 8.5. International standardisation of methods for microbiological drinking water analyses

ISO numbers refer to a published standard, () standard proposal not yet published
or [] published standard under revision

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
Coliform bacteria, Thermotolerant coliforms, <i>Escherichia coli</i>	[ISO 9308-1]	Membrane filtration on a selective medium; incubation (after resuscitation) at 36°C for 1 day (coliforms) or at 44°C for 1 day (thermotolerant coliforms); for confirmation of coliforms subculturing for lactose fermentation and gas production at 36°C for 2 days; for confirmation of thermotolerant coliforms subculture for lactose fermentation and gas production at 44°C for 1 day; for confirmation of <i>E. coli</i> subculturing for indole production at 44°C for 1 day is additionally needed; oxidase test	Poor selectivity; target colonies difficult to score
Coliform bacteria, Thermotolerant coliforms, <i>Escherichia coli</i>	[ISO 9308-2]	Liquid culturing in a selective medium; incubation at 36°C for 2 days; for confirmation (gas production) subculturing in BGGB at 36°C for 2 days for coliforms and in EC medium at 44°C for 1 day for thermotolerant coliforms, and additionally testing for indole production at 44°C for 1 day; oxidase test	A choice from several selective media is allowed in this outdated standard; revision is delayed due to lack of validation data on different media, coliforms as the target group taxonomically too heterogenic; time-consuming enumeration; material not expensive but labour costs significant

Table 8.5. International standardisation of methods for microbiological drinking water analyses (*continued*)

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
Faecal enterococci	ISO 7899-2	Membrane filtration on m-enterococcus agar, incubation at 36°C for 2 days; <i>in situ</i> aesculin hydrolysis test on BEAA at 44°C for 2 hours	Ecology of the target group should be re-evaluated due to new taxonomy; time consuming enumeration; material not expensive
Faecal enterococci	[ISO 7899-1]	Cultivation in liquid medium, azide glucose broth, incubation at 36°C for 1 and 2 days; subculturing on BEAA at 44°C for 2 days; catalase test	Ecology of the target group should be re-evaluated due to new taxonomy; time consuming enumeration; material not expensive
Sulphite-reducing clostridia, spores	[ISO 6461-2]	Normal or modified membrane filtration on sulphite-iron or tryptose-sulphite agar anaerobically at 37°C for 1 and 2 days	Pasteurisation of the sample enhances spore germination as well as their selection by killing vegetative cells; the target group poorly defined; material costs increase if anaerobic jars are used
Sulphite-reducing clostridia, spores	[ISO 6461-1]	Liquid culturing in DRCM anaerobically at 37°C for 2 days	Pasteurisation of the sample enhances spore germination as well as their selection by killing vegetative cells; the target group poorly defined; material not expensive
<i>Pseudomonas aeruginosa</i>	[ISO 8360-2]	Membrane filtration on Drake's medium 19, incubation at 37°C for 2 days; for confirmation subculturing on milk agar at 42°C for 1 day (growth, casein hydrolysis, fluorescence and pyocyanine)	Atypical isolates should be further identified; material not expensive but labour costs significant; revision carried out within CEN

Table 8.5. International standardisation of methods for microbiological drinking water analyses (*continued*)

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
<i>Pseudomonas aeruginosa</i>	[ISO 8360-1]	Liquid culturing in Drake's medium 10 at 37°C for 2 days; for confirmation subculturing on milk agar at 42°C for 1 day (growth, casein hydrolysis, fluorescence and pyocyanine)	Atypical isolates should be further identified; material not expensive but labour costs significant
<i>Legionella species</i>	ISO 11731	Spread plating on GVPC medium with antibiotics at 36°C for 10 days; subculturing on BYCE and BCYE-cys; serological testing of isolates growing on BYCE but not on BCYE-cys ; identification by fatty acids, isoprenoid quinones, indirect or direct immunofluorescent antibody assay, slide or latex bead agglutination, genus-specific monoclonal antibody or enzyme-linked immunosorbent assay	With and without sample pretreatment; background growth interferes; antibiotics and identification increase costs
<i>Legionella species</i>	(ISO/DIS 11731-2)	A screening method based on membrane filtration	

Table 8.5. International standardisation of methods for microbiological drinking water analyses (*continued*)

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
<i>Salmonella</i> species	[ISO 6340]	Liquid pre-enrichment in buffered peptone water at 36°C for 1 day, enrichment in modified Rappaport-Vassiliadis broth at 42°C for 1 day, selection on brilliant green/phenol red lactose and xylose lysine deoxycholate agar at 36°C for 1 day and optionally on bismuth sulphite agar at 36°C for 2 days; isolation of typical colonies for confirmation using biochemical and serological tests	<i>S. typhi</i> needs another pre-enrichment medium; time and many media needed which increases costs
Culturable microorganisms	ISO 6222	Pour plate technique, yeast extract agar, incubation at 36°C for 2 days and at 22°C for 3 days	All microorganisms are not expected to generate colonies; changes in colony forming units (cfu) relevant; cheap method
F-RNA phages	ISO 10705-1	Double layer pour plate, <i>Salmonella typhimurium</i> strain WG49, phage type 3 Nair (F' 42 <i>lac</i> ::Tn5): NCTC 12484; <i>Escherichia coli</i> K12 Hfr: NCTC 12486 or ATCC 23631 as host bacteria, TYGB and TYGA at 37°C for different periods depending on step; with and without RNase	AQC on the host bacterium necessary, count of RNA phages on the basis of subtraction of DNA phages from total plaque forming units (pfu); RNA phages produce small plaques
Somatic coliphages	ISO 10705-2	Double layer pour plate, ATCC 13706 strain as host bacterium, MSB and MSA at 37°C for different periods depending on step	Most sensitive of all the phage methods; multiplication of somatic coliphages possible, but appears not to be significant.

Table 8.5. International standardisation of methods for microbiological drinking water analyses (continued)

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
<i>Bacteroides fragilis</i> phages	(ISO 10705-4)	Double layer pour plate, defined strain as host bacterium, BPRM at 37°C for different periods depending on step, incubation anaerobically	Low numbers compared with somatic coliphages; host bacterium fastidious
Concentration of bacteriophages	(ISO/DIS10705-3)	Validation method described	
Enteroviruses	CEN/TC 230	Cultivation on mono-layer of BGM cells at 37°C for 7 days; 5% of CO ₂ in the atmosphere	Detects a range of enteroviruses, but BGM cells may be inhibited by components from environmental samples
Evaluation of membrane filters	ISO 7704	Comparison of relative recoveries for a method	
Evaluation of colony count media	ISO 9998	Comparison of relative recoveries for a method	
Validation of microbiological cultivation methods	(ISO TR 13843)	Characterisation of methods and confirmation of the detection of the target organism	
Equivalence testing of microbiological cultivation methods	(ISO/CD 17994)	Comparison of relative recoveries of target organisms between different methods	

Key: AQCC: Analytical Quality Control. ATCC: American Type Culture Collection (Manassas, VA, USA). BCYE: Buffered Charcoal Yeast Extract agar. BCYE-cys: BCYE without cysteine. BEAA: Bile Esculin Azide Agar. BGGB: Brilliant Green Bile Broth. BGM: Buffalo Green Monkey. BPRM: Bacteroides Phage Recovery Medium. DRCM: Clostridium Differential Bouillon. EC: selective broth for coliforms and E. coli (containing bile salts). GVPC: BCYE with Legionella selective supplements. MSA: Modified Scholtens' Agar. MSB: Modified Scholtens' Broth. NCTC: National Collection of Type Cultures and Pathogenic Fungi (Public Health Laboratory Service, UK). TYGA: Tryptone Yeast Extract-Glucose Agar. TYGB: Tryptone Extract-Glucose Broth.

8.3.2 Detection and identification

Molecular methods targeting nucleic acids are the necessary tools for unveiling microbial diversity and can be used in detection and identification. Basic nucleic acid methods are hybridisation, restriction, amplification, cloning, and sequencing and these are summarised in Box 8.2.

Box 8.2. Molecular methods targeting nucleic acids

Hybridisation is a reaction involving two complementary nucleic acid strands, which bind to form a double-stranded molecule. Often, one of the nucleic acids is denatured total bacterial DNA and the other is a nucleic acid fragment used as a probe. The probe is either a cloned DNA fragment or, more conveniently, a synthetic oligonucleotide (usually 15 - 25 nucleotides long). The hybridisation reaction can easily be followed when the probe is labelled. Non-radioactive label is visualised by an immuno-enzymatic reaction or a cascade of reactions involving avidin and a biotinylated enzyme, when the label is biotin. Visualisation is colorimetric, fluorescent, or luminescent. Alternatively, oligonucleotide probes can be bound to a support (filter, microtiter plate, micro-chip) and unknown DNA labelled.

Some parameters (temperature, ionic strength) must be controlled in order for the hybridisation to work properly (Grimont, 1988). For given reacting sequences and ionic strength, there is an optimal temperature allowing maximum binding of the probe. A stringent temperature allows the best removal of incompletely reassociated nucleic acid while retaining enough perfectly bound probe to allow the detection of an unambiguous signal (e.g. colour). A low ionic strength allows the use of a lower temperature. Optimal (or stringent) temperature and ionic strength depend on the length of perfectly hybridised nucleic acid. This means that optimal temperature for a given probe will allow some partial hybridisation (cross-reaction) when probe and target sequences do not match perfectly (heterologous nucleic acid). For a given probe, the specificity index is defined as the dilution needed to lower the homologous reaction to a level similar to that of a heterologous reaction (Grimont *et al.*, 1985). In other terms, excessive numbers of non-target bacteria may give false positive results. This is probably why hybridisation works well for confirmation of culture identification (when nucleic acid amounts are controlled) but is often inconclusive when used on field samples with unknown amounts of nucleic acid or mixtures of unknown numbers of bacterial species. It should also be noted that probes targeting DNA do not differentiate between live and dead bacteria.

Box 8.2. Molecular methods targeting nucleic acids (*continued*)

Restriction endonucleases are enzymes that recognise short specific palindromic sequences and cleave double stranded DNA at these sites. Digesting a DNA molecule with a given restriction endonuclease yields a finite number of DNA fragments. Electrophoresis is used to separate restriction fragments by size. Restriction of a bacterial genome often produces too many fragments to be analysed. This problem has been solved in two ways: one way is to use restriction enzymes that recognise rare cleavage sites thus generating a few very large fragments. The latter can be separated with the help of a special technique referred to as pulse field gel electrophoresis. The other way is to visualise a subset of fragments after hybridising with a specific probe (Southern method). When the probe targets 16 and 23S rRNA genes, the method is applicable to all bacteria and is often referred to as ribotyping (Grimont and Grimont, 1986). Alternatively, a DNA fragment can be amplified (see below) and digested by a restriction endonuclease to give a simple pattern (Kilger and Grimont, 1993). Restriction methods are best applied to purified DNA extracted from pure culture (bacterial identification and typing) and are not currently used on field samples with complex bacterial flora.

Amplification is a method in which a chosen nucleic acid sequence (DNA or RNA) is copied many times. Currently, the polymerase chain reaction (PCR) is the most widely used principle (see Box 8.3). A major problem with this technique relates to the high sensitivity of PCR, which allows the amplification of contaminating polynucleotides when careful procedures are not implemented. Furthermore, dead bacteria (*e.g.* autoclaved or disinfected) can still be detected by PCR. On the other hand, sample specific substances can interfere with the PCR reaction and may seriously affect the detection limit (Wilson, 1997).

The above methods can be combined. Restriction and hybridisation are used by the Southern method. Restriction of amplified products are used for identification (when rRNA genes are amplified) or typing (*e.g.* flagellin genes). Selective amplification of restriction fragments is used in the method called Amplified Fragment Length Polymorphism (AFLP).

An emerging technology consists of using arrays of probes bound to a support (membrane or microchip). Amplified target DNA is hybridised with the bound probes and individual reactions are scored either using some electronic device or by image analysis. Although the molecular techniques used are not new (Rijpens *et al.*, 1995), and unable to distinguish between live and dead bacteria when DNA is targeted, probe multiplicity (several thousand) and miniaturisation are interesting in many fields, especially the identification of alleles of many genes in a given bacterial strain.

Cloning is a method in which a restriction fragment is inserted in an autoreplicating vector (plasmid, phage, and cosmid) and thus biologically amplified.

Sequencing often uses a cloned or amplified gene and oligonucleotides (which hybridise to part of the gene), DNA polymerase (which copies the gene) and nucleotide analogues (which randomly stop elongation when adding a given nucleotide type). The result is a family of fragments all ending with a given nucleotide type. These are separated by electrophoresis. Sequences can be read automatically and compared with those contained in databases. Several databases are available on the Internet.

8.3.2.1 Polymerase chain reaction (PCR) – based detection

Polymerase chain reaction (PCR)

A basic laboratory infrastructure is essential to perform PCR (see Box 8.3). Various kits are commercially available from different suppliers which provide all protocols and reagents needed to carry out PCR based assays. In addition, a thermocycler for the PCR reaction and appropriate equipment for separation (e.g. power supplies, electrophoresis units) and detection/visualisation of nucleic acids are required.

Box 8.3. Amplification of nucleic acids

Among a variety of nucleic acid amplification technologies the **Polymerase Chain Reaction (PCR)** is the most prominent example. This method, carried out *in vitro* in a thermocycler, takes advantage of the thermostability and fidelity of DNA polymerases from certain thermophilic bacteria. Using DNA probes as templates and two oligonucleotide primers that bind to complementary sequences flanking the target, this method allows the exponential multiplication of nucleic acid fragments, in the presence of deoxynucleotides, within several hours. PCR amplification of DNA occurs in three steps: denaturation, annealing and extension of the primers. From the analysis of such amplicons, which can be separated in an electrophoretic step, it is possible to draw conclusions on the microorganisms that were present in the original sample (e.g. water). Thus, depending on the target sequence and the choice of primers, this technology allows the indirect detection of large groups of organisms or alternatively the identification of specific (sub)species. This can be achieved by using primers that bind to more conserved targets (e.g. regions encoding 16S rRNA) or to regulatory DNA sequences or genes that might be associated with specific functions such as virulence determinants (e.g. streptococcal pyrogenic exotoxins), respectively.

In order to prove that the amplified sequence is indeed the desired target a second PCR step can be included. **Semi-nested PCR** is performed with one external primer (used in the original amplification) and one internal primer that is designed from sequences contained in the expected first amplicon. In **nested PCR** two internal primers are used. This double PCR technique can increase the sensitivity of detection by one to two orders of magnitude.

Duplex PCR and **multiplex PCR** involve the use of two or multiple sets of primers resulting in two or multiple amplification products. When all products of amplification are diagnostic for a particular species it is possible to distinguish between closely related (sub)species. Alternatively, more than one organism can be detected when primers are used that are specific to different genomes.

Introducing molecular “tags”, such as digoxigenin (DIG) or biotin-labelled dUTP into the PCR product can provide an invaluable tool for diagnostics. Such labelled PCR products may either be used as hybridisation probes or be detected by use of capture probes. For instance, with PCR-generated DIG-labelled hybridisation probes, it is possible to detect and quantify minute amounts of a pathogen.

Box 8.3. Amplification of nucleic acids (*continued*)

As DNA can survive for long periods after cell death the PCR reaction does not distinguish between viable and non-viable organisms. In contrast to DNA, messenger RNA (mRNA) which is transcribed from DNA is very labile with a typical half-life of only a few minutes. A technique that offers potential for assessing viability and the presence of mRNA is **reverse transcriptase (RT) PCR**. There are two steps:

- Reverse transcription which produces DNA fragments from RNA templates, and
- PCR, which produces multiple copies of the target DNA.

In the first step a reverse transcriptase enzyme is used to extend an oligonucleotide primer hybridised to a single-stranded RNA containing the message of interest, producing a complementary DNA strand (cDNA). DNase I is used to remove any contaminating DNA that may result in false positive results. The entire process usually takes around three hours. Total RNA, messenger RNA (mRNA), transfer RNA (tRNA) or ribosomal RNA (rRNA) from a variety of sources (bacteria, viruses, parasites, yeast, plants, etc.) can be used as templates for reverse transcription. As in PCR, the amplified DNA segments corresponding to the target sequences can be detected using standard detection methods such as agarose gel electrophoresis or membrane hybridisation with specific DNA probes or by using ELISA.

Random Amplified Polymorphic DNA (RAPD) or Arbitrarily Primed PCR (AP-PCR) is a specialised form of PCR. It differs from normal PCR in that a single short primer (generally ten base pairs long) of a random sequence is utilised for the amplification of genomic DNA. This single short primer can anneal randomly at specific sites within a genome. Priming sites are randomly distributed throughout a genome and polymorphisms in such sites result in differing amplification products, detected by the presence or absence of fragments.

In principle, nucleic acid might be detected by PCR from all waterborne viruses and (micro)organisms, as long as their envelopes (capsids, membranes, cell walls) can be disrupted to make the nucleic acids accessible to the enzymatic reaction. For the release of nucleic acids from viruses and microorganisms different methods such as freeze-thaw cycles, boiling, addition of detergents, digestion with enzymes are applied.

The complete procedure including sample preparation is fast compared to the 'classical' methods, with results available in three to four hours. The amplified DNA fragments can be easily detected by gel electrophoretic separation and subsequent staining techniques, and could be analysed further. PCR is very flexible, and allows highly specific detection of particular (sub)species (*e.g. Escherichia coli*: EHEC, ETEC, STEC, UPEC), certain groups of microorganisms (*e.g. Enterobacteriaceae*) or can be used to study aspects of biodiversity in water samples. Since the nucleic acid region that is flanked by the primers does not have to be known completely, uncultured pathogenic microorganisms might be discovered in broad-range PCR approaches.

PCR techniques also have limitations. Although PCR is very sensitive samples have, in most cases, to be concentrated (*e.g.* by flocculation, filtration, centrifugation, precipitation, immuno magnetic separation, adsorption to particles followed by sedimentation and elution). The method may generate false positive results, especially when carried out without a pre-cultivation step of the original water sample, since it does not discriminate between viable and non-viable organisms. In addition, DNA molecules can survive in the environment for long periods of time, this may also result in a number of false positive reactions. The basic procedure does not allow quantitation of the number of the amplifiable DNA (RNA) fragments in the original sample. The PCR reaction is sensitive to inhibition by compounds that are present in environmental water samples (*e.g.* divalent cations, fulvic and humic acids) and might vary depending on thermocyclers and reagents used from different suppliers. Despite these limitations, in the coming years PCR and PCR-based methods are likely to be further automated, allow quantitation and be used in routine laboratories.

Reverse transcriptase (RT – PCR)

The indication of the viability of microorganisms in a given sample would be of enormous significance for food, industrial, environmental and medical applications. RT-PCR is useful to detect the presence of specific messenger RNA (mRNA) or ribosomal RNA (rRNA) sequences (see Box 8.3). Messenger RNA is turned over rapidly in living bacterial cells. Most mRNA species have a half-life of only a few minutes (Belasco, 1993). Detection of mRNA by RT-PCR might therefore be a good indicator of living cells or those only recently dead at the time of sampling (Sheridan *et al.*, 1998). This method has been used to determine the viability of *Legionella pneumophila*, *Vibrio cholerae* as well as that of *Giardia* cysts and *Cryptosporidium* oocysts through the detection of heat shock protein hsp70 mRNA (Bej *et al.*, 1991; Bej *et al.*, 1996; Stinear *et al.*, 1996; Abbaszadegan *et al.*, 1997) in environmental water samples. The method, combined with calcium carbonate flocculation to concentrate samples, induction of hsp70, and purification by immunomagnetic separation has been shown to be able to detect a single oocyst. However there are still disadvantages using this technology. The method is qualitative not quantitative and as the oocysts are broken subsequent counting is not possible.

Despite their large advantages, RNA based approaches face technical difficulties, particularly the extraction of detectable levels of intact RNA (a molecule that is significantly less stable than DNA). In order to minimise this problem a number of commercial kits for extraction and purification of RNA have been developed. The enzyme, reverse transcriptase, like the polymerases

for PCR is highly susceptible to a number of inhibitory contaminants commonly found in water (e.g. humic compounds). Therefore, considerable efforts have to be made in order to remove these compounds prior to testing. Immunomagnetic capture, as well as nucleic acid capture have proven to be successful for this purpose. Oligonucleotide probe-linked magnetic beads combined with RT-PCR have been used for the detection of viable *Giardia* and *Cryptosporidium* in water samples containing PCR-inhibiting substances. Although, as in the case of PCR, loss of microorganisms during the concentration and recovery can greatly reduce the detection sensitivity of the method.

Direct PCR does not distinguish between infectious and non-infectious viral sequences. The integrated technique involves inoculation of the concentrated sample onto cell monolayers, which are then incubated for a minimum of 24 hours. This allows virus RNA to be amplified in tissue culture making RT-PCR on cell culture lysate more sensitive. The technique is known as integrated cell culture RT-PCR (ICC RT-PCR).

Quantification using RT-PCR is still difficult, laborious and inaccurate, and requires skilled operators and large amounts of materials. Because neither PCR nor RT-PCR provides reliable means for quantification, commonly RT-PCR detection of pathogens in water has only been used as a qualitative presence/absence test. In recent years advances in technology and products have been made towards quantification of PCR and RT-PCR. These developments, (e.g. the TaqMan™ and LightCycler™ systems) are very promising for ‘in-tube’ detection and quantification.

8.3.2.2 Fluorescence in situ hybridisation (FISH)

With the help of *in situ* hybridisation techniques organisms can be detected in their natural habitat without the need for pre-culture techniques. The method involves fixation of the cells in their natural state followed by permeabilisation of the cell wall. This enables all the reagents, including the species-specific oligonucleotide probes, to move into the cell and hybridise to their target. The probes are labelled with fluorescent dyes to enable the hybridised target within the cell to be viewed by epifluorescence microscopy or laser scanning electron microscopy. Fluorescent oligonucleotide probes can react with, for example, all bacteria of a given phylogenetic branch, a genus, or a single species (Amann *et al.*, 1990, 1995). Different fluorescent labels can be used enabling multicolour reactions. Possible targets for hybridisation are genes, mRNA and rRNA. Genes cannot be detected by *in situ* hybridisation unless some *in situ* PCR step is used, as some 10 000 labelled molecules are typically required for ‘visualisation’.

Detection of bacteria by FISH

A typical *in situ* hybridisation protocol includes filtration of a water sample through a membrane, fixation of bacterial cells on the membrane, permeation of cells (to allow the probe to access its target), hybridisation with a fluorescent probe, washing to eliminate unbound probe, and microscopic examination.

rRNA molecules are universally present in bacteria, have diversely conserved portions of their sequences, and occur in about 30 000 copies per 'active' cell, as such they are perfect targets for *in situ* hybridisation. Databases contain many rRNA sequences. However, since sequences are not available for all described species, probes must be tested against a collection of reference microorganisms.

The fluorescent signal given by a cell depends on the number of targets and the accessibility of the target. For a given probe targeting rRNA, the signal is in relation to the number of ribosomes (*i.e.* the physiological state of the bacterium). When a probe is designed, attention should be given to target accessibility as the fluorescent signal varies widely depending on the sequence position of the target on the rRNA (Fuchs *et al.*, 1998). The fluorescent signal is stronger when the probe is longer with multiple labels (Trebesius *et al.*, 1994) or when a peptide nucleic acid (PNA) is used as a probe (Prescott and Fricker, 1999). However, for a given probe sequence, PNA probes are less specific than regular oligonucleotide probes and mismatches must be introduced to raise specificity. Signal amplification systems, such as Tyramide Signal Amplification (TSA) accumulate fluorescent compounds in cells where a probe reacted. This gives a very strong signal and may allow detection and enumeration of fluorescent cells by scanning cytometry. PNA probes and TSA have been combined, and the use of PNA probes targeted against the 16S rRNA molecule for the specific detection of *E. coli* have been shown to offer a fast efficient alternative to conventional approaches (Prescott and Fricker, 1999). In this method, bacteria are captured by filtration of the water samples through metallic membranes. The cells are then fixed by placing the membranes on a filter pad pre-soaked with paraformaldehyde solution. They are then treated with lysozyme, washed and overlaid with hybridisation solution containing a biotinylated PNA oligonucleotide probe specific for the detection of *E. coli*. The biotin PNA-RNA complex is detected by incubation in streptavidin horseradish peroxidase (HRP) followed by the addition of fluorescein tyramide. The HRP catalyses the deposition of fluorescein and the cells are detected by epifluorescence microscopy. The test requires no specialised equipment and is easy to perform in two to three hours. The procedure could be performed directly on the water sample without the need for culture techniques. Unfortunately, dead bacteria can also be detected after signal amplification.

Problems and shortcomings have been identified when FISH is applied to the detection of bacteria in water:

- **Detection is strictly taxonomic.** Molecular detection and identification of bacteria work in the framework of molecular taxonomy. Taxonomic groups that are not confirmed by molecular methods may not be properly identified. As an example, coliforms (whether faecal or not) do not constitute a taxon in molecular terms, therefore no nucleic acid probe can detect them. It is possible to use a probe or PCR system targeting the beta-galactosidase gene. Such probes, however, will not react with all coliforms but rather with coliform species that are phylogenetically close to *E. coli*, irrespectively of their habitat. Moreover, all *Shigella* species and serotypes, except *S. boydii* 13, belong to the *E. coli* genomic species and *Shigella* spp. are seen as entero-invasive clones of *E. coli*. Therefore, no taxonomic probe can distinguish *Shigella* spp. from *E. coli*. Probes (or PCR systems) can target the invasivity genes. These probes will detect invasive strains of *Shigella* and *E. coli* but will not detect *Shigella* strains that have lost the invasivity plasmid.
- **Bacteria in water are often starved or stressed.** Starved or stressed bacteria are less reactive and often occur as tiny cells. As such, they are difficult to distinguish among some inanimate material which may bind probes non-specifically. Furthermore, naturally fluorescent bacteria or objects may occur. A major problem with molecular methods is to distinguish live from dead bacteria. A bacterial state has been described in which bacteria are viable but nonculturable (VBNC) (Roszak and Colwell, 1987; Colwell and Grimes, 2000). However, clear definitions of bacterial life and death are needed (Villarino *et al.*, 2000). When starved or stressed bacteria are incubated in the presence of nutrients, yeast extract, and nalidixic acid (or ciprofloxacin), the cellular machinery is restarted, ribosomes accumulate while the cell elongates (nalidixic acid or ciprofloxacin prevent cell division, not elongation), thus demonstrating viability. Such cells are easily differentiated from inanimate material and from dead cells. The method called Direct Viable Count, or DVC (Kogure *et al.*, 1979) was adapted to FISH (Kalmbach *et al.*, 1997; Nishimura *et al.*, 1993; Regnault *et al.*, 2000) and proved to be the most accurate viability marker (Villarino *et al.*, 2000). The DVC was also used to first detect VBNC bacteria in water (Xu *et al.*, 1982).
- **The major drawback of microscopic methods is sensitivity.** In order to reach sufficient microscopic sensitivity to detect one cell per 100 ml, bacteria should be concentrated from volumes of 100 litres. Alternatively, machines scanning the whole filter surface for fluorescent objects could

be used together with automatic positioning of the microscope above detected fluorescent objects.

Detection of protozoa by FISH

Probes targeting the 18S rRNA and used in the hybridisation assay can be synthesised to the genus or species level. The use of FISH as an alternative technique would enable the specific detection of *Cryptosporidium parvum* as traditional methods such as antibody staining are unable to distinguish between different species within the genus (Vesey *et al.*, 1998). However fluorescence labelling does not produce fluorescence bright enough to be used for primary detection since autofluorescence particles like algae fluoresce more brightly. A combination of fluorescence and secondary antibodies should improve detection systems.

8.4 Emerging procedures

8.4.1 Laser scanning analysis

With the development of test procedures on the basis of chromogenic (Table 8.4) and fluorogenic substrates for the detection and enumeration of coliform bacteria and *E. coli*, the analysis can be performed in 24-48 hours. Several approaches have been investigated to enhance the sensitivity of enzymatic reactions using instrumentation instead of the traditional visual approach. Spectrophotometry has been shown to reduce the 24hr Colilert® test by 6 hours (Rice *et al.*, 1993). Using fluorometry one faecal coliform can be detected within 7 hours. In addition the 'ChemScan®' instrument from 'Chemunex®' has been used with membrane filtration tests for the detection of fluorescent microcolonies. Samples are filtered onto Cycloblack-coated polyester filters that are then incubated on a prefilter saturated with Colicult® medium. After the membrane has been transferred to a second prefilter saturated with a fluorogenic substrate the membrane is analysed by the ChemScan® instrument. The ChemScan® is a laser-scanning device with a motorised stage attached to an epifluorescence microscope. Each fluorescent event detected can be validated by microscopy. Initial experiments indicate that this method can be performed within 3.5 hours and yield results equivalent to those of standard methods. This technology allows the detection of any bacteria and protozoa that can be labelled with a fluorescent substrate linked to an antibody or nucleic acid probe. An alternative semi-quantitative system for the detection of coliforms is also available from Colifast®. This instrument called the CA-100 system also utilises the ability of coliforms to cleave galactoside conjugates to yield

fluorescent products. The level of fluorescence is measured at given time intervals and is directly proportional to the number of coliform bacteria present.

8.4.2 DNA – chip array

The future holds endless possibilities for the detection of both indicators and pathogens alike. On the horizon are methods based on microarrays and biosensors. Biosensors in the medical area have largely been based on antibody technology, with an antigen triggering a transducer or linking to an enzyme amplification system. Biosensors based on gene recognition, however, are looking very promising in the microarray format for detecting microorganisms. There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity:

- Probe cDNA (500~5 000 bases long) is immobilized to a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in a mixture. This method was originally called DNA microarray and was developed at Stanford University (Ekins and Chu, 1999).
- An array of oligonucleotide (20~25-mer oligos) or peptide nucleic acid probes is synthesised either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilisation. The array is exposed to labelled sample DNA, hybridised, and the identity/abundance of complementary sequences are determined. This method, originally called GeneChip® arrays or DNA chips, was first developed at Affymetrix Inc. (Lemieux *et al.*, 1998; Lipshutz *et al.*, 1999).

Microarrays using DNA/RNA probe-based rRNA targets may be coupled to adjacent charged couple device detectors (Guschin *et al.*, 1997). Eggers *et al.* (1997) have demonstrated the detection of *E. coli* and *Vibrio proteolyticus* using a microarray containing hundreds of probes within a single well (1cm²) of a conventional microtiter plate (96 well). The complete assay with quantification took less than one minute.

The microarray under development by bioMerieux (using Affymetrix Inc. GeneChip technology) for an international water company (Lyonnaise des Eaux, Paris, France) is expected to reduce test time for faecal indicators from the current average of 48 hours to just four hours. In addition, the cost for the standard water microbiology test is expected to be ten times less than present methods. The high resolution DNA chip technology is expected to target a range of key microorganisms in water. The prototype GeneChip® measures

about 1 cm², on which hybridisation occurs with up to 400 000 oligonucleotide probes.

8.4.3 Biosensors

The biosensor relies on optics, immunoassays and other chemical tests, which may be directed to detect microorganisms. To date, most work has focused on bacterial pathogens (Wang et al., 1997). In general, there is an immunoaffinity step to capture and concentrate bacteria on beads, membranes or fibre optics probe tips, followed by detection by laser excitation of bound fluorescent antibodies, acoustogravimetric wave transduction, or surface plasmon resonance.

Several types of biosensors are currently under development, especially to detect foodborne pathogens in, for example, meat and poultry. As an example, one type is described (Georgia Tech Research Institute, 1999):

The biosensor operates with three primary components - integrated optics, immunoassay techniques and surface chemistry tests. It indirectly detects pathogens by combining immunoassays with a chemical sensing scheme. In the immunoassay, a series of antibodies selectively recognise target bacteria. The 'capture' antibody is bound to the biosensor and captures the target bacteria as it passes nearby. A set of 'reporter' antibodies (which bind with the same target pathogen) contain the enzyme urease, which breaks down urea that is then added resulting in the production of ammonia. The chemical sensor detects the ammonia, affecting the optical properties of the sensor and signalling changes in transmitted laser light. These changes reveal both the presence and concentration of specific pathogens in a sample at extremely minute levels.

The method is currently unable to distinguish viable from non-viable microbes, and it will be necessary to increase the sensitivity in order to apply this technique to water testing. Nonetheless this methodology has a great potential for future application, especially as it is extremely fast.

8.4.4 Solid state biochips

The idea of rapid detection (minutes) of a number of toxins and actual microbial cells on a solid state biochip is a visionary approach currently being developed. This approach does not require isolation and characterisation of

nucleic acids from the microorganisms and does not rely on capturing of antibodies. Further characteristics are: no lengthy incubation times, no labelling and no washing are needed. The technique is not yet available, so limitations cannot be determined.

8.5 Performance and validation of methods

8.5.1 Limitations and characteristics of microbiological methods

The low numbers of target organisms in microbiological and especially drinking water analyses increase measurement uncertainty. Even if assuming homogenous distribution of target in the sample, the numbers detected are defined by the Poisson distribution. Therefore, the uncertainty of measurement is related to each individual measurement result and method specific values are not satisfactory alone. To be of use in validation a clear specification should be available. This should include an exact description of the working conditions and media used, upper counting limit, recovery, working limits within which the method can be used, selectivity with the respect to the target organism, specificity, robustness and limitations of the method.

The aim of the selective recovery of target organisms from samples is challenging. In growth dependent methods the viability of a target organism is defined by growth of this organism under specified conditions (*i.e.* by the method itself, a non-selective method or a reference method). It is nearly impossible to determine the true number of viable target organisms that are present in a sample (even when the sample is spiked). Therefore, absolute recovery cannot be defined and for a new method only a relative recovery can be given by relating it to that obtained with other (reference) methods. Similar problems occur in molecularly based methods. Microbiological methods are not robust in the sense that chemical methods are. The target and many contaminants in the sample are living entities and therefore unexpected effects and phenomena can occur. Robustness is affected by many different factors including the physical, chemical and microbiological properties of sample itself. In the analyses based on nucleic acids, it has been repeatedly observed that sample specific inhibitors interfere and decrease sensitivity (Wilson, 1997). All the methods are affected by sample storage before analysis (*e.g.* cold-shocks), incubation conditions and the competence of the personnel executing the analysis (*e.g.* time needed to perform certain steps).

8.5.2 *Statistical issues*

In samples (even in well-mixed laboratory samples) particles, including target organisms, are unevenly distributed and this results in a random basic variation of the results obtained which cannot be avoided (Tillett and Lightfoot, 1995). Due to experimental imperfections the variations observed in practice in parallel determinations are even larger than predicted by the Poisson distribution (the mathematical law which describes the ideal distribution they should follow). This effect is called over-dispersion. In contrast to chemical analysis where even at low concentrations the number of target molecules in a sample volume is high this over-dispersion cannot be avoided in microbiological testing because the number of target microorganisms is usually low. It is therefore important to analyse a sufficient number of samples to obtain a convincing result. Although statistical theory provides clear information (*e.g.* Cochran, 1977; ISO/TR 13843, 2000) as to how many samples would be required for a certain testing scenario, it is often not possible to meet these requirements (*e.g.* because of cost reasons) and statistical considerations usually become guidelines only. However, generally it should be remembered that too few samples may be a waste of effort, time and money.

8.5.3 *Validation of methods*

Method validation provides evidence that a specific method is capable of serving the purpose for which it is intended (*i.e.* that it does detect or quantify a particular microbe [or group of organisms, or a viral particle] with adequate precision and accuracy). A new, or inadequately characterised, method is initially investigated in a primary validation process to establish its operational limits. Primary validation should result in an unambiguous and detailed quantitative description of the results the method can deliver. Primary validation of a new method is typically performed by the laboratory that has developed it. When the method is implemented in another laboratory secondary validation takes place (also referred to as verification). Here, it is established whether the specifications described in primary validation can be met. Usually only selected and simplified forms of the procedures used in the primary validation process are used, but over an extended period of time and/or more samples. It should be pointed out that validation should simulate the later routine as closely as possible and natural samples should be used as the main test material wherever possible.

For both primary and secondary validation it is of course essential that strict analytical quality control is used, because application of valid methods does not necessarily ensure valid results. The methods of analytical quality

control include, replications at different levels, inclusion of reference materials (qualitative and quantitative), intercalibrations and spiked samples (Lightfoot and Maier, 1988; McClure, 1990).

In case equivalent methods already exist, the justification for introducing a new method always requires careful comparison with one or more established methods in parallel on the same samples. Since every method usually consists of several steps, method performance includes many different aspects. For example, one method might be superior in specificity but inferior in recovery. One method might give highest recovery of target organisms but require confirmation of positive results in routine test. Hence, for routine use a method giving lower recovery but not requiring confirmation of positives is probably preferable. This indicates that it is frequently difficult to numerically specify the superiority of one method over the other.

Collaborative tests in which several laboratories participate are considered essential for the validation of microbiological methods as well as the performance of individual laboratories. These tools were developed for chemical analytical methods but many of the principles are now also applied to microbiological testing. These collaborative tests are mainly of two types (Horwitz, 1988; McClure, 1990):

- **Intercalibration exercises** which allow laboratories to compare their analytical results with those of other participating laboratories.
- **Method performance tests** that yield precision estimates (repeatability, reproducibility) when several laboratories analyse identical samples with strictly standardised methods. In such tests ‘artificial’ samples (*i.e.* certified reference materials and spiked samples) are included in the samples to be analysed by the participating laboratories.

Experience from chemical collaborative testing indicates that it is important that the participating laboratories have in-depth knowledge and experience with the methods to be tested and collaborative method performance tests are not used as laboratory proficiency tests and training exercises. It is important to note that a number of established microbiological methods (*e.g.* Endo agar for total coliforms or mFC for thermotolerant coliforms), although used for decades by hundreds of laboratories, have not been assessed in collaborative tests.

Whether or not a validated method is successful in practice may depend on political and/or commercial issues. For example, within ISO, acceptance and publication of a method as a standard method requires approval of at least 75%

of the member bodies casting a vote. On the other hand, stimulation of the use of certain methods by the development and promotion of easy to handle kits by commercial companies is also possible.

8.6 Summary

Table 8.6 summarises the predominant characteristics, advantages and limitations of the main detection methods that are described in this chapter.

Table 8.6. Methods for the detection of microbial contamination in drinking water

Method	Characteristics/advantages	Limitations/disadvantages	Application: status quo and future perspective
Cultivation of bacteria	• Cultivation media mostly inexpensive • Easy to perform • Qualitative and quantitative results obtainable • Differentiation and preliminary identification possible on selective solid media • Detection of bacteria occurring in low numbers possible (in combination with concentration techniques, e.g. filtration)	• Time consuming • Not all bacteria of interest can be cultivated • Large sample volumes cause problems for some of the methods • Does not detect 'viable but non-culturable' organisms • Selectivity for the detection of certain indicators often not sufficient (false positive species) • No information on infectivity of a pathogen • Biosafety issues	• Standardised (ISO, CEN, APHA) for a number of species(groups) • Improved media might be developed in order to obtain faster growth and to increase sensitivity and selectivity of the assays
Cultivation of bacterial viruses (bacteriophages)	• Assays inexpensive and easy to perform • Quantitation possible • Similar to bacterial methods • Minimal biosafety issues (host cells)	• No direct correlation in numbers of phages and viruses excreted by humans • Phages can be useful as faecal indicators, as well as models or surrogates for enteric viruses in water environments, but care is needed in interpreting the results.	• Standardised methods available (ISO) for major groups

Table 8.6. Methods for the detection of microbial contamination in drinking water (continued)

Method	Characteristics/advantages	Limitations/disadvantages	Application: status quo and future perspective
Cultivation of animal/human viruses	• Several enteric viruses can be propagated in cell culture (a variety of cell lines have been tested and used) • Quantitation possible • Growth indicates infectivity	• Requires some level of training and specialised laboratories • Various cell lines may need to be used for the detection of a larger number of virus types • Biosafety issues	• Standardised (ISO, CEN, APHA) for a number of species(groups) • New cell lines are being developed and new media formulation may increase sensitivity
Cultivation of protozoa	• Excystation in vitro can be taken (to a certain extent) as indication for viability	• Does not provide information on infectivity for man • Time consuming • Propagation of most organisms in vitro using cell cultures is extremely poor • Not all protozoa of interest can be cultivated • Biosafety issues	• At present, the only available infectivity assay depends on animal hosts, which is costly and very time-consuming
Immunological detection of antigenic structures associated with microorganisms	• Qualitative and quantitative results regarding the number of microorganisms possible (to a certain extent) • Relatively specific for target organism	• Often needs pre-cultivation step which is time consuming • Lack of sensitivity • Selectivity can be a problem due to cross-reacting antibodies • Without pre-cultivation, currently no discrimination between viable and non-viable microorganisms • No information on infectivity of a pathogen.	• Assays allow standardisation and automation

Table 8.6. Methods for the detection of microbial contamination in drinking water (*continued*)

Method	Characteristics/advantages	Limitations/disadvantages	Application: status quo and future perspective
Immunomagnetic separation (IMS)	• Faster and more specific than other concentration methods • Sound basis for other detection methods (PCR, RT-PCR, FACS, FISH) as well as cultivation methods	• Sensitivity, robustness, consistency can be affected by environmental conditions • Selectivity can be a problem due to cross reacting antibodies • No information on infectivity of a pathogen	
Polymerase chain reaction (PCR)	• In principle highly sensitive (but see limitations) • Selective • Specific • Can detect 'non-culturable' microbes • Faster than cultivation methods (3-4 hours) • Sound basis for further analyses of nucleic acids (sequencing, RFLP, RAPD) •	• Limited reliability (at present the detection of an individual microbe cannot be guaranteed due to inconsistencies in performance of the technique) • Sufficient quantity of nucleic acids from the targeted microbe has to be recovered • Negatively affected by certain environmental conditions • Basic procedure does not allow quantitation of the number of amplifiable DNA/RNA fragments • At present no discrimination between viable and non-viable microorganisms • No information on infectivity of a pathogen	• Currently no standardisation • Potential for automation • Potential for quantitation

Table 8.6. Methods for the detection of microbial contamination in drinking water (*continued*)

Method	Characteristics/advantages	Limitations/disadvantages	Application: status quo and future perspective
RT-PCR	• As PCR • Good indication for living organisms with mRNA as target • Can provide information on pathogenic potential of an organism when mRNA of a virulence gene is assayed	• As PCR (except discrimination between viable and non-viable microorganisms with mRNA as target) • Extraction of detectable levels of intact RNA molecules is problematic due to their instability	• Currently no standardisation • Potential for automation • Potential for quantitation
Flow cytometry, fluorescence-activated cell sorting (FACS)	• Faster than cultivation methods • Detection of non-culturable organisms	• No information on infectivity of a pathogen • Expensive technology • Limited reliability for the detection of microbes that are present in extremely low concentrations	
Fluorescence in-situ hybridisation (FISH)	• Faster than cultivation methods • No pre-cultivation needed • Detection of non-culturable organisms • Can detect individual cells when ribosomal RNA is target • Different (multicolour) fluorescent labels allow detection of different microbes • Can be used in combination with machines that do automated scanning of filter surfaces for fluorescent objects	• Lack of sensitivity with chromosomal genes or mRNA as target • Detection is strictly taxonomic • Differentiation between living and dead cells is often difficult • Not applicable to detect 1 indicator per 100 ml without concentration/filtration	• Potential for automation

Table 8.6. Methods for the detection of microbial contamination in drinking water (continued)

Method	Characteristics/advantages	Limitations/disadvantages	Application: status quo and future perspective
Molecular fingerprinting (ribotyping, RFLP, RAPD, AP-PCR)	• Faster than cultivation methods • Excellent tool for differentiation of strains or isolates within a species	• At present no discrimination between viable and non-viable microorganisms • RAPD requires the use of pure isolates	
DNA chip array	• Micromanufacturing techniques allows testing of up to several thousand sequences in one assay on a single "chip" • Sensitive, selective and specific to the desired level to detect groups of organisms or (sub)-species, respectively • Fast (2-4 hours)	• At present very cost intensive • Highly trained personnel needed • Absolute quantitation might be problematic	• Technique not yet widely available
Biosensors	• Immunoaffinity step to bind microorganisms to surfaces; detection by laser excitation of bound fluorescent antibodies, acoustogravimetric wave transduction, or surface plasmon resonance • Rapid, but depends on culturable microorganisms		• Currently unable to discriminate between viable and non-viable microbes
Solid state biochip	• Aim of the method: rapid detection (minutes) of a number of toxins and microbial cells • Approach does not require isolation and characterisation of nucleic acids	• Limitations cannot be determined yet	• Technique not yet available, visionary approach under development

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